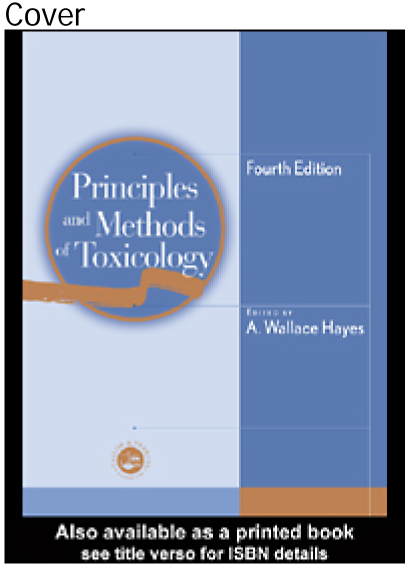
Cover



title: Principles and Methods of Toxicology 4Th Ed.
author: Hayes, A. Wallace
publisher: Taylor & Francis Routledge
isbn10 | asin: 0203362837
print isbn13: 9780203379639
ebook isbn13: 9780203362839
language: English
subject Toxicology, Toxicology--methods, Poisoning, Poisons, Toxicity Tests, Toxicología, Toxicologie , Toxicité
publication date: 2001
lcc: RA1211.P74 2001eb
ddc: 615.9
subject: Toxicology, Toxicology--methods, Poisoning, Poisons, Toxicity Tests, Toxicología, Toxicologie , Toxicité

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PRINCIPLES AND METHODS OF TOXICOLOGY

Fourth Edition

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PRINCIPLES AND METHODS OF TOXICOLOGY

Fourth Edition

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This edition published in the Taylor & Francis e-Library, 2005.

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USA Publishing Office: TAYLOR & FRANCIS
325 Chestnut Street
Philadelphia, PA 19106
Tel: (215) 625–8900
Fax: (215) 625–2940
Distribution Center: TAYLOR & FRANCIS
7625 Empire Drive
Florence, KY 41042
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PRINCIPLES AND METHODS OF TOXICOLOGY, Fourth Edition

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2 3 4 5 6 7 8 9 0
Cover design by Curtis Tow Graphics.
A CIP catalog record for this book is available from the British Library.
∞ The paper in this publication meets the requirements of the ANSI Standard Z39.48–1984 (Permanence of Paper).
Library of Congress Cataloging-in-Publication Data
Principles and methods of toxicology/[edited] by A.Wallace Hayes.—4th ed.
p.; cm.
Includes bibliographical references and index.
ISBN 1-56032-814-2 (alk. paper)
1. Toxicology. I.Hayes, A.Wallace (Andrew Wallace), 1939–
[DNLM: 1. Toxicology-methods. 2. Poisoning. 3. Poisons. 4. Toxicity Tests. QV 600
P957 2000]
RA1211.P74 2000
615.9–dc21
00–037719
ISBN 0-203-36283-7 Master e-book ISBN
ISBN 0-203-37963-2 (OEB Format)
ISBN 1-56032-814-2 (Print Edition) (case)

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file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_viii.html[2010/10/28 20:30:52]

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Foreword to the Third Edition

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

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

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Preface to the Fourth Edition

The First Edition of *Principles and Methods of Toxicology* was written to deal with evaluation of toxicological data. It described many of the testing procedures available at that time. The Second Edition included a more systematic approach to toxicology without loosing its methodological origin. It described not only current testing protocols but offered useful guidance for data interpretation. The Third Edition was expanded and revised to reflect current needs in toxicology. This Edition continues the tradition of earlier editions by providing detailed testing procedures but with an expanded insight regarding evaluation of data. The Fourth Edition has new chapters on epidemiology for toxicologists, on exposure assessment, and a chapter on repeat dosing that combines chapters that previously subdivided multiple dosing into arbitrary intervals. As before, every effort to reflect the needs and issues in toxicology and to keep the book suitable for use as a textbook in graduate education has been made.

Classically, toxicology is the study of adverse effects of chemicals and physical agents on living systems. Nonetheless, it should be remembered that Paracelsus said, "It is the dose that determines what is not a poison." Based on this important observation, toxicology must look beyond such a simple definition and focus its attention on determining a safe dose from a harmful or detrimental dose. In order to determine the safe use of a chemical or physical agent, it is necessary to have a sound understanding of biologic mechanisms and the methods employed to define these mechanisms. The vastness of the field of toxicology and the rapid accumulation of data preclude the possibility of absorbing and retaining more than a fraction of these techniques and information. However, an understanding of the principles underlying these methods is not only manageable but essential.

This tome was designed for courses in general and advanced toxicology. The framework of the Fourth Edition follows that of earlier editions. The history of toxicology opens the book and is followed by a section which covers basic toxicological principles. Chapters in this section include metabolism, toxicokinetics, physiologically based pharmacokinetic modeling, statistics, exposure assessment, quantitative aspects of interspecies extrapolation, and a chapter on epidemiology for the toxicologist. Also included in this section are chapters on the regulatory process and the toxicologic assessment of pharmaceutical and biotechnology products. The next section covers agent to toxicity including food-borne toxicants, solvents and vapors, pesticides, metals, and radiation. The third section covers basic toxicological testing methods including many of the test protocols now required to meet regulatory standards. Organ systems, tissue culture, and cell systems are dealt with in this section. Each method or procedure is discussed from the standpoint of technique and interpretation of data. A state-of-the-art approach is emphasized as well as discussions involving various problems and pitfalls that may be encountered in performing each procedure. Each chapter contains information that allows one to perform an experiment or test a hypothesis and provides insight into the rationale behind the experiment. Consideration is given in a number of the chapters to the need for validated alternative methods to animal testing. The last section contains a glossary of terms important in toxicology. The organization of the book should facilitate its use both by the student of toxicology and the more advanced researcher. A number of new authors allowed us to provide a broader coverage of the ever-changing field of toxicology.

The Fourth Edition of *Principles and Methods of Toxicology* will be useful as a text for courses in toxicology and as a valuable, timely review for practicing toxicologists. Research scientists who have used earlier editions as a reference source will find updated materials in areas of their special or peripheral interests.

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Foreword to the Fourth Edition

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

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

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

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

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Preface to the Second Edition

The First Edition of this textbook was designed primarily for courses dealing with an evaluation of toxicologic data with a particular emphasis on those methodologies used in toxicology. This Second Edition has been expanded to include a more systematic approach to toxicology without losing its methodological basis. This edition describes current testing procedures, offers useful guidelines on data interpretation, and highlights major areas of controversy. Every effort has been made to keep the book simple and suitable for use as a textbook for graduate teaching.

Since toxicology is the study of the harmful action of chemicals on biologic tissues, it necessitates an understanding of biologic mechanisms as well as the methods employed to examine these mechanisms. However, the vastness of the field of toxicology and the rapid accumulation of data preclude the possibility of any one individual absorbing and retaining more than a fraction of these techniques. There are, however, specific methods that are applicable to a large number of chemicals. An understanding of the principles underlying these methods is not only manageable but essential. Thus, individuals who are not directly involved with the day-to-day activity of toxicology, or who have not yet entered a specialized field in toxicology, will find this book a valuable resource in acquiring a broad understanding of toxicological approaches available.

This volume has been designed to serve as a textbook for, or adjunct to, courses in general as well as advanced toxicology. The overall framework of the Second Edition follows that of the initial volume with the exception that major sections or principles related to toxicology have been added. A number of new authors have been added to this edition to broaden input and provide coverage of the ever-changing field of toxicology. New chapters have been added on metabolism, food-borne toxins, solvents, pesticides, and on the regulatory process as it relates to toxicology.

The only true “facts” in biology are the results of individual experiments carried out under control conditions by carefully defined methodology. Although it is not the purpose of this volume to catalog or to discuss these biologic “facts,” it is the purpose of this book to present those methodologies which can generate these facts. Achievement of this goal requires the more or less arbitrary resolution to select methods and testing protocols from the current literature. The bibliography of each chapter will carry the reader beyond the techniques and methods presented in the book.

This volume has been organized to best facilitate its use. The first section covers basic toxicologic principles including the philosophies underlying testing strategies. The second section covers basic toxicologic testing methods and includes most of the testing procedures now required to meet regulatory standards. The third section deals with specific organ systems and contains chapters on kinetics and effects on cellular organelles and target organs. Each method or procedure is discussed from the standpoint of technique and interpretation of data. A state-of-the-art approach is emphasized as are the various problems and pitfalls encountered. Each chapter contains information that allows a person to perform an experiment or test a protocol, and also provides insight into the rationale behind the experiment.

Principles and Methods of Toxicology, Second Edition, will be useful as both a text for introductory courses in toxicology and as a valuable, timely review for the practicing toxicologist. Research scientists who have used the first edition as a reference source will find updated material in areas of their special or peripheral interest.

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Preface to the Third Edition

The First Edition of *Principles and Methods of Toxicology* was designed for courses dealing with an evaluation of toxicological data with a particular emphasis on methodologies used in toxicology. The Second Edition was expanded to include a more systematic approach to toxicology without loosing its methodological basis. This Third Edition, as did the First and Second Editions, describes current testing procedures, offers useful guidelines on data interpretation, and highlights major areas of controversy. In addition, the Third Edition has been expanded and revised to reflect current needs and issues in toxicology. Every effort has been made to keep the book simple and suitable for use as a textbook for graduate teaching.

Since toxicology is the study of harmful effects of chemicals and physical agents on living systems, it necessitates an understanding of biologic mechanisms, as well as the methods employed to examine these mechanisms. However, the vastness of the field of toxicology and the rapid accumulation of data preclude the possibility of any one individual absorbing and retaining more than a fraction of these techniques. There are, however, specific methods that are applicable to a large number of chemicals. An understanding of the principles underlying these methods is not only manageable but essential. Thus, individuals who are not directly involved with the day-today activity of toxicology, or who have not yet entered a specialized field in toxicology, will find this book a valuable resource in acquiring a broad understanding of toxicological approaches available.

This volume has been designed to serve as a textbook for, or adjunct to, courses in general as well as advanced toxicology. The framework of the Third Edition follows that of earlier editions with the exception that several major sections on principles related to toxicology have been added, including chapters on environmental toxicology, pharmaceuticals and biotechnology products, metals, radiation, and risk assessment. New chapters on methods involving cellular and molecular techniques, instrumentation in toxicology, and physiologically based pharmacokinetics are included in the Third Edition. A number of new authors have been added to this edition to broaden input and provide coverage of the ever-changing field of toxicology. The history of toxicology now opens the book.

The only true "facts" in biology are the results of individual experiments carried out under controlled conditions by carefully defined methodologies. Although it is not the purpose of this volume to catalog those biologic—facts,—it is the purpose of this book to present those methodologies which can generate these facts. Achievement of the broad goal requires the more or less arbitrary resolution to select methods and testing protocols from the current literature. The bibliography of each chapter will carry the reader beyond the techniques and methods presented in this book.

This volume has been organized to best facilitate its use. As Abraham Lincoln so ably stated, "None seemed to think the injury arose from the use of a bad thing but from the abuse of a very good thing." The sixteenth century physician, Paracolsas, further pointed out that all substances can be poisonous with the difference between safe use and toxicity being the dose. Such often is the case in toxicology. The first section covers basic toxicological principles, including the history of toxicology and the philosophies underlying testing strategies. The second section covers agents of toxicity including food-borne toxicants, solvents and vapors, pesticides, metals, and radiation. The third section covers basic toxicological testing methods and includes many of the testing protocols now required to meet regulatory standards. Specific organ systems also are dealt with in this section. Each method or procedure is discussed from the standpoint of technique and interpretation of data. A state-of-the-art approach is emphasized as are various problems and pitfalls encountered with the various methodologies. Each chapter contains information that allows a person to perform an experiment or test a protocol and also provides insight into the rationale behind the experiment. Consideration is given in a number of the chapters to the need for, and yet the lack of, acceptable, validated alternative methods to animal testing.

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The Third Edition of *Principles and Methods of Toxicology will* be useful as both a text for introductory courses in toxicology and as a valuable, timely review for practicing toxicologists. Research scientists who have used earlier editions as reference sources will find updated materials in areas of their special or peripheral interests.

A.Wallace Hayes, Ph.D, D.A.B.T., FATS

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Chapter 1

The Art, the Science, and the Seduction of Toxicology: An Evolutionary Development

Joseph F.Borzelleca

Principles and Methods of Toxicology,
Fourth Edition, edited by A.Wallace Hayes.
Taylor & Francis, Philadelphia © 2001.

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"We can never be fully in possession of a science until we know the history of its development." (Charles Greene Cumston)

"Continuity with the past is a necessity, not a duty." (Oliver Wendell Holmes, Jr.)

"History is bunk." (Henry Ford)

Toxicology! What an exciting word with interesting connotations. It initially evoked thoughts of poisons, poisoners, intrigue, cloak-and-dagger, villains, victims and perpetrators, and plants and chemicals as instruments of ill. What does the word conjure up today? Polluted water, air laden with noxious gases and particulates, foods contaminated with pesticides, soil loaded with heavy metals permanently handicapping children. There are still victims and perpetrators. In earlier times, the act of poisoning was deliberate and usually involved one or several people. Today, exposure to agents/chemicals is often unintentional (e.g., a spill), although it may be deliberate (e.g., suicide attempts) and may involve one or several individuals (e.g., in the workplace) or larger numbers of people (e.g., a community as a result of a spill). It is unfortunate that the same term, ***poisoning,*** is used whether exposure is deliberate or not. Poisonings in earlier times were probably not well reported for several reasons, including the inability to identify the poisoners or even that the cause of death was due to a poison, and to limited means of communication. Today, due to the significant advances in analytical chemistry and to better and faster means of communication, there is a faster and greater dissemination of information. Unfortunately, there is also a profusion of misinformation, and this is unnecessarily creating problems for the public. Industry is usually portrayed as the villain (by the media), and the unsuspecting public as the victim. Industry is also portrayed as profiting at the expense of the public health. If only the truth could be presented to the public in an unbiased manner! This is a challenge for modern toxicologists. Let those of us capable (by training and/or experience) of interpreting relevant data do so objectively and fairly. We must accept this challenge lest the public perish in a cesspool of media-fabricated hype. Will toxicologists rise to the occasion? The dedicated and concerned ones will.

How did we arrive at the present situation? What has happened as humans evolved from nomadic huntergatherers and cave dwellers to a relatively stable society of workers involved in many and varied activities? How can we explain the change from the direct use of poisons by individuals to kill one or several to the insidi-

ous exposure to presumed poisons to large groups by industry or other groups? An appreciation of the evolution of toxicology may be helpful.

Without definitions, meaningful discourse and science are impossible. "In the right definition of names lies the first use of speech, which is the acquisition of science; and in the wrong, or no definitions, lies the first abuse, from which proceed all false or senseless tenets" (Thomas Hobbes).

What is toxicology and how has it evolved to the eminent position it now enjoys? ***Toxicology*** is the study of the adverse effects of chemical or physical agents on biological systems; it is the science of poisons. A poison is any substance (chemical, physical, or biological) that is harmful or destructive to a biological (living) system. A poison derived from a biological source is a ***toxin,*** and the study of toxins is ***toxinology***.

"Toxin" was originally ***tekw,*** a word meaning to run or flee, later becoming ***toxsa*** in Persian and ***toxon*** in Greek, meaning bow and arrow; the toxin meaning may have come from the poison used to tip the arrows, or, as Robert Graves suggested, from the yew tree ***taxus,*** from which arrows were best made and whose berries were long known to be poisonous. The word for poison came by a devious route, like a long-delayed afterthought. It derives from ***poi,*** to drink, becoming ***potare*** in Latin, whence "potion" (and also "symposium" from ***sym,*** together, plus ***posis,*** to drink). The venomous meaning did not come until the notion of love potions evolved, and the idea of poison came to consciousness. There is the same strange history behind the word "venom." This began as the simple word ***wen*** meaning to wish or will, leading more or less directly to "win." Along the way, a fork led to "venus," "venery" and "venerate," all indicating varieties of love. The love potion was called venin, and somehow this gradually acquired today's sense of venom. Nobody can explain why "poison" and "venom" come from love potions. Perhaps it was because the pharmacology of the day was primitive and chancy, a very fine line from toxicology. Or maybe there was a commonsense consensus that any sort of chemical additive intended to induce false love, is by nature, a fundamental poison. It tells something important about the good taste of earlier human beings that venom and poison were taken resentfully out of the hands of artificial lovers and transferred to the stings of insects and the fangs of serpents.

"Noxious," incidentally, came from ***nek*** meaning death, by way of ***necare*** and ***nocere*** in Latin, providing "necropsy" and cognate words for us; nectar was the drink of the gods because it prevented death (***tar,*** meaning to overcome). Chance (origin is cadence). "Cadence" comes from ***kad,*** meaning to fall. Kad led to ***cadere*** in Latin and ***cad*** in Sanskrit, also meaning to fall, sometimes to die. Incidentally, "hazard" also came from ***dice,*** by way of Old French ***hasard*** and Spanish ***azar,*** from the Arabic ***yasara,*** to play at dice (55).

The development of toxicology reflects the history of the development of society; that is, a progression from simplicity to sophistication, from crude to cultured, from elemental to elegant. Consider killing. Killing animals for sustenance and survival predates recorded history. The biblical directive to Adam is clear. "Then God said: 'Let us make man in our image, after our likeness. Let them have dominion over the fish of the sea, the birds of the air, and the cattle, and over all the wild animals and all the creatures that crawl on the ground'" (Genesis 1:26). "God blessed them saying: 'Have dominion over the fish of the sea, the birds of the air, and all living things that move on the earth.'" God also said: "See, I give you every seed-bearing plant all over the earth and every tree that has seed-bearing fruit on it to be your food; and to all the animals of the land, all the birds of the air, and all the living creatures that crawl on the ground, I give all the green plants for food. And so it happened" (Genesis 1:28–30). Animals, including humans, were, and still are, born with a strong basic instinct for survival (and control) that involves eating and drinking. It became (and still is) necessary to kill plants and animals in order to survive. Humans also have an instinct to control their own destinies. Occasions arose when it became necessary to remove (i.e., to kill) other humans (for control, not nutritional survival). The methods used to kill humans were the same as those used to kill animals. The usual instruments of kill were clumsy physical weapons that required strength for effectiveness. Small and less powerful humans were at a great disadvantage. Even though later developments, such as the bow and arrow, required more skill and less physical strength, something more (an equalizer) was needed for the less physically endowed. Early humans probably learned through experience about the harmful properties of insects and animals (including venoms). The women studied plants and determined which were beneficial and which were poisonous. The poisonous ones were then used by the men as aids in hunting (e.g., arrow poisons). Poisons proved to be very useful in killing animals. Could they be the equalizer needed by the less powerful to solve other problems? Could humans be dispatched as readily as animals with the use of poisons? It was worth testing. Killings were sometimes performed for reasons other than for survival, such as control, frustration, anger, or convenience. For example, to satisfy their lust for power, wealth,

pleasure, and new excitements, the "power class" (wealthy landowners, rulers) found it necessary to kill other humans, but killing other humans was not limited to the "power class." In general, genteel, subtle, and undetectable means of killing were desirable (such as dispensing with a spouse to be able to enjoy a new lover), but these were not available until professional poisoners appeared on the scene. When used properly, poisons could be (and often were) the perfect solution to difficult problems and detection was impossible.

Once the value of poisons was recognized, they became very attractive as "fit instruments of ill." Humans then and now are seduced by things attractive (and useful). Poisoning as a solution to difficult problems was very appealing and had a great deal to recommend it; it could

be fast or slow, painful or painless, poisons were inexpensive and readily available; and, as noted earlier, defied detection. The seduction of toxicology had begun! Once the need had been established, suppliers and practitioners were needed for proper implementation. The amateur or do-it-yourself poisoners were soon replaced by professional poisoners (early applied toxicologists), who offered advice and/or performed the required services. The agents used were initially naturally occurring; these were supplemented by new agents developed by alchemists/chemists. Thus, toxicology evolved into an art and then into a science.

The popularity of poisoning grew until it reached epidemic proportions in some countries. This popularity was enhanced by the inability to detect poisons and to prove that poisoning had occurred. However, once the chemists turned their skills from developing new poisons to detecting poisons, the popularity of poisons as killer agents began to wane. Poisoners could now be identified. Although poisons are still used today, more subtle and ingenious means of killing are available. Today, poisoning conjures up images of large groups of individuals unintentionally exposed to chemicals; however, detection and source/cause identification of the exposure are possible and responsible party (parties) identified. In earlier times, the poisoner was usually an individual; today, poisoning/pollution may involve a corporation.

A man without a sense of history, without memory of the past, who is forced to reconsider his place in the world, a man deprived of the historic experience of his own and other peoples lacks any perspective and can only live in the present. (Chingiz Aitmatou, 1983)

POISONS ARE IN!

With respect to the art of poisoning, de Quincey wrote:

The bowl needs for its effective management a scientific precision of plan and a subtlety of execution that place it in a different category from the vulgar methods of the knife, the bullet, or the bludgeon. The process can be almost indefinitely prolonged, giving large opportunities for the exercise of skill, resourcefulness, craft, and daring; and with allowing time for the enjoyment of the full passion of deliberate crime, which finds imperfect gratification in the hasty thrust of the knife. There have been persons who poisoned, not out of covetousness or hatred or wanton cruelty, but for the pleasure of the thing. They were true artists, practicing their art for its own sake, with a proper scorn for the limitations which an irrelevant morality would impose in its exercise.

Do poisons have any redeeming value; in other words, are there uses for poisons beyond killing? Very definitely! Poisons have contributed to the health and safety of humankind and to the advancement of biological sciences (including medicine) in a number of ways. In 1878, Claude Bernard, an outstanding early physiologist and probably the first and foremost mechanistic toxicologist wrote:

Poisons can be used as agents for the destruction of life or as means to cure disease; but in addition to these uses—there is a third which particularly interests the physiologist. For him the poison becomes an instrument which dissociates and analyses the most delicate phenomena of the living machine and by careful study of the mechanism of death in different poisonings, he can gain knowledge, indirectly, of the physiological mechanism of life. (i.e. poisons can be used to explain physiological events).
(translation of P.N.Mage, 1865)

For interesting discussions of these artist-poisoners, including Locusta, Toffana, the Marquise de Brinvilliers, Ezra Wharton, Florence Maybrick, the Borgias, Catherine de Medici, Sainte Croix, and Catherine Deshayes (La Voisine), the reader is referred to articles by Gallo and Doull, Decker, Thompson, and Osius.

Claude Bernard used curare to study the physiology of the neuromuscular junction. Radiation, a "physical poison," has been an invaluable tool in elaborating some of the basic events in mutagenesis and carcinogenesis. Identifying the role of mixed-function oxidases (MFOs) and cytochrome P450 (P450) and exploring hepatic mechanisms at the molecular level would have been delayed had it not been for hepatotoxins. Low doses of certain toxins may be useful in therapeutics: for example, botulinum toxin in the management of strabismus, blepharospasm, and spasmodic torticollis. Poisons are not all bad!

Farming (food production) has evolved from a very primitive but necessary human activity to feed a few people to a very sophisticated process that feeds multitudes. In earliest times, it involved the use of simple tools, few crops, and primitive methods. With the introduction of power (animal, then mechanical), larger areas could be cultivated. More crops were developed. Efficiency improved but there was considerable room for improvement. Enriching the soil resulted in increased yields, but pests competed with humans for crops thereby reducing effective availability. Pesticides are chemicals (usually) that are used to kill unwanted subhuman organisms. The judicious use of these materials has resulted in increased food production and storage and subsequently in better health and prolonged life

expectancy. The introduction of genetically modified plants that are diseaseand pest-resistant and have a better nutrient profile and improved organoleptic properties has resulted in diminished exposure to chemical agents. There is every reason to believe that this trend will continue.

With the introduction of chemical power to enrich the soil, treat the seeds, and eliminate pests, unprecedented yields were realized. Humans began to manipulate, to control, part of their environment; they worked with Nature to their benefit. But there were occasions when the interaction with Nature was not symbiotic—when air,

water and/or soil were abused, often due to ignorance but sometimes to greed.
Food preparation and storage evolved in a similar manner. Prior to the introduction of heat (fire power), food was eaten raw. Cooking provided an opportunity to broaden one's choices and to improve the bioavailability of certain nutrients. Heat (sun, fire) was also used to preserve food by drying. Chemical power (e.g., salt) was also used to preserve food. Add to drying and chemical preservation the power of radiation and food could be maintained safe for human consumption for unbelievably long periods of time. In addition, chemicals (e.g., flavors and colors) could be used to enhance the organoleptic properties of foods. Again, humans were controlling the immediate environment to their benefit. The preparation of food (a renewable resource) for consumption was/is usually less destructive of nature.
And the need for safe drinking water! It took a long time to associate contaminated water with disease. Or did it? The Egyptians used a physical method (sand bag) to purify water, a technique used today (sand banks). But it was not until chemical power was introduced in the form of chlorine gas that waterborne diseases were finally eliminated.
Other natural products have played a role in the cultural/sociological/hedonistic aspects of human activities. These include sugar, salt, coffee, tea, rosemary, garlic, pepper, opium, digitalis, alcohol, and tobacco.
Toxicology is an evolving discipline: from art to science, from using chemicals that hurt to chemicals that help, or at least prevent hurting, and from taking lives to saving lives. It is not precisely known when the first human used a plant toxin, a phytotoxin (plant poison), to kill another human, but it probably did not require too great a leap of faith to extrapolate from effects in animals to humans. The age of poisoning, of practical toxicology, had begun. As poisoning developed into an art, its practitioners became famous/infamous. Identification of the culprit was extremely difficult since determination of the cause of death (proof of poisoning) required yet-to-be-developed analytical techniques.
Natural products were used (and are still used) to prevent and/or to treat disease. Women were the original naturopaths; they learned the biological properties (effects) of plants and used plants and/or their constituents with appropriate activity in the management of disease (to heal), while those with poisonous properties were used by the men in hunting (to kill). (The adverse effects of venomous insect stings and animal bites were probably also noted, but the practical utility of these venoms was limited.)
Interest in plant poisons (phytotoxins) and animal poisons (venoms), as nuisances to health and as tools for vindication, continued to grow. Lists (catalogs) of poisons and their effects in humans began to appear. Each culture/civilization appears to have had a list or lists of these. The use of plants, including herbs, for medicinal purposes also continued to evolve. The cures (healing or killing) for the problems of humankind were to be found in nature (a concept that has again found favor with many)! With time, the lists began to include detailed descriptions of preparation, use, and effects of biologically active plant materials. Understandably, concerns about prevention and treatment of poisoning began to emerge.
Prevention of poisoning was accomplished by the use of appropriate bioassays (e.g., official tasters of prepared food and drink) and by the development of tolerance/adaptation through the repeated ingestion of small doses of toxins. Initially, treatment (antidotes) was shrouded in folklore and mysticism. Only when mechanisms of toxicity were understood and toxicology became a recognized science did treatment have a rational, scientific basis.
Advances in chemistry led to the development of analytical chemistry. The application of analytical techniques to the detection of poisons was the beginning of the science of forensic toxicology. This had a chilling effect on the use of poisons. Now poisoning could be proved. Practitioners became more sophisticated in attempts to avoid detection, but they were no match for the dedicated chemists who continued to develop exquisitely sensitive and specific analytical methods. Perpetrators/villains could now be identified and appropriate action taken. The development of ultrasensitive analytical methods has continued into the present. Contaminants in soil, air, and water are now easily identified and quantified. The origins and the originators can be identified and appropriate action taken. Ultrasensitive analyses continue to challenge toxicologists to assess the biological/health significance of the presence of chemicals at parts per billion or parts per trillion in body fluids or tissues. Dose-response relationships were established as correlations between the level of the chemical in blood and/or in tissues and biological activity were made. Concomitant with advances in analytical/forensic toxicology were efforts to elucidate the mechanisms of action of chemical agents. This was followed by the development of rational therapy for poisonings, including the development of specific antidotes.

POISONS ARE OUT!

In light of this scientific onslaught, the deliberate use of poisons to kill declined rapidly. The practitioners of the art of poisoning disappeared to be replaced by a new breed of toxicologist, the scientist who

understood the basis of toxicity from the whole animal to the molecular level, could appreciate all of its ramifications, and could

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extrapolate to the human situation. Quantification of the responses to toxic agents and the relationship of structure to biological activity became, and still is, the basis for a great deal of scientific activity.
When it became known that nondeliberate exposure to chemicals could produce adverse health effects (e.g., in the workplace), efforts were directed to the prevention of the adverse effects of chemicals by defining safe conditions of exposure to protect humans and other life forms from chemical and physical injury. This was followed by the identification and quantification of the risk of adversity following exposure (risk identification, assessment, and management). Quantifying a risk, assigning a number to it, tends to decrease the uncertainty of extrapolation and to provide a comfort factor.
In maturing to a scientific discipline, toxicology passed through a number of phases, including observation/ phenomenology (lists of poisons and antidotes), experimentation/deduction/mechanisms/analytical (including dose-response), and application (TLV, ADI, safety/ uncertainty factor) and quantification (quantitative risk assessment)/prognostication). A transition from using chemicals to kill to finding uses for chemicals/poisons that would benefit humankind (e.g., pesticides and therapeutic agents) to identifying, quantifying, and preventing adverse effects of chemicals (e.g., establishing safe exposure conditions in the workplace and safe levels of chemicals in foods and water) occurred. Toxicology had evolved to another stage wherein it became a respected member of the scientific biomedical community.
The toxicologist was needed when poisons were in and continues to be needed now that poisoning is out. From supplying poisons to studying their mechanisms of action to developing analytical methods to identifying and quantifying poisons in body fluids and tissues to developing rational antidotes to establishing safe limits of exposure from carefully designed and executed studies to quantifying and predicting adverse effects—the toxicologist continues to play a critical role in the advancement of humankind.

AGE OF OBSERVATION/RECORDING OF PHENOMENA

Biblical

Although most of the references to poisons in the Bible appear to be limited to venoms, chemicals and food regulations are also mentioned. In the Book of Genesis, God is portrayed as the Supreme Regulator and protector; God proscribed certain foods. The regulated were Adam and Eve. Had they blindly accepted the regulations imposed upon them (had they had more faith in their Regulator), there would have been no dire consequences. Is there a lesson here for us today? The bitter water to test the fidelity of a wife suspected of unfaithfulness is described in Numbers 5:11. Venoms and plant poisons appear in Deuteronomy 32:24, "with the venom of reptiles gliding in the dust," and 32:31, "poisonous are their grapes and bitter their clusters." Arrow poisons are mentioned in Job 6:4, "for the arrows of the Almighty pierce me, and my spirit drinks in their poison," and in 20:16, "The poison of asps he shall drink in; the viper's fangs shall slay him." "The venom of asps lies behind their lips" appears in Romans 3:13. In Psalms 58:5, "theirs is poison like a serpent's, like that of a stubborn snake that stops its ears," and Psalms 140:4 "they make their tongues sharp as those of serpents; the venom of asps is under their lips." Jeremiah mentions chemical and biological poisons, "he has given us poison to drink" (8:14) and "I will send against you poisonous snakes against which no charm will work when they bite you" (8:17). In James 3:8, we find "the tongue no man can tame. It is restless evil, full of deadly poison." There appears to be an awareness of the natural occurrence of poisons and the effects produced, but no mention of the deliberate use of them. Antidotes are not mentioned, only charms. There is no list of poisons in the Bible, although there are proscriptions about foods and food practices (e.g., Deuteronomy 14, Leviticus 11, 17, 19), apparently based on potential adverse health effects.

Egyptian

The first list of poisons and antidotes appears in Egyptian writings. This is not unusual since the Egyptians were the intellectual leaders of the world and Egyptian medicine was reputed to be the most advanced. Menes (Mena, Meni, Min), the first king of unified Egypt and the founder of Memphis, the capital, was reported in the Egyptian papyri to have had an interest in poisons. He cultivated and studied the effects of poisonous and medicinal plants somewhere between 3500 and 3000 BC. Unfortunately, there is no detailed written history of these activities. His son Athothis, a physician, wrote a textbook on medicine in which sanitation was stressed.
A papyrus discovered in Thebes (Luxor) in 1872 by the German egyptologist Georg Moritz Ebers (1837–1898), named appropriately in his honor the Ebers Papyrus, was written between 1553 and 1500 BC. It is more than 20 m long and contains 110 columns of hieratic (priestly) script (about 110 pages). More than 700 drugs (medicinal substances) are identified in about 875 to 900 formulas (quantitative recipes). The formulas also contain specific indications and dosages, together with appropriate spells

and/or incantations. Forty-seven case histories are pre-

sented. Modes of administration include snuffs, inhalations, gargles, pills, troches, suppositories, enemas, fumigations, lotions, ointments, and plasters. Drugs were identified on the basis of origin as plant (e.g., acacia, castor bean, wormwood, fennel, garlic), animal (e.g., milk, excrement) or mineral (e.g., alum, iron oxide, limestone, sodium bicarbonate, salt, sulfur). Vehicles used included beer, wine, milk, and honey. There is also a great deal of information on the toxicity of opium, hellebore, aconite, hyoscyamus, hemlock, lead, antimony, and copper. Insect and animal venoms were described. Antidotes, including incantations, were also mentioned. To assure recovery, Egyptian physicians used combination therapy: chemical and or biological materials (rational therapy) plus mysticism (requests for assistance from the gods; irrational therapy?). Today, some pray for assistance only after conventional therapy has been unsuccessful. Perhaps simultaneous is more effective than sequential!

There are other papyri detailing medical practices in early Egypt. The Edwin Smith Papyrus was discovered at the same time and place as the Ebers Papyrus, but it was probably written earlier. The Hearst Papyrus was written about 1400 BC and was discovered in upper Egypt in 1899. The Kahun Papyrus was written between 2000 and 1800 BC and was discovered by Sir Flinders Petrie in the Faiyum. It deals primarily with gynecology. The Berlin Papyri (two, the same period as Ebers Papyrus) and the Brugsch Papyrus were the latest to be discovered.

For example, the Egyptians used chemicals in the administration of justice. The Penalty of the Peach involved having the accused ingest the distillate from crushed pits of peaches (high in hydrocyanic acid); if the accused died, it was a presumption of guilt; if the accused lived, it was a presumption of innocence. This practice of using chemicals in the administration of justice was used by other cultures (e.g., Greek) and persists to the present; lethal injections of chemicals are used for executions in the United States.

Egyptian practitioners of medicine believed that respiration was the most important function of the body followed by circulation (blood and heart); that most diseases were caused by parasites (which is not surprising considering conditions in Egypt); that personal and social hygiene were very important in maintaining good health; and that therapeutics should be both rational and mystical. They covered all bases! The Egyptian contribution to toxicology includes lists of poisons and antidotes.

Chinese

On the other side of the world, the Chinese were also developing a culture that was advanced for the period. The second of China's mythical emperors, Shen Nung, is considered the father (founder) of Chinese medicine, materia medica, and agriculture. He is credited with inventing the cart, the plow, and the yoking of horses. He taught the Chinese how to clear land with fire to increase farmland. Shen Nung wrote a 40-volume herbal entitled ***Pen Ts'ao or Pun Tsao*** (the Great Herbal or Chinese Materia Medica) around 2735 BC. It contained lists of poisonous plants, plants with medicinal value (365), and drugs (265, 240 of which are vegetable in origin). The effects of plants and drugs and appropriate antidotes were described. Included among the drugs were iodine, aconite (also used as an arrow poison), opium, cannabis, rhubarb, alum, camphor, iron, sulfur, and mercury. He was also reputed to have discovered a number of drugs and experimented upon himself. Phytotoxicology appeared to be well developed. Here is the second list of poisons and antidotes. Like the Egyptians, drugs and poisons were presented together. (Pharmacology and toxicology joined together at this early age! Did this presage the development of toxicology?) Another emperor, Hwang Ti (2650 BC), wrote ***Nei Ching,*** the Book of Medicine, the basis for most Chinese medical writings.

In addition to the lists of drugs and poisons and their effects (the early Chinese contribution to toxicology and pharmacology), the Chinese made other significant contributions to medicine. These include discovering the circulation of blood; the yang and yin principles (two opposing forces that control everything including ebb and flow, male and female, life and death, moon and sun, heat and cold, strength and weakness (parasympathetic and sympathetic, cholinergic and adrenergic nervous systems?); the five elements of the human body (earth, fire, water, wood, metal); the five organs of the body (heart, liver, spleen, lung, kidney); five colors, and five heavenly bodies—five appeared to be a magic number in this culture. Other cultures had magical numbers; for the Greeks, it was four or seven. Health was the result of balance between the forces and elements, a concept that still has its adherents.

Hindu

The most significant contribution of the Hindus to medicine was in the field of surgery. Like the Chinese and the Egyptians, they also had their list of poisons and antidotes. The Rig-Veda, a Sanskrit document written between 1500 and 1200 BC, is the earliest written account of Hindu medicine. It contains many references to alchemy and science and magic in the treatment of disease. Included are discussions of many diseases including cough, fever, diarrhea, seizures, tumors, and skin diseases. There are

treatments for specific diseases. These

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treatments, like the Egyptian, include spells and incantations, again the combination of rational and mystical therapies! Medicinal and poisonous plants and antidotes (e.g. for snake bites) are listed. The influence of gold as a therapeutic agent and on longevity is discussed. A later work, the Ayur-Veda, the Veda of long life, a Sanskrit document written about 700 BC, discussed medicine and all its branches in eight parts; drugs and poisons were also mentioned.

"He who knows only one branch of his art is like a bird with one wing". (Susruta)

Susruta, a Hindu surgeon, authored a medical/surgical text called Susruta-Samhita, in the last centuries of the pre-Christian period. The text was divided into six sections. He identified 1120 diseases and gave fever great importance. He stressed the importance of hygiene and presented many surgical procedures in great detail. The section on drugs listed 760 indigenous medicinal plants, of which many were used externally as ointments, baths, sneezing powders, inhalations; it also listed animal and mineral remedies. The fifth section, the Kalpa Sthana, was the section on toxicology and contained mostly antidotes. The Hindus were keenly interested in poisons and antidotes, especially for bites and stings and aphrodisiacs (interesting; any message here?). Other topics discussed included malaria and the role of the mosquito and plague and the influence of rats.

Greek

There are many Greek legends and myths involving gods and goddesses concerned with poisons and poisoning. For example, Hecate used aconite; Medes, colchicine; and Hercules was poisoned with cantharidin applied to his shirt. Like other cultures, the Greeks also had their lists of poisons, lists that were consulted by citizens and by the government. Other and more significant contributions to the advancement of toxicology were made, including detailed descriptions of the effects of various agents in humans, antidotes, and principles for the management of poisonings.

The Greeks made many significant contributions to the advancement of humankind. For example, they developed a system of philosophy (***philos,*** friend; ***sophia,*** wisdom) that defined the place of humans in nature; they attempted a rational explanation for nature and natural phenomena including medicine and the healing arts; and they believed that there exists a single fundamental principle, a prime force, from which everything in nature developed. The Greeks perceived medicine as both an art, the careful examination and accurate observation of the patient, and a science, part of the science of humans and their place/role in nature. The Greeks had a great deal of knowledge about poisons (especially plant poisons), metals (especially arsenic, antimony, mercury, gold, copper, and lead), and antidotes (e.g., hot Oil and vomiting). The Greeks also executed criminals with poisons (e.g., hemlock, which was the state poison), which presaged the use of lethal injections in the United States today. Suicide by poisoning was not uncommon since poisons were readily available.

The father of the Pythagorean theorem (proposition), Pythagoras (580–489 BC), was born in Samos, and lived in southern Italy (Crotona) for many years. He was a mathematician who developed the theory of numbers and became known as the father (founder) of arithmetic, a physician and scientist who was especially interested in procreation and animal physiology, an astronomer, a philosophical-religious leader who tried to reform the political, social, and moral ills of the time, and a numerologist who considered the number seven highly significant. He believed the earth was a sphere, that animals possessed souls, and that both animal and human souls were immortal and transmigrated. His doctrine of numbers was probably the basis for the four elements and four humors and the four critical days in illness described by Hippocrates (this is surprising since he valued the number seven). His theory of harmony may be the basis for the theory that health is the result of a balance among the various elements and humors. He believed that health is perfect harmony and disease is a disruption of this harmony. His most significant contribution to medicine and to toxicology was the importance of causality and the need for critical thinking. His other contributions to toxicology include his studies of the effects of metals (e.g., tin, iron, mercury, silver, lead, gold, copper) in the body. Since he left few, if any, writings, all of his teachings have come through his disciples.

Empedocles of Agrigentum (490–430 BC) was the son of Meto, an activist politician; he was born in Sicily and died in Peloponnese, Greece. He was of the Pythagorean school, a physiologist, physician, philosopher, religious teacher, poet, and politician who believed in the unity of things. He also believed that all matter consisted of four elements (the Chinese believed in five), earth, fire, air, water; that nothing is destroyed (accord/ love holds things together, discord/strife [stress?] tends to dissociate things). These two forces, one internal (accord) and one external (discord), are antagonistic (yang and yin?). His teachings became the basis for the four body humors—blood, phlegm, yellow bile (choler), black bile (melancholy)—and for the theory that health was the result of harmony among these humors and among the four elements (cf. Pythagoras). He was a strong advocate of hygiene, personal and

social, and of public health measures to prevent epidemics (e.g., draining of swamps). Although Aristotle is often credited with identifying the four elements, he was merely a strong proponent of the four elements and not the first to identify them.

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Empedocles believed the heart was the most important organ since it distributed the "pneuma" throughout the body. (Egyptians considered respiration most important.) He established the basis for sense perception. He was considered a wonder worker due to his great healing skills and to his abilities to prophesy. According to tradition, he cast himself into the crater of Mt. Etna to prove something or other.

"There is only one good, knowledge; there is only one evil, ignorance." (Socrates)

Socrates (470–399 BC), son of Sophroniscus, a sculptor who was dispatched with hemlock, the state poison, is probably the most famous victim of poisoning in history. He developed the Socratic method of inquiry, a series of questions designed to elicit clear expressions or answers. He had a strong contempt for conventional ideas and life-styles that ultimately led to his demise. His idealistic philosophy was passed on through the writings of Plato, his most famous student; Socrates left no writings of his own.

"Life is short, and the Art long; opportunity fleeting; experiment dangerous, and judgment difficult." (Hippocrates; was he thinking of toxicologists when he wrote this?)

Hippocrates (460–355 BC), the father of medicine, was born on the island of Cos, the son of Heraclides, a physician, and Phenarete. It is said that he was a member of the family of Asclepiadae. He was well educated and traveled extensively. He was a contemporary of Sophocles, Euripedes, Aristophanes, Pindar, Socrates, Plato, Herodotus, Thucydides, Phidias, and Polygnotus. He had two sons, Thessalus and Dracon, and many pupils. His contributions to the advancement of medicine are legendary, due in great measure to his belief that the causes of diseases were natural and not supernatural. In addition, he stressed the importance of nutrition/diet and believed that too little or too much food was equally harmful. What he lacked in instrumentation was more than compensated for by his use of the scientific method, sound observation, and logical reasoning. He is the presumed author of a number of significant texts and treatises characterized by advanced scientific and practical thinking and skillful clinical observation. Like other Greek physicians, he believed that health was the result of an equilibrium or balance in the body among the humors (blood, black bile, yellow bile, and mucus) and that disequilibrium resulted in ill health.

He apparently was the first physician of record (a clinical ecologist?) who believed that environmental factors should be considered as probable causes of disease. For example, in his book ***Airs, Waters and Places,*** he argued that environmental factors (overall weather, local weather conditions, and drinking water) can influence health. "Every disease has its own nature and arises from external causes, from cold, from the sun, or from changing winds." According to Hippocrates, the first step in treating disease, including poisonings, should be to purify the body of disease-producing humors by purgation (catharsis, purification) by diet or by drugs. This cleansing is the first step in restoring equilibrium (including the management of poisoning). Hippocrates probably foreshadowed the current practice of the clinical ecologists, who espouse thorough cleansing of xenobiotics from the body as the first step in restoring health.

Hippocrates taught that the body is maintained by air and nutriments, that it is nature that heals, and that the role of the physician is to assist nature in the healing process by increasing nature's healing forces ("help nature to help herself'; was he portending the current "back-to-nature" movement?). This can best be done by diet and modifying one's life-style, usually to get more rest and appropriate exercise. The key to good health is proper diet, sufficient exercise, and adequate rest (makes a great deal of sense even today!). Drugs may be used to assist the dietetic cure.

Hippocrates identified about 400 drugs, mostly of plant origin, that included narcotics (e.g., poppy, henbane, mandragora), purgatives, and sudorifics. He also advocated the use of emetics and enemas (as part of the cleansing process). The patient is a unit and must be treated as such (that is, as an individual), there must be very careful observation (good case history and physical examination?), and the approach to treatment should be appropriate, simple, and rational. Mysticism was not a part of his therapeutic regimen. His advice to physicians is summarized in his ***Aphorisms,*** which include being prepared "to do the right thing at the right time." He also established a code of medical ethics, the Hippocratic oath, which has survived to the present. His contributions to toxicology include the use of the scientific method, sound observation, logical reasoning ("the scientific method"), and basic approaches to the management of intoxication (decrease absorption; if ingested, induce vomiting) and the use of proper antidotes. He died in Thessaly in 355 BC.

"For where there is the love of man [humans], there is also love of the art." (Hippocrates)

The second most famous Greek physician, Diocles of Carystus (375–300 BC), was loved for his kindness toward his fellow humans and greatly admired for his oratorical skills. He wrote a number of famous texts including ***Anatomy*** (the first systematic textbook on animal anatomy), ***Dietetics, Physiology,***

Embryology, and ***Rhizotomikon*** (considered to be the first work on botany that included the names of the plants, their habitat, means of collection, and medical uses). His second book on plants described those used for food and his third book

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dealt with poisonous plants. His works indicate that serious studies on the pharmacology and toxicology of plants had begun. Another famous book by Diocles was one that dealt with personal and social hygiene.

Diocles' recommendations for good health included cleaning teeth, massaging gums, walking and exercising before breakfast (which was a light meal), drinking water before meals, napping in the afternoon, exercising before dinner (just before sunset and followed by white wine diluted with water and honey), walking, and early retiring. Good advice!

Hippocrates and Diocles extended toxicology beyond merely listing poisons and antidotes. Rational methods for the study of the effects of poisons and the treatment of poisoning were proposed. Experimental studies to assess the biological effects of plants had begun.

"Nature does nothing without a purpose." (Aristotle)

Aristotle (384–322 BC) was the son of the court physician to Amyntas II, a student of Plato, a teacher of Alexander the Great, and a philosopher and scientist (though not a physician) who stressed the importance of biology as a science (pre-med curriculum?) and thereby influenced medicine. A scientific genius, he established the foundations for comparative anatomy and embryology. His "Ladder of Nature" was the beginning of the concept of evolution (presaging Darwin). Like other Greek scientists and physicians of his day, he believed that the human body possessed four qualities—hot, cold, dry, and moist; that it was composed of four humors—blood, phlegm, yellow bile, and black bile; and that disease resulted from an imbalance of these. His contributions to toxicology are related to his contributions to science, especially biology.

Theophrastus (372–287 BC), one of Aristotle's most famous pupils, was a philosopher and scientist (probably the most famous Greek botanist/herbalist) who wrote ***Theoretical Botany*** and ***De Historia Plantarum*** in 300 BC (two volumes, inquiry into plants and growth of plants). They included the natural history and descriptions of medicinal and poisonous plants and are considered the beginning of modern botany and an excellent medical botany text. Indications for the use of medicinal plants were presented. He was also the first to record adulteration of food. He was interested in food preservation and found that certain soils preserved wheat but in so doing it became adulterated. Others adulterated wheat with soil to increase its weight. His contributions to toxicology include a list of poisonous plants and the recognition of adulteration of food (might he be considered the founder of food toxicology?).

Cato (234–149 BC) was interested in food preservation (salting) and in detecting adulterations, expecially of wine.

Nicander of Colophon (185–135 BC) was a Greek physician, poet, and grammarian who wrote, among other things, two hexameter poems, ***Alexipharmaca*** (properties of poisonous substances and antidotes) and ***Theriaca*** (bites and stings of venomous animals and antidotes). Although there were some fanciful parts, much was accurate and reflected upon his powers of observation and his experiences. Theriac has come to mean antidote against poisons. It survived into the 19th century and was considered by some to be a tonic and a means of maintaining good health. His contribution to toxicology is his list of poisons and antidotes.

King Mithridates VI Eupator the Great, king of Pontus, polyglot king (120–63 BC), was considered a military genius and a dabbler in poisons. He evaluated poisons and potential antidotes in slaves and in prisoners (without implied consent or institutional review boards and to eliminate the need for extrapolation!). He was a student of toxicology but was obsessively possessed with a fear of poisons. To provide protection, he took daily doses of poisons (beginning with very small doses and increasing the amounts ingested) to develop polyvalent tolerance (the first recorded successful case); he drank the blood of ducks fed toxic chemicals and took mixtures of antidotes. Mithridatium, his universal antidote, which was presumably invented by one of his physicians, Zopyrus, consisted of 20 leaves of rue, one walnut, a grain of salt, and two dried figs and was to be taken each morning before breakfast to effectively prevent poisoning. The Mithridaticum of Celsus consisted of 36 ingredients, Pliny's 54, and Galen's 73; each pill was the size of a grape and 10 had to be taken before and after food. Mithridates was captured and tried to commit suicide by selfpoisoning but proved immune to the actions of the various poisons. He was eventually killed with a sword (a throwback). The term ***mithridatics,*** antidotes or preventives for poisoning containing many ingredients, immortalizes his contribution to toxicology. He was probably the first to systematically study poisons in humans and thus became the first clinical toxicologist (could be considered the founder of clinical toxicology except for the nature of his clinical studies).

Heraclides Pontius of Tarentum (240 BC), a philosopher and student of Plato, was reported to have spent a great deal of time studying poisons and antidotes, but little remains of his contributions. He

used opium to induce sleep and was concerned with cosmetology. His greatest contribution to science was probably his contention that the earth rotated on its own axis.

Andromachus the Elder (ca. 60 AD) was an archiater, the public physician who treated the poor, and the royal physician to Nero. He added squills, viper's flesh, and opium to the mithridatium and administered it, in honey, to Nero. This became known as the Theriaca of Andromachus, Theriaca Andromachi, or Venice Treacle; it contained 70 substances and was used until the 18th

century. Interestingly, he wrote 175 Greek iambic verses describing it.

Dioscorides (Pedanius Dioscorides) (40–90 AD), was born in Anazarbia in Cilicia. He was a Greek physician and pharmacologist, physician and surgeon to Nero's army, and the originator of materia medica. He is the author of many texts, including one of the greatest works (if not the greatest) on materia medica of ancient times (77–78 AD). He took advantage of his military travels to study medicinal properties of plants and minerals, which he described in a five-volume series, *De Materia Medica*, *De Universa Medica*, the leading text in pharmacology for 16 centuries. Included are descriptions of about 600 plants and 1000 simple drugs. His approach differed from that in Eber's Papyrus, in which each disease had listed all the remedies for that disease. Dioscorides described a plant and then listed all the diseases it might cure. Also discussed are the dietetic and therapeutic value of animal products (e.g., milk, honey) and mineral drugs (e.g., mercury, arsenic, lead acetate, calcium hydrate, copper oxide). He also described a surgical anesthetic made from opium and mandragora and one made from an alcoholic extract of mandrake. He was the first to recognize the toxicity of mercury. His contributions to toxicology include classifying poisons into three major classes (animal, plant, mineral), identifying antidotes, and recommending decreasing absorption to control intoxication (for example, by inducing vomiting or purgation; cf. Hippocrates).

Galen of Pergamum (129–199 AD), considered by some to be the greatest Greek physician and surgeon, was the son of an engineer (Nicon) and was very well educated. He traveled widely to increase his knowledge of medicine (as did many others), studied philosophy, and began to write. He eventually became the most celebrated ancient medical writer and influenced medicine for 1500 years. His fame as an outstanding healer in Rome began with the successful treatment of the Aristotelian philosopher Eudemus, an influential Roman, who was dying despite the care of the best physicians in the city. Galen's practice flourished and his patients included the most influential Romans of the period. At this time, he was given a dissecting room in which to study comparative anatomy (using the bodies of slain gladiators). His benefactor, Flavius Boethus, also provided him with secretarial assistance, and several anatomy books followed. Galen is considered the founder of experimental physiology. He was the first to prove that the arteries carried blood and not air and conducted other experiments involving the nervous system, heart, and liver. He taught and practiced that it is essential to select the right drug for the right condition for the right patient; he introduced rationality into drug therapy.

Galen argued that although apothecaries knew drugs, only the physician understood both the drug and the patient, and further, that drugs are tools only for physicians (hence, few experimental nonphysician pharmacologists in Greece). He recommended mixtures of drugs for treating disease, which is the basis for the term *galenicals.* The foundation of his writings was the teachings of Hippocrates. Galen tried to amalgamate the various doctrines espoused by Hippocrates, including qualities, four humors, pneuma, and physis. Galen also believed, as did many early physicians, that health depended upon a balance of the various bodily humors (phlegm, blood, yellow bile, black bile). He identified four personality types (phlegmatic, sanguine, choleric, melancholic) based on these four humors. He tried to establish a composite system of medicine, one that would include formulas/principles that would remove uncertainty from the decisions of practicing physicians. He further developed the theriac, the universal antidote, to include 100 substances, which was to be administered in honey and wine. His advice to physicians (also valid for toxicologists): "If anyone wishes to gain fame through these, and not through clever talk, all that he needs is, without more ado, to accept what I have been able to establish by zealous research."

Paul of Aegina (625–690 AD), celebrated Byzantine physician, authored a seven-volume medical encyclopedia, *Epitome.* It is based on the 70 books by Oribasius and other writings. Book 5 deals with toxicology, specifically bites and wounds of venomous animals. Paul was probably the "last Greek compiler."

The contributions of the Greeks to the advancement of toxicology include comprehensive lists of poisons and antidotes, experimental studies of the biological effects of plants (plant constituents) and metals, and rational methods for the management of poisonings.

Roman

The Romans also had an intense interest in poisons. They derived a great deal of their knowledge of medicine from the Greeks and from other cultures. Aurelius Cornelius Celsus (30 BC-AD 50), the "Cicero of physicians," "Hippocrates of the Romans," the greatest Roman medical writer and an armchair physician, was a Roman nobleman of the Cornelii family, a man of letters, and an encyclopedist who authored the outstanding encyclopedia of his day and a number of books on medical subjects. He provided a systematic survey of medicine, presented sound principles of good surgery, and stressed the

application of common sense to medical issues. Only eight volumes of his classic *De Medicina* survived. It was a medical classic and included discussions of many diseases. The importance of dietetics in treatment was stressed. He was the first to identify the four signs of inflammation: calor, rubor, tumor, and dolor.

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He also stressed cleanliness, the use of vinegar (currently experiencing a renaissance) and thyme oil to cleanse wounds, the use of ligatures to stop bleeding, and the use of skin from other parts of the body for facial plastic surgery. He believed that "the best medicament is food opportunely given." He also introduced the nutrient enema. *De Medicina* is considered basically Hippocratic with some methodism. Book 5, *Toxicology and Rabies,* included the works of Nicander and Dioscorides and poisons and antidotes. Celsus was not interested in mechanisms or theories of poisoning. He cited others who believed that poisons and animal venoms depressed the vital factor resulting in the loss of innate heat and chilling. Incidentally, *De Medicina* was one of the first medical works to be published after the introduction of the printing press (1478).

Consistent with Hippocratic teaching, Celsus also advocated getting rid of the poison as quickly as possible. He proposed free bleeding, sucking the wound, amputation, promoting evacuation, eliciting heating action (by administering hot, peppery materials or by the application of the warm flesh of a freshly killed fowl or small animal to a wound), and "poisoning the poison" (acrid materials applied to wounds, cupping severe wounds, suction with palms of the hand, and the use of hypertonic salt solutions). In addition, he recommended the use of appropriate antidotes (including the antidote of Mithridates, 37 ingredients in honey). His contributions to toxicology include his list of poisons and antidotes and the management of poisoning.

Pliny the Elder, Gaius Plinius Secundus (23–79 AD), was born in Como. He was a famous Roman naturalist, historian, military tactician, philosopher, and one of the most learned men of his time. He wrote 160 books. Although he was not trained in medicine, he chronicled medical history. His quest for knowledge was insatiable (it is said that he died while trying to observe an eruption of Vesuvius). He collected data about all living things. He was skeptical of the value of mithridatics. He described poisonous plants and animals and the types of injury they cause in his famous *Historia Naturalis* (Natural History), a compendium of information that consisted of 37 books. He advocated the doctrine of signatures, which stated that the therapeutic usefulness of a plant, mineral, or animal was based on its resemblance to the signs of a disease. This doctrine persisted for many centuries and still has a few proponents. He was also interested in adulteration of foods and developed methods for the detection of adulteration (e.g., chalk in flour, herbs, and spices). Like Hippocrates and others, he believed the "the greatest aid to health is moderation in food." His contributions to toxicology include lists of poisons and their biological effects and his questioning of the value of nonspecific antidotes like mithridatics.

Arab

The Arabs made significant contributions to the health professions. For example, their pharmacies are considered to be the forerunners of modern pharmacies. Since Avicenna believed in the importance of keeping patients happy, he instructed pharmacists to make medicines pleasant. To this end, the pharmacists developed sugar-coated pills, mixed rose water or perfumes with medicines, and were the first to wrap medicines (pellets) in silver foil. To make the physician's job easier and to promote more effective healing, Arabian pharmacists are credited with developing or perfecting tinctures, confections, syrups, pomades, plasters, and ointments.

Avicenna (Abu Ali Husain ibn Abdullah ibn Sina, 980–1037), the "Prince of Physicians," was born in Bokhara, Persia. He was a child prodigy who knew the Koran by heart at the age of 10. He studied philosophy, jurisprudence, and mathematics; at the age of 16 he turned to the study of medicine. By 18, his fame as a physician was so great that he was appointed physician to the prince and became physician-in-chief to the hospital in Baghdad. Avicenna was given access to the library of the prince as a token of gratitude and appreciation for healing the prince of a serious ailment. He was also the personal physician to other caliphs. By the age of 21, he had written a 20-volume encyclopedia. He developed a philosophy (avicennism) that was partly Aristotelian and partly new platonic. His most significant medical works were his *Book of Heal-ing* and *Canon of Medicine,* a five-volume treatise that included *The Theory of Medicine, Simpler Drugs, Special Pathology and Therapeutics, General Diseases,* and *Pharmacopoeia*. He also discussed oral and parenteral poisons, bites and stings and their treatment, and classified and discussed poisons as plant, animal, or mineral (cf. Ebers Papyrus). He was also the first to describe the fetid odor exhaled by those poisoned by mercury (Ecobichon, personal communication). In addition, he developed his own psychiatry and believed that psychic alterations were the result of changes in the brain (pathology due to humors [neurotransmitters?]).

His books were logically arranged (an example of "Aristotelian dialectic and Arabian scholasticism"). He was a logical thinker and an astute observer. Some have referred to him as a second Aristotle. Avicenna wanted to develop a system of medicine, to make medicine "a quasi-mathematical discipline." This would remove uncertainty from medical decisions (cf. Galen). His contributions to toxicology include

mechanisms of action of poisons including neurotoxicity and metabolic effects. He also recommended the bezoar stone as an antidote for venoms and preventive of disease. Despite his many accomplishments, Avicenna was not "a dryasdust;" he was "a man of the world, he loved wine, women, and

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song—more ardently, perhaps, than was good for his health.... But before he reached the age of fifty-eight his bodily powers were exhausted, and he died" (some believe of an overdose of opium). His work was the authoritative text on poisons and antidotes for 500 years.
Note that the Arabs translated most of the important Greek works. The Arabs excelled in chemistry and invented distillation, sublimation, and crystallization. They introduced camphor, benzoin, saffron, laudanum, and naphtha.
Arabian doctors as a rule watched each other jealously. Disagreements were at times settled by duel-by-poison. In such duels each doctor was expected to take his opponent's poison, then find a quick antidote. Two court physicians once tried it: The first doctor's draught was fierce enough to "melt black stone," but his rival parried with an antidote. Then the second doctor picked a rose, mumbled an incantation, and asked his antagonist to sniff the flower. The first doctor complied, and promptly fell dead. Fright had killed him, for the rose was only a rose.
The Persians also believed in the "Poison Maiden." An attractive young girl was fed poisons (increasing doses of a number of poisons) so she became very "venomous" (double seduction). A kiss or sexual intercourse with her would prove fatal to her lover. Avicenna mentions such a girl; she was so venomous that insects that bit her were poisoned and died.
"Man should believe nothing that is not attested to (1) by rational proof as in mathematical science, (2) by evidence of the senses or (3) by authority of prophets and saints." (Maimonides)
Rabbi Moses ben Maimon (Moses Maimonides, 1135–1204), was a famous Jewish philosopher and physician, court physician to Saladin, and rabbi of Cairo. His book on poisons, *Poisons and Antidotes/Upon Poisoning and Its Treatment,* was translated into Latin by Armend and Blasii in 1305, into French in 1865, and into German in 1873 by Steinschneider. He described poisonous insects and animals and noted that the most dangerous bite was that of a fasting human. His treatment of poisons included "ligature of the bite, sucking out the poison by means of cupping glasses or with the oiled lips, again extending Hippocratic teaching to decrease absorption, and the use of external (e.g., salt, onions, asafetida) and internal remedies (e.g., emetics)." It was a much-cited text. His books on health were very advanced and are considered modern. He believed in the importance of preventive medicine and stressed the importance of hygiene; he wrote a four-volume treatise upon hygiene and diet *(Sepher Rephuoth)*. His aphorisms are as pertinent today as when they were written.

Others

Pietro de Abano (1250–1316) was born in Abano near Padua. He was a teacher of science and medicine at the University of Padua and one of the most famous teachers and skillful physicians of his time, and the author of *De Venemis,* a book of poisons. Like others before him, he classified poisons as mineral, vegetable, and animal, and he was the first to identify sound as a potential poison. He further noted that poisons can be absorbed from air and through the skin ("poisoning by touch"). The book was very popular and went through 14 editions. He also wrote *Conciliator Differentiarium,* an attempt to reconcile Greek and Arabic medicine. This tradition in toxicology has been maintained even until today, when Marcello Lotti directs an internationally recognized institute of occupational/medicine and toxicology.
The age of observations (recording phenomena) and categorizing and listing poisons gave way to the period of challenge and active investigation—experimental toxicology—through the efforts of one of the most controversial yet influential figures in medicine, Paracelsus. Although others made observations in humans (often after deliberately administering a poison), Paracelsus encouraged the use of animals to study poisons. He also developed and promulgated certain basic principles of the action of chemicals (e.g., dose-response) that still form the scientific underpinnings of modern experimental toxicology.

THE AGE OF EXPERIMENTAL TOXICOLOGY

"The universities do not teach all things." (Paracelsus)
Philippus Theophrastus Aureolus Bombastus von Hohenheim (Paracelsus, 1492–1541) was born near the village of Einsiedeln near Zurich, Switzerland, on November 10 (or 14), the son of Wilhelm Bombast von Hohenheim, a German physician/alchemist. Following the death of his mother when he was still very young, Paracelsus with his father moved to Villach in southern Austria, where his father taught chemistry, practiced medicine, and became interested in the health problems of the local miners, eventually becoming an expert in occupational medicine. Paracelsus attended the universities of Basel, Tubingen, Wittenberg, Leipzig, Heidelberg, Cologne, and Vienna, from which he received a baccalaureate in medicine in 1510, at the age of 17. He received his doctorate from the University of Ferrara in 1516. Since it was the custom of the humanists at that time to Latinize their names after they received their degree, he began using the name Paracelsus (*para* Celsus, above Celsus), since he considered himself greater than Celsus. Deichmann et al. (1986) claimed that Paracelsus "is a Greco-Roman translation of

Hohenheim and says 'next to heaven.'" He traveled throughout Europe, England, Scotland, Egypt, the Holy Land, and Constantinople, attempting to learn the most effective means of medical treatment and the latest findings in alchemy.

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He wanted to discover "the latent forces of nature" and wrote, "He who is born in imagination discovers the latent forces of Nature…besides the stars that are established, there is yet another—Imagination—that begets a new star and a new heaven." He returned to Villach in 1524 and became town physician and lecturer in medicine at the University of Basel in 1527. His fame had spread, and students flocked to his lectures.

Paracelsus believed all physicians who preceded him were incompetent, liars, or fakers. "I am to be the monarch, and the monarchy will belong to me." He defied tradition (a young Turk!). Paracelsus drastically and permanently changed the course of medicine. Although a theosophist and an iconoclast, he was a keen student of human behavior, a forerunner of Freud, a chemical anatomist, the founder of medicinal chemistry, and the "godfather of modern chemotherapy," who believed that practicing physicians needed to use common sense, gain experience, travel, and practice humility (good advice that is relevant and sound today). He was a peripatetic physician and was always trying to learn more medicine. His approach to medicine and the body was chemical. For example, he taught that it was more important to learn about the chemical composition of the body than about the muscles. Since God created (caused) diseases, God (Nature) also provided cures. It was the role of the alchemist (chemist) to find these and convert them to effective remedies. Paracelsus began with simple materials, the metals. When the "conservative physicians" warned that metals would poison patients, Paracelsus replied, "This poison, as you call it, has a far better effect than the wagon grease…with which you are so fond of smearing your patients."

His disdain for established authorities, for everything that had been said by his predecessors, reached its climax on 24 June 1527 when he publicly burned the books of Avicenna and Galen in front of the university. He discarded the old ways, including humoral pathology, but upheld Hippocrates; he attacked medical principles of his time, trusted only his own observations, ideas, and works, and tried to bring chemistry into therapeutics by encouraging the use of mineral salts, acids, and chemically prepared therapeutic agents ("better living through chemistry"), since he believed that the body was a chemical laboratory.

Paracelsus was a free thinker. He developed his own system of medicine and boasted about his contempt for science. One of his methods of learning was theosophical intuition; that is, all knowledge is the result of mystical insight, all wisdom comes directly from God, and one should be in intimate contact with God and God's creation. Submit to the will of God and all knowledge will flow. This intuitive process of learning was also part of gnosticism. He appeared to be contemptuous of the established way of thinking and believed that man was a little world (microcosm) that contained all knowledge. This was based on man's direct descendancy from Adam, who had within himself all sciences since he contained the germs of all creatures. In his book *Paramirum,* Paracelsus described his system of medicine. Health, disease, and human destiny depend upon five entia (cf. Shen Nung); disease is caused by the five entities: ens astrale (influence of the stars), ens venini (influence of nutrition/poisons in food), ens naturale (nature and functions of the body), ens spirituale (spirits, demons), and ens Dei (acts of God directly upon us to restore order and health). His theory of humors included three elementary principles: salt (representing stability), sulfur (representing combustibility), and mercury (representing liquidity). Disease is a separation of one principle from the other two (disequilibrium among humors/ principles?). He also believed, according to Cole, that there are five phlegms, five hydropathies, five jaundices, five fevers, five cancers, and so forth. Diseases tend to be localized in a particular "target" organ. Although he tried to bring more chemistry into medicine (e.g., by the use of inorganic salts), he also believed that God will provide cures for us since God is benevolent.

Paracelsus wrote that "nature hints at cures." This is the basis for the doctrine of signatures (cf. Pliny the Elder, "that an agent of nature shows by its external forms its unique qualities"). For example, since turmeric is yellow, use it to treat jaundice, foxglove for heart, figwort for scrofula, and hepatica for liver disease. Cope (1957) described Paracelsus as arrogant and conceited "almost to the point of insanity…extremely effective in [his] criticisms of the then accepted doctrines…reveled in the wildest speculations and taught [his] mad conjectures as unassailable truths…bitter and unscrupulous controversialist…mystic…his writings…so confused and obscure as to be often quite unintelligible… braggart, scorner of authority…that Paracelsus scarcely ever lectured except when he was half drunk, or attended a patient until he was wholly drunk." Paracelsus defended himself in his *Seven Arguments, Answering to Several of the Detractions of His Envious Critics,* written in 1537.

But what of Paracelsus's positive contributions? He was an original but eccentric thinker. He is considered by some to be the father (founder) of chemistry and/or medicinal chemistry and the reformer of materia medica. He did not support the humoral basis for disease; he believed that diseases

were specific/discrete conditions and are cured by specific/discrete treatments. He taught that observation and experience are essential for success in medicine (and in science?). He is credited with the introduction of the following into the practice of medicine of his day: mineral baths, laudanum, mercury, lead, arsenic, copper sulfate, and iron. He forever destroyed the doctrine of the four humors. His principal works include

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Chirurgia magna (1536), *De gradibus* (1568), and *A Treatise on Diseases of Miners* (1567). In one of his books, *Paragranum,* he presents the four pillars upon which medicine should be based: philosophy (knowledge of nature; disease and healing are part of nature); astronomy (heaven paternalistically deals with us); chemistry (provide drugs and insight into biological events; "nature is the ideal chemist"); and virtue (love is the foundation of medicine). In his *Third Defense,* he wrote, "What is there that is not poison? All things are poison and nothing (is) without poison. Solely, the dose determines that a thing is not a poison." (Deichmann et al., 1986). This is often misquoted as "the dose makes the poison." This concept has been expanded to include no-effect level, threshold, extrapolation, and dose-response relationship. His other contributions include target-organ toxicity, animal experimentation to study the effects of chemicals, and the use of inorganic salts in medicine. He was thoroughly seduced by the complexity of chemical-biological interactions and spent his lifetime trying to solve the mysteries of these interactions.

Paracelsus's teachings in psychiatry are often overlooked and may be as significant as his contributions in other medically related areas. For example, he believed that in man there are two antagonistic forces, animal and godly, and that man has to suppress the animal spirit if he is to be successful (yin and yang, id and superego?). He also believed that psychoses are not demonic in origin, the mind (will or spirit) can influence the state of the body (cure or cause some diseases—psychosomatic medicine?) and not the existence of a subconscious, and that women are different from men and must be treated differently ("men are from Mars and women are from Venus").

Paracelsus was a deeply religious man. "He was intensely concerned with the eternity, or soul, in man, and felt that a doctor was neither "pillmaker" nor businessman, but a legate of God, the supreme physician. Medicine was therefore a divine mission, and the doctor must raise his eyes from "excrements and salvepots to the stars." The perfect physician, he felt, was a philosopher, an astrologer, an alchemist and above all, a virtuous man. The character of such a doctor, Paracelsus proclaimed, was far more effective than mere mechanical skill (Amen!) (Bettman, 1959).

Paracelsus died prematurely at age 49. Some say he died in a brawl at the White Horse Tavern in Salzburg on 24 December 1541, presumably exhausted. Despite his early death, he made an indelible mark on medicine and especially on toxicology.

The French physician, poet, and playwright Jacques Grevin (1538–1570), "the father of modern biotoxicology," published his classical work, *Deux Livres des Venins,* in 1568 and further developed the concept of chemical-biological interactions.

Some of the concepts developed by Paracelsus were further developed by others. For example, Felice Abate Fontana (1720–1805), an abbot, physician, physiologist, naturalist, and professor of philosophy at Pisa and director of the Natural History Museum at Florence, advanced the concept of target-organ toxicity; that is, the symptoms of poisoning are the result of poisons acting on a particular organ. He is considered the first modern scientist to study venoms (*Ricerche fisiche sopra il veleno della vipera,* 1767). He is also known for the spaces of Fontana (*Dei moti dell'iride,* 1765). Although Paracelsus drew attention to the plight of miners, little attention was focused on the effects of nondeliberate exposure to chemicals, for example, in the workplace. It was the brilliant Italian physician Bernardino Ramazzini (1633–1714) who effectively and convincingly brought the workplace situation to the attention of the world, especially to the field of medicine. He was the first to describe, in a comprehensive, systematic, and detailed fashion, industrial health problems in his *De Morbis Artificium Diatriba* and his famous *Diseases of Tradesmen/Workers,* which was originally published in 1700; the English edition appeared in 1705. As an astute scientist and physician, he had remarkable powers of observation. He first described "stone mason's consumption [silicosis], potter's sciatica, gilder's ophthalmia and lead poisoning." He is the founder of occupational/industrial medicine. He also advocated the use of cinchona bark to treat malaria, which ran counter to Galen's recommendation that it be treated with purgatives.

The observations of Ramazzini concerning the relationship between workplace exposure and disease were strengthened and extended by the classical studies of Sir Percival Pott (1714–1788), the famed British physician and surgeon to St. Bartholomew's Hospital, who achieved fame in two areas, occupational medicine/ toxicology and orthopedics. He sustained an ankle fracture following a fall and described it so well that it became known as Pott's fracture (*Treatise on Fractures,* 1750/1769). Pott's disease (caries of the spine) was first described by Percival Pott. He later described the relationship between scrotal cancer and soot in chimney sweeps (1775). His contributions to toxicology include the identification of chemical carcinogenesis in humans and noting the increased sensitivity of children to chemicals (a concept currently enjoying a resurgence).

Attempts to explain the action of toxins attracted the attention of some of the intellectual giants in the

biomedical sciences (intellectual seduction). A few will be mentioned. Ambrose Pare (1510–1590) has been called "the greatest surgeon of the 16th century," the founder of modern surgery, and one of the most famous anatomists of all time. He introduced the use of ligatures of blood vessels as opposed to cauterization and authored many

books on medical topics, including anatomy, surgery, malformations, and obstetrics. He investigated CO poisoning and published a report in 1575. On the other side of the channel, Richard Mead (1673–1754), a British physician with a medical degree from Padua who worked at St. Thomas' Hospital and was also physician to the royal family, attempted to explain the action of poisons (venoms) in his book *A Mechanical Account of Poisons* (1702). He described snake poisoning and noted that the venom is only effective parenterally. To prove this, he swallowed the venom and (fortunately) nothing happened. He also authored a book on the influence of the sun and moon on human bodies. The formal beginning of mechanistic toxicology, however, was to await the experiments of Magendie and Bernard, discussed later.

Forensic toxicology, the application of analytical techniques to the detection of poisons, had its beginning with Joseph Jacob Plenck (1738–1807), who noted in his text, *Elementa Medicinae et Chirurgiae Forensis,* that "the only certain sign is the chemical identification of the poison in the organs of the body," which is still a basic principle of forensic toxicology. Unfortunately, his works were not accepted by the medical or scientific communities of his day.

The application of analytical chemistry to matters of food and drug safety formally began with Friedrich Accum (1769–1838), although earlier attempts were made by Theophrastus (370–285 BC), Cato (234–129 BC), Pliny the Elder (23–79), Dioscoridies (40–90), and Galen (131–201). Born in Buckebourg, Germany, Accum moved to London in 1797 as a pharmacist and in 1801 began working with Sir Humphrey Davy. Being entrepreneurial, he also set up his own contract laboratory and supply house. He was the first to use analytical chemistry to detect adulterants in food and published *A Treatise on Adulterations of Food and Culinary Poisons* in 1820, a very successful book that was acclaimed worldwide. He later published *An Attempt to Discover the Genuineness and Purity of Drugs and Medicinal Preparations.* He left England and returned to Germany because of the many (false) charges directed against him.

THE AGE OF MECHANISTIC AND ANALYTICAL TOXICOLOGY

When you can measure what you are speaking about, and express it in numbers, you know something about it; but when you cannot measure it, when you cannot express it in numbers, your knowledge is of a meager and unsatisfactory kind; it may be the beginning of knowledge, but you have scarcely in your thoughts, advanced to the stage of science. (William Thomson, Lord Kelvin)

The adverse effects of chemicals (including poisons) in humans and extensive lists of poisons and antidotes had been recorded, some poisons had been evaluated in animals, and analytical techniques were being applied to toxicological problems. Toxicology was being recognized as a scientific discipline. But little was being done to answer the basic question, "How do poisons kill?" The era of mechanistic toxicology formally began with the classical studies of the two most famous physiologists in medical history, François Magendie and his pupil Claude Bernard. Contributions had been made by others, but these were limited and not as systematic, fundamental, and far-reaching as those of Magendie and Bernard.

François Magendie (1783–1855), French physician and experimental physiologist, contributed significantly to the advancement of physiology, medicine, and toxicology. For example, he demonstrated the functioning of spinal nerves and he studied blood flow, swallowing, and vomiting. He is credited with the introduction of strychnine, iodine, and bromine compounds into medicine. His interest in the functioning of the nervous system led him to establish the mechanisms of action of emetine and strychnine and the dynamics of movement across body membranes. He was also the first, or one of the first, to observe and describe anaphylactic shock. His most famous pupil, Claude Bernard (1813–1878), the son of a Burgundian vinegrower, studied pharmacy and enjoyed science, but wanted to be a playwright; his critics told him to study medicine and fortunately for humankind, he accepted their advice. He went to Paris and was accepted as an assistant by François Magendie at the Hotel Dieu. He enthusiastically endorsed Magendie's philosophy that physiologists must discover the laws of "vital manifestations" or physiological functions and that observation and experimentation were the only methods of investigation. He received his degree in 1843; his thesis dealt with gastric juice and digestion. He and Magendie did much to advance physiology, especially of the nervous system (autonomic) and the gastrointestinal tract, including the liver. Bernard studied both normal and pathological physiology. His contributions to toxicology include furthering the concept of target organ toxicity, establishing approaches to defining the mechanism of action of drugs and other chemicals (e.g., curare, nicotine, carbon monoxide), demonstrating that the basic principles of pharmacology and toxicology are identical, and showing that drugs and other chemicals can modify the function and structure of tissues. He believed that "the physiological analysis of organic systems…can be done with

the aid of toxic agents" (a new use for poisons!). His works were published in 18 volumes. One of his most famous, *An Introduction to the Study of Experimental Medicine,* was published in

1865 and translated into English in 1949. It is a classic in the field of experimental biology and "must" reading for all students of biology and medicine.

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

Bernard's work stimulated others to experimentally establish the mechanisms of action of toxic agents and to publish textbooks. For example, Francesco Rognetta (1800–1857), an Italian physician and scientist, significantly advanced our knowledge of the mechanisms of action of toxic agents, especially arsenic. The Florentine physician and scientist R.Bellini (1817–1878) authored the first experimental toxicology text, entitled *Lezioni Sperimentali de Tossicologia*. The discipline of toxicology was also advanced by the outstanding research efforts of such noted pharmacologists as Rudolph Buchheim, Oswald Schmiedeberg, and Lewis Lewin (again noting the closeness of these two disciplines).

The time had come for analytical techniques to be formally incorporated into toxicology. It had been difficult to establish poisons as the cause of death since they could not be identified in tissues, the only scientifically valid proof. Analytical (forensic) toxicology had its formal origins in the outstanding work of Orfila. His investigations were also the forerunner of modern pharmaco- and toxicokinetics and dynamics.

"Measure what can be measured; make measurable what cannot" (Galileo).

Mathieu Joseph Bonaventure Orfila (1787–1853) was born on the island of Minorca. He was educated in Valencia and Barcelona and studied chemistry and medicine in Paris, receiving his medical degree from the University of Paris in 1811. In 1813–1815, he published his classical and monumental two-volume work, *Traité de Toxicologie: Traité des poisons tirés des regnes mineral, vegétal at animal ou toxicologie générale considerée sous les rapports de la physiologie, de la pathologie et de la médecine légale* (Crochard, Paris). This is probably the first book devoted entirely to toxicology and thereby established toxicology as an experimental science different from pharmacology. Orfila classified poisons into six classes including animal, vegetable, and mineral (not unique; cf. Ebers Papyrus and others). He presented the chemical, physical, physiological, and toxic properties of each chemical, methods of treatment and chemical tests for their identification. This classic work, the first of its kind, effectively combined forensic and clinical toxicology with analytical chemistry. It was translated into English in 1817. In 1816, Orfila published *Eléments de chimie médecale* and in 1818, *Secours à donner aux personnes empoisonnées ou asphyxixés*. He provided a rational basis for some antidotes. He demonstrated the toxicity of strychnine in numerous experiments on dogs. At that time, strychnine was widely used in prescriptions and in tonics and was considered by practitioners of medicine to be a safe drug (Magendie later established the mechanism of action of strychnine). He later published *Leçons de médecine légale* (1821), in which he classified poisons as "irritants, narcotics, narcotico-acrids and putrefiants."

Orfila's books were translated into many languages and this helped him to internationalize toxicology (presaging the efforts of Coulston, Doull, Golberg, Zbinden, and others). He was appointed professor of legal medicine at the University of Paris and became dean of the faculty in 1831. He founded the Musée Orfila of Comparative Anatomy. He retired in 1848 and died in 1853. Orfila was an excellent analytical chemist and very capable physician; he was an experimentalist and administered known doses of poisons to animals, carefully observed effects produced, examined organs for evidence of toxicity (target-organ toxicity), and chemically analyzed tissues and body fluids to establish relationships between dose, response, and tissue levels. He was able to demonstrate conclusively and quantitatively that poisons are absorbed from the gastrointestinal tract and accumulate in tissues. His significant contributions to toxicology include the chemical detection of poisons in tissues and fluids, thereby permitting better diagnoses; furthering the concept of target organ toxicity by evaluating tissues grossly and histologically; relating symptoms to specific tissue injury; and extending the concept of dose-response.

His influence on modern toxicology is legendary and probably secondary only to that of Paracelsus and Bernard. His books were published in many languages and used in many countries (including the United States). He spawned other works in toxicology and brought to toxicology the recognition it deserved and needed. For example, Sir Robert Christison (1797–1882), a noted Scottish physician with a medical degree from Edinburgh, studied toxicology with Orfila and became professor of forensic medicine and materia medica at Edinburgh. He published *A Treatise on Poisons* in 1829. The fourth edition, published in 1845, became the first American edition. He also strove to provide a further scientific basis for toxicology. His works helped develop the basis for expert witnessing (and created opportunities for

consultants!). Alfred Swaine Taylor (1806–1880), famous British physician and the founder of British forensic medicine, is also the founder of modern medical jurisprudence, a natural sequel to the development of forensic toxicology. He received a diploma from the Apothecaries Society in 1828, his certificate to practice from the Royal College of Surgery in 1830, and presented the first course in medical jurisprudence in England in 1831. He taught chemistry and medical jurisprudence

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at Guy's Hospital. He was probably the most famous expert witness of his time and published his *Manual of Medical Jurisprudence* in 1842. It became very popular, and the tenth edition was published in 1879. Other forensic toxicologists of note include the following: Henry Coley, New York City forensic toxicologist, published *Poisons and Asphyxia* in 1832. Included in this book were mineral acids, caustic alkalis, ammonia, nitrates, phosphorus, cyanide, metals, and alkaloids, as well as their chemistry, uses, signs and symptoms of poisoning, cause of death, postmortem findings and treatments. James M.Marsh, developed a test for arsenic (1836). Duflos, developed a test for systematically searching for mineral poisons that involved wet ashing with chlorine (1838). Hugo Reinsch, developed a test for arsenic and mercury (1842). Frensenius and von Bobo reported on a systematic scheme for detection of mineral poisons using wet ashing with chlorine (1844); Jean Servais Stas developed a method for extracting alkaloids from cadavers (1850). T.Graham and A.W.Hofman reported on the adsorbing properties of charcoal (adsorbed strychnine from beer) and proposed utilizing adsorption in toxicological analyses (1853). Theodore George Wormley published *Microchemistry of Poisons,* the first American toxicology textbook (1867). Lieben reported on an iodoform test for alcohol (1870). K.L. Dey developed a test for opium in toxicological analysis. A.W.Blyth published *Poisons: Their Effects and Detection,* an excellent analytical toxicological text (1884). Rudolph A.Witthaus and Tracy C.Becker edited a four-volume text, *Medical Jurisprudence, Forensic Medicine and Toxicology,* which became the standard reference text in the field (1894–1896). Walter S.Haines and Frederick Peterson wrote a toxicology text (1903). Alexander Gettler, who probably influenced the development of forensic toxicology in America more than anyone else, began working in the Office of the Chief Medical Examiner in New York City (1918). Rolla Harger developed the Drunkometer for testing drivers presumed to be under the influence (1937).

Advances in chemistry, physiology, pathology, and clinical medicine in the 18th and 19th centuries resulted in significant advances in toxicology. The discipline of toxicology was recognized in the scientific community as a distinct entity, separate from pharmacology and drawing upon chemical, biological, and physical sciences. Today, toxicology continues to benefit from advances in all the sciences.

The advances in forensic toxicology paralleled advances made in understanding the basic mechanisms of action of chemicals and drugs. The mechanistic studies of Claude Bernard were furthered by many scientists including the brilliant German chemist, microbiologist, and immunologist Paul Ehrlich (1854–1915) who significantly advanced mechanistic toxicology (toxicodynamics) and pharmacology (pharmacodynamics). His keen interest in chemistry and biological structure and function led him to propose the concept of a receptor as the sensitive site for chemical-biological interaction; that "chemical substances in organisms had specific points of attachment" (receptors), and once these were known, specific remedies could be developed. His most famous remedy was the use of arsenic in the management of syphilis (Compound 606, arsphenamine). He subsequently identified several receptors. His successful bout with tuberculosis stimulated his interest in immunity (as did his association with Koch), and he subsequently formulated the concepts of active and passive immunity and the side-chain theory of immunity. His contributions to toxicology and pharmacology include developing the receptor theory (the founder of the receptor theory), underscoring the importance of mechanistic studies and structure-activity relationships. He shared the Nobel prize for physiology and medicine with E.Metchnikoff in 1908.

Rudolph Kobert (1854–1918), a student of Oswald Schmiedeberg (one of the founders of modern pharmacology and toxicology and himself a student of Rudolph Buchheim, the founder of modern pharmacology), published a toxicology textbook (1893). A contemporary, Louis Lewin (1854–1929) reported on the toxicology of alcohols, chloroform, opiates, and plant-derived hallucinogens and also wrote a toxicology text (1929).

Mechanistic studies led to a better understanding of the toxic action of many chemicals and to the development of specific antidotes: for example, the use of BAL (dimercaprol) as an antidote for arsenic (based on the studies of Carl Voegtlin [1923] and Rudolph Peters [1945]), nitrite and thiosulfate for cyanide (K.K.Chen [1934], one of America's most distinguished pharmacologists), and organophosphorus compounds and the use of 2-PAM (Wilson [1951, 1955] and W.Lange and G.Schrader [1952]).

Occupational medicine and industrial toxicology were identified by Paracelsus, systematized and advanced by the pioneering efforts of Bernardo Ramazzini, and further advanced by one of America's foremost physicians, Alice Hamilton (1869–1970). Physician and pathologist, she researched occupational diseases, publicized the hazards of industrial chemicals to workers, and wrote several books on industrial toxicology. She was the foremost woman occupational physician and industrial hygienist, the first woman faculty member of the Harvard Medical School, and the only woman to serve on the Health

Committee of the League of Nations. She graphically described the history of industrial toxicology/occupational medicine in the United States in her autobiography, *Exploring the Dangerous Trades* (1943). Others who contributed significantly to this field include Cecil Drinker (1887–1919) of Harvard; R.Bohm

(who believed that toxicological information was accumulating very rapidly; mechanisms of toxicity were being elaborated and exposure to chemicals was increasing due to advances [?] in manufacturing); and Ethel Browning (1891–1979), who received her doctorate in medicine in 1927 and wrote the *Toxicity of Industrial Organic Solvents* in 1937. Interestingly, this was the first book on this subject and was written when Browning had no personal occupational medical experience. Her other publications included *Ionizing Radiations* (1959), *Toxicity of Industrial Metals* (1961), and her greatest work, *Toxicity and Metabolism of Industrial Solvents* (1965).

The stage was set for the application of toxicological principles and findings to protection of the public and especially workers from the adverse effects of chemical exposure. Consumers also needed protection from the potentially adverse effects of chemicals found in air, water, foods, and other consumer products.

AGE OF SAFETY EVALUATION, QUANTIFICATION, AND PROGNOSTICATION

"In no single thing do men approach the gods more readily than in giving of safety to mankind" (Cicero).

In the United States, Harvey Washington Wiley (1844–1930), physician and chemist, served as head of the Bureau of Chemistry of the U.S. Department of Agriculture from 1883 to 1912. His main goal was to provide effective food and drug legislation to protect the unsuspecting public. His efforts culminated in the first U.S. Food and Drug Act (1906), which has been expanded and which formed the basis for food safety legislation worldwide. He issued a number of bulletins summarizing his studies of the effects of food chemicals in human subjects, the "Poison Squad." He wrote, "Injury to public health, in my opinion, is the least important question in the subject of food adulteration, and it is the one which should be considered last of all. The real evil of food adulteration is deception of the consumer." Wiley also served as Director of Foods, Health and Sanitation for Good Housekeeping magazine from 1912 to 1930. He wrote a number of books, including *Principles and Practices of Agricultural Analysis* (three volumes, 1894–1897), *Foods and their Adulteration* (1907), and *History of a Crime Against the Food Law* (1929). Another prominent regulatory pharmacologist/ toxicologist was Arnold J.Lehman (1900–1979), who earned his PhD from the University of Washington in Seattle in 1930 and his MD from Stanford in 1936. He taught at a number of universities and joined the U.S. Food and Drug Administration as Director of the Division of Pharmacology in 1946. He and his staff published *Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics,* in 1955, the first attempt by the agency to provide guidelines for toxicological studies (this presaged the Redbooks). He and his colleagues, most notably O.Garth Fitzhugh, developed the concept of safety factors (a number applied to the highest dose that did not elicit an adverse effect in a properly designed and executed toxicological study, the no-observed-adverse effect level, NOAEL). He is also renowned for the expression on his office wall: "You too can become a toxicologist in two easy lessons, each ten years long." He was succeeded by Leo Friedman (formerly of MIT), who advocated the use of in utero exposure in lifetime studies. Sandford Miller (also of MIT) served as successor to Friedman. To assist toxicologists concerned with the safety of food and color additives, the U.S. Food and Drug Administration issued a series of guidelines, Redbook I and II. The Organization for Economic Cooperation and Development (OECD) issued similar guidelines. Both of these guidelines are designed to encourage sound science and the conservation of resources while providing adequate data for determining safe exposure limits for consumers. The first comprehensive text to address the principles and practices of toxicology was edited by A.Wallace Hayes. At the international level, the World Health Organization (WHO), through the enlightened efforts of Frank Lu and Gaston Vettorazzi, applied sound toxicological thinking to establishing safe exposure conditions for food chemicals including pesticides. They developed the concept of an acceptable daily intake (ADI) based on sound toxicological data and the proper use of appropriate safety factors (SF). This concept is recognized and used worldwide and has been found very effective. There have been no significant problems with food chemicals evaluated in this manner. The evaluations are conducted through the auspices of the International Programme on Chemical Safety (IPCS), and implemented by the Joint Expert Committee on Food Additives (JECFA) and the Joint Meeting on Pesticide Residues (JMPR), under the chairmanship of John Herrman.

"Even while they teach, men [they] learn" (Seneca).

"Toxicology is the ultimate Renaissance science." (Gillett, 1987)

"Irrespective of its location and source of identity, toxicology as a field must studiously cross many traditional boundaries.… The science of toxicology is located somewhere between medicine and the sciences, but drawn toward law by forensic uses and the need to regulate various human activities. Societal values and needs tug the field more into the center of the tetrahedron." (Gillett, 1987).

As toxicology became a recognized scientific discipline and more were seduced by its scientific and public health

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appeal, training programs began. Although it was very difficult to develop programs that could address the many facets of toxicology including chemistry and biochemistry, physiology and pharmacology, pathology, statistics, and epidemiology, excellent training programs were developed at some of the most prestigious universities. Examples included, in the United States, California, Chicago, Harvard, Iowa, Kansas, Medical College of Virginia, New York University, Rochester, and Vanderbilt; in Germany, Freiburg, Hannover, Tubingen, and Wuurzburg; in Sweden, Karolinska; in Denmark, Copenhagen; in Switzerland, Zurich; in Italy, Bologna, Milan, and Padua; in England, Guy's Hospital, London, and St. Mary's, Surrey; in Ireland, Dublin; and in Australia, Canberra. A list of current training programs in the United States appears in the Appendix following Chapter 37. The continuing (and increasing) popularity of toxicology is due to many factors including its intellectual and emotional appeal. It is multidisciplinary in nature, challenging and demanding, and its experimental findings are relevant and applicable to public health issues, including improving the quality of life and (hopefully) extending it.

"Knowledge is of two kinds. We know a subject ourselves or we can find information on it." (Samuel Johnson)

The need for a standard textbook became evident. Although several texts were available, none appeared adequate. This issue was addressed and resolved by Louis J.Casarett and John Doull. Dr. Casarett received his PhD in 1958 from the University of Rochester, where he studied respiratory toxicodynamics and morphological changes following exposures to potentially toxic materials, especially to polonium. In 1967 he moved to the University of Hawaii, where he developed a program in toxicology. His research involved drugs of abuse and pesticides. He was considered an excellent researcher and teacher. Dr. Doull received both his PhD in pharmacology and his MD from the University of Chicago. He remained at Chicago for a number of years, then moved to the University of Kansas Medical Center, where he established one of the most outstanding programs in toxicology in the world (and attracted and trained a number of internationally famous toxicologists). Casarett and Doull published *Toxicology, the Science of Poisons* in 1975. It is now in its fifth edition.

Significant contributions to the advancement of toxicology came and continue to come from many sources, academic, governmental/regulatory, and industrial. Government/regulatory toxicologists have made significant contributions especially in the area of safety evaluation, including the quantification of risk. Regulatory agencies demand adequate data of high quality to serve as the basis for establishing safe exposure levels. The extent of testing was and is often determined by the depth of the science, as well as the chemical and physical properties of the agent and the extent of exposure. For example, if absorption, distribution, metabolism, and excretion (ADME) studies were conducted properly, and it was determined that the test material was not absorbed from the gastrointestinal tract, then the need for long-term studies was not compelling. This caused toxicologists who were conducting safety studies to design programs that addressed basic issues early, often resulting in considerable saving of resources. If the agencies had not recommended or required these data, they probably would not have been conducted, or at least not early in the program. The regulators appeared to be driving the science!

Academic and industrial and governmental research laboratories continue to advance the frontiers of toxicology by seeking the molecular basis for toxic action. Contract toxicology laboratories have also made a significant contribution to toxicology by providing unique opportunities for those interested in the pragmatic (applied) aspects of toxicology, namely, the conduct of appropriate tests to establish safe conditions of exposure. These studies must consider the latest developments and advances in toxicology and related disciplines and the needs of regulators internationally. This is especially challenging today in this era of increased international trade and harmonization.

As with other recognized, independent scientific disciplines, toxicologists realized a need for a learned society to provide a forum for the exchange of scientific information. Toxicology continued to attract (to seduce) more students and practitioners, and this resulted in more research. The need for an appropriate journal in which to publish, and thus disseminate, the results of investigations was acute. The journal, *Toxicology and Applied Pharmacology* was founded by Fred Coulston; this was followed by *Fundamental and Applied Toxicology* (now *Toxicological Sciences*). Other journals included *Food and Cosmetic* (now *Chemical*) *Toxicology* (founded by Leon Golberg in 1963), *Journal of Applied Toxicology, Human and Experimental Toxicology*, and others.

The Society of Toxicology was founded in 1965. This was the first international society for and by toxicologists. Since its founding and as a result of the tremendous growth of toxicology, other societies of toxicology have been established. Almost every developed country has its own society of toxicology, a testimony of the recognition of its importance and its growth.

Toxicology continues to grow. Its uniqueness continues to attract (yes, to seduce by virtue of its

attractiveness) some of the brightest students. There is something for everyone: from the molecular to the macro to the modeler, from the gene to the whole animal to the human, from SAR to QSAR. Toxicology's uniqueness and strengths derive from the integration of many chemical and biological sciences and supporting disciplines. This

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provides a strong base from which to extrapolate data to humans. Toxicology is also one of the few sciences in which academic, industrial, and regulatory scientists can effectively interact to protect the public. The importance of toxicology is recognized by governments worldwide. Toxicology has evolved from listing poisons to protecting the public from the adverse effects of chemicals; from simply identifying effects (qualitative toxicology) to identifying and quantifying human risks from exposure (quantitative toxicology); and from observing phenomena to experimenting and determining mechanisms of action of toxic agents and rational management for intoxication. As Claude Bernard noted:

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

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

It is fitting to close with the daily prayer of Moses Maimonides (appropriate for practicing toxicologists): "May there never rise in me the notion that I know enough, but give me strength and leisure to enlarge my knowledge.... Thou hast chosen me in Thy grace to watch over the life."

"It is your work in life that is the ultimate seduction." (Pablo Picasso)

QUESTIONS

1. Define poisons and describe several functions for poisons.
2. How has toxicology evolved from an art into a science?
3. How has the receptor concept influenced the development of toxicology?
4. Discuss some of the similarities and differences between toxicology and pharmacology.
5. Should Claude Bernard or Paracelsus be credited with the title, founder of (modern) toxicology? Defend your choice.

SELECTED READINGS

1. Accum, F. (1820): *A Treatise on Adulterations of Food and Culinary Poisons.* ABM Small, Philadelphia, PA (cited in ref. 40).
2. Ackerknecht, E.H. (1982): *A Short History of Medicine.* Johns Hopkins University Press, Baltimore.
3. Albert, A. (1985): *Selective Toxicity.* 1st ed., Methuen, London, 1951; 7th ed., Chapman & Hall, New York, 1985.
4. Baas, J.H. (1889): *Outlines of the History of Medicine,* trans. H.E. Handerson. J.H.Vail, New York.
5. Beeson, B.B. (1930): Orfila: Pioneer toxicologist. *Ann. Med. Hist.,* 2:68–70.
6. Bernard, C. (1865): *An Introduction to the Study of Experimental Medicine,* trans. H.C.Greene. Dover, New York, 1957 edition.
7. Bettmann, O.L. (1979): *A Pictorial History of Medicine.* Charles C.Thomas, Springfield, IL, 5th printing.
8. Breathnach, C.S. (1987): Orfila. *Irish Med. J.* 80:99.
9. Casarett, L.J. (1975): Origin and scope of toxicology. In: *Toxicology: The Basic Science of Poisons,* edited by L.J.Casarett, and J.Doull. Macmillan, New York.
10. Castiglioni, A. (1941): *A History of Medicine,* trans. E.B. Krumbhaar. Alfred A.Knopf, New York.
11. Christison, R.A. (1845): *A Treatise on Poisons.* Barrington & Howell, Philadelphia, PA.
12. Clendening, L. (1942): *Source Book of Medical History.* Paul B. Hober, New York; Dover, New York, 1960.
13. Cope, Z. (1957): *Sidelights on the History of Medicine.* Butterworth, London.
14. Debus, A.G. (1999): *Paracelsus and the Medical Revolution of the Renaissance; A 500th Anniversary Celebration. National Library of Medicine, Paracelsus, Five Hundred Years; Three American Exhibits.*
15. Decker, W.J. (1987): Introduction and history. In: *Handbook of Toxicology,* edited by T.J.Haley and W.O.Berndt. Hemisphere, Washington, DC.
16. Deichmann, W.B., Henschler, D., Holmstedt, B., and Keil, G. (1986): What is there that is not poison? A study of the Third Defense by Paracelsus. *Arch. Toxicol.,* 58:207–213.
17. Doull, J., and Bruce, M.C. (1986): Origin and scope of toxicology. In: *Casarett and Doull's Toxicology: The Basic Science of Poisons,* 3rd ed., edited by C.D.Klaassen, M.O.Amdur, and J.Doull. Macmillan, New York.

18. DuBois, K., and Geiling, E.M.K. (1959): *Textbook of Toxicology.* Oxford University Press, New York.
19. Eckert, W.G. (1980): Historical aspects of poisoning and toxicology. *Am. J. Forens. Med. Pathol.*, 1:261–264.
20. Gallo, M.A., and Doull, J. (1991): History and scope of toxicology. In: *Casarett and Doull's Toxicology*, 4th ed., edited by C.D.Klaassen, M.O.Amdur, and J.Doull. Pergamon Press, New York.
21. Garrison, F.H. (1929): *An Introduction to the History of Medicine*, 4th ed. W.B.Saunders, Philadelphia, PA.
22. Gettler, A.O. (1953): The historical development of toxicology. *J. Forens. Sci.*, 1:1–25.
23. Gillett, J. (1987): *The ICET Newsletter*.
24. Glaister, J. (1954): *The Power of Poison.* William Morrow, New York.
25. Godon, B.L. (1959): *Medieval and Renaissance Medicine.* Philosophical Library, New York.
26. Goulding, R. (1978): Poisoning as a fine art. *SO Med. Leg. J.*, 46:6–17.
27. Goulding, R. (1987): Poisoning as a social phenomenon. *J. R. Coll. Phys. London*, 21:282–286.
28. Gunther, R.T. (1959): *The Greek Herbal of Disocorides*. Hafner, New York.

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29. Guthrie, D.A.(1946): *A History of Medicine.* J.B.Lippincott, Philadelphia, PA.
30. Haggard, H.W. (1933): *Mystery, Magic and Medicine.* Doubleday, Doran, Garden City, NY.
31a. Hamilton, A. (1943): *Exploring the Dangerous Trades; The Autobiography of Alice Hamilton, MD.* Little, Brown, Boston.
31b. Hamilton, A. (1925): *Industrial Poisons in the United States.* Macmillan, New York.
31c. Hamilton, A. (1934): *Industrial Toxicology.* Harper & Brothers, New York.
32. Holmstedt, B., and Liljestrand, G. (1981): *Readings in Pharmacology.* Raven Press, New York.
33. Hutt, P.B., and Hutt, P.B. II (1984): A history of governmental regulation of adulteration and misbranding of food. *Food Drug Cosmet. Law J.*, 39:2–73.
34. LaWall, C.H. (1924): *Four Thousand Years of Pharmacy.* J.B. Lippincott, Philadelphia, PA.
35a. Lewin, L. (1920): *Die Gifte in der Weltgeschichte. Toxikologische, allgemeinverstandliche Untersuchungen der historischen Qhellen.* Springer, Berlin.
35b. Lewin, L. (1929): *Gifte und Vergiftungen.* Stilke, Berlin.
36. Loomis, T.A. (1978): *Essentials of Toxicology.* Lea & Febiger, Philadelphia, PA.
37. Macht, D.J. (1931): Louis Lewin: Pharmacologist, toxicologist, medical historian. *Ann. Med. Hist.*, 3:179–194.
38. Massengill, S.E. (1943): *A Sketch of Medicine and Pharmacy.* S.E. Massengill, Bristol, TN.
39. Meek, W.J. (1954): *Medico-Historical Papers: The Gentle Art of Poisoning.* Department of Physiology, University of Wisconsin, Madison.
40. Mettler, C.C., and Mettler. F.A. (1947): *History of Medicine.* Blakiston, Philadelphia, PA.
41. Neuberger, A., and Smith, R.L. (1983): Richard Tecwyn Williams: The man, his work, his impact. *Drug Metab. Rev.*, 14:559–607.
42. Neuburger, M.(1910): *History of Medicine*, trans. Ernest Playfair. Oxford University Press, London.
43. Olmsted, J.M.D. (1938): *Claude Bernard: Physiologist.* Harper & Brothers, New York.
44. Oser, B.L. (1987): Toxicology then and now. *Regul. Toxicol. Pharmacol.*, 7:427–443.
45. Osius, T.G. (1957): The historic art of poisoning. *Univ. Mich. Med. Bull.*, March: 111–116.
46. Pagel, W. (1982): *An Introduction to Philosophical Medicine in the Era of the Renaissance.* Karger. Basel, Switzerland.
47. Peters, R.A., Stocken, L.A., and Thompson, R.H.S. (1945): British anti-lewisite (BAL). *Nature*, 156:616–619.
48. Ramazzini, B. (1713): *Diseases of Workers.* Latin text translated by W.C.Wright.
49. Rhodes, P. (1985): *An Outline History of Medicine.* Buttersworth, London.
50. Rosenfield, L. (1985): Alfred Swaine Taylor (1806–1880), pioneer toxicologist—and a slight case of murder. *Clin. Chem.*, 31:1235–1236.
51. Sigerist, H.E. (1958): *The Great Doctors. A Biographical History of Medicine.* Doubleday, New York.
52. Sonnedecker, G. (1976): *Kremers and Urdang's History of Pharmacy*, 4th ed. J.B. Lippincott, Philadelphia, PA.
53. Talbott, J.H. (1970): *A Biographical History of Medicine. Excerpts and Essays on the Men and Their Work.* Grune & Stratton, New York.
54. Temkin, C.L., Rosen, G., Zilboorg, G., and Sigerist, H.W. (1996): *Four treatises of theophrastus von Hohenheim called Paracelsus*, edited by H.W.Sigerist. Johns Hopkins University Press, Baltimore. MD.
55. Thomas, L. (1979): *The Medusa and the Snail.* Viking Press. New York.
56. Thompson, C.J.S. (1931): *Poisons and Poisoners. With Historical Accounts of Some Famous Mysteries in Ancient and Modern Times.* H. Shaylor. London.
57. Voegtlin, C., Dyer, H.A., and Leonard C.S. (1923): On the mechanism of the action of arsenic upon protoplasm. *Public Health Rep.*, 38:1882–1912.
58. von Oettingen. W.F. (1952): *Poisoning: A Guide to Clinical Diagnosis and Treatment.* Paul B. Hoeber, Harper & Brothers, New York.
59. Williams, R.T. (1959): *Detoxication Mechanisms.* John Wiley, New York.
60. Wooton. A.C. (1910): *Chronicles of Pharmacy.* Macmillan, London.

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Chapter **2**

The Use of Toxicology in the Regulatory Process

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Principles and Methods of Toxicology,
Fourth Edition, edited by A.Wallace Hayes.
Taylor & Francis, Philadelphia © 2001.

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BACKGROUND

Regulatory toxicology is that area of toxicology directed at protecting public health by regulating exposure to potentially harmful materials. Historically, regulatory toxicology has developed in a manner that has reflected humankind's ability to relate exposure to certain agents with adverse health effects. Thus, early regulatory attention generally focused on preventing the acute effects of chemical agents, because these effects were observable and could be easily associated with exposure. Once the germ theory of disease was developed and the disease potential of human and animal waste was recognized, the disposal of these materials began to be regulated. Food and drugs were also the focus of early regulation, due, no doubt, to the relative ease in associating acute health effects, such as food poisoning, with exposure to materials in the diet or in medications. Hutt (82) notes that adulteration of the food supply was a serious problem in the ancient world, and quotes Pliny the Elder. writing in the first century AD, as saying, "So many poisons are employed to force wine to suit our taste—and we are surprised that it is not wholesome!"

Occupational exposures were also an early focus of regulation, due again to the fact that the relationship between exposure and effect was often observable. Early industrial hygiene efforts were typically intended to prevent overt or frank effects of materials in the workplace. Some of the first observations of the effects of chronic human exposures to certain chemicals were also made in occupational settings. Hutt (82) notes that during the 16th century, Paracelsus wrote about diseases characteristic of miners. The fact that certain chronic occupational hazards affected the exposed

individual at the point of contact also made the connection between agent and effect easier to discern. The first epidemiological study linking human cancer to a specific cause is attributed to Sir Percival Pott, who identified occupational exposure to soot as being responsible for scrotal cancers in young British chimney sweeps (153).

The development of regulatory toxicology during the 20th century has continued to shadow the ability to detect both chemicals and effects; that is, as we have become able to detect chemicals at lower and lower levels and to detect smaller biochemical and physiological changes, we have turned regulatory attention to these "new problems." For example, small increases in airway resistance following exposure to certain air pollutants are currently used as one basis for regulating these air pollutants; historically, no one would have been aware of these subtle effects. The practice of regulatory toxicology has evolved from historical concern about overt effects at high exposure levels to current concern about subtle effects at very low exposure levels, thus paralleling our ability to detect smaller amounts of chemicals and more subtle effects. For example, guidelines for occupational exposures to benzene have decreased by two orders of magnitude from 100 ppm in 1927 to the current Occupational Safety and Health Administration (OSHA) standard of 1 ppm. Ambient criteria for nonoccupational benzene exposures are currently as low as 0.03 ppm in some states (141).

Because of the recent dramatic increase in our ability to detect smaller effects and lower concentrations, programs to regulate chemicals in the environment have increased at an astronomical rate during the last 25 years. Factors contributing to the recent increase in regulatory activity include:

- The realization of the vast number of chemicals that humans have dispersed into the environment and to which humans have been exposed. Approximately 60,000 chemicals that are used commercially have been identified and listed under the Toxic Substances Control Act (TSCA) (214). Advances in analytical chemistry have allowed parts-per-billion levels of chemicals to be detected in "pristine" areas, in wildlife, in food products, and in human body tissues. This message was delivered initially by Rachel Carson in 1962 with the publication of *Silent Spring* (34).
- The realization that historical chemical management practices might not have been protective for the health effects we are concerned with today (effects at low doses), although such practices were consistent with the state of knowledge at the time. For example, during the 1970s, residents of Love Canal realized that they had unknowingly been exposed to chemicals that had migrated from a nearby landfill into their basements. The Comprehensive Emergency Response, Compensation, and Liability Act (CERCLA), also known as Superfund, was enacted shortly after the Love Canal incident.
- The establishment of causal relationships between certain diseases and chronic chemical exposures, such as leukemia and benzene, or mesothelioma and asbestos.
- The reduction in illness and mortality due to microbial diseases and the improved standard of living, which have focused increasing attention on other causes of ill health.

The rapid increase in the number and complexity of regulatory programs to address potential health effects from chemical exposures is also a result of the increased scientific uncertainty about toxicology and risk that has evolved with our increased understanding of these subjects (i.e., the more we learn, the more we realize how much more there is to learn). As we have come to better understand the complexities of toxicology, we have developed more complex procedures for characterizing toxic responses. An example of this is provided in the sixth section of this chapter, which describes probabilistic risk assessment methods.

In 1958, when the Delaney Clause forbidding the addition to food of any substance found to induce cancer in animals or humans was passed (156), the public generally believed that the intent of the law to provide a "zero risk" food supply was achievable. No one foresaw that, 20 years later, scientists would have identified over 500 animal carcinogens, been able to detect chemical concentrations between two and five orders of magnitude lower than they could detect in the 1950s, and found that many naturally occurring chemicals in food could be considered animal carcinogens (213). These developments have forced the recognition that absolute safety is impossible to achieve, even in products regulated to assure safety. Thus, Scheuplein (167) has noted that "the vast improvement in our methods of analytical detection have [sic] exposed carcinogens in the food supply in amounts too minuscule for our carcinogen bioassays and our risk assessment procedures to evaluate with comparable precision. We are capable now, more than before, of asking scientific questions that we cannot answer." (p. 244)

CURRENT REGULATORY FRAMEWORK

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

It is clear from Table 2.1 that there is a broad range of chemical exposures with which federal

regulatory auth-

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Table 2.1 Federal laws related to exposures to toxic substances

Legislation	Agency	Area of Concern
Food, Drug and Cosmetics Act (1906, 1938, amended 1958, 1960, 1962, 1968, 1976)	FDA	Food, drugs, cosmetics, food additives, color additives,new drugs, animal and food additives, and medical devices
Federal Insecticide, Fungicide and Rodenticide Act (1948, amended 1972, 1975, 1978)	EPA	Pesticides
Dangerous Cargo Act (1952)	DOT, USCG	Water shipment of toxic materials
Atomic Energy Act (1954)	NRC	Radioactive substances
Federal Hazardous Substances Act (1960, amended 1981)	CPSC	Toxic household products
Federal Meat Inspection Act (1967); Poultry Products Inspection Act (1968); Egg Products Inspection Act (1970)	USDA	Food, Feed, color additives, and pesticide residues
National Environmental Policy Act (1970); amended 1975, 1985, 1987, 1989, 1996, 1997)	EPA	Ecosystems and natural resources
Occupational Safety and Health Act (1970; amended 1974, 1978, 1982, 1983, 1984, 1986, 1987, 1990, 1992, 1995, 1996, 1997)	OSHA, NIOSH	Workplace toxic chemicals
Poison Prevention Packaging Act (1970, amended 1981)	CPSC	Packaging of hazardous household products
Clean Air Act (1970, amended 1974, 1977, 1990)	EPA	Air Pollutants
Hazardous Materials Trasportation Act (1972)	DOT	Transport of hazardous materials
Clean Water Act (formerly Federal Water Pollution Control Act; 1972, amended 1977, 1978, 1987)	EPA	Water pollutants
Marine Protection, Research and Sanctuaries Act (1972)	EPA	Ocean dumping
Consumer Product Safety Act (1972, amended 1981)	CPSC	Hazardous consumer products
Lead-based Paint Poison Prevention Act (1973, amended 1976)	CPSC, HEW (HHS), HUD	Use of lead paint in federally assisted housing
Safe Drinking Water Act (1974, amended 1977) and subsequent amendments	EPA	Drinking Water, Contaminants
Resource Conservation and Recovery Act (1976, amended 1984)	EPA	Solid waste, including hazardous wastes
Toxic Substances Control Act (1976); Asbestos Information Act (1988)	EPA	Hazardous chemicals not covered by other laws, includes premarket review
Federal Mine Safety and Health Act (1977)	DOL, NIOSH	Toxic substances in coal and other mines
Comprehensive Environmental Response, Compensation, and Liability Act (1981); Superfund Amendments and Reauthorization Act (1986); Emergency Planning and Community Right-to-know Act (1986)	EPA	Hazardous, substances, pollutants and contaminants
Radon Gas and Indoor Air Quality Research Act of 1986	EPA	Indoor air
Oil Pollution Act (1990)	DOT	Oil pollution
Pollution Prevention Act (1990)	EPA	Toxics use reduction
Food Quality Protection Act-amendments to FDCA and FIFRA	EPA	Pesticides in food

Note. Adapted from Office of Science and Technology Policy (147)

orities are concerned. Chemicals may be regulated on the basis of environmental medium (e.g., air, water), activity (e.g., food manufacture, chemical transport, ocean dumping), and type of exposure environment (e.g., workplace, residential). While the statutes in Table 2.1 represent almost 100 years of federal legislative history, 17 of the 23 have been written since 1970, illustrating the recent increase in public concern about chemical exposures.

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

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

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

The Federal Food, Drug, and Cosmetic Act (FFDCA) is another example of a law requiring health-based standards for limiting the public's exposure to chemicals. Section 409 of the FFDCA requires the sponsor of a food additive to show that no harm to consumers will result when the additive is put to its intended use, and contains the Delaney Clause (discussed earlier), a special provision that forbids the use of any food additives that have been found to induce cancer in humans or animals. The Delaney Clause essentially specifies that the acceptable risks from carcinogens as food additives is zero. This bright line has proven to be very difficult for both the FDA and (until passage of the Food Quality Protection Act in 1996) for the EPA to implement, because the law does not allow the implementing agencies to specify *de minimis*, or acceptable, levels of risk. The EPA regulates pesticides under both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the FFDCA. Under the FIFRA, the agency was required to balance the risk from the pesticide with the benefit associated with its use. However, under the FFDCA, the EPA was bound by the Delaney Clause with regard to pesticides that may concentrate in processed foods above the level allowed on the raw agricultural commodity. This dichotomous standard (known as the "Delaney Paradox") forced the EPA to regulate to zero risk for pesticides applied to foods that may concentrate during processing, while regulating using risk-benefit analysis to regulate the same pesticides on raw agricultural commodities (134). In 1992, a Circuit Court ruled that the Delaney Clause does not allow the EPA to permit use of carcinogenic pesticides under the FFDCA, even if their use is associated with negligible risk (1). The Delaney Clause has also been difficult for the FDA to implement as more chemicals (including naturally occurring chemicals in foods) have been determined to be carcinogens, and the FDA has searched for ways to establish acceptable risk levels for food additives. In 1988, the U.S. Court of Appeals for the District of Columbia struck down an effort by the FDA to interpret the Delaney Clause as allowing the agency to set a *de minimis* risk level for two color additives for use in cosmetics and drugs (212).

Other laws require the implementing agency to balance the risks and benefits of alternative regulatory choices. Examples of balancing statutes include Section 408 of the FFDCA, which, until the Food Quality Protection Act (FQPA) of 1996, required tolerances for pesticide residues on raw agricultural commodities to be set at levels necessary to protect the public health, while considering the need for "an adequate, wholesome, and economical food supply" (134). Section 6 of the Toxic Substances

Control Act (TSCA) requires the EPA to consider the potential benefits of using a chemical and the economic consequences of restricting its use when determining whether the manufacture, distribution, use, or disposal of a substance presents an unreasonable risk of injury to health or the environment.

Language in the Occupational Safety and Health Act specifies that the Occupational Safety & Health Administration (OSHA) must "adequately assure(s) to the extent feasible...that no employee will suffer material impairment of health or functional capacity" (145). This statutory language also requires balancing of risks and costs, but is particularly interesting because the dual requirements of "feasibility" and "no employee will suffer" may be impossible to reconcile in certain situations (163).

While the narrative terms *unreasonable risk* and *ample margin of safety* have not been clearly or consistently defined across agencies or across statutes, over the past decade, agencies have generally interpreted this language as requiring a qualitative, and frequently quantitative, estimate of the health risks associated with an exposure and the reduction in risks resulting from regulatory action. A major factor in the increased use of risk analysis by regulatory agencies was the 1980 Supreme Court decision in Industrial Union Department v. American Petroleum Institute. In this case, OSHA proposed lowering the occupational standard for benzene from 10 ppm to 1 ppm on the basis that benzene was a carcinogen, that any reduction in exposure would result in a reduction in risk, and that 1 ppm was technologically feasible. The Supreme Court did not find for the Union, stating that "Before he can promulgate any permanent health or safety standard, the Secretary [of Labor] is required to make a threshold finding that a place of employment is unsafe—in the sense that significant risks are present and can be eliminated or lessened by a change in practices" (85). The Court left the decision of what constitutes a "significant risk" to the OSHA. This landmark decision has had a major impact on agencies in addition to OSHA, resulting in an increase in the development and use of tools to quantify risks from exposure to environmental chemicals.

Perhaps one of the most far-reaching (and likely precedent-setting) statutes in recent years is the Food Quality Protection Act (FQPA) of 1996. This statute, which addresses risks from pesticides in food through the setting of tolerance limits, is primarily risk based with limitations on the extent to which EPA can consider benefits. This is in contrast to the risk/benefit balancing requirements of the FFDCA noted earlier. Only in certain narrow circumstances under the FQPA can EPA set pesticide tolerance levels that do not meet health-based criteria. Specifically, the circumstances comprise those situations where the use of the pesticide prevents even greater risks from occurring to consumers (a risk/risk balancing) or where the lack of the pesticide would result in "a significant disruption in domestic production of an adequate, wholesome, and economical food supply." In addition, the FQPA eliminates certain aspects of the "Delaney Paradox" discussed earlier. Tolerance limits for pesticides in both raw agricultural products and processed foods, for both carcinogens and noncarcinogens (the Delaney clause considered carcinogens only), are now to be based on health only. Other important provisions of the FQPA include the requirement that EPA specifically consider exposures and risks to infants and young children in setting pesticide tolerance limits, allowing up to an additional 10-fold safety factor, the need to consider all pathways of exposure (e.g., drinking water, soil/dust ingestion, etc.) to a pesticide in setting tolerance limits for that pesticide in food; the need to consider the cumulative risk for multiple pesticides that act via a common mechanism of action when setting a tolerance limit for any single pesticide of the "common mechanism" class; and the establishment of a very ambitious comprehensive screening program for pesticides that exert estrogenic and possibly other endocrine effects (190). The statute represents a landmark piece of legislation not only in terms of the regulatory implications, but also with respect to the advancement in scientific understanding required to implement the statute.

The combined effect of the increasing use of risk assessment to help make regulatory decisions and the significant uncertainty that accompanies most quantitative estimates of toxicological risk has resulted in considerable debate about the practice of risk assessment. On one side of the debate, the EPA, the FDA, and other agencies have been criticized by the Office of Management and Budget (OMB) and many representatives of the regulated community for being too conservative in their risk assessment procedures (146). On the other side, environmental advocacy groups such as Greenpeace have claimed that "In the real world, quantitative risk assessments are used almost exclusively to justify pollution" (175). Others have noted that "current risk estimates are by no means routinely exaggerated, either for the entire populations they apply to or for highly exposed or highly susceptible individuals within those populations" (58). Much of this difference in opinion is perhaps due to the fact that risk estimates are frequently inadequately defined and presented. Often, risk assessors produce single-value estimates of risk that may apply to some unknown percentage of the population. Because the variability in the exposure and dose-response characteristics of a population are so large, the risk estimates for a small, highly exposed or sensitive subpopulation will generally be very different from the estimates of the most likely risks for the entire population. Although the risk assessment results are supposed to be qualified and uncertainty discussed, the risk number is often used without appropriate explanation of the inherent

variability. In a recent effort to address this problem, the EPA prepared guidance to risk assessors on the need to provide fuller, more explicit descriptions of risk when providing such information not only to risk

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managers but also to the general public (199). For example, the guidance recommends multiple descriptions of risk (e.g., sensitive receptors and general population) as well as clear descriptions of the uncertainties in the risk assessment.

Governmental and nongovernmental agencies other than those already discussed can influence the regulatory process as well. The American Conference of Governmental Industrial Hygienists (ACGIH) sets exposure limits based solely on health protection for approximately 600 workplace chemicals. These exposure limits, known as threshold limit values or TLVs (7), do not carry any regulatory weight, but it is not uncommon for workplaces to adhere to the TLVs for chemicals that OSHA does not regulate or which have an exposure limit that has not been revised since the inception of the OSHA in 1970. The TLVs have also been used by several state environmental agencies to derive acceptable ambient levels for toxic air pollutants.

Agencies in the Department of Health and Human Ser-vices that influence the regulatory process include the National Cancer Institute; the National Institute of Environmental Health Sciences, in particular the National Toxicology Program; the National Institute for Occupational Safety and Health (NIOSH) and the Center for Environmental Health (part of the Centers for Disease Control and Prevention, CDC); and the Agency for Toxic Substance and Disease Registry (ATSDR) (183). These agencies affect the regulatory process in several ways, ranging from decisions on which chemicals to test in long-term cancer bioassays, to defining principles for evaluating carcinogens, to conducting site-specific (as with a hazardous waste site) and chemical-specific risk assessments. International organizations such as the World Health Organization (WHO) and the International Agency for Research on Cancer (IARC) also have a significant role in the use of information by regulatory agencies.

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

Some states have also developed their own risk assessment procedures or standards. In the absence of emissions standards for hazardous air pollutants at the federal level, many states developed their own emissions standards for hazardous air pollutants. Under the CWA, states can develop water quality criteria for certain water bodies in the state. Many states also have Superfund-type laws for the cleanup of abandoned hazardous waste sites, and have consequently developed risk assessment procedures for these sites.

RISK ASSESSMENT PARADIGM

In response to a directive from the United States Congress, the FDA contracted with the National Research Council (NRC) of the National Academy of Sciences to evaluate the risk assessment process in the federal government and to make recommendations on how the process could be improved. As a result of this effort, the Committee on the Institutional Means for Assessment of Risks to Public Health published a book in 1983 entitled *Risk Assessment in the Federal Government: Managing the Process* (136). The book summarized past experiences, and although it did not propose new ways to evaluate risks from environmental chemicals, it has nevertheless had an important effect on the use of scientific information by regulatory agencies in its codification of the risk assessment process.

The report has been particularly influential in two areas: (a) the separation of the risk assessment process from the risk management process, and (b) the classification of the risk assessment process into four broad components: *hazard identification, dose-response assessment, exposure assessment,* and *risk characterization.* These concepts are illustrated in Figure 2.1.

More recently, in response to Congressional mandates, both the Committee on Risk Assessment of Hazardous Air Pollutants (established by the NRC under the direction of the EPA) and the Presidential/Congressional Commission on Risk Assessment and Risk Management (CRARM) have reevaluated risk assessment and risk management approaches. The findings of the NRC committee are published in a 1994 book entitled *Science and Judgment in Risk Assessment* (133), and the findings of CRARM are published in a 1997 report entitled *Risk Assessment and Risk Management in Regulatory*

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Decision-Making (155). While the four components making up the basic risk assessment paradigm and the separation of risk assessment and risk management remain key underlying principles, both committees recommended refinements in risk assessment and risk management

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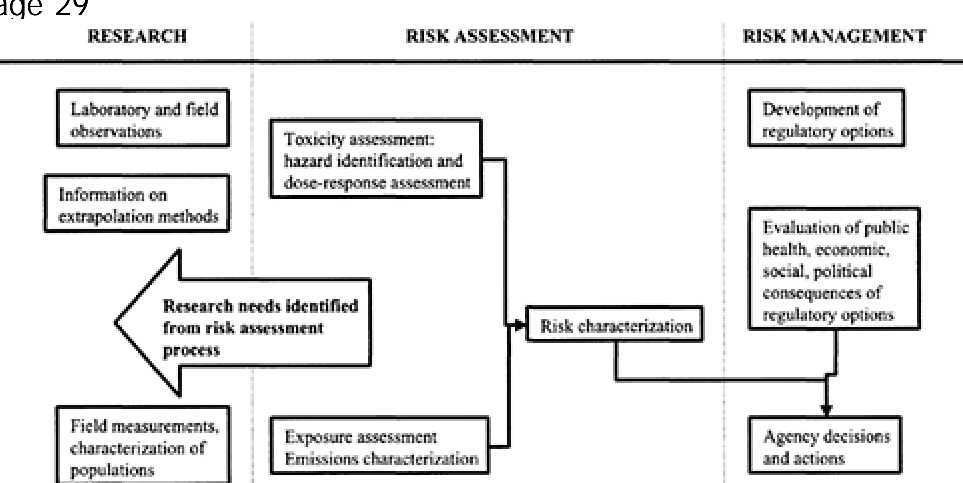

FIG. 2.1. NAS/NRC risk assessment/management paradigm. Adapted from NRC (133).

approaches. For example, the NRC committee highlights the importance of an iterative approach to risk assessment to reduce uncertainties, with each iteration incorporating fewer default assumptions and more specific information, balancing the use of "better science" with the constraints of the available resources (133). CRARM proposes a framework for risk management that encourages early and frequent involvement of all groups affected by the risk management problem, and decision making based on consideration of the risk management problem in the context of the broader, real-world goals of risk reduction and improved health status (155).

Risk assessment is defined as the "systematic, scientific characterization of potential adverse effects of human or ecological exposures to hazardous agents or activities," and involves assessment of the strength of the evidence as well as evaluation of the uncertainties associated with risk estimates (155). In contrast, risk management is "the process of identifying, evaluating, selecting, and implementing actions to reduce risk to human health and ecosystems" (155). Risk managers choose actions that will mitigate risks, considering not only the information derived from risk assessment, but also cultural, ethical, political, social, economic, and engineering information in the decision process.

The distinction between risk assessment and risk management is critical (136). The influence of risk management issues, such as the economic significance of a product, on the risk assessment process can seriously undermine the credibility of the risk assessment. This concern is exemplified in the separation between NIOSH and OSHA. NIOSH, part of the Department of Health and Human Services, is responsible for recommending health-based standards for workplace exposures to OSHA, part of the Department of Labor. As the federal agency actually responsible for setting standards for work place exposures and for implementing them, OSHA is also required to consider feasibility in the choice of exposure limits. It is not uncommon to find that exposure levels permitted by OSHA are different from NIOSH recommended exposure levels (182).

Of course, the distinction between risk assessment and risk management is not nearly so clear in practice. This is because each component of a chemical risk assessment is associated with considerable uncertainty. In the face of this uncertainty, regulatory officials have generally resorted to erring on the side of caution by including health-protective assumptions in their risk assessments. For example, the choice of a low-dose extrapolation model for carcinogens, which leads to a higher estimate of risk than other models, represents a risk management decision as much as a science policy decision. That is, the approach is conservative and provides the regulator with a greater level of confidence that the true risk to the human population is likely to be less than that expressed through the model. This approach has historically been justified as consistent with prudent public health policy when uncertainty is so great that it is difficult to provide a precise estimate of risk (i.e., in the face of uncertainty, it is easier to say the risk is less than *x* than to say the risk equals *y*). However, this practice can lead to inconsistent levels of protection for different chemicals and may direct resources away from the more significant risks (142). For example, the potential cancer risks associated with chemical disinfectants should be compared to the risks of waterborne microbial diseases when making decisions about treating public drinking water supplies, yet such risk/risk trade-offs

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cannot be accurately weighed if health-protective assumptions have been used to different extents in the underlying risk assessments (67). As noted earlier, the practice of using health-protective assumptions in conducting risk assessments has been identified as an inappropriate application of risk management to the risk assessment process (146).

Although risk assessments are commonplace at many federal and state agencies, there are no uniform guidelines that specify how regulatory officials should calculate chemical risks. There are also no uniform criteria that indicate how the findings of a risk assessment should influence regulatory decisions (165). As a result, potency estimates developed by different regulatory agencies for the same chemical can vary substantially (12). Furthermore, the level of risk sufficient to trigger regulatory action has varied considerably between agencies and even between different programs within a single agency (180). EPA's postregulatory "acceptable" risk levels for arsenic under one statute (the CAA) vary by four orders of magnitude (179).

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

The result is a scientific judgment that the chemical can, at some exposure concentrations, cause a particular adverse health effect in humans. The result is not a simple yes-or-no evaluation but a weight-of-evidence estimation of the likelihood that the particular chemical has the potential to cause the particular effect. For example, studies showing that ozone can suppress pulmonary defenses against microbial agents in several species of animals (129), and information on similarities in pulmonary defenses between humans and animals (68), would lead to the conclusion that ozone exposure in humans could, under certain conditions, result in an increased susceptibility to infection (205).

The hazard identification process has been codified mainly for carcinogens. as exemplified in the classification schemes from a variety of agencies including IARC (111), EPA (208), and OSHA (144). These schemes are discussed in more detail later in this chapter.

Dose-response evaluation, the second component of the risk assessment process, involves quantitative characterization of chemical potency, that is, the relationship between the dose of a chemical administered or received and the incidence or severity of an adverse health effect in the exposed population. Characterizing the dose-response relationship involves understanding the importance of the intensity of exposure, the concentration×time relationship, whether a chemical has a threshold, and the shape of the dose-response curve. The metabolism of a chemical at different doses, its persistence over time, and an estimate of the similarities in disposition of a chemical between humans and animals are also important aspects of a dose-response evaluation. While the National Academy of Science report considers dose-response estimates mostly in terms of carcinogens, the evaluation of the dose-response relationships has long been a key component of pharmacology and toxicology for many chemicals (136). Recent advances in dose-response assessment for noncancer risk assessment are described in chapter 8 by Rodericks et al. and in a later section of this chapter.

In *exposure assessment,* the third component of the risk assessment process, a determination is made of the amount of a chemical to which humans are exposed. Data are often very limited for exposure assessment. Measures of chemicals in environmental media, such as air or soil, or in food may be available; however, the extrapolation of those levels to a dose received by humans has many uncertainties. Models exist that can describe the movement of chemicals through a particular medium, and assumptions can be made regarding inhalation, ingestion, or dermal contact rates and the bioavailability of the chemical. This information can then be used to derive an estimate of the dose taken up by humans. Host factors, such as exercise, the use of certain consumer products, or the consumption of particular foodstuffs, will complicate the exposure assessment (see chapter 9, Exposure Assessment).

The use of biological monitoring such as measurement of volatile organic chemicals in exhaled breath for example (219)—and the use of personal sampling devices, such as respirable particulate monitors (178), represent new ways in which the uncertainties of exposure assessment can be reduced. Blood lead testing is another example of biological monitoring that has the ability to reduce the uncertainty in

quantifying exposure and in extrapolating from exposure to dose.

The last stage of the risk assessment process, *risk characterization*, involves a prediction of the frequency and severity of effects in the exposed population. That is, the information from the dose-response evaluation (what dose is necessary to cause the effect?) is combined with the information from the exposure assessment (what dose is the population receiving?) to produce an estimate

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of the likelihood of observing the effect in the population being studied. Most risk assessments, particularly for cancer, performed in the regulatory arena produce a single-number estimate of risk (e.g., lung cancer risk of 1 in a million). These are often designed to represent the risk to the reasonable maximally exposed (RME) individual in a potentially exposed population.

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

In addition to population variability, there is also significant *uncertainty* present in risk estimates, due to uncertainty in many of the risk assessment components (e.g., model and measurement error). It is critical that the risk characterization step of the risk assessment process describes the biological and statistical uncertainties in the final estimation and identify which component of the risk assessment process (hazard identification, dose-response, or exposure) involved the greatest degree of uncertainty. For example, the dose-response evaluation is generally highly uncertain. This is often due to the model error in extrapolating from animals to humans or short-term to life-time exposures. Information may not be available to characterize the active species, mechanism of effect, the effective dose, or absorption, metabolism, and excretion rates. McKone and Bogen (126) determined that 65% of the variance in the risk assessment results for a case study of tetrachloroethylene in groundwater was due to variance in the estimate of the chemical potency. Because the degree of uncertainty varies greatly among risk assessments for different chemicals, lack of consideration of uncertainty can lead to inappropriate levels of concern for different chemicals.

Monte Carlo uncertainty analysis techniques have been applied to the risk assessment process as one method of attempting to more fully characterize the distribution of potential risks in a population. Rather than using single values to represent input parameters such as contaminant ingestion rates, body weights, and chemical potencies, Monte Carlo analysis uses the range of potential values for each particular input parameter, as well as an estimate of how these values are distributed (termed the probability density function). These individual probability density functions are then used to calculate a probability density function for the risk estimate (47, 174, 193). Figure 2.2 shows a probability density function, produced by a Monte-Carlo analysis, which was calculated using probability density functions for individual input parameters. Although these techniques provide more information on the distribution of potential risk than a single number risk estimate, they are limited by the availability of information with which to characterize the input probability density functions. Particularly uncertain are estimates of chemical potency, which can vary by orders of magnitude depending upon beliefs regarding carcinogenic mechanisms.

The Committee on Risk Characterization, convened by the NRC, makes recommendations for improving the risk characterization process in its 1996 book entitled *Understanding Risk, Informing Decisions in a Democratic Society* (132). Rather than just a presentation of numerical risk results and associated uncertainties, a risk characterization should also convey the information in a clear and easily understandable way that is useful to risk managers in making informed decisions and that addresses the concerns of interested and affected parties. Therefore, the rigorous scientific analyses involved in risk characterization must be performed in conjunction with frequent deliberations with all interested and affected parties. As explained by the NRC Committee on Risk Characterization, "developing an accurate, balanced, and informative synthesis" involves "getting the science right," "getting the right science," "getting the right participation," and "getting the participation right" (132). The EPA adopted similar values in its 1995 risk characterization guidance (196).

TOXICOLOGY INFORMATION USED IN THE REGULATORY PROCESS

Three main categories of scientific information are employed by agencies in the evaluation and regulation of toxic chemicals in the environment: (a) epidemiology, (b) controlled clinical exposures, and (c) animal toxicology. In vitro studies and structure-activity relationships are typically used by regulatory agencies to support the interpretation of information from the three major categories, and are only occasionally used as a primary source of information.

Epidemiology, studies of clinical exposures, and animal toxicology provide qualitatively different

information, with unique advantages and limitations. Environmental epidemiology studies, which attempt to associate disease or other adverse health outcomes with an environmental exposure, have the advantage of measuring an effect in

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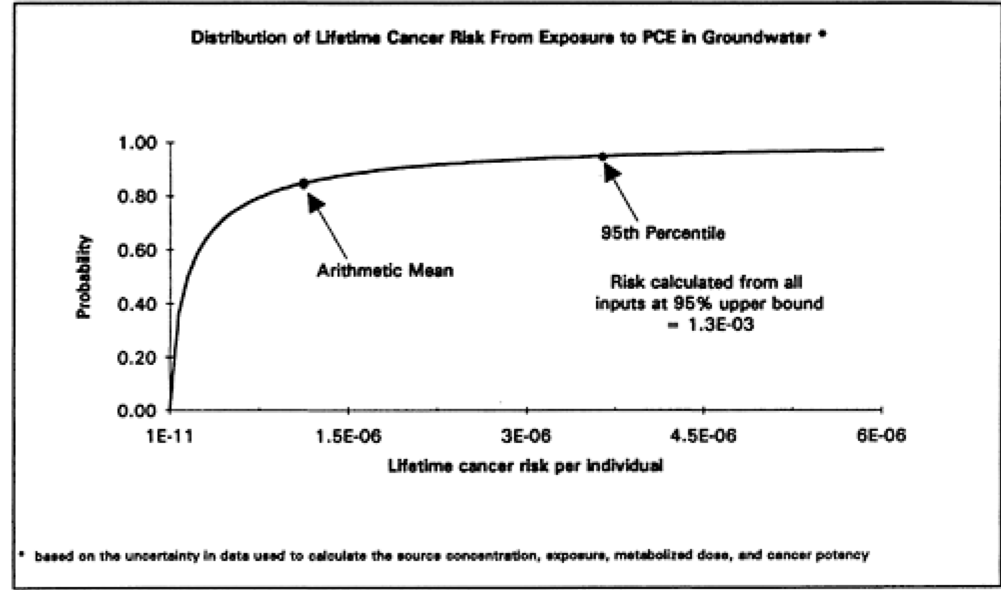

Selected Input Parameters and Distributions

Parameter	Units	Distribution Type	Min	Max	Arithmetic Mean	Arithmetic Std. Dev.	Geometric Std. Dev.
PCE concentration in water	mg/L	empirical			0.0003	0.00035	2.5
Breathing rate per unit body weight	m3/kg-day	lognormal			0.4	0.5	2.7
PCE metabolized cancer potency	kg-day/mg	empirical			0.11	0.14	4.8
Exposure time in house	h/day	uniform	8	20	14		
Skin permeability	m/h	uniform	0.004	0.01	0.007		

FIG. 2.2. Monte Carlo analysis of risk: tetrachloroethylene (PCE) in groundwater. Adapted from McKone and Bogen (126).

humans at exposure conditions that are by definition realistic. The first demonstration that benzene was a carcinogen came from epidemiological studies of rubber workers (86). It wasn't until several years after these studies that benzene was shown to cause cancer in animal studies (124). Studies of the London smog pollution episode in 1952 demonstrated that high levels of pollution from coal combustion could cause mortality, particularly in the very young, the elderly, and those individuals with preexisting cardiopulmonary disease (122). Evaluation of similar effects in animal studies would be difficult, given the complexity of the exposure in London and the lack of good animal models for susceptible populations, such as asthmatics. In general, epidemiology has been particularly helpful in the evaluation of working environments or other environments where exposure concentrations are relatively high.

Several factors limit the use of epidemiological studies by regulatory agencies. One of the major limitations is the lack of well-defined exposure information, both for chemical species and for actual concentrations. For example, the lack of accurate total exposure information limits the ability to quantify the effects of ambient air pollution in the United States. The Harvard University Six City Study showed that outdoor NO_2-monitoring devices are inadequate in accurately assessing total exposure to NO_2, due to the importance of indoor exposure (157). Subsequently, the EPA conducted Total

Exposure Assessment Measurement (TEAM) studies in which personal exposure to VOCs and pesticides were measured and found to be significantly higher than exposures estimated using stationary monitors for indoor or outdoor air (218). In some cases, the use of biological markers of exposure, such as measurements of arsenic levels in urine or lead levels in blood, can provide more accurate information about exposure and help reduce uncertainties in the results of epidemiological studies.

It is also difficult to define the causal element in epidemiological investigations, particularly when complex exposures are involved. For example, several indicators of pollution were measured during the London smog episodes that occurred between 1958 and 1972. Initial evaluations focused on the role of total particulate and SO_2 as causative agents for the elevated mortality levels; however, subsequent analyses of the London studies, as well as studies from other cities, indicate the importance of acid sulfates on mortality (176).

Another limitation is that epidemiological studies are frequently of worker populations, and such studies can be difficult to apply to prediction of health effects in the general population. Occupational studies, in general, focus on healthy adult male workers. The general population is more heterogeneous than the worker population and, for some pollutants, may exhibit a greater range in susceptibility. In general, only more recent epidemiology studies have considered adverse health effects of chemicals specific to women and children, such as developmental, reproductive, or hormonally mediated effects, including cancer. An example of the limitations of occupational epidemiology involves studies of peripheral nerve function in lead-exposed workers, which would underestimate risk of lead exposure in young children, for whom the primary concern is neurobehavioral effects resulting from relatively low-level exposures (217). At the same time, it must be recognized that the young and elderly are not always more susceptible to the effects of chemicals. As noted by Calabrese (25), adults, rather than the young or the elderly, are more susceptible to the renal toxicity of fluorides and mercury. High-risk subgroups are discussed in more detail in a later section of this chapter.

Epidemiology studies are frequently limited by the need for a relatively large increase in disease incidence (twofold or more), given the sample sizes generally available for such investigations. Enterline (55) notes that it would require a large population (1000 deaths, using the Peto model) to detect a 50% excess in deaths from lung cancer at an asbestos level of 2 fibers/cm^3 air.

Controlled clinical studies of humans exposed to pollutants address some of the difficulties of epidemiology studies. The exposures can be controlled and quantified, the effects are observed in humans, and the exposed population can be chosen to consist of susceptible individuals, such as asthmatics or exercising individuals. Thus, changes in airway resistance in asthmatics exposed to SO_2 during exercise (18, 164) have been important in the EPA evaluation of the National Ambient Air Quality Standard (NAAQS) for SO_2 (210), because these effects reflect the response of the susceptible population, using an appropriate exposure concentration and a relevant averaging time. Given the subtlety of these changes (nonsymptomatic bronchoconstrictions) and the fact that they occur only in a selected subset of the general population (asthmatics constitute about 4% of the total population), these effects would not have been detectable in the general population.

One of the advantages of controlled clinical exposure studies, that they are performed with humans, is also a major limitation. Since these studies must be limited to short-term effects that are readily reversible, they cannot be used to evaluate the potential of a chemical to cause chronic disease. Furthermore, because of the mildness of the changes observed in these studies, one may question their clinical significance. For example, how does one interpret the change in resistance observed with SO_2 exposure, given that, if not perceptible, the relationship to physical performance may be questionable (72)? Also, although some susceptible populations, such as mild asthmatics, can be tested, individuals with a greater degree of impairment, such as asthmatics who require continual medication, are usually not considered to be appropriate subjects for these studies because of the greater potential for harm during exposure. Later sections in this chapter address the questions of severity of effect on susceptible populations in greater detail.

Animal toxicology studies constitute the third major source of information for assessing the toxicity of chemicals. Animal toxicology studies allow the investigator the greatest degree of control over the exposure conditions, the population exposed, and the effects measured. One can readily evaluate subtle effects of acute and chronic exposure. For example, morphological and numerical changes in the pulmonary type I and type II cell populations, as well as interstitial fibrosis, have been observed in rats exposed to 0.06 to 0.25 ppm O_3 (35, 63). It would have been very difficult to describe this effect with other experimental approaches, and yet the effect is clearly of concern for humans who are exposed to comparable concentrations of ozone in certain urban environments.

In animal experiments, the ability to manipulate the experimental conditions permits the evaluation of many variables on the response to toxic chemicals. Thus, Elsayed and Mustafa (53) were able to demonstrate the protective effect of vitamin E on the acute toxicity of NO_2 in mice. In addition, the role of metabolism in susceptibility to polycyclic aromatic hydrocarbon-induced carcinogenesis has been evaluated in

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studies of genetic variants in mice (120, 138). Such studies can be important in predicting modifiers of toxicity in humans and in identifying the susceptible human populations.

The limitations of animal studies fall into two broad categories: (a) those due to uncertainties in extrapolating from animals to humans, and (b) those due to uncertainties in extrapolating from the high exposures in animal studies to the lower exposures typically experienced by humans. Interspecies extrapolation is complicated by the greater homogeneity of laboratory animals than humans, the controlled conditions of housing and diet, innate genetic factors, and other variables. The relevance of trichloroethylene (TCE)-induced hepatocarcinogenesis in the mouse to humans has been questioned on the basis of differences in peroxisomal proliferation in the liver in the two species (52). Similarly, high exposure concentrations typically used in animal studies may result in saturation of detoxification pathways and thus may produce effects that are not relevant to effects produced at ambient exposure concentrations, where detoxification pathways are not saturated. Increased numbers of macrophages and impairment of alveolar clearance are observed in rats exposed to relatively high concentrations of diesel particulates (80). The significance to humans who are exposed to ambient levels of diesel particulates much lower than those employed in the animal studies is uncertain (143).

Historically, in vitro studies and analysis of structure-activity relationships have been used to help set priorities for chemical testing. For example, structure-activity relationships have been used to predict mutagenicity, lethality, and carcinogenicity (54). This type of information can be very useful, for example, in selecting compounds for longer term testing in animals or eliminating chemicals being considered for potential industrial or pharmaceutical applications due to toxicological concerns.

Short-term tests have typically been used indirectly in the regulatory process to support decision making, rather than as a basis per se for decision making. For example, evidence that a chemical is a point mutagen in an in vitro test system might be used to support the classification of a chemical as a possible human carcinogen or the use of a linear dose-response model for carcinogenesis. Metabolism, pharmacokinetic, and mechanistic studies can also provide information to reduce uncertainties in the use of toxicology information. For example, metabolic studies showing that a critical reactive metabolite in rodents is also formed in humans could reduce uncertainties in extrapolating from animals to humans, while mechanistic studies could indicate whether a subtle effect observed in a clinical study is a precursor for later, more serious health endpoints, and therefore of concern as a biomarker of effect.

In some circumstances, short-term tests and structure-activity relationships may be used directly to provide a basis for decision making. For example, Beck and co-workers (16) estimated permissible levels for alkylphenols in water, based on the ability of different alkylphenols to inhibit cyclooxygenase and by comparison with toxic effects of aspirin, a well known cyclooxygenase inhibitor. A critical element in this example is that adequate toxicological data were available for some members of the classes of chemicals being studied and that estimates of risk were applied within a class of chemicals of similar physical/chemical properties.

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

We can conclude from the preceding discussion that there is no “best” source of information for regulatory agencies. The rational approach is to examine all avail-

Table 2.2 Advantages and disadvantages of epidemiological studies

Advantages	Disadvantages
Exposure conditions realistic	Costly and time-consuming
Occurrence of interactive effects among individual chemicals	Post facto, not protective of public health*a*
Effects measured in humans	Difficulty in defining exposure, problems with confounding exposure
Full range of human susceptibility frequently expressed	Increase in risk must frequently be about twofold to be detected
	Effects measured often relatively crude (morbidity, mortality)

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

Table 2.3 Advantages and disadvantages of controlled clinical studies

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

Table 2.4 Advantages and disadvantages of animal toxicology studies

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

able sources of information in the evaluation of toxic chemicals. Some kinds of information may be especially useful in hazard identification, the likelihood that a chemical will be toxic to humans, whereas other types of information will be more appropriately applied to the estimation of the dose-response relationship.

EVALUATION OF CARCINOGENS

Background

The public demand for zero risk has made the regulation of carcinogens a formidable challenge. Within the scientific community, there is still no consensus on how to define a potential human carcinogen, much less how to estimate cancer risks under practical conditions of chemical exposure. This uncertainty is due largely to the fact that mechanisms of carcinogenesis for many chemicals are still poorly understood, and different carcinogens act in different ways to induce cancer. The task of regulating carcinogens has been complicated, rather than simplified, by many of the mechanistic discoveries of recent years. The simple picture of the 1950s, when only a very small number of chemicals were thought to be carcinogens, has been replaced by the realization that chemical carcinogenesis takes place in multiple stages, some with reversible steps, which have different dose-response relationships. Essential nutrients and hormones can be carcinogenic in some circumstances. The same chemical can promote or inhibit carcinogenesis, depending on the circumstances of exposure (75, 121). Public pressure to regulate carcinogens, even where very little toxicological information exists, has in many circumstances compelled regulatory agencies to treat carcinogens as though they all act by the same mechanisms, even as it has become apparent that they do not. Despite virtual consensus in the scientific and regulatory communities that carcinogens should be regulated in a way that reflects their mechanism of action, sufficient information to allow this to be done does not exist for many chemicals.

From a public health standpoint, the regulatory agencies have generally regulated carcinogens at exposure levels that reflect a very low probability of tumor production (e.g., excess cancer risk of 1 per million exposed). However, for practical reasons, it is impossible to conduct animal studies of a size that would allow observation of effects following treatment at such low doses. The practice has therefore been to conduct animal studies at high dose levels and then extrapolate the results from high to low dose and from animals to humans. Thus, the chronic animal bioassay results, the extrapolation from high to low doses, and the extrapolation across species

are used to derive potency factors (i.e., indicators of carcinogenic potency) for carcinogens. These potency factors enable one to relate a dose in humans to a probability of tumor occurring as a result of that dose. It should be noted that even with established human carcinogens, extrapolation procedures are still used to extrapolate carcinogenic response from high to low dose or from one type of exposure condition (e.g., intermittent, subchronic) to another exposure condition (e.g., continuous chronic).

This section on carcinogens first provides some basic information on mechanisms of carcinogenesis. We then describe some of the key issues that agencies address in the interpretation and application of scientific data on carcinogens. These issues fall into the categories of hazard identification and dose-response assessment (136). Hazard identification for carcinogens addresses two questions: (a) What is the evidence that a particular chemical is an animal carcinogen? (b) What is the likelihood that an animal carcinogen is a human carcinogen? Dose-response assessment attempts to determine the probability of tumor production, given a particular exposure or dose level. The dose-response assessment section of this chapter discusses mathematical models used to extrapolate from high to low doses, physiologically based pharmacokinetic (PBPK) modeling to relate administered and effective doses in animals and humans, and issues concerning the relationship between effective dose and response.

Mechanisms of Carcinogenesis

Carcinogenesis is currently understood to be a multistage process that has been described as involving the initiation, promotion, and progression of normal cells into neoplastic cells. Chemicals can act at one or more of these stages, and can act directly (e.g., mutagen) or indirectly (e.g., immune suppression).

Initiation is generally understood to be a permanent and irreversible event involving DNA mutation, and the first step in the process of carcinogenesis. Many genotoxic agents are considered to be initiators, thus having the potential to begin the transition from normal to cancer cells. Genotoxic materials have been considered to act via a nonthreshold mechanism, and this belief has formed the basis for linear extrapolation of effects seen at high doses down to low doses. Inferences as to the absence of a threshold for initiating agents comes from the study of mutations that result from these agents. In addition, studies investigating the number of preneoplastic focal lesions induced by an initiating agent did not find a measurable threshold (149). Ionizing radiation is an example of an initiating agent. In addition, certain chemicals (e.g., aflatoxin B1, diethylnitrosamine, tobacco smoke) are considered to be complete carcinogens, capable of initiation, pro motion, and progression. Potential factors modifying the efficiency of initiation include rates of cell division and DNA synthesis as well as the rate of metabolism of a chemical to its active form or rate of metabolic detoxification. (It should therefore be noted that even the no-threshold concept may not be applicable to all genotoxic carcinogens, due to the existence of repair mechanisms and other factors that reduce or eliminate responses at low exposure levels.)

The promotion stage is characterized by clonal expansion of the initiated cells. Promoting agents can act by various mechanisms to increase rates of cell proliferation or decrease rates of cell death. For example, cell proliferation can be induced by cytoxic agents or mitotic agents. Interference with intercellular communication may also be responsible for clonal expansion of initiated cells (181). An important feature of this stage is its reversibility and, in some cases, the existence of a threshold for the effect. In many cancer model systems, withdrawal of the promoting agent halts the development of tumors. The promotion stage is also easily modulated by environmental factors including frequency of dosing, age of test animal, and diet (149, 195). Promoting agents are generally thought to exhibit a threshold (or inflection point) in the dose-response curve. Examples of promoting agents include hormones, alcohol, and dietary fat.

Progression is an irreversible stage characterized by the development of malignant neoplasms, and is understood to require a second genetic mutation. Agents that act only during progression, or advance a cell from promotion to progression, have not yet been definitively characterized. It has been hypothesized that malignant neoplasms may all exhibit an abnormal expression of one or more proto- and cellular oncogenes (149). In this scenario, initiation is defined by the first mutation event and progression as the second mutation, resulting in homozygosity at the anti-oncogene locus, and total loss of growth control (130).

Hazard Identification

The question of how to decide whether a particular chemical is a potential human carcinogen is currently the subject of considerable scientific debate. It is an important question because the act of labeling some chemicals "carcinogens" and not labeling others has profound regulatory and societal implications (66). This regulatory paradigm, whereby chemicals are regulated either as carcinogens or as noncarcinogens, requires that the question of whether a particular chemical is a carcinogen or not be

answered with a "yes" or "no." In the United States, most regulatory agencies have historically regulated all carcinogens as though they operate via the same no-threshold mechanism. However,

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different chemicals may act in different ways during the various stages of cancer formation to impact the development of the tumor. These various mechanisms of tumor formation are not all consistent with the mechanistic assumptions that form the basis of the regulatory framework for carcinogens. Thus, the more we learn about carcinogenesis, the less the simple regulatory approach is able to accommodate the new information. A chemical may be carcinogenic via certain routes of exposure and not others, or only above certain dose levels. More flexible classification approaches are being developed (e.g., ref. 195) that allow the incorporation of more science into the classification process.

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

Animal Studies

The evidence that a chemical is an animal carcinogen frequently derives from long-term animal bioassays. Such studies usually consist of exposing groups of about 50 animals (typically rats or mice) to two concentrations of a chemical over the lifetime of the animals. Sex- and age-matched unexposed animals constitute the control group. At the termination of the bioassay, the animals are killed and the number of tumor-bearing animals and the number and type of tumors per animal are quantified. Interim examinations may be performed, particularly on animals that appear moribund.

The maximum tolerated dose. Dose selection plays a key issue in the design and interpretation of the animal bioassay. Animals are typically exposed at two dose levels: the maximum tolerated dose (MTD) and half the MTD. The MTD is predicted from subchronic toxicity studies as the dose "that causes no more than a 10% weight decrement, as compared to the appropriate control groups, and does not produce mortality, clinical signs of toxicity or pathologic lesions (other than those related to a neoplastic response) that would be predicted [in the long-term bioassay] to shorten an animal's natural lifespan" (171). The MTD is not a nontoxic dose and is expected to produce some level of acceptable toxicity to indicate that the animals were sufficiently challenged by the chemical. The MTD has been justified as a means of increasing the sensitivity of an animal bioassay involving limited numbers of animals so as to be able to predict risks in large numbers of humans (76).

An objection to the use of MTDs has been that metabolic overloading may occur at high dose levels, leading to an abnormal handling of the test compound (131). For example, toxic metabolites could be produced as a consequence of saturation of detoxification pathways. Organ toxicity could occur that might not happen at lower concentrations of the chemical (127), particularly at those concentrations to which humans are typically exposed. Thus, it has been argued that nongenotoxic agents that are determined to be positive in rodent carcinogenicity bioassays, due to target organ toxicity and subsequent cell proliferation, should not be assumed to be carcinogenic at low doses (37).

Ames and co-workers (9,10) have suggested that target-organ toxicity and subsequent mitogenesis is responsible for the fact that over half of all chemicals tested in chronic bioassays at the MTD are determined to be carcinogens in rodents. They observed that both genotoxic and nongenotoxic agents tested at the MTD cause increased rates of mitogenesis, thus increasing the rate of mutation. For several chemicals, induction of tumors was more strongly correlated with cell division than with DNA adducts or mutagenic activity. Others have reported that cancer potency and MTD are inversely correlated and that, consequently, the potency estimate is simply an artifact of the experimental design (161). Goodman and Wilson (65) found that cancer potency and the MTD were more strongly related for nonmutagens than for mutagens in rat bioassays, indicating that the carcinogenic effect and toxicity were more closely associated for nonmutagens than for mutagens. However, they noted that even for most mutagens, their findings suggested that, at high doses, carcinogenicity is induced via mechanisms associated with toxicity.

Haseman and Lockhart (77) compared the sensitivity of the rodent bioassay for detecting carcinogens at the MTD as compared to lower doses. Approximately two-thirds of the chemicals that were positive at the MTD in rodents were also positive at the lower dose, albeit often for fewer tumor sites. One tumor site that was affected disproportionately was the kidney, where a positive response was observed at the lower dose only about one third of the time as compared to the higher dose.

The EPA (195) noted that bioassay results at doses that exceeded the MTD can be rejected if toxic damage to target organs compromises study interpretation. The reason is that dosing above the MTD in a study may result in tumor production secondary to tissue damage rather than a direct carcinogenic

influence of the agent tested.
Thus, use of information from testing at fractional doses of the MTD may yield results that are more relevant to human risk. Overall there would be a somewhat modest (except for kidney-only carcinogens) reduction in the total number of carcinogens. It should also be noted that

it is still possible that mechanisms may occur at fractional MTD levels that would not occur at the typically lower human exposure levels.

Other issues in hazard identification. Another key issue in the evaluation of animal bioassays is the analysis of the tumors themselves. Considerations include the categorization of benign tumors and whether tumor analysis should be site-specific or based on all sites. The position of the IARC (110) is that "few, if any chemicals exist which produce only benign tumors and no malignant tumors in any species" and that chemicals that cause a marked increase in the number of benign tumors "are now viewed with almost as much suspicion as potential human hazards as they would have been if the induced tumors had been malignant." Thus, it has been the general policy of regulatory agencies to accord almost the same weight to benign tumors as to malignant tumors, especially if there is evidence that the benign tumors could progress to malignancy (208).

It is sometimes stated that one should consider only the overall incidence of tumors, since, from a public health perspective, the concern is with total cancer risk for humans rather than risk at any one site. While this position has an innate appeal, it is difficult to apply in practice for two main reasons:

- This approach greatly decreases the ability of the bioassay to detect a positive effect, given the high background incidence of some tumor types in rodents. For example, the incidence of testicular tumors can be as high as 82% in rats and liver tumors can be as high as 25% in mice (74).
- The grouping of tumor types that do not share a common cellular origin is of questionable biological relevance, since the mechanisms involved in the production of the different tumor types could differ. Furthermore, the metastatic potential of different tumor types is highly variable and would have an important influence on the lethality of a particular type of cancer.

It should also be noted that reductions in tumor incidence are frequently observed in the same cancer bioassays in which tumor increases are observed. A recent analysis by Linkov and co-workers (121) indicates the anticarcinogenic effects observed in rodent bioassays are not explained by random effects. The basis for the reduction is not known and could be a consequence of perturbations in the animal's physiology. These observations lend credence to the concept that animal bioassays must be interpreted with special attention as to whether biological phenomena are induced at high doses that may not occur (or occur with a much lower frequency) at low doses. A similar observation is found in the evaluation of some human carcinogens, in particular those which act through hormonal processes. For example, oral contraceptives are associated with an increased risk of breast cancer, but a decreased risk of ovarian and endometrial cancer (Table 2.5). Anticarcinogenic properties of carcinogens are typically not considered as part of the regulatory process for carcinogens.

In addition, the standard NTP-type 2-year cancer bioassay may not be sensitive to hormonally regulated cancers such as breast cancer. This is because the mouse and rat strains used to perform these bioassays are selected because they are known to be susceptible to liver, kidney, or lung tumors in particular. It has not been demonstrated that these strains are also susceptible to cancers at hormonally sensitive sites. Thus, these bioassays may not detect certain cancer effects that are hormonally regulated (172).

Carcinogen Classification Schemes

The IARC, the European Union (EU), the EPA, the National Toxicology Program (NTP), the German Commission for Investigation of Health Hazards, Health Canada, and the ACGIH have developed classification schemes for carcinogens, based on a weight-of-evidence or strength-of-evidence evaluation of available human and animal studies. These seven classification systems are shown in Table 2.6. The EPA developed guidelines in 1986 (208) and proposed new guidelines in 1996 (195) (Tables 2.7–2.10). Some classification schemes are based on the weight of evidence considering positive and negative evidence (e.g., EPA), whereas others (e.g., IARC) classify chemicals as carcinogens, based on a strength-of-evidence (positive evidence only) basis. The different guidelines use mechanistic information to different degrees.

The EPA (208), IARC (111), and other agencies typically conclude that a chemical demonstrating "sufficient evidence of carcinogenicity" from animal experiments is a potential human carcinogen. To some degree this conclusion is supported by evaluation of known human carcinogens in animal bioassays. For the 67 chemicals, processes or environmental factors associated with cancer indication in humans by IARC (112, 177), more than half of those that have been tested have also been positive in animal bioassays (Table 2.5). Recent understanding of carcinogenesis indicates that this assumption is not valid for all animal carcinogens. Species-specific responses or high-dose-only effects indicate that positive animal results are not always evidence of human carcinogenicity.

In 1996, EPA proposed new cancer guidelines (195). These proposed guidelines, in their flexibility and

incorporation of new science, represent a significant advance in carcinogen classification schemes. The new guidelines take a weight-of-evidence approach in which human (Table 2.7), animal (Table 2.8), and other relevant

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Table 2.5 Chemicals, industrial processes, and environmental factors associated with cancer induction in humans: target organs and main routes of exposure in humans and degree of supporting evidence in animals (IARC)

Chemical or industrial process	Humans: Main type of exposure[a]	Humans: Target organ(s)[b]	Animals, degree of evidence for carcinogenicity
1-(2-Chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (methyl-CCNU)	Medicinal	Leukemia	Limited
1,4-Butanediol dimethanesulfonate (Myleran)	Medicinal	Leukemia	Limited
2-Naphthylamine	Occupational	Bladder (liver)	Sufficient
4-Aminobiphenyl	Occupational	Bladder	Sufficient
8-Methoxypsoralen (Methoxsalen) plus UV radiation	Medicinal	Skin	Sufficient
Aflatoxins	Environmental	Liver (lung)	Sufficient
Alcoholic beverages	Cultural	Oral cavity, pharynx, larynx, esophagus, liver (breast)	Inadequate
Aluminum production	Occupational	Lung, bladder (lymphoma, esophagus, stomach)	No data
Arsenic compounds[c]	Occupational, medicinal, and environmental	Skin, lung (liver, hematopoietic system, gastrointestinal tract, kidney)	Limited
Asbestos	Occupational	Lung, pleura, peritoneum, gastrointestinal tract, larynx	Sufficient
Auramine manufacture	Occupational	Bladder	No data
Azathioprine	Medicinal	Lymphoma, skin, mesenchymal tumors, hepatobiliary system	Limited
Benzene	Occupational	Leukemia	Sufficient
Benzidine	Occupational	Bladder	Sufficient
Beryllium and beryllium compounds[d]	Occupational	Lung	Sufficient
Betel quid (with tobacco)	Cultural	Oral cavity (pharynx, larynx, esophagus)	Limited
Bis(chloromethyl) ether and chloromethyl methyl ether (technical grade)	Occupational	Lung	Sufficient
Boot and shoe manufacture and repair	Occupational	Leukemia, nasal sinus (bladder, digestive tract)	No data
Cadmium and cadmium compounds[e]	Occupational	Lung	Sufficient
Chlorambucil	Medicinal	Leukemia	Sufficient
Chromium compounds (hexavalent)[f]	Occupational	Lung (gastrointestinal tract)	Sufficient
Ciclosporin[e] (U.S.: cyclosporin)	Medicinal	Lymphoma, Kaposi's sarcoma	Limited
Coal tars and pitches, mineral and shale oils, and soots[f]	Occupational, environmental	Skin, lung, bladder (gastrointestinal tract, leukemia, colon)	Sufficient
Cyclophosphamide	Medicinal	Bladder, leukemia	Sufficient
Diethylstilbestrol	Medicinal	Cervix/vagina, breast, testis (endometrium)	Sufficient
Epstein–Barr virus[h]	Environmental	Lymphomas, nasopharyngeal carcinoma	Sufficient
Erionite	Environmental, cultural	Pleura, peritoneum	Sufficient
Estrogens (steroidal, nonsteroidal)[e]	Medicinal	Endometrium, breast, cervix/vagina, testis	Sufficient
Estrogen therapy, postmenopausal[f]	Medicinal	Endometrium, breast	No data
Ethylene oxide[e]	Occupational	Lymphatic and hematopoietic systems	Sufficient
Furniture and cabinet making	Occupational	Nasal sinus	Inadequate
Helicobacter pylori[h]	Environmental	Stomach	No data
Hematite mining (with radon exposure)	Occupational	Lung	Inadequate
Hepatitis B virus[f]	Environmental	Liver	Inadequate

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Chemical or industrial process	Humans: Main type of exposure[a]	Humans: Target organ(s)[b]	Animals, degree of evidence for carcinogenicity
Hepatitis C virus[m]	Environmental	Liver	Inadequate
Human immunodeficiency virus Type 1[e]	Environmental	Kaposi's sarcoma, non-Hodgkin's lymphoma	Inadequate
Human papilloma virus Types 16 and 18[e]	Environmental	Cervix	None
Human T-cell lymphotropic virus Type I[f]	Environmental	Adult T-cell leukemia/lymphoma	Inadequate
Ionizing radiation	Environmental	Leukemia, skin, various internal organs	Sufficient
Iron and steel founding	Occupational	Lung (digestive tract, genito-urinary tract, leukemia)	No data
Isopropyl alcohol manufacture (strong acid process)	Occupational	Nasal sinus (larynx)	Inadequate
Magenta manufacture	Occupational	Bladder	Inadequate
Melphalan	Medicinal	Leukemia	Sufficient
MOPP and other combined chemotherapy including alkylating agents	Medicinal	Leukemia	No data
Mustard gas	Occupational	Lung, larynx, pharynx	Limited
Nickel and nickel compounds[f]	Occupational	Nasal sinus, lung (larynx)	Sufficient
N, *N*-Bis(2-chloroethyl)-2-naphthylamine (Chlornaphazine)	Medicinal	Bladder	Limited
Opisthorchis viverrini[g]	Environmental	Liver	Limited
Oral contraceptives, combined[i,j]	Medicinal	Liver (also protective effect against cancers of the ovary and endometrium)	Sufficient
Oral contraceptives, sequential	Medicinal	Endometrium	Sufficient
Painters (occupational exposures as)	Occupational	Lung (esophagus, stomach, bladder)	No data
Phenacetin (in analgesic mixtures)	Medicinal	Renal pelvis/ureter, bladder	Limited
Radon and its decay products	Environmental	Lung	Sufficient
Rubber industry	Occupational	Bladder, leukemia (lymphoma, lung, renal tract, digestive tract, skin, liver, larynx, brain, stomach)	Inadequate
Salted fish[e]	Environmental	Nasopharynx	Limited
Schistosoma haematobium[g]	Environmental	Urinary bladder	Limited
Silica, crystalline[e]	Occupational	Lung	Sufficient
Solar radiation[e]	Environmental	Skin	Sufficient
Talc containing asbestos fibers	Occupational	Lung (pleura)	Inadequate
Tamoxifen[e]	Medicinal	Endometrium (reduces risk for contralateral breast cancer in women with previous diagnosis of breast cancer)	Sufficient
2,3,7,8-Tetrachlorodibenzo-*para*-dioxin[e]	Occupational	Multi-site with no site predominating	Sufficient
Thiotepa[l]	Medicinal	Leukemia	Sufficient
Tobacco products, smokeless	Environmental, cultural	Oral cavity (pharynx, esophagus)	Inadequate
Tobacco smoke	Environmental, cultural	Lung, bladder, oral cavity, larynx, pharynx, esophagus, pancreas, renal pelvis (stomach, liver, cervix)	Sufficient
Treosulphan	Medicinal	Leukemia	No data
Vinyl chloride	Occupational	Liver, lung, brain, lymphatic and hematopoietic system (gastrointestinal tract)	Sufficient
Wood dust[m]	Occupational	Nasal cavity, paranasal sinus	Inadequate

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Note. From IARC (112), Tomatis et al. (177), IARC (115), and references in other footnotes.

[a] The main types of exposure mentioned are those by which the association has been demonstrated; exposures other than those mentioned may also occur.

[b] Suspected target organs in parentheses.

[c] The evaluation of carcinogenicity to humans applies to the group of chemicals as a whole and not necessarily to all individual chemicals within the group.

[d] Source: International Agency for Research on Cancer (87).

[e] Source: International Agency for Research on Cancer (88).

[f] Not all chemicals in this group are associated with all cancers listed.

[g] Source: International Agency for Research on Cancer (89).

[h] Source: International Agency for Research on Cancer (90).

[i] Source: International Agency for Research on Cancer (91).

[j] Source: International Agency for Research on Cancer (92).

[k] Source: International Agency for Research on Cancer (93).

[l] Source: International Agency for Research on Cancer (94).

[m] Source: International Agency for Research on Cancer (95).

[n] Source: International Agency for Research on Cancer (96).

[o] Source: International Agency for Research on Cancer (97).

[p] Source: International Agency for Research on Cancer (98).

[q] Source: International Agency for Research on Cancer (99).

[r] Source: International Agency for Research on Cancer (100).

[s] Source: International Agency for Research on Cancer (101).

[t] Source: International Agency for Research on Cancer (102).

[u] Source: International Agency for Research on Cancer (103).

[v] Source: International Agency for Research on Cancer (104).

[w] Source: International Agency for Research on Cancer (105).

[x] Source: International Agency for Research on Cancer (106).

[y] Source: International Agency for Research on Cancer (107).

[z] Source: International Agency for Research on Cancer (108).

[aa] Source: International Agency for Research on Cancer (109).

toxicological (Table 2.9) evidence are evaluated. As part of this evaluation, the quality of an individual study, as well as the overall consistency across studies, is considered. Demonstration of cancer in humans at the same organ site in multiple studies with well-characterized exposure in the absence of confounding exposures enhances confidence that a chemical is the likely cause of cancer. In contrast to the 1986 guidelines, other evidence relevant to carcinogenicity, such as mechanistic information in animals that attests to the relevance (or lack of relevance) of a particular tumor response, is explicitly considered. Data from human, animal, and other sources are combined to weigh the totality of evidence (Table 2.10) to classify the human carcinogenic potential of a particular chemical. Three descriptors have been proposed:

• Known/likely—Applies to chemicals for which there is convincing human evidence of carcinogenicity or limited (or no) human evidence combined with strong animal evidence that is relevant to the human response.

• Cannot be determined—Applies to chemicals where a conclusion regarding carcinogenicity cannot be drawn for reasons including the presence of conflicting or inadequate data, or suggestive data not sufficiently strong to draw any conclusion.

• Not likely—Applies to chemicals for which there is experimental evidence (in the absence of human data suggesting a carcinogenic potential) that indicates a lack of human hazard potential. Types of evidence include demonstration that a response in animals (e.g., male rat kidney cancer due to $\alpha 2\mu$-globulin nephropathy) is not relevant to humans or that well-conducted animal cancer bioassays are negative for carcinogenicity. One noteworthy aspect of the "not likely" category is that, in contrast to the 1986 guidelines, this category allows for conditional classification. For example, a chemical may be classified as likely to be a human carcinogen only with respect to a particular route of exposure, or only above a certain dose level.

Perchloroethylene provides a useful example of how different agencies classify carcinogens. For example, IARC considers perchloroethylene a probable human carcinogen (category 2A), based on limited evidence in humans and sufficient evidence in animals (114). In contrast, Health Canada (123) and ACGIH (6) both consider perchloroethylene as probably not carcinogenic to humans (categories A5 and Group V, respectively). IARC placed greater weight on often times conflicting epidemiological evidence than did ACGIH or Health Canada. For example, IARC considered the positive association between perchloroethylene and certain types of cancer (e.g., non-Hodgkin's lymphoma) as unlikely to be due to chance, while still acknowledging the possible role of confounding factors (e.g., exposure to other solvents). In contrast, Health Canada (123) considered the weaknesses in the epidemiological studies sufficiently large as to make the studies inadequate for drawing any conclusions on perchloroethylene carcinogenicity. With respect to animal studies, both IARC and Health Canada concluded that perchloroethylene causes liver cancer in mice. However, Health Canada concluded that the liver tumors in mice are unlikely to be relevant to humans, based on metabolic differences between humans and mice [humans produce much less trichloroactic acid (TCA) the relevant metabolite in the liver] and species-specific response differences to TCA (humans show little, if any, peroxisomal proliferation, which is likely to play a critical role in hepatic carcinogenesis in response to TCA). Overall, IARC gave greater weight to positive evidence than to negative or conflicting evi-

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Table 2.6 Summary of the classification schemes for carcinogens

Agency	Classification	Meaning
DFG/MAK	A1:	Induces malignant tumors in humans.
	A2:	Clearly carcinogenic in animal studies.
	B	Justifiably suspected of having carcinogenic potential.
EU	1	Carcinogenic to humans.
	2	Should be regarded as if carcinogenic to humans.
	3	Cause for concern in humans.
		3A. Substances that are well investigated.
		3B. Substances that are insufficiently investigated.
IARC	1	Carcinogenic to humans.
	2	Reasonably anticipated to be a carcinogen.
		2A. Probably carcinogenic in humans: limited human evidence, sufficient animal evidence.
		2B. Possibly carcinogenic in humans: limited human evidence in the absence of sufficient animal evidence.
	3	Not classified.
	4	Probably not carcinogenic to humans.
ACGIH	A1	Confirmed human carcinogen.
	A2	Suspected human carcinogen, limited human evidence and sufficient relevant animal evidence
	A3	Confirmed animal carcinogen with unknown relevance to human, epidemiologic studies do not confirm risk to humans.
	A4	Not classifiable.
	A5	Not suspected as human carcinogen, based on properly conducted epidemiologic studies or evidence in animal studies.
Health Canada	Group I	Carcinogenic to humans.
	Group II	Probably carcinogenic to humans. Inadequate epidemiologic evidence; sufficient evidence in animal species.
	Group III	Possibly carcinogenic to humans. Inadequate or flawed epidemiologic studies. Limited animal evidence, or adequate animal evidence, but involves epigenetic mechanisms.
	Group IV	Unlikely to be carcinogenic in humans. No evidence in adequate epidemiologic studies; positive animal studies of limited or unlikely relevance to humans.
	Group V	Probably not carcinogenic in humans. No evidence in adequate epidemiologic studies. No evidence or inadequate evidence in animal studies.
U.S. EPA	–A	Human carcinogen. Sufficient epidemiologic evidence.
	–B	Probable human carcinogen.
		B1. Limited epidemiologic evidence, sufficient animal evidence.
		B2. Inadequate or no epidemiologic evidence, sufficient animal evidence.
	–C	Possible human carcinogen. Limited animal evidence, no epidemiologic evidence.
	–D	Not classifiable. Inadequate or no human or animal evidence.
	–E	Evidence of non-carcinogenicity for humans. No evidence for carcinogenicity in at least two adequate animal species in both adequate epidemiologic and animal studies.
NTP	–1	Known to be a carcinogen.
	–2	Reasonably anticipated to be a carcinogen.
		A. Limited evidence in human studies indicating credible causal relationship evidence in human studies.
		B. Sufficient evidence in animal studies.

Note. DFG/MAK, Deutsche Forschungsgemeinschaft/maximale arbeitsplatz-Konzentration (German Commission for the Investigation of Health Hazards of Chemical compounds in the work area) (49, as cited in 140). EU, European Union (56, as cited in 140). IARC, International Agency for Research on Cancer (111,113,115). ACGIH/TLV, American Conference of Governmental Industrial Hygienics, threshold limit values (5). Health Canada (79), Human Health Risk Assessment for Priority Substances, as cited in http://www.tera.org/.ter/methods/cancer.htm. U.S. EPA (206), see Table 7.10 and text for proposed modifications. NTP, National Toxicology Program, as cited in ref. 5.

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Table 2.7 Factors for weighing human evidence

Increase Weight	Decrease Weight
Number of independent studies with consistent results	Few studies Equally well designed and conducted studies with null results
Most causal criteria satisfied: •Temporal relationship •Strong association •Reliable exposure data •Dose response relationship •Freedom from bias and confounding or results cannot be explained by bias and confounding •Biological plausibility •High statistical significance	Few causal criteria satisfied

Note. Adapted from U.S. EPA (195).

Table 2.8 Factors for weighing animal evidence

Increase Weight	Decrease Weight
Number of independent studies with consistent results	Single study; inconsistent results
Same site across species, similar response with structural analogues	Single site/species/sex
Multiple positive observations across species, sexes, tumor sites	
Severity and progression of lesions	Benign tumors only
• Early in life tumors/malignancy	
• Lesion progression through malignancy	Hight background of incidence tumors
• Uncommon tumor	
Route of administration like human exposure	Route of administration unlike human exposure

Note. Adapted from U.S. EPA (195).

dence on mechanistic data. In contrast, Health Canada took more of a weight-of-evidence approach, similar to that proposed by EPA in the 1996 guidelines (195).

Dose-Response Assessment

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

Table 2.9 Factors for weighing other key evidence

Increase Weight	Decrease Weight
A rich set of other key data are available	Few or poor data
Physicochemical data (e.g., electropholicity) support hazard potential	or
Data indicate reactivity with macromolecules	Inadequate data necessitating use of default assumptions
Structure-activity relationships support hazard potential	or
Comparable metabolism and toxicokinetics across species	Mechanistic and other data show that animal findings are not relevant to humans
Toxicological and human clinical data support tumor findings	
Biomarker data support attribution of effects to agent	
Mode-of-action data support causal interpretation of human evidence or relevance of animal evidence	

Note. Adapted from U.S. EPA (195).

Table 2.10 Factors for weighing totality of evidence

Increase weight	Decrease weight
Evidence of human causality	Data not available or do not show causality
Evidence of animal effects relevant to humans	Data not available or not relevant
Consistency across studies	Conflicting data
Comparable metabolism and toxicokinetics between species	Metabolism and toxicokinetics not comparable
Mode of action comparable across species	Mode of action not comparable across species

Note. Adapted from U.S. EPA (195).

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

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

Low-Dose Extrapolation

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

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Threshold versus nonthreshold mechanisms. The determination of whether carcinogenesis is a threshold or nonthreshold phenomenon is a key consideration in the choice of the model used to characterize the dose-response relationship. It is considered plausible that carcinogenesis could be a nonthreshold phenomenon for genotoxic agents, particularly those that act directly to cause mutations. For example, the human carcinogen vinyl chloride is an electrophilic agent and is understood to interact with DNA (220). However, there is much debate over whether carcinogenesis is a threshold phenomenon for many chemicals that do not interact directly with DNA (not directly genotoxic) and that may induce cancer through epigenetic mechanisms.

In addition, for many carcinogens, it is unclear whether the dose-response relationship observed at high doses is necessarily the same as the dose-response relationship that might occur at low doses. Because the measure of a chemical's carcinogenic potency is typically determined by fitting a model to the observed data and then extrapolating to low doses, the implicit assumption is that the dose-response relationship is the same at high and low doses. For many chemicals that cause cell damage at high doses, or for chemicals for which detoxification pathways become saturated at high doses, it is likely that a different dose-response relationship will be observed at high and low doses, even for those chemicals where a non-zero slope is plausible at any dose.

Butterworth (22) has noted that there are many different classes of non-DNA-reactive carcinogens, and that the characterization of carcinogens as either genotoxic or nongenotoxic is too simple to adequately reflect the numerous mechanisms by which nongenotoxic carcinogens exert their effects. For example, the potent promoter TCDD, which is characterized as a hormone-type carcinogen, acts by binding to a specific receptor, resulting in enzyme induction via an apparent no-threshold mechanism. Thus, Portier and co-workers have postulated that TCDD may cause cancer by a nonthreshold mechanism despite its characterization as a nongenotoxic carcinogen (152). Other types of nongenotoxic carcinogens include phenobarbital, a non-DNA-reactive carcinogen that is understood to act by altering growth control (increasing mitogenesis), and saccharin, which appears to exhibit initiating/promotional and/or carcinogenic activity as secondary events to the cytotoxicity and increased cell proliferation caused by the high dose levels used in animal bioassays (22). Thus, saccharin would not be anticipated to increase tumor production at doses that do not cause cytotoxicity, and phenobarbital might not be expected to cause cancer at doses that do not affect growth control.

Zeise et al. (222) reviewed the experimental evidence for various shapes of dose-response relationships for carcinogens. They concluded that "reliable high dose data from human studies contains examples of superlinearity (radium injections and bone cancer), linearity (various radiation exposures), and sublinearity (smoking)." (p. 301). Their analysis of animal studies indicated that the "variety of shapes of dose-response curves observed for humans was also seen for animals." Zeise et al. noted that there are no data to indicate the shape of dose-response relationships at doses corresponding to lifetime risks of one in a million; in humans there are some data for incidence rates as low as 1%, and in animals there are two large studies that provide data at lifetime risks of a few tenths of a percent. Because carcinogens act by different mechanisms, it is very likely that different carcinogens, or classes of carcinogens, will exhibit different types of dose-response relationships. Moreover the same carcinogen may cause cancer at different tumor sites via different mechanisms.

A striking example of different dose-response relationships for a single carcinogen is the example of 2-acetylaminofluorene (2-AAF) (40). 2-AAF is a potent mutagenic carcinogen. The dose-response relationship for 2-AAF-induced liver cancer exhibits the expected (for a genotoxic carcinogen) linear dose-response relationship, whereas the dose-response relationship for bladder cancer is highly nonlinear, demonstrating an apparent threshold. The mechanistic basis for the different dose-response relationships appears to involve differences in the relative importance of genetic damage (the likely key event in liver cancer) versus genetic damage *and* hyperplasia of the bladder urothelium (the likely key events in bladder cancer). Thus, selection of the appropriate shape of the dose-response relationship for *any* chemical requires understanding of the mechanism by which tumors are induced.

Mathematical models. The choice of the low-dose extrapolation model can have a major impact on the estimate of risk at low exposure levels. Figure 2.3 shows the estimate of risk from 2-acetylaminofiuorene at low exposure levels, using different models. The level of risk varies by many orders of magnitude at the same exposure level, depending on the model chosen to characterize the dose-response curve in the unobservable region. One of the more common models used by regulatory agencies particularly in the United States has been the linearized multistage model (12). The EPA has used the upper 95% confidence limit of this model on the basis of its biological plausibility (it assumes a nonthreshold) and its conservatism (it is unlikely to underestimate risk at low exposure levels) (208) to develop cancer

slope factors (CSF), in units of $(mg/kg\text{-}day)^{-1}$. CSFs relate dosage (mg/kg-day) to the probability of an individual developing cancer. Cancer potency estimates for chemicals are highly dependent on model choice. Anderson (12) analyzed cancer potency estimates for TCDD, derived by using different low dose extrapolation

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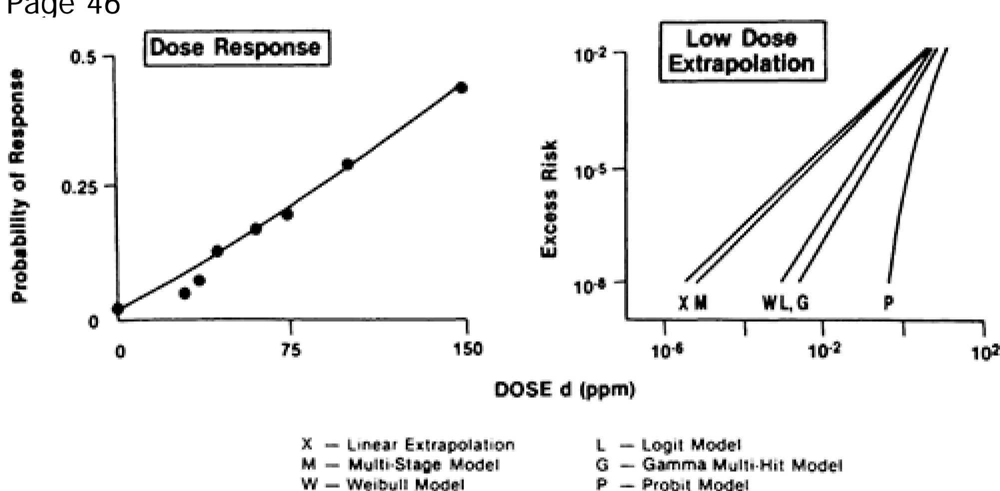

FIG. 2.3. Low-dose extrapolation for 2-acetylaminofluorene under several mathematical models. From Bickis and Krewski (19a).

models and different selection and treatment of bioassay data, and found that model choice alone (Weibull, multistage, log probit) would account for a difference in calculated cancer potency of 13 orders of magnitude.

One difficulty with the use of the upper 95% confidence limit of this model is that this approach basically reduces the model to worst-case curve fitting and does not take basic carcinogenic mechanisms into account. Thus, Cook et al. (43) have stated that if the multistage model as they defined it for carcinogenesis were correct, then the value of *k*, or the number of stages of cellular transformation, would generally be from four to six. However, the algorithm used in the calculation of cancer risk by the EPA for the multistage model requires *k* to assume a value not greater than the number of dose levels used in the study. Because three dose levels are usually employed—the MTD, half the MTD, and controls—the value of *k* is restrained to be, at most, equal to 3. Furthermore, when the MTD dose has been overestimated and excessive mortality occurs, the MTD cannot be used for risk assessment, thereby reducing *k* from 3 to 2. Allowing the value of *k* to be determined by the number of dose levels in a study, rather than on an understanding of the process of carcinogenesis, results in risk estimations of the multistage model that have reduced biologic relevance (43).

Because of the uncertainties in dose-response modeling for low dose cancer risk and the growing acceptance of a threshold dose-response relationship for some carcinogens, in the proposed 1996 cancer risk assessment guidelines, the EPA revised the approach to dose-response assessment (195). If sufficient data are available, a biologically based dose-response model rep resents the most appropriate method for evaluating the observed data and for extrapolating to exposures below the observed dose range. Data are not available for most carcinogens for development of biologically based models. In this case, a "point of departure" approach is recommended. The point of departure represents a dose, within the range of observed data, associated with a 10% extra tumor risk. The point of departure is developed using the linearized multistage model and expressed as the lower 95% confidence limit on the dose associated with 10% extra risk (LED10). Risks below the LED10 are characterized either through linear extrapolation (for chemicals believed to act via a linear dose-response relationship (e.g., genotoxic carcinogens), or through a margin-of-exposure analysis (for chemicals whose dose-response relationship is likely to be either threshold or nonlinear). For chemicals where data might support either linear extrapolation or a margin-of-exposure analysis, both analyses are to be presented.

A panel organized by the International Life Sciences Institute (ILSI) recently applied the 1996 EPA guidelines to an assessment of the dose-response relationship for chloroform (64). The ILSI panel evaluated the large database of information relevant to the mode of action by which chloroform induces liver tumors in rodents. The group identified a number of key elements important to chloroform's likely mode of action: lack of evidence of genotoxicity; tumor induction at doses associated with frank toxicity at the tumor sites; and the role of cytotoxicity and compensatory cell proliferation in tumor induction. The group concluded that the evidence did not support a linear dose-response relationship. A margin-of-exposure analysis was considered to represent

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the most appropriate method for evaluating the potential hazards of chloroform at low doses. The significance of such an approach is potentially quite large with respect to regulatory decision making. In the case of chloroform, use of the margin-of-exposure analysis would yield a permissible level in drinking water in the United States of 300 μg/L, a 60-fold increase beyond the present permissible level of 5 μg/L.

The linearized multistage model is clearly not appropriate for estimating the low-dose carcinogenic potency of some carcinogens. This is largely because experimental evidence indicates that for most chemicals, the dose-response relationship at the high doses used for model fitting is likely to be different than that at low doses. Pharmacokinetic and pharmacodynamic models can and should be used to more accurately characterize the dose-response relationship at low doses. In the absence of information to perform such assessments, alternative approaches, such as those proposed by the U.S. EPA (195), represent a reasonable interim measure.

EVALUATION OF SYSTEMIC TOXICANTS

In its broadest sense, systemic toxicity refers to all adverse effects, but in general it is applied only to chemicals that are postulated to induce an adverse effect through a threshold mechanism. That is, for these chemicals, there is a level of exposure below which there is minimal, if any, chance for an adverse effect. The effects range from skin and eye irritation to subchronic or chronic damage to any organ system, such as pulmonary fibrosis.

The underlying hypothesis for the threshold model for systemic toxicants is that multiple cells must be injured before an adverse effect is experienced, and that the injury must occur at a rate that exceeds the rate of repair. This is in contrast to the commonly used paradigm for carcinogenesis, in which a genotoxic insult to a single cell is theoretically sufficient to allow that cell to grow to a malignant tumor (215). An example of a threshold-type injury can be seen with pulmonary fibrosis due to mineral dust exposure. Fibrotic areas may be present and observed as radiographic or histopathologic changes in the lungs of miners as a consequence of mineral dust exposure in the absence of any physiological impairment such as reduced forced expiratory volume in 1s (FEV1) or in the absence of changes in lung volume. Physiological impairment will occur as the fibrosis increases and the fibrotic areas begin to coalesce (223).

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

The RfD and Uncertainty Factors

The general approach for setting exposure limits for systemic toxicants differs from that commonly used for carcinogens. Basically, appropriate uncertainty factors are applied to an experimental exposure to yield a level classically defined as the *acceptable daily intake* (ADI). The ADI represents a daily intake level of a chemical in humans that is associated with minimal or no risk of adverse effects. The ADI is expressed in terms of milligrams of chemical per kilogram of body weight per day (135). The EPA refers to such an exposure level as the *risk reference dose* or RfD. The basis for the change in terminology is that the ADI does not represent a magic dividing line between safe and nonsafe, but represents an estimated dose, derived through a consistent methodology, at which the chance of adverse effects is estimated to be negligible. The lack of precision is reflected in EPA's description of the RfD as having an uncertainty perhaps spanning an order of magnitude (200).

An ADI or RfD is typically based on either a no-observed-adverse-effect level (NOAEL) or a lowest-observed-adverse-effect level (LOAEL) from an epidemiology or animal toxicology study. "Uncertainty" factors (UFs), also termed "safety" factors, are then applied to the NOAEL or LOAEL to account for uncertainties in the relationship between exposure to a chemical in an animal study and a particular effect, and the relationship between lifetime daily exposure to the same chemical in the general population of humans and the likelihood of a particular effect.

The history as well as the experimental support for UFs in developing a permissible dose for production against systemic toxicity in humans have been described by Dourson and Stara (51). These authors describe four categories of UFs:

- UFH: An up to 10-fold uncertainty factor to account for variations in susceptibility in humans. Thus, if a NOAEL was defined from a long-term study in humans, this would be the only UF applied. In a later section of this chapter we discuss in detail the variability in human responsiveness to environmental pollutants and its relevance to the regulatory process.

• UFA: An up to 10-fold uncertainty factor to extrapolate from animal data to human data. This factor is used for animal studies and is based on the assumption that some humans may be more susceptible than experimental animals to

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a particular chemical. The default assumption is that the magnitude of the increased susceptibility is within a factor of 10.
• UFS: An up to 10-fold uncertainty factor to extrapolate from a subchronic exposure to a chronic exposure. This factor is used for studies that involve less than lifetime exposure and is based on the assumption that if the chemical were given over the lifetime of the animal rather than over a fraction of the lifetime, a smaller amount of chemical would result in the same NOAEL.
• UFL: An up to 10-fold uncertainty factor to extrapolate from a LOAEL to a NOAEL. This factor is used for studies in which a NOAEL was not identified. The default assumption is that a dose at 1/10 the LOAEL would result in a NOAEL.
More recently, EPA has developed a fifth uncertainty factor, UFD, which may be applied when the database is incomplete (203). The assumption here is that when the database for a chemical is limited, there is uncertainty as to whether the identified NOAEL might be significantly lower if other studies were performed, or whether a different NOAEL might have been identified if additional health endpoints (such as reproductive toxicity) had been evaluated. A complete database is defined as having two chronic mammalian studies, one mammalian multigeneration study, and two mammalian developmental toxicity studies. If these five studies are available, then there is a high degree of confidence that one has approximated the lowest NOAEL.
Uncertainty factors are used multiplicatively. To derive the RfD, EPA divides the exposure level from the toxicity study by the UFs. Mathematically, this is represented as

$$\mathrm{RfD} = \frac{\mathrm{LOAEL\ or\ NOAEL}}{\mathrm{UF}_1 \times \mathrm{UF}_2 \ldots \mathrm{UF}_n}$$

The use of all five UFs (UFH, UFA, UFS, UFL, UFD), each representing an order of magnitude, could in theory lead to a total uncertainty factor of 100,000. This would occur if data were from a subchronic animal study that identified only a LOAEL and the database was limited. However, since the multiplication of four or five factors of 10 each is likely to yield unrealistically conservative RfDs, EPA has restricted the total uncertainty factor calculation as follows: when uncertainty exists in four areas, EPA uses a 3000-fold total uncertainty factor, and when uncertainty exists in five areas, EPA uses a 10,000-fold total uncertainty factor (203).
Although 10 represents the default value for a UF, values less than 10 may also be used, depending upon the nature of the available information. For example, in deriving an RfD for chromium(VI), a factor of 3 was applied for the UFs, to account for the fact that the one year exposure duration in the principal study was less than lifetime, but longer than typical subchronic studies in rodents (186). No UFs were used in developing the RfD for fluoride because the NOAEL for the critical effect (dental fluorosis) was observed in the sensitive population (children) for a sufficiently long exposure duration (188).
In addition, a modifying factor (MF) that is greater than 0 and less than or equal to 10 may be used to address uncertainties not addressed in the other factors. For example, the use of a very large number of animals in a study may enhance certainty in the RfD, resulting in the use of a MF less than 1, but greater than 0. Alternatively, when an RfD is based on a very limited number of animals, an MF greater than 1 but less than or equal to 10 may be appropriate (203).
The RfD approach represents a generally accepted (NAS, FDA, and EPA, among others) method for setting lifetime exposure limits for humans, and the use of default 10-fold uncertainty factors has some experimental support, particularly as upper bound estimates (51). For example, the ratio between the subchronic and chronic NOAEL or LOAEL for 52 chemicals was less than 10 in 96% of the cases, as described in the analysis by Dourson and Stara (51). Thus, the uncertainty factor of 10 would be an underestimate for only 4% of these chemicals. A similar analysis regarding subchronic to chronic was performed by Lewis (15), who observed that for 18 chemicals, the ratio of the subchronic to the chronic NOAEL had a ratio of 3.5 or less for 14 chemicals and only 1 had a ratio of greater than 10. If the chemical with a ratio greater than 10 were excluded from the analysis, the mean subchronic to chronic NOAEL ratio was 3.3. Thus, the default UF of 10 for extrapolating from subchronic to chronic exposures would be very protective for most chemicals, and an UF of 3 may be more appropriate than the default value of 10 for many chemicals.
There are several limitations in the RfD approach, the net result of which is that exposures resulting in the same RfD do not imply the same level of risk for all chemicals, and that exposures above the RfD do not represent the same increase in risk for all chemicals. First, the choice of a LOAEL or a NOAEL does not take into consideration the greater experimental confidence associated with, for example, studies using more experimental animals. An exposure concentration defined as a NOAEL in one experiment

could turn out to be a LOAEL, had more experimental animals been used (i.e., an effect might have been detected using more animals). As a result, poor experiments may yield anticonservative RfDs, since studies using fewer animals may result in a higher RfD than studies using larger numbers of animals (46).

In addition, the RfD approach does not make use of dose-response information, which is a key determinant in assessing the likelihood of effects. Thus, a chemical

with a steep dose-response curve would be associated with a greater likelihood of effects as exposure increased above the RfD and a smaller likelihood of effects with exposures below the RfD than would a chemical with a more shallow dose-response curve (50).

There are also difficulties with the implications of specific UFs. The default value of 10 for the interspecies uncertainty factor (UF*A*) is a reasonable assumption in some cases, but in other cases may not be appropriate. For chemicals for which metabolism is a key determinant of toxicity, interspecies differences may be due mainly to physiological and metabolic differences across species (32). Under this assumption, interspecies differences are believed to scale according to allometric principles, that is, when the dose is expressed on a dose-per-unit surface area, different species are presumed equally sensitive to a chemical (51). According to this interpretation, then, a scaling factor based on surface area (body weight2/3) should account for interspecies differences (32, 51). For rodent toxicity studies (most commonly used in toxicological risk assessments), such a scaling factor would be about 8 for rats and 13 for mice, similar to the default value of 10 for the UF*A*. However, using this interpretation the default value of 10 would not be adequately protective for much smaller animals, and would be overly protective for much larger animals.

If, however, pharmacokinetic modeling has been used to estimate a biologically effective dose and to extrapolate dose across species, the use of an interspecies UF*A* may be unnecessary. For example, this may apply to chloroform, for which a mechanistically based dose-response model for hepatotoxicity has been developed (42). A similar conclusion has been reached by Jarabek and co-workers, who have developed a methodology for estimating the *reference concentration,* or RfC (116). The RfC is an air concentration of a chemical that is expected to be associated with minimal risk, if any, for adverse effects in humans, including susceptible populations (116, 204). As such, the RfC is the functional equivalent of the RfD, except that it is based on an air concentration, rather than an administered dose. Overton and Jarabek (148) noted that when dosimetric adjustments (e.g., through physiologically based pharmacokinetic modeling) are made, use of the value of 10 for the UF*A* for cross-species extrapolation may be inappropriate. This is because the dosimetric adjustment has already addressed some of the basis for interspecies variability. (Additional description of the RfC methodology is provided in chapter 8 by Rodricks et al.)

Differences in metabolism, however, are insufficient to explain all interspecies differences in toxicity. For some chemicals, innate differences in responsiveness are responsible. For example, the difference in susceptibility to the lethal effects of 2,3,7,8-tetrachlorodibenzodioxin is about 10,000-fold greater in guinea pigs than in hamsters and is likely a reflection of differences in Ah receptor activity and other genetic elements (158). Another example is seen in studies involving the fibrogenic mineral dust alpha-quartz. Macrophages of different animal species, when exposed to the fibrogenic mineral dust, alpha-quartz, do not demonstrate the same response in terms of levels or characteristics of fibroblast stimulating factor (73, 81). These differences are consistent with differences in the nature and extent of the fibrotic response to intratracheally instilled alpha-quartz (3, 21, 70).

Alternative Approaches to the RfD

There are alternative approaches to the standard RfD approach. By employing dose-response modeling and statistics, these alternative approaches can address issues of experimental quality, the shape of the dose-response curve, and other limitations of the RfD approach. Examples include the *benchmark dose* method (57, 198), probabilistic RfD approaches (13, 169), and distributional population approaches. These methods are described next.

The EPA defines a *benchmark dose* (BMD) as "a statistical lower confidence limit on the dose producing a predetermined level of change in adverse response compared with the response in untreated animals" (198). For example, a BMD could represent the 95% lower confidence limit on a dose that produces a 10% increase in a particular adverse health effect. The BMD is then used like a NOAEL or LOAEL, and appropriate UFs are applied to derive an RfD based on a BMD. Calculation of a BMD is illustrated in Figure 2.4.

The BMD approach overcomes many of the weaknesses of the RfD approach (57, 198). Because BMDs are determined based on statistical modeling of dose-response data, the approach incorporates information on the sample size and the shape of the dose-response curve, information that is not taken into account using the standard RfD approach. Unlike NOAELs or LOAELs, BMD values are not constrained to be based on one of the experimental doses tested, and are less dependent on the study design. Also, the BMD approach can be used for both threshold and nonthreshold adverse health effects, as well as for both quantal and continuous toxicity data. The BMD approach allows for greater consistency between values derived for different chemicals. A further benefit of the BMD approach is

that it allows for possible future harmonization of cancer and noncancer risk assessment methods (45). For any particular chemical, uncertainty factors applied to NOAELs or LOAELs using the standard RfD approach may not be appropriate to apply to a

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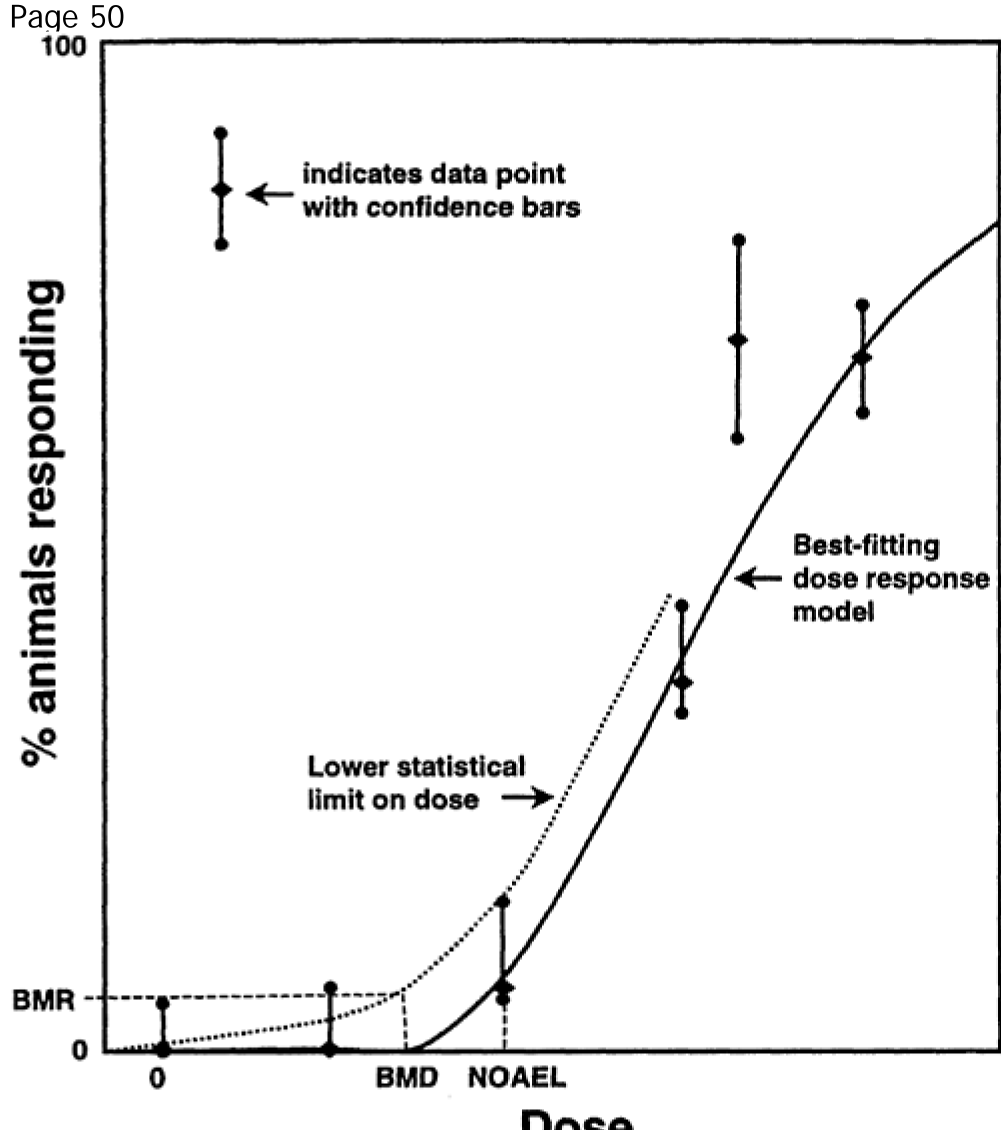

FIG. 2.4. Example of calculation of a BMD. Source: U.S. EPA (198).

BMD. This is because additional dose-response information has already been incorporated into the BMD. For example, when using a BMD, a steeper dose-response curve or a less severe critical effect may warrant a smaller value for UF*L* than would a more shallow dose-response curve or a more severe effect (50).

Recently, the EPA has adopted the BMD approach in developing RfDs for a number of chemicals, including beryllium, methylmercury, and tributyltin oxide (185). Similarly, benchmark concentrations (BMC) have been used to develop RfCs for antimony trioxide, carbon disulfide, methylene diphenyl diisocyanate, methyl methacrylate, phosphoric acid, 1,1,1,2-tetrafluoroethane, and chromium(VI) particulates (185). For all chemicals listed, a 10% relative change was chosen as the benchmark response. Although the EPA has performed a benchmark dose analysis for naphthalene, the resulting RfD (3×10^{-2} mg/kg-day) was very similar to the value derived using a standard NOAEL/RfD approach (2×10^{-2} mg/kg-day), and the EPA decided to use the NOAEL/RfD approach in deriving the final EPArecommended value (189).

A probabilistic approach for developing RfD or ADI values, combining distributions for each uncertainty factor using a probabilistic approach to generate an overall distribution for the no-adverse-effect level in sensitive human subpopulations, has been recommended by the Netherlands National Institute of Public Health and the Environment (169). This approach allows consideration of all available data on various uncertainties, and minimizes the conservatisms introduced by multiplying numerous UFs. A similar approach has also been recommended by Baird and co-workers (13).

For compounds that have been well studied, particularly in humans (both in terms of exposure and in terms of toxicity), distributional population approaches have been used to evaluate toxicity and to

provide input into the decision-making process. Such approaches have been

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applied mainly to evaluation of the National Ambient Air Quality Standards (NAAQS)—standards for moderately toxic air contaminants that are ubiquitous in the United States. Much of the basis for selection of a NAAQS is based upon human toxicological data, although animal data are used in a supporting role.

An example of a distributional population approach can be seen in the recent evaluation of carbon monoxide (CO) toxicity and exposure by EPA's Ambient Standards Branch (201). As part of this assessment, EPA reviewed several studies that evaluated the relationship between exposure to carbon monoxide, using carboxyhemoglobin (COHb) in blood as an indicator of exposure, and percent decrease in time to angina or pain in the chest, as an indicator of effect. Most of the studies showed an impact of low COHb levels on angina. However, there was no consistent dose-response relationship when studies were analyzed in the aggregate. This may have been due to differences in study design, study populations, and other factors. Because of the lack of a clear dose relationship, EPA evaluated the impact of different concentrations of CO in air upon various "cutoff' points of COHb, from 2.1 to 3.0%. These cutoff points are conceptually similar to the LOAEL used in RfD development. Levels of CO that result in COHb of 2.9 to 3.0% or higher might constitute frank effect levels (FEL). This is because levels of COHb of 2.9 to 3.0% or higher in persons with heart disease are considered as possibly increasing the risk of myocardial ischemia and diminishing blood flow to the heart.

The risk of CO exposure to people with heart disease in Denver, CO (36, 345 individuals at the time), was estimated under different carbon monoxide levels (201). The number of person-days where individuals might have at least one hourly COHb level greater than or equal to a defined percent COHb was estimated. Table 2.11 presents some of the results of this analysis. For example, under conditions at the time (considering both indoor and outdoor sources of carbon monoxide), there were approximately 488 person-days in which the Denver population with preexisting heart disease would experience COHb levels greater than 2.1%. If only ambient air is considered, then the person-days drops to 72. If the NAAQS for CO is attained, then the person-days drops to 457 for all sources and 0 for ambient air only. This type of analysis is useful in showing the benefits of CO reduction, as well as identifying the significance of different sources.

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

Endpoints—Developmental Toxicity

Recent refinements in the evaluation of systemic toxicants have focused on specific target-organ systems, such as the nervous system or the developing fetus. In this section, we focus on developmental toxicity as illustrative of the use of a specific endpoint in regulatory toxicology, including, for example, classification schemes and approaches toward setting permissible levels. Evaluation of developmental toxicants presents some unique challenges in risk assessment. This is because standard chronic and subchronic toxicity tests cannot be used to provide information on developmental effects in the offspring of exposed mothers. Rather, animal selection and exposure conditions must be designed specifically to assess developmental effects.

Developmental toxicity has been defined to include "any detrimental effect produced by exposures during embryonic stages of development" (135). These effects may include structural abnormalities, functional abnormalities, growth retardation, or lethality (202).

Table 2.11 Heart person-days with at least one hourly COHb estimate greater than or equal to value for four alternative scenarios

	"As is" air quality		"Just attain" air quality	
Exposure indicators	"Ambient air" plus internal sources	"Ambient air" without internal sources	"Ambient air" plus internal sources	"Ambient air" without internal sources
COHb≥2.1%	488	72	457	0
COHb≥3.0%	37	0	24	0

Note. Adapted from U.S. EPA (201).

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The effects may be reversible, such as a temporary reduction in growth rate, or irreversible, such as an overt physical malformation.

We discuss the evaluation of developmental toxicants in the regulatory process by focusing on four main issues:

• Selection of testing protocols.
• Relevance of animal studies to hazard identification in humans, including weight of evidence classifications.
• Use of the RfD approach to assess risk.
• Use of other approaches to assess risk.

Selection of Testing Protocols

The use of animal studies is particularly critical in evaluating developmental toxicants, as compared to chemicals that induce other effects. Reasons for this include the emphasis on adult males in occupational epidemiology studies, the lack of a long-term national registry of birth defects, and the difficulty in identifying certain endpoints, such as resorptions.

Types of studies used to evaluate developmental toxicity include the conventional "segment 2" study, in which the dams are exposed typically during the period of fetal organogenesis, and litters are evaluated for a number of endpoints, including number of viable offspring, types and incidence of skeletal or visceral malformations or variations, and body weight (119, 202). Segment 1 tests focus on fertility and reproductive performance of males and females. Segment 3 tests include perinatal and postnatal study after treatment of females only and, as such, may be considered sequential to the segment 2 test. In addition, multigeneration studies are also performed to assess fertility, reproductive performance, and, sometimes, teratology (119).

In the segment 2 tests, maternal toxicity endpoints, such as organ weights and clinical histopathology, are also evaluated. Of particular concern are those compounds that induce toxicity in the offspring in the absence of significant maternal toxicity.

There are significant differences in the protocols for segment 2 tests, particularly between countries. These include differences in animal species, dosing regimen, and specific endpoints evaluated. For example, the Japanese protocol requires that some females be allowed to litter and the pups in the litter are examined for physical, reproductive, and functional development (119), which contrasts with typical U.S. protocols where littering does not occur and only in utero pups are examined.

These differences may be significant, with potential implications for regulatory action. For example, functional deficits in offspring could be observed in the Japanese segment 2 protocol, but not in the standard U.S. segment 2 protocol, where additional testing (segment 3) would be required to detect such effects.

Because of the implication of test differences between countries, an expert panel of scientists has proposed that efforts be made toward "harmonization" of guidelines for reproductive and developmental toxicity testing (119). Harmonization would result in international guidelines for reproductive and developmental toxicity testing to improve the comparability of data from studies. In addition, harmonization of testing schemes would allow for more efficient use of resources and possibly would reduce the need for animal testing, since one country could more readily use the results of studies performed in other countries than is now possible.

In addition to the standard methods, specialized developmental toxicity methods involving, for example, developmental neurotoxicity testing, are available. These can be either an addition to a segment 2 test, or a distinct test. Developmental neurotoxicity testing may be especially critical in evaluating certain chemicals such as lead (217) or polychlorinated biphenyls (PCBs) (216), which have been associated with subtle neurobehavioral changes in offspring from relatively low-level prenatal exposures.

Short-term screening tests and in vitro tests, such as the Chernoff/Kavlock (36), have also been used to evaluate developmental toxicants. In general, these tests are insufficient for performing quantitative risk assessments. However, they may be useful in selecting chemicals for further analysis and in helping to guide the nature of further analyses.

Short-term tests are of particular use when one considers the complexities of animal testing and the relatively high frequency of developmental abnormalities. For example, about 3% of infants are born with major congenital malformations that are recognized in the first year of life (125).

Relevance of Animal Studies to Hazard Identification in Humans, Including Weight-of-Evidence Classifications

Significant species differences have been observed with respect to susceptibility of chemicals to induce developmental toxicity. Perhaps the classic example of species differences is thalidomide. Thalidomide

exposure induces comparable target-organ specificity for limb defects in rabbits and various primates, but not in rats. Had initial toxicity tests on thalidomide involved more appropriate animal species, the human tragedy of thalidomide might have been mitigated (125).

Attempts have been made to develop categorical classification schemes and to provide interpretative descriptions of developmental toxicity data. Overall, the aim of such evaluations is to assess the likelihood that a chemical can cause developmental effects in humans. Although not as codified as the cancer classification

Table 2.12 Classification of chemicals based on teratogenic potential

Criteria	Category A	Category B	Category C	Category D
1. Ratio: minimum maternotoxic dose to minimum teratogenic dose	Much greater than 1	Generally greater than 1, teratogenic range starts below the maternotoxic dose range*a* and overlaps it	≤1	No teratogenicity even at maternotoxic doses
2. Incidence of malformations	Dose-related and high	Dose related and high	Dose relatedness of each malformation less obvious, incidence low	—
3. Type of malformation at lower doses	Organ systems involved are specific	Characteristics, possibly specific, generally multiple	Nonspecific involving different organ systems	—
4. Target cell	Specific cells	Specific cells	Nonspecific and generalized	Not known
5. Range of safety factor	1–400	1–300	1–250	1–100

a The maternotoxic dose range extends between the dose initiating signs of toxicity and the dose causing 50% mortality (LD50). Source: Khera et al. (119).

schemes described earlier, the use of such schemes for developmental toxicants could be further developed to form a more integral part of the regulatory process. For example, weight-of-evidence schemes could be used for developing regulatory priorities.

A classification scheme for developmental toxicants is shown in Table 2.12. This scheme categorizes chemicals, based on teratogenic potential. Given equal exposure levels, chemicals in category A would present the greatest concern for teratogenic potential. This particular scheme includes several elements: relationship of maternally toxic dose to developmentally toxic dose; shape of the dose-response curve; and the nature of the malformations. The scheme differs from most carcinogen classification schemes in that it takes into consideration elements of the dose-response relationship. For example, the scheme considers the relationship between the maternally toxic and the developmentally toxic dose, and proposes a range of "safety factors" for extrapolating to human risks, depending on category.

An alternative to classification schemes for developmental toxicants is the use of text descriptors of hazard and other risk elements. This approach was recently used by the Institute for Evaluating Health Risk (IEHR) in the report "An Evaluative Process for Determining Human Reproductive and Developmental Toxicity of Agents" (83). This document contains the deliberations of an ad hoc group of industry and government scientists. Using a hypothetical chemical "terminator", the relevance of animal toxicological data for human risk was evaluated, using expert opinion and consensus development, considering factors such as pharmacokinetic differences between humans and animals, absorption potential through different body interfaces, and biological monitoring data. As noted earlier, rather than yield a rigid categorization scheme, the analysis resulted in text descriptors to assist in the interpretation of animal toxicological studies, with respect to potential for hazard in humans.

The use of classification schemes or interpretive text descriptors to evaluate the relevance of animal developmental studies to humans, as described earlier, could significantly improve the use of developmental toxicity data in the regulatory process. In addition, similar approaches are warranted for other endpoints, such as immunotoxicity.

Use of the RfD to Assess Risk

RfDs may be derived for developmental effects, using essentially the same methodology as described earlier for systemic toxicants in which case the value is an RfDDT (202). The RfDDT is derived from a LOAEL or NOAEL from a developmental toxicity study. However, because the relevant exposure period is not chronic, but is the in utero and possibly earlier time period, the RfDDT applies not to a lifetime exposure but only to the study exposure period. At present, RfDDT values are available for only a limited number of chemicals. In general, the use of RfDs for specific endpoints and periods of exposure duration could allow greater comparability of RfDs and better use of toxicological information.

Use of other Methods to Assess Risk

Alternative methods to the RfDDT have been developed to assess risks or to develop protective levels for developmental toxicants. One method described earlier in Table 2.12 involves the use of different

safety factors applied to the minimum teratogenic dose depending upon the

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teratogenic potential category (A-D) to which the chemical belonged. The use of variable "safety" (now termed *uncertainty*) factors for developing permissible levels from a NOAEL or a LOAEL allows better use of information about the specific developmental endpoint and the likelihood of hazard for humans. Nonetheless, this approach still suffers from the basic flaws as the RfD approach, such as the difficulty in evaluating excursions above the RfD level.

The benchmark dose (BMD) approach has also been proposed to improve assessments of developmental toxicants (117). Briefly, the benchmark dose (defined in a previous section) is developed through the use of dose-response modeling and reflects the dose (or the lower confidence limit on the dose) associated with a certain percent response in the population.

Incorporating Information on Severity of Effect

A critical difference in evaluating risks for carcinogenicity versus risks for systemic effects is that, from a regulatory perspective, almost all types of cancer are considered equally severe, while the severity of systemic effects can vary significantly. In general, there is little basis from a regulatory perspective for distinguishing among carcinogens on the basis of malignancy or tumor type. Despite advances in earlier diagnosis and treatment, the fatality rate for cancer is still relatively high. For example, the relative 5-year survival rate for all cancers from 1986 to 1993, excluding nonmelanoma skin cancer, was 60% for whites and 44% for blacks (4). Nonmelanoma skin cancer includes squamous- and basal-cell carcinoma, which can be induced by agents such as ultraviolet light and arsenic and has relatively low (<10%) fatality rates, even when untreated (206).

In contrast, target-organ effects range greatly in severity. For example, using the same target organ and susceptible population—namely, airways in asthmatics—responses may range from imperceptible mild bronchoconstriction induced by low levels of SO_2 to a fatal asthmatic response, as may have been due to acid sulfate pollution in the London smog episode (176, 210).

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

In 1985, the American Thoracic Society (ATS) defined an adverse respiratory effect (8). Rather than providing a clear demarcation between nonadverse and adverse, the ATS described a continuum of respiratory effects from mild effects of limited, if any, medical significance (e.g., occasional cough, runny nose) to effects of obvious adverseness and medical significance (e.g., an asthmatic attack). To the extent that the effect caused discomfort and impaired daily function and quality of life, it was viewed as more adverse.

Similarly, EPA has considered severity in its evaluation on the effects of ozone as part of the NAAQS setting process. For example, based on lung function changes, duration of effect, symptoms, and impact on activity level, EPA categorized ozone responses into four categories: mild, moderate, severe, and incapacitating. The mild category includes FEV_1 declines of 5 to 10% and no impact on activity. The EPA (205) recommended that the responses in the mild category *not* be considered an adverse respiratory effect in adults for purposes of defining the NAAQS for ozone.

As noted earlier, the concept of severity of effect is also incorporated into the process for developing the RfD. Specifically, the RfD is based on an effect that, by definition, considers adverseness. The critical effect is adverse, because it may result in functional or structural impairment or is a precursor state to irreversible toxicity (203). For example, fatty infiltration of the liver or a greater than 10% reduction in weight gain versus controls would be considered adverse effects, and the associated dose would be a LOAEL. As the dose increases, the fraction of that population experiencing such effects would increase. Frank effect levels (FELs) are dose levels that result in overt, often clinically apparent toxicity, and are considered "too adverse" to be used in the development of the RfD. Examples of frank effects include liver necrosis or cirrhosis, which are severe and may be irreversible. FELs are not considered appropriate for RfD development because the protection level would be inadequate.

Information on severity has been incorporated into the *reportable quantity* (RQ) definition, under CERCLA. Under this statute, releases of chemicals in amounts greater than some predetermined level, defined as the RQ, require that EPA be notified of the release (48). The amount of release that triggers notification is based on an assessment of the potency of the chemical and the severity of the effect at the dose level where the potency was quantified. The ranking of severity is shown in Table 2.13 (48), where it can be seen that effects range from slight biochemical changes through gross toxicity, including lethality. Unlike the RfD process, this scoring is not restricted to data sets containing information on

mildly adverse effects, from subchronic or chronic studies. The RQ process can result in development of scoring indicators from lower quality data sets, involving shorter time periods of exposure and more severe toxicity.

Table 2.13 Rating values for NOAELS, LOAELS, and FELs used to rank chronic toxicity

Rating	Effects
1	Enzyme induction or other biochemical change with no pathologic changes and no change in organ weights.
2	Enzyme induction and subcellular proliferation or other changes in organelles, but no other apparent effects.
3	Hyperplasia, hypertrophy, or atrophy, but no change in organ weights.
4	Hyperplasia, hypertrophy, or atrophy with changes in organ weights.
5	Reversible cellular changes: cloudy swelling, hydropic change, or fatty changes.
6	Necrosis, or metaplasia with no apparent decrement of organ function. Any neuropathy without apparent behavioral, sensory, or physiologic changes.
7	Necrosis, atrophy, hypertrophy, or metaplasia with a detectable decrement of organ functions. Any neuropathy with a measurable change in behavioral, sensory, or physiologic activity.
8	Necrosis, atrophy, hypertrophy, or metaplasia with definitive organ dysfunction. Any neuropathy with gross changes in behavior, sensory or motor performance. Any decrease in reproductive capacity. Any evidence of fetotoxicity.
9	Pronounced pathologic changes with severe organ dysfunction. Any neuropathy with loss of behavioral or motor control or loss of sensory ability. Reproductive dysfunction. Any teratogenic effect with maternal toxicity.
10	Death or pronounced life shortening. Any teratogenic effect without signs of maternal toxicity.

Note. Source: deRosa et al. (48).

The RQ process demonstrates the use of severity information in both risk assessment (developing RQ indicators) and risk management (defining release levels requiring notification as associated with defined RQ values).

Recent efforts involving the use of categorical exposure response modeling demonstrate additional approaches towards consideration of severity. For example Guth and co-workers (as cited in ref. 15) analyzed acute effects resulting from methyl isocyanate exposures of less than 8 h in duration (as seen in Figure 2.5). Effects were separated into three categories: NOAEL (circles), adverse effect level (AEL) (triangles), and lethal (squares). Effect categories were then analyzed on the basis of concentration and time, using logistic regression. Figure 2.5 presents a line above which there is a 90% probability that the true NOAEL lies. This method allows the use of data from a range of severity endpoints and considers various combinations of exposure level and exposure duration. Conceivably, this type of approach could lead to the development of concentration time nomograms for definition of NOAELs for different exposure durations.

PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELS

One of the areas of recent regulatory attention is that of physiologically based pharmacokinetic (PBPK) models and their potential use in risk assessment. This issue is discussed in depth in the chapter 5 by Krishnan and Andersen and chapter 8 by Rodricks et al., we discuss the topic here in terms of regulatory implications.

PBPK models are essentially mechanistic models that describe quantitatively the pharmacokinetic processes affecting the disposition of a chemical and its metabolism from the time it is absorbed to the interaction with different and various body tissues. Once it is determined whether the parent compound or its metabolites are the likely cause of a carcinogenic response, a PBPK model may be developed to quantify the magnitude and the time course of exposure to this agent at the critical target site in the animal model. After the estimates of target tissue dose in the animal model have been made and validated, the information can then be scaled to the human to obtain an estimate of target-organ dose in humans. This estimate may then be used to predict human cancer risk under dif-

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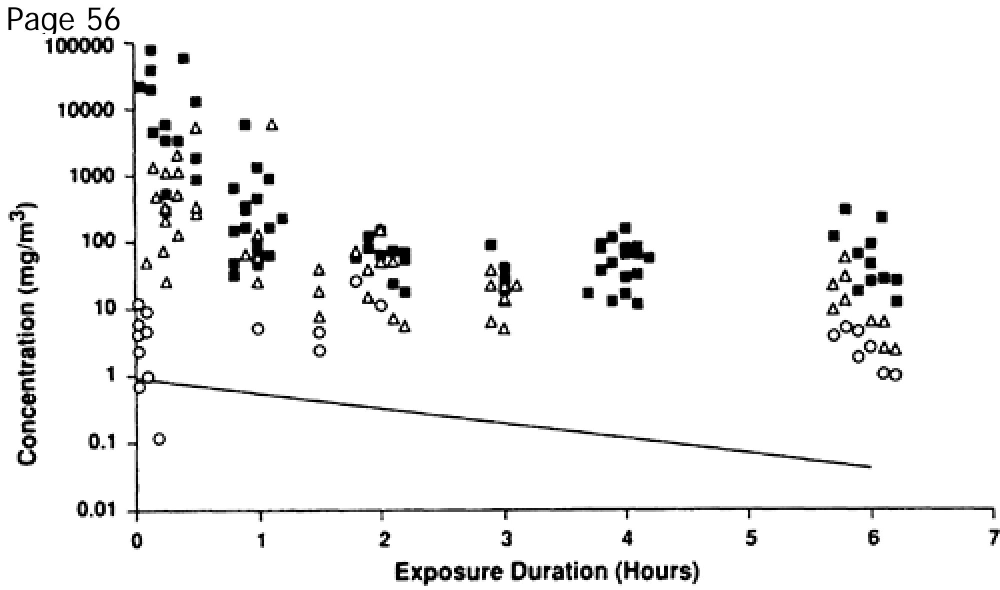

FIG. 2.5. Categorical data from published results on methyl isocyanate for exposures of less than 8 h in duration and shown as NOAEL (circles), AEL (triangles), or lethality (squares). The maximum likelihood model fit is shown by the line representing the model prediction of *p*=.1 that severity is greater than the NOAEL category at the corresponding exposure concentration and duration.

ferent exposure conditions or to develop more precise estimates of the reference dose. It should be emphasized that the PBPK model does not offer an explanation of the most appropriate dose-response relationship, once the dose to target is estimated (11, 24). Furthermore, full validation of the model at the relatively low levels of environmental chemicals to which humans are exposed can be difficult. Despite these limitations, PBPK modeling does offer an important tool for researchers and regulators alike. PBPK models can be used to quantify target organ doses between species and to extrapolate from high to low doses. Of added significance is that new information about the pharmacokinetics of a chemical can be incorporated into the model without affecting the basic structure of the model, thus enhancing its predictive capability.

The use of PBPK models also provides important advantages over conventional pharmacokinetic analyses (11, 24). In typical pharmacokinetic modeling, timecourse curves are determined for the concentration of the administered agent or its metabolite(s) in blood or some other body compartment. The resulting curves are then described by curve-fitting biostatistical techniques. The approach of conventional pharmacokinetics may be criticized for being more dependent on the mathematical model than on the biological system it purports to represent. However, PBPK models are designed to predict kinetic behavior over a wide range of doses and exposure conditions, and are based on basic physiologic and metabolic parameters. This modeling requires many data on anatomical and physiological parameters, the partitioning of test agents into selected tissues, and the biochemical constants for tissue binding and metabolism in various organs. From these data, a series of mass balance differential equations can be written to describe the interactions between the chemical and the animal model.

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

PBPK modeling has been applied to several agents, including methylene chloride and ethylene dichloride (11, 24, 38). A look at the methylene chloride case illustrates the powerful implications of this approach. Anderson et al. (11) developed a PBPK model based on data indicating two routes of metabolism, one dependent on oxidation by mixed-function oxidase (MFO) and the other dependent on glutathione *S*-transferase (GST) in four species (mouse, rat, hamster, human).

Models were designed to quantify the contributions of the two metabolic pathways in the lung and liver and to allow for extrapolation from rodents to humans. Kinetic constants for the model were obtained from experiments or the literature, with model validation involving a comparison of predicted blood concentration time-course data in rats, mice, and humans, with experimental data from these species. The capacity of methylene chloride to cause tumors in mice was associated with the target tissue dose and was closely related to the amount of methylene chloride metabolized by the GST but not the MFO pathway. Using the PBPK model, the target tissue doses in humans exposed to low concentrations of methylene chloride were between approximately 50- and 200-fold lower than would have been predicted by the linear extrapolation and body surface area factors used in conventional risk assessment methods. Thus, the PBPK analysis suggested that conventional risk analysis greatly overestimated the risk to humans exposed to low levels of methylene chloride. One of the major uncertainties, however, is the metabolic capacity of the body at low exposure levels where metabolism may not be saturated. Also, the dominant pathway for methylene chloride metabolism at other organ sites has not been determined. Still, the PBPK approach represents an attractive development, since it can increase the biological plausibility of predictive approaches while still incorporating biomathematical approaches for low-dose risk prediction. The EPA has incorporated this pharmacokinetic information into its cancer risk assessment for methylene chloride exposure via inhalation (187).

It is important to note that there are substantial uncertainties in PBPK modeling. For example, Hattis et al. (78) compared PBPK models for perchloroethylene developed by seven different authors and found appreciable differences among the model predictions. Given identical exposure levels in humans, the range of values for metabolized perchloroethylene span a 50-fold range, with 6 of the 7 models having predictions with a 14-fold range. With respect to methylene chloride, Clewell (38) noted the importance of the tissue distribution of GST enzyme activity across species, especially in humans as a source of model uncertainty. Studies to refine estimates of GST enzyme activity across species and within the human population will serve to provide more refined estimates of dose across humans and hence of potential differences in susceptibility.

ROLE OF HIGH-RISK GROUPS

In a previous section, we described the RfD concept as used by regulatory agencies to estimate acceptable levels for noncancer effects. One of the factors in the derivation of this level was to account for variations in population susceptibility. The purpose of this section is to expand upon that issue, to describe the basis for variations in susceptibility and the magnitude of that variation, and to demonstrate the relationship of this issue to the regulatory process. (For more detail the reader is referred to refs. 14, 25, and 139).

There is a high degree of variability in the response of humans to different exposure levels of environmental pollutants (25, 44). In fact, the variability in the dose-response relationship in a heterogeneous population makes it difficult to estimate an acceptable level for chemical contaminants that would be protective of the whole population. Perhaps the most critical question is not what is a "safe" numerical standard, but how many individuals are adversely affected at different levels of exposure (33).

Knowing which groups of individuals are at high risk with respect to pollutants is very important in answering this question, since these individuals will be the first to experience morbidity and mortality as pollutant levels increase. If the high risk segments are protected, then the entire population is also protected. Information concerning both the identification and quantification of high-risk groups should play an integral role in the derivation of environmental health standards.

Use of Uncertainty Factors for High-Risk Groups

In trying to assess the role of high-risk groups in the derivation of environmental health standards, it is useful to consider the extent to which the EPA has utilized the concept of high risk groups within the standard setting process. Perhaps the most common approach utilized by the EPA and other regulatory agencies has been the implementation of uncertainty factors for noncancer endpoints. While this approach implicitly recognizes that certain people are more sensitive to pollutants than others, it is inherently imprecise. The precise difference in sensitivity between a statistically "normal" individual and groups at increased risk will vary for the different causes of the high-risk condition and for different pollutants.

The EPA has utilized the uncertainty factor approach in attempting to deal with protection of high risk individuals, as illustrated by the national drinking-water standards for noncarcinogenic chlorinated hydrocarbon insecticides (25). These substances were tested in two animal species, the rat and the dog. Chronic toxicity testing provided an estimate of the lowest level of pollutant (on a milligram of dose per

kilogram of body weight) that the animal could ingest with either minimal or no toxic effects. In the absence of data to indicate a basis for

an alternative choice, the species that was the most sensitive to the substances was chosen to derive the standard, implying that humans are as sensitive as the most sensitive animal species. In the absence of supporting human exposure data, an uncertainty factor of 500 was applied to the minimally toxic dose in the most sensitive animal species (i.e., the minimally toxic dose was divided by 500). It should be noted that this methodology differs from that of the RfD approach described earlier, in which case a factor of 100 would have been applied. This number was taken to be the total amount of insecticide to which a human could be exposed each day, over an unspecified period of time, without suffering any adverse health effects.

Several questions occur when evaluating such a methodological scheme. For example, on what basis can we assume that the most sensitive humans have the same degree of responsiveness as the most sensitive animals? Why was 500 chosen as a safety factor? What assurances exist that it would provide sufficient protection for the general population as well as high risk groups for these chemicals? Who, in fact, are the groups considered at increased risk?

The main problem with an uncertainty factor approach is its lack of specificity in identifying susceptible subpopulations, the extent of their susceptibility, and, most importantly, what fraction is protected by different standard levels. It should also be realized, however, that when only limited data are available, imprecise safety factors are the only realistic options available. Still, this approach will result in uncertain levels of protection. Alternative approaches must be developed to reduce the magnitude of that uncertainty.

Consideration of Specific High-Risk Groups

A better approach, when data are available to support it, is to consider specific groups at high risk on a chemical-by-chemical basis. There are clear examples of groups more susceptible to particular chemicals and cancer and non-cancer health effects (reviewed in refs. 20, 60, and 69). These include:

- Individuals with genetic variations in metabolism. For example, a slow acetylator phenotype is associated with an increased risk of bladder cancer following exposure to aromatic amine dyes (69).
- Individuals with enzymatic genetic polymorphisms. For example, polymorphisms in cytochrome P450 enzymes can result in differential detoxification or bioactivation of environmental chemicals (60).
- Individuals with inherited genetic defects. For example, xeroderma pigmentosum, an autosomal recessive disease, results in altered DNA repair capacity and increases the risk of skin cancer by more than 1500-fold (60).
- Individuals with preexisting illness. For example, asthmatics may be more susceptible to ozone (20), and those with hepatitis B are more susceptible to liver cancer (69).

Other factors that can affect susceptibility to environmental chemicals include gender, age, and life-style (i.e., cigarettes, alcohol, diet). The role of diet and certain types of cancer is shown in studies demonstrating an inverse relationship between the amount of vitamin A in the diet and susceptibility to hydrocarbon-induced epithelial cancers (41). Also, certain subgroups may be at greater risk, not because of an inherent difference in toxicological susceptibility, but because they are more likely to be exposed. For example, young children are at greater risk from soil contaminants because they tend to accidentally ingest more soil and dust than older children and adults, due to their significant hand-to-mouth activity. Thus, it is likely that, even given the same exposure, individuals are not equally susceptible to the induction of cancer and other adverse health effects, and in many cases the differential susceptibility may be very large.

Recently, there has been increasing attention on the potential for children to be more susceptible to environmental chemicals. In 1996, the EPA emphasized its focus of protecting infants and children in a report entitled "A National Agenda to Protect Children's Health from Environmental Threats" (194). The 1996 FQPA requires use of an additional 10-fold UF for pesticides to account for potential prenatal and postnatal developmental toxicity (191). As noted by Roberts (162), children may be more susceptible because many cells and organs are undergoing growth and development and have not yet matured. A child's diet and physical environment, and therefore his or her exposure potential, may vary significantly from that of an adult. For many routes of exposure (air, food, water, and dermal), chemical intake (on a per kilogram body weight basis) is generally greater for infants and children than adults (150).

However, a subgroup at high risk for one chemical is not necessarily at high risk for other chemical exposures. For example, although children are often assumed to be more sensitive than adults, this is not always the case. Reactions to pharmaceuticals, since they are more widely studied than responses to environmental exposures, can be considered as examples. Acute overdoses of acetaminophen result in less hepatotoxicity in children than in adults with comparable plasma concentrations, possibly due to differences in metabolism (118).

There is currently debate about whether current risk assessment methods adequately account for more highly susceptible groups. In general, setting of levels for

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carcinogen exposure has not addressed the role of population variability in susceptibility to carcinogens. Consequently, groups at high risk to environmental carcinogens, with the obvious exceptions of smoking as a risk factor for exposure to asbestos, uranium, and coke-oven emission-related cancer, have not generally been addressed. It should be noted, however, that the conservatism of the cancer risk assessment process might result in adequate protection of high-risk groups.

For noncancer effects, there is debate over the appropriate uncertainty factors to account for high-risk subgroups. At a conference organized by the ILSI and the EPA (71), "it was suggested that, in many cases, genetic variation in human susceptibility may be greater than an order of magnitude when comparing differences between children and adults." A coalition of farm food, manufacturing, and pest management organizations concluded that the additional UF of 10 required by the FQPA is not necessary to use "across the board" to protect infants and children (84). They also concluded that the standard default UFs are adequate for a pesticide with a complete and reliable database, and that an additional UF "should only be applied to an endpoint that is relevant to protection of fetuses, infants, and/or children" (84). The EPA is looking into establishing criteria for appropriate use of the 10-fold additional FQPA uncertainty factor (191,192). Overall, the best approach is to consider more susceptible subgroups on a caseby-case basis when data are available, for both carcinogens and noncarcinogens.

Regulatory Applications

The role of population variability should be considered by regulatory agencies in risk assessments for both carcinogens and noncarcinogens. Identification and quantitative characterization of susceptible populations could provide decision makers with a theoretical framework on which to base regulatory action. For example, Tamplin and Gofman (173) have employed knowledge of susceptible populations in predicting the incidence of cancer from radiation pollution in drinking water to help define acceptable levels of exposure. They assumed that the latency period is shorter for in utero exposure than for all radiation exposure beyond birth (i.e., 5 years vs. 15 years). Consideration of the increased susceptibility of the fetus to radiation-induced cancer resulted in greater estimates of cancer risk as compared with traditional methodological approaches, which predict carcinogenic effects at low doses, based on high levels of exposure in adults (160).

The EPA has specifically evaluated the increased sensitivity of specific high-risk groups in setting National Ambient Air Quality Standards for carbon monoxide, lead, nitrogen dioxide, ozone, particulates, and sulfur dioxide, and in establishing drinking-water standards for some environmental chemicals. Examples of the high-risk groups considered are shown in Table 2.14. For instance, the NAAQS for lead considers high-risk populations in a more quantitative way by estimating the fraction of the susceptible subpopulation (children) that would be protected at different air levels of lead (209). Following is a detailed description of the EPA consideration of high-risk groups in the derivation of drinking-water standards for nitrates and cadmium.

Nitrates in Drinking Water

The drinking water standard of 10 mg nitrat (NO_3^2) as milligrams nitrogen per liter is designed to prevent the formation of elevated levels of methemoglobin (MetHb) in infants. In the presence of nitrite (NO_2^-), formed from nitrate in infants, hemoglobin is oxidized to MetHb, which is not able to reversibly combine with oxygen. Levels of 1–2% and 2–5% MetHb are typical in the blood of adults and infants, respectively. When concentrations are less than 5% MetHb, there are no obvious indications of toxicity. However, with levels of MetHb from 5 to 10%, clinical signs of toxicity (e.g., cyanosis) may appear (25). Infants are at considerable risk for nitrate-related toxicity, as compared to adults. Factors that predispose infants to the development of MetHb formation include:

1. The incompletely developed ability to secrete gastric acid. This permits the gastric pH to be high enough (5–7 pH) to permit the growth of nitrate-reducing bacteria in the gastrointestinal tract, thereby converting nitrate to nitrite before absorption into the circulation (184).
2. The higher levels of fetal hemoglobin in infants. This form of hemoglobin is more susceptible than adult hemoglobin to oxidation to MetHb (19).
3. The diminished enzymatic capability of infants to reduce MetHb to hemoglobin (166).

Research has revealed that levels of nitrate beyond 20 mg/L resulted in a marked upshift in the frequency of methemoglobinemia in infants but not in adults (25). Consequently, a standard of 10 mg/L is principally designed to prevent the occurrence of elevated levels of MetHb in infants. Concentrations twice as great would still protect adults.

Cadmium

Studies with rats show that at a kidney concentration of 200 ppm cadmium (Cd) (211), renal damage is

initiated. The EPA calculated that humans would need to ingest 50 g Cd/day for 50 years to reach a level of 200 ppm in their kidneys. In the derivation of the Cd drinking-water standard, the EPA assumed a daily Cd

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Table 2.14 High-risk groups in the derivation of standards by the U.S. EPA

A. Drinking-Water Standards

Substance	High-risk condition considered
Arsenic	None
Barium	No specific groups; but a safety factor of 2 incorporated to account for variation (or increased susceptibility) within the human population
Cadmium	None
Fluoride	Children—to prevent mottling of teeth
Lead	Children—to prevent neurological disorders
Mercury	Based on humans who exhibited toxicity at the lowest level of exposure from a group of mercury-poisoned adults
Nitrate	Infants—to protect against methemoglobinemia
Selenium	None
Sodium (no standard)	Individuals with heart and kidney disease
Chlorinated hydrocarbon insecticides (noncarcinogenic)	None
Chlorophenoxy herbicides (noncarcinogenic)	None

B. National Ambient Air Quality Standards

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

exposure of 75 μg from the diet and 20 μg from water. This 20 μg Cd/day from drinking water would occur at a level of 0.01 mg/L. The total daily Cd exposure is therefore approximately 95 μg Cd/day, and thus a safety factor of 4 was assumed.

In proposing their drinking water standard for cadmium, the EPA requested feedback from the public as to whether the standard should include additional protection for cigarette smokers, since smoking is a source of appreciable cadmium exposure (e.g., approximately 1.5 μg Cd/cigarette) (25). It is interesting to note that of the 52 comments received by the EPA on this issue, only 3 suggested that this standard be modified to include protection for the cigarette smokers. The EPA decided not to incorporate additional safety factors to protect smokers (208). Thus, this example describes a situation

in which protection of a high-risk group was not taken into account in derivation of a standard.

HORMESIS

As described in the sections on evaluation of carcinogens and of systemic toxicants, the risk assessment process, as articulated by most regulatory agencies, has generally (but not always) assumed that the shape of the dose-response curve is either, for noncarcinogens, a threshold response below which no toxicity is observed or, for carcinogens, a linear relationship for which there is no exposure without an estimated effect.

Such risk assessment assumptions concerning the nature of the dose-response relationship for different biological endpoints have been challenged in recent years by the recognition that there are many exceptions to the threshold and linearity assumptions. A key exception to the two basic assumptions is that of the U- or inverted U-shaped dose-response relationship (Figure 2.6). Whether a dose-response relationship takes the U or inverted U form is dependent on the endpoint measured. In the case of U-shaped dose-response relationships, the endpoints could be various biological effects or diseases, such as mutation rate, birth defects, and cancer incidence (see ref. 30 for review). In the case of inverted U-shaped dose-response relationships, the types of endpoints include growth rate, fecundity (see ref. 27 for review), and longevity, among others. The phenomenon of stimulatory effects of low-level exposures has been termed *hormesis* and has been applied to both chemicals and radiation. Typical extrapolation processes for both threshold and nonthreshold phenomena ignore such possible U- or inverted U-shaped responses in the low-dose zone. A key implication of U- and the inverted U-shaped dose-response relationships is that, when such phenomena occur, the low-dose response (e.g., disease, incidence, longevity) cannot be readily predicted by responses at higher doses. The rest of this section presents background information on chemical hormesis and discusses some practical implications for both study design and risk-based standards development.

Although the concept of hormesis was seemingly well established by the 1920s at least as far as chemical hormesis is considered, the concept became marginalized within the scientific community in the 1930s and never had any serious impact on the modern concept of the dose-response relationship (26). The principal factors for the demise of the concept are complex, but include the following. One difficulty in differentiating the low-dose stimulatory phenomenon from normal variation was the lack of a study design with an adequate number of appropriately placed doses in the low dose range; this difficulty was further magnified by the fact that

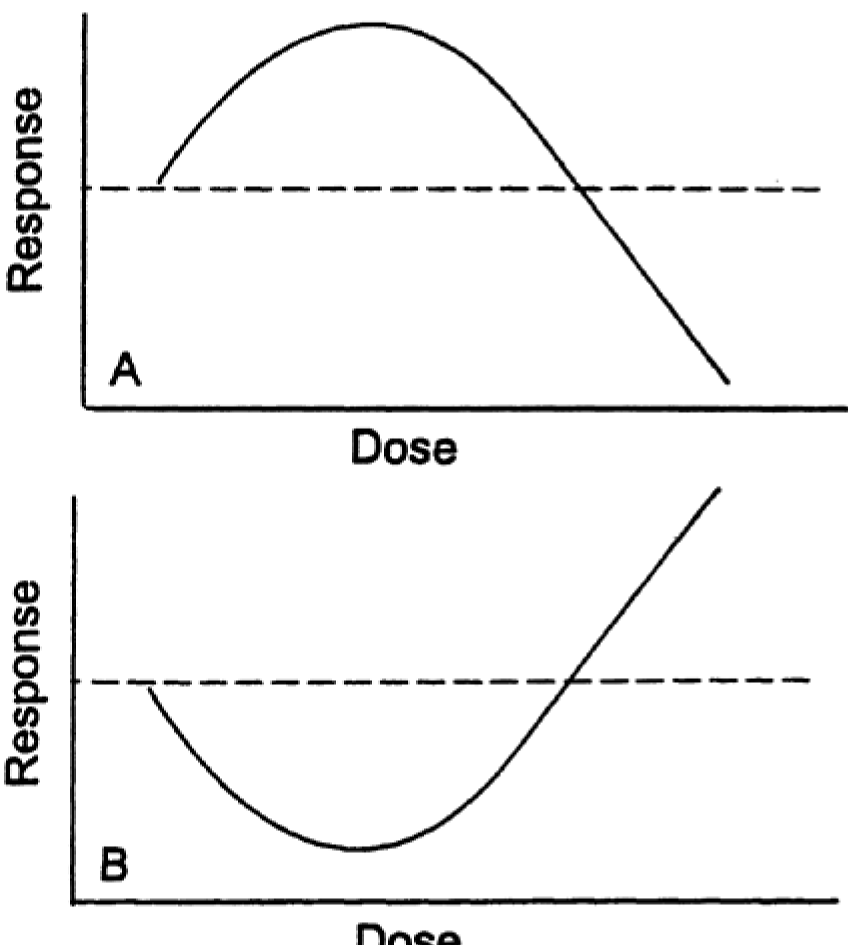

FIG. 2.6. Dose-response curves for hormesis. (A) The most common form of the hormetic dose-response curve depicting low-dose stimulatory and high-dose inhibitory responses, the *β* or inverted U-shaped curve. Examples of endpoints demonstrating the *β*- or inverted U-shaped dose-response curve include growth, longevity, fecundity, and weight gain. (B) The hormetic dose-response curve depicting low-dose reduction and high-dose enhancement of adverse effects, the J- or U-shaped curve. Examples of endpoints demonstrating the J- or U-shaped dose-response curves include mutations, cancer incidence, and birth-defects incidence. Source: Calabrese and Baldwin (28).

the magnitude of the low-dose stimulatory response has a maximum average of about 30–60% above the control (Figure 2.7). Lack of appreciation for what the hormetic dose-response was coupled with inadequate study design features led to criticism that the phenomenon was simply normal variation. Over the past several years, Calabrese and Baldwin (30, 31) have reinvestigated the hormesis hypothesis and developed rigorous a priori criteria for its evaluation. This investigation has uncovered several thousand examples of well-designed studies published in the peer-reviewed literature that are consistent with the hormesis phenomenon. The hormesis phenomenon has been proposed to result from a modest overcompensation to a disruption in homeostasis. More specifically, the investigation of minor damage induces a generalized repair response. In order to assure that the damage is adequately

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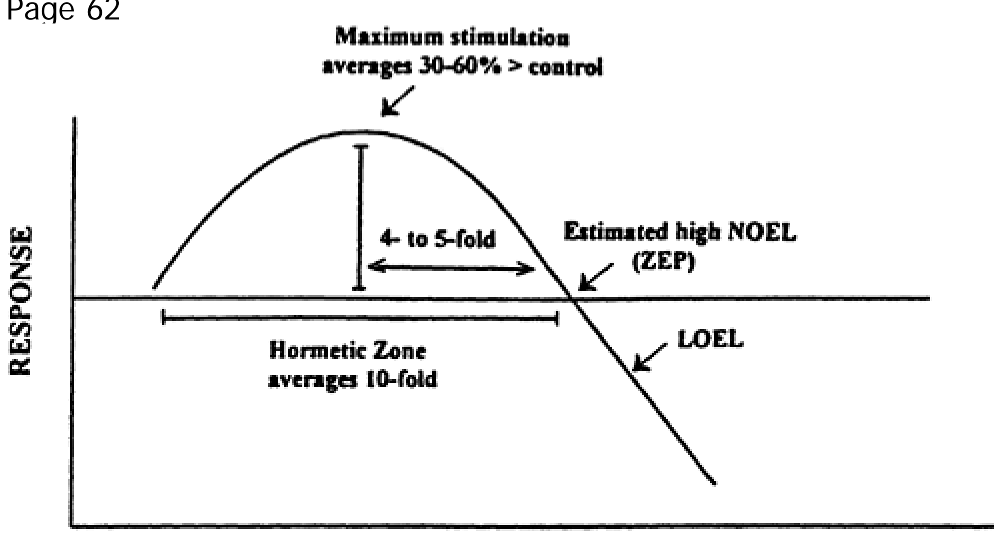

FIG. 2.7. Characteristics of the chemical hormetic zone. Abbreviations: NOEL, no-observed-effect level; LOEL, lowest-observed-effect level; ZEP, zero equivalent point. Source: Calabrese and Baldwin (31).

repaired, there is a slight overcompensation in repair responses. As the dose increases, the capacity to repair the damage decreases and eventually the capacity to repair equals the level of damage when the zero equivalent point (ZEP) is reached [i.e., the no observed adverse effect level (NOAEL)]. At higher doses, frank toxicity occurs. This process provides a framework to understand the basis for the observation that the stimulatory range is modest by up to about 10-fold, that the maximum stimulation averages about 50% above the control, and that the NOAEL/ZEP is only three- to five-fold greater than the maximum stimulatory response. Hormetic responses appear to be generalizable with respect to biological model, chemical class, and toxicological endpoint measured, including critical risk assessment endpoints such as tumor incidence, longevity, and various disease incidences.

Even in the situation where U-shaped dose-response relationships may exist. study design and other factors may reduce the capacity to detect such a relationship (Table 2.15). For example, it is not possible to assess hormesis with the use of certain toxicological parameters such as liver damage as reflected by increased serum enzymes.

To the extent that hormesis exists for a specific chemical, there are several implications for both noncarcinogenic and carcinogenic risk assessment. (a) The dose-response curve would be similar for both the carcinogenic and noncarcinogenic responses. (b) Thresholds would exist for both carcinogens and noncarcinogens. (c) At certain doses below the NOAEL (or ZEP) there would be less damage in the exposed group than observed in the control group. (d) Based on a review of hormetic dose-response relationships, the initial disruption in homeostasis typically occurs approximately 5- to 10-fold below the traditional NOAEL. However, this initial disruption is not only adequately repaired by the induction of adaptive mechanisms, but then background biological damage is also reduced by the induced adaptive mechanisms. (e) Current experimental approaches to assess the dose-response relationship in the hazard assessment process are inadequate since many studies only emphasize the relatively high-dose aspect of the dose-response curve. Thus, the hormetic perspective suggests that new study

Table 2.15 Factors contributing to the inability to detect a hormetic response when one exists

Inappropriate dose range.
Inappropriate spacing of doses.
Inappropriate number of doses.
Inappropriate temporal measurements in relationship to endpoint.
Inappropriate endpoint selection (e.g., serum enzyme levels as bioindicators of liver damage).
Condition of model organism.
Interindividual variability.
Low background in relationship to showing a U- or inverted U-shaped dose-response curve.

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design criteria become incorporated into the hazard assessment protocol and that new default parameters become incorporated into the risk assessment framework incorporating the hormetic assumption.

Practical Implications of Hormesis for Quantitative Risk Assessment

Hormesis and Carcinogens

In a recent issue of the BELLE Newsletter, Sielken and Stevenson (168) identified seven ways in which the concept of hormesis should affect quantitative risk assessment (Table 2.16). The seven factors provided by Sielken and Stevenson (168) offer an important and clear blueprint for how the current approaches for both chemical and radiation cancer risk assessment modeling could both include and take advantage of the concept of hormesis and its underlying database. Many of these recommended changes reflect a less biased approach as well. For example, the parameters in the multistage model are typically required for regulatory purposes to be nonnegative values. This restriction determines that the probabilities in the multistage model be increasing as

Table 2.16 Implications of hormesis for quantitative risk assessment

Dose-response models need greater flexibility to fit the observed shape of the dose-response data; such models should not be constructed to be forced to always be linearly decreasing at low doses.

Hazard assessment evaluations need to incorporate greater opportunity to identify the hormetic portion of the dose-response relationship.

New dose metrics should be used that incorporate age or time dependence on the dose level rather than a lifetime average daily dose or its analog for a shorter time period.

Low-dose risk characterization should include the likelihood of beneficial effects and the likelihood that a dose level has reasonable certainty of no appreciable adverse health effects.

Exposure assessments should fully characterize the distribution of actual doses from exposure rather than the just upper bounds.

Uncertainty characterizations should include both upper and lower bounds.

Risk should be characterized in terms of the net effect of a dose on health instead of a single dose's effect on a single disease endpoint (i.e., total mortality rather than a specific type of fatal disease).

Note. Adapted from Sielken and Stevenson (168).

the dose increases. Sielken and Stevenson (168) argue that such nonnegativity restrictions for the parameters in the multistage model should be removed so that the fitted model may reflect the shape of the reported dose-response data. Furthermore, such a change would allow the multistage model enhanced flexibility to explicitly address hormetic effects seen in actual reported data. In addition, when an hormetic effect is present the risk at low dose would be less than for the control group. The lower bound on the "added" risk in the case of hormesis is no longer the zero risk as is now assumed, but would be negative. Clearly, therefore, both upper and lower bounds of uncertainty should be incorporated.

Hormesis and Noncarcinogens

The role of hormesis in affecting the risk assessment process for noncarcinogens has also been discussed (28, 29, 62, 197). As discussed in earlier sections, independent of whether hormesis exists or not, the major goal of the hazard assessment process is the derivation of the NOAEL (or in some cases the BMD).

Calabrese and Baldwin (29) have noted that the estimation of the BMD or the NOAEL can be affected by the existence of U-shaped dose-responses in two ways: (a) doses residing in the hormetic zone by affecting the modeling involved in the BMD derivation process, or (b) a dose within the hormetic zone could be selected as the NOAEL. With respect to the former possibility (i.e., BMD derivation), the occurrence of hormetic responses would tend to flatten the model-based dose-response relationship, thereby requiring a higher response to achieve the BMD05/10 response. In the latter case (i.e., where the highest dose is not different from the control), a dose with a value lower than the zero equivalent point (ZEP) would be designated the NOAEL. This may be illustrated in Figure 2.8, which shows a typi-

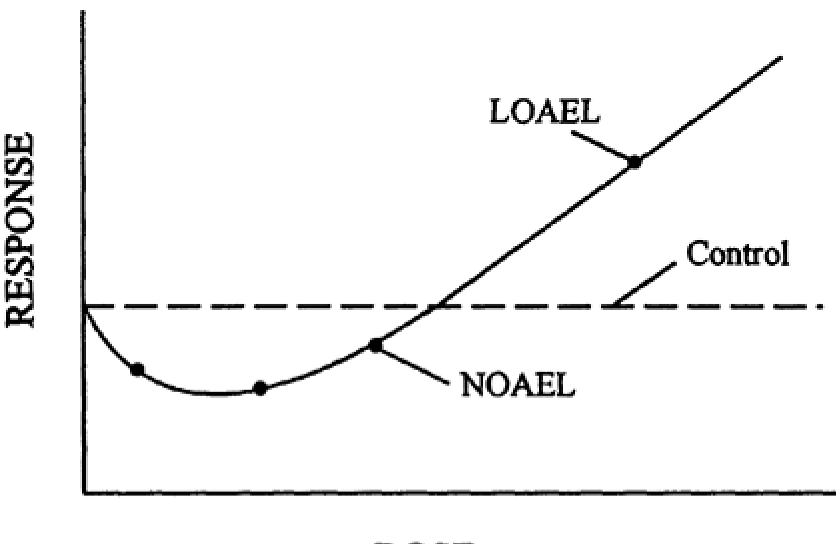

FIG. 2.8. Dose-response relationship presenting NOAEL in the hormetic zone. Source: Calabrese and Baldwin (29).

cal U-shaped dose-response relationship. The highest dose causes a significant toxic effect and is designated the LOAEL. The dose immediately below the highest dose exhibits a response lower than the reference or control group. This hormetic dose would become the NOAEL since it is the highest dose not significantly different with regard to an adverse effect and satisfies the above definitional criteria. Therefore, in these two cases, the concept of the NOAEL is (a) affected by the presence of hormetic doses in the application of the BMD process and (b) being explicitly incorporated into the hormetic portion of the dose-response curve (i.e., traditional NOAEL derivation process).

While the U-shaped dose-response is often seen and appears to be highly generalizable, it is not seen in numerous instances (Table 2.15). For example, it is not possible to assess hormesis if tumor incidence of the background (or control) is very low. This lack of ability to make judgments about hormesis is further seen with respect to other toxicological parameters such as liver damage, which is inferred by increased serum enzymes. Even though the current methods cannot assess hormesis under these conditions, this does not suggest that it does not exist. In fact, hormesis may be predicted to exist independent of the biological incidence of the disease under study.

If hormetic effects were to have an opportunity to affect the RfD derivation process, the hazard assessment process would have to expand the toxicological testing process into the low dose zone by increasing the number of doses from 2–3 to 6–7 doses with an assurance that at least three doses were below the traditional toxicological NOAEL (31). This would help insure that the hormetic phenomenon would have been properly evaluated. Such an expansion of the hazard assessment process would add considerable cost and time to the testing process. An alternative to the expanded testing strategy would be to incorporate hormesis as a default assumption in the RfD derivation process. If hormesis were a default parameter, the current hazard assessment process could remain as is. The RfD process would have to proceed by first adjusting the NOAEL value to equal the ZEP dose by a model-based procedure, thereby deriving an adjusted ZEP-based NOAEL, and then employing a hormesis optimization scheme to facilitate the RfD derivation (23, 62).

Calabrese and Baldwin (29) have offered the following considerations in order to establish a decision-making framework for assessing whether hormesis could achieve default parameter status and be incorporated into RfD development. The concept of default parameter status implies a highly generalizable phenomenon with regard to chemical class, biological model, and endpoint, which is linked to the risk assessment process. Consequently, the concept of hormesis would first need to permeate the broad classes of inorganic and organic contaminants. especially those containing agents that are of regulatory concern. Second, the phenomenon of hormesis must be generalizable across species to ensure that hormesis for a particular chemical does not reflect a species- or strain-specific response. The third factor for assessing hormesis as a default parameter in the RfD derivation process concerns biological endpoint. Regulatory actions focus predominantly on adverse endpoints such as organ-specific toxicity, biomarkers of toxic response (e.g., blood lead levels, serum cholinesterase activity), and teratogenic and other reproductive effects, as well as tumor formation. The influence of hormesis is not only in the reduction of already indicated adverse effects and biomarker responses, but also in the enhancement of outcomes such as increased longevity, improved performance, and reduction of disease incidence below background. The ability to incorporate what society considers both beneficial and harmful effects of a chemical into decision making presents a challenge to regulators. Consider, for example, the difficulties in making recommendations on alcohol consumption where moderate levels can reduce risk of heart disease, but excessive drinking clearly takes a toll in terms of morbidity and premature mortality.

The recently developed chemical hormesis database (30) evaluates toxicological investigations for their capacity to demonstrate evidence consistent with the hormesis hypothesis based on study design attributes, response criteria, statistical power, and reproducibility. This database provides evidence that hormesis is likely to be generalizable across the three critical areas listed here and that its relationship to the NOAEL is readily estimated, thus allowing practical incorporation into the risk assessment process. Based on this information, we recommend that regulatory agencies consider the concept of hormesis as a default parameter in the risk assessment process. What would be the practical implication if hormesis were accepted as a default assumption for the RfD derivation process? First, consideration of hormesis would redefine the concept of NOAEL for regulatory purposes. In addition, consideration of hormesis would allow an optimizing process to estimate the dose providing the best balance between preventing the agent-induced adverse effects and enhancing hormetic/ adaptive responses within the population.

IMPLICATIONS OF CHEMICAL INTERACTIONS FOR THE REGULATORY PROCESS

One of the major difficulties in current environmental public health practice is that the focus is on a

limited number of environmental contaminants, with limited consideration of interactive effects among pollutants. In fact, the number of environmental pollutants in differ-

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ent media is large, making it difficult to estimate the degree of public health protection afforded by our present regulatory apparatus. Still, it is clear that the scientific and regulatory communities must address the issue of multiple chemical exposures. In fact, animal models and human epidemiological studies show that interactions do occur among chemicals and that this can result, under certain circumstances, in greater than additive effects. For example, uranium miners who smoke have a fourfold greater risk of cancer than nonsmokers. However, uranium miners who smoke display a 40-fold greater cancer risk than the general population of nonsmokers (137).

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

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

In testing for possible interactions, at least two important considerations must be addressed. These include temporal (time) factors and response-endpoint considerations.

Time Factor

While most screening tests for interactions employ simultaneous exposure, this type of exposure approach has the chance of reducing the likelihood of detecting some potential interactions. For example, two agents may affect the same cellular mechanism but may have markedly different times of onset to expression. If a critical threshold of reversible cellular injury is required for the adverse effect, tests of acute toxicity of combinations given simultaneously may show antagonism, whereas an additive action would be observed if the dosing and observation periods were spaced to cause the maximum effect.

Toxic Effect

Since most toxic substances have multiple toxic effects, the nature of any chemical interaction may vary, depending on the measured responses. For example, since chlorinated insecticides and halogenated solvents produce liver injury independently, it is plausible that they could under certain circumstances act in an additive or synergistic manner when combined. However, the insecticide is likely to be a central nervous system stimulant, whereas the solvent may be a central nervous system depressant. Thus, as measured by neurological tests, these chemicals could interact in an antagonistic way.

Predictive Models

Development of predictive models of chemical interaction must rely on an understanding of the basic toxicological principles concerning kinetics of reactions of chemicals with primary sites of action (tissue receptor sites) and with secondary tissue sites of reaction. Four factors have been identified as of central importance:

1. Relative affinities of the individual chemicals for sites of action (e.g., target enzymes, cellular membranes, etc.).
2. Relative affinities for sites of loss of the chemical (e.g., detoxifying enzymes, nonvital tissue binding sites, pathways of excretion, and storage sites).
3. Intrinsic activity of the agents at their sites of action.
4. Sites of bioactivation.

While many of the examples are derived from the pharmacology literature, similar interactions could occur among environmental chemicals.

Pharmacokinetic Drug/Pollutant Interactions

The four factors listed allow us to predict how toxicological interactions may occur. The biological damage caused by a toxic agent is proportional to the amount of the biologically active form of the agents able to react with critical cellular macromolecules. An interaction may occur when the availability of an active chemical is altered by the presence of another agent, or when its reactivity with critical macromolecules is altered by the presence of another agent. The first case involves a site of loss of active chemical, whereas the second involves an interaction at a site of action. Thus, considerable

research activity has investigated the capacity of a chemi-

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cal to affect the absorption, distribution, metabolism, and excretion of another chemical.

Absorption

Absorption of an agent may be affected by a second drug that alters pH or gut motility. For example, aspirin is absorbed more rapidly at low pH because more of the drug is present in the readily diffusible nonionized lipid soluble form. Agents that cause an increase in the pH will slow down the gastric absorption of aspirin when taken simultaneously. Similarly, the absorption of tetracycline is reduced by aluminum hydroxide gels and readily ionized salts of calcium and magnesium. In contrast, the gastrointestinal absorption of acetaminophen is enhanced in the presence of sorbitol.

Protein Binding

Drugs may compete for the same protein binding sites in plasma. When this occurs, the effective biological concentrations of the displaced drug can rise markedly. For example, usually 98% of the anticoagulant drug warfarin is bound to the plasma protein albumin, so that only 2% of the total drug in the plasma is biologically active. If the effect of another drug competing for the same plasma albumin site is to reduce the binding of warfarin from 98 to 96%, the concentration of pharmacologically active warfarin would be doubled. This interaction would have approximately the same effect on clotting time as would doubling the dose of the anticoagulant. This type of interaction with an anticoagulant drug has resulted in a number of clinical incidents, with some resulting in fatal hemorrhagic complications.

Metabolism

Many chemicals, including drugs and environmental contaminants, enhance the metabolic capacity of the liver. Other chemicals may diminish the metabolic capacity of the liver. These interactions could have profound implications. In fact, it is now recognized that several of the insecticide synergists (i.e., agents that, when administered along with insecticides, markedly enhance the insecticide's ability to kill insects) act by blocking the enzymes normally affecting insecticide detoxification (221). For example, the toxicity of the insecticide carbaryl against susceptible female houseflies is enhanced by over 200-fold by certain chemical synergists. Thus, knowledge of synergy has been used to develop more effective insecticide formulations.

Insecticides provide another example of how agencies use information on chemical interactions. In 1957, Frawley et al. (61) reported the first synergistic interaction of two organophosphate insecticides (i.e., malathion and ethyl *p*-nitrophenyl phenyl phosphonothionate or EPN), which led to the development of the FDA requirement that all newly registered organophosphate insecticides be evaluated for possible synergisms with all the already-registered organophosphate insecticides. As more organophosphate insecticides were developed, this regulatory requirement became an extreme testing burden. However, with elucidation of the biochemical mechanism1 for this interaction, it became possible to assess possible interactions of organophosphate insecticides via biochemical means and thereby circumvent the time-consuming and costly toxicological testing of whole animals. In both examples, that of making a more efficient insecticidal preparation and that of predicting adverse public health effects from multiple agent exposures, chemical interaction predictions were markedly enhanced with a clear understanding of the mechanisms of toxicity.

Implications

The examples just given represent ideal situations, since the mechanisms of toxicity of the insecticides were very well characterized. More frequently, little information is available on toxic mechanisms. Regulatory agencies need to develop approaches in such situations when reasonable mechanistic predictions cannot be made. To this end, Finney (59) developed a theoretical mathematical approach for predicting the degree of toxicity derived from various types of chemical interactions. Pozzani et al. (154) indicated that only 2 of the 36 pairs of mixtures of industrial vapors tested for acute toxicity in rats deviated significantly from the calculations of Finney's theoretical approach for additive joint toxicity. According to Smyth et al. (170), the study by Pozzani et al. (154) supported the hypothesis that the acute toxicity of chemical mixtures randomly chosen has a high likelihood of being accurately predicted by Finney's theoretical formula for additive joint toxicity. In an attempt "to evaluate the overall confidence that can be placed on the prediction of the joint toxicity of many chemical pairs," Smyth et al. (170) studied the toxicity of 27 industrial chemicals in all possible pairs to rats. Their results were consistent with the prediction of Finney (59) that most interactions should be considered as additive until proven otherwise. Smyth et al. (170), in agreement with the general findings of Pozzani et al. (154), concluded that approximately 5% of the various combinations tested exhibited more or less than additive effects.

Most studies have evaluated synergism at only very high or acute levels of exposure. It is important, however, to ask whether synergisms would occur at lower (more realistic) concentrations. Recent

investigations using complex mixtures, tested at relatively low levels of

1 EPN inhibits the nonspecific enzyme carboxyesterase that detoxifies malathion. Thus, in the presence of EPN, malathion is more persistent and causes a greater effect as a cholinesterase inhibitor than would have occurred had enzymatic detoxification mechanisms not been affected.

exposure, indicate that the potential for interactions is likely dose dependent.
Their studies indicated that synergistic interactions, the type of interactions of greatest regulatory concern, may be less likely to occur at environmentally relevant exposure levels than at higher (e.g., pharmaceutical or some occupational) exposure levels.

Approaches Used by Regulatory Agencies to Assess Interactions

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

The Hazard Index Approach

Perhaps the simplest approach is the assumption of additivity of hazard. In this approach (applied to noncarcinogens), hazard indices (the ratio between the estimated dose and the reference dose; see section on evaluation of systemic toxicants) are summed across chemicals and across routes of exposure to obtain a total hazard index for a particular exposure setting (207). If the decision criterion of a total hazard index of 1 is exceeded, further review is performed to determine which chemicals act at the same target organ. A subsequent summation is performed for those chemicals only. While this approach is useful as a screening approach, it has several limitations that must be considered when interpreting the results. These include (but are not limited to) the following:

- Reference doses (the denominator in the hazard index) for different chemicals contain different types and magnitudes of uncertainty factors. Thus, differences in the magnitude of a hazard index for a particular chemical or a pathway of exposure may reflect intrinsic differences in hazard as well as differences in the uncertainty of a particular toxicity value.
- Different types of interactive effects are possible, even for chemicals that act at the same target organ. For example, organophosphates that act via the inhibition of acetylcholinesterase at nerve endings would generally be presumed to act in an additive manner (128). In contrast, trichloroethylene (TCE) and alcohol, both of which affect the central nervous system, can when consumed simultaneously, act synergistically (e.g., producing “degreaser’s flush”); however, chronic alcohol consumption can, by induction of metabolizing enzymes, diminish the response to TCE (2).

The Toxicity Equivalency Factor Approach

The toxicity equivalency factor (TEF) approach has been applied to a mixture that contains toxicologically and structurally similar chemicals. Perhaps one of the best known examples of the TEF approach is the approach developed by the U.S. EPA (17) for polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs). This approach is based on the assumption that PCDDs and PCDFs exert their toxicity through binding to the Ah receptor with subsequent effects on transcription and translational events responsible for toxicity. The most potent PCDD, 2,3,7,8-tetrachlorodibenzodioxin, has the greatest affinity for the Ah receptor and, hence, is the most potent member of this class. TEFs are developed for individual PCDDs and PCDFs, expressed as a fraction (typically in orders of magnitude) of that of TCDD, which is given a TEF of 1.0. Thus, RfDs and cancer slope factors (CSFs) are calculated as a ratio to the RfD and CSF for 2,3,7,8-TCDD. While there is experimental support, based on mixtures of PCDDs and PCDFs, for this approach, there are few, if any, data from long-term studies (17). Other uncertainties in this approach include the assumption of additivity, where competitive interactions may occur at sufficiently high doses, and the choice of a particular TEF value, which can be influenced by selection of endpoint, exposure duration, and dose (151).

The Complex Mixture Approach

There are certain classes of chemicals for which toxicological data exist primary for the complex mixture itself, with limited data for individual constituents. An example of this type of mixture is polychlorinated biphenyls or PCBs. PCBs were manufactured in the United States under the trade name of Aroclors for use in electrical capacitors. Different Aroclor mixtures contained different percentages of chlorine. For example, Aroclor 1242 contained approximately 42% chlorine (159). Much of the toxicity testing of PCBs consists of studies of different Aroclor mixtures (39, 159). As a result, toxicity criteria for PCBs are typically expressed as Aroclor-specific values. In the case of Aroclor 1254, for example, the Agency for Toxic Substances and Disease Registry (ATSDR) developed a chronic minimum risk level or MRL, a value conceptually to the RfD, based on immunological effects in monkeys exposed to Aroclor 1254 in feed for 23 months (151). While this approach does not require assumptions on how individual constituents will interact, it does assume that the characteristics of the mixture in the environment are the same as in the laboratory studies. Unfortunately, this assumption is not always correct, since complex mixtures

frequently undergo chemical transformations in the environment. Moreover, the individual constituents may partition differently in the environment. In the case of PCBs, for example, the more chlorinated forms bioaccummulate in fish more readily than the less chlorinated forms (39).

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

CONCLUSIONS

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

In addition, toxicology is used in the regulatory process to help assess potential risk associated with defined exposure levels. The traditional paradigm for assessing such risks is as follows: For carcinogens, potential risk is defined as an upper bound estimate of excess cancer risk based on cancer incidence at high dose levels, and for noncarcinogens, potential risk is defined as the ratio of the estimated exposure to an exposure level associated with negligible, if any, risk. Recent advances in the understanding of toxicological mechanisms indicate that these methodologies are not appropriate in all circumstances. Some carcinogens, such as those that operate by receptor-mediated or cytotoxic mechanisms, may exhibit a threshold or nonlinear dose-response relationship, and thus exposure levels associated with virtually zero risk might be defined. Examples of chemicals with these types of dose-response relationships are saccharin and phenobarbital. The U.S. EPA recent cancer risk assessment guidelines, which consider different dose-response relationships for different carcinogens, represent an important development in this area. Our understanding of certain noncarcinogenic effects, such as angina associated with CO exposure, is reasonably advanced; in this example, risks from CO are more fully described in terms of number of individuals with heart disease who might be expected to exceed defined COHb levels under certain exposure conditions. Benchmark dose and categorical exposure-response modeling represent additional examples of recent advances in noncarcinogenic risk assessment.

Toxicology is frequently applied in the regulatory context of developing permissible exposure levels in different exposure media, such as ambient air, drinking water, or food. As discussed in this chapter, definition of the health-based permissible exposure level is only one part of developing a regulatory standard. Other important factors in the regulatory process include risk management issues such as the definition of acceptable risk, the weighing of the costs and technical feasibility of reducing risk, the availability of alternatives, and the new risks possibly created by reducing the original risk (e.g., use of a less well tested substitute chemical). Issues of equity and whether certain members of the population are unfairly burdened by chemical exposure represent other considerations.

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

QUESTIONS

1. How do different approaches used for noncancer risk assessment (e.g., benchmark dose, reference dose, and the distributional population approach) address susceptible populations?
2. Under what circumstances would one use the following approach for assessing risks of complex mixtures: the hazard index approach, the toxicity equivalency factor approach, and the complex mixture?
3. What are some of the advantages and disadvantages of using cancer bioassay results at the

maximum tolerated dose (MTD) for interpreting likelihood of carcinogenicity to humans?

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REFERENCES

1. Abelson, P.H. (1993): Pesticides and food. *Science*, 259:1235.
2. Agency for Toxic Substances and Disease Registry. (1997): Toxicological profile for trichloroethylene—Update. Prepared by Sciences International, Inc., for U.S. Public Health Services, U.S. EPA. NTIS PB-101165/XAB.
3. Allison, A.C., Harrington, J.S., and Birbeck, M. (1966): An examination of the cytotoxic effects of silica on macrophages. *J. Exp. Med.*, 124:141–154.
4. American Cancer Society. (1998): *Cancer Facts & Figures*—1998. Publication 98–300M-No. 5008.98. American Cancer Society, Inc., Atlanta, GA.
5. American Conference of Governmental Industrial Hygienists. (1998): *Guide to Occupational Exposure Values—1998.* ACGIH, Cincinnati, OH.
6. American Conference of Governmental Industrial Hygienists. (1996): *Documentation of the Threshold Limit Values and Biological Indices.* American Conference of Governmental Industrial Hygienists, Cincinnati, OH. 6th edn. Supplement—Perchloroethylene.
7. American Conference of Governmental Industrial Hygienists. (1992): *Threshold Limit Values for Chemical Substances and Physi-cal Agents*. ACGIH, Cincinnati, OH.
8. American Thoracic Society. (1985): Guidelines as to what constitutes an adverse respiratory health effect with special reference to epidemiologic studies of air pollution. *Am. Rev. Respir. Dis.*, 131:666–669.
9. Ames, B.N., and Gold, L.S.S. (1990): Too many rodent carcinogens: Mitogenesis increases mutagenesis. *Science*, 249:970–971.
10. Ames, B.N., Swirsky-Gold, L., and Shigenaga, M.K. (1996): Cancer prevention, rodent high-dose cancer tests, and risk assessment. *Risk Anal.*, 16:613–617.
11. Andersen, M.E., Clewell, H.J. III, Gargas, M.L., Smith, F.A., and Reitz, R.H. (1987): Physiologically based pharmacokinetics and the risk assessment process for methylene chloride. *Toxicol. Appl. Pharmacol.*, 87:185–205.
12. Anderson, P.D. (1988): Scientific origins of incompatibility in risk assessment. *Stat. Sci.*, 3(3):320–327.
13. Baird, S.J.S., Cohen, J.T., Graham, J.D., Shlyakhter, A.I., and Evans, J.S. (1996): Noncancer risk assessment: A probabilistic alternative to current practice. *Hum. Ecol. Risk Assess.* 2(1):79–102.
14. Beck, B.D. (1997): The use of information on susceptibility in risk assessment: State of the science and potential for improvement. *Environ. Toxicol. Pharmacol.*, 4:229–234.
15. Beck, B.D., Connolly, R.B., Dourson, M.L., Guth, D., Hattis, D., Kimmel, C., and Lewis, S.C. (1993): Improvements in quantitative noncancer risk assessment. *Fundam. Appl. Toxicol.*, 20:1–14.
16. Beck, B.D., Toole, A.P., Callahan, B.G., and Siddhanti, S.K. (1991): Utilization of quantitative structure activity relationships (QSARs) in risk assessment. *Regul. Toxicol. Pharmacol.*, 14:273–285.
17. Bellin, J.S., Barnes, D.G., Kutz, F.W., and Bottimore, D.P. (1989): Interim procedures for estimating risks associated with exposures to mixtures of chlorinated dibenzo-p-dioxins and dibenzofurans (CDDs and CDFs) and 1989 update. Prepared for U.S. EPA, Risk Assessment Forum. EPA-625/3–89–016. NTIS PB90–145756 INZ.
18. Bethel, R.A., Epstein, J., Sheppard, D., Nadel, J.A., and Boushey, H.A. (1983): Sulfur dioxide-induced bronchoconstriction in freely breathing exercising, asthmatic subjects. *Am. Rev. Respir. Dis.*, 128:987–990.
19. Betke, J., Kleihaver, E., and Lipps, M. (1956): Vergleichende Untersucheg uber Sportanozydation von Nabelschnur and Erwachsenenhamoglobin. *Ztschr. Kinderh.* 77:549.
19a. Bickis, M., and Kreski, D. (1985): Statistical design and analysis of the long-term carcinogenicity bioassay. In: *Toxicological Risk Assessment*, Vol. 1, edited by D.B.Clayson, D.Krewski, and I.Munro, pp. 125–147. CRC Press, Boca Raton, FL.
20. Bromberg, P.A. (1998): Risk assessment of the effects of ozone exposure on respiratory health: dealing with variability in human responsiveness to controlled exposures. In: *Human Variability in Response to Chemical Exposure*, edited by D.A.Neumann and C.A.Kimmel, pp. 139–163. ILSI Press, Washington, DC.
21. Burns, C.A., Zarkower, A., and Ferguson, F.G. (1980): Murine immunological and histological changes in response to chronic silica exposure. *Environ. Res.*, 21:298–307.
22. Butterworth, B.E. (1990): Consideration of both genotoxic and nongenotoxic mechanisms in predicting carcinogenic potential. *Mutat. Res.*, 239:117–132.
23. Calabrese, E.J. (1996): Expanding the RfD concept to incorporate and optimize beneficial effects

while preventing toxic responses from non-essential toxicants. *BELLE Newslett.*, 4:1–10.
24. Calabrese, E.J. (1987): Animal extrapolation: A look inside the toxicologist's black box. *Environ. Sci. Technol.*, 21:618–623.
25. Calabrese, E.J. (1978): *Pollutants and High Risk Groups*. John Wiley, New York.
26. Calabrese, E.J., and Baldwin, L.A. (1999): The marginalization of hormesis. *Toxicol. Pathol.*, 27:187–194.
27. Calabrese, E.J., and Baldwin. L.A. (1999): Significant biological effects below the NOAEL in reproductive toxicology. In: *Environmental and Human Risk Assessment*, edited by H.Salem, pp. 95–106. Taylor and Francis, Washington, DC.
28. Calabrese, E.J., and Baldwin. L.A. (1998): Can the concept of hormesis be generalized to carcinogenesis? *Regul. Toxicol. Pharmacol.*, 28:230–241.
29. Calabrese, E.J., and Baldwin, L.A. (1998): Hormesis as a default parameter in RfD derivation. *Hum, Exp. Toxicol.*, 17:444–447.
30. Calabrese, E.J., and Baldwin, L.A. (1997): The dose determines the stimulation (and poison): Development of a chemical hormesis database. *Int. J. Toxicol.*, 16:545–559.
31. Calabrese, E.J., and Baldwin, L.A. (1997): A quantitatively-based methodology for the evaluation of chemical hormesis. *Hum. Ecol. Risk Assess.*, 3:545–554.
32. Calabrese, E.J., Beck, B.D., and Chappell, W.R. (1992): Does the animal-to-human uncertainty factor incorporate interspecies differences in surface area? *Regul. Toxicol. Pharmacol.*, 15:172–179.
33. Carnow, B.W. (1976): Panel discussion on TLV's—Lead. In: *Health Effects of Occupational Lead and Arsenic Exposure: A Symposium*, edited by B.W.Carnow, p. 197. U.S. PHS, NIOSH. Washington, DC.
34. Carson, R.L. (1962): *Silent Spring.* Houghton Mifflin, Boston.
35. Chang, L.-Y., Huang, Y., Stockstill, B.L., Graham, J.A., Grose, E.C., Menache, M.G., Miller, F.J., Costa, D.L.. and Crapo, J.D. (1992): Epitheliel injury and interstitial fibrosis in the proximal alveolar regions of rats chronically exposed to a simulated pattern of urban ambient ozone. *Toxicol. Appl. Pharmacol.*, 115:241–252.
36. Chernoff, N., and Kavlock. R.J. (1982): An in vivo teratology screen utilizing pregnant mice. *J. Toxicol. Environ. Health*, 10:541–550.
37. Clayson, D.B., and Clegg, D.J. (1991): Classification of carcinogens: Polemics, pedantics, or progress? *Regul, Toxicol. Pharmacol.*, 14:147–166.
38. Clewell, H.J. (1995): The use of physiologically based pharmacokinetic modeling in risk assessment: A case study with methylene chloride. In: *Low-Dose Extrapolation of Cancer Risks*,

edited by S.Olin, W.Farland, C.Park, L.Rhomberg, R. Scheuplein, T.Starr, and J.Wilson, pp. 199–222. ILSI Press, Washington, DC.
39. Cogliano, V.J. (1996): PCBs: Cancer dose-response assessment and application to environmental mixtures. Prepared for U.S. EPA, National Center for Environmental Assessment. NTIS PB96–140603. EPA/600/P-96/001A. NTIS PB96–140603.
40. Cohen, S.M., and Ellwein, L.B. (1995): Biological theory of carcinogenesis: Implications for risk assessment. In: *Low-Dose Extrapolation of Cancer Risks,* edited by S.Olin, W.Farland, C.Park, L.Rhomberg, R.Scheuplein, T.Starr, and J.Wilson, pp. 145–161. ILSI Press, Washington, DC.
41. Colditz, G.A., Stampfer, M.J., and Green, L.C. (1988): Diet. In: *Variations in Susceptibility to Inhaled Pollutants,* edited by B. D.Brain, A.J.Waven, and R.A.Shaiker, pp. 314–331. Johns Hopkins University Press, Baltimore, MD.
42. Conolly, R.B., and Butterworth, B.E. (1995): Biologically based dose response model for hepatic toxicity: a mechanistically based replacement for traditional estimates of noncancer risk. *Toxicol. Lett.,* 82/83:901–906.
43. Cook, P.J., Doll, R.., and Fellingham, S.A. (1969): A mathematical model for the age distribution of cancer in man. *Int. J. Cancer,* 4:93–112.
44. Cooper, W.C. (1973): Indicators of susceptibility to industrial chemicals. *J. Occup. Med.,* 15(4):355.
45. Crump, K.S., Clewell, H.J., and Andersen, M.E. (1997): Cancer and non-cancer risk assessment should be harmonized. *Hum. Ecol. Risk Assess.,* 3(4):495–499.
46. Crump, K.S. (1984): A new method for determining allowable daily intake. *Fundam. Appl. Toxicol.,* 4:854–871.
47. Cullen, A.C., and Frey, H.C., eds. (1999): *Probabilistic Techniques in Exposure Assessment. A Handbook for Dealing with Variability and Uncertainty in Models and Inputs.* Plenum Press, New York.
48. deRosa, C.T., Stara, J.F., and Durkin, P.R. (1985): Ranking chemicals based on chronic toxicity data. *Toxicol. Ind. Health,* 1:177–192.
49. Deutsche Forchungsgemeinschaft. (1996): List of Max and BAT values for 1996. VCH Verlagsgeschschaft, Weinheim. As cited in Neumann, H.-G., Thielmann, H.W., Filser, J.G., et al. (1997): Proposed changes in the classification of carcinogenis chemicals in the work area. *Regul. Toxicol. Pharmacol,* 26:288–295.
50. Dourson, M.L. (1986): New approaches in the derivation of acceptable daily intake (ADI). *Comments Toxicol.,* 1:35–48.
51. Dourson, M.L., and Stara, J.F. (1983): Regulatory history and experimental support of uncertainty (safety) factors. *Regul. Toxicol. Pharmacol.,* 3:224–238.
52. Elcombe, C.R., Rose, M.S., and Pratt. I.S. (1985): Biochemical, histological, and ultrastructural changes in rat and mouse liver following the administration of trichloroethylene: Possible relevance to species differences in hepatocarcinogenicity. *Toxicol. Appl. Pharmacol.,* 79:365–376.
53. Elsayed, N.M., and Mustafa. M.G. (1982): Dietary antioxidants and the biochemical response to oxidant inhalation. I. Influence of dietary vitamin E on the biochemical effects of nitrogen dioxide exposure in rat lung. *Toxicol. Appl. Pharmacol.,* 66:319–328.
54. Enslein. K. (1987): Computer-assisted prediction of toxicity. In: *Toxic Substances and Human Risk. Principals of Data Interpretation,* edited by R.G.Tardiff, and J.V.Rodricks, pp. 317–338. Plenum Press, New York.
55. Enterline, P.E. (1983): Epidemiologic basis for the asbestos standard. *Environ. Health Perspect..* 52:93–97.
56. European Union. (1993): Annex VI general classification and labeling requirements for dangerous substances and preparation, communication directions 93/21/EEC of April 27. 1993. *Official Journal of the European Communities* L110A 5/4/1993. As cited in Neumann, H.-G., Thielmann, H.W., Filser, J.G., et al. (1997): Proposed changes in the classification of carcinogenis chemicals in the work area. *Regul. Toxicol. Pharmacol.,* 26:288–295.
57. Faustman, E.M. (1996): Review of Noncancer Risk Assessment: Application of Benchmark Dose Methods. Prepared for the Commission on Risk Assessment and Risk Management. Washington, DC. June.
58. Finkel, A.M. (1991): Testimony of Adam M.Finkel, Resources for the Future, before the U.S. House of Representatives Committee on Science, Space, and Technology. Subcommittee on Environment. Hearing on Risk Assessment: Strengths and Limitations of Utilization for Policy Decisions, May 21, 1991. Washington, DC.
59. Finney, D.J. (1952): *Probit Analysis.* Cambridge University Press, London.

60. Frame, L.T., Ambrosone, C.B., Kadlubar, F.F., and Lang, N.P. (1998): Host-environment interactions that affect variability in human cancer susceptibility. In: *Human Variability in Response to Chemical Exposure*, edited by D.A.Neumann and C.A. Kimmel, pp. 165–204. ILSI Press, Washington, DC.
61. Frawley, J.P., Fuyat, H.N., Hagan, E.C., Blake, J.R., and Fitzhugh, O.G. (1957): Marked potentiation in mammalian toxicity from simultaneous administration of two anti-cholinesterase compounds. *J. Pharmacol. Exp. Ther.*, 121:96.
62. Gaylor, D. (1998): Safety assessment with hormetic effects. *Hum. Exp. Toxicol.*, 17:251–253.
63. Germolec, D.R., Yang, R.S., Ackermann, M.F., Rosenthal, G. J., Boorman, G.A., Blair P., and Luster, M.I. (1989): Toxicology studies of a chemical mixture of 25 groundwater contaminants. II. Immunosuppression in B5C3F1 mice. *Fundam. Appl. Toxicol.*, 13:377–387.
64. Golden, R.J., Holms, S.E., Robinson, D.E., Julkunen, P.H., and Reese, E.A. (1997): Chloroform in mode of action: implications for cancer risk assessment. *Regul. Toxicol. Pharmacol.*, 26:142–155.
65. Goodman, G., and Wilson, R. (1992): Comparison of the dependence of the TD50 on maximum tolerated dose for mutagens and nonmutagens. *Risk Anal.*, 12(4):525–533.
66. Graham, J.D. (1992): *Recommendations for Improving Cancer Risk Assessment.* Center for Risk Analysis, Harvard School of Public Health, Boston.
67. Graham, J.D., and Wiener, J.B., eds. (1995): *Risk vs. Risk: Tradeoffs in Protecting Health and the Environment.* Harvard University Press, Cambridge, MA.
68. Green, G.M. (1984): Similarities of host defense mechanisms against pulmonary disease in animals and man. *J. Toxicol. Environ. Health*, 13:471–478.
69. Grassman, J.A., Kimmel, C.A., and Neumann, D.A. (1998): Accounting for variability in responsiveness in human health risk assessment. In: *Human Variability in Response to Chemical Exposure*, edited by D.A.Neumann and C.A.Kimmel, pp. 1–26. ILSI Press, Washington, DC.
70. Gross, P., de Villiers, A.J., and de Treveille, R.T.P. (1967): Experimental silicosis: The "atypical reaction" in the Syrian hamster. *Arch. Pathol.*, 84:87–94.
71. Guzelian, P.S., and Henry, C.J. (1992): Conference summary; similarities and differences between children and adults: Implications for risk assessment (November 5–7, 1990, Hunt Valley, Maryland). In: *Similarities and Differences Between Children and Adults: Implications for Risk Assessment*, edited by P.S. Guzelian, C.J.Henry, and S.S.Olin, pp. 1–3. ILSI Press, Washington, DC.

72. Hackey, J.D., and Linn, W.S. (1983): Controlled clinical studies of air pollutant exposure: Evaluating scientific information in relation to air quality standards. *Environ. Health Perspect.*, 52:187–191.
73. Harington, J.S., Ritchie, M., King, P.C., and Miller, K. (1973): The *in-vitro* effects of silica-treated hamster macrophages on collagen production by hamster fibroblasts. *J. Pathol.*, 109:21–37.
74. Hart, R.W., and Fishbein, L. (1985): Interspecies extrapolation of drug and genetic toxicity data. In: *Toxicological Risk Assessment*, Vol. I, edited D.B.Clayson, D.Krewski, and I.Munro, pp. 3–40. CRC Press, Boca Raton, FL.
75. Hart, R.W., and Turturro, A. (1988): Introduction. In: *Banbury Report 31: Carcinogen Risk Assessment: New Directions in the Qualitative and Quantitative Aspects*, edited by R.W.Hart and F.D.Hoerger., pp. 1–14. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
76. Haseman, J.K. (1985): Issues in carcinogenicity testing: Dose selection. *Fundam. Appl. Toxicol.*, 5:66–78.
77. Haseman, J.K., and Lockhart, A.-M. (1994): The relationship between use of the maximum tolerated dose and study sensitivity for detecting rodent carcinogenicity. *Fundam. Appl. Toxicol.*, 22:382–391.
78. Hattis, D., White, P., Marmarstein, L., and Koch, P. (1990): Uncertainties in pharmacokinetic modeling for perchloroethylene: I. Comparison of model structure, parameters. and predictions for low-dose metabolism creates for models derived by different authors. *Risk Analysis*, 10(3):449–458.
79. Health Canada (1994): Human Health Risk Assessment for priority substances. As cited in http://www.tera.org/iter/methods/cancer.htm. Downloaded 2/27/99.
80. Heinrich. U., Muhle, H., Takenaka, S., Ernst, H., Fuhst, R., Mohr, U., Pott, F., and Stober, W. (1986): Chronic effects on the respiratory tract of hamsters, mice and rats after long-term inhalation of high concentrations of filtered and unfiltered diesel engine emissions. *J. Appl Toxicol.*, 6:383–395.
81. Heppleston, A.G. (1984): Pulmonary toxicology of silica, coal and asbestos. *Environ. Health Perspect.*, 55:111–127.
82. Hutt, P.B. (1985): Use of quantitative risk assessment in regulatory decision making under federal health and safety statutes. In: *Risk Quantitation and Regulalory Policy*, edited by D.G.Hoel, R.A.Merrill, and F.P.Perera, pp. 15–29. Banbury Report 19, Cold Spring Harbor Laboratory, Cold Spring Harbor. N.Y.
83. Institute for Evaluating Health Risks. (1992): An evaluative process for determining human reproductive and developmental toxicity of agents, draft version. Institute for Evaluating Health Risks, Washington, DC.
84. Implementation Working Group. (1998): *A science-based, workable framework for implementing the Food Quality Protection Act. Implementation Working Group's "Road Map" Report.* Prepared by Jellinek, Schwartz & Connolly, Inc., McDermott, Will & Emery and Morgan, Lewis & Bockius. (Washington, DC).
85. Industrial Union Department. (1980): AFL-CIO v. American Petroleum Institute, 448 U.S. 60165 L. Ed. 2d 1010, 100 S. Ct. 2844.
86. Infante, P.F., Rinsky, R.A., Wagoner, J.K., et al. (1977): Benzene and leukemia. *Lancet*, p. 867–870.
87. International Agency for Research on Cancer. (1999): *IARC Monograph Summary for Beryllium and Beryllium Compounds (Group 1) (updated 5/4/99).* Downloaded from IARC web site http://193.51.164.11/cgi/iHound/chem/iH_chem_frames.html
88. International Agency for Research on Cancer. (1999): *IARC Monograph Summary for Cadmium and Cadmium Compounds (Group 1) (updated 5/4/99).* Downloaded from IARC web site http://193.51.164.11/cgi/iHound/chem/iH_chem_frames.html
89. International Agency for Research on Cancer. (1999): *IARC Monograph Summary for Cyclosporin (Group 1) (updated 5/4/99).* Downloaded from IARC web site http://193.51.164.11/cgi/iHound/chem/iH_chem_frames.html
90. International Agency for Research on Cancer. (1999): *IARC Monograph Summary for Epstein-Barr Virus (Group 1) (updated 5/4/99).* Downloaded from IARC web site http://193.51.164.111/cgi/iHound/chem/iH_chem_frames.html
91. International Agency for Research on Cancer. (1999): *IARC Monograph Summary for Oestrogen Replacement Therapy (Group 1) (updated 5/4/99).* Downloaded from IARC web site http://193.51.164.11/cgi/iHound/chem/iH_chemJrames.html
92. International Agency for Research on Cancer. (1999): *IARC Monograph Summary for Ethylene Oxide (Group 1) (updated 5/4/99).* Downloaded from 1ARC web site http://193.51.164.11/1/cgi/iHound/chem/iH_chem_frames.html
93. International Agency for Research on Cancer. (1999): *IARC Monograph Summary for Infection with Helicobacter Pylori (Group 1) (updated 5/4/99).* Downloaded from IARC web site http://193.51.164.11/cgi/iHound/chem/iH_chem_frames.html

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_71.html[2010/10/28 18:31:07]

94. International Agency for Research on Cancer. (1999): *IARC Monograph Summary for Hepatitis B Virus (Group 1) (updated 5/4/99).* Downloaded from IARC web site http://193.51.164.11/1/cgi/iHound/chem/iH_chem_frames.html
95. International Agency for Research on Cancer. (1999): *IARC Monograph Summary for Hepatitis C Virus (Group 1) (updated 5/4/99).* Downloaded from IARC web site http://193.51.164.11/cgi/iHound/chem/iH_chem_frames.html
96. International Agency for Research on Cancer. (1999): *IARC Monograph Summary for Human Immunodeficiency Viruses: HIV-1 (Group 1); HIV-2 (Group 2B) (updated 5/4/99).* Downloaded from IARC web site http://193.51.164.11/cgi/iHound/chem/iH_chem_frames.html
97. International Agency for Research on Cancer. (1999): *IARC Monograph Summary for Human Papilloma Viruses (HPV): HPV types 16 and 18 (Group 1): HPV types 31 and 33 (Group 2A); Some HPV types other than 16, 18, 31 and 33 (Group 2B) (updated 5/4/99).* Downloaded from IARC web site http://193.51.164.11/cgi/iHound/chem/iH_chem_frames.html
98. International Agency for Research on Cancer. (1999): *IARC Monograph Summary for Human T-Cell Lymphotropic Viruses: HTLV-I (Group 1); HTLV-II (Group 3) (updated 5/4/99).* Downloaded from IARC web site http://193.51.164.11/cgi/iHound/chem/iH_chem_frames.html
99. International Agency for Research on Cancer. (1999): *IARC Monograph Summary for Infection with Liver Flukes (Opisthorchis viverrini, Opisthorchis felineus and Clonorchis sinensis): Opisthorchis viverrini (Group 1); Opisthorchis felineus (Group 3); Clonorchis sinensis (Group 2A) (updated 5/4/99).* Downloaded from IARC web site http://193.51.164.11/cgi/iHound/chem/iH_chem_frames.html
100. International Agency for Research on Cancer. (1999): *IARC Monograph Summary for Hormonal Contraception and Postmenopausal Hormonal Therapy (Vol. 72) (2–9 June, 1998) In prep-aration (updated 5/4/99).* Downloaded from IARC web site http://193.51.164.11/cgi/iHound/chem/iH_chem_frames.html
101. International Agency for Research on Cancer. (1999): *IARC Monograph Summary for Oral Contraceptives, Combined (Group 1) (updated 5/4/99).* Downloaded from IARC web site http://193.51.164.11/cgi/iHound/chem/iH_chem_frames.html
102. International Agency for Research on Cancer. (1999): *IARC Monograph Summary for Salted Fish: Chinese-style salter fish (Group 1); Other salted fish (Group 3) (updated 5/4/99).* Downloaded from IARC web site http://193.51.164.11/cgi/iHound/chem/iH_chem_frames.html
103. International Agency for Research on Cancer. (1999): *IARC Monograph Summary for Infection with Schistomsomes*

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_71.html[2010/10/28 18:31:07]

(Schistosoma haematobium, Schistosoma mansoni and Schistosoma japonicum): Schistosoma haematobium (Group 1); Schistosoma mansoni (Group 3); Schistosoma japonicum (Group 2B) (updated 5/4/99). Downloaded from IARC web site http://193.51.164.11/cgi/iHound/chem/iH_chem_frames.html
104. International Agency for Research on Cancer. (1999): *IARC Monograph Summary for Silica: Crystalline silica-inhaled in the form of quartz or cristobalite from occupational sources (Group 1); Amorphous silica (Group 3) (updated 5/4/99).* Downloaded from IARC web site http://193.51.164.11/cgi/iHound/chem/iH_chem_frames.html
105. International Agency for Research on Cancer. (1999): *IARC Monograph Summary for Solar and Ultraviolet Radiation: Solar radiation (Group 1), Ultraviolet A radiation (Group 2A), Ultraviolet B radiation (Group 2A), Ultraviolet C radiation (Group 2A), Use of sunlamps and sunbeds (Group 2A), Exposure to fluorescent lighting (Group 3) (updated 6/2/99).* Downloaded from IARC web site http://193.51.164.11/cgi/iHound/chem/iH_chem_frames.html
106. International Agency for Research on Cancer. (1999): *IARC Monograph Summary for Tamoxifen (Group 1) (updated 5/4/99).* Downloaded from IARC web site http://193.51.164.11/cgi/iHound/chem/iH_chem_frames.html
107. International Agency for Research on Cancer. (1999): *IARC Monograph Summary for Polychlorinated Dibenzo-para-Dioxins: 2,3,7,8-Tetrachlorodibenzo-para-dioxin (Group 1); Polychlorinated dibenzo-para-dioxins (other than 2,3,7,8-Tetrachlorodibenzo-para-dioxin): 2,7-DCDD, 1,2,3,6,7,8-/1,2,3,7,8,9-HxCDD, 1,2,3,4,6,7,8-HpCDD (Group 3); Dibenzo-para-dioxin (Group 3) (updated 5/4/99).* Downloaded from IARC web site http://193.51.164.11/cgi/iHound/chem/iH_chem_frames.html
108. International Agency for Research on Cancer. (1999): *IARC Monograph Summary for Thiotepa (Group 1) (updated 5/4/99).* Downloaded from IARC web site http://193.51.164.11/cgi/iHound/chem/iH_chem_frames.html
109. International Agency for Research on Cancer. (1999): *IARC Monograph Summary for Wood Dust (Group 1) (updated 5/4/99).* Downloaded from IARC web site http://193.51.164.11/cgi/iHound/chem/iH_chem_frames.html
110. International Agency for Research on Cancer. (1980): *Long-term and Short-term Screening Assays for Carcinogens: A Critical Appraisal.* IARC Monographs, Suppl. 2. International Agency for Research on Cancer, Lyons, France.
111. International Agency For Research on Cancer. (1982): *Evaluation of Carcinogenic Risk of Chemicals to Humans.* IARC Monographs, Suppl. 4. International Agency for Research on Cancer, Lyons, France.
112. International Agency For Research on Cancer. (1987): *Overall Evaluations of Carcinogenicity: An Updating of IARC Monographs Volumes 1 to 42.* IARC Monographs, Suppl. 7. International Agency for Research on Cancer, Lyons, France.
113. International Agency for Research on Cancer. (1992): Meeting Report: Working Group on Mechanisms of Carcinogenesis and the Evaluation of Carcinogenic Risks. *Cancer Res.*, 52:2357–2361.
114. International Agency for Research on Cancer. (1997): *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Dry Cleaning, Some Chlorinated Solvents and Other Industrial Chemicals.* IARC Monographs, Vol. 63. International Agency for Research on Cancer, Lyons, France.
115. International Agency for Research on Cancer. (1999): *Overall Evaluations of Carcinogenicity to Humans.* Updated by IARC January 20, 1999. http://193.51.164.11/monoeval/crthal.html, downloaded 2/9/99.
116. Jarabek, A.M., Menache, M.G., Overton, J.H., Jr., Douson, M. L., and Miller, F.J. (1990): The U.S. Environmental Protection Agency's inhalation RfD methodology: risk assessment for air toxics. *Toxicol. Ind. Health*, 6:279–301.
117. Kavlock, R.J., Schmid, J.E., and Setzer, R.W. (1996): A simulation study of the influence of study design on the estimation of benchmark doses for developmental toxicity. *Risk Anal.*, 16(3):399–410.
118. Kauffman, R.E. (1992): Acute acetaminophen overdose: An example of reduced toxicity related to developmental differences in drug metabolism. In: *Similarities and Differences Between Children and Adults: Implications for Risk Assessment*, edited by P.S. Guzelian, C.J.Henry, and S.S.Olin, pp. 97–103. ILSI Press, Washington, DC.
119. Khera, K.S., Grice, H.C., and Clegg, D.J. (1989): *Current Issues in Toxicology, Interpretation and Extrapolation of Reproductive Data to Establish Human Safety Standards.* Springer-Verlag, New York.
120. Kouri, R.E., and Nebert, D.W. (1977): Genetic regulation of susceptibility to polycyclic hydrocarbon induced tumors in the mouse. In: *Origins of Human Cancer*, edited by H.H.Hiatt, J.D.Watson, and J.A.Winstyen, pp. 811–835. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
121. Linkov, I., Wilson, R., and Gray, G.M. (1998): Anticarcinogenic responses in rodent cancer

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_72.html[2010/10/28 18:31:08]

bioassays are not explained by random effects. *Toxicol. Sci.*, 43:1–9.
122. Lipfert, F.W. (1980): Sulfur oxides, particulates and human mortality: Synopsis of statistical correlations. *J. Air Pollut. Control Assoc.*, 30:366–371.
123. Liteplo, R.G., and Meek, M.E. (1994): Tetrachloroethylene: Evaluation of risks to health from environmental exposure in Canada. *Environ. Carcinogen. & Ecotoxicol. Rev.*, C12(2): 493–506.
124. Maltoni, C., Conti, B., and Cotti, G. (1983): Benzene: A multi-potential carcinogen. Results of long-term bioassays performed at the Bologna Institute of Oncology. *Am. J. Ind. Med.*, 4:589–630.
125. Manson, J.M., and Wise, L.D. (1991): Teratogens. In: *Casarett and Doull's Toxicology*, edited by M.O.Amdur, J.Doull, and C.D.Klaassen, pp. 226–254, Pergamon Press, New York.
126. McKone, T.E., and Bogen, K.T. (1991): Predicting the uncertainties in risk assessment. *Environ. Sci. Technol.*, 25(10): 1674–1681.
127. Melnick, R.L., Boorman, G.H., Haseman, J.K., and Huff, J. (1984): Toxicity and carcinogenicity of melamine in F344 rats and B5C3F1 mice. *Toxicol. Appl. Pharmacol.*, 72:292–303.
128. Mileson, B.E., Chambers, J.E., Chen, W.L., Dettbarn, W., Ehrich, M., Eldefrawi, A.T., Gaylor, D.W., Hamernik, K., Hodgson, E., Karczmar, A.G., Padilla, S., Pope, C.N., Richardson, R.J., Saunders, D.R., Sheets, L.P., Sultatos, L. G., and Wallace, K.B. (1998): Commom mechanism of toxicity: A case study of organophosphorus pesticides. *Toxicol. Sci.*, 41(1):8–20.
129. Miller, F.J., Illing, J.W., and Gardner, D.E. (1978): Effect of urban ozone levels on laboratory-induced respiratory infections. *Toxicol. Lett.*, 2:163–169.
130. Moolgavkar, S.H. (1986): Carcinogenesis modeling: from molecular biology to epidemiology. *Ann. Rev. Public Health*, 7:151–169.
131. Munro, I.C. (1977): Considerations in chronic toxicity testing: The chemical, the dose, the design. *J. Environ. Pathol. Toxicol.*, 1:183–197.
132. National Research Council. (1996): *Understanding Risk: Informing Decisions in a Democratic Society*, edited by P.C.Stern and H.V.Fineberg. National Academy Press, Washington, DC.
133. National Research Council. (1994): *Science and Judgment in Risk Assessment*. National Academy Press, Washington, DC.

134. National Research Council. (1987): *Regulating Pesticides in Food: The Delaney Paradox.* National Academy Press, Washington, DC.
135. National Research Council. (1986): *Drinking Water and Health,* Vol. 6. National Academy Press, Washington, DC.
136. National Research Council. (1983): *Risk Assessment in the Federal Government: Managing the Process.* National Academy Press, Washington, DC.
137. National Research Council. (1980): *Principles of Toxicological Interactions Associated with Multiple Chemical Exposures.* National Academy Press, Washington, D.C.
138. Nebert, D.W. (1989): The Ah locus: Genetic differences in toxicity, cancer, mutation. *Crit. Rev. Toxicol.,* 20:153–174.
139. Neumann D.A., and Kimmel, C.A., eds. (1998): *Human Variability in Response to Chemical Exposure.* ILSI Press, Washington, DC.
140. Neumann, H.-G., Thielmann, H.W., Filser, J.G., Gelbke, H.-P., Greim, H., Kappus, H., Norpoth, K.H., Reuter, U., Vamvakas, S., Wardenbach, P., and Wichmann, H.-E. (1997): Proposed changes in the classification of carcinogenic chemicals in the work area. *Regul. Toxicol. Pharmacol.,* 26:288–295.
141. New York State Department of Environmental Conservation. (1986): *New York State Air Guide-1: Guidelines for the Control of Toxic Ambient Air Contaminants.* Division of Air Resources, Albany.
142. Nichols A.L., and Zeckhauser, R.J. (1986): The perils of prudence; how conservative risk assessments distort regulation. *Regulation,* November/December: 13–24.
143. Oberdorster, G., and Yu, C.P. (1990): The carcinogenic potential of inhaled diesel exhaust: a particle effect? *J. Aerosol Sci., Suppl.* 1(21):397–401.
144. Occupational Safety and Health Administration. (1980): Identification, classification, and regulation of potential occupational carcinogens. *Fed. Reg.,* 45:5002–5296.
145. Occupational Safety and Health Act of 1970. 29 U.S.C. 655.
146. Office of Management and Budget. (1990): Current regulatory issues in risk assessment and management. In: *Regulatory Program of the United States Government.* Executive Office of the President, Washington, DC. April 1, 1990-March 31, 1991.
147. Office of Science and Technology Policy. (1986): Chemical carcinogens: A review of the science and its associated principles. U.S. Interagency Staff Group on Carcinogens. *Environ. Health Perspect.,* 67:201–282.
148. Overton, J.H., and Jarabek, A.M. (1989): Estimating equivalent human concentrations of no observed adverse effect levels: A comparison of several methods. *Exp. Pathol.* 37:89–95.
149. Pitot, H.C., and Dragan, Y.P. (1991): Facts and theories concerning the mechanisms of carcinogenesis. *FASEB J.,* 5:2280–2286.
150. Plunkett, L.M., Turnbull, D., and Rodricks, J.V. (1992): Differences between adults and children affecting exposure assessment. In: *Similarities and Differences Between Children and Adults: Implications for Risk Assessment,* edited by P.S.Guzelian, C. J.Henry, and S.S.Olin, pp. 79–94. ILSI Press, Washington, DC.
151. Pohl, H.R., Hansen, H., and Chou, C.H. (1997): Public health guidance values for chemical mixtures: current practice and future directions. *Regul. Toxicol. Pharmacol.,* 26(3):322–329.
152. Portier, C., Tritscher, A., Kohn, M., Sewall, C., Clark, G., Edler, L., Hoel, D., and Lucier, G. (1993): Ligand/receptor binding for 2,3,7,8-TCDD: Implications for risk assessment. *Fundam. Appl. Toxicol.,* 20:48–56.
153. Pott, P. (1779): Cancer scroti. In: *Chirurgical Works, A New Edition in Three Volumes.* Vol. I. London.
154. Pozzani, U.S., Weil, C.S., and Carpenter, C.P. (1959): The toxicological basis of TLVs: 5. The experimental inhalation of vapor mixtures by rats, with notes upon the relationship between single dose inhalation and single dose oral data. *Am. Ind. Hyg. Assoc. J.,* 20:364–369.
155. Presidential/Congressional Commission on Risk Assessment and Risk Management. (1997): Risk Assessment and Risk Management in Regulatory Decision-Making (Final Report, Vol. 2). Washington, DC.
156. Public Law 85–929. (1958): Food Additives Amendment of 1958.
157. Quakenboss, J.J., Kanarek, M.S., Spengler, J.D., and Letz, R. (1982): Personal monitoring for nitrogen dioxide exposure: Methodological considerations for a community study. *Environ. Int.,* 8:249–258.
158. Research Triangle Institute. (1998): Toxicological profile for chlorinated dibenzo-p-dioxins. National Technical Information Service, Prepared for Agency for Toxic Substances and Disease Registry. NTIS

PB99–121998.
159. Research Triangle Institute. (1998): Toxicological profile for polychlorinated biphenyls (PCB) (Update—Draft for public comment). Prepared for U.S. Public Health Service, Agency for Toxic Substances and Disease Registry *(ATSDR),* Atlanta, GA.
160. Riddiough, C.R., Musselmann, R., and Calabrese, E.J. (1977): Is EPA's radium-226 drinking water standard justified? *Med. Hypoth.,* 3(5): 171.
161. Rieth, J.P., and Starr, T.B. (1989): Chronic bioassays: Relevance to quantitative risk assessment of carcinogens. *Regul. Toxicol. Pharmacol.* 10:160–173.
162. Roberts, R.J. (1992): Overview of similarities and differences between children and adults: implications for risk assessment. In: *Similarities and Differences Between Children and Adults: Implications for Risk Assessment,* edited by P.S.Guzelian, C. J.Henry, and S.S.Olin, pp. 11–15. ILSI Press, Washington, DC.
163. Rodricks, J.V., and Taylor, M.R. (1989): Comparison of risk management in U.S. regulatory agencies. *J. Haz. Mater.,* 21:239–253.
164. Roger, L.J., Kehrl, H.R., Hazucha, M., and Horstman, D.H. (1985): Bronchoconstriction in asthmatics exposed to sulfur dioxide during repeated exercise. *J. Appl. Physiol.,* 59:784–791.
165. Rosenthal, A., Graf, G.M., and Graham, J.D. (1992): Legislating acceptable cancer risk from exposure to toxic chemicals. *Ecol. Law Q.,* 19:269–362.
166. Ross, J.D., and Des Forges, J.F. (1959): Reduction of methemoglobin by erythrocytes from cord blood. Further evidence of deficient enzyme activity in newborn period. *Pediatrics,* 23:218.
167. Scheuplein, R.J. (1987): Risk assessment and food safety: A scientist and regulator's view. *Food Drug Cosmet. Law J.,* 42:237–250.
168. Sielken, R.L., Jr., and Stevenson, D.E. (1998): Some implications for quantitative risk assessment if hormesis exists. *Hum. Exp. Toxicol.,* 17:259–262.
169. Slob, W., and Pieters, M.N. (1997): A probabilistic approach for deriving acceptable human intake limits and human health risks from toxicological studies: General framework. Report 620110 005. National Institute of Public Health and the Environment, BiHhoven, the Netherlands.
170. Smyth, H.F., Jr., Weil, C.S., West, C.P., and Carpenter, J.S. (1969): An exploration of joint toxic action: 27 Industrial chemicals in rats in all possible pairs. *Toxicol. Appl. Pharmacol.,* 14:340–347.
171. Sontag, J.M., Page, N.P., and Sanotti, U. (1976): Guidelines for carcinogen bioassays in small rodents. DHHS publ. (NIH) 76–801. National Cancer Institute, Bethesda, MD.
172. Strauss, H.S. (1993): Sex biases in the risk assessment of toxic chemicals. Presented at the Annual Meeting of the American Association for the Advancement of Science, Boston, February 12.
173. Tamplin, A.R., and Gofman, J.W. (1970): *Population Control Through Nuclear Pollution.* Nelson-Hill, Chicago.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_73.html[2010/10/28 18:31:08]

174. Thompson, K.M., Burmaster, D.E., and Crouch, E.A.C. (1992): Monte-Carlo techniques for quantitative uncertainty analysis in public health risk assessments. *Risk Anal.*, 12(1):53–63.
175. Thorton, J. (1991): Written testimony of J.Thornton, Greenpeace U.S.A., for the U.S. House of Representatives Committee on Science, Space, and Technology, Subcommittee on Environment. Hearing on Risk Assessment: Strengths and Limitations of Utilization for Policy Decisions, May 21.
176. Thurston, G.D., Ito, K., Lippman, M., and Hayes, C. (1989): Reexamination of London, England mortality in relation to exposure to acidic aerosols during 1963–1972 winters. *Environ. Health Perspect.*, 79:73–83.
177. Tomatis, L., Aitio, A., Wilbourn, J., and Shuker, L. (1989): Human carcinogens so far identified. *Jpn. J. Cancer Res.*, 80:795–807.
178. Tosteson, T., Spengler, J.D., and Weber, R.A. (1982): Aluminum, iron, and lead content of respirable particulate samples from a personal monitoring system. *Environ. Int.*, 2:265–268.
179. Travis, C.C., and Hattemer-Fry, H.A. (1988): Determining an acceptable level of risk. *Environ. Sci. Technol.*, 22(8):873–876.
180. Travis, C.C., Richter, S.A., Crouch, E.A.C., Wilson, R., and Klema, E.D. (1987): Cancer risk management. *Environ. Sci. Technol.*, 21(5):415–420.
181. Trosko, J.E., and Chang, C.C. (1988): Nongenotoxic mechanisms in carcinogenesis: Role of inhibited intercellular communication. In: *Banbury Report 31: Carcinogen Risk Assessment: New Directions in the Qualitative and Quantitative Aspects*, edited by R. W.Hart and F.D.Hoerger, pp. 139–170. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
182. U.S. Department of Health and Human Services. (1986): NIOSH recommendations for occupational safety and health standards. *Morbid. Mortal. Weekly Rep.*, 35:1S-33S.
183. U.S. Department of Health and Human Services. (1985): Risk assessment and risk management of toxic substances. Report to the Secretary of DHHS from the Executive Committee of the DHHS Committee to Coordinate Environmental and Related Programs. Washington, DC.
184. U.S. Department of Health, Education and Welfare, Public Health Service. (1962): Public Health Drinking Water Standards. Rockville, MD.
185. U.S. Environmental Protection Agency. (1999): Integrated Risk Information System. http://www.epa.gov/iris/
186. U.S. Environmental Protection Agency. (1999): IRIS substance file for chromium(VI); CASRN 18540–29; 9/3/98 (updated 5/21/99). Downloaded from http://www.epa.gov/ngispgm3/iris/subst/0144.htm
187. U.S. Environmental Protection Agency. (1999): IRIS substance file for dichloromethane; CASRN 75–09–2; 1/31/87 (updated 6/14/99). Downloaded from http://www.epa.gov/ngispgm3/iris/subst/0070.htm
188. U.S. Environmental Protection Agency. (1999): IRIS substance file for fluorine (soluble fluoride); CASRN 7782–41–4; 1/31/87 (updated 6/14/99). Downloaded from http://www.epa.gov/ngispgm3/iris/subst/0053.htm
189. U.S. Environmental Protection Agency. (1999): IRIS substance file for naphthalene; CASRN 91–20–3; 9.17/98 (updated 5/21/99). Downloaded from http://www.epa.gov/ngispgm3/iris/subst/0436.htm
190. U.S. Environmental Protection Agency. (1999): Summary of FQPA amendments to FIFRA and FFDCA. Downloaded from http://www.epa.gov/oppfeadl/fqpa/fqpa-iss.htm
191. U.S. Environmental Protection Agency. (1998): Framework for addressing key scientific issues presented by the Food Quality Protection Act (FQPA) as developed by the Tolerance Reassessment Advisory Committee (TRAC). *Fed. Reg.*, 63(209):58038–58045.
192. U.S. Environmental Protection Agency. (1998): Presentation for FIFRE Scientific Advisory Panel by Office of Pesticide Programs, Health Effects Division on FQPA Safety Factor for infants and children (draft 3/9/98, updated 3/12/98). Downloaded from http://www.epa.gov/pesticides/SAP/march/10x.htm
193. U.S. Environmental Protection Agency. (1997): Guiding principles for Monte Carlo analysis. EPA/630/R-97/001. USEPA, Risk Assessment Forum (Washington, DC), NTIS PB97–18810 6I.NZ.
194. U.S. Environmental Protection Agency. (1996): A national agenda to protect children's health from environmental threats (ipdated 9/11/96). Downloaded from http://occ-env-med.mc.duke.edu/oem/content/epa.htm
195. U.S. Environmental Protection Agency. (1996): Proposed guidelines for carcinogenic risk assessment; Notice. *Fed. Reg.*, 61(79):17960–18011.
196. U.S. Environmental Protection Agency. (1995): Guidance for risk characterization. Science Policy Council, USEPA, Washington, DC. February 1995.
197. U.S. Environmental Protection Agency. (1995): Proposed guidelines for neurotoxicity risk

assessment. *Fed. Reg.*, 60:52032–52056.
198. U.S. Environmental Protection Agency. (1995): The use of the benchmark dose approach in health risk assessment. Risk Assessment Forum, Office of Research and Development, Washington, DC. EPA/630/R-94/007.
199. U.S. Environmental Protection Agency. (1992): Memorandum from F.H. Habicht to assistant administrators and regional administrators, Re: Guidance on risk characterization for risk managers and risk assessors.
200. U.S. Environmental Protection Agency. (1993): Reference dose (RfD): Description and use in health risk assessments (background document 1A), 3/15/93 (updated 5/21/99). Integrated Risk Information System. Downloaded from http://www.epa.gov.ngispgm3/iris/rfd.htm
201. U.S. Environmental Protection Agency. (1992): Review of the National Ambient Air Quality Standards for carbon monoxide 1992 reassessment of scientific and technical information, OAQPS staff paper.
202. U.S. Environmental Protection Agency. (1991): Guidelines for developmental toxicity. *Fed. Reg.*, 56(234):63798–63826.
203. U.S. Environmental Protection Agency. (1990): General quantitative risk assessment guidelines for noncancer health effects. Technical Panel for the Development of Risk Assessment Guidelines for Noncancer Health Effects. ECAO-CIN-538.
204. U.S. Environmental Protection Agency. (1990): Interim methods for development of inhalation reference concentrations (review draft). Environmental Criteria and Assessment Office. EPA/600/8–90/066A.
205. U.S. Environmental Protection Agency. (1989): Review of the National Ambient Air Quality Standard for ozone: Assessment of scientific and technical information. Office of Air Quality Planning and Standards. Research Triangle Park, NC. NTIS PB92–190446. February 26, 1992.
206. U.S. Environmental Protection Agency. (1988): Special report on ingested inorganic arsenic: Skin cancer; nutritional essentiality. Risk Assessment Forum. EPA-625/3–87–013F.
207. U.S. Environmental Protection Agency. (1988): Technical support document on risk assessment of chemical mixtures. National Technical Information Service. EPA/600/8–90/064.
208. U.S. Environmental Protection Agency. (1986): Guidelines for carcinogen risk assessment. *Fed. Reg.*, 51:33992–34003.
209. U.S. Environmental Protection Agency. (1986): *Air Quality Criteria for Lead*, vols. I–IV. EPA-600/8–83/028adf.
210. U.S. Environmental Protection Agency. (1986): Review of the National Ambient Air Quality Standards for sulfur oxides: Updated assessment of scientific and technical information-

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addendum to the 1982 OAQPS staff paper. Office of Air Quality, Planning and Standards. EPA-450/5–86–013.
211. U.S. Environmental Protection Agency. (1975): Interim primary drinking water standards. *Fed. Reg.*, 40(51):11990–11998.
212. U.S. Food and Drug Administration. (1988): Color additives: Denial of petition for listing of D&C red no. 19 for use in externally applied drugs and cosmetics. *Fed. Reg.*, 53(136):26831–26883.
213. U.S. Food and Drug Administration. (1986): Listing of D&C orange no. 17 for use in externally applied drugs and cosmectics. Final rule. *Fed. Reg.*, 51(152):28331–28346.
214. U.S. General Accounting Office. (1990): Toxic substances: Effectiveness of unreasonable risk standards unclear. Report to the Chairman, Subcommittee on Health and the Environment, Committee on Energy and Commerce, House of Representatives. GAO/RCED-90–189.
215. U.S. Interagency Staff Group of Carcinogens. (1986): Chemical carcinogens: A review of the science and its associated principals. *Environ. Health Perspect.*, 67:201–282.
216. U.S. Public Health Service, ATSDR. (1989): Toxicological profile for selected PCBs (Arochlor-1260, -1254, -1248, -1242, -1232, -1221, and -1016). Report ATSDR/TP-8821. NTIS PB89–225403.
217. U.S. Public Health Services, Centers for Disease Control. (1991): Preventing lead poisoning in young children: A statement by the Centers for Disease Control. Dept. of Health & Human Services, Public Health Service, Centers for Disease Control, Atlanta, GA.
218. Wallace, L.A. (1991): Comparison of risks from outdoor and indoor exposure to toxic chemicals. *Environ. Health Perspect.*, 95:7–13.
219. Wallace, L.A., Pellizzari, E.D., Hartwell, T.D., Sparacino, C., and Zelon, H. (1983): Personal exposure of volatile organics and other compounds indoors and outdoors: The TEAM study. Proceedings of the 76th Annual Meeting of the Air Pollution Control Association. Air Pollution Control Associatoin, Pittsburgh, PA.
220. Waring, M.J. (1981): DNA modification and cancer. *Annu. Rev. Biochem.*, 50:159–192.
221. Wilkinson, C.F. (1971): Effects of synergists on the metabolism and toxicity of anticholinesterase. *Bull. WHO,* 40:171–190.
222. Zeise, L., Wilson, R., and Crouch, E.A.C. (1987): Dose-response relationships for carcinogens: A review. *Environ. Health Perspect.*, 73:259–308.
223. Ziskind, M., Jones, R.N., and Weil, H. (1976): Silicosis. *Am. Rev. Respir. Dis.*, 113:643–665.

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Chapter **3**

Metabolism: A Determinant of Toxicity

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Principles and Methods of Toxicology,
Fourth Edition, edited by A.Wallace Hayes.
Taylor & Francis, Philadelphia © 2001.

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As stated in the title of this chapter, the metabolism of *xenobiotics*, compounds foreign to the normal biochemistry of cells, is a major determinant of their toxicity. Most often, toxicologists are interested in the metabolism of potentially toxic compounds to which species may be exposed; however, many of these same enzymes are

involved in the detoxication and metabolism of *endobiotics*, which are compounds produced during the normal biochemical reactions that maintain life. For example, some of these enzymes are involved in the detoxication of reactive oxygen species produced during aerobic metabolism that can produce oxidative stress and tissue damage. A toxicologist must have a fundamental knowledge of these detoxication systems to understand, predict, and determine the potential toxicity of a compound. It is our purpose to provide an overview of these various systems that can serve as a foundation for a more comprehensive study of their role in toxicology.

EVOLUTION OF XENOBIOTIC METABOLISM

As the first macromolecules began to organize into complex arrays with the basic attributes of life, environmental factors were present that represented disruptive forces. These forces were physical and chemical. Chemicals present in the early milieu of life could interact with these life forms, disrupting the delicate balance through which they maintained their integrity. Those life forms that developed protective mechanisms, such as the cell membrane, the ability to store energy, and motility, were able to survive. It does not stretch the imagination to hypothesize that these early life forms manufactured molecules capable of reacting with environmental chemicals, including increasing concentrations of oxygen, thereby decreasing their biological activity. An example of such a molecule is glutathione. This nucleophile can react nonenzymatically with many electrophilic chemicals to reduce their toxicity. An early step in the evolution of protective mechanisms may have been the development of macromolecular catalysis to chemically alter disruptive chemicals. These macromolecular catalyses were the first detoxification enzymes. Evidence for such a scenario exists in the occurrence of certain detoxication enzymes in both animal and plant species ranging from simple, single cellular to complex, multicellular organisms.

The cytochrome P450 (P450) monooxygenase system (discussed in detail later) is a large superfamily of hemoproteins whose evolutionary roots have recently been investigated (36, 59). This superfamily serves as an example of the evolution of enzymes capable of protecting the organism from toxic chemicals. In fact, its current nomenclature is based on evolutionary and genetic relationships between its isozymes. The P450 superfamily of hemoproteins consists of about 20 gene families, and the functional P450 genes in a mammalian species are estimated to be over 200. The amino acid sequences of more than 100 P450 hemoproteins are known. Phylogenetic trees based on these sequences have been developed to aid in understanding the evolution of these important enzymes (see (58, 95) for reviews).

The original ancestral P450 gene may date back 3 billion years or more. The wide distribution of these genes in prokaryotic and eukaryotic organisms emphasizes their importance in maintaining life. About 400 million years ago there appears to have been a major increase in the number of new genes in one of the P450 families (the CYP2 family). It is believed that this evolutionary burst of new genes was associated with animal colonization of land during the Devonian era. In an attempt to protect themselves, plants began to develop toxins to deter their consumption by land animals. This resulted in coevolution of animal cytochromes P450 to detoxify these plant toxins (58). As species began to fill specific ecological niches with unique environmental and dietary chemical challenges, the species diversity in expressed P450 genes that we encounter today developed. The evolution of this single xenobiotic metabolism system illustrates the significance of xenobiotic detoxication systems in the development and maintenance of the diversity of life we encounter on earth today.

An alternative to the hypothesis that the enzymes developed to metabolize foreign compounds is that they represent enzymes of normal anabolic and catabolic metabolism. These enzymes, in addition to functioning in their normal biochemical role, also function in xenobiotic metabolism. Examples of such enzymes certainly exist and include the methyltransferases involved in DNA synthesis and in detoxication, such as the metabolism of nicotine; however, it would appear fortuitous that normal anabolic and catabolic enzymes could have evolved to detoxify the broad array of chemical structures that are foreign to organisms. Another factor that diminishes the confidence in the alternative hypothesis is that many of the xenobiotic metabolizing enzymes can rapidly respond with increased activity to the presence of xenobiotics and environmental change. This would be unlikely if they simply represented enzymes of normal metabolism with the ability to metabolize alternative substrates. Many of the enzymes involved in xenobiotic metabolism are also involved in specific aspects of the metabolism of normal cellular biochemical constituents. Generally these enzymes are isozymes with higher substrate specificity for the endogenous compounds than the isozymes involved in xenobiotic metabolism. This could likely represent a process by which the cell used a preexisting xenobiotic metabolizing enzyme for metabolism of endogenous substrates instead of vice versa. Whatever the evolutionary source of the

xenobiotic metabolizing enzymes, it is obvious that they represent a distinct mechanism of metabolizing the thousands of naturally occurring and synthetic chemicals to which cells and tissues are exposed.

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GENERAL FEATURES AND BASIC CONCEPTS OF XENOBIOTIC METABOLISM

The majority of organisms studied have *biotransformation* enzymes, although there is diversity in the occurrence, function, and rates of specific enzymes. Certain bacteria contain more primitive or less highly developed systems and may lack certain pathways altogether. Even mammals demonstrate diversity in the activity or rates of specific systems, and, as would be expected of genetically controlled functions, there are species and individual differences. This diversity extends to the organ level in multicellular organisms. Specific organs show different levels of activity, and specific cell types within organs demonstrate variation in biotransformation capability. There is even subcellular diversity in that certain of these enzymes are compartmentalized whereas others are free in the cytoplasm.

The variety of chemicals to which organisms may be exposed requires that the biotransformation enzymes have broad substrate specificity. This characteristic is not shared by the majority of enzymes involved in anabolic and catabolic metabolism. In addition, the types of reactions catalyzed are diverse, as shown in Table 3.1, including oxidation, reduction, epoxidation, deamination, hydroxylation, sulfoxidation, dehalogenation, and conjugation with endogenous compounds, to name a few. Although it is logical to initially focus on one xenobiotic metabolizing system at a time, in many cases, xenobiotic metabolism involves more than a single metabolic route. In addition, the eventual toxicity of a xenobiotic may be modified by a number of factors, including age, gender, physiological status, nutrition, diet, and the presence or absence of disease, among others.

Exposure of an animal to certain xenobiotics can result in the *induction* of specific enzymes associated with xenobiotic metabolism. When induced, their activity can dramatically increase, compared to their basal level. Induction is sometimes coordinated with more than one enzyme induced. Induction results in an increase in the ability of animals to metabolize a xenobiotic, and in most cases this reduces their susceptibility to its toxicity. Induction generally lasts only a few days. When exposure ceases, the enzymes return to their basal levels.

Because xenobiotic metabolism does not always represent detoxication, the term *biotransformation* has come into general use to denote the actions of xenobiotic metabolizing enzymes, although it is still not semantically specific for xenobiotic metabolism. Biotransformation is divided into two distinct phases. *Phase I* reactions result in *functionalization,* the addition or the uncovering of specific functional groups that are required for subsequent metabolism by *phase II* enzymes. Phase II reactions are biosynthetic. These phase I and II reactions

Table 3.1 Examples of types of reactions and enzymes that participate in xenobiotic metabolism

Phase I Reactions	
Oxidation	Ester hydrolysis
P450 monooxygenase	Carboxylesterases
Xanthine oxidase	Amidases
Peroxidases	
Amine oxidase	Dehydrogenases
Monoamine oxidase	Alcohol dehydrogenases
Dioxygenases	Aldehyde dehydrogenases
Semicarbizide sensitive amine oxidase	
Reduction	Superoxide dismutase
P450 monooxygenase	
Ketoreductase	
Glutathione peroxidases	
Hydration	
Epoxide hydrolase	
Phase II Reactions	
Glucuronosyltransferase	Methylation
Sulfotransferase	*O*-Methyltransferases
Glutathione *S*-transferase	*N*-Methyltransferases
Glucosyltransferase	*S*-Methyltransferases
Thioltransferase	
Amide synthesis (transacylase)	Acetylation
	N-Acetyltransferase
	Acyltransferases
	Thiosulfate sulfurtransferase

(rhodanase)

are often coordinated, with the product of phase I reactions becoming the substrate for phase II enzymes. A commonality of biotransformation reactions is the conversion of hydrophobic xenobiotics into more polar, more easily excreted compounds. Because the composition of cells is more lipophilic than their environment, nonpolar compounds tend to accumulate. This could lead to bioconcentration of chemicals within the cell to levels higher than that of the environment and increase the likelihood of a cytotoxic event; however, conversion of nonpolar chemicals to more polar metabolites allows them to be more easily excreted by the cell. *Conjugation* of a xenobiotic with an endogenous compound, a phase II reaction, increases water solubility, and, in some cases, the added chemical group is recognized by specific carrier proteins or proteins involved in facilitated diffusion or active transport. This increases the cell's ability to remove the xenobiotic.

There are many diverse examples of xenobiotics whose toxicity is directly dependent on the activity of the biotransformation enzymes. For most chemicals,

increases in the activity of these enzymes result in decreases in toxicity, whereas decreases in activity result in increased toxicity; however, there are examples in which the product of xenobiotic metabolism is more toxic than the parent compound. Conversion of a foreign compound to a more toxic metabolite is termed *metabolic activation*. For example, the majority of genotoxic and carcinogenic chemicals require metabolic activation to highly reactive species capable of interacting with DNA. The enzymes that protect the animal from the toxicity of certain compounds may be responsible for the toxicity of others. An organism's susceptibility to the toxicity of a particular chemical is dependent, in many cases, on the delicate balance between detoxication and metabolic activation that exist during exposure to the xenobiotic. Due to the sensitivity of the enzymes of xenobiotic biotransformation to both endogenous and exogenous factors, this balance may differ among individuals and at different points in time.

BIOLOGICAL OXIDATION

Cytochrome P450-dependent Monooxygenase System

The P450-dependent monooxygenase system is central to the metabolism of most xenobiotics. Not only is it the primary enzymatic system for metabolism of many xenobiotics, but it is also involved as the initial functionalization step in the further metabolism of many others. Consequently, P450 plays essential roles in several areas of research, including biochemistry, pharmacology, toxicology, physiology, and medicine. Several names for the P450 system exist in the literature. The names most commonly encountered include

1. Mixed function oxidase
2. P450 system
3. P450-dependent monooxygenase system

Generally these names are related either to a specific function or are descriptive of a biochemical mechanism. Currently, it generally is referred to in terms of a monooxygenase system to denote its ability to incorporate one atom of molecule oxygen into its substrates.

Components of the Cytochromes P450 System

The history of the discovery of P450 and the elucidation of its functions and mechanisms of action are intriguing and have recently been reviewed by Estabrook (43). P450 was first described independently in 1958 by Klingenberg (86) and Garfinkel (52) in microsomes isolated from rat liver homogenates and pig liver homogenates, respectively. Klingenberg (86) noted that during 1955 in the laboratory of Britton Chance at the Johnson Foundation G.R.Williams was the first to observe the pigment. The name P450 derived from the occurrence of a pigment that, when reduced and treated with carbon monoxide, yielded a spectrophotometric Soret band at *450* nm. Six years after the publication of the original description of the P450 peak in hepatic microsomes, Omura and Sato (131, 132) published their pivotal papers describing P450 as a b-type hemocytochrome. Their work demonstrated that the cytochrome was located in hepatic *microsomes,* which form from the endoplasmic reticulum upon cellular disruption. Upon isolation from the membrane, using proteases, P450 is converted to a form whose reduced carbon monoxide complex produces a spectrophotometric peak at 420 nm. This form was termed *cytochrome P420* (P420) and was found inactive in metabolism. Conversion of P450 to the inactive cytochrome P420 upon isolation from the membrane was one of the major limitations encountered in early attempts to understand the mechanism of this membrane-bound monooxygenase.

Before the discovery of P450, Julius Axelrod and his colleagues (6), in the laboratory of Chemical Pharmacology at the National Heart Institute, were involved with studies on the metabolic disposition of drugs (6). They found that the oxidative metabolism of amphetamine required the co-factor NADPH and the presence of oxygen. An important publication by Estabrook et al. (42) brought together studies of P450 biochemistry and studies of biotransformation. They established that P450 was the terminal oxidase involved with the C-21 hydroxylation of steroids in adrenal cortical microsomes. This was followed by a study from Cooper (32) that demonstrated the involvement of P450 in both steroid and drug metabolism by hepatic microsomes. Many individuals and laboratories have played major roles in the development of the current knowledge concerning P450. Readers who become acquainted with the original literature will no doubt recognize the role of these pioneers in the development of a new field of study: xenobiotic biotransformation.

It soon became obvious that although P450 played a major role in the activity of the monooxygenase, it did not act alone. In 1950, Horecher (70) isolated a flavoprotein from the liver, but no function was identified. This flavoprotein used reducing equivalents from NADPH and was termed *NADPH-cytochrome c reductase* (EC 1.6.2.4). In 1955, La Du et al. (89) showed that cytochrome c could inhibit dealkylation of aminopyrine. This was followed by the studies of Gillette et al. (54) in 1957, which presented additional evidence that cytochrome c reductase was involved in xenobiotic metabolism. Williams and

Kamin (188) and Philips and Langdon (136) reported in 1962 that NADPH-

cytochrome c reductase occurred in the endoplasmic reticulum of liver cells and might be involved in drug and steroid oxidation. Further proof of the involvement of the flavoprotein came in 1969, when it was shown that antibodies to the reductase inhibited xenobiotic metabolism (88, 130), and Lu et al. (100, 101) demonstrated its requirement in monooxygenase activity reconstituted from isolated components.

One confusing aspect concerning this flavoprotein is its nomenclature. As previously mentioned, it is sometimes referred to as NADPH-cytochrome c reductase; however, no cytochrome c occurs in the endoplasmic reticulum, and cytochrome c is not its normal substrate. The more appropriate name is NADPH-cytochrome P450 reductase, indicating its natural substrate and function.

Although the major components of the P450-dependent monooxygenase system appear to be P450 and P450 reductase, other components may also be involved with metabolism of specific xenobiotics. Cytochrome b5 reductase has been proposed to participate in monooxygenase activity through electron transport to cytochrome b5 and, subsequently, to P450; however, several systems of electron transport in the endoplasmic reticulum and isolated microsomes, as well as other activities, such as peroxidation, have greatly complicated the elucidation of the role of cytochrome b5. Cytochrome b5 may affect P450-mediated xenobiotic metabolism by shunting electrons either toward or away from P450 (119). An elucidation of the role of cytochrome b5 must await further understanding of the complex electron transfer pathways that exist in the endoplasmic reticulum.

Although the catalytic activity of the monooxygenase system appears to require only two proteins—NADPH P450 reductase and P450—it is capable of carrying out a variety of different reactions on a large number of substrates. This ability is based on the occurrence of a variety of P450 isozymes, but it also is based on the basic reaction mechanism of the cytochromes and their apparent overlapping substrate specificity. The nonspecificity of the monooxygenase provides important flexibility to xenobiotic metabolism, but this flexibility comes with a price. Generally, the enzymatic reactions of anabolism and catabolism are both extremely specific in substrate specificity and catalytically efficient, resulting in high activity and high substrate turnover number. The turnover number and efficiency of P450 are considerably lower than most enzymes. This is probably related to the inefficient electron transfer due to the presence of a water molecule at the active site. Some substrates are more efficiently oxidized because they exclude water from the active site (137). Inefficiency of metabolism is more than made up for by the ability to metabolize a variety of chemical structures and the ability to catalyze a variety of reactions. An additional factor that compensates for the relatively low substrate turnover number is the high concentration of the system in organs important in detoxication.

Before a discussion of the various reactions catalyzed by P450, a discussion of the catalytic cycle is appropriate. Knowledge of the catalytic cycle will assist in understanding the various reactions catalyzed by the system and in predicting metabolic pathways for specific xenobiotics.

Catalytic Cycle of the P450-dependent Monooxygenase System

The reaction catalyzed by the cytochrome P450– dependent monooxygenase system and its stoichiometry is illustrated in Figure 3.1. One molecule of substrate reacts with one molecule of molecular oxygen and NADPH to yield oxidized substrate containing one atom from molecular oxygen, water (containing the other oxygen atom), and oxidized NADPH. The incorporation of one oxygen atom from molecular oxygen into the substrate is the source of the term *monooxygenase*. Oxidation of substrate and concomitant reduction of one atom of oxygen to water is the source of the name *mixed function oxidase*. Although the reaction stoichiometry appears simple, obtaining this stoichiometry in the laboratory is difficult (60). The main difficulty is the number of oxidation-reduction reactions that occur simultaneously in the endoplasmic reticulum. These reactions use oxygen and NADPH and may yield water and oxidized NADP. When these diverse reactions have been accounted for, the predicted stoichiometry has been obtained.

It is recommended that the reader carefully follow the reaction sequence illustrated in Figure. 3.2 during this discussion of the catalytic cycle. The initial step of the cycle is binding of the substrate (represented by S in Figure 3.2) to P450. As previously mentioned, P450 exists as a series of closely related isozymes, each of which demonstrates a degree of substrate specificity. This substrate specificity is not absolute, and some overlapping is evident. At any one time, several isozymes of P450 exist in the endoplasmic reticulum. This is dependent on the specific genetic, environmental, and physiological conditions of the organism. Therefore, binding of the substrate to the active site of P450 may represent binding to a single isozyme predominantly but not exclusively. The activity of the catalytic process, as well as the specific metabolites produced, is a function of the particular isozyme profile. Although our understanding of the structure of the active

$$RH + O_2 + NADPH + H^{\oplus} \longrightarrow ROH + H_2O + NADP^{\oplus}$$

FIG. 3.1. Reaction and stoichiometry of P450-dependent monooxygenase.

FIG. 3.2. Catalytic cycle of the P450-dependent monooxygenase. The second electron insertion step may be from b5 (as shown) or from NADPH P450 reductase.

site of P450 is developing (95, 96), more needs to be learned. From the nature of the hemoprotein and its substrates, the active site contains the heme and a hydrophobic region. The substrate must have a specific orientation within the active site.

As occurs with many other enzymes, the binding of the substrate to the hemoprotein appears to produce conformational alterations in the enzyme that assist its catalytic activity. For instance, substrate binding results in P450 being more easily reduced by P450 reductase, in part, by lowering its redox potential. Binding of the substrate to the active site changes the absorption spectrum of the cytochrome. Because the oxidized heme iron is paramagnetic, electron paramagnetic resonance (EPR) can be applied to probe the environment of the iron in the heme. These studies have revealed alterations in the EPR signal that correlate with the blue shift in the Soret band from about 419 nm to 390 nm, observed when substrates bind the cytochrome. EPR and visible spectra changes result from the substrate binding in close proximity to the heme iron with a concomitant displacement of a water molecule from the iron. Substrate binding is rapid, the heme is transformed from its low-spin form to the high-spin form, and the substrate is placed in close spatial proximity to the oxygen activation site on the heme. The relationships between the spin state of the cytochrome, interaction with the amino acids at the binding site, and substrate binding is more complex than described here. The reader is referred to discussions of changes in the spin state of P450 in (157, 95, 140, 137).

The next step in the catalytic cycle after substrate binding is the one-electron reduction of the substrate-P450 binary complex. As mentioned, substrate binding and the concomitant alterations in P450 may facilitate this reduction step. The ferric (Fe^{3+}) hemocytochrome P450-substrate complex is reduced by a single electron to the ferrous (Fe^{2+}) hemocytochrome P450-substrate complex. This electron is provided by NADPH through P450 reductase. This flavoprotein contains two flavins: flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). The flavoprotein appears to exist in its half-reduced (one-electron reduced) form, and upon reaction with NADPH, is fully reduced (two-electron reduced). The intramolecular electron flow appears to be from FAD to FMN. It is interesting that whereas the flavoprotein is a one-electron donor, its substrate, NADPH, provides two electrons. The mechanism for the two-electron shuttle by the one-electron donor flavoprotein is incompletely understood (7).

The flavoprotein has at least two domains, one of which is imbedded in the endoplasmic reticulum membrane and the other above the plane of the membrane on the cytosolic side. The domain solubilized in the membrane consists mainly of hydrophobic amino acids. The actual interaction with NADPH and oxidation-reduction takes place outside the membrane. Another interesting aspect of P450 reductase is that the quantity of P450 is in large excess to the quantity of reductase (as much as 15-fold to 20-fold or more, dependent upon conditions). This means that each flavoprotein must reduce several P450 molecules, indicating that the interaction between the

reductase and P450 is an important consideration, as discussed later.

Upon reduction of the ferric hemocytochrome P450-substrate binary complex to the ferrous state by the reductase, the complex binds oxygen. This results in the ternary complex, ferrous hemocytochrome P450-substrate-oxygen shown in Figure 3.2. The oxygen binds at the free ligand of the heme iron and is believed to be oriented spatially with the substrate binding portion of the active site. Uncoupling (interrupting the flow of electrons) the catalytic cycle at this point can produce the oxidized ferric P450 and a reduced form of oxygen, the superoxide radical. Other reactive oxygen species can be generated by P450 including hydrogen peroxide and the hydroxyl radical. Generation of active oxygen species by P450 has been reviewed in (8).

At this stage of the catalytic cycle, highly critical reactions take place that are still incompletely understood (140). The major event is the activation of the oxygen molecule. The ternary complex accepts a second electron required for reaction. The source of this electron can be either NADH or NADPH, dependent upon the mediator of electron transport. Because the purified, reconstituted system consisting of isolated P450, P450 reductase and phospholipid requires only the presence of NADPH, NADPH P450 reductase can mediate this step; however, as previously mentioned, in some systems it appears that cytochrome b5 can mediate the electron transfer employing reducing equivalents from NADH through NADH cytochrome b5 reductase. Whichever the source of the second electron, it results in the production of the peroxy P450-substrate complex, which has a net charge of −2. Determination of the various oxy complexes shown in brackets in Figure 3.2 is critical to understanding the oxygen activation reaction. Of the variety of mechanisms proposed for oxygen activation and insertion into the substrate, two appear to be generally accepted. The first mechanism involves heterolytic cleavage of diatomic oxygen with the abstraction of hydrogen from the substrate and the insertion of oxygen into the substrate. The second mechanism involves homolytic cleavage, whereby two oxygen radicals are generated. Whatever the mechanism, one atom of this reactive oxygen is introduced into the substrate, whereas the other is reduced to water. The oxidized substrate and water are released, regenerating the oxidized ferric P450, which can again initiate the catalytic cycle.

It must be emphasized that other pathways of electron transport in the endoplasmic reticulum can have significant impact on the catalytic activity of the monooxygenase by altering the availability of reducing equivalents. The interested reader is encouraged to consult other sources (7, 133) for a more comprehensive discussion of these pathways.

This catalytic cycle is common to cytochromes P450-dependent monooxygenase activity associated with xenobiotic metabolism in a variety of organs and among different species; however, certain of these monooxygenases, especially the more specific forms associated with anabolic and catabolic metabolism, have different mediators of electron transport. For example, the adrenal cortex mitochondria systems use a non-heme iron protein in addition to the P450 reductase in the electron transport chain, as does the monooxygenase in certain microorganisms (137, 147).

The P450 system is not totally independent, and its activity is affected by a number of factors. One of these factors is the availability of reducing equivalents. The monooxygenase is primarily dependent on NADPH, as previously discussed, and possibly, to a lesser extent, on NADH. NADPH is generated from the pentose-phosphate shunt, isocitrate dehydrogenase, and the malate enzyme. Under most conditions, these pathways provide saturating levels of NADPH; however, certain conditions can stress the ability of the cell to provide NADPH, and it may become rate limiting. Under conditions of high monooxygenase activity, starvation may reduce the activity toward certain substrates due to reduced levels of NADPH. It is generally believed that the decreased activity due to limiting NADH is an unlikely condition. A discussion of these and other factors that regulate monooxygenase activity can be found in the (171).

An additional factor that influences monooxygenase activity is the endoplasmic reticulum membrane. The asymmetric nature of the protein components of the system in respect to the membrane surface, coupled with the disproportionality of the concentrations of the components (i.e., a 1-to-15–20 ratio between the flavoprotein and P450), indicates an interesting topology and interaction between the components. The membrane topology of the P450 system has been a topic of research for a number of years. The interaction between the protein components of the system and the interaction of these components with the lipid matrix of the membrane are important in the overall reactions of this system. P450 appears to be anchored into the membrane of the endoplasmic reticulum by an anchor peptide at the NH2-terminal end of the protein with the anchor peptide transversing the membrane. The active site, including the heme, is on the cytoplasmic side of the membrane. The active site portion is rich in alpha helix content, globular in nature, and not associated with membrane lipids. The area around the active site may be associated with the cytosolic surface of the membrane, providing a somewhat rigid

character.
P450 appears to exist as multicomponent complexes of six P450 molecules clustered around a single P450 reductase. The NH_2-terminal regions on the opposite side

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of the membrane may interact to anchor this complex together. This allows for a catalytic advantage because of the close association of the components. This organization implies that each reductase would be capable of sequentially reducing several P450s. P450 may form a transient complex with the reductase that has an extremely short, non-rate-limiting half-life (10, 64).

Isozyme Heterogeneity and Substrate Specificity of Cytochrome P450

For many years, P450's apparent lack of substrate specificity intrigued investigators. It appeared that one of the major features of substrate specificity was lipid solubility. There appeared to be few other structural restraints for substrates. Intensive research on the nature of the hemoproteins has revealed that much of this apparent lack of substrate specificity results from the existence of multiple families and multiple subfamilies of P450 isozymes.

As the array of individual isozymes grew in number, nomenclature became a problem. It was sometimes difficult for investigators to know what exact P450 they were working with because of inconsistencies in nomenclature. This led to attempts to develop a systematic nomenclature for the isozymes. P450 nomenclature has evolved from identifications based on spectra peaks to speciesdependent nomenclature based on isolated and semipurified P450s to the current system, which is based on amino acid sequences that result from specific gene sequences (124). P450 are now placed in families, which are further divided into subfamilies. Names are based on the root CYP derived from cytochrome P450. The CYP is followed by a number identifying the gene family to which it belongs, such as CYP1, CYP2, CYP3, etc. The number for the gene family is followed by a letter denoting the subfamily to which the P450 belongs, such as CYP1A and CYP2A. The subfamilies are further defined by the addition of a number identifying the gene, such as CYP1A1 and CYP1A2. Thus, P450 nomenclature is based on genetic relationships defined by protein and gene sequences. All P450s within a single family must exhibit a protein sequence similarity greater than 40%. P450s within the same subfamilies have sequence similarities greater than 55% within the same species. Subfamilies have sequence similarity that may be somewhat less than 55% when comparing species that are more distantly related. Members of subfamilies within a species appear to be located on a specific chromosome, and different subfamilies within a gene family may be clustered on the same chromosome. As is generally found in biology, there are exceptions to the classification system resulting from P450s that do not fit the usual patterns. Although this nomenclature provides infor mation on genetic and evolutionary relationships, it provides little information about substrate specificities and the reactions catalyzed by the different P450s. In fact, more is known about the protein and gene sequences of many P450s than about their specific roles in metabolism. With the advent of new methodologies, such as polymerase chain reaction (PCR) techniques, our ability to sequence specific P450s has outgrown our ability to define their specific roles in the metabolism of xenobiotics and endogenous compounds.

Different species may contain a CYP gene or protein that appears to be highly related; these are termed *orthologous genes*, or *orthologs*. Orthologs are believed to have evolved from a single gene that existed before the two species diverged from a single species. Although these genes and their proteins may contain a high degree of sequence homology, it is not necessarily true that they share a catalytic similarity, or vice versa. A small change in an important amino acid sequence can result in a large change in the activity of a P450. Humans and rats have the CYP2 family and the CYP2D subfamily. The rat subfamily contains five genes, one of which is CYP2D1. This P450 has catalytic activity toward debrisoquine metabolism. The human P450 that has the highest catalytic activity toward debrisoquine is CYP2D6, which makes up less than 5% of the complement of P450 in the liver (Figure 3.3). Because these rat and human P450s have similar substrate specificity, it might be assumed they have a high degree of sequence similarity. This was found not to be true. Therefore, even though these two isozymes have similar catalytic activity, they may have been derived from different ancestral genes. Sequence orthologs do not always predict similar catalytic activities. This is important for toxicologists who extrapolate toxicity from animal models to humans, as noted later. It also indicates that such information is important when toxicologists do interspecies extrapolation of toxicity, such as from mice to rats.

The section that follows provides a brief description of the major P450 families involved in xenobiotic metabolism. Those families predominately involved in metabolism of endogenous substrates have been excluded. For a more complete discussion of the P450 families, the reader is referred to the excellent reviews in (73, 159).

CYP1 Family

The CYP1 family contains two subfamilies of P450s: CYP1A and CYP1B. CYP1A is much better characterized than the CYP1B subfamily.

CYP1A Subfamily. The CYP1A subfamily contains CYP1A1 and CYP1A2, which appear to occur in all mammals. These two P450s may have been derived from a common ancestor approximately 120 million years ago.

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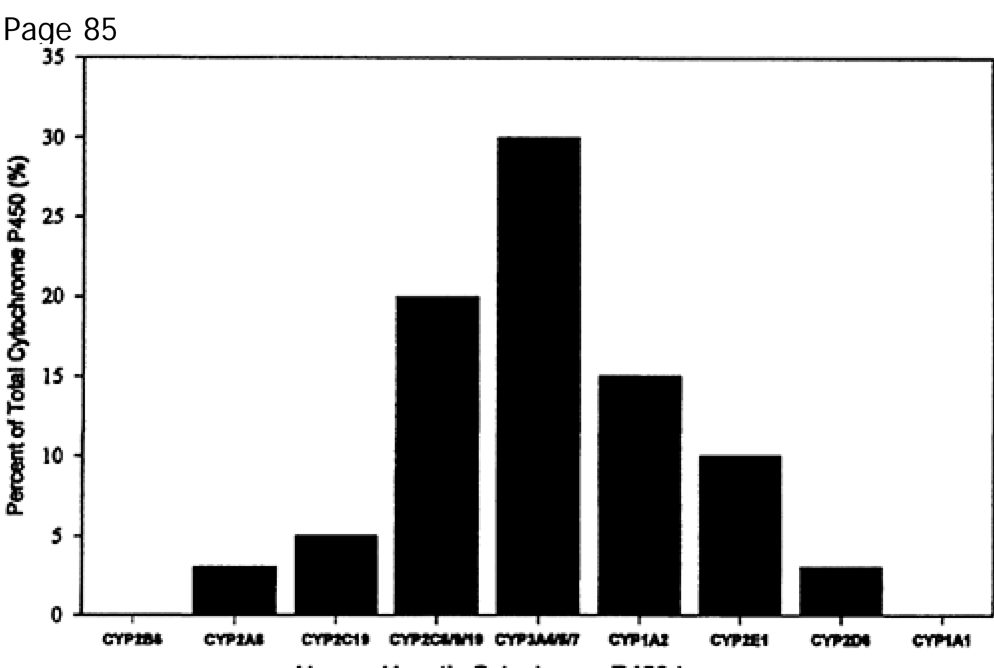

FIG. 3.3. Estimated percentage of distribution of P450 enzymes in human liver. Individual humans may demonstrate significant deviations from this average distribution. CYP2B6 and CYP1A1 may be in very low concentrations and appear only after enzyme induction. Modified from Reference 134.

Both P450s are important in the metabolism of environmental xenobiotics. Although these hemoproteins share a number of physiochemical characteristics, such as similar primary structures, they demonstrate different substrate specificities. For example, CY1A1 is highly active in the metabolism of benzo(a)pyrene (Figure 3.4), whereas CYP1A2 is active in the metabolism of acetanilide. These two P450s were first known as P448, and their classical enzyme inducer is 3-methylcholanthrene. Isosafrole is a specific inducer of CYP1A2. Another characteristic of these P450s is that CYP1A2 is mainly a liver enzyme in humans and animals, whereas CYP1A1 occurs in the liver (Figure 3.3) but also in extrahepatic tissues, such as the lung and kidney. For a number of years there has been an interest in the role of CYP1A1 in the metabolic activation of polycyclic hydrocarbons, such as benzo(a)pyrene. Although many of the investigations on the role of CYP1A1 in activation of polycyclic hydrocarbons have been done in animals with induced CYP1A1, polycyclics may be metabolized by other P450s in uninduced animals. Because its concentration in the liver is low, CYP1A1 may be more important in the metabolic activation of polycyclics in extrahepatic tissues, such as the lung. In humans, CYP1A1 demonstrates genetic polymorphism (discussed later). CYP1A2 has been shown to be associated with the mutagenic activation of heterocyclic amines, such as 4-aminobiphenyl and 2-aminonaphthalene. It also 0-dealkylates phenacetin and 4-hydroxlates acetanilide. Humans can show large differences in the activity of this P450, suggesting it also may be polymorphic. CYP1A2 catalyzes the N3-demethylation of caffeine to paraxanthine. This reaction has been used in vivo as a *substrate probe* for CYP1A2 activity.

CYP2 Family

The CYP2 family contains a number of subfamilies important in xenobiotic metabolism, including CYP2A, CYP2B, CYP2C, CYP2D, and CYP2E.

CYP2A. The CYP2A subfamily contains at least 12 members that differ in their substrate specificity, tissue distribution, and response to inducers and inhibitors, and demonstrate species differences. CYP2A1, CYP2A2, and CYP2A3 are rat P450s, Cyp2a4, Cyp2a5, and Cyp2a12 are found in mice, whereas humans have CYP2A6 and CYP2A7. Rat CYP2A1, along with CYP2A2, hydroxylates testosterone, progesterone, and androsteredione. They can also metabolize aminopyrine, benzphetamine, ethylmorphine, aniline, acetanilide, and N-nitrosodimethylamine. CYP2A1 has low activity toward 3-hydroxylation of benzo(a)pyrene, 7-ethoxycoumarin O-deethylation, and does not metabolize 7-ethoxyresorufin. It occurs in liver and testis, but not kidney and lung. In adult rats it predominates in females and appears to be under endocrine control. CYP2A3 appears to be lung specific, and its substrate specificity has not been well characterized. Human CYP2A6 demonstrates coumarin 7-hydroxylase activity but has

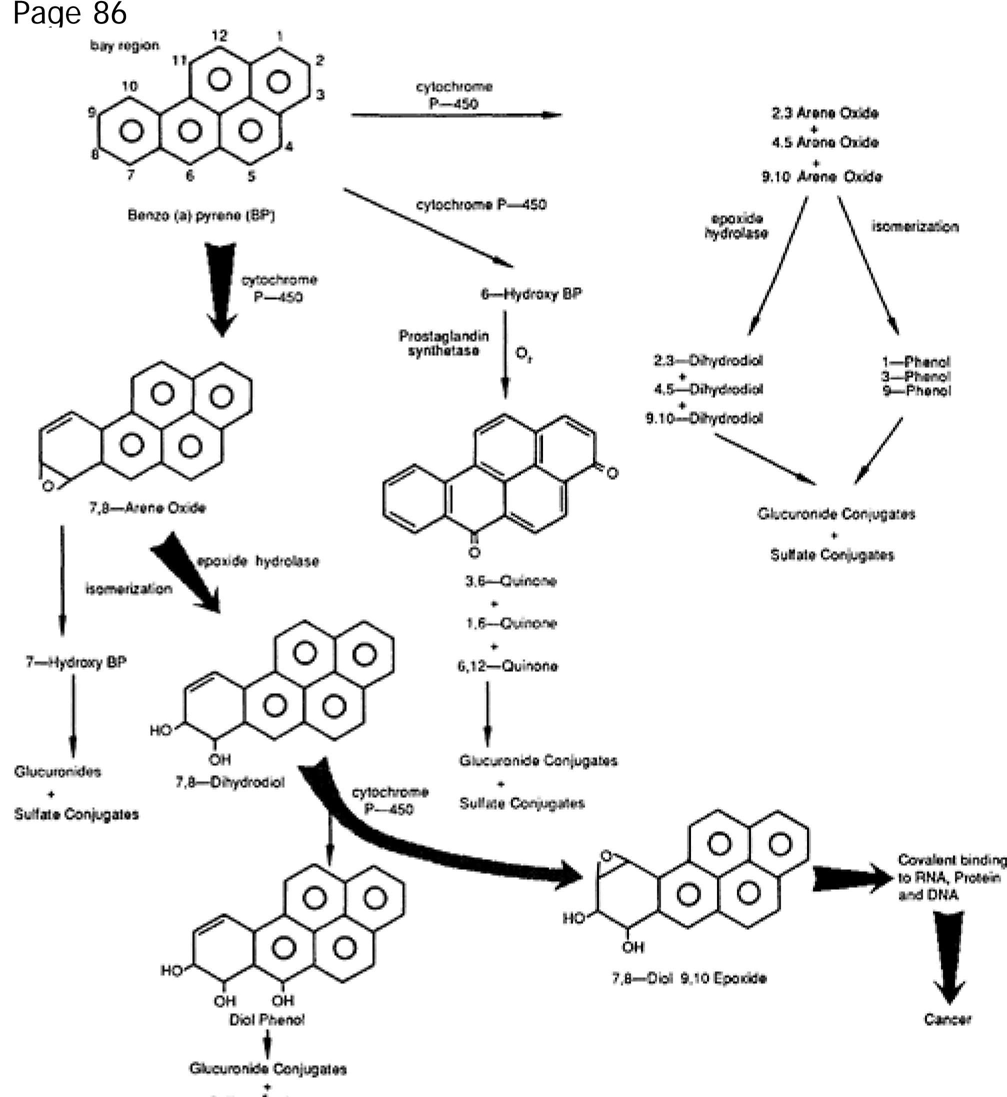

FIG. 3.4. Biotransformation and metabolic activation *(heavy arrows)* of the carcinogen benzo(a)pyrene. (BP).

no activity toward testosterone, in contrast to rat CYP2A1 and CYP2A2. It has no activity toward substrate probes such as 7-ethoxyresorufin, 7-benzyloxyresorufin, ethylmorphine, and testosterone. Although it shows wide variation in activity, there is no evidence that this is a polymorphic P450. Human liver microsomal studies have indicated this P450 may play a role in the metabolic activation of a number of nitrosamines and possibly activation of aflatoxin B1 to its hepatocarcinogenic epoxide.

CYP2B. This subfamily contains P450s, such as rat CYP2B1 and rat CYP2B2, that are highly induced by phenobarbital. Although the rat P450s in the CYP2B subfamily have been studied for a number of years, the

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CYP2B subfamily may have limited importance in humans. The human CYP3A subfamily may be more important in respect to phenobarbital-type induction in humans. Hydroxylation of testosterone at the 16β position is used as a specific substrate probe for this P450 in rats. Other substrates for this subfamily include benzyloxyresorufin, ethoxycoumarin, and pentoxyresorufin. In rats, CYP2B1 has been detected in the lung, adrenal gland, testis, and brain, whereas CYP2B2 occurs in liver and brain. Its role in xenobiotic metabolism has not been thoroughly investigated, although the CYP2B subfamily can metabolically activate xenobiotics, such as bromobenzene, carbon tetrachloride, benzo(a)pyrene, aflatoxin B1, and some nitrosamines in the rat.

CYP2C. Four CYP2C subfamily members have been identified in humans, CYP2C8, CYP2C9, CYP2C18, and CYP2C19. At least eight members of this subfamily have been identified in rats. Rat CYP2C P450s are gender specific, whereas this is not true for humans. In rats, members of this family are expressed in hepatic and extrahepatic tissues. In humans, CYP2C9 and CYP2C8 are present in the small intestine, and CYP2C8 has been detected in skin. Human CYP2C18 catalyzes the 4-hydroxylation, and CYP2C8 catalyzes the 4- and 7-hydroxylation of warfarin, but at a much lower rate. CYP2C8 also hydroxylates benzo(a)pyrene.

CYP2D. Rats have six members in the CYP2D subfamily, whereas three have been identified in humans: CYP2D6, CYP2D7, and CYP2D8. Mice have five CYP2D members, and this subfamily has been identified in other mammals. Human CYP2D6 was the first human P450 shown to be polymorphic, and is discussed later. Many drugs are metabolized by CYP2D6, and the reactions range from aryl hydroxylation to *N*- and *O*-dealkylation.

CYP2E. The CYP2E subfamily is one of particular interest to toxicologists. Currently, CYP2E1 is the only member of this subfamily in rats, mice, and humans. CYP2E1 appears restricted to mammals, and may have evolved more recently than certain other gene families. It is expressed in liver and kidney and occurs at low levels in a number of other tissues. Although it normally represents less than 10% of total P450 in human liver, it is induced by a broad array of its substrates. Its hepatic concentration can vary up to 50% between different humans. Rat and human forms of CYP2E1 share many similarities, including similar substrate specificities. It is known to metabolize more than 70 different chemicals with diverse structures. Structural requirements for CYP2E1 substrates appear limited to small molecules with hydrophobic character. It does not appear to be active in the metabolism of many drugs but does metabolize a wide array of alcohols, aldehydes, alkanes, aromatic hydrocarbons, ethers, fatty acids, halogenated hydrocarbons (including anesthetics), heterocyclics, and ketones. There are no specific substrate probes for CYP2E1, but aniline hydroxylation, p-nitrophenol hydroxylation, and carbon tetrachloride-dependent lipid peroxidation have been used to follow its activity in vitro.

Interest in the role of CYP2E1 as a mediator of toxicity comes from two of its actions. First, it is known to be important in the metabolic activation/detoxication of a number of carcinogens and heptatoxins. Second, it may have an important role in free-radical production and oxidative stress. For instance, it is believed to be involved in the metabolic activation associated with the carcinogenicity of benzene, nitrosamines, and azoxymethane, and the hepatotoxicity of nitrosammes, acetaminophen, halothane, and enflurane.

In respect to free radical production, it is involved in the formation of a reactive hydroxyethyl radical produced during its metabolism of ethanol to acetaldehyde. This hydroxy radical is believed to play a role in ethanol-related liver damage. It also appears to be involved in the production of a trichloroethyl radical produced by chlorine removal during the metabolism of carbon tetrachloride. This radical may initiate membrane lipid peroxidation associated with carbon tetrachloride-induced hepatotoxicity. An additional mechanism by which CYP2E1 could produce reactive radicals is associated with its potential for futile cycling in the absence of substrate. It appears more loosely coupled than some of the other P450s. Oxygen activation during the catalytic cycle in the absence of substrate results in the production of highly reactive hydroxyl radicals, superoxide anions, and hydrogen peroxide. If these reach concentrations that overcome cellular protection mechanisms, they can initiate oxidative stress leading to tissue damage.

P450s are not evenly expressed in the liver but occur in specific zones. The highest concentration of P450 is generally found in a layer surrounding the terminal hepatic venules. This is especially true for induced CYP2E1. Enhanced CYP2E1 activity in the centrilobular region appears related to the centrilobular necrosis produced by hepatotoxins, such as ethanol, carbon tetrachloride, benzene, nitrosamines, and acetaminophen. It may appear that CYP2E1 is predominately involved with metabolic activation; however, this is not necessarily true. As noted before, P450–mediated xenobiotic metabolism is generally associated with production of less toxic metabolites, but in some cases, more toxic

metabolites are produced. CYP2E1 is no exception to this rule and participates in not only metabolic activation, but also detoxication.

CYP3 Family

The CYP3 family of P450s encompasses CYP3A1 and CYP3A2 in rats, Cyp3a-11 and Cyp3a-13 in mice, and

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CYP3A3, CYP3A4, CYP3A5, and CYP3A7 in humans, along with others from rabbits, dogs, and other species. The CYP3 family contains P450s that are important in the metabolism of many xenobiotics, especially drugs.

CYP3A. This subfamily contains at least four genes in humans. In many cases, it has been difficult to separate the activities of human CYP3A3 and CYP3A4. CYP3A4 is the major form of P450 expressed in human liver. It is also the major P450 expressed in the human gastrointestinal tract. Small amounts are found in several other organs, such as the kidney and skin. CYP3A4 and other members of the CYP3A subfamily are induced by a number of drugs. These isozymes do not demonstrate a high degree of structural selectivity with respect to their substrates. Generally, their substrates are relatively large, highly lipophilic molecules. Over 150 drugs from 38 classes can be metabolized by CYP3A4. They are also capable of metabolically activating carcinogens, such as aflatoxin B1 and benzo(a)pyrene. Because of the large number of drugs metabolized by CYP3A4, it may play a role in a number of drug-drug interactions that may result in adverse effects.

An example of how dietary constituents can affect specific isozymes is provided by the interaction between the consumption of grapefruit juice and CYP3A4. Consumption of grapefruit juice can cause an increase in the oral availability of a number of drugs that are CYP3A4 substrates. This affect appears to be associated with intestinal CYP3A4 more so than hepatic CYP3A4. Increased bioavailability is produced by inhibition of intestinal CYP3A4 activity by 6′,7′-dihydroxyberga-mottin, which is a component of grapefruit juice. This dietary compound is a mechanism-based inhibitor of CYP3A4 that results in the rapid partial loss of CYP3A4 activity (150). Inhibition of metabolism of the CYP3A4 substrates during their intestinal absorption accounts for the higher-than-anticipated plasma concentration of the drugs.

CYP3A5. This P450 may be polymorphically expressed in humans and has been detected in only 25%-30% of adults. It does not appear to have the broad substrate specificity of CYP3A4 and has lower activity.

Other P450s. There are a large number of P450 families and subfamilies not discussed here. Most of these are involved with the metabolism of endogenous substrates or occur in species that are beyond the scope of the topic of this chapter (see (73) for a more complete discussion).

Reactions Catalyzed by the Cytochromes P450-dependent Monooxygenase System

On first inspection, it appears that P450 can catalyze a bewildering number of reactions (Table 3.2); however,

Table 3.2 Major oxidative reactions catalyzed by the P450 monooxygenase system

Aliphatic hydroxylation
Aromatic hydroxylation
Epoxidation
N-Dealkylation
O-Dealkylation
S-Dealkylation
Deamination
Sulfoxidation
N-Oxidation
Oxidative dehalogenation
Desulfuration

on closer inspection, there is a degree of commonality among these reactions. The first area of commonality is that most of the reactions represent oxidations. Second, the reactions convert lipophilic substrates to more hydrophilic products. Third, many of the reactions can be represented as hydroxylations, as pointed out by Mannering (106). For a review of P450 reactions, see (62).

Aliphatic Hydroxylation

Examination of aliphatic hydroxylation reactions is illustrative of several important aspects of monooxygenase activity. The reaction mechanism, which may be common to several other types of monooxygenase metabolism, appears to occur by a hydrogen (or electron) abstraction mechanism. Oxygen activation produces a $[FeO]^{3+}$ at the heme of P450. Hydrogen abstraction from the substrate results in production of the carbon radical. This radical interacts with activated oxygen (through oxygen rebound) to yield hydroxylation. Other reactions, such as O-dealkylation of ethers and carboxylic acid esters, may proceed through this mechanism with decomposition of unstable hydroxylation products. Hydrogen abstraction is site selective, resulting in a nonrandom hydroxylation. The specific hydroxylation site is determined by structure and the specific spacial orientation of the substrate at the active site. Different isozymes of P450 show different degrees of site selectivity. For example, n-hexane

hydroxylation can occur at C-l, C-2, C-3, C-4. P450 isozymes induced by phenobarbital metabolized n-hexane to yield a four- to fivefold increase in the 2-,3-, and 4-hydroxylated metabolites and only a slight increase at the 1 position. On the other hand, benzo(a)pyrene-induced isozymes result in decreased yields of the 1- and 2-hydroxylated products but increased yields of the 3- and 4-hydroxylated products (49). Hydroxylation of aliphatic compounds is generally considered detoxication because of the greater water solubility of the products, but one must be cautioned

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against overgeneralization, as products that are more toxic could be produced by subsequent metabolism.

Aromatic Oxidation

Aromatic oxidation reaction mechanisms are not completely understood but may occur by several mechanisms. The exact mechanism may be based on a number of factors, such as the steric features of the substrate and the configuration of the active site of the specific P450. Potential mechanisms include direct oxygen insertion into the C-H bond to form an epoxide through radicaloid reactions and/or through intermediates bonded to $(FeO)^{+3}$.

An example of a compound that is hydroxylated by both direct insertion of oxygen at the C-H bond and oxygen addition at the C=C bond is p-chlorobenzene. Both 3- and 4-chlorobenzene oxides are formed by the addition reaction to yield the arene epoxides. These spontaneously rearrange to form the *o*-chlorophenol and *p*-chlorophenol. The occurrence of the *m*-chlorophenol as a metabolite is an example of the direct insertion reaction. The production of arene oxides has been widely studied because of their importance in the formation of epoxide ultimate carcinogens. These epoxides can also be formed in nonaromatic systems, yielding reaction products as illustrated by the metabolism of aflatoxin B1. This mycotoxin is metabolized to a number of hydroxylated products and to the 8,9-epoxide. It is generally agreed that this epoxide is the ultimate carcinogen of aflatoxin B1.

Heteroatom Oxidation

P450 not only oxidizes carbon atoms but also nitrogen and sulfur atoms. A number of nitrogen-containing compounds can be oxidized to stable *N*-oxides. Another hepatic enzyme, flavin monooxygenase (FMO), can also catalyze this reaction. P450 and FMO may form *N*-oxides from the same xenobiotic; however, FMO generally prefers substrates with an electron-deficient nitrogen, whereas P450 prefers an electron-rich nitrogen. P450–mediated *N*-oxidation is possible with primary and secondary aromatic amines to produce hydroxylamines. Sulfoxidation can also be catalyzed by P450 and FMO. Sulfoxidation can produce a sulfoxide (SO) that can be further oxidized to a sulfone (SO_2). The mechanism associated with these reactions is believed to be electron abstraction from the heteroatom by $(FeO)^{3+}$.

Heteroatom Dealkylation

P450-dependent heteroatom dealkylation begins like heteroatom oxidation with electron abstraction from N, S, or O. This is followed by abstraction of H^+ from the carbon attached to the heteroatom. This α-carbon is hydroxylated followed by cleavage of the α-carbon and its rearrangement to an aldehyde or ketone. Sulfur atoms generally are not as readily dealkylated as nitrogen and oxygen atoms.

Ether compounds can also be dealkylated. The O-dealkylation is similar to N-dealkylation and is believed to proceed through the initial oxidation of the carbon adjacent to oxygen (i.e., carbon oxidation). The products of this reaction are again an alcohol and aldehyde, analogous to those produced by N-dealkylation.

Oxidative Deamination, Desulfuration, and Dehalogenation

Primary amines can be deaminated by the elimination of ammonia and the formation of an aldehyde or ketone. In a similar manner, P450 can catalyze desulfuration and dehalogenation, with the heteroatom being replaced with oxygen.

Reduction Reactions

An interesting series of reactions in which P450 may participate under special conditions are reductive reactions. These appear to involve transfer of electrons from Fe^{+2} to the substrate. Examples of such reactions are nitro reduction, azo reduction, arene oxide reduction, and reductive dehalogenation. These reactions generally are studied in vitro under anaerobic conditions in the presence of isolated microsomes and NADPH. Because these reactions require low oxygen tension to progress, their in vivo role (if any) is not well understood. Whether or not these reactions represent simply a curious phenomenon associated with P450 or a viable metabolic pathway is not known. It may be possible that under certain cellular conditions of low oxygen tension these reactions could proceed in vivo.

Induction and Inhibition of Cytochromes P450

Induction

When animals are exposed to certain xenobiotics, their ability to metabolize a variety of xenobiotics is increased. This phenomenon is termed *induction*. Induction produces a transitory resistance to the toxicity of many compounds; however, this may not be the case with compounds that require metabolic activation because their toxicity may increase. The exact toxicological outcome of this increased metabolism will be dependent on the specific xenobiotic and its metabolic pathway. Because the toxicological outcome of a xenobiotic exposure can depend on the balance between those reactions that

represent detoxication and those that represent activation, increases in metabolic capacity may, at times, produce unpredictable results. Induction of P450 has been reviewed in (17).

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One of the initial reports of increased metabolic capacity associated with xenobiotic exposure suggests how induction may provide a survival advantage. In 1954, Brown et al. (19) were studying the metabolism of methylated aminoazo dyes and found that xenobiotics in the animal diets enhanced the P450-dependent demethylation of these compounds. Free-living animals consume a variety of feeds that may contain toxic constituents. If the animal can respond rapidly to these toxic compounds by developing resistance, it can continue to use the feed source and obtain a survival advantage. One mechanism of rapidly developing such resistance is through increased detoxication resulting from stimulation of xenobiotic metabolizing enzyme activity.

Conney (31) published a pivotal review in 1967 that indicated more than 200 chemicals could induce P450–dependent metabolism, and most of these chemicals were monooxygenase substrates. Although the induction of the P450 enzymes has been most intensively investigated, other enzymes of biotransformation can be induced, such as the UDP-glucuronosyltransferases.

The classical definition of enzyme induction requires transcriptional activation at the level of DNA and increased production of mRNA, followed by an increase in the synthesis of the enzyme. The term has taken on a broader definition when used in respect to xenobiotic metabolism. This broader definition includes mechanisms such as mRNA and enzyme stabilization, all of which are associated with xenobiotic "induction." There are a number of "classes" of P450 inducers, which are listed in Table 3.3.

The polycyclic aromatic hydrocarbon class of inducers includes 3-methylcholanthrene, benzo(a)pyrene, and 2, 3,7,8 tetrachlorodibenzo-p-dioxin, and their mechanism of induction in animals has been extensively investigated. These inducers induce CYP1A1, which occurs in the liver and extrahepatic tissues of rats and extrahepatic tissues in humans. The low constitutive hepatic concentrations of CYP1A1 result from suppression of transcription by a nuclear repressor protein. Within the cytoplasm of the hepatocyte, there exists a receptor protein termed the *Ah receptor* that is complexed with heat-shock protein (hsp90). When a polycyclic hydrocarbon-type inducer enters the hepatocyte, it binds and activates the Ah receptor, resulting in release of hsp90. The Ah receptor is phosphorylated and subsequently binds to the Ahr nuclear translocator protein (Arnt), which is also activated by phosphorylation. This complex then moves to the nucleus of the hepatocyte. In the nucleus, this complex binds to a DNA regulatory sequence termed the *xenobiotic responsive element* (XRE). A DNA segment similar to rat XRE has been found in mouse and human cells. The XRE has also been found in genes of other xenobiotic metabolism enzymes, such as glutathione *S*-transferase, aldehyde dehydrogenase, and UDP-glucuronosyltransferase, where it may be involved in regulation of their expression. Binding of the ligand-bound-Ah-Arnt complex to XRE enhances transcription of the CYP1A1 gene, resulting in increased quantities of CYP1A1 mRNA followed by an increase in the hepatic concentration of CYP1A1.

In contrast to the polycyclic hydrocarbon class of inducers, no cytoplasmic receptor has been found for the phenobarbital-type inducers. Phenobarbital and other compounds of diverse structure induce expression of CYP2B1 and CYP2B2 and, to a lessor extent, CYP2A1, CYP2C6, CYP3A1, and CYP3A2. It also increases the quantity of endoplasmic reticulum in hepatocytes, increases total microsomal protein, and increases NADPH-cytochrome P450 reductase as well as other xenobiotic metabolizing enzymes, such as UDPGglucuronosyltransferase and epoxide hydrolase. Its induction of P450s is at the transcription level and is believed to be associated with the removal of a repressor

Table 3.3 Inducers of P450

Class of Inducer	Primary Example	Other Examples	Examples of Induced P450s
Polycyclic hydrocarbon type	3-Methylcholanthrene	Benzo(a)pyrene, β-naphthoflavone, TCDD, chlorpromazine, isosafrole, ketoconazole	CYP1A1, CYP1A2
Phenobarbital type	Phenobarbital	Phenytoin, griseofulvin, chlorpromazine, ketoconazole, dieldrin, butylated hydroxy-toluene	CYP2A1, CYP2B1, CYP2B2, CYP2C6, CYP3A2
Ethanol type	Ethanol	Acetone, heptane	CYP2E1
Glucocorticoid type	Dexamethasone	Pregnenolone-16a-carbonitrile, spironolactone, clotrimazole, prednisolone, methylprenisolone, rifampicin	CYP3A1, CYP3A2, CYP3A4
Clofibrate type	Clofibrate		CYP4

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protein from an enhancer region of the DNA, allowing an increase in transcription. Compounds in the phenobarbital inducer class, such as terpenes, organochlorine pesticides, and polychlorinated biphenyls, may act through a common pathway of induction (51).

CYP2E1 induction has been studied in detail in the rat and represents an interesting situation where induction is controlled at the transcription, mRNA stabilization, translation, and enzyme stabilization levels. In addition, diet and pathophysiological conditions can produce induction of this P450. CYP2E1 is induced by the ethanol-type inducers. Although not true for all P450s, the CYP2E1 inducers generally are substrates for the isozyme. In many cases, the regulation of expression of CYP2E1 is controlled by stabilization of CYP2E1 mRNA and stabilization of the enzyme apoprotein, along with possible increased efficiency of translation. Cycloheximide, which blocks translation, blocked the increase in CYP2E1 apoprotein when mRNA was unchanged, indicating the increase in apoprotein was related to increased translation. Actinomycin D, which blocks transcription, did not block the apoprotein increase, indicating it was not transcription related. Many of the CYP2E1 inducers act by post-translational stabilization, including acetone, low ethanol doses, pyridine, and pyrazole. With these inducers, CYP2E1 concentration increases, whereas there is no change in mRNA. This indicates that CYP2E1 degradation decreases while synthesis remains constant, with the net result being increased CYP2E1.

Nutritional factors and disease conditions can also result in increased activity of CYP2E1. High-fat diets and starvation produce an induction of CYP2E1, as does insulin-dependent diabetes and obesity. One common factor in all of these conditions is increased plasma ketone body concentrations. Whether or not this induction is produced by increased ketone bodies, including acetone, or by other factors is currently under investigation.

The glucocorticoid-type inducers, such as dexamethasone and pregnenolone-16α-carbonitrile, induce CYP3A1 and CYP3A2. Induction is associated with transcription activation and mRNA stabilization. The exact mechanism of induction is unknown and its relationship to the glucocorticoid receptor, if any, is uncertain. CYP3A isozymes are involved in the metabolism of a number of drugs, and induction could effect drug-drug interactions.

Clofibrate-type inducers induce the CYP4A subfamily, that is, in most part, associated with metabolism of endogenous compounds. They also cause hepatocyte peroxisome proliferation in rodents. Many compounds that cause peroxisome proliferation in rodent liver are also hepatocarcinogens; however, humans are resistant to peroxisome proliferation, and these compounds do not appear to be a hepatocarcinogenic risk to humans. The low concentrations of CYP4A in human liver and its limited number of xenobiotic substrates, reduce its role in drug-drug interactions in humans. Induction by compounds such as clofibrate appears associated with a peroxisome proliferator-activated receptor (PPAR) involved in activation of transcription and resulting apoprotein synthesis.

Inhibition

Just as induction of xenobiotic metabolism can have important toxicological ramifications, inhibition of the ability to metabolize a xenobiotic can result in profound changes in its toxicity. Inhibition of a compound's metabolism can result in a higher plasma concentration than predicted and unexpected toxicity. During multiple drug treatment, unexpected adverse effects can be produced through drug-drug interactions where one drug inhibits the metabolism of another, resulting in higher than expected plasma concentrations.

Four mechanisms are generally associated with inhibition of P450-mediated detoxication. First, two xenobiotics may be substrates for the same P450 isozyme and will compete for the active site of the enzyme. This is an example of competitive inhibition. A second mechanism of competitive inhibition is the binding of a xenobiotic to the active site of a P450, although it is not a substrate for that P450. The presence of the nonsubstrate at the active site blocks the binding of the true substrate, inhibiting its metabolism. A third mechanism of inhibition involves the metabolism of a xenobiotic to a product that has a higher affinity for the active site than the parent compound. The active site is then occupied and additional substrate cannot bind. This essentially makes the enzyme inactive and is an example of noncompetitive inhibition. The fourth mechanism is another example of noncompetitive inhibition resulting from the production of a highly reactive metabolite that binds (often covalently) to the heme or apoprotein of P450, destroying its activity. This type of inhibitor is termed a *mechanism-based inhibitor* or a *suicide substrate*.

Other, less common mechanisms can result in inhibition of P450-mediated xenobiotic metabolism, including compounds that may modify protein or heme synthesis or degradation, those that may uncouple electron transport to P450, those that may interfere with cofactor availability, and those that may directly inhibit NADPH P450 reductase activity. Just as some substrates may demonstrate a higher

affinity for specific P450s and others may not, inhibitors may show a narrow or broad range of affinity for a specific P450. Inhibitors have been useful tools in determining mechanisms associated with xenobiotic metabolism and in attempts to predict specific drug-drug interactions. Induction and inhibition

of human cytochromes P450 have been recently reviewed in (135).
Species, Strain, and Gender Differences in Monooxygenase Activity
The activities of cytochromes P450 play a central role in the expression of the toxicity of the majority of xenobiotics. Knowledge of the rates at which a xenobiotic is metabolized and the chemical and biological nature of its metabolites assist not only in understanding its toxicity, but also its mechanism of action. This provides essential information to enable toxicologists to predict its toxicity. One factor that complicates extrapolation of toxicity between species is the quantitative and qualitative differences in how species metabolize xenobiotics. Generally, the basic reactions and major metabolites of a xenobiotic are similar between species; however, subtle differences in metabolism can lead to major differences in susceptibility to the toxicity of a xenobiotic.
A variety of mechanisms can be associated with differences between species in their response to a xenobiotic, including (1) adsorption, (2) distribution, (3) organ differences in metabolic capacity, (4) qualitative and quantitative differences in P450 isozymes and other xenobiotic metabolizing enzymes, (5) excretion, and (6) insensitivity of either the target organ or biochemical target site. Although all of these factors may be more or less important in understanding species differences in the susceptibility to the xenobiotic, it appears that the dominant factor is xenobiotic metabolism.
Mechanisms that may account for species differences include (1) lack of a metabolic pathway or a genetic "defect" in a particular metabolic pathway, (2) differences in the Km and Vmax of specific enzymes, (3) the existence of different isozymes and differences in the ratios of specific isozymes of important enzymes, such as P450, and (4) differences in the ratio of activities of separate enzyme systems that act together to metabolize a specific xenobiotic.
The lack of a specific pathway or a defect in a pathway will make a species or strain susceptible to xenobiotics that are detoxicated via that pathway. Conversely, when the xenobiotic is metabolically activated by that pathway, the species will be resistant. Although there does not appear to be a mammalian example of the lack of the monooxygenase system, examples exist for other enzymes, such as the lack of glucuronidation in cats. A more common explanation of species difference is the variation in the activity (Km, Vmax) and substrate specificity of isozymes associated with xenobiotic metabolism. At low doses or environmental exposures, these differences will be expressed in detoxication and species susceptibility. Caution needs to be used in both the design and interpretation of studies to investigate species differences. As mentioned, doses below enzyme saturation may not reveal species differences. Care must be used when investigating these differences in vitro, as this activity may not mimic in vivo activity.
When one metabolite represents a metabolically activated form and another a detoxicated form, the ratio of these metabolites can dictate a species susceptibility to a xenobiotic. This type of species difference is most commonly encountered when the P450-dependent monooxygenase acts in coordination with another pathway. Species may differ in either the initial monooxygenase functionalization reaction or in the activity of the secondary pathway. This is illustrated by the metabolic activation of benzo(a)pyrene (BP) (see Figure 3.4) in rats and mice. The metabolic activation of BP requires initial epoxidation by the P450-dependent monooxygenase at the 7,8 position, followed by hydration of the epoxide by epoxide hydrolase to yield the 7,8-diol. This diol is then epoxidated by the monooxygenase to yield the ultimate carcinogen of BP, the 7,8-dihydrodiol 9,10-oxide. When mouse hepatic microsomes were used for metabolic activation in the Ames assay for mutagenicity, BP was highly mutagenic, indicating a high degree of metabolic activation; however, when rat hepatic microsomes were employed in the same assay, only slight mutagenicity was evident. This indicates a significantly lower ability for the rats to metabolically activate BP in vitro (127). Although mice do metabolize BP to a greater extent than rats, rats have six- to sevenfold more epoxide hydrolase activity. Further studies (126, 127) indicated that both species have adequate monooxygenase to metabolically activate BP and that higher epoxide hydrolase activity in the rat may have been responsible for the lower mutagenicity. Therefore, the species differences in the secondary pathway, epoxide hydrolase, may have controlled the mutagenicity, as opposed to differences in the monooxygenase activity.
Just as species demonstrate differences in metabolism that alters toxicity, different strains of the same species may demonstrate differences in metabolism. For instance, if a different strain of mouse had been used in the studies described above, the data may have been different. It is important to recognize these strain differences when designing toxicological studies. The mechanisms associated with strain differences may be diverse. As with any genetically controlled activity, differences in metabolism are not unusual. Laboratory animals are generally bred in distinct groups, and without extensive outbreeding strain differences can develop quite rapidly. In the wild, such strain differences would be less likely, but

individual differences may be greater. Other factors, such as diet, environment, etc., may result in what appears to be strain differences.

Species differences in metabolism and, consequently, susceptibility lead to an important concept in toxicology,

that of selective toxicity. Ideally, a pesticide should be toxic only to the organism against which it is directed. This concept of selective toxicity has resulted in efforts to develop selective pesticides. For these activities to be fruitful, it is important that species differences in metabolism are understood and this information is used.

For the toxicologist, the species differences of major importance are those between humans and those species used in toxicological testing. Without an understanding of these species differences, it will be difficult to extrapolate toxicological studies performed with animals to humans. Studies of species differences in animals are difficult to design and interpret, and those involving humans are even more complex. This complexity results from the large differences in xenobiotic biotransformation found in humans. Many factors contribute to these individual differences in metabolism, including the following: (1) humans are free-living and have few restraints to reproductive diversity, diminishing the development of small genetic pools that result in genetically less diverse, more homogeneous control of metabolism; (2) environmental factors, such as diet, nutrition, xenobiotic exposure, etc., are diverse among humans; and (3) humans generally have more control and probably more interest in consumption of varied non-nutritive materials, such as alcohol, drugs, etc. These, as well as other factors, result in a large diversity in susceptibility to xenobiotic exposure. This is, in part, why such large safety factors are employed in risk or hazard assessments of xenobiotics to which humans may be exposed. These safety factors are used to attempt to protect the vast majority of individuals at risk. For further discussions of species differences, the reader is directed to the articles by Walker and Oesch (182) and by Caldwell (22). A discussion of intraindividual and interindividual differences can be found in the monograph by Vesell and Penno (178). For detailed reviews of P450 in a number of species, including plants, see (149).

Gender differences in xenobiotic metabolism may be an important factor in gender-dependent differences in toxicity. The best example of gender differences in xenobiotic metabolism, especially cytochrome P450-mediated metabolism, is the rat. Because the rat is commonly used in toxicological safety assessments, it is important to realize the gender differences in this species and understand how it relates to the extrapolation of rat data to humans.

Generally, male rats have a higher capacity to metabolize xenobiotics than females. This difference is, in most part, related to the cytochromes P450. Although females have 10%–30% less total P450 than males, this difference is not high enough to explain the 2- 20-fold difference seen in metabolism. Much of the differential seen between males and females can be explained by differences in P450 isozymes between the sexes. For instance, males express CYP2C11 whereas females do not. Isozymes that predominate in males are CYP2A1, CYP2A2, and CYP3A2. Adult females also have predominant P450s, such as CYP2C12, which occurs in juvenile and older males but not in young adult males. These differences are under hormonal control and can be altered by procedures such as castration and administration of sex hormones. They also are developmentally controlled, and the stage of life at which these procedures are done can influence their outcome. Neonatal castration of male rats results in different expression of P450 when they become adults. The adult expression of P450s can actually be "imprinted" during the neonatal period. Although sex hormones play an important role in the expression of P450 in rats, other hormones, including growth hormone, thyroxine, insulin, and somatostatin, may play important roles.

These differences between male and female rats also show an age dependency. As male rats age, their P450 isozyme profiles begin to appear more like females. Toxicologists using rats as a model in safety assessments need to be cognizant of these gender- and age-dependent changes. The toxicity data from young rats, generally used in toxicity studies, may not reflect the toxicity seen in old rats. During chronic toxicity and carcinogenicity studies, the response of rats to the toxicity of a test material may change as the study progresses. This is especially true of carcinogenicity studies that begin with in utero exposure. Early developmental changes in P450 in animals and humans may be important in responses to teratogens and embryotoxic compounds (112).

If gender differences in xenobiotic metabolism in rats can complicate toxicity assessments, what about other species, including humans? Rats have been the most intensively investigated species in regard to gender differences; however, studies with other species suggest that they generally do not demonstrate such large gender differences. Mice, another species important in toxicology studies, generally do not show the exaggerated gender differences in xenobiotic metabolism seen in rats. Gender differences in mice seem to be dependent on the specific strain of mouse investigated. Where gender differences do exist in mice, it is generally the female that has the higher metabolic capacity, but the differences are not as great as that seen in rats.

Other species used in toxicological investigations, such as dogs, appear to demonstrate some gender differences in the expression of P450 isozymes, but, again, they are not as exaggerated as in rats. Although there are few reported studies, monkeys have not been reported to demonstrate significant gender differences in xenobiotic metabolism.

Humans have not been shown to demonstrate gender-dependent differences in the expression of P450 isozymes. Although there can be significant differences

between human males and females in xenobiotic biodisposition, these appear to be more based on anatomical and physiological differences that affect absorption, distribution, and excretion. There can be large differences between individual humans based on life-style factors and exposure to environmental chemicals, food, and drugs; however, there do not appear to be inherent gender differences in the expression of P450.

This raises the question as to how species, such as rats, can be used to predict toxicity in humans. With care and knowledge of the differences between rats and humans, the rat can serve as a model for human toxicity. This has been shown through decades of use. For instance, rats and humans share similarities between the CYP isozyme subfamilies CYP1A1, CYP1A2, and CYP2E1, and these subfamilies are not expressed in a highly gender-dependent manner in rats. Xenobiotics that are substrates for these isozymes may be metabolized in a similar manner in rats and humans. Gender-dependent differences in xenobiotic metabolism are but one of the reasons toxicologists must use both sexes in the safety assessment of chemicals. Gender differences in xenobiotic metabolism have recently been reviewed (120).

Pharmacogenetics, Human Polymorphism of P450 Isozymes, and Their Toxicological Significance

Pharmacogenetics is the study of the hereditary basis of the observed differences in response (both therapeutic and adverse) to drugs by individuals and populations. The term can be expanded to include not only drugs but also dietary and environmental chemicals. This field has seen a large expansion over the last decade, as the understanding of the genetics, genetic regulation, and interindividual variations in P450 and other xenobiotic metabolism enzymes has increased. New methodologies from molecular biology and refinement of other methodologies to study genetic differences in individuals and populations have spurred interest in the pharmacogenetics of xenobiotic metabolism.

Many studies of the adverse effects of chemicals in animals and humans have indicated there are highly significant differences between animal strains, individual animals, and especially differences in individual humans and different human population groups. These genetic *polymorphisms* can result in unexpected drug and environmental toxicities and complicate safety assessments and the extrapolation of data from animal studies to humans. As discussed later, this has led to recommendations that the specific family and subfamily of P450 that metabolizes a specific drug candidate be determined during early drug discovery efforts. This could avoid unexpected interactions and suggest potential adverse effects before additional developmental efforts with a drug or other chemical product are undertaken.

Several human P450s have been shown to be polymorphically expressed, including CYP1A1, CYP2A6, CYP2C9, CYP2C19, CYP2D6, and CYP2E1. The chromosomal locations of these P450 genes have been identified and the genetic basis for the altered P450 activity is becoming understood. For instance, several alleles in the CYP2D6 family are known to contain specific nucleotide deletions that result in inactive genes and a lack of production of the CYP2D6 protein (34). Individuals homozygous for these gene variations will be “poor metabolizers” of CYP2D6 substrates. In contrast, some individuals have multiple copies of the CYP2D6 gene, possibly due to gene duplication (80). These individuals have enhanced capability to metabolize CYP2D6 substrates and are “ultra-rapid metabolizers” (9). A chemical whose detoxification depends on CYP2D6 would be more toxic than expected in the poor metabolizers, but less toxic than expected in the ultra-rapid metabolizers. In contrast, a chemical that is metabolically activated would be less toxic in poor metabolizers and more toxic in ultra-rapid metabolizers. To predict toxicity, it is obvious that not only must the role of metabolism in the toxicity of a compound be known, but also the potential genotype of exposed individuals.

Major advances have been made in determining the genotypes of individuals in respect to P450 isozymes. These range from the in vivo administration of probe drugs and determination of their metabolites as probes of metabolic capability and P450 genotype to the tools of modern molecular biology. For instance, the CYP2D6 genotype of individuals and populations can be probed using polymerase chain reaction (PCR) technology, followed by restriction endonuclease digestion, as well as other techniques (159). Genotyping studies have shown that the most common CYP2D6 mutations occur in about 7% of Caucasians and less than 1% of Asians.

CYP2D6 represents one of the first P450 polymorphisms discovered and one of the most extensively studied from the aspects of genetics, biochemistry, and clinical significance. It also is an excellent example of how all of these factors come into play to define a P450’s role as a determinate of toxicity. Smith and his colleagues were among the first to suggest that the high incidence of side effects associated with debrisoquine, an antihypertensive, might be due to genetic differences in debrisoquine metabolism (103). The early studies led to the knowledge that debrisoquine was metabolized to its 4-

hydroxy metabolite by CYP2D6. Investigations of the substrate specificity of this P450 have indicated that its preferred substrates are basic arylalkylamines with an ionized nitrogen at physiological pH. The site of

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oxidation is generally on or near a planar aromatic system 5–7 A from the basic nitrogen (160). A proposed strong ion pairing between the substrate nitrogen and an aspartic acid in the active site of the P450 suggest it would have a high affinity for its substrates. This is supported by its low Km and Ki, compared to other P450s.

Although the requirements for a compound to be a substrate for CYP2D6 appear stringent, it has been shown to metabolize over 30 prescribed and overthe-counter drugs, including members of a variety of drug classes such as β-adrenergic blocking agents, antiarrhythmics, and antidepressants, among others. From this listing, it is obvious that CYP2D6 polymorphism is of clinical significance. In addition, it has been linked with increased risk of cancer in several organs, Parkinson's disease, and dopamine neurotransmission.

Regulatory and Product Development Aspects of Xenobiotic Metabolism

International regulatory agencies require some information about the metabolism of drugs and other chemicals that fall under their jurisdiction. The amount of data required will depend on the use of the chemical, its potential exposure to humans, and the potential role of metabolism in its efficacy and toxicity. Early information on the metabolism of drugs is becoming essential for the selection of drug candidates for further development. With the development of combinatorial synthetic chemistry and high-throughput pharmacological screening, the number of potential drug candidates that need to be rapidly screened for their potential metabolism has increased dramatically. This is leading to the development of rapid methods to predict metabolism and potential drug-drug interactions. Data are needed from in vitro and in vivo animal models that will be used in safety assessments to allow the toxicologist to design appropriate studies during the preclinical phase of a safety assessment. Data are also needed concerning the potential human metabolism of the drug candidate to allow the toxicologist to extrapolate animal safety data to humans. In the recent past, it was difficult to obtain in vitro data from human tissues. With the current knowledge of the human P450 isozymes and the commercial availability of human hepatic preparations and cells that express human P450s, it is possible to obtain data concerning human metabolism of drug candidates.

The U.S. Food and Drug Administration (FDA) has recently released a guidance document concerning in vitro drug metabolism and drug-drug interaction studies during the drug development process (173). This document stresses the importance of obtaining infor mation on a drug candidate's metabolism during the early stages of development. This information is important in predicting potential individual differences based on polymorphic expression of xenobiotic metabolism enzymes and in predicting drug-drug interactions. The guidance document is based on the following general observations:

1. The concentrations of a drug or its active metabolite circulating in the body determine the extent of its desirable and/or adverse effects.
2. A major determinant of a drug's concentration is clearance, and metabolism is a major determinant of clearance.
3. Drugs that are not substantially metabolized may impact the metabolism of other drugs.
4. Large differences in blood concentrations can occur because of polymorphic metabolism. Drug-drug interactions can also produce large changes in the blood concentration of a drug.
5. Major advances have been made in availability of human tissue and recombinant enzymes for in vitro studies of drug metabolism.

The guidance document suggests that the goals of in vitro metabolism and interaction studies should be (1) to identify major metabolic pathways and the specific isozymes involved, and (2) to explore and extrapolate the effects of the drug candidate on the metabolism of other drugs and the effect of other drugs on the candidate's metabolism. To accomplish these goals, the FDA suggests starting from human hepatic microsomes, now commercially available, then moving to expression systems that express specific human P450s, which are also commercially available. They note it is possible to move to hepatocytes and precision-cut liver slices but realize there are technical difficulties with these preparations.

Co-incubation of the drug candidate with substrate probes with known metabolic pathways can be used to determine its effects on their metabolism. In addition, assessment of the drug candidates' metabolism by CYP3A4 may provide information on intestinal metabolism that can affect drug bioavailability. Parallel studies using preparations from animal models can aid the toxicologist in choice of species for the toxicological studies. The document points out that in vitro studies currently cannot replace in vivo studies but can give direction for the proper design of in vivo studies. For instance, if the drug candidate is not metabolized by CYP2D6, then it will not be important to study the impact of the slow metabolizer phenotype on potential adverse effects. If the drug candidate is not a substrate for CYP3A4, then there

is less concern that inhibition of CYP3A4 by drugs such as ketoconazole and erythromycin or

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induction by rifampin and anticonvulsants could cause problems.

Role of the Cytochrome P450-dependent Monooxygenase in Toxicity

The toxicity of any agent is dependent on its concentration at its target site. This is a function of many factors, including the route of exposure, the pharmacokinetics of the xenobiotic, the excretion of both the parent compound and its metabolites, and the sensitivity of the target site. The ability of the organism to clear the xenobiotic through excretion will have a profound influence on the concentration at the target site. Directly associated with the ability to clear many xenobiotics is the ability to metabolize the xenobiotic to more water-soluble metabolites.

Without doubt, the P450-dependent monooxygenase plays a pivotal role in the metabolism of xenobiotics. It is the prime metabolic route for the majority of xenobiotics, acting either directly in detoxication or indirectly by priming the xenobiotic for further metabolism through functionalization, as illustrated in other sections of this chapter.

The original interest in the P450 system was associated with its ability to metabolize drugs and decrease both their toxicity and duration of action. It soon became evident that, in certain cases, this enzyme system converted certain drugs from pharmacologically inactive forms to active forms. Examples of the metabolic activation of toxicants, such as the in vivo conversion of the inactive insecticide parathion to its active form, paraoxon, were soon encountered. It was also discovered that this enzyme system could activate stable molecules such as benzo(a)pyrene to highly reactive metabolites capable of damaging cellular macromolecules, as shown in Figure 3.4. Further studies have indicated that metabolic activation plays an important role in the toxicity of a number of xenobiotics.

Studies undertaken to understand the biochemistry of P450 played a large role in the development of the modern fields of biochemical and molecular toxicology. Currently, much effort is placed on the determination of the balance between metabolite activation, detoxication, and detoxication of activated metabolites. This is providing new insight for the toxicologist in understanding the toxicity of xenobiotics. Studies on the active sites of P450 and other xenobiotic metabolism enzymes, and the factors that influence their activity and their expression, are bringing toxicologists closer to being able to predict potential toxicity with more accuracy. These efforts are also aiding toxicologists in the difficult task of predicting human toxicity from studies done with cellular and animal models.

Other Enzymes Associated with Oxidative Metabolism

Microsomal Flavin-containing Monooxygenase

Since 1960, it has been apparent that a microsomal monooxygenase other than P450 could catalyze the oxygenation of nucleophilic nitrogen, sulfur, and phosphorus compounds. Purification to homogeneity indicated it was an NADPH-dependent, flavin-containing monooxygenase that does not contain P450. This monooxygenase (EC 1.14.13.8) has been referred to as amine oxidase, Ziegler enzyme, dimethylaniline monooxygenase, and flavin-containing monooxygenase. This enzyme may be a good example of proteins involved in normal anabolic and catabolic metabolism being recruited for xenobiotic metabolism. The flavin prosthetic group that is characteristic of these enzymes is especially versatile at carrying out redox functions.

The catalytic cycle for the flavin-containing monooxygenase is shown in Figure 3.5. NADPH reduces the FAD of the enzyme, and the oxidized NADP+ remains bound. Oxygen then binds to yield FAD peroxide followed by substrate binding. An oxygen atom from the peroxide is transferred to the substrate leaving the hydroxyflavin. The final and rate-limiting step of the cycle is the dehydration to regenerate FAD, yield water, and release the bound NADP+. NADH can substitute for NADPH, but with lower affinity and activity.

There are at least five isoforms of the flavin-containing monooxygenase, designated as FMO1-FMO5, whose genes are expressed across several species and tissues. These forms have different substrate specificities and are probably related to the species-dependent toxicity

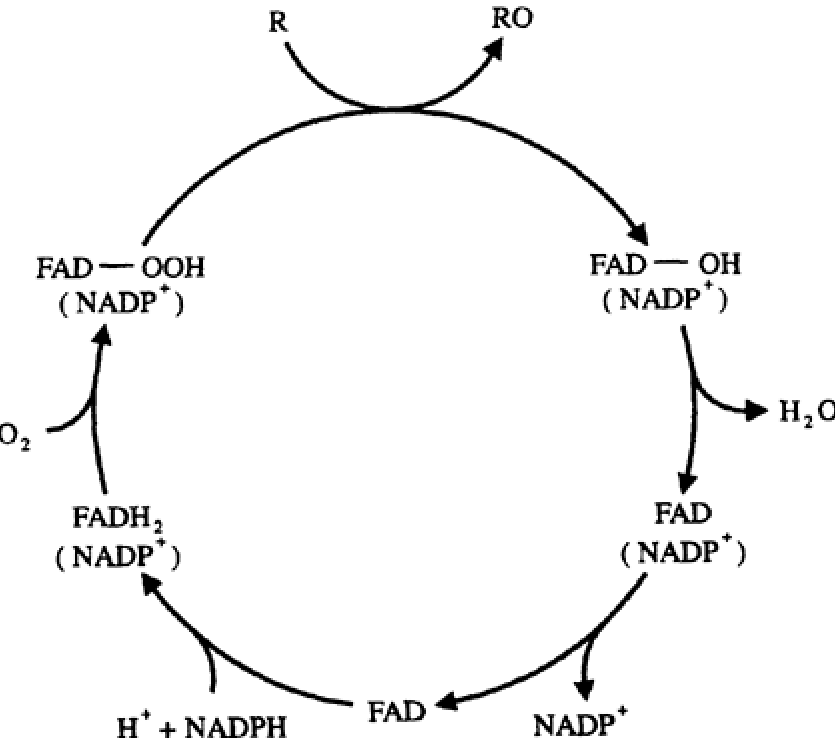

FIG. 3.5. Catalytic cycle of the flavin-containing monooxygenase. Adapted from References 194 and 195.

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of certain substrates, such as the pyrrolizidine alkaloids. Humans, rats, and mice have relatively high activity of FMO1 in kidney. Humans and mice have low activity for this form in the liver, whereas the rat has high activity. Humans and mice have high activity of FMO3 in the liver, whereas the rat has low activity. No FMO3 activity has been detected in human kidney but rat and mouse kidney show high activity. In mice, only females express FMO3 and have two- to threefold higher activity of FMO1 compared to males. FMO5 shows no gender differences. Male rats have higher total FMO than females, and two- to threefold more FMO1 than females, but no differences are seen in FMO3 (27). Humans can show considerable individual differences, but no gender differences have been detected. This illustrates the species, gender, and tissue differences that can be encountered with this monooxygenase and emphasizes the importance of choosing an appropriate animal model for toxicological studies of compounds that are potential substrates for this enzyme.

This microsomal enzyme catalyzes the oxidative attack on the nucleophilic nitrogen and sulfur heteroatom of a variety of xenobiotics (195) (See Table 3.4). It was once believed that oxidations of basic aliphatic and tertiary aromatic amines were carried out by the flavin-containing monooxygenase while primary aromatic amines and the acidic nitrogens of amides were catalyzed by P450, whereas secondary amines were oxidized by both enzyme systems. More recent studies with the purified enzymes have demonstrated that there is no clear division between the types of substrates preferred by the two enzymes. Therefore, the metabolism of each nitrogen-containing xenobiotic must be considered on an individual basis. The thermal instability of the flavin monooxygenase in the absence of NADPH (above 35°C) has provided a tool to separate the activity of this enzyme in vitro from that of P450; however, defining the relative contribution of the flavin monooxygenase and P450 to the metabolism of many xenobiotics is difficult because some inhibitors of P450, such as SKF-525A, are substrates for the flavin monooxygenases. More selective inhibitors of P450, such as N-benzylimidazole and aminobentotriazole, are a better choice for distinguishing these two enzymes (195). Antibodies to specific P450 isozymes can be used to inhibit P450 and determine the role of the flavin-containing monooxygenase.

Many nitrogen- and sulfur-containing xenobiotics are metabolized by this phase I enzyme, as seen in Table 3.4. *N*-oxidation of nucleophilic tertiary amines yields *N*-oxides. Primary and secondary amines are oxidized to hydroxylamines. In addition, primary amines can be oxidized to oximes and secondary amines to nitrones. Thiols, thioethers, and other xenobiotics containing sulfur can be oxidized to sulfur oxides. The flavincontaining monooxygenase has a relatively broad substrate specificity, but individual isozymes demonstrate some specificity. Broad substrate specificity and its occurrence in several tissues indicate that it can be a major determinant in oxidative xenobiotic metabolism.

Amine Oxidases

Amine oxidases can play a significant role in the metabolism of specific xenobiotics (167). Monoamine oxidase (MAO) (EC 1.4.3.4) and related amine oxidases catalyze the oxidative deamination of endogenous amines. They can also be involved in the metabolism of primary, secondary, and tertiary xenobiotic amines. Two of the amine oxidases (MAO and semicarbazide-sensitive amine oxidase) will be used as examples of amine oxidases.

Most tissues express two forms of the mitochondrial enzyme MAO, termed MAO-A and MAO-B, each expressed by a separate gene. MAO is a flavoprotein capable of oxidative deamination of primary, secondary, and tertiary amines. Metabolism of primary amines yields an aldehyde and ammonia, whereas secondary amines yield an aldehyde and a primary amine. The aldehyde products may be further metabolized by other enzymes to carboxylic acids or alcohols. Unlike the monooxygenases, the oxygen used in the reaction is derived from water. During the oxidation, the FAD prosthetic group is reduced (FAD→FADH2) then reoxidized by oxygen with the production of hydrogen peroxide.

A well-publicized example of an MAO-related toxicity was initiated by individuals attempting to synthesize a narcotic related to demerol. Instead of the intended product, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) resulted from the synthesis. Individuals who self-administered MPTP demonstrated Parkinson's disease-like symptoms. This was related to neuro-cytotoxicity in dopaminergic neurons produced by brain MAO-B metabolism of MPTP to l-methyl-4-phenyl-2, 3-dihydropyridine (MPDP+), which oxidizes to the neurotoxic l-methyl-4-phenylpyridine (MPP+). The cytotoxicity of MPP+ results from its inhibition of mitochondrial respiration.

A number of amine drugs have been shown to be substrates for MAO. Some of these act as pro-drugs and require MAO metabolism to produce the active form; others have their activities limited by MAO metabolism. MAO-A and MAO-B have different substrate specificities, but there can be overlap in specificity.

Semicarbazide-sensitive amine oxidases (SSAO) (EC 1.4.3.6), like monoamine oxidase, catalyzes the oxidative deamination of endogenous amines, but also can metabolize xenobiotic amines (102, 167). The SSAO do not contain a flavin but do contain copper. They demonstrate a more limited activity than MAO by only catalyzing deamination of primary aliphatic and aro-

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Table 3.4 Functional groups oxidized by flavin-containing monooxygenase

Functional group	Compound class	Example
	Sulfur Oxidation	
$RSH \rightarrow RSSR$	Thiols	Cystamine→cysteamine
$RSSR \rightarrow RS(=O)SR$	Disulfides	
$RSR \rightarrow RS(=O)R \rightarrow RS(=O)_2R$	Sulfides	Cimetidine, aldicarb
$-N-C(SH)-NH \rightarrow -N-C(=S{=}O)-NH_2 \rightarrow -N-C(SO_2H)=NH$	Thiocarbamides	
$R-C(S)-NH_2 \rightarrow R-C(=S{=}O)-NH_2 \rightarrow R-C(SO_2H)=NH_2$	Thioamides	Thioacetamide, thiobenzamide
$(-N)(=N-H)SH \rightarrow (-N{=})(=N-H)SOH \rightarrow (-N{=})(=N-H)SO_2H$	Mercaptopurines and pyrimidines	
	Nitrogen Oxidation	
Acrylic $R-N(R_1)(R_2) \rightarrow R-N(OH)(R_1)-R_2$ *Cyclic* $R-(ring)N-CH_3 \rightarrow R-(ring)N(\rightarrow O)-CH_3$	Tertiary amines	Chloropromazine, cocaine, fluphenazine, nicotine
$R-NH-R_1 \rightarrow R-N(OH)-R_1 \rightarrow R=N(\rightarrow O)-R_1$	Secondary amines	Desipramine, *N*-methylaniline
$R-N(NH_2)-R_1 \rightarrow R+N(OH)(R_1)-NH_2$	Hydrazines	Dimethylthydrazine, procarbazine, benzylthydrazine

$$\text{Ar}-\text{NH}-\text{R} \rightarrow \text{Ar}-\text{N(OH)}-\text{R}$$

N-Alkylarylamines 2-Acetylaminofluorene

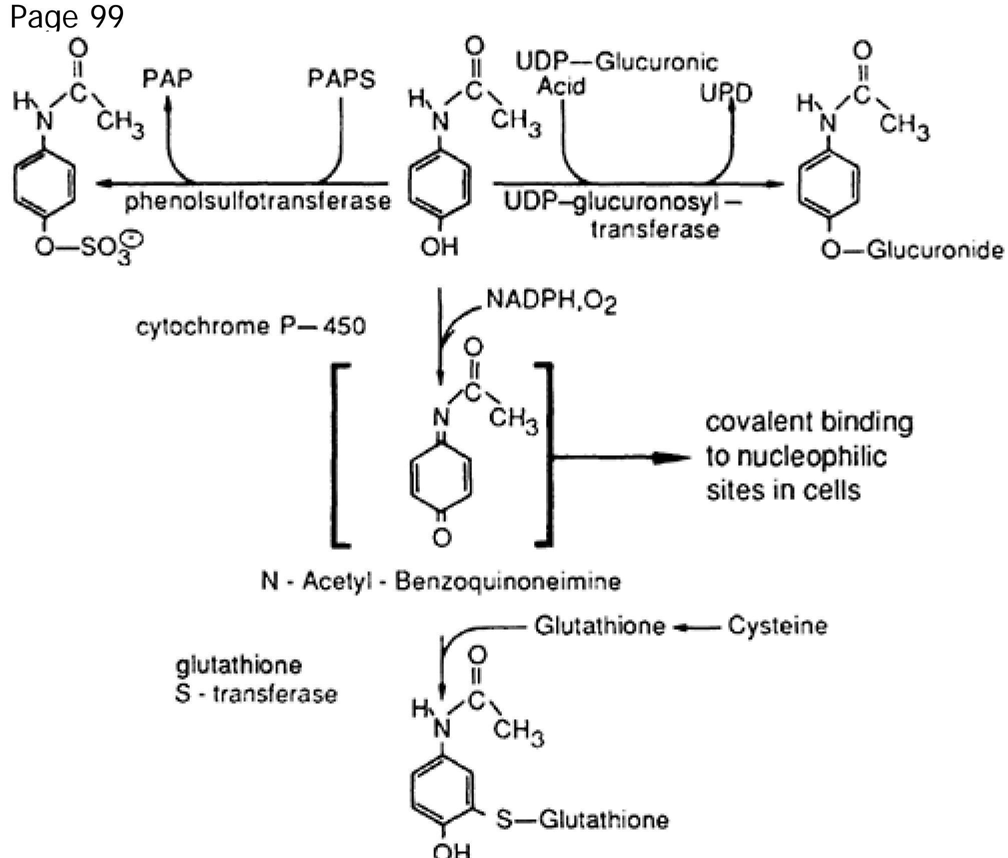

FIG. 3.6. Biotransformation of the analgesic acetaminophen.

matic monoamines. They are sensitive to inhibition by semicarbazide but insensitive to the classic MAO inhibitors. The products of their reaction are an aldehyde, ammonia, and hydrogen peroxide. They occur in most species, including bacteria, fungi, plants, and animals. In animals, they occur in plasma and bound to tissues. Although they can metabolize several endogenous substrates (191), their exact physiological role is currently unknown. There are considerable species differences with SSAO. For instance, rats have relatively low concentrations of plasma SSAO compared to humans. SSAO can metabolize certain xenobiotics to more toxic metabolites. 3-Aminopropene has been used in the manufacture of pharmaceuticals and in rubber vulcanization. Chronic exposure to this compound can produce lesions similar to acute myocardial necrosis and atherosclerosis. SSAO appears to metabolize 3-aminopropene to 2-propenal (acrolein), which alkylates glutathione S-transferase and allows excessive peroxidative damage (66). Damage occurs in the heart and aortic tissue, which have high SSAO activity (30).

Co-oxidation of Xenobiotics by Prostaglandin Synthase H

Pathways other than the monooxygenases may be involved in xenobiotic oxidation. Marnett and Reed (107) demonstrated that prostaglandin H synthetase, an enzyme system responsible for prostaglandin biosynthesis, was capable of oxidizing benzo(a)pyrene to quinones. Two catalytic activities co-purify with the synthase: fatty acid cyclooxygenase and prostaglandin hydroperoxidase. The cyclooxygenase catalyzes arachidonic acid oxidation to prostaglandin G2, and the hydroperoxidase reduces the hydroperoxidase (–OOH) to the corresponding alcohol in prostaglandin H2, as shown in Figure 3.7. Oxidation of xenobiotics results from a one-electron pathway involving an oxidizing agent produced during the hydroperoxidase-catalyzed reduction of prostaglandin G2 to the hydroxy endoperoxide, prostaglandin H2. Prostaglandin synthetase is a major source of alkyl hydroperoxides produced during normal metabolism (108). Most tissues possess prostaglandin synthetase activity and are capable of oxidizing certain xenobiotics, even if the tissue is low in P450 content. In fact, acetaminophen, which is activated to a reactive intermediate by P450 (Figure 3.6), can be activated by prostaglandin synthetase in the medulla of the kidney (Figure 3.7). This tissue is low in P450 activity, but in the presence of arachidonic acid, the medulla activates acetaminophen to a reactive intermediate that covalently binds to tissue macromolecules (14). Other compounds that undergo co-oxidation include aminopyrine, benzphetamine, oxyphenbutazone, benzidine, and benzo(a)pyrene.

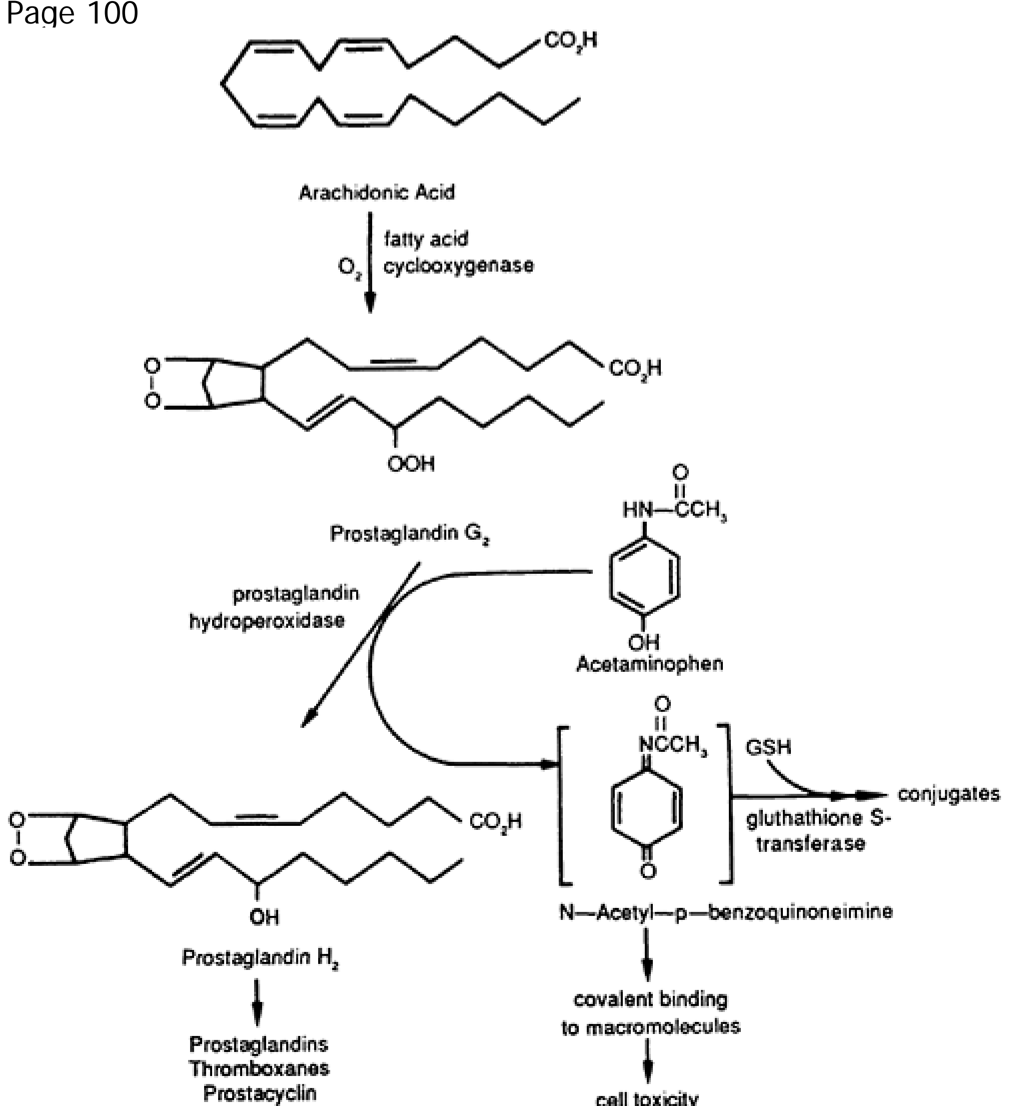

FIG. 3.7. Co-oxidation of acetaminophen by prostaglandin endoperoxide synthetase.

The bladder also possesses high prostaglandin synthetase activity. Mattammal et al. (109) proposed that several structurally diverse renal and bladder carcinogens are metabolically activated by prostaglandin synthetase. For example, the bladder carcinogen 2-amino-4-(5-nitro-2-furyl)thiazole is believed to be activated by prostaglandin synthetase co-oxidation in bladder transitional epithelium to metabolites capable of covalently modifying RNA and DNA. Feeding aspirin to rats can inhibit the bladder lesion induced by 5-nitrofuran, the ultimate carcinogen. This suggests that prostaglandin synthetase is involved in the metabolic activation, as aspirin is a specific inhibitor of prostaglandin synthetase.

Use of the analgesic p-phenetidine has declined because of reports of kidney damage in man following prolonged use of the drug. Andersson et al. (5) proposed a mechanism by which phenetidine is activated by prostaglandin synthetase in the kidney. The primary amine nitrogen of phenetidine undergoes a one-electron oxidation similar to that shown in Figure 3.7 for acetaminophen. This leads to hydrogen abstraction yielding a reactive nitrenium radical. This radical is postulated based on its rate of reaction with reduced glutathione. Benzene can be hydroxylated to phenol in the liver by P450, and the phenol can be further oxidized to hydroquinone. The phenol and hydroquinone can enter the blood stream and be distributed to other tissues. In the bone marrow, the phenol stimulates prostaglandin synthetase peroxidative activation of hydroquinone to reactive metabolites that form adducts with nucleophiles, such as protein and DNA. This is believed to result in the bone marrow suppression seen with chronic exposure to benzene. Phenolic compounds may be converted to reactive phenoxyl radicals by the one-electron oxidative process. Other peroxidases, such as lactoperoxidase and myeloperoxidase, may be involved in xenobiotic metabolism.

BIOCHEMICAL CONJUGATIONS

Mammals can synthesize xenobiotic conjugates that are more polar and readily excreted, compared with the parent compound. Conjugate synthesis is finely controlled through various feedback pathways. Two major reactants are required for conjugate synthesis: a xenobiotic with the appropriate functional group and a cosubstrate that can be conjugated with the xenobiotic. If the xenobiotic does not have a functional group amenable to conjugation, such as a hydroxyl group, it may be oxidized (functionalized) by cytochromes P450. The oxidized product and the cosubstrate must be simultaneously available for conjugation. Both functions must be tightly integrated for rapid excretion of the xenobiotic. Although the forthcoming sections will discuss each conjugating system as a separate entity, it must be emphasized that in vivo metabolism is integrated. Examples showing the integration of the conjugating systems with related pathways will be described.

Glucuronidation: Uridine Diphosphoglucuronosyltransferases

P450s are the principal phase I oxidative enzymes. Similarly, uridine diphospho(UDP)glucuronosyltransferases (also known as UDP-glycosyltransferases) are the principal phase II enzymes. Glucuronosyltranferases can use monooxygenase products to form glucuronides; however, it is not a necessity for substrates of the glucuronosyltransferase to be monooxygenase products. Significant numbers of xenobiotics and certain endobiotics possess the necessary functional groups for glucuronidation and do not require functionalization. These enzymes belong to a superfamily of related genes, and over 30 glucuronosyltransferases have been purified or cloned and expressed. Whereas the multienzyme complex of the P450 monooxygenase is termed a *system* because the enzymes are closely linked, the multiple enzymes of glucuronidation are not linked, but are interdependent. The general reaction mechanism of the conjugating enzymes involves the activation of an endogenous molecule. Subsequent reaction of this activated form of the endogenous molecule with the xenobiotic produces the conjugate. Activation may occur in a different cellular compartment than conjugation, as is the case with glucuronidation. Activation of glucose occurs in the cytosol, whereas conjugation occurs in the lumen of the endoplasmic reticulum. UDP-glucuronosyltransferases occur in several tissues, but their highest activity is found in the liver. Other tissues containing these enzymes include kidney, intestine, lung, skin, adrenals, and spleen. Extrahepatic glucuronosyltransferases may be differentially expressed and demonstrate different isoforms compared to the liver (123, 165).

Although the products of P450 are more water soluble than their parent compounds, some still possess considerable lipophilicity. Subsequent conjugation produces metabolites with higher water solubility. These metabolites can generally be readily excreted in the bile or urine. Transport proteins can recognize the glucuronic acid moiety of the glucuronide and aid in excretion from the liver and kidney. An additional method by which glucuronidation produces less toxic metabolites is via the addition of a bulky moiety to the xenobiotic. This can result in both the shielding of reactive portions of the xenobiotic and in the blocking of reactions between the xenobiotic and the site responsible for the toxicological sequelae. In some cases, the product of glucuronidation has more biological activity than the parent compound, and conjugation can be considered metabolic activation, although examples are far fewer than with P450 oxidation.

Glucuronides are either secreted by the liver into the bile, and consequently found in the feces, or by the kidney into the urine. The excretion route is generally dependent upon the molecular weight of the xenobiotic. The rat excretes glucuronides of xenobiotics with molecular weights greater than about 250–300 in the bile and those with lower molecular weights in the urine. Higher molecular weight xenobiotics, such as morphine, chloramphenicol, and endogenous steroids, are excreted in bile and enter the intestine. Bililary excretion can result in enterohepatic circulation, which can cause prolonged plasma half-lives for some compounds. The intestine contains β-glucuronidase, from the intestinal microflora, that catalyzes the hydrolysis of glucuronides. This releases the xenobiotic in the intestine, where it can be absorbed into the blood. The xenobiotic can then be taken up by the liver, where it is reconjugated and excreted into the bile, where the cycle is again initiated. This can cause prolonged exposure to target organs, such as the liver, and result in unanticipated toxicity.

History of the discovery of glucuronidation and the glucuronosyltransferases is interesting, and the reader is directed to Williams' classic book on detoxication mechanisms (189) and the comprehensive discussions of this enzyme (40, 170).

Nomenclature for UDP-Glucuronosyltransferase Gene Superfamily

Nomenclature for the UDP-glucuronosyltransferases has progressed similar to that for the P450 superfamily. It has been proposed that each gene be identified by the root symbol UGT for *U*DP-glucuronosyltransferase.

D—Glucose → ∝—D—Glucose—1—phosphate

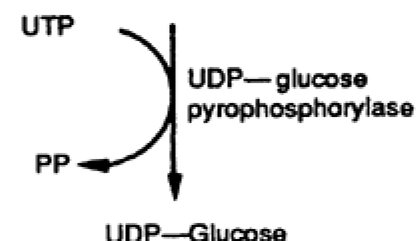

FIG. 3.8. Glucuronidation of phenol. An example of the pathway leading to production of glucuronic acid conjugates.

The gene family is identified by a number and a letter is added to designate the subfamily (UGT2B) followed by a number to identify the gene (UGT2B1). This system, as with the P450 nomenclature, is an attempt to provide isoforms with a name that is not only specific but reflects the evolutionary divergence of the genes. As new discoveries are being made, the nomenclature for these enzymes is evolving.

Biochemistry of Glucuronidation

Glucuronidation (illustrated in Figure 3.8) requires the availability of three reactants:

1. UDP-α-D-glucuronic acid (UDPGA), generated in the cytoplasm.
2. UDP-glucuronosyltransferase (UDPGT, EC 2.4.1.17), bound to the endoplasmic reticulum.
3. Substrate with the requisite functional group and some hydrophobic character.

Maximal enzyme activity is dependent on optimal concentrations of these reactants at the membrane site of catalysis.

As seen in Figure 3.8, D-glucose is the original precursor of UDPGA. During anabolic metabolism, D-glucose is converted to α-D-glucose-1-phosphate. This compound serves as substrate for UDP-glucose pyrophosphorylase (EC 2.7.7.9), which catalyzes its reaction with uridine triphosphate to yield the high-energy phosphate containing UDP-D-glucose and pyrophosphate. UDP-D-glucose then reacts with nicotine adenine dinucleotide (NAD) catalyzed by UDP-glucose

dehydrogenase (EC 1.1.1.22) to yield UDP-D-glucuronic acid, which completes glucose activation. This compound is termed the *glycone,* indicating its source. The xenobiotic that is conjugated is termed the *aglycone*. Glucose activation occurs within the cytoplasm, whereas glucuronidation of the aglycone occurs at the endoplasmic reticulum. Because UDP-D-glucose is also used in glycogen synthesis, it generally is available in the cell. This is not true for all conjugation reactions, and may be one of the reasons that glucuronidation is a major conjugation pathway.

The topology of UDP-glucuronosyltransferases is important for understanding substrate specificity and the need to disrupt microsomes with detergents or other means before assaying these enzymes in vitro. It is believed that large interlaboratory variation of in vitro glucuronidation data comes from variation in detergent-released latency. UDP-glucuronosyltransferases are oriented in the endoplasmic reticulum in such a way that the majority of the protein protrudes into the lumen of the endoplasmic reticulum. The intraluminal portion of the protein possesses the UDP-glucuronic acid-binding domain as well as the xenobiotic or endobiotic (endogenous substrates) binding domain. This means that UDP-glucuronic acid must pass through the membrane, possibly by carrier mediation, and that the substrate must also pass through the membrane (63). Molecular biology studies indicate that the C-terminal half of the protein is highly conserved among different UDP-glucuronosyltransferases, whereas the N-terminal region is highly variable. The C-terminal half of the protein contains the transmembrane sequences that anchor the enzyme within the membrane and the short portion of the C terminus that protrudes from the outside surface of the endoplasmic reticulum into the cytoplasm. The C-terminal half of the enzyme may contain a UDPglucuronic acid binding site. The broad substrate specificity is believed to come from variation in the primary sequence of the N-terminal region where the substrate-binding domain resides (110).

UDP-glucuronic acid and the aglycone (xenobiotic or endobiotic) must be present for the conjugation reaction to be initiated. The number of xenobiotics that have been shown to be substrates for UDPGTs is large and continues to grow (113). The major functional groups forming glucuronides are (a) hydroxyl, (b) carboxyl, (c) amino, and (d) sulfhydryl. The substituents to which these functional groups are attached can be quite variable (see Table 3.5). Similar to the substrate requirements for monooxygenases of the endoplasmic reticulum, the aglycone must be somewhat lipid soluble to be a substrate for the UDPGTs. This requirement reflects the need for the xenobiotic to penetrate the endoplasmic reticulum to gain access to the active site. All of the endobiotics associated with normal metabolism and homeostasis that are substrates for the UDPGTs are lipid soluble and include bilirubin, catechols, such as 3-O-methyladrenaline, serotonin, and 17-hydroxy-containing steroids.

As noted earlier, UDP-glucuronosyltransferases constitute a multigene family, divided into subfamilies, consisting of a number of genes, all but one of which encode for an individual UDPGT isozyme. Evidence accumulated from differential induction and developmental studies, enzyme purification, immunochemical analysis, and the cloning of UDPGT cDNAs suggests the existence of at least 12 different UDPGT isozymes in the rat. Rat isozymes are divided into two gene families. The UGT1 family contains isozymes that appear related to a single gene, and each member of the UGT2 family is related to a unique gene. Some of the UGTI family are inducible by 3-methylcholanthrene and others are inducible by phenobarbital. Two members of the UGT2 family are inducible by phenobarbital.

A similar approach has been applied to human UDPGTs, and the heterogeneity of the enzyme in humans is now recognized. Compared to the rat, the characterization of the isozymes in humans is less complete. The expression of human liver UDPGT cDNAs in tissue culture is providing useful information on the substrate specificity of individual isozymes. Site-directed mutagenesis is proving useful to determine which amino acid sequences are modifying substrate specificity among these isozymes. Human isozymes are also divided into UGT1, which contains at least six isozymes from a single gene, similar to the rat. The human UGT2B family contains at least six isozymes from unique genes.

Reactions Catalyzed by the UDP-glucuronosyltransferases

As with many of the enzymes of detoxication, the glucuronosyltransferases have a low order of substrate specificity. This lack of substrate specificity makes them ideally suitable as detoxication enzymes. Whether or not they evolved as detoxication enzymes or represent enzymes of normal metabolism whose lack of specificity make them suitable for detoxication is open to debate. Of interest in this respect is that they occur only in higher organisms. Glucuronosyltransferases have been found in all mammals, birds, and reptiles that have been investigated, although their specific activities toward specific substrates may vary among different species and strains. Unlike the monooxygenase, they have not been found in bacteria and less developed species. This fact, among others, lends support to Dutton's hypothesis that these transferases evolved to metabolize endogenous compounds, such as bilirubin, catecholamines, and steroids, and not as detoxication enzymes (39).

Table 3.5 Functional groups forming glucuronides

Functional group	Compound class	Example
Hydroxyl → *O*-glucuronide	Alcohols	
—C—OH → —C—O—Glucuronic acid	Aliphatic	Trichlorethanol
	Alicyclic	Hexobarbital
	Benzylic	Methylphenylcarbinol
N—OH → N—O—Glucuronic acid	Phenolic	Phenol
	Enols	4-Hydroxycoumarin
	Hydroxyamines	*N*-hydroxy-2-acetylaminofluorene
Carboxyl → *O*-glucuronide	Carboxylic acids	
	Aliphatic	2-Ethylhexanoic acid
C(=O)—OH → C(=O)—O—Glucuronic acid		
	Aromatic	Benzoic acid
Amine → *N*-glucuronide	Arylalkyl	Phenylacetic acid
N-glucuronic acid	Heterocyclic	Nicotinic acid
—O—C(=O)—N(H)-glucuronic acid		
(R_3)—N^+—glucuronic acid		
	Aromatic	Aniline
R—SO_2—N(H)—glucuronic acid	Carbamate	Meprobamate
	Aliphatic tertiary	Tripelennamine cotinine
	Amine	
	Sulfonamide	Sulfadimethoxine
Sulfhydryl→S-glucuronide		
O—*S*—glucuronic acid	Heterocyclic	Sulfisoxazole
C—*S*—glucuronic acid	Arylthiol	Thiophenol
Carbon→C—glucuronide		
C—glucuronic acid		
	Dithiocarbamic acid	
	1,3-Dicarbonyl system	Phenylbutazone

Modified from Reference 81.

Table 3.5 illustrates the functional groups, generally nucleophilic heteroatoms, that form glucuronides, and examples of the reactions. The glucuronides formed from these functional groups have different properties. Stability is among the most important with respect to detoxication. Breakdown of the glucuronide can lead to reformation of the parent compound, and in certain cases the production of highly reactive electrophilic species. These reactive species may be responsible for the production of acute and chronic toxicity by covalent binding to nucleophilic sites on tissue macromolecules.

Among the most commonly encountered glucuronides are those involving linkage of glucuronic acid and

the xenobiotic through an oxygen atom. These Oglucuronides may form with a number of chemical classes including, aryl, alkyl, and acyl compounds, as illustrated in Table 3.5.
The alkyl-O-glucuronides are ether-linked glucuronides that can form from a variety of primary, secondary, and tertiary alcohols. Although generally stable at physiological conditions, they can be hydrolyzed under acidic conditions.
The enolic glucuronides are formed from aglycones without a free hydroxyl group. Glucuronides are formed from the enolized keto group. These conjugates lack the stability of the ether glucuronides and are susceptible

to both acid and alkaline hydrolysis. They are more stable at neutral and alkaline pH than in acid conditions. Ester glucuronides can be produced from a variety of carboxylic acids, including primary, secondary and tertiary aliphatic acids and both aryl and heterocyclic compounds. They generally are stable in acidic conditions but are susceptible to alkaline hydrolysis.

The chemical properties of *N*-glucuronides are different from those of *O*-glucuronides. One of the most important of these is their lack of stability. They are especially unstable at pH below neutrality. The instability of these compounds may have important biological consequences; examples are discussed in more detail later. Quanternary ammonium N-glucuronides are formed by *N*-glucuronidation of cyclic and acyclic tertiary amines. These charged metabolites can be major metabolites of certain xenobiotics in higher primates while not being found in other animal models, such as the rat.

The *S*-glucuronides are not as commonly encountered as the *O*-glucuronides, but they represent important detoxication pathways for thiolic compounds. Their stability is similar to that of the Oglucuronides.

The *C*-glucuronides represent recently recognized conjugates, and only a few examples are known, such as phenylbutazone. Generally, they appear to be formed by the transferase, but other possible mechanisms of formation have been suggested.

Role of UDP-glucuronosyltransferases in Detoxication and Metabolic Activation

The foregoing discussion indicates that the UDP-glucuronosyltransferases play a critical role in the metabolism and detoxication of xenobiotics. Some substrates require functionalization by the monooxygenase before metabolism by the transferase, whereas others can be directly conjugated. The conjugates are more water soluble than the parent xenobiotic, and some readily form salts. Addition of the glycone may enable some of the conjugates to be more readily excreted through carrier-mediated mechanisms. Mechanisms other than increased excretion rates may also be important. The addition of the relatively bulky glycone may hide or hinder the biological reactivity of particular functional groups on the xenobiotic. In addition, binding of the toxicant to particular receptors responsible for toxicity may be blocked. Overall, these mechanisms represent an efficient system for detoxication. On the other hand, glucuronidation of certain compounds represents a metabolic activation where the product is more toxic than the parent compound.

Aromatic amines are among the most studied examples of the role glucuronidation plays in metabolic activation of carcinogens. These glucuronides transport the proximate carcinogen to the target site, where it decomposes to the species that react with cellular macromolecules producing the biochemical lesion responsible for generating the pathological lesion.

Several of the arylamines are potent bladder carcinogens, including 4-aminobiphenyl, 1-naphthylamine, and benzidine. Metabolic activation of these carcinogens to the ultimate carcinogen appears similar and requires the action of UDP-glucuronosyltransferase. Metabolic activation begins with P450-dependent activation of the arylamine to the proximate carcinogen, an *N*-hydroxyarylamine. Other specific ring hydroxylated forms may be produced and may represent more stable products. The unstable *N*-hydroxyarylamines are then converted to stable N-glucuronides. These *N*-glucuro-nides are transported to the bladder. In the bladder, the *N*-glucuronides are subject to *β*-glucuronidase activity, which splits off the glycone. They are also subject to hydrolysis in acidic urine producing the *N*-hydro-xyarylamine. The *N*-hydroxyarylamine spontaneously converts to the electrophilic arylnitrenium ion as illustrated in Figure 3.9.

The electrophilic arylnitrenium ion can then react with nucleophilic centers on macromolecules of the bladder epithelium, especially DNA, to initiate tumor formation. The concentration of the glucuronide in the bladder, in combination with the time the glucuronide remains in

FIG. 3.9. Metabolic activation of aromatic amines via glucuronidation.

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the bladder, can modify the potential for tumor formation. Glucuronides may function in this manner with a number of carcinogens and be important in explaining why certain target organs are susceptible to a specific carcinogen and others are not susceptible. In the above example, glucuronidation may protect the liver but make the bladder, the target organ, susceptible.

Glucuronidation has also been implicated in adverse drug reactions of certain carboxylic drugs, which resulted in a toxic immunological response. It is believed that a reactive glucuronide covalently binds to cellular proteins that act as haptens, producing an anaphylactic reaction.

Stable expression of cloned UDP-glucuronosyltransferases in tissue culture cell lines is providing a powerful new tool for understanding their role in the biotransformation of xenobiotics (21).

Species, Gender, and Genetic Differences in UDP-glucuronosyltransferase Activity

Studies of species, strain, and gender differences in glucuronidation are complicated by a number of factors. Activity may be affected by age, hormonal status, environmental exposure to xenobiotics in the diet and other sources, and by nutrition status. Factors associated with the methodology to determine differences in glucuronidation also play a role, including substrate, assay method, method of freeing latent activity, and the method of isolating the preparation employed to measure activity. This has led to a number of reports of differences in activity that could be artifactual. However, the large number of reports concerning differences in glucuronidation among species, strains, and the sexes indicate that certain of these differences are real and may have a genetic basis.

As mentioned previously, lower animals, including prokaryotes and invertebrates, do not produce glucuronides. Fish and reptiles do demonstrate glucuronidation of xenobiotics, but vary dramatically in activity, which is generally 10-fold or more lower than mammalian activity. Birds have glucuronidation ability similar to that of mammals.

Differences among mammalian species in their ability to glucuronidate a xenobiotic may be quite large; however, as mentioned, in some cases this could be artifactual. The guinea pig generally has higher activity than most other laboratory species. This higher activity may be associated with less latent enzyme activity, as its UDPG-glucuronosyltransferases can be activated by much gentler methods than other species. Cats are well known for their extremely low transferase activity. Although capable of forming glucuronides with endogenous compounds, they form only low levels or no glucuronides with xenobiotics.

Glucuronidation of amines is divided into two groups: nonquaternary N-conjugates and quaternary N-conjugates. There does not appear to be major species differences with the nonquaternary N-conjugate group, sulfonamides, arylamines, and cyclic and heterocyclic amines, although quantitative differences do exist. Quaternary glucuronidation occurs in primates, including humans, but not in other species. In humans, quaternary ammonium-linked glucuronides of aliphatic amines appear to be produced by UGT1A3 and UGT1A4 (28, 61).

A well-known example of a strain difference is the almost complete lack of bilirubin glucuronidation in the Gunn rat. This rat strain also has low activity toward a number of xenobiotic substrates, but normal activity toward others. There is a genetic component to this, with the low activity being autosomally recessive. The mutation in the Gunn rat responsible for its lack of bilirubin conjugation occurs in the UGT1 family and affects this entire group of isozymes. A frameshift mutation occurs because of a deleted guanine that results in a TGA stop codon occurring sooner than normal. This mutation results in a protein missing 115 amino acids that constitute a hydrophobic region associated with insertion of the protein into the membrane. Lack of insertion negates the activity of this enzyme form and results in degradation of the incomplete protein. The genes in the UGT2 family are normally expressed in the Gunn rat.

Similar defects occur in humans and are known as *unconjugated hyperbilirubinemias*. Gilbert's syndrome is a milder form of the disease that occurs in 2%–5% of the population. This large prevalence in the population makes it an important human genetic deficiency when considering interindividual variation in xenobiotic metabolism. These patients are characterized by mild, chronic, unconjugated hyperbilirubinemia that produces jaundice and an impaired ability to metabolize menthol. Decreased clearance of several drugs, including tolbutamide, rifamycin, josamycin, and paracetamol, has been observed. Crigler-Najjar syndrome is a familial form of severe unconjugated hyperbilirubinemia. Infants often developed severe neurological damage from bilirubin encephalopathy (kernicterus). Patients are divided into two types. Type I is more severe (unconjugated bilirubin >20 mg/dl) and not responsive to barbiturate or glutethimide therapy. Type II patients respond to induction by phenobarbital, which suggests a fundamental difference from type I in the molecular basis of the genetic defect. Type I results from mutations in the UGT1 family that produces a loss of bilirubin conjugation (29), whereas

less severe mutations occur in type II that produce a decrease, but not a loss, of activity.
Gender differences appear hormonally related (166) and can be substrate dependent. Although it is sometimes stated that males have higher glucuronidation activity

than females, this is substrate dependent, and no general classification should be made. Like monooxygenase activity, activity may be sensitive to imprinting or programming during the neonatal period. As with species and strain differences, care must be taken when extrapolating data obtained with one substrate to other substrates.

Glucuronidation of estradiol and estrone is higher in female rats than male rats (193). Paracetamol, oxazepam, and diflunisal are cleared 30%-50% faster in males, due primarily to enhanced glucuronidation.

Induction of the Glucuronosyltransferases

UDP-glucuronosyltransferases are inducible enzymes, much like cytochrome P450. They are inducible by some of the same inducers. Evidence of a true induction process involving *de novo* protein synthesis and increases in mRNA has been observed for induction of the UDP-glucuronosyltransferases by phenobarbital. Most inducers of CYP1A, CYP2B, CYP3A, and CYP4A can induce these transferases. Rat UGT1A6 and UGT1A7 and human UGT1A6 and UGT1A9 are polycyclic hydrocarbon-inducible transferases. Induction appears mediated by the Ah receptor. Rat UGT1A7 and human UGT1A9 have high activity toward the phenolic and diphenolic metabolites of polycyclics, such as benzo(a)pyrene (11). Few specific inducers of the transferases that do not also induce the monooxygenase are known. For example, trans-stilbene oxide and ethoxyquin appear to only induce the transferases, but more studies are needed to determine if this is a true induction. Induction of the transferases modifies the toxicity of xenobiotics in a manner similar to induction of P450, as previously discussed.

Sulfation: Sulfotransferases

As early as 1815, it was recognized that mammals excrete organic sulfates in their urine (68). The discovery that these organic sulfates were esters produced in the mammalian body by conjugation of endogenous organic compounds with inorganic sulfate came about 50 years later (16). The sulfate conjugates were believed to be ethers formed between the organic aryl group and the inorganic sulfate group. Therefore, they called the urinary fraction containing these metabolites *ethereal sulfates;* however, these metabolites are actually esters of sulfuric acid, and the term ethereal sulfate is only of historical significance (121).

Sulfation, or more appropriately, sulfonation, of xenobiotics and endobiotics is catalyzed by a set of enzymes called *sulfotransferases* (EC 2.8.2). These enzymes belong to a multigene family and occur in prokaryotes, plants, and animals. Some of the enzymes are membrane bound and others occur in the cytosol. The membrane-bound sulfotransferases are found in the Golgi membranes and are involved in the sulfation of endogenous compounds, such as glycosaminoglycans, glycoproteins, and proteins, and peptides secreted by the Golgi apparatus; they are not involved in xenobiotic metabolism. The soluble sulfotransferases can also sulfate endogenous compounds, such as steroids and thyroid hormones, and are involved in the metabolism of xenobiotics. For the most part, sulfation of xenobiotics results in metabolites that are less toxic than the parent compound; however, the sulfotransferases, like many xenobiotic metabolism enzymes, can produce metabolically activated products that have mutagenic and carcinogenic potential.

Until recently, the sulfotransferases have not been as intensely investigated as some of the other xenobiotic metabolism enzymes. Currently there is renewed interest in these enzymes based, in part, on attempts to understand their role in metabolic activation. Utilization of the tools of molecular biology has provided new insight into their roles in metabolism and has revealed the complexity of their gene family (38, 139). The ability to sequence the sulfotransferases and identify new isoforms of these enzymes has progressed faster than our understanding of their individual roles in xenobiotic metabolism. The availability of expression systems that express single and multiple sulfotransferases is providing toxicologists a powerful tool to investigate their roles in xenobiotic metabolism.

Biochemistry of Sulfation

A limiting factor in the sulfation of xenobiotics by the sulfotransferases is the availability of 3-phosphoadenosine 5′-phosphosulfate (PAPS) (reviewed in (85)). As illustrated in Figure 3.10, PAPS is synthesized in a two-step process. The first step is formation of adenosine 5′-phosphosulfate (APS) catalyzed by ATP-sulfurylase. Although the synthesis of APS from sulfate and ATP is not energetically favored, the rapid hydrolysis of pyrophosphate and the rapid utilization of APS as a substrate for APS-kinase drives the reaction toward APS synthesis. APS-kinase catalyzes synthesis of PAPS from APS and ATP. This enzyme is tightly coupled with the ATP-sulfurylase, which results in the rapid utilization of APS.

Tissue concentration of PAPS is relatively low (4–80 ng/g tissue), compared to UDPGA, the active form of glucuronic acid used in glucuronidation (200 nmol/g liver). During active sulfation, PAPS becomes

rapidly depleted. For example, the sulfotransferase has a high affinity of acetaminophen, which forms a sulfate con

ATP + $SO_4^{=}$ → (ATP—sulfurylase) Adenosine 5'—phosphosulfate (APS) + Pyrophosphate (PPi)

APS + ATP → (APS—kinase) 3'—Phosphoadenosine 5'—phosphosulfate (PAPS) + ADP

2ATP + SO_4^{2-} → ADP + PAPS + PPi

Overall Stoichiometry

FIG. 3.10. Reactions catalyzing the formation of PAPS from inorganic sulfate and ATP.

jugate. (See Figure 3.6). At low doses, rats excrete the sulfated acetaminophen as a major urinary metabolite. As the dose of acetaminophen is increased, the sulfate metabolite does not increase, whereas the glucuronide of acetaminophen increases dramatically; this is believed to be due to the limited availability of PAPS. The limitation in the synthesis of PAPS is sulfate. The major sources of sulfate include diet and degradation of sulfur amino acids (methionine and cysteine). These sources are inadequate to maintain sulfate concentrations for PAPS synthesis during rapid sulfotransferase activity. In the mouse, sulfation appears more limited by sulfotransferase activity than by PAPS and sulfate.

Reactions Catalyzed by Sulfotransferases

As mentioned, sulfotransferases esterify a variety of endogenous substrates, including steroids, carbohydrates, and proteins. Sulfation also plays a role in the disposition of hormones. Sulfation directs lipophilic compounds, such as the steroidal hormones, to more polar environments, including the active sites of enzymes and to body fluids. For example, sulfation enhances the elimination of steroids from the adrenal gland (116). Sulfation also facilitates deiodination of thyroid hormone and is a rate-limiting step in one of the elimination pathways of thyroid hormone (179).

Xenobiotic conjugation with sulfate (Figure 3.11) is an important route for conversion of lipophilic xenobiotics to more readily excreted polar metabolites (Table 3.6) (79, 151). Sulfation of xenobiotics with an aliphatic or aromatic hydroxyl group readily occurs. For example, phenol is excreted as its sulfate conjugate (Figure 3.12). Often it is necessary for phase I metabolism to functionalize a xenobiotic with a hydroxyl group before it can be sulfated. For example, toluene is oxidized to benzyl alcohol before conjugation with sulfate (Figure 3.12).

Role of Sulfotransferases in Detoxication and Metabolic Activation

Alcohols, phenols, aliphatic and aromatic amines, and aromatic hydroxyamines and hydroxyamides can be

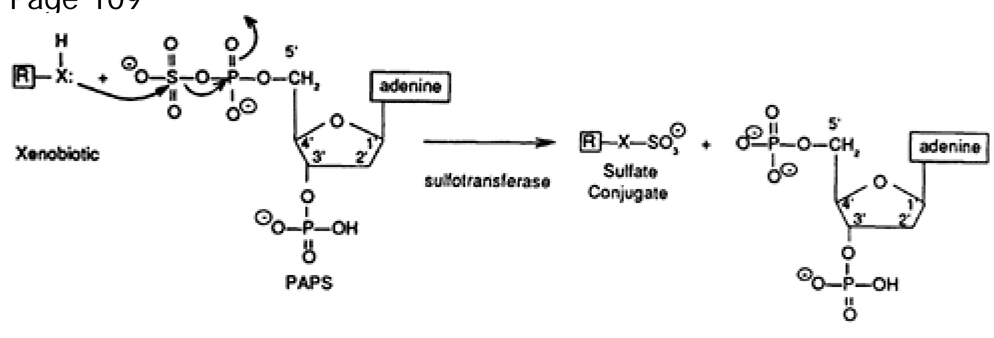
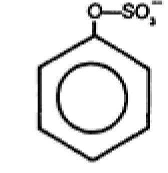


FIG. 3.11. Synthesis of a sulfate conjugate from a model xenobiotic by sulfotransferase.

Table 3.6 Sulfotransferases involved in the metabolism of xenobiotics

EC No.	Name	Example substrates
2.8.2.1	Arylsulfotransferase	2-naphthol, phenol, substituted phenols, serotonin, acetaminophen
2.8.2.2	Alcohol sulfotransferase (also called hydrosteroid sulfotransferase)	Primary and secondary aliphatic alcohols, nonaromatic hydroxysteroids
2.8.2.4	Estrone sulfotransferase	Estrone and other aromatic hydroxysteroids
2.8.2.9	Tyrosine ester sulfotransferase	Tyrosine methyl ester, 2-cyanoethyl-Nhydroxythioacetamide
2.8.2.14	Bile salt sufotransferase	Conjugated and unconjugated bile acids



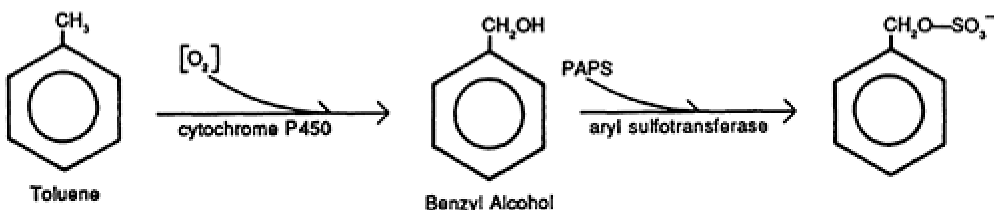


FIG. 3.12. Sulfotransferase-catalyzed sulfation of phenol and toluene.

sulfated. These same groups can form glucuronides. At low doses, sulfation may play an important role in detoxication of xenobiotics; however, as acetaminophen demonstrates, at high doses glucuronidation becomes more important because of sulfate limitations. Secondary effects may be produced by sulfation lowering sulfate availability for the sulfation of endogenous substrates. Sulfotransferases can be involved in the conversion of pro-drugs to their active forms. For instance, minoxidil is sulfoconjugated to its active form, which is more active as an antihypertensive and hair-growth stimulate than the parent drug.

Sulfotransferases can be involved in the metabolic activation of a number of mutagens and carcinogens. One of the best known examples is the metabolic activation of the carcinogen 2-acetylaminofluorene (illustrated in Figure 3.13). *N*-hydroxylation of the amide nitrogen by monooxygenases is followed by sulfation of the *N*-hydroxy group. The sulfate ester is unstable and decomposes to an electrophilic nitrenium ion-carbonium resonance ion that can form covalent adducts at nucleophilic sites on macromolecules. Support for the hypothesis that the sulfate conjugate of 2-AAF is the reactive metabolite comes from studies indicating factors that modulate sulfotransferase activity also modulate 2-AAF carcinogenicity. Male rats have higher sulfotransferase activity and develop more 2-AAF-induced tumors than females. Reduction of sulfotransferase activity in male rats by castration, hypophysectomy, thyroidectomy, or steroid hormones

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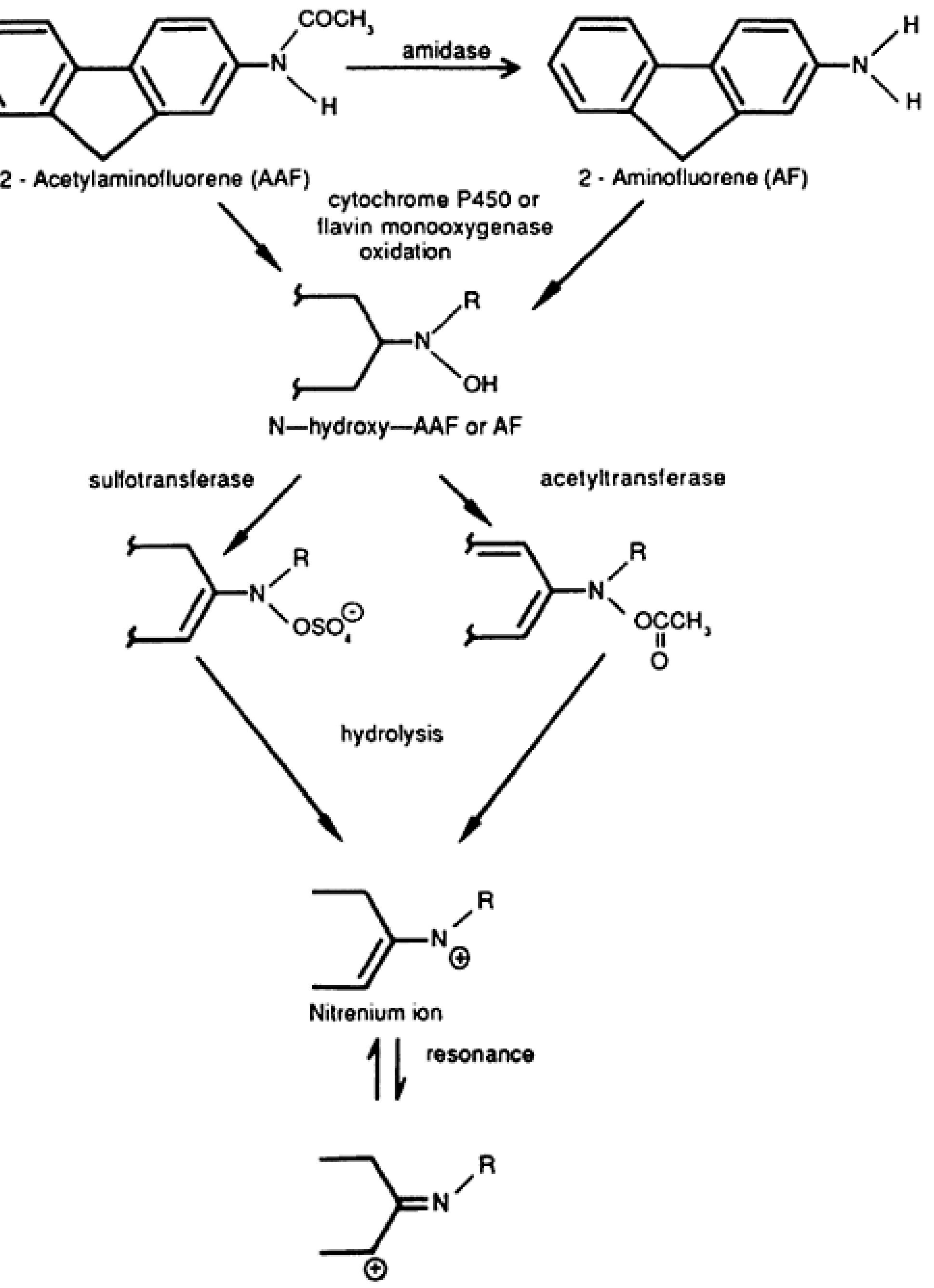

FIG. 3.13. Metabolic activation of 2-acetylaminofluorene to a reactive metabolite capable of covalent modification of macromolecules.

reduces 2-AAF covalent adducts. These results are consistent with the hypothesis that sulfation of 2-AAF is required for covalent modification of DNA. This mechanism is at least partially responsible for the activation of several other xenobiotics, including aromatic amines, mono- and dinitrotoluene, *N*-hydroxyphenacetin, I′-hy-droxysafrole, *N*3-hydroxyxanthine, and other N-hydroxyarylamides (122). Secondary nitroalkanes, such as 2-nitrobutane and 3-nitropentane, can be metabolically activated by aryl sulfotransferase to mutagens and hepatocarcinogens. Primary nitroalkanes, such as 1-butane and 1-nitropentane, are not activated by aryl sulfotransferase (46).

Sulfotransferases can metabolically activate certain products of CYP1A1 metabolism of polycyclic hydrocarbons. For example, 9-hydroxymethylbenzo(a)pyrene (*see* Figure 3.4) can be sulfated to yield a highly reactive sulfate ester that is heterolytically cleaved to produce an electrophilic cation that damages DNA, RNA, and protein. In addition, 6-hydroxymethylbenzo(a)pyrene can be activated to the carcinogenic 6-sulfooxymethylbenzo(a)pyrene by rat and mouse sulfotransferase (47). Other examples include 5-hydroxymethylchrysene and 7,12-dihydroxymethyl benz(a)anthracene (184). The potential for sulfotransferases to metabolically activate xenobiotics has resulted in the development of new assay systems for genotoxicity. For instance, Glatt's laboratory (55, 56) has developed *Salmonella* strains that express rat and human sulfotransferase activity for use in Ames assays. They have also developed Chinese hamster V79-derived cells that express rat sulfotransferase activity.

Sulfotransferase Isoforms, Genetics, and Species Differences

Sulfotransferases belong to a multigene family that produces a number of distinct enzymes that have different, but overlapping, substrate specificities. Some of these enzymes demonstrate species and even tissue specificity in their expression. It is expected that more detailed analysis will identify additional sulfotransferases.

The nomenclature used to describe these enzymes is still evolving, and there is no universal naming system, as yet. Weinshilboum et al. (186) have divided 30 of the known cDNA sequences into three families that have at least a 45% amino acid sequence identity, then into subfamilies with at least 60% homology. Two of the families are expressed in animals and one in plants. The two animal families represent phenol-sulfotransferases and hydroxysteroid sulfotransferases, and the phenol-sulfotransferase are divided into the phenol group and estrogen group. The phenol group contains three human genes, two mouse genes, and one rat gene. The estrogen group contains one human gene, two rat genes, and one mouse gene. The hydroxysteroid family contains one human gene, three rat genes, and three mouse genes. Undoubtedly, more genes will be discovered and the classification system refined; however, this classification demonstrates the diversity of genes in the sulfotransferase family.

Amino acid sequence data indicate that four regions of sulfotransferases have been highly conserved among species. One region, near the carboxy terminus of the proteins, is believed to be the PAPS binding site. A critical lysine may be involved in stabilization of an intermediate form during catalysis.

Humans demonstrate sulfotransferase genetic polymorphisms, which help explain some of the differences between individuals in response to specific xenobiotics. Because sulfotransferases do not appear to be as sensitive to xenobiotic induction of their activity, exposure to xenobiotics may not be as important as with some of the other xenobiotic metabolizing enzymes in producing individual variations in metabolism.

Sulfation occurs in most species, including mammals, birds, reptiles, amphibians, fish, and invertebrates. The most notable exception to this is the low sulfotransferase activity in the pig. Members of the cat family are deficient in glucuronyltransferase activity but have high sulfotransferase activity. This balance of glucuronyltransferase and sulfotransferase must always be kept in mind when evaluating the activity of either enzyme system. A deficiency in one pathway can shift metabolism, as similar functional groups are conjugated by the two enzyme systems. In addition, sulfation appears to have high affinity but low capacity for phenols, whereas glucuronidation has low affinity and high capacity for these substrates.

A large part of our understanding of sulfotransferases comes from study of purified rat enzymes. There are at least six different phenol sulfotransferases and seven different forms of rat liver steroid/bile acid sulfotransferases. By contrast, only three distinct isozymes of cytosolic sulfotransferase have been isolated: two phenol-sulfotransferases and one bile acid sulfotransferase. The two phenol sulfotransferases have been referred to as the monoamine-sulfating form and the phenol-sulfating form. Dopamine, epinephrine, and levodopa are substrates for the monoamine form, and 4-nitrophenol, minoxidil, and acetaminophen are substrates for the phenol form (45).

There is wide species variation in sulfation of two model substrates: isoprenalin and harmol. For example, the activity toward isoprenalin in mouse liver is 10 times that in monkey liver. Sulfation of acetaminophen is limited by PAPS availability in rats. In mice, acetaminophen sulfation is limited by

lower sulfotransferase activity. Although mice have lower PAPS synthetic capability than

< previous page page_111 next page >

rats, lower sulfotransferase activity is the major limiting factor in mice (98). The activity of acetaminophen sulfotransferase and 17 α-ethinylestradiol sulfotransferase in hepatic preparations from monkeys, dogs, and humans were compared. Rhesus and cynomolgus monkeys and dogs had higher activity acetaminophen sulfotransferase than humans (152).

Four cytosolic sulfotransferases, distinguished by their substrate specificity, have been found in human tissues. Dehydroepiandrosterone or hydroxsteroid sulfotransferases appear to be involved in sulfation of steroids and may be important in regulating their activity and in bile acid sulfation and excretion. Estrogen sulfotransferase is involved in the inactivation of estrogens in target tissue. Other sulfotransferases can also sulfate estrogen, but with lower activity. This transferase does not appear to play an important role in xenobiotic metabolism.

The other two cytosolic sulfotransferases belong to the phenol sulfotransferase group and are involved in xenobiotic metabolism. Phenol-sulfotransferases (PST) are found in a number of tissues, and their discovery in blood platelets has provided an important source for their study. They are divided into two groups: P-PST and M-PST. P-PST sulfates phenols, such as p-nitrophenol and α-naphthol, and aromatic amines, such as 2-naphthylamine. M-PST sulfates monoamine neurotransmitters. Again, substrate specificity overlaps as demonstrated by acetaminophen, which can be sulfated by both sulfotransferases. As mentioned, nomenclature for the sulfotransferases is currently under development and can cause confusion. Some investigators use the term *aryl sulfotransferase* for a gene family that includes sulfotransferases found in rats, humans, and mice that have a 70% sequence homology and catalyze the sulfation of dopamine, p-nitrophenol, estradiol, and other phenols.

Factors Modifying Metabolism

Sulfotransferases are not induced by the classical inducers, phenobarbital and 3-methylcholanthrene, and these compounds may actually suppress their expression (146). Several inhibitors of sulfotransferase have been discovered and exploited experimentally to study these enzymes. Pentachlorophenol and 2,6-dichloro-4-nitrophenol are potent sulfotransferase inhibitors. Only 0.2 μM pentachlorophenol is required for 50% inhibition of 2-dichloro-4-nitrophenol sulfation by purified arylsulfotransferase (77). Pentachlorophenol and 2,6-dichloro-4-nitrophenol are effective inhibitors because the *ortho*- and *para*-aromatic ring positions are substituted with electron-withdrawing groups. This effect is consistent with the mechanism whereby the sulfotransferases facilitate electrophilic attack of the hydroxyl oxygen by the sulfur (Figure 3.11).

Gender Differences

There are major gender differences in the sulfate conjugation of steroid hormones. For example, female rats have fivefold higher activity for cortisol metabolism than do male rats (154). This gender difference in cortisol metabolism is apparently due more to suppression of sulfotransferase by male hormone levels than to stimulation by the ovaries (153, 155). Three steroid sulfotransferases have been isolated from rat liver, and it is the relative amounts of these isozymes that account for the large gender difference. Aryl sulfotransferase concentrations in the livers of male rats were higher than in females. In contrast, hydroxysteroid sulfotransferase concentration was higher in the liver of female rats compared to males (26).

Lower sulfotransferase activity observed in neonatal rats has been attributed to sexual immaturity because as gonads develop, sulfotransferase activity increases. Newborn infants, who characteristically exhibit pronounced immaturity in glucuronidation, have a fully developed phenol sulfotransferase activity. For example, newborns excrete acetaminophen as a sulfate conjugate, whereas adults primarily excrete it as a glucuronide conjugate. Chloramphenicol is extremely toxic in neonates because it is a poor substrate for sulfotransferase and is primarily cleared by glucuronidation in adults.

Glutathione S-transferases

A family of cytosolic enzymes known as *glutathione S-transferases* is capable of conjugating relatively hydrophobic electrophilic molecules with the nucleophile-reduced glutathione (Figure 3.14) (24). These enzymes are found in highest concentrations in the liver, kidney, intestines, and lung, but they occur in most tissues. Glutathione conjugates have higher molecular weights and are more water soluble and more likely to be excreted in urine and bile than are the parent compounds. Conjugation with glutathione makes it unlikely the xenobiotic will react with toxicological targets.

Glutathione *S*-transferases involved in xenobiotic metabolism are cytosolic proteins that catalyze the conjugation of glutathione with a substrate bearing an electrophilic atom (74). The transferases facilitate the nucleophilic attack of glutathione thiolate ion (GS−) on the electron-deficient atom of a relatively hydrophobic electrophilic xenobiotic. There is little specificity in the active site for the xenobiotic other than it must possess hydrophobic character. Substrates for the glutathione *S*-transferases can react with

glutathione nonenzymati

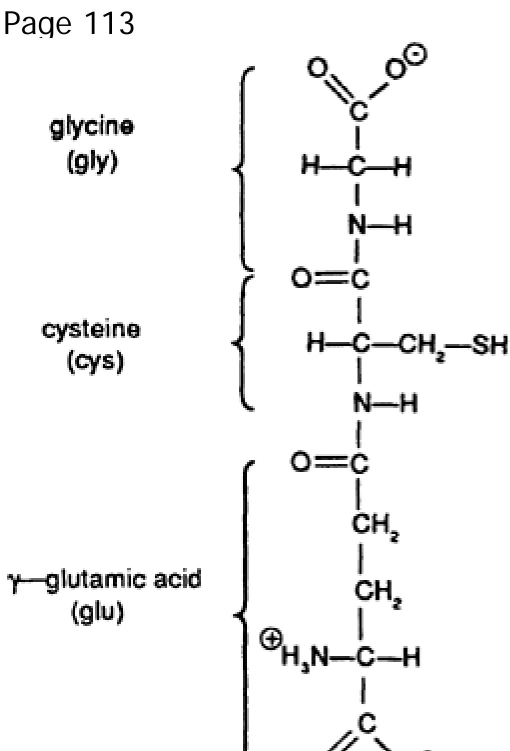

FIG. 3.14. Structure of reduced glutathione (MW 307).

cally but at slower rates than the enzyme-catalyzed reaction.

In addition to catalyzing the conjugation of xenobiotics with glutathione, glutathione *S*-transferases are capable of binding the chemical on the enzyme surface. This binding may or may not inhibit the catalytic activity of the enzyme, but it prevents the xenobiotic from interacting with other critical cellular sites, such as proteins and nucleic acids. A glutathione *S*-transferase possessing this property has been called *ligandin* (161, 162). Glutathione *S*-transferases can form covalent bonds between reactive xenobiotics and the enzyme's active site. Binding inactivates the enzyme but also inactivates the reactive xenobiotic and represents an additional detoxication mechanism (76). This process is called *suicide inactivation* and is seen with other detoxication enzymes, such as P450.

Because of these three activities of the glutathione *S*-transferases, the enzymes have been called "a triple threat in detoxification" by Jakoby and Keen (76). Glutathione and the glutathione S-transferases can detoxify a broad spectrum of compounds that the organism either may encounter in its environment or generate during normal cellular metabolism. Although not highly efficient in its reactions (operates at relatively high concentrations of the xenobiotic), glutathione S-transferases are capable of catalyzing or reacting with a number of reactive chemical functional groups. Any lack of efficiency is made up by the high cellular concentration of glutathione and glutathione *S*-transferase. Liver glutathione concentrations are high (10 mM) and glutathione S-transferases can represent as much as 10% of the total hepatocellular proteins (2); however, it is possible for glutathione conjugation to become capacity limited at high doses of xenobiotics. Glutathione utilization can be faster than its synthesis, resulting in decreased conjugation and increased toxicity.

Bromobenzene metabolism is an example of a compound whose glutathione conjugate protects the liver from the toxicity of its P450-generated epoxide. At doses that deplete the cytosolic store of glutathione, the epoxide produces severe hepatotoxicity. Another example is acetaminophen. It is a very safe drug, but at extremely high doses, where glutathione is depleted by reaction with its activated metabolite, *N*-acetyl-benzoquinoneimine (see Figure 3.6), it produces hepatic necrosis.

Glutathione *S*-transferase enzymes are dimers of two protein subunits. They may exist as homodimers, where each subunit is identical, or as heterodimers, where the subunits are expressed by different genes. These transferases are inducible by the classical P450 inducers phenobarbital and 3-methylcholanthrene. The region of the gene that controls the expression of some of these transferases contains the xenobiotic-responsive element (XRE) previously discussed for P450 induction. In the case of the transferases that are heterodimers, the expression of each subunit may be independently regulated. Different glutathione *S*-transferases have substrate preferences but not a high order of substrate

specificity. Substrate selectivity of these enzymes can be based on small changes in the primary structure of the enzymes. For instance, a glutathione *S*-transferase of the P-class contains a tyrosine at a site important for selectivity, whereas a transferase of the A-class contains a valine. When the tyrosine of the P-class enzyme is replaced with a valine, its substrate selectively is changed toward that of the A-class (125).

Biochemistry of Glutathione S-transferases

Although the urinary metabolites of glutathione conjugation, mercapturic acids, were first described in the 19th century (16), it was not until the 1950s that glutathione (Figure 3.14) was identified as the source of the cysteine in the mercapturic acid (189). Glutathione is synthesized in the cytosol of most cells by the gamma-glutamyl cycle, a series of six, tightly controlled, enzyme-catalyzed reactions (Figure 3.15). The three amino acids that comprise glutathione—cysteine, glycine, and glutamic acid—can enter the cycle from several biochemical pathways, although the cycle depicts them arising from glutathione. Some xenobiotics contain sufficiently electrophilic groups to react directly with glutathione, whereas others must first undergo phase I metabolism. Most xenobiotics react with glutathione through the catalytic activity of

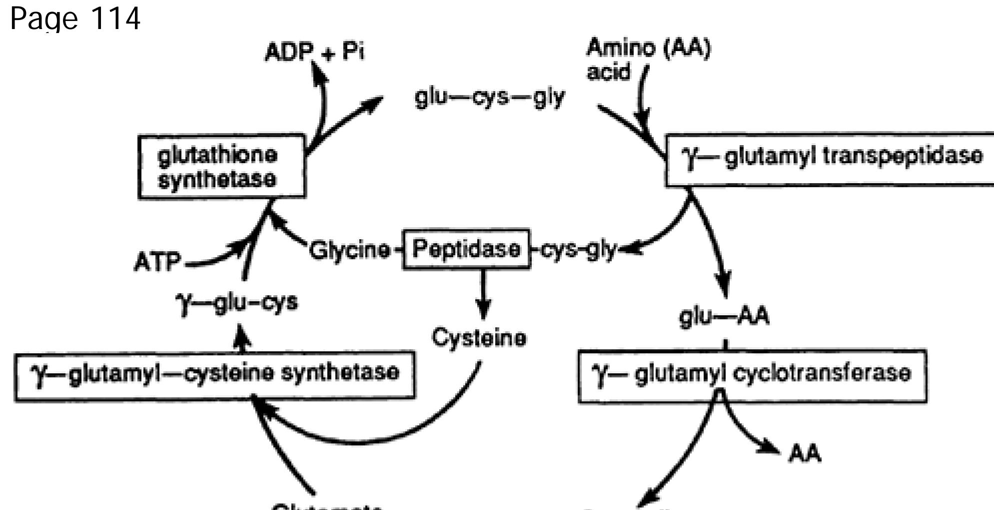

FIG. 3.15. The γ-glutamyl cycle responsible for the biosynthesis of reduced glutathione.

the glutathione S-transferases. Glutathione *S*-transferase reactions fall into four broad categories, as depicted in Figure 3.16: reaction with (1) electrophilic carbon, (2) nitrogen, (3) sulfur, and (4) oxygen (50, 65, 75).

Reaction with Electrophilic Carbon

The reactions of glutathione with electrophilic carbon can be divided into three types, as shown in Figure 3.16: (a) displacement reactions, (b) opening of strained rings, and (c) addition to activated double bonds.

(a) *Displacement of leaving groups such as halides, sulfates, sulfonates, phosphates, and nitro groups from saturated carbon or heteroatoms.* Displacement is facilitated if the saturated carbon atom is allylic or benzylic. Displacement of halide or nitro groups on aromatic rings occurs if there are sufficient electron-withdrawing groups that predispose the ring system toward nucleophilic substitution. The rate of formation of a carbanion intermediate of the aromatic ring governs the overall rate of the reaction. Functional groups that withdraw electrons stabilize the carbanion and

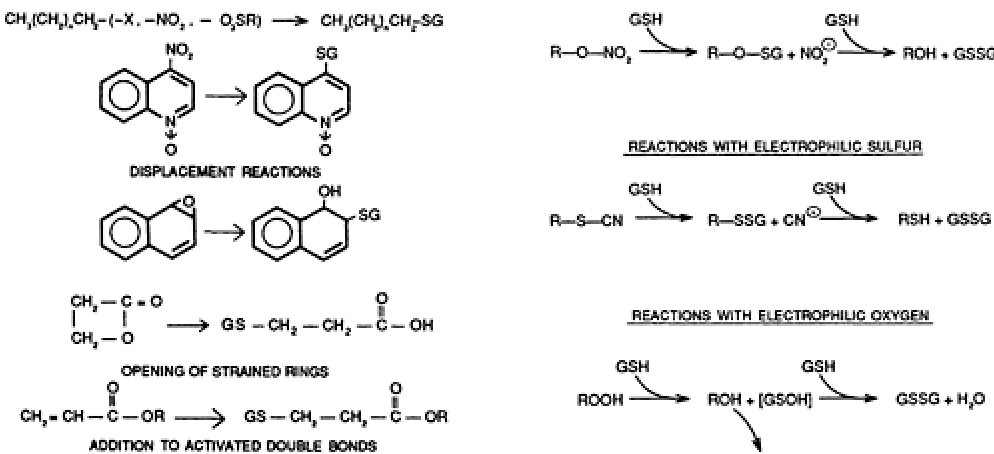

FIG. 3.16. Examples of the reactions catalyzed by the glutathione S-transferases.

FIG. 3.17. Putative reaction mechanisms for the glutathione *S*-transferase-catalyzed nucleophilic attack of the glutathione thiolate anion on electrophilic xenobiotics where X represents a halogen and R and A represents the listed substituents.

are considered "good leaving groups." Those that donate electrons to the ring deactivate the ring, making displacement of the leaving group by glutathione less likely (Figure 3.16).

(b) *Opening of strained rings, such as epoxides and 4-membered lactones.* As shown in Figure 3.16, the 1,2-epoxide of naphthalene is opened, resulting in a 1-napthol conjugate of glutathione. These reactions can be stereoselective. Epoxide products of P450 are detoxified by this reaction and are an example of a phase II conjugation of a phase I activated metabolite.

(c) *Addition to activated double bonds via Michael addition.* The glutathione thiolate anion will also attack *β*-unsaturated xenobiotics due to the partial positive charge on the *β*-carbon, as shown in Figure 3.17 (addition reaction). Figure 3.22 illustrates the addition of glutathione to the β-carbon of an acrylate ester.

Reaction with Electrophilic Nitrogen

The reaction sequence and overall stoichiometry for the reaction of organic nitrate esters with glutathione is shown in Figure 3.16. This reaction was once attributed to a glutathione reductase but is now known to be catalyzed by glutathione *S*-transferases. The transferases catalyze the removal of nitrite from the ester, generating an R-O-S conjugate with glutathione. The vasodilators, nitroglycerin and erythrityl tetranitrate, are two organic nitrate ester substrates for this reaction. As seen in Figure 3.16, the alcohol-glutathione conjugate can react nonenzymatically with another reduced glutathione molecule. This yields an alcohol and oxidized glutathione.

Reaction with Electrophilic Sulfur

Alkyl and aryl thiocyanates are substrates for glutathione S-transferase catalyzed conjugations, as shown in Figure 3.16. Products of this nucleophilic attack of the thiolate ion on the sulfur of the xenobiotic result in a mixed disulfide and hydrogen cyanide. The mixed disulfide can react nonenzymatically with another molecule of glutathione to yield a thiol of the xenobiotic (RSH) and oxidized glutathione (GSSG).

Reaction with Electrophilic Oxygen

Figure 3.16 illustrates how glutathione reacts with organic hydroperoxides in a two-step sequence. The first step is catalyzed by glutathione *S*-transferase and forms an alcohol or phenol and a glutathione sulfenic acid intermediate (G-SOH). Another glutathione reacts nonenzymatically with the sulfenic acid to form oxidized glutathione and water. An example of reaction with endogenous hydroperoxides is the conversion of hydroperoxy-PGF2α to PGFa. Cumene hydroperoxide is metabolized as rapidly by purified glutathione *S*-transferases as the classical transferase substrate probe 1-chloro-2,4-dinitrobenzene.

Glutathione S-transferases Nomenclature

The glutathione *S*-transferases were originally named by their substrate specificity, such as S-epoxide trans

ferase and *S*-alkyltransferase. When purification techniques and homogeneous preparations of the transferases became available, it was found that the transferases had overlapping substrate specificity. Nomenclature based on substrate specificity was no longer appropriate. They were then termed based on their elution from carboxymethylcellulose columns used to purify them (78). As the various enzyme subunits were cloned and sequenced, the older nomenclature was inappropriate. A nomenclature that is being used by many investigators is based on the four major classes of cytosolic enzymes (alpha, mu, pi, and theta) and their subunit classifications. A glutathione *S*-transferase in class Alpha with identical class 1 subunits would be GSTAI-1. A heterodimer transferase from the Mu class with class 1 and 2 subunits would be GSTM1-2.

Mercapturic Acid Formation

Mercapturic acids are *N*-acetylated, *S*-substituted, cysteine conjugates that arise from conjugation of a xenobiotic with glutathione (15).

The glutathione conjugates formed in the liver and other tissues as shown in pathway 1 of Figure 3.18 are polar and partition into the aqueous phase of cells and blood. Because 25% of the blood flow passes through the kidney, glutathione conjugates are transported to the kidney via systemic circulation. There, the glutathione conjugate undergoes a series of reactions, shown in pathway 2 of Figure 3.18, which result in mercapturic acid formation.

The initial step in mercapturic acid synthesis is cleavage of glutamic acid from cysteine catalyzed by γ-glutamyltranspeptidase (EC 2.3.2.2). This enzyme is located in the brush border of the proximal tubules in the kidney (187). Evidence that this enzyme is involved in glutathione degradation comes from observations of pronounced glutathionemia and glutathionuria (high levels of glutathione in blood and urine, respectively) in patients who lack detectable γ-glutamyltranspeptidase. This enzyme not only hydrolyzes the glutathione moiety, but also transfers the γ-glutamyl group to a variety of amino acids and dipeptides. These two reactions have been shown to proceed at equivalent rates under physiological conditions (190).

Next, the glycine group is cleaved from the resulting cysteinylglycine conjugate by aminopeptidase M yielding the *S*-substituted cysteine conjugate of the xenobiotic. The cysteine conjugate is a substrate for N-acetyltransferase that acetylates the free amino group of cysteine to yield the mercapturic acid, which is excreted in the urine (pathway 3, Figure 3.18). These two enzymes, γ-glutamyltransferase and aminopeptidase M, are also responsible for the normal turnover of glutathione in mammalian cells previously shown in Figure 3.15.

Role of Glutathione S-transferase in Detoxication

Free reactive electrophilic intermediates of xenobiotics can produce damage to important cellular constituents. Reduced glutathione and the glutathione *S*-transferases protect cells from this damage by capturing the reactive electrophiles before they can react at nucleophilic sites critical to cell viability.

The metabolism of acetaminophen, an analgesic that at high doses can produce hepatic necrosis, serves as an example of this protective system. A large body of work has shown that one of the principal ways in which acetaminophen produces its hepatotoxicity is via the reactive intermediate, N-acetyl-p-benzoquinone imine, as shown in Figure 3.6. This intermediate is an electrophile that reacts readily with the nucleophile glutathione. As long as the amount of glutathione present at the site of activation of acetaminophen is sufficient to bind the reactive intermediate, no toxicity ensues; however, as demonstrated in the classic study by Mitchell (115), when glutathione is depleted by pretreatment with diethyl maleate, the benzoquinone imine covalently binds to tissue proteins resulting in tissue necrosis. Mitchell (115) was among the first to propose that glutathione plays a fundamental role in protecting tissues against electrophilic attack by xenobiotics.

Since these early studies demonstrating the protective role of glutathione, many compounds have been shown to form conjugates with glutathione. For a comprehensive review of these reactions, see (25, 87).

Factors Affecting Metabolism

Glutathione *S*-transferases have been found in most species, including reptiles, birds, insects, amphibians, and plants. Factors that influence the availability of reduced glutathione drastically alter the effectiveness of glutathione *S*-transferases. As was discussed previously, the toxicity of acetaminophen is modulated by the availability of reduced glutathione. Most xenobiotics that are highly reactive nonenzymatically with glutathione can deplete glutathione. Other mechanisms can also lower glutathione availability. For example, certain individuals have genetic defects in the γ-glutamyl cycle, resulting in low tissue concentrations of glutathione. These individuals generally are anemic due to the lack of glutathione and the resulting loss of protection from oxidative damage to erythrocytes (111).

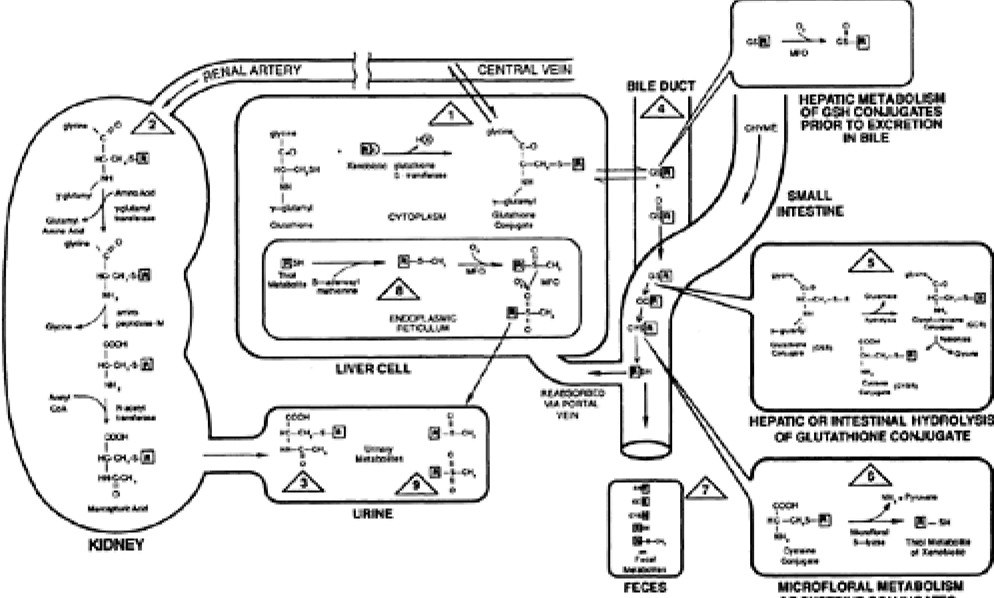

FIG. 3.18 Integration of glutathione conjugate disposition in mammals. Pathway numbers referred to in the text as shown in the triangles.

Cysteine is the limiting factor for synthesis of glutathione via the cycle shown in Figure 3.15. Nutritional factors that limit sulfur amino acid availability decrease glutathione *S*-transferase activity by reducing the availability of glutathione (168). Methionine is an essential amino acid that can be used to synthesize cysteine and cystine via the transsulfuration pathway. If diets low in sulfur amino acids are fed, the availability of glutathione for conjugation with reactive intermediates of xenobiotics can be decreased.
Glutathione *S*-transferases are inducible by phenobarbital and 3-methylcholanthrene. Dietary ingredients, such as cruciferous vegetables, specific components of coffee, butylated hydroxyanisole, and organosulfur compounds of allium vegetables, can also induce glutathione *S*-transferases. For example, when cafestol and kahweol, diterpenes found in coffee, were administered to rats for up to 90 days, DNA adducts produced by aflatoxin B1 were inhibited 50%. This appeared related to induction of glutathione *S*-transferase and a decrease in P450 isozymes involved in the metabolic activation of aflatoxin (23). Coffee consumption has been shown to increase salivary concentrations of the glutathione *S*-transferases in humans (163). Induction may be specific for one or more of the transferases and may be tissue specific. Triphenyltinchloride, bromosulfophthalein, cibracon blue, and hematin are selective inhibitors of the transferase isozymes (175).

Polymorphisms of Glutathione S-transferases

Glutathione *S*-transferases exhibit polymorphic expression in humans, especially GSTM1. A proportion of a population will show low GSTM activity, whereas the majority will show normal activity. Because these enzymes play a critical role in protecting the cell from cytotoxic and mutagenic damage, a number of population studies have been done to determine relationships between genotype and disease. Studies have attempted to correlate lung cancer risk and transferase expression with mixed results. A better correlation has been found between transferase genotype and diseases associated with oxidative stress, especially for GSTM1 or GSTT1 polymorphism and colon cancer (53) and esophageal cancer (97). No correlations were found between breast cancer and GSTM1 polymorphism (1). It appears that some of the highest correlations between genotype and cancer susceptibility are those where P450 genotype and transferase genotype are combined for analysis. This, again, emphasizes the close relationship between metabolic activation by phase I enzymes and detoxication of reactive metabolites by phase II enzymes.

Species and Gender Differences

As mentioned earlier, glutathione *S*-transferases have been found in most species investigated. Species differences in the expression, substrate specificity, and activity of these transferases can have a significant role in the toxicity of xenobiotics. For instance, rats are susceptible to the potent hepatocarcinogen aflatoxin B1 (AFB1), whereas mice are extremely resistant. This species difference results from the expression of mGSTA3–3 in mice, which has a high activity toward the P450-generated activated metabolite of AFB1 (the 8,9 epoxide). Although rats express a closely related transferase (rGSTA3–3), it has low activity toward the epoxide. Although these two transferases have equivalent activity toward a probe substrate (l-chloro-2,4-dinitrobeneze), the rat form has 1,000-fold less activity toward the AFB1 epoxide compared to the mouse. This difference in activity between the transferases from the two species appears to be based on differences in as few as six critical amino acids (177).
Hepatic glutathione *S*-transferase activities are low in prepubertal male and female rats. As the rats reach sexual maturity between 30 and 50 days of age, glutathione-conjugating activity toward dichloronitrobenzene is two- to threefold higher in males than in females (91). This difference in glutathione *S*-transferase activity was not related to sex steroids but was dependent on pituitary secretions. Growth hormone may play a role in establishing glutathione *S*-transferase activities (90), as it does with P450. Although growth hormone is important in regulating adult levels of glutathione *S*-transferase in the rat, it appears that other factors also play a role. The student of toxicology should be aware of the multifaceted way that xenobiotics can affect organisms. For example, monosodium glutamate, which produces lesions in the arcuate nucleus of the hypothalamus, can lower the glutathione *S*-transferase activity in male rats. This, in turn, could increase their sensitivity to electrophilic chemicals.
Gender differences in the expression of glutathione *S*-transferase have been suggested to be responsible for the higher susceptibility of female mice to the carcinogenicity of benzo(a)pyrene compared to males. Males express higher mGSTP1–1, mGSTA3–3, mGSTM1–1, and mGSTA4–4 compared to females. At higher doses of benzo(a)pyrene this gender difference is lost, possibly by the higher doses overcoming the protective role of the higher transferase activity in males (156).
Some studies suggest that humans do not demonstrate gender differences in glutathione *S*-transferases. No gender or age differences were seen in GSTM and GSTP activity in human lymphocytes, but an age-dependent decrease in glutathione was detected (176).

Role of Glutathione S-transferases in Metabolic Activation

Glutathione conjugation does not always produce an innocuous and readily excreted metabolite. For example, Elfarra and Anders (41) compiled a list of 1,2-dihaloalkanes and halogenated alkenes whose glutathione or cysteine conjugates were nephrotoxic (Figure 3.19). Glutathione reacts with these 1,2-dihaloalkanes via a glutathione *S*-transferase-catalyzed reaction that yields sulfur mustards. An electrophilic episulfonium ion can be formed from the mustard when the second halogen atom is displaced by a cellular nucleophile. The episulfonium ion intermediate has been implicated in the toxicity of these chemicals. The major DNA adduct resulting from exposure to the carcinogen, 1,2 dibromoethane, was *S*-2-*N*-7-guanylethylglutathione (72). A brief review of this bioactivation pathway is included in (4, 174).

As shown in Figure 3.18, glutathione and cysteine conjugates (GSR and CySR, respectively) formed in the liver (pathway 1) can be excreted in the bile (pathway 4). Glutathione conjugates can be hydrolyzed to cysteine conjugates by pancreatic peptidases in the small intestine (pathway 5). Cysteine conjugates originating from the bile and those formed by hydrolysis of glutathione con-

FIG. 3.19. Halogenated hydrocarbons that form glutathione and cysteine *S*-conjugates that are nephrotoxic. Asterisks indicate the position where the glutathione *S*-transferase-catalyzed displacement (SN2) occurs.

jugates are good substrates for microfloral *β*-lyase (pathway 6). *β*-Lyase, an enzyme found in liver, kidney, and in intestinal microflora, cleaves thioether linkages in cysteine conjugates of xenobiotics (169). The resulting thiol compounds are more hydrophobic than the conjugates and can be readily absorbed in the small intestine. These thiol metabolites return to the liver via the portal circulation and act as substrates for thiol S-methyltransferase that methylates the thiol group (pathway 8). Enterohepatic circulation (pathways 1→4→5→6→8) of glutathione conjugates accounts for some of the unusual sulfur-containing metabolites (pathway 9) that have been found in the urine of animals treated with xenobiotics, such as propachlor (138). A portion of the glutathione-derived sulfur-containing metabolites formed in the small intestine is excreted in the feces (pathway 7 of Figure 3.18).

Reactions of glutathione and cysteinyl conjugates shown in Figure 3.19 are believed to play a role in the nephrotoxicity of several xenobiotics. For example, the cysteinyl conjugate of trichloroethylene, *S*-(l,2-dichlorovinyl)-L-cysteine (DCVC), is a potent nephrotoxin and a *β*-lyase substrate. Inhibition of renal β-lyase with aminooxyacetic acid, an inhibitor of pyridoxyl phosphate-dependent enzymes, protected

against DCVC-induced nephrotoxicity (41).

In general, glutathione conjugate synthesis results in readily excreted polar metabolites; however, in some cases (depicted in Figure 3.18), the residence time of a glutathione conjugate in the body is prolonged. This can result in formation of metabolites that are more reactive than the original, parent xenobiotic, or the glutathione conjugate. If these reactive metabolites interact with critical cellular sites, toxicity can ensue. For a review, see (3, 118).

Glutathione S-Transferases as Markers of Liver Damage

Glutathione *S*-transferases may be valuable as an adjunct to serum aminotransferases for detecting acute liver damage. These transferases constitute as much as 3% of cytosolic protein in the hepatocyte and are uniformly distributed across the liver lobule, compared with aminotransferases, which are located periportal. Their plasma half-life is less than 60 minutes, compared to 48 hours for the alanine aminotransferase. Selective use of these different characteristics between aminotransferases and glutathione *S*-transferases have led to more accurate diagnosis of hepatic damage produced by xenobiotics (67). Recently, it has been suggested that determination of glutathione *S*-transferase be included in toxicology studies, and validated methods for rats and dogs have been developed (84).

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N – Methylation

Aromatic Azaheterocycle N – Methyltransferase - catalyzed

R – (+) – Nicotine → R – N – Methylnicotinium ion

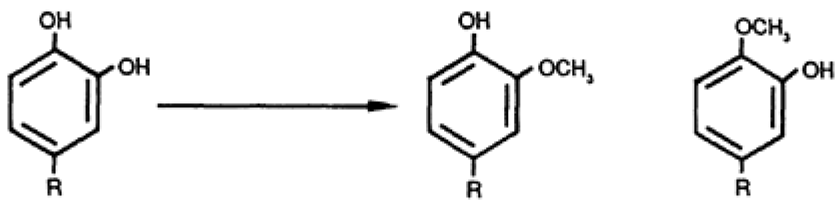
Histamine → 1 – Methylhistamine

Indolethylamine N—Methyltransferase - catalyzed

Indoleamines

O - Methylation

Catechol O - Methyltransferase - catalyzed



Catechols

S - Methylation

Thiol S - Methyltransferase - catalyzed

Thiouracil → S - Methyl Thiouracil

$(C_2H_5)_2N-C(=S)-SH$ → $(C_2H_5)_2N-C(=S)-S-CH_3$

Diethylthiocarbamyl Sulfide

FIG. 3.20. Methylation reactions.

Methylation

Methyl conjugation is an important pathway in the metabolism of many neurotransmitters, drugs, and xenobiotics. Methylation of endogenous substrates, such as histamine, amino acids, proteins, carbohydrates, and polyamines, is important in the regulation of normal cellular metabolism and accounts for the presence of this activity in mammalian cells. Only when a xenobiotic fits the requirements for the enzymes involved in these normal reactions does methylation become important in the metabolism of foreign compounds.

Methylation can be achieved by two routes. First and foremost is the methyltransferase-catalyzed methylation that requires *S*-adenosylmethionine (SAM) as a cosubstrate. Most biological methylations require SAM as the methyl donor; however, there are methyl transferases that require SAM as a cosubstrate but vary in other requirements for optimal activity (105). Reactions involving four of these SAM-dependent methyltransferases are shown in Figure 3.20. Two enzymes catalyze nitrogen methylation: aromatic azaheterocycle N-methyltransferase and indolethylamine N-methyltransferase. Oxygen methylation is primarily catalyzed by catechol *O*-methyltransferase and sulfur methylation thiol *S*-methyltransferase. A secondary source of methylation is the N5-methyltetrahydrofolate (5-CH3-THF)catalyzed methylation. This methylation is important in the synthesis of nucleic acids; however, S-CH3-THF is 1000 times less reactive toward soft nucleophiles (shown in Figure 3.20) than SAM, suggesting that it plays a smaller role in xenobiotic metabolism.

Methylation is a major route for nicotine biodisposition in the guinea pig. The enzyme responsible for nicotine methylation is an aromatic azaheterocycle *N*-methyltransferase that normally methylates histamine (Figure 3.20). Guinea pigs are well known for their ability to methylate histamine. They represent a good animal model for studying xenobiotic methylation. Nicotine is an example of a xenobiotic that can be metabolized by an enzyme of normal cellular metabolism. Because nicotine is a weak base, methylation of the pyridyl nitrogen results in charges at both nitrogens at physiologic pH, increasing water solubility and urinary excretion.

Methylation reactions can be stereoselective. For example, the R(+) enantiomer of nicotine is preferentially methylated over the S(−) enantiomer (33). Stereoselective metabolism of xenobiotics can be important in understanding the metabolic basis of toxicity. Because most biotransformation reactions are catalyzed by enzymes, there is always the possibility that the active site will select one orientation around a chiral center over another.

Individual differences in S-methylation activity have been observed, with activity being trimodal. These data suggested that S-methylation is controlled by a single gen etic locus with two alleles, one controlling low activity and one controlling high activity (185). Inherited variations in "methylator" status should be included among those factors responsible for individual variations in the metabolism of thiopurine and catechol xenobiotics.

Amide Synthesis

Amide biosynthesis can take place via two principal routes:

1. Conjugation of a carboxylic acid-containing xenobiotic with the free amino group of an amino acid such as glycine.
2. The acetylation of a xenobiotic containing a primary amine ($-NH_2$).

Amino Acid Conjugation

Xenobiotics that contain a carboxylic acid moiety are susceptible to conjugation with endogenous amino acids. Xenobiotic conjugation occurs in hepatic mitochondria. The free carboxylic acid is activated by reaction with ATP followed by reaction with acetyl coenzyme A (CoA), as shown in reactions 1 and 2 of Figure 3.21. For example, the carboxylic acid of benzoic acid is activated to a thioester CoA intermediate that reacts with the primary amine of glycine to form the amide, hippuric acid.

Glycine has historical significance in xenobiotic conjugation because it is one of the earliest reactions attributed to xenobiotic metabolism. Keller (83), in 1842, administered benzoic acid to himself and then isolated and characterized the major metabolite, hippuric acid, a glycine conjugate. This reaction has been used as a liver function test in humans. The liver is the principal site of glycine conjugation. Amino acids other than glycine can be used for conjugating aromatic and heterocyclic carboxylic acids. For example, arginine is used by arachnids, glutamine by chimpanzees, and ornithine by certain birds.

Acetylation

Acetylation, catalyzed by *N*-acetyltransferases, is the principal pathway of amide formation for primary aromatic amines, endogenous primary aliphatic amines, anutrient amino acids, hydrazines, hydrazides, and sulfonamides. The activated acetyl group is derived from acetyl-CoA. Mercapturic acid formation in the kidney is an example of acetylation that has been presented. In this reaction, the primary amine

group of the cysteine conjugate of the xenobiotic is acetylated to form the mercapturic acid. This is an exception to the rule that aliphatic primary amines generally are not good substrates

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Activation of the Carboxyl Group

1. RCOOH + ATP —acylsynthetase or thiokinase→ RCO-AMP + Pyrophosphate
2. RCO-AMP + CoA-SH —acylthiokinase→ RCO-S-CoA + AMP
3. RCO-S-CoA + R'NH_2 —transacylase→ RCO NHR' + CoA-SH

Examples of Amide Synthesis

Benzoic Acid (COOH) —CH_2NH_2COOH / CoA—SH→ Hippuric Acid (C(=O)—NH—CH_2—COOH)

Sulfanilamide (SO_2NH_2, NH_2) + CH_3—C(=O)—S—CoA (Acetyl CoA) → N^4—Acetylsulfanilamide (SO_2NH_2, NH—C(=O)—CH_3) + HS—CoA

FIG. 3.21. Series of reactions leading to amide formation from either a xenobiotic containing a carboxylic functional group (RCOOH) or a primary amine group (R′NH2).

for the N-acetyltransferases. It is worth noting that the use of the -uric suffix to denote an acidic metabolite was once common. Mercapturic acids, hippuric acid, and salicyluric acid all share this common suffix that is probably derived from the fact that these were acids. Uric acid had similar characteristics and all were isolated from urine.

The N-acetyltransferases are cytosolic enzymes that occur in many tissues. Some species, including humans, express two independently regulated transferases, NAT1 and NAT2, whereas other species express three. NAT1 and NAT2 are similar proteins but have different substrate specificities, although there is some overlap. NAT2 is primarily a liver and intestinal enzyme, whereas NAT1 is expressed in a number of organs. The *N*-acetyltransferases are important in the metabolic disposition of drugs and can also be involved in the metabolic activation of xenobiotics (e.g., aromatic amines).

Human Amide Synthesis Polymorphism

The main route of metabolism of isoniazid (1-isonicotinyl hydrazide) in humans is conjugation with acetyl-CoA to form the amide metabolite (106). Isoniazid is eliminated much faster from the body once it is acetylated. With the widespread use of isoniazid to control tuberculosis, it became obvious that there were major differences in the rates at which individuals eliminated the drug (44). Careful studies of hundreds of patients showed that there were two distinct populations: the fast and the slow acetylators. Slow acetylators inherited the trait as a homozygous recessive allele.

NAT polymorphisms have been reported in several species. Although it is possible that NAT1 may be polymorphic, NAT2 has received the most interest. Slow acetylation is associated with mutations, in the NAT2 gene. Not all slow acetylators show identical mutations, and there is a spectrum of activity in the slow acetylator phenotype. Some mutations affect enzyme activity, whereas others decrease the stability of the enzyme. A number of population studies have been done to determine if the NAT polymorphism alters susceptibility to carcinogens. In some studies, no differences have been reported, whereas in others, associations have been suggested (18). The number of enzymes involved in carcinogen biodisposition and the polymorphisms associated with some of these enzymes makes the task of identifying specific polymorphisms associated with increased cancer risk difficult. As more is learned about the complex interactions of these enzymes, populationbased studies will provide valuable information.

HYDROLYSIS

Many xenobiotics and their phase I metabolites contain either a carboxyl ester, an amide bond, or an epoxide that mask hydrophilic functional groups, such as alcohols, carboxylic acids, and amines. The rate at which an organism can hydrolyze these bonds and unmask these function groups can influence their toxicity. In fact, pesticides and therapeutic drugs have been synthesized with intent to modulate the bioavailability of the active species by affecting the rate of hydrolysis of the parent compound. Hydrolysis normally competes with other detoxication reactions. An example of competition is demonstrated by the metabolism of acrylate esters (Figure 3.22). Most acrylate esters either react with glutathione via a glutathione *S*-transferase-catalyzed pathway or are hydrolyzed by B-esterases to acrylic acid and the corresponding alcohol (Figure 3.22).

Epoxide Hydrolase

Organisms may be exposed to epoxides in the environment, or, they may be produced during the oxidative metabolism of specific xenobiotics from their environment. Epoxides generally are reactive electrophilic compounds due to the highly strained oxirane ring (Figure 3.23). Excess strain energy can be released by ring opening in the presence of nucleophiles. Ring opening may follow either a SN1-type mechanism with the formation of an intermediate with carbonium ion character or an SN2 mechanism with bond formation with the attacking nucleophile. The latter case has important toxicological consequences when the nucleophile is on a critical tissue macromolecule, such as DNA, because it results in covalent modification of the macromolecule. Modification of DNA results in a biochemical lesion that may be the precursor to a number of pathological lesions, including cancer. Reaction of the epoxide with cellular nucleophiles, such as proteins, could also lead to other mechanisms producing acute or subchronic toxicity.

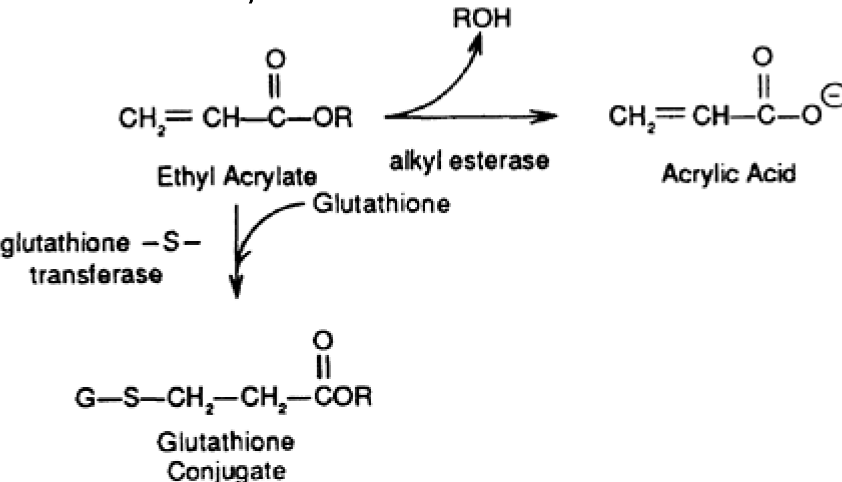

FIG. 3.22. Routes of disposition of ethyl acrylate.

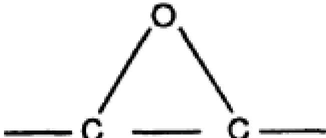

FIG. 3.23. An example of a strained epoxide oxirane ring.

The chemical reactivity and, consequently, the biological activity of epoxides are influenced by the constituents attached to the oxirane ring carbons. Epoxides with asymmetric carbon atoms can exhibit optical activity and exist as enantiomers in a racemic mixture. Reaction of epoxides with nucleophiles with an asymmetrical center will produce diastereoisomers with different spatial orientations around the carbon center. Production of diastereoisomers is important in biological activity in respect to both reaction at critical biochemical sites and subsequent metabolism. For instance, one isomer may react more efficiently at the toxigenic site and be a poor substrate for subsequent metabolism when compared to another isomer.

Although not always the case, the epoxides that are formed in vivo appear to be more toxicologically important than those that occur in the environment. Highly reactive epoxides would most likely interact with nucleophilic sites in the environment, such as proteins in food, and not be absorbed in their active form. Epoxides formed in vivo are produced close to their sites of action and require only diffusion or short transport to their target. Epoxides most frequently formed in vivo represent alkene and arene

oxides produced by P450. Their efficient detoxication is important to cellular survival.
Detoxication of epoxides may follow several routes:
(1) Spontaneous decomposition.
(2) Nonenzymatic reaction with glutathione.
(3) Reaction with glutathione catalyzed by glutathione transferase.
(4) Hydration by epoxide hydrolase.
(5) Minor mechanisms such as a P450 hydrolysis.
Nonenzymatic and enzymatic conjugations with glutathione have been previously discussed.
A major route for biodisposition of epoxides is hydration catalyzed by epoxide hydrolase. This enzyme was previously referred to as epoxide hydrase and epoxide hydratase, and readers will sometimes encounter these terms still in use. This microsomal enzyme catalyzes the biotransformation of arene oxides and aliphatic epoxides to vicina (Latin: *vicinalis*, neighboring) dihydrodiols. In most cases, this enzymatic pathway results in less reactive diol metabolites that are more readily excreted from the organism, either as the diol or as a glucuronide or sulfate conjugate of the diol.

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Epoxide hydrolases occur as membrane-bound proteins located in the endoplasmic reticulum and as a soluble enzyme in the cytosol of most mammalian cells. The mechanism of the soluble and microsomal epoxide hydrolase-catalyzed reaction appears to be a nucleophilic attack by an amino acid at the active site, possibly asparagine, on the epoxide to form an α-hydroxy-acyl-enzyme intermediate. Water hydrolysis of the acyl-enzyme occurs at the carbonyl carbon of the ester bond yielding the active enzyme and diol (12, 172). This stereoselective attack usually results in the diols having a transconfiguration. Hydrolysis of the ester is the rate-limiting step in catalysis.
Membrane-bound epoxide hydrolase has a 20 amino acid sequence at the N-terminal end that anchors it to the membrane. The active site of the enzyme occurs outside of the membrane. Unlike P450, if the anchor sequence of the protein is not present, the enzyme retains a portion of its catalytic activity (48).
Epoxide hydrolase has been found in a variety of tissues, including liver, kidney, lung, skin, intestine, colon, testis, ovary, spleen, thymus, heart, and brain. The activity of liver microsomal enzyme is relatively low in newborn rats and increases during neonatal development until adult males have about twice the activity of females. This sexual dimorphism is remarkably similar to that seen in the rat for P450. In contrast, the renal epoxide hydrolase of male and female rats does not demonstrate age-dependent changes or gender differences. Human hepatic microsomal epoxide hydrolase activities increase during gestation, but there does not appear to be a gender difference in humans (129).
Humans demonstrate considerable variation in epoxide hydrolase activity. This, in part, is associated with the inducibility of epoxide hydrolase and environmental exposures and life-style differences among individuals. In addition, there is evidence that there are genetic polymorphisms with the enzymes that result from amino acid sequence differences. These human polymorphisms may not result in significantly altered enzyme activity nor post-transcriptional regulation (93), although more work is needed in this area. Polymorphic expression of epoxide hydrolase has been related to specific human diseases (92, 158).
The activity of this enzyme is induced by the classical inducers of cytochromes P450. Although *trans*-stibene oxide has been shown to be an inducer of epoxide hydrolase, no specific inducer of epoxide hydrolase has been reported. Two widely used inhibitors of epoxide hydrolase are trichloropropane oxide and cyclohexene oxide.
Because of its localization in the endoplasmic reticulum, microsomal epoxide hydrolase is ideally situated to catalyze the detoxication of lipophilic epoxides formed by P450; however, it can also be involved in meta bolic activation. One example of metabolic activation is the biotransformation of benzo(a)pyrene to the ultimate mutagen benzo(a)pyrene trans-7, 8-dihydrodiol-9–10-oxide, which is shown in Figure 3.4 and described under metabolic oxidations. These diol epoxides are poor substrates for further metabolism by epoxide hydrolase and, as shown in Figure 3.4 react with critical cellular macromolecules.
An immunologically distinct epoxide hydrolase has also been identified in the cytosol of some species. This enzyme may play a role in hydrolysis of more water-soluble epoxides that partition out of the endoplasmic reticulum. As discussed earlier, this enzyme completes with glutathione transferases for cytosolic epoxides. The activity of the cytosolic epoxide hydrolase appears to be highest in mice and rabbits and relatively low in rats.

Esterases and Amidases

Hydrolysis of xenobiotics containing ester linkages and amide bonds is catalyzed by a group of enzymes with broad substrate specificity. In general, these enzymes perform endogenous functions and appear to metabolize xenobiotics that have structural similarities to endogenous substrates.
The reactions carried out by this diverse group of enzymes are illustrated in Figure 3.24. Specificity of carboxylesterases depends on the nature of the R groups rather than on the atom (*O*, *N*, or *S*) adjacent to the carbonyl carbon (69). The esterases have been broadly grouped into three categories based on their reactivity with organophosphorous compounds (181). Those esterases preferring carboxylesters with aryl groups in the R position and that can use organophosphate esters as substrates are classified as A-esterases (Table 3.7). Those esterases preferring esters with alkyl groups in

$$R{-}C({=}O){-}O{-}R' + H_2O \longrightarrow R{-}C({=}O){-}OH + HOR'$$

Carboxylester hydrolysis

$$R{-}C({=}O){-}N(R''){-}R' + H_2O \longrightarrow R{-}C({=}O){-}OH + HNR'R''$$

Carboxyamide hydrolysis

$$R{-}C({=}O){-}S{-}R' + H_2O \longrightarrow R{-}C({=}O){-}OH + HSR'$$

Carboxythioester hydrolysis

FIG. 3.24. Reactions catalyzed by esterases and amidases.

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Table 3.7 Classification of esterases by how they interact with organophosphates and substrate specificity

Esterase	Interaction with organophosphates	Substrates	Examples
A-esterases (arylesterases)	Substrates	$R-C_6H_4-C(=O)-O-R'$ Aromatic Esters	Organophosphate and carbamate insecticides
B-esterases (carboxyltesterases including cholinesterases)	Inhibitors	$R-CH_2-C(=O)-O-R'$ Aliphatic Esters	Acetylcholine, acrylate esters, succinylcholine, propanidid
C-esterases (acetylesterases)	No interaction	$CH_3-C(=O)-O-R'$ Acetate Esters	*p*-Nitrophenyl acetate, *n*-propylchloroacetate

the R position and that are inhibited by organophosphate esters are classified as B-esterases. Another group of esterases that prefer acetate esters and do not interact with organophosphates are referred to as C-esterases. This classification has been devised to help organize this multifarious group of enzymes. It also has some practical value in toxicology. The mechanism of organophosphate and carbamate insecticide toxicity is inhibition of acetylcholinesterase, a B-type esterase. Organophosphate insecticides, such as malathion, are detoxified in mammals by A-esterase hydrolysis. Many insects have lower levels of A-esterases than mammals. The selective toxicity of malathion in birds and insects can be explained by the low activity of A-esterases compared to mammals (182).

MICROFLORA METABOLISM

Xenobiotic metabolism by microorganisms can be divided into reactions occurring in the environment and reactions occurring inside the body (141). Metabolism of chemicals by microorganisms in the environment has become familiar through the use of microorganisms to degrade chemical spills (164). The in vivo metabolism of chemicals by microorganisms is not as familiar. Mammals are colonized by microorganisms (only those animals raised in a germ-free environment (gnotobiotic) are microbe-free). The metabolic reactions carried out by these microorganisms are dependent on the substrate and environment in which they are growing. Microbes growing in an aerobic environment are capable of cleavage of aromatic nuclei and can use these xenobiotics as sole carbon sources for biosynthetic reactions and growth. Microbes growing in an anaerobic environment are more likely to carry out reductive metabolism. The hallmark of metabolism by organisms colonizing the intestinal tract of mammals is reduction (Table 3.8).

Because the majority of microbes that colonize various surfaces of the mammalian body reside in the intestinal tract, most of this discussion will center around intestinal microflora metabolism. The intestinal microflora can alter xenobiotic bioavailability by metabolizing the parent compound to a metabolite that may be absorbed to a greater or lesser extent. Intestinal microflora can also metabolize products of xenobiotic biotransformation that are secreted into the intestine either directly from the blood or via the bile, saliva, or by swallowing respiratory tract mucus. Metabolism of secreted metabolites is a common mechanism by which microflora influence xenobiotic toxicity.

Xenobiotic Biotransformation by Microbes Colonizing Mammals

The intestinal tract of mammals contains a variety of microorganisms. The location, total number, and species diversity of microflora vary among mammals. Microflora can range from ruminants that have evolved to be dependent on microflora metabolism for energy needs to monogastric mammals, such as humans, that have great

Table 3.8 Types of metabolic reactions carried out by intestinal bacteria

Reaction	Representative Substrate
Hydrolysis	
Glucuronides	Estradiol-3-glucuronide
Glycosides	Cycasin
Sulfamates	Cyclamate, amygdalin
Amides	Methotrexate
Esters	Acetydigoxin
Nitrates	Pentaerythritol trinitrte
Dehydroxylation	
C-Hydroxy groups	Bile Acids
N-Hydroxy groups	N-Hydroxyfluorenylacetamide
Decarboxylation	Amino acids
N-Demethylation	Biochanin A
Deamination	Amino acids
Dehydrogenase	Cholesterol, bile acids
Dehalogenation	DDT
Reduction	
Nitro groups	*p*-Nitrobenzoic acid
Double bonds	Unsatrated fatty acids
Azo groups	Food dyes
Aldehydes	Benzaldehydes
Alcohols	Benzyl alchols
N-Oxides	4-Nitroquinoline-1-oxide
Other Reactions	
Nitrosamine formation	Dimethylnitrosarnine
Aromatization	Quinic acid
Acetylation	Histamine
Esterification	Gallic acid

numbers of bacteria only in the large intestine. Because of this variation in location within the intestinal tract, the types of microorganisms present, and hence the types of microflora metabolism, vary with the mammalian species being studied.

Another factor that relates to the location of the microflora is the disposition of the xenobiotic and its microflora metabolites. Chemicals metabolized by microflora located in the stomach will be distributed differently than chemicals metabolized in the large intestine.

The majority of mammals have a gradient of microflora that increases in numbers and species diversity along the intestinal tract from the foregut to the hindgut. Most research on microflora metabolism has focused on microorganisms that colonize the large intestine of humans, as most of the research in toxicology is directed toward understanding the toxicity of chemicals in humans. In vivo and in vitro models have been developed for studying human colonic flora (145).

Role of Diet and Other Factors in Modulating Microflora Metabolism

The microflora colonizing the digestive tract of mammals play a major role in the digestion of plant cell wall constituents that are indigestible by mammalian enzymes. These dietary fibers provide energy substrates that support the large bacterial populations in the gut. These energy sources also influence the microflora metabolism of xenobiotics. Certain types of dietary fiber, such as the fermentable carbohydrate pectin, can influence the toxicity of xenobiotics that require microflora metabolic activation by increasing the number of anaerobic bacteria colonizing the large intestine (35). This diet-induced elevation in the number of bacteria increases the total metabolic capacity of the large intestine for metabolizing xenobiotics. For reviews of this topic, see (143, 144).

Examples of Xenobiotics Whose Toxicity is Dependent on Microflora Metabolism

Nitroaromatics

A body of literature has now accumulated indicating that the toxicity of many nitroaromatic compounds is dependent on microflora metabolism. One of the most studied nitroaromatics is 2,6-dinitrotoluene (DNT), which is hepatocarcinogenic in male rats (94). DNT is metabolized to the 2,6-dinitrobenzylalcohol glucuronide conjugate that is preferentially excreted in the bile of male rats (99) (Figure 3.25). The glucuronide conjugate is hydrolyzed by gut microflora β-glucuronidase, and one or both of the nitro groups are reduced by microflora nitroreductase to a reduced aglycone. The resulting aminobenzyl

alcohol is relatively nonpolar and reabsorbed in the intestine, where it returns to the liver via the portal circulation. In the liver, the aglycone is activated to the putative proximate carcinogen by N-hydroxylation of the amine functional group followed by sulfation of the N-hydroxy group (82). Evidence that intestinal microflora were required for the activation of DNT was provided by studies indicating the genotoxicity of DNT in hepatocytes isolated from rats treated with DNT was dependent on the presence of bacteria in the intestinal tract (114). Rats raised in a germ-free environment showed minimal levels of genotoxicity. Additional evidence emphasizing the role of microflora in the metabolic activation of DNT was the observation that DNT was not genotoxic when tested in vitro in isolated hepatocytes (114). These results indicated that liver metabolism was not sufficient to activate the molecule to the ultimate carcinogen. The genotoxicity of DNT to liver cells only occurred when the compound was

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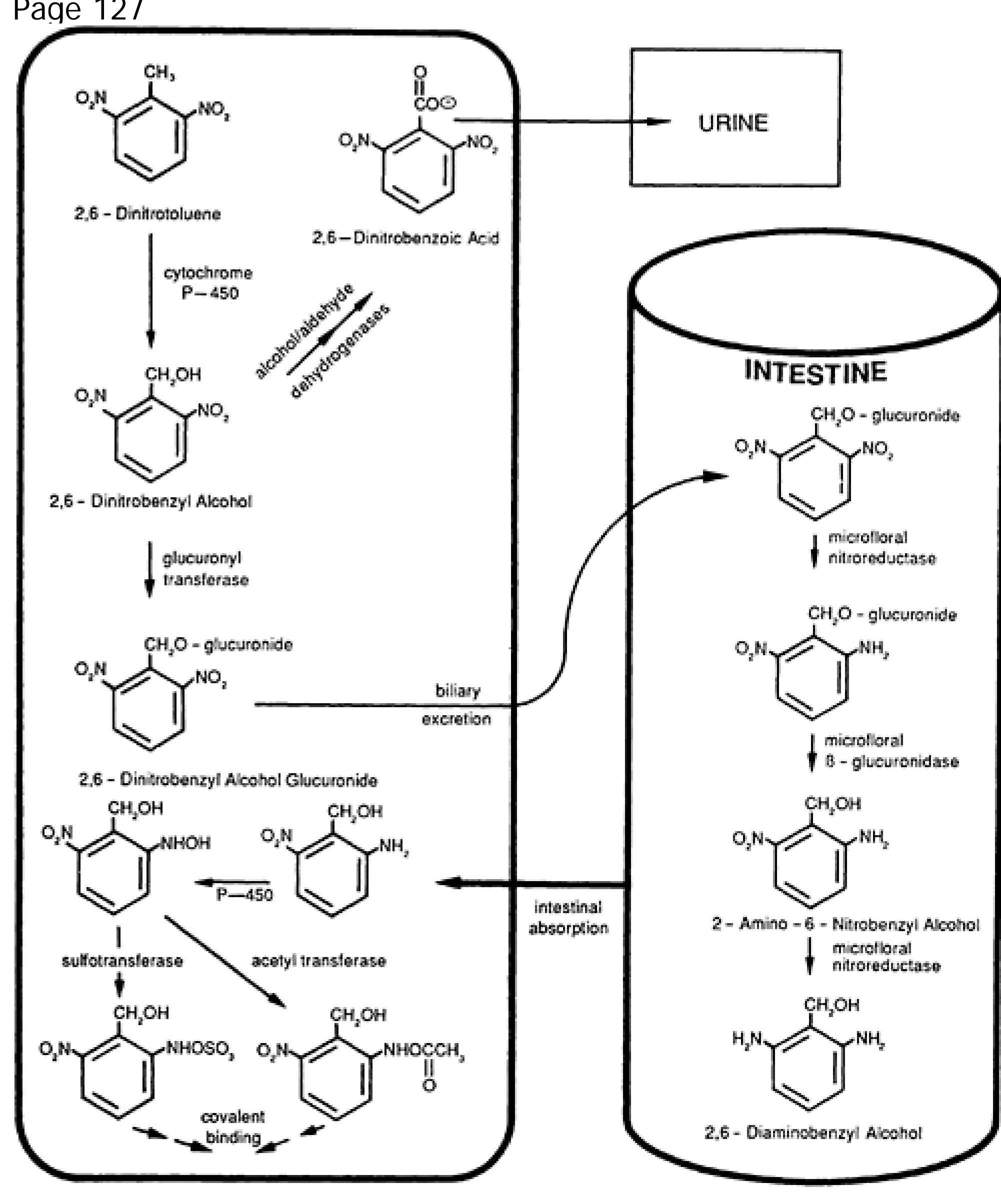

FIG. 3.25. Putative route of disposition of 2,6-dinitrotoluene.

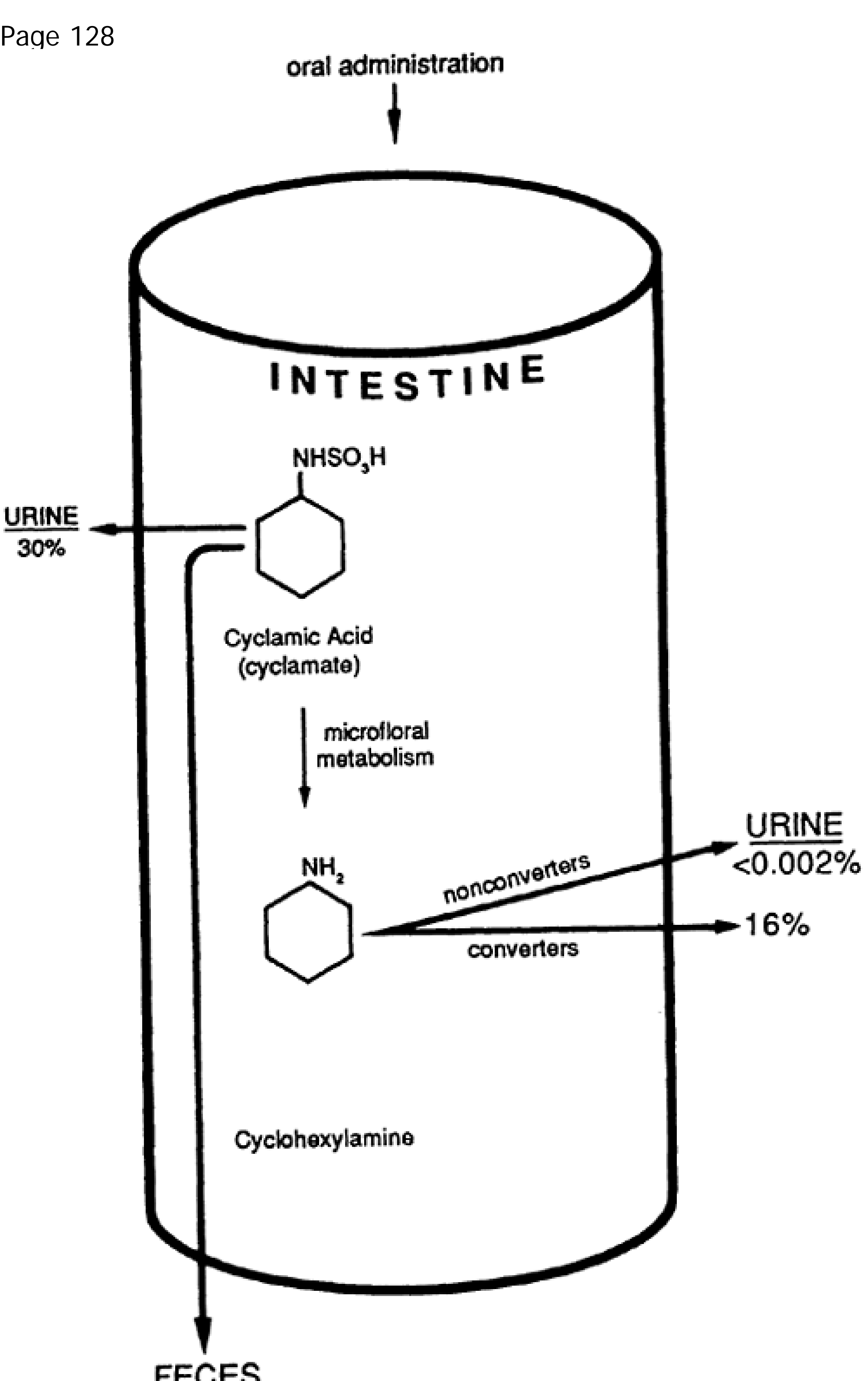

FIG. 3.26. Metabolic degradation of cyclamic acid by intestinal microflora.
administered to the animal and allowed to undergo enterohepatic circulation involving intestinal microflora.

The level of DNT-derived radioactivity covalently bound to DNA, RNA, and protein isolated from the livers of rats treated with DNT was also dependent on the presence of intestinal microflora (99). Dietary treatments that increased the microbial metabolic capacity of the rat's large intestine also increased the covalent binding of DNT-derived radioactivity to hepatic macromolecules (35).

The importance of complementary in vitro short-term toxicity test with suitable in vivo test for predicting a chemical's toxicity is well illustrated by this example, where the toxicity of DNT was dependent on the disposition within the host rather than metabolic activation within a single organ.

Cyclamate

The sodium and calcium salts of cyclamic acid (cyclohexylsulfamic acid) were used as an artificial

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sweetening agent until 1969 in the United States, when it was removed from the market because a metabolite, cyclohexylamine, was suspected of being a bladder carcinogen.

Most of the hydrolysis of cyclamate to cyclohexylamine takes place in the gut by the microflora, as shown in Figure 3.26 (142). Cyclohexylamine is more lipophilic than the parent acid and is readily absorbed from the intestine and excreted in the urine. Minor urinary metabolites include cyclohexanol and trans-cyclohexane-1,2-diol.

Although only trace amounts of the cyclohexylamine could be detected in humans administered cyclamate, chronic exposure to the acid increased the capacity to produce this metabolite (104). It was found that certain individuals possessed a greater capacity to metabolize cyclamate to cyclohexylamine; these individuals were called converters. Thus, cyclamate is a good example of how prior exposure to a xenobiotic can alter the disposition of the xenobiotic. For additional reading on intestinal microflora xenobiotic metabolism, see (57, 148).

INTEGRATION OF METABOLIC PATHWAYS

To understand a complex system, it is necessary to reduce it to its basic components and study each component separately. After achieving an understanding of the components, it is important to integrate them back into the whole. Xenobiotic metabolism is a complex system. Now that the reader has examined each component of the system in detail, let us examine how they act in concert to protect an organism from toxic injury. Specific examples of integrated biotransformations will be presented.

Bromobenzene

Bromobenzene, an industrial solvent, produces centrilobular necrosis in the rat liver. Bromobenzene's hepatotoxicity results from metabolic activation of the parent compound via epoxidation (Figure 3.27) catalyzed by P450. Bromobenzene-3,4-epoxide is stable enough to

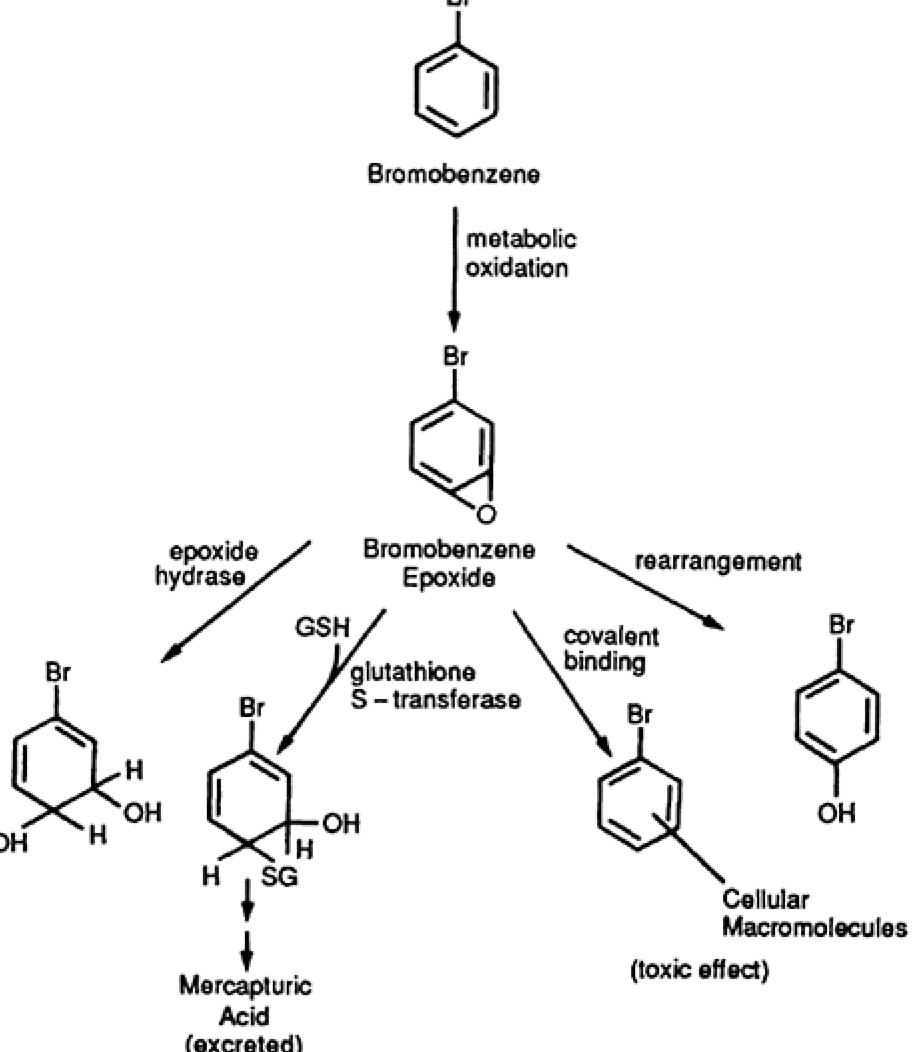

FIG. 3.27. Routes of disposition of bromobenzene. From Reference 93.

diffuse from its site of formation in the endoplasmic reticulum. It can then follow several biodisposition routes, depending on a variety of factors. The epoxide is a substrate for epoxide hydrolase, which would catalyze its hydration to the diol (Figure 3.27). It is also a substrate for glutathione *S*-transferase, which catalyzes thioether formation. The glutathione conjugate is processed by the kidney into its corresponding mercapturic acid, which is excreted in the urine (Figure 3.18). The epoxide can also undergo spontaneous rearrangement to form *p*-bromophenol. This metabolite can cycle through oxidation by P450. If the phase II reactions do not trap the reactive epoxide metabolite formed from the phase I metabolism of bromobenzene, the electrophilic carbon of the epoxide will react nonenzymatically with nucleophilic sites on cellular macromolecules. The ability of electrophilic metabolites to react covalently with critical cellular macromolecules is responsible for many chemical-induced toxicities, including chemical carcinogenicity. The delicate balance between detoxication and metabolic activation determines whether a chemical will be toxic to an organism and in what tissue. Toxic doses of bromobenzene produce centrilobular hepatic necrosis, whereas benzo[a]pyrene, which is activated via a similar mechanism, produces carcinogenicity. Many xenobiotics that are activated to mutagens and are tumorigenic are also cytotoxic.

Multiple Pathways for Xenobiotic Activation

Three different pathways in which chemicals can be nephrotoxic have been presented: oxidation by P450, formation of an episulfonium ion of a cysteine conjugate, and oxidation by prostaglandin synthetase. Very often, the toxicity of a xenobiotic results from more than one route of activation, even within the same organ. For example, acetaminophen has been associated with analgesic nephropathy. Acetaminophen can be activated by P450 and prostaglandin synthetase to a reactive metabolite capable of binding to macromolecules. The realization that a pathway other than the wellcharacterized P450 route was involved occurred through observations that acetaminophen was covalently bound in the inner medulla of the rabbit kidney, a site nearly devoid of P450 activity (117); however, large in vivo doses of acetaminophen produce only damage in the kidney cortex because prostaglandin synthetase is inhibited by these large doses. The P450 pathway is not inhibited and is responsible for the cortical damage. Aspirin and indomethacin are very specific inhibitors of prostaglandin synthetase. Analgesic preparations containing aspirin and one of the analgesics known to be activated by prostaglandin synthetase would probably show considerably less toxicity to the medullary tissue of the kidney. This gradient of oxidation pathways across the kidney, with the cortex possessing higher P450 activity than prostaglandin synthetase, the outer medulla being intermediate, and the inner medulla possessing far greater prostaglandin synthetase activity, results in certain xenobiotics being more toxic to one region of the kidney than another (192). These examples stress the importance of understanding the metabolic activation of a toxicant in explaining its mechanism of action.

Another example of competing pathways is acrylate ester metabolism. Many acrylate esters are good substrates for both alkyl esterase and glutathione S-transferase, and can react with glutathione nonenzymatically. Depending on the dose and route of administration of these compounds, these pathways compete for acrylate metabolism. The alkyl esterase hydrolyzes the acrylate ester to acrylic acid, and the corresponding alcohol (Figure 3.22) and glutathione adds to the β-carbon of the acrylate ester. Both pathways are considered detoxication pathways; however, at high doses glutathione can be depleted faster than it can be resynthesized. Elimination of the glutathione pathway can result in saturation of the esterase pathway and an accumulation of the acrylate ester at the site of administration producing tissue damage.

QUESTIONS

1. During the development of a new pesticide, it was decided that the introduction of a hydroxyl group onto the molecule would make the compound more water soluble. This had advantages in making the spraying of the pesticide simpler. An initial study of the plasma concentrations of the nonhydroxylated analogue in rats had been completed in anticipation of beginning a subchronic toxicity study. When the plasma concentrations of the less lipophilic, hydroxylated pesticide were determined, it was found that the plasma concentrations were maintained for a longer period of time than with the nonhydroxylated analogue. How would you explain this finding?
2. As a toxicologist, you have been asked to design a program to assess the potential hazard of a chemical. What type of information concerning its metabolism would you want to have before you design the hazard assessment program? Based on the metabolism information you have requested, how would you choose the species to be used in the hazard assessment program?
3. A cancer chemotherapeutic drug has been shown effective in treating a specific type of cancer; however, the drug is also cytotoxic and produces severe side effects if it is not rapidly metabolized

by cytochromes P450. Therefore, it is important to not treat a patient with doses of the drug that are too high for the patient's capacity to rapidly metabolize it to the less toxic product. What characteristics of the patient should be considered when attempting to choose a dose that will minimize the side effects?

4. A compound is functionalized by the P450 system and then forms sulfate and glucuronide conjugates and a mercapturic acid before being excreted. How may its metabolism be altered as the dose is increased from a No Observable Effect Level (NOEL) to a dose that produces severe toxicity?

5. Many chemical carcinogens are metabolized by routes that represent detoxication and by other routes that represent metabolic activation. What are the various phenomena that may shift the balance between detoxication and metabolic activation?

REFERENCES

1. Ambrosone, C.B., Coles, B.F., Freudenheim, J.L., and Shields,P. G. (1999): Glutathione S-transferase (GSTM1) genetic polymorphisms do not affect human breast cancer risk regardless of dietary antioxidants. *J. Nutr.*, 129:565–568.

2. Akerboom, T.P.M., and Sies, H. (1981): Assay of glutathione, glutathione disulfide, glutathione mixed disulfides in biological samples. In: *Methods in Enzymology*, *Vol. 77*, *Detoxication and Drug Metabolism: Conjugation and Related Systems.*, edited by S.P.Colowick and N.O.Kaplan, p. 376. Academic Press, New York.

3. Anders, M.W. (1988): Glutathione-dependent toxicity: Biosynthesis and bioactivation of cytotoxic S-conjugates. *ISI Atlas Pharmacol.*, 2:99–104.

4. Anders, M.W., Lash, L., Dekant, W., Wlfarra, A.A., and Dohn, D.R. (1988): Biosynthesis and biotransformation of glutathione S-conjugates to toxic metabolites. *CRC Crit. Rev. Toxicol.*, 18:311–341.

5. Andersson, B., Nordenskjold, M., Rahimtula, A., and Moldeus, P. (1982): Prostaglandin synthetase-catalyzed activation of phenacetin metabolites to genotoxic products. *Mol. Pharmacol.*, 22:479–485.

6. Axelrod, J. (1983): The discovery of the microsomal drug-metabolizing enzymes. In: *Drug Metabolism and Distribution: Current Reviews in Biomedicine 3,* edited by J.W. Lamble, pp. 1–6. Elsevier Biomedical Press, New York.

7. Backes, W.L. (1993): NADPH-cytochrome P450 reductase: Function. In: *Cytochrome P450*, edited by J.B.Schenkman and H.Greim, pp. 15–34. Springer-Verlag, Berlin.

8. Bernhardt, R. (1995): Cytochrome P450: Structure, function, and generation of reactive oxygen species. *Rev. Physiol. Biochem. Pharmacol.*, 127:137–221.

9. Bertilsson, L., Dahl, M.L., Sjoqvist, F., Abergwisted, A., Humble, M., Johansson, I., Lundqvist, E., and Ingelman-Sundberg, M. (1993): Molecular basis for rational megaperscribing in ultrarapid hydroxylators of debrisoquine. *Lancet*, 341:63.

10. Blanck, J., and Ruckpaul, K. (1993): Lipid-protein interactions. In: *Cytochrome P450*, edited by J.B.Schenkman and H.Greim, pp. 581–597. Springer-Verlag, Berlin.

11. Bock, K.W., Gschaidmeier, H., Heel, H., Lehmkoster, T., Munzel, P.A., Raschko, F., and Bock-Hennig, B. (1998): Ah receptor-controlled transcriptional regulation and function of rat and human UDP-glucuronosyltransferase isoforms. *Adv. Enzyme Regul.*, 38:207–222.

12. Borhan, B., Jones, A.D., Pinot, F., Grant, D.G., Kurth, M.J., and Hammock, B.D. (1995): Mechanism of soluble epoxide hydrolase: Formation of an α-hydroxy ester-enzyme intermediate through ASP-333. *J. Biol. Chem.*, 270:26923–26930.

13. Boxenbaum, H.G., Bedersky, I., Jack, M.L., and Kaplan, S.A. (1979): Influence of gut microflora on bioavailability. *Drug Metab. Rev.*, 9:259–279.

14. Boyd, J.A., and Eling, T.E. (1981): Prostaglandin endoperoxide synthetase-deficient cooxidation of acetaminophen to intermediates which covalently bind in vitro to rabbit renal medullary microsomes. *J. Pharmacol. Exp. Ther.*, 219:659–664.

15. Boyland, E., and Chasseaud, L.F. (1969): The role of glutathione and glutathione S-transferases in mercapturic acid biosynthesis. *Adv. Enzymol*, 32:173–219.

16. Braumann and Preusse, (1879): Ueber bromophenylmercaptursaure. *Ber. Dtsch. Chem. Ges.*, 12:806–810.

17. Bresnick, G. (1993): Induction of cytochromes P450 1 and P450 2 by xenobiotics. In: *Cytochrome P450*, edited by J.B.Schenkman and H.Greim, pp 503–524. Springer-Verlag, Berlin.

18. Brockmoller, J., Cascorbi, I., Kerb, R., Sachse, C., and Roots, I. (1998): Polymorphisms in xenobiotic conjugation and disease predisposition. *Toxicol. Lett.*, 102–103:173–183.

19. Brown, R.R., Miller, J.A., and Miller, E.C. (1954): The metabolism of methylated aminoazo dyes. IV. Dietary factors enhancing demethylation in vitro. *J. Biol. Chem.*, 209:211–217.

20. Burchell, B., and Coughtrie, M.W.H. (1989): UDPglucuronosyltransferases. *Pharmacol. Ther.*, 43:261–289.
21. Burchell, B., Ebner, T., Baird, S., Bin Senati, S., Clark, D., Brierley, C., and Sutherland, L. (1994): Use of cloned and expressed human liver UDP-glucuronosyltransferases for analysis of drug glucuronide formation and assessment of drug toxicity. *Environ. Health Perspect*, 102 Suppl 9:19–23.
22. Caldwell, J. (1980): Comparative aspects of detoxication in mammals. In: *Enzymatic Basis of Detoxication*, edited by W.B. Jakoby, pp. 85–114. Academic Press, New York.
23. Calvin, C., Holzhauser, D., Constable, A., Huyggett, A.C., and Schilter, B. (1998): The coffee-specific diterpenes cafestol and kahweol protect against aflatoxin B1 induced genotoxicity through a dual mechanism. *Carcinogenesis*, 19:1369–1375.
24. Chasseaud, L.F. (1973): The nature and distribution of enzymes catalyzing the conjugation of glutathione with foreign compounds. *Drug Metab. Rev.*, 2:185–220.
25. Chasseaud, L.F. (1978): The role of glutathione and glutathione S-transferases in the metabolism of chemical carcinogens and other electrophilic agents. *Adv. Cancer Res.*, 29:175–274.
26. Chen, G., Baron, J., and Duffet, M.W. (1995): Enzyme and sex-specific differences in the interlobular localization and distributions of aryl sulfotransferase IV (tyrosine-ester sulfotransferase) and alcohol (hydroxysteroid) sulfotransferase a in rat liver. *Drug Metab. Dispos.*, 12:1346–1353.
27. Cherrignton, J.J., Cao, Y., Cherrington, J.W., Rose, R.L., and Hodgson, E. (1998): Physiological factors affecting protein expression of flavin-containing monooxygenases 1, 3 and 5. *Xenobiotica*, 28:673–682.
28. Chiu, S.H., and Huskey, S.W. (1998): Species differences in N-glucuronidation. *Drug Metab. Dispos.*, 26:838–847.
29. Ciotti, M., Obaray, R., Martin, M.G., and Owens, I.S. (1997): Genetic defects at the UGT1 locus associated with Crigler-Naffar type I disease, including a prenatal diagnosis. *Am. J. Med. Genet.* 68:173–178.

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30. Conklin, D.J., Langford, S.D., and Boor, P.J. (1998): Contribution of serum and cellular semicarbazide-sensitive amine oxidase to amine metabolism and cardiovascular toxicity. *Toxicol. Sci.*, 46:386–392.
31. Conney, A.H. (1967): Pharmacological implications of microsomal enzyme induction. *Pharmacol. Rev.*, 19:317–350.
32. Cooper, D.Y. (1964): Photochemical action spectrum of the terminal oxidase of mixed function oxidase systems. *Science*, 147:400–402.
33. Cundy, K.C., Sato, M., and Crooks, P.A. (1985): Sterospecific in vivo N-demethylation of nicotine in the guinea pig. *Drug Metab.*, 13:175–185.
34. Daly, A.K., Brockmuller, J., Broly, F., Eichelbaum, M., Evans,W. E., Gonzalez, F.J., Huang, J, D., Idle, J.R., Ingelman-Sundberg, M., Ishizaki, T., Jacqzaigrain, E., Meyer, U.A., Nebert,D. W., Steen, V.M., Wolf, C.R., and Zanger, U.M. (1996): Nomenclature for human CYP2D6 alleles. *Pharmacogenetics*, 6:193–201.
35. deBethizy, J.D., Sherrill, J.M., Rickert, D.E., and Hamm, T.E., Jr. (1983): Effects of pectin containing diets on the hepatic macromolecular covalent binding of 2,6-dinitro[3H]toluene in Fischer-344 rats. *Toxicol. Appl. Pharmacol.*, 69:369–376.
36. Degtyarenko, K.N., and Archakov, A.I. (1993): Molecular evolution of P450 superfamily and P450-containing monooxygenase systems. *Fed. Eur. Biochem. Soc. Let.*, 332:1–8.
37. De Meio, R.H. (1975): Sulfate activation and transfer. In: *Metabolism Pathways, Vol.* 7, Edition 3, edited by D.M.Greenberg, pp. 287–385. Academic Press, New York.
38. Dooley, T.P., and Huang, Z. (1996): Genomic organization and DNA sequences of two human phenol sulfotransferase genes (STP1 and STP2) on the short arm of chromosome 16. *Biochem. Biophys. Res. Commun.*, 228:134–140.
39. Dutton, G.J. (1971): Glucuronide-forming enzymes. In: *Handbook of Experimental Pharmacology. Part 2*, p. 378. SpringerVerlag, New York.
40. Dutton, G.J. (1980): *Glucuronidation of Drugs and Other Compounds*. CRC Press, Boca Raton, Florida.
41. Elfarra, A.A., and Anders, M.W. (1984): Commentary: Renal processing of glutathione conjugates—Role in nephrotoxicity. *Biochem. Pharmacol.*, 33:3729–3732.
42. Estabrook, R.W., Cooper, D.Y., and Rosenthal, O. (1963): The light reversible carbon monoxide inhibition of the steroid C-21-hydroxylase system of adrenal cortex. *Biochem. Z.*, 338:741–755.
43. Estabrook, R.W. (1996): Cytochrome P450: From a single protein to a family of proteins—with some personal reflections. In: *Cytochromes P450: Metabolic and Toxicological Aspects.*, edited by C.Ioannides, pp. 3–28. CRC Press, Boca Raton.
44. Evans, D.A.P. (1989): N-Acetyltransferase. *Pharmacol. Ther.*, 42:157–234.
45. Falany, C.N. (1991): Molecular enzymology of human liver cytosolic sulfotransferases, *Trends Pharmacol. Sci.*, 12:255–259.
46. Fiala, E.S., Sodum, R.S., Hussain, N.S., Rivenson, A., and Dolan, L. (1995): Secondary nitroalkanes: Induction of DNA repair in rat hepatocytes, activation by aryl sulfotransferase and hepatocarcinogenicity of 2-nitrobutane and 3-nitropentane in male F344 rats. *Toxicology*, 99:89–97.
47. Flesher, J.W., Horn, J., and Lehner, A.F. (1997): 6-sulfooxymethylbenzp(a)pyrene is an ultimate electrophilic and carcinogenic form of the intermediary metabolite 6-hydroxymethylbenzo(a)pyrene. *Biochem. Biophys. Res. Commun.*, 234:554–558.
48. Friedberg, T., Lollmann, B., Becker, R., Holler, R., and Oesch, F. (1994): The microsomal epoxide hydrolase has a single membrane signal anchor sequence which is dispensable for the catalytic activity of this protein. *Biochem. J.*, 303:967–972.
49. Frommer, U., Ullrich, V., Staudinger, H., and Orrenius, S. (1972): The monooxygenation of n-heptane by rat liver microsomes. *Biochem Biophys. Acta*, 280:487–494.
50. Fukami, J.I. (1984): Metabolism of several insecticides by glutathione S-transferase. *Int. Encycl. Pharmacol. Ther.*, 113:223–264.
51. Ganem, L.G., Trottier, E., Anderson, A., and Jefcoate, C.R. (1999): Phenobarbital induction of CYP2B1/2 in primary hepatocytes: Endocrine regulation and evidence for a single pathway for multiple inducers. *Tox. Appl. Pharmacol.*, 155:32–42.
52. Garfinkel, D. (1958): Studies on pig liver microsomes. I. Enzymic and pigment composition of different microsomal fractions. *Arch. Biochem. Biophys.*, 77:493–509.
53. Gertig, D.M., Stampfer, M., Haiman, C., Hennekens, X.H., Kelsey, K., and Huner, D.J. (1998): Glutathione S-transferase GSTM1 polymorphisms and colorectal cancer risk: A prospective study. *Cancer*

Epidemiol. Biomarker Prev., 11:1001–1005.
54. Gillette, J.R., Brodie, B.B., and La Du, B.N. (1957): The oxidation of drugs by liver microsomes: On the role of TPNH and oxidase. *J. Pharmacol. Exp. Ther.*, 119–540.
55. Glatt, H. (1997): Bioactivation of mutagens via sulfation. *FASEB J.*, 11:314–321.
56. Glatt, H., Bartsch, I., Coughtrie, M.W., Falany, C.N., Hagen, M., Landsiedels, R., Pabel, U., Phillips, D.H., Seidel, A., and Yamazoe, Y. (1998): Sulfotransferase-mediated activation of mutagens studied using a single membrane signal anchor sequence which is dispensable for the catalytic activity of this protein. *Chem. Biol Interact.*, 109:195–219.
57. Goldman, P. (1978): Biochemical pharmacology of the intestinal flora. *Annu. Rev. Pharmacol.*, 18:523–539.
58. Gonzalez, F.G., and Nebert, D.W. (1990): Evolution of the P450 gene superfamily: Animal-plant warfare, molecular drive and human genetic differences in drug oxidation. *Trends in Genetics*, 6:182–186.
59. Gonzales, F. (1993): Cytochrome P450: Evolution and nomenclature. In: *Cytochrome P450*, edited by J.B.Schenkman and H.Greim, pp. 211–219. Springer-Verlag, Berlin.
60. Gorsky, L.D., Koop D.R., and Coon, M.J. (1984): On the stoichiometry of the oxidase and monooxidase reaction catalyzed by liver microsomal cytochrome P450. *J. Biol. Chem.*, 259:6812–6817.
61. Green, M.D., and Tephly, T.R. (1998): Glucuronidation of amine substrates by purified and expressed UDP-glucuronosyltransferase proteins. *Drug Metab. Dispos.*, 26:860–867.
62. Guengerich, F.P. (1996): The chemistry of cytochrome P450 reactions. In: *Cytochrome P450: Metabolic and Toxicological Aspects*, edited by C.Ioannides, pp. 55–74. CRC Press, Boca Raton.
63. Gueraud, F., and Paris, A. (1998): Glucuronidation: A dual control. *Gen. Pharmacol.*, 31:683–688.
64. Gut, J. (1982): Rotation of cytochrome P450 II: Specific interactions of cytochrome P450 with NADPH-cytochrome P450 reductase in phospholipid vesicles. *J. Biol. Chem.*, 257:7030–7036.
65. Habig, W.H. (1982): Glutathione S-transferases: Versatile enzymes of detoxification. In: *Radioprotectors and Anticarcinogens*, edited by O.F.Nygaard, pp. 169–190. Academic Press, New York.
66. Haenen, G.R.M., Vermeulen, N.P.E., Tai Tin Tsoni, J.N.L., Regetti, H.M.N., Timmerman, H., and Bast, A. (1988): Activation of the microsomal glutathione S-transferase and reduction of the glutathione dependent protection against lipid peroxidation by acrolein. *Biochem. Pharmacol.*, 37:1933–1938.

67. Hayes, P.C., Bouchier, I.A.D., and Becket, G.J. (1991): Glutathione S-transferases in human health and disease. *Gut*, 32:813–818.
68. Henry, W. (1815): *The Elements of Experimental Chemistry*. Vol. II, Edition 7, p. 352. Baldwin, Cradock and Joy, London.
69. Heyman, E. (1982): Hydrolysis of carboxylic esters and amides. In: *Metabolic Basis of Detoxication*, edited by W.B.Jakoby, J.R. Bend, and J. Caldwell, pp. 229–245. Academic Press, New York.
70. Horecher, B.L. (1950): Triphosphopyridine nucleotide cytochrome c reductase in liver. *J. Biol. Chem.*, 183:593–605.
71. Huttner, W.B. (1982): Sulphation of tyrosine residues: A widespread modification of proteins. *Nature*, 299:273–276.
72. Inskeep, P.B., Koga, N., Cmarik, J.L., and Guengerich, F.P. (1986): Covalent binding of 1,2-dihaloalkanes to DNA and stability of the major DNA adduct, S-[2-(N7-guanyl)ethyl] glutathione. *Cancer Res.*, 46:2839–2844.
73. Ioannides, C. (1996): *Cytochromes P450: Metabolic and Toxicological Aspects.* CRC Press, Boca Raton.
74. Jakoby, W.B. (1978): The glutathione S-transferases: A group of multifunctional detoxification proteins. *Adv. Enzymol. Relat. Areas Mol. Biol.*, 46:383–414.
75. Jakoby, W.B., and Habig, W.H. (1980): Glutathione transferases. In: *Enzymatic Basis of Detoxification, Vol. II*, edited by W.B. Jakoby, pp. 63–94. Academic Press, New York.
76. Jakoby, W.B., and Keen, J.H. (1977): A triple-threat in detoxification: The glutathione S-transferases. *Trends Biochem. Sci.*, 2:229–231.
77. Jakoby, W.B., Duffel, M.W., Lyon, E.S., and Ramaswamy, S. (1984): Sulfotransferases active with xenobiotics: Comments on mechanism. In: *Progress in Drug Metabolism, Vol. 8,* edited by J.W.Bridges and L.F.Chasseaud, pp. 11–33. Taylor and Francis, London.
78. Jakoby, W.B. (1976): Glutathione S-transferases: Catalytic aspects. In: *Glutathione Metabolism and Function*, edited byI. M.Arias and W.B.Jakoby, pp. 189–211. Raven Press, New York.
79. Jakoby, W.B. (1980): Sulfotransferases. In: *Enzymatic Basis of Detoxification, Vol.* II., edited by W.B.Jakoby, pp. 199–228. Academic Press, New York.
80. Johansson, I., Lundquist, E., Bertilsson, L., Dahl, M.-L., Sjoqvist, F., and Ingrelman-Sundberg, M. (1993): Inherited amplification of an active gene in the cytochrome P450 CYP2D locus as a cause of ultrarapid metabolism of debrisoquine. *Proc. Nat. Acad, Sci.*, USA, 90:11825–11829.
81. Kasper, C.B., and Henton, D. (1980): Glucuronidation. In: *Enzymatic Basis of Detoxification*, Vol. II, edited by W.B.Jakoby, pp. 3–41. Academic Press, New York.
82. Kedderis, G.L., Dyroff, M.C., and Rickert, D.E. (1984): Hepatic macromolecular covalent binding of the hepatocarcinogen 2,6-dinitrotoluene and its 2,4-isomer in vivo: Modulation by the sulfotransferase inhibitors pentachlorophenol and 2, 6-dichloro-4-nitrophenol. *Carcinogenesis*, 5:1199–1204.
83. Keller, W. (1842): Ueber verwandlung der Benzoesaure in hippursaure. Justus Liebig's *Ann. Chem.*, 43:108–111.
84. Kilty, C., Doyle, S., Hassett, B., and Manning, F. (1998): Glutathione S-transferases as biomarkers of organ damage: Applications of rodent and canine GST enzyme immunoassays. *Chem. Biol. Interact.*, 112:123–135.
85. Klaassen, C.D., and Boles, J.W. (1997): The importance of 3′-phosphoadenosine 5′-phosphosulfate (PAPS) in the regulation of sulfation. *FASEB J.*, 11:404–418.
86. Klingenberg, M. (1958): Pigments of rat liver microsomes. *Arch. Biochem Biophys.*, 75:379–386.
87. Koob, M., and Dekant, W. (1991): Bioactivation of xenobiotics by formation of toxic glutathione conjugates. *Chem. Biol. Interact.*, 77:107–136.
88. Kuriyama, Y., Omura, T., Siekevitz, P, and Palade, G.E. (1969): Effects of phenobarbital on the synthesis and degradation of protein components of rat liver microsomal membranes. *J. Biol. Chem.*, 244:2017–2026.
89. La Du, B.N., Gaudette, L., Trousof, N., and Brodie, B.B. (1955): Enzymatic dealkylation of aminopyrine (Pyramidon) and other alkylamines. *J. Biol. Chem.*, 214:741–752.
90. Lamartiniere, C.A. (1981): The hypothalamic-hypophyseal-gonadal regulation of hepatic glutathione S-transferases in the rat. *Biochem. J.*, 198:211–217.
91. Lamartiniere, C.A., and Lucier, G.W. (1983): Endocrine regulation of xenobiotic conjugation enzymes. *Basic Life Sci.*, (Organ Species Specif. Chem. Carcinog.): 295–312.
92. Lancaster, J.M., Brownlee, H.A., Bell, D.A., Futreahs, R.A., Marks, J.R., Berchuchk, A., Wiseman, R.W., and Taylor,J. A. (1996): Microsomal epoxide hydrolase polymorhphisms as a risk factor for ovarian

cancer. *Mol. Carcinog.*, 3:160–162.
93. Laurenzana, E.M., Hassett, C., and Omiecinski, C.J. (1998): Post-transcriptional regulation of human microsomal epoxide hydrolase. *Pharmacogenetics*, 8:157–167.
94. Lenoard, T.B., and Popp, J.A. (1981): Investigation of the carcinogenic initiation potential of dinitrotoluene: Structure-activity study. *Proc. Am. Assoc. Cancer Res.*, 22:82.
95. Lewis, D.F.V. (1996): *Cytochromes P450: Structure, Function and Mechanism.* Taylor and Francis, London.
96. Lewis, D.F.V. (1998): The CYP2 family: Models, mutants and interactions. *Xenobiotica*, 28:617–661.
97. Lin, D.X., Tang, Y.M., Peng, Q., Lu, S.X., Ambrosone, C.B., Kadlubar, F.F. (1998): Susceptibility to esophageal cancer and genetic polymorphisms in glutathione S-transferases T1, P1 and Ml and cytochrome P450 2E1. *Cancer Epidemiol. Biomarkers Prev.*, 11:1013–1018.
98. Lui, L., and Klaassen, C.D. (1996): Different mechanism of saturation of acetaminophen sulfate conjugation in mice and rats. *Toxicol. Appl. Pharmacol.*, 139:128–134.
99. Long, R.M., and Rickert, D.E. (1982): Metabolism and excretion of 2, 6-dinitro-[14C]toluene in vivo and in isolated perfused rat livers. *Drug Metab. Dispos.*, 10:455–458.
100. Lu, A.Y.H, Strobel, H.W., and Coon, M.J. (1969): Hydroxylation of benzphetamine and other drugs by a solubilized form of cytochrome P450 from liver microsomes: Lipid requirement for drug demethylation. *Biochem. Biophys. Res. Commun.*, 36:545–551.
101. Lu, A.Y.H., Strobel, A.H.W., and Coon, M.J. (1970): Properties of a solubilized form of the cytochrome P450-containing mixed-function oxidase of liver microsomes. *Mol. Pharmacol.*, 6:213–220.
102. Lyles, G.A. (1996): Mammalian plasma and tissue-bound semicarbazide sensitive amine oxidase: Biochemical, pharmacological and toxicological aspects. *Int. J. Biochem. Cell Biol.*, 28:259–274.
103. Mahgoub, A., Idles, J.R., Dring, L.G., Lancaster, R., and Smith, R.L. (1977): Polymorphic hydroxylation of debrisoquine in man. *Lancet*, 2:584–586.
104. Mallett, A.K. (1985): Metabolic adaptation of rat faecal microflora to cyclamate in vitro. *Fd. Chem. Toxicol.*, 23:1029–1034.
105. Mandell, H.G. (1981): Pathways of drug biotransformation: Biochemical conjugations. In: *Fundamentals of Drug Metabolism and Drug Disposition*, edited by B.N.La Du, H.G.Mandel, and E.L.Way, pp. 169–171. Robert E.Kreiger, Malabar, Florida.
106. Mannering, G.T. (1971): Microsomal enzyme systems which catalyze drug metabolism. In: *Fundamentals of Drug Metabolism and Drug Disposition*, edited by B.N.La Du, H.G.Mandel, and E.L.Way, pp. 206–252. Robert E.Kreiger, Malabar, Florida.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_133.html[2010/10/28 18:41:03]

107. Marnett, L.J., and Reed, G.A. (1979): Peroxidative oxidation of benzo(a)pyrene and prostaglandin biosynthesis. *Biochemistry*, 18:2923–2929.
108. Marnett, L.J., Reed, G.A., and Johnson, J.T. (1977): Prostaglandin synthetase dependent benzo(a)pyrene oxidation: Products of the oxidation and inhibition of their formation by antioxidants. *Biochem. Biophys. Res. Commun*., 79:569–576.
109. Mattammal, M.B., Zenser, T.V., and Davis, B.B. (1981): Prostaglandin hydroperoxidase-mediated 2-amino-4-(5-nitro-2furyl)[14C]thiazole metabolism and nucleic acid binding. *Cancer Res.*, 41:4961–4966.
110. Meech, R., and Mackenzie, P.I. (1997): Structure and function of uridine diphosphate glucuronosyltransferases. *Clin. Exp. Pharmacol. Physiol.*, 24:907–915.
111. Meister, A., and Tate, S.S. (1976): Glutathione and related gamma-glutamyl compounds: Biosynthesis and utilization. *Annu. Rev. Biochem.*, 45:559–604.
112. Miller, M.S., Juchau, M.R., Guengerich, P., Nebert, D.W., and Raucy, J.L. (1996): Symposium overview: Drug metabolism enzymes in developmental toxicology. *Fund, Appl Toxicol.*, 34:165–175.
113. Miners, J.O., and Mackenzie, P.I. (1991): Drug glucuronidation in humans. *Pharmacol. Ther.*, 51:347–369.
114. Mirsalis, J.C., and Butterworth, B.E. (1982): Induction of unscheduled DNA synthesis in rat hepatocytes following in vivo treatment with dinitrotoluene. *Carcinogenesis*, 3:241–245.
115. Mitchell, J.R. (1973): Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. *J. Pharacol. Exp. Ther.*, 187:211–217.
116. Miyazaki, M., Yoshizawa, I.I., and Fishman, J. (1969): Direct methylation of estrogen catechol sulfates. *Biochemistry*, 8:1669–1672.
117. Mohandas, J., Duggin, G.G., Horvath, J.S., and Tiller, D.J. (1981): Metabolic oxidation of acetaminophen (paracetamol) mediated by cytochrome P450 mixed-function oxidase and prostaglandin endoperoxide synthetase in rabbit kidney. *Toxicol. Appl. Pharmacol.*, 61:252–259.
118. Monks, T.J., Anders, M.W., Dekant, W., Stevens, J.L., Lau, S.S., and van Bladeren, P.J. (1990): Glutathione conjugate mediated toxicities. *Toxicol. Appl. Pharmacol.*, 106:1–19.
119. Morgan, E.T., and Coon, M.J. (1984): Effects of cytochrome b5 on cytochrome P450-catalyzed reactions: Studies with manganese-substituted cytochrome b5. *Drug Metab. Dispos.*, 12:358–364.
120. Mugford, C.A., and Kedderis, G.L. (1998): Sex-dependent metabolism of xenobiotics. *Drug Metab. Rev.*, 30:441–498.
121. Mulder, G.J. (1981): Introduction. In: *Sulfation of Drugs and Related Compounds*, edited by G.J.Mulder, pp. 1–3. CRC Press, Boca Raton, Florida.
122. Mulder, G.J. (1981): Generation of reactive intermediates from xenobiotics by sulfate conjugation: Their potential role in chemical carcinogenesis. In: *Sulfation of Drugs and Related Compounds*, edited by G.J.Mulder, pp. 213–226. CRC Press, Boca Raton, Florida.
123. Munzel, P.A., Bruck, M., and Bock, K.W. (1994): Tissue-specific constitutive and inducible expression of rat phenol UDP-glucuronosyltransferase. *Biochem. Pharmacol.*, 8:1445–1448.
124. Nelson, D.R., Koymang, L., Kamataki, T., Stegeman, J.J., Feyerelsin, R., Waxman, D.J., Waterman, M.R., Gotoh, O., Coon, M.J., Estabrook, R.W., Gunsalus, I.C., and Nebert, D.W. (1996): P450 superfamily: Update on new sequences, gene mapping, accession numbers, and nomenclature. *Pharmacogenetics*, 6:1–42.
125. Nuccetelli, M., Mazzetti, A.P., Rossjohn, J., Parker, M.W., Beard, P., Caccuri, A.M., Federiai, G., Ricci, G., and LoBello, M. (1998): Shifting substrate specificity of human glutathione transferase (from class Pi to class alpha) by a single point mutation. *Biochem. Biophys. Res. Commun*., 252:184–189.
126. Oesch, F. (1980): Species differences in activating and inactivating enzymes related to in vitro mutagenicity mediated by tissue preparations from these species. *Arch. Toxicol.*, Suppl. 3:179–194.
127. Oesch, F., Bentley, P., and Glatt, H.R. (1970): Prevention of benzp(a)pyrene induced mutagenicity by homogenous epoxide hydratase. *Int. J. Cancer*, 18:448–452.
128. Oesch, F., and Glatt, H.R. (1976): Evaluation of the importance of enzymes involved in the control of mutagenic metabolites. *IARC Sci. Publ.*, 1232:255–274.
129. Omiecinski, C.J., Aicher, L., and Swenson, L. (1994): Developmental expression of human microsomal epoxide hydrolase. *J. Pharmacol. Exp. Ther.*, 269:417–423.
130. Omura, T. (1969): Discussion. In: *Microsomes and Drug Oxidations*, edited by J.R.Gillette, pp. 160–161. Academic Press, New York.
131. Omura, T., and Sato, R. (1964): The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.*, 239:2370–2378.
132. Omura, T., and Sato, R. (1964): The carbon monoxide-binding pigment of liver microsomes. II.

Solubilization, purification and properties. *J. Biol. Chem.*, 2379–2385.
133. Ortiz de Montellano, P.R. (1986): Oxygen activation and transfer. In: *Cytochrome P450 Structure, Mechanism and Biochemistry*, edited by P.R.Ortiz de Montellano, pp. 217–271. Plenum Press, New York.
134. Ortiz de Montellano, P.R. (1986): *Cytochrome P450 Structure, Mechanism and Biochemistry.* Plenum Press, New York.
135. Pelkonen, O., Maenpan, J., Taavitsainen, P., Rautio, A., and Rautio, H. (1998): Inhibition and induction of human cytochrome P450 (CYP) enzymes. *Xenobiotica*, 28:1203–1253.
136. Phillips, A.H., and Langdon, R.G. (1962): Hepatic triphosphopyridine nucleotide-cytochrome reductase: Isolation, characterization and kinetic studies. *J. Biol. Chem.*, 237A:2652–2660.
137. Poulas, T.L., and Raag, R. (1992): Cytochrome *P450cam:* Crystallography, oxygen activation, and electron transfer. *FASEB J.*, 6:674–679.
138. Rafter, J.J. (1983): Studies on the reestablishment of the intestinal microflora in germ-free rats with special reference to the metabolism of N-isopropyl-alpha-choloracetanilide (Propachlor). *Xenobiotica*, 13:171–178.
139. Raftogianis, R.B., Her, C., and Weinshiboum, R.M. (1996): Human phenol sulfotransferase pharmacogenetics: STP1 gene cloning and structural characterization. *Pharmacogenetics,* 6:473–478.
140. Rein, H., and Jung, C. (1993): Metabolic reactions: Mechanism of substrate oxidation. In: *Cytochrome P450*, edited by J.Schenkman and H.Greim, pp. 106–122. Springer-Verlag, Berlin.
141. Renwick, A.G. (1977): Microbial metabolism of drugs. In: *Drug Metabolism: From Microbe to Man*, edited by D.V.Parke and R.L.Smith, pp. 169–189. Proceeding of the International Symposium, Guilford, England. Taylor and Francis, London.
142. Renwick, A.G., and Williams, R.T. (1972): The fate of cyclamate in man and other species. *Biochem. J.*, 129:869–79.
143. Rowland, I.R., and Wise, A. (1985): The effect of diet on the mammalian gut flora and its metabolic activities. *CRC Crit. Rev. Toxicol.*, 16:31–103.
144. Rowland, I.R. (1988): Factors affecting metabolic activity of the intestinal microflora. *Drug Metab. Rev.*, 19:243–261.

145. Rumney, C.J., and Rowland, I.R. (1992): In vivo and in vitro models of the human colonic flora. *CRC Food Sci. Nutr.*, 31:299–331.
146. Runge-Morris, M.A. (1997): Regulation of expression of the rodent cytosolic sulfotransferases. *FASEB J.*, 11:109–117.
147. Sanglard, D., and Kappeli, O. (1993): Cytochrome P450 in unicellular organisms. In: *Cytochrome P450*, edited by J. Schenkman and H.Greim, pp. 325–349. Springer-Verlag, Berlin.
148. Scheline, R.R. (1973): Metabolism of foreign compounds by gastrointestinal microorganisms. *Pharmacol. Rev.*, 25:451–523.
149. Schenkman, J., and Greim, H. (1993): *Cytochrome P450*. Springer-Verlag, Berlin.
150. Schmiedlin-Ren, P., Edwards, D.J., Fitzsimmons, M.E., He, K., Lown, K.S., Woster, P.M., Rahman, A., Thummel, K.E., Fisher, J.M., Hollenberg, P.F., and Watkins, P.B. (1997): Mechanisms of enhanced oral availability of CYP3A4 substrates by grapefruit constituents: Decreased interocyte CYP3A4 concentration and mechanism-based inactivation by furanocoumarins. *Drug Metab. Disp.*, 25:1228–1233.
151. Sekura, R.D., Marcus, C.J., Lyon, E.S., and Jakoby, W.B. (1979): Assay of sulfotransferases. *Anal. Biochem.*, 95:82–86.
152. Sharer, J.E., Shipley, L.A., Vanderbranden, R.R., Binkley, S.N., and Wrighton, S.A. (1995): Comparisons of phase I and phase II in vitro hepatic enzyme activities of human, dog, rhesus monkey and cynomolgus monkey. *Drug Metab. Dispos.*, 11:1231–1241.
153. Singer, S.S., and Brun, L. (1978): Enzymatic sulfation of steroids—V11. Hepatic cortisol sulfation and glucocorticoid sulfotransferases in old and young male rats. *Exp. Gerontol.*, 13:425–429.
154. Singer, S.S., Giera, D., Johnson, J., and Sylvester, S. (1976): Enzymatic sulfation of steroid. I. The enzymatic basis for the sex difference in cortisol sulfation by rat liver preparations. *Endocrinology*, 98:963–974.
155. Singer, S.S., and Sylvester, S. (1976): Enzymatic sulfation of steroids. II. The control of the hepatic cortisol sulfotransferase activity and of the individual hepatic steroid sulfotransferases of rats by gonads and gonadal hormones. *Endocrinology*, 99:1346–1352.
156. Singh, S.V., Benson, P.J., Hy, X., Pal, A., Srivastava, S.K., Awasthi, S., Zaren, H.A., and Orchard, J.L. (1998): Gender-related differences in susceptibility of A/J mouse to benzo(a)pyrene-induced pulmonary and forestomach tumorigenesis. *Cancer Lett.*, 128:197–204.
157. Sligar, S.G., and Murray, R.I. (1986): Cytochrome P450cam and other bacterial P450 enzymes. In: *Cytochrome P450: Structure, Mechanism, and Biochemistry*, edited by P.R.Ortiz de Montellano, pp. 429–503, Plenum Press, New York.
158. Smith, C.A., and Harrison, D.J. (1997): Association between polymorphism in gene for microsomal epoxide hydrolase and susceptibility to emphysema. *Lancet*, 350:630–633.
159. Smith, G., Stubbins, M.J., Harries, L.W., and Wolf, C.R. (1998): Molecular genetics of human cytochrome P450 monooxygenase superfamily. *Xenobiotica*, 28:1124–1165.
160. Smith, D.A., and Jones, B.C. (1992): Speculations on the substrate structure activity relationship (SSAR) of cytochrome P450 enzymes. *Biochem. Pharmacol.*, 44:2089–2094.
161. Smith, G.J., and Litwack, G. (1980): Roles of ligandin and the glutathione S-transferases in binding steroid metabolites, carcinogens and other compounds. *Rev. Biochem. Toxicol.*, 2:1–47.
162. Smith, G.J., Ohl, V.S., and Litwack, G. (1977): Ligandin, the glutathione S-transferases, and chemically induced hepatocarcinogenesis: A review. *Cancer Res.*, 37:8–14.
163. Sreeravan, L., Hedge, M.W., and Sladek, N.E. (1995): Identification of a class 3 aldehyde dehydrogenase in human saliva and increased levels of this enzyme, glutathione S-transferases, andDT-diaphorase in the saliva of subjects who continually ingest large quantities of coffee or broccoli. *Clin. Cancer Res.*, 1:1153–1163.
164. Stirling, L.A. (1980): Microorganisms and environmental pollutants. In: *Introduction to Environmental Toxicology*, edited by F.E.Guthrie and J.J.Perry, pp. 329–342. Elsevier North Holland, New York.
165. Strassburg, C.P., Nguyen, N., Manns, M.P., and Tukey, R.H. (1998): Polymorphic expression of the UDP-glucuronosyltransferase UGT1A gene locus in human gastric epithelium. *Mol. Pharmacol.*, 54:647–654.
166. Strasser, S.I., Smid, S.A., Mashford, M.L., Desmond, P.V. (1997): Sex hormones differentially regulate isoforms of UDP-glucuronosyltransferase. *Pharm. Res.*, 14:1115–1121.
167. Strolin Benedetti, M., and Dostert, P. (1994): Contribution of amine oxidases to the metabolism of xenobiotics. *Drug Metab. Rev.*, 26:507–535.
168. Tateishi, N., and Sakamoto, Y. (1983): Nutritional significance of glutathione in rat liver. In:

Glutathione: Storage, Transport and Turnover in Mammals, edited by S.Y.Sakamoto, T.Higashi, and N.Tateishi, pp. 13–38. Japan Science Society Press, Tokyo/VNH Science Press, Utrecht.
169. Tateishi, M., Suzuki, S., and Shimizu, H. (1978): Cysteine conjugate beta-lyase in rat liver: A novel enzyme catalyzing formation of thiol-containing metabolites of drugs. *J. Biol. Chem.*, 253:8854–8859.
170. Tephly, T.R., and Burchell, B. (1990): UDP-glucuronosyltransferases: A family of detoxifying enzymes. *Trends Pharmacol. Sci.,* 11:276–279.
171. Thurman, R.G. (1987): Regulation of monooxygenation in intact cells. In: *Mammalian Cytochromes P450, Vol. 11*, edited byF. P.Guengerich, pp. 131–152. CRC Press, Boca Raton, Florida.
172. Tzeng, H.F., Laughlin, L.T., and Armstrong, R.N. (1998): Semifunctional site-specific mutants affecting the hydrolytic half-reaction of microsomal epoxide hydrolase. *Biochemistry*, 37:2905–2911.
173. U.S. FDA (1997): *Guidance for Industry: Drug metabolism/drug interaction studies in the drug development process: Studies in vitro.* The Drug Information Branch, Center for Drug Evaluation and Research, Rockville, Maryland.
174. Vamvakas, S., and Anders, M.W. (1990): Formation of reactive intermediates by phase II enzymes: Glutathione-dependent bioactivation reactions. In: *Biological Reactive Intermediates IV.* edited by C.M.Witmer, et al., pp. 13–24. Plenum Press, New York.
175. vanBladeren, P.J., and van Ommen, B. (1991): The inhibition of glutathione S-transferases: Mechanisms, toxic consequences and therapeutic effects. *Pharmacol. Ther.*, 51:35–46.
176. van Lieshout, E.M., and Peters, W.H. (1998): Age and gender dependent levels of glutathione and glutathione S-transferases in human lymphocytes. *Carciongenesis*, 19:1873–1875.
177. Van Ness, K.P., McHugh, T.E., Bammler, T.K., and Eaton, D.L. (1998): Identification of amino acid residues essential for high aflatoxin BI-8,9-epoxide conjugation activity in alpha class glutathione S-transferases through site-directed mutagenesis. *Toxicol. Appl. Pharmacol.*, 152:166–174.
178. Vesell, E.S., and Penno, M.B. (1983): Intraindividual and interindividual variations. In: *Biological Basis of Detoxication*, edited by J.Caldwell and W.B.Jakoby, pp. 369–410. Academic Press, New York.
179. Visser, T.J., van Buuren, J.C.J., Rutger, M., Rooda, S.J.E., and deHerder, W.W. (1990): The role of sulfation in thyroid hormone metabolism. *Trends Endocrinol. Metab.*, 1:211–218.

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180. Vos, R.A.M.E., and Van Bladeren, P.J. (1990): Glutathione S-transferase in relation to their role in the biotransformation of xenobiotics. *Chem. Biol. Interactions*, 75:241–265.
181. Walker, C.H., and Mackness, M.I. (1983): Esterases: Problems of identification and classification. *Biochem. Pharmacol.*, 32:3265–3269.
182. Walker, C.H., and Oesch, F. (1983): Enzymes in selective toxicology. In: *Biological Basis of Detoxication*, edited by J. Caldwell and W.B.Jakoby, pp. 349–368. Academic Press, New York.
183. Watabe, T., Hiratsuka, A., and Okuda, H. (1985): Sulfate conjugations. *Tok Foramu*, 8:264–277.
184. Watabe, T. (1985): Metabolic activation of 7,12-dimethyl-benz(a)anthracene and 7-methylbenz(a)anthracene via hydroxymethyl sulfate esters by P450-sulfotransferase. *Gann Monogr.*, 30:125–139.
185. Weinshilboum, R. (1992): Methylation pharmacogenetics: Thiopurine methyl transferase as a model system. *Xenobiotica*, 22:1055–1071.
186. Weinshilboum, R.M., Otterness, D.M., Aksoy, I.A., Wood,T. C., Her, C., and Raftoginnis, R.B. (1997): Sulfotransferase molecular biology: cDNAs and genes. *FASEB J.* 11:7–14.
187. Wendel, A., Heinle, H., and Silbernagl, S. (1977): The degradation of glutathione derivatives in the rat kidney. *Curr. Probl. Clin. Biochem.*, 8:73–84.
188. Williams, C.H., Jr., and Kamin, H. (1962): Microsomal triphosphopyridine nucleotide-cytochrome c reductase of liver. *J. Biol. Chem.*, 237:587–595.
189. Williams, R.T. (1959): *Detoxication Mechanisms*, Edition 2. Chapman and Hall, London.
190. Wood, J.I. (1970): Biochemistry of mercapturic acid formation. In: *Metabolic Conjugation and Metabolic Hydrolysis, Vol. 2,* edited by W.H.Fishman, pp. 261–299. Academic Press, New York.
191. Yu, P.H., and Zuo, D.-M. (1997): Formation of formaldehyde form adrenaline: A potential risk factor for stress-related antipathy. *Neurochem. Res.*, 22:615–620.
192. Zenser, T.V., Mattammal, M.B., and Davis, B.B. (1979): Demonstration of separate pathways for the metabolism of organic compounds in rabbit kidney. *J. Pharmacol. Exp. Ther.*, 208:418–421.
193. Zhu, B.T., Suchar, L.A., Huang, M.T., and Conney, A.H. (1996): Similarities and differences in the glucuronidation of estradiol and estrone by UDP-glucuronosyltransferase in liver microsomes from male and female rats. *Biochem Parmacol.*, 51:1195–1202.
194. Ziegler, D.M. (1990): Flavin-containing monooxygenases: Enzymes adapted for multisubstrate specificity. *Trends Pharmacol. Sci.*, 11:321–324.
195. Ziegler, D.M. (1993): Recent studies on the structure and function of multisubstrate flavin-containing monooxygenase. *Ann. Rev. Pharmacol. and Toxicol.*, 33:179–199.

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Chapter **4**

Toxicokinetics: Pharmacokinetics in Toxicology

Andrew Gordon Renwick

Principles and Methods of Toxicology,
Fourth Edition, edited by A.Wallace Hayes.
Taylor & Francis, Philadelphia © 2001.

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The term pharmacokinetics is derived from the Greek words *pharmako* (medicine, drug, or poison) and *kinetikos* (motion or movement). Thus, pharmacokinetics is the study of the movement of drugs within the body, that is, the absorption, distribution via the blood, metabolism, and excretion. This term is in contrast to *pharmacodynamics,* which is concerned with the pharmacological actions of the drug within the body, that is, its effects at the site of action/receptor. The word drug is popularly associated with medicines or therapeutic agents, although for certain subjects, for example, drug metabolism, this word has long been applied to any environmental anutrient, that is, drugs, pesticides, environmental contaminants, and plant products. Because the processes concerned with the absorption, distribution, and elimination of therapeutic drugs are nonspecific and shared with other types of anutrients, the principles of pharmacokinetics apply to any environmental anutrient (xenobiotic). It is thus valid to apply the term *pharmacokinetics* to all foreign compounds, although other terms, such as "chemobiokinetics" (32), have been proposed to describe the application of these principles to nontherapeutic substances. *Toxicokinetics* is receiving increasing and even international (122) usage; it has useful connotations with respect to the nonspecific nature of the toxicant and the implicit requirement for kinetic data at toxic doses. However, most of the basic principles were established in relation to therapeutic drugs, and the introductory chapters of many clinical pharmacology textbooks contain much useful basic information.

The term *toxicokinetics* is frequently misused and applied to nonspecific data, such as autoradiography, and radiolabeled excretion and metabolism studies. Toxicokinetics, in the present context, is the application of pharmacokinetic principles to animal toxicity studies and to human toxicity data in order to provide information on exposure to the parent compound and its metabolites, and other aspects such as accumulation during chronic exposure. The incorporation of data from animal studies into risk assessment requires data from related studies in humans at appropriate doses.

The understanding and interpretation of toxicological findings requires information on two key areas: (a)

delivery of the compound to its site of action (toxicokinetics) and (b) the mechanism of action and potency of the chemical at the site of action (toxicodynamics) (Figure 4.1). Such information may assist in understanding the dose-response relationship in animal toxicity studies and its relevance to humans as well as assisting in the identification of potentially at-risk subgroups of

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IN VIVO DOSE-RESPONSE

TOXICOKINETICS TOXICODYNAMICS

ADMINISTERED DOSE → COMPOUND AT SITE OF ACTION → TOXIC RESPONSE

ABSORPTION
DISTRIBUTION
METABOLISM
EXCRETION

BIOACTIVATION
CELLULAR CHANGES
CYTOPROTECTION

FIG. 4.1. The relationship between in vivo response and toxicokinetics and toxicodynamics.

the exposed human population. Risk assessment has traditionally used different approaches for cancer (and other nonthreshold effects), compared with toxicity believed to show a biological threshold. For cancer, and similar effects, the dose-response relationship in animals is extrapolated down to a very low level of risk, or virtually safe dose; toxicokinetics are frequently incorporated into the process by the use of a physiologically based pharmacokinetic model (PBPK). For threshold toxicity, the approach is to estimate a level of human intake without appreciable health effects; the acceptable daily intake (ADI), or tolerable daily intake (TDI), or reference dose (RfD), for human exposure is derived by dividing the intake of animals treated at the no-observed-adverse-effect level (NOAEL) (on a mg/kg basis) by an appropriate safety or uncertainty factor. A safety factor of 100 is usually applied with a 10-fold factor to allow for extrapolation from animals to humans and a 10-fold factor to allow for interindividual differences in the exposed human population (75, 123). A scheme was proposed (76) that would allow appropriate toxicokinetic or mechanistic data to be incorporated into the derivation of a data-derived safety factor by the replacement of part of the relevant 10-fold default factor. The use of such a scheme would produce a more secure basis for the establishment of an ADI/TDI/RfD, while providing a direct return for the investment necessary to produce more than the minimum toxicity database required for regulatory purposes. The greater use of toxicokinetic data in regulatory decisions will provide a more scientific basis for risk assessment, and will encourage the increased generation of such data.

Toxicokinetic studies are important in compound development, and such information is regarded as necessary before proceeding with long-term and carcinogenicity tests (101). If the kinetic evidence indicates tissue accumulation on prolonged dosing, saturation of elimination at subtoxic doses, or the formation of chemically reactive metabolites, chemical analogs without these problems may be selected for development, because these properties mitigate against a high therapeutic index (for drugs) or ADI (for food additives).

Toxicokinetics is concerned with the relationship between the external dose, as usually measured in toxicity studies (e.g., mg/kg body weight per day) and a measure of the internal dose of active compound delivered to the target for toxicity (Figure 4.1), such as the concentration in the general circulation or at the target for toxicity. Frequently the compound administered has to pass many lipid and metabolic barriers prior to reaching the target, as shown in Figure 4.2. An understanding of the extent and nature of these processes may be derived from serial analysis of the concentrations of the chemical in plasma and urine. A knowledge of the concentrations of the parent compound and any metabolites in plasma and tissues, allied to the rate of change on further dosing or cessation of administration, allows logical selection of the animal species most appropriate for toxicity testing, and extrapolation of any toxicity observed in animals to the likely risk for man (2, 17, 29, 65, 75, 76, 122, 123, 124).

Therefore, toxicokinetic studies are designed to produce information on the profile of exposure of the site of toxicity to the active moiety, under the conditions which produce the toxicity, and which are the basis for determining the NOAEL. Important toxicokinetic data relate to:

1. Exposure (or internal dose) in animals based on plasma or blood concentrations of the parent compound or its active metabolite
2. Relationship between the dose given to the animals and exposure
3. Relationship between plasma or blood concentrations and those at the site of toxicity
4. Information on appropriate blood/plasma data after the administration of tracer doses to human volunteers in order to allow extrapolation of animal data to humans

The aim of this chapter is to introduce the underlying principles in both biological and mathematical

terms, and subsequently to describe methods of obtaining suitable samples from certain animal species, primarily the rat. The final section of the chapter covers the analysis of data, with examples to illustrate the type of information and insights that may be obtained using these techniques. In all cases, the examples and data processing described have been restricted to simple analyses of results, in order to illustrate principles. References are provided for further reading, because the analysis of data using computer programs and more complex mathematical models are not covered in this chapter.

In the past, animal and human disposition studies during drug development utilized slightly different approaches, with absorption, distribution, metabolism, and excretion studies (ADME studies) in animals, which were usually based on the fate of the radiolabeled drug,

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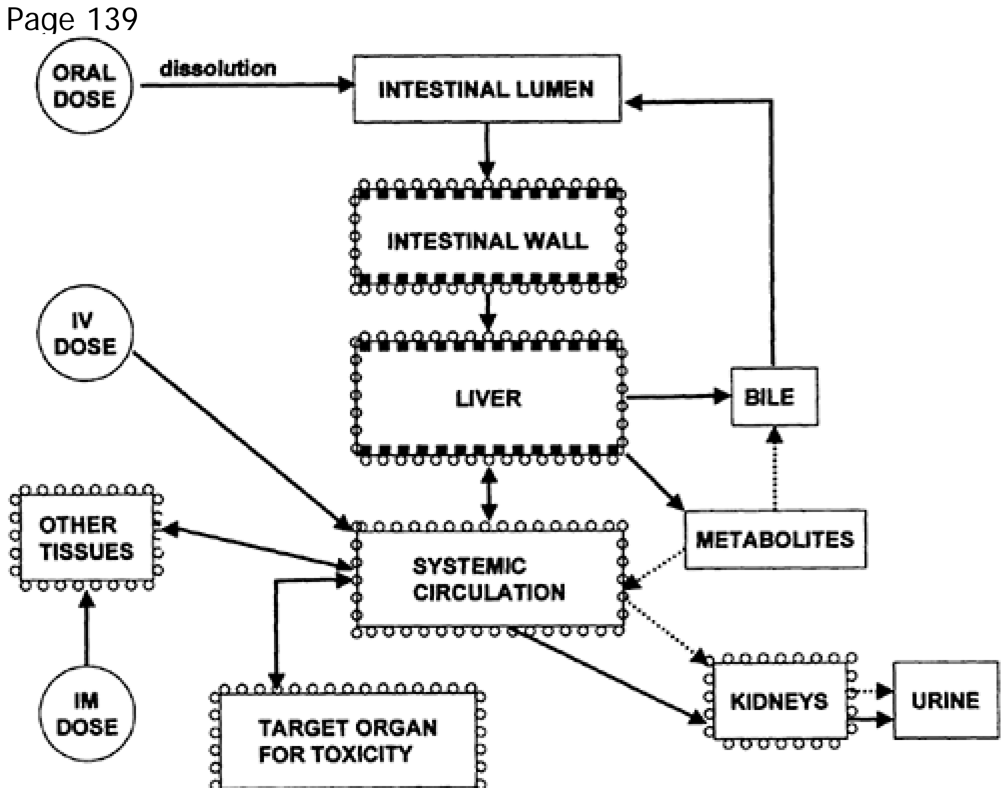

FIG. 4.2. Toxicity in relation to pharmacokinetics. The chemical may be given orally or by injection or inhalation. The concentration at the target organ is in equilibrium with that in the systemic circulation, which is itself in dynamic equilibrium with a large number of other physiological processes tending to increase or decrease that concentration. The transfer from one tissue to another usually involves transfer across a lipid membrane *(open circles)* and frequently entails entering a tissue with high elimination capacity *(solid squares)* such as the liver.

whereas similar radiolabeled studies in humans were frequently supplemented with data on plasma concentration-time curves. The basis for the interpretation of plasma concentration-time curves was the formulation of suitable mathematical models which allowed the derivation of rates of absorption, metabolism, and excretion in humans. It was thus possible, using pharmacokinetic methods, to gain an insight into the rates and extents of processes in humans, which had been shown to occur in animals using serial sacrifice and tissue analysis, and which had been detected in metabolic studies (measurement of urinary metabolites or incubation of the drug with liver microsomes).

However, with the development of automated analytical techniques of high sensitivity and specificity (such as HPLC, LC-MS, and LC-MS-MS), the expansion of laboratories undertaking plasma drug analyses, and the full potential of pharmacokinetics to reveal information on in vivo drug absorption, distribution, and elimination has resulted in these techniques being applied increasingly to toxicology problems in laboratory animals. For therapeutic drugs, the impetus to develop toxicokinetic data in animals has been increased by recent international harmonization initiatives which promote the use of kinetic data in animals and humans to establish maximum dosage levels for toxicity studies. Although there is not acceptance that the top dose for nondrugs can be based on kinetic principles, there is increasing recognition of the value of such data for the interpretation of toxicity studies in which the top dose has been established by tolerability, for example the maximum tolerated dose. Problems of accumulation on repeated dosing and saturation of elimination are particularly pertinent to high-dose animal toxicity studies, and information on these areas can be obtained only from suitably designed in vivo toxicokinetic studies. It must be emphasized at the outset that the key to successful kinetic studies is the development of an assay of high specificity that measures the chemical without interference by its metabolites, and that is of sufficient sensitivity to define the terminal slope accurately (see below).

BIOLOGICAL PRINCIPLES

Certain general principles governing the disposition of therapeutic drugs are applicable to nearly all foreign compounds that are not substrates for normal intermediary

metabolism. These general properties of absorption, distribution, and elimination are valuable concepts, but it should be emphasized that they are not universally applicable, and investigators must be alert for exceptions. Exceptions arise when the foreign compound is structurally similar to an endogenous body constituent, because it may then undergo a specific carrier-mediated uptake process or metabolism. Good examples of compounds showing such characteristics include the antiparkinsonian drug levodopa, the amino acid metabolites of the intense sweetener aspartame, and the purine and pyrimidine base analogs used in cancer chemotherapy and as antiviral agents, many of which not only undergo active uptake into cells but also may be metabolized to phosphorylated products, which accumulate within the cells of the body.

Absorption

Absorption describes the processes involved in the transfer of the drug from the site of administration into the systemic blood circulation. Because most toxicity studies are performed by the oral route, absorption from the gut is of greatest importance, although absorption from other sites is appropriate for certain toxicological studies.

Absorption from the Gut

Significant absorption occurs once the compound has dissolved and is present in the gut lumen as a molecular solution. Slow dissolution and release of drugs from oral sustained-release formulations can be useful for therapeutic drugs that are eliminated very rapidly. Dissolution may be slow and be the rate limiting process, especially when high doses have to be administered as a suspension. Slow dissolution of the compound in the gut lumen can affect the rate of absorption, the peak concentrations, and even the magnitude of acute effects.

The extent of absorption is determined largely by the pH of the gut lumen and the pKa and lipid solubility of the compound. Other biological variables, such as the presence of food, gastric emptying time, intestinal transit time, and the gut microflora, may also play important roles in limiting the rate of absorption and the amount of compound absorbed unchanged. The absorption of chemicals requires passage across lipid membranes (Figure 4.2), which can involve (a) passive diffusion through the membrane, (b) passage through membrane pores, and (c) specialized carrier-mediated processes.

The rate of diffusion of a chemical across a membrane, given by Fick's law, is proportional to the concentration gradient, the membrane surface area, and the permeability coefficient of the compound. The permeability coefficient depends on the diffusivity of the molecule through the membrane, the membrane/aqueous medium partition coefficient, and the thickness of the membrane (21), and thus it is a characteristic for that particular compound and corresponds to a rate constant. Most environmental anutrients are absorbed in the small intestine because of its large surface area. For weak acids and bases, the membrane/aqueous partition coefficient varies with the pH of the medium. For such compounds, the diffusivity and partition coefficient of the ionized molecular species may be regarded as insignificant compared with the uncharged or un-ionized species.

Because it is the uncharged species that readily diffuses across membranes, absorption is faster under conditions in which ionization is suppressed, that is, at low pH for acids and high pH for bases. When the two compartments separated by the lipid membrane are kept at different pH values, the total concentrations in each compartment at equilibrium are different. The extent of ionization of a weak acid may be related to the environmental pH and its p*Ka* by the Henderson-Hasselbalch equation:

$$\mathrm{pH} = \mathrm{pK_a} + \log\frac{[\text{conjugate base}]}{[\text{conjugate acid}]}$$

At equilibrium, the concentrations of the diffusible form (un-ionized) on each side of the membrane are equal, and the concentration of the ionized form is given by the Henderson-Hasselbalch equation (Figure 4.3).

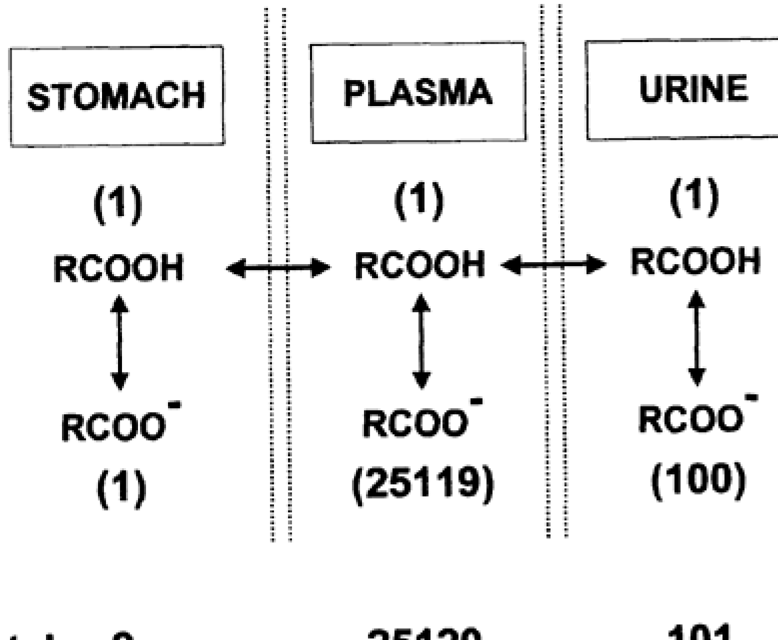

FIG. 4.3. pH partitioning. The numbers give the relative concentration of un-ionized and ionized species in each compartment, as determined by the HendersonHasselbalch equation for a weak acid (p*Ka* 3.0) at the pH of stomach (3.0), plasma (7.4), and urine (5.0). The total concentration is the concentration of compound in each compartment at equilibrium, assuming that the ionized form undergoes negligible diffusion.

It is apparent from Figure 4.3 that weak acids should be absorbed rapidly and extensively in the low pH of the stomach, whereas weak bases should undergo absorption in the intestine and not the stomach. Although this is true for bases, absorption of acids from the stomach is limited, possibly due to the relatively small surface area of the gastric mucosa. Strong organic acids and bases frequently show incomplete absorption from the gut, because they are extensively ionized at all pH values of the gut.

The absorption of foreign compounds by passage through membrane pores, which are about 4 A in diameter, is largely applicable to small water-soluble molecules (<200 Da) (21). Bulk passage of water across the membrane may act as a driving force and carry small molecules with it (66), and this should be borne in mind when studying the absorption kinetics of large doses of sparingly water-soluble compounds. Under such circumstances, the oral administration of high doses in large volumes of hypotonic solution could result in enhanced absorption. This situation is the opposite of the case for compounds undergoing carrier-mediated absorption, because high concentrations may saturate the carrier, and the rate and extent of absorption may be reduced at high doses. Food can also affect carrier-mediated absorption by competition with the natural substrate for the carrier. Examples of foreign compounds undergoing active absorption are rare and usually apply when the chemical resembles a nutrient (e.g., levodopa).

A number of factors may limit the amount of a compound that reaches the systemic circulation as the original or parent compound after oral administration (bioavailability; see later).

1. Extremes of pH, which may affect the stability of the compound. Species differences can then arise between rats (gastric pH 3.8–5.0) and rabbits (gastric pH 3.9) (98) and humans (gastric pH 1–2).
2. Hydrolytic enzymes. The gut is rich in nonspecific proteases and lipases, which may affect foreign compounds.
3. Gut microflora. The gut flora can perform a wide range of largely degradative metabolic reactions on foreign compounds (91), which may reduce the amount available for absorption or result in the formation of potentially toxic metabolites (72). Species differences in gut flora are seen in both types of organism present and their distribution along the gut (98), with larger numbers in the stomach and upper intestine of rats and mice, compared with rabbits, dogs, and humans, in which the upper intestinal tract is almost sterile because of the low gastric pH.
4. Metabolism by the gut wall. The gut wall has the capacity to inactivate metabolically certain compounds prior to their reaching the hepatic portal vein (first-pass effect) (16). The intestinal wall is rich in enzymes catalyzing general hydrolysis and conjugation reactions (such as glucuronidation and sulfation), monoamine oxidase, and some oxidative enzymes, particularly CYP3A4/5.
5. Metabolism by the liver. Many compounds are effectively removed from the hepatic portal vein by a single passage (first-pass effect) (36). The liver is the main site of foreign compound metabolism in the body, and represents the main metabolic barrier to the parent compound reaching the general circulation.
6. Food present in the gut lumen, which may affect the absorption rate, gastric pH, or gut motility.
7. P-Glycoprotein, which is expressed on the luminal surface of the intestinal epithelium, and can act as an efflux pump for compounds entering the enterocyte.

These barriers to the establishment of effective plasma levels of the compound may be associated with suppression of systemic pharmacological and toxic properties (35) and thus render dietary administration an inappropriate route for the toxicity testing of compounds for which exposure of humans is parenteral.

Absorption from the Nasal Cavity

The nasal cavity has various interesting properties as a route of administration. Although it has relatively small surface area, the mucous membrane is highly permeable, so that after nasal administration even quaternary ammonium compounds, which are poorly absorbed from the gut, show blood levels approaching those present following intravenous administration (103). In addition, the nature of this site and its venous drainage directly into the systemic circulation allow increased absorption of compounds extensively metabolized in the gut lumen (e.g., proteins) or in the liver. Local toxicity may be a problem with this route at high doses (103).

Absorption from the Lung

The lung represents a poor barrier to a chemical entering the blood, as it has a large surface area of thin membrane, a limited capacity to metabolize foreign compounds, and an excellent blood supply. The epithelium acts as a limited permeability barrier, allowing only slow absorption of highly water-soluble compounds (106), although the rate may be greater than that from the gastrointestinal tract. The lung is a major site of inactivation of circulating local hormones such as peptides and prostaglandins; however,

for toxicity testing, similar substances would not be likely to be given by this route. Major problems exist with the quantitative analysis of the extent and rate of absorption from the lung, due to poor measurement of the dose given by this route. Par

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ticulate matter is largely trapped by the cilia and passed back to be absorbed in the gut. Volatile compounds are absorbed only partially, and the unabsorbed fraction is eliminated in the expired air and not retained for subsequent absorption, as occurs in the gut (37).

Percutaneous Absorption

The extent of percutaneous absorption is highly dependent on the lipophilicity of the compound, because the stratum corneum of the epidermis acts as an effective barrier (37, 54). This route is important for the therapeutic administration of potent lipophilic drugs which undergo extensive first-pass metabolism (e.g., organic nitrates in angina) and for workers exposed to environmental aerosols and particulates. Studies in animals and humans suggest that the rate-limiting step is the initial penetration of the stratum corneum (40), which may result in very slow absorption and "flip-flop" kinetics (see following).

Distribution

Foreign compounds are distributed largely via the blood, although the lymphatic system may be important in the initial distribution of some very lipid-soluble chemicals given orally. The rate of uptake of a compound by the tissues may be limited by either diffusion rate or perfusion rate.

1. Diffusion rate. If the diffusion of the chemical across membranes is slow, the rate of entry into tissues is limited by this property of the molecule.
2. Perfusion rate. If the diffusion of the chemical across membranes is rapid, the rate of entry is limited by the rate of delivery to the tissue, that is, the perfusion rate.

As a generalization, diffusion rate limitation applies to highly water-soluble compounds, whereas perfusion rate limitation applies to the entry of lipid-soluble compounds into slowly perfused systems, such as adipose tissue. The perfusion rates of the major organ systems of man (Table 4.1) can be readily divided into well and poorly perfused tissues.

The extent to which chemicals leave the blood and enter tissues depends on their relative affinities for each system. Thus compounds highly bound to plasma protein but not to tissue show a relatively high concentration in the plasma, whereas drugs with a high affinity for tissue components such as proteins or fat have a low plasma concentration. However, it should be remembered that it is the *relative* affinity that determines the extent of distribution to tissues. The dye Evans Blue has a high affinity for plasma protein, and its distribution (Table 4.2) is restricted to the plasma volume (3 L in man); the β-blocker propranolol is highly bound to plasma protein (95%), but it also shows a higher affinity for the tissues,

Table 4.1 Relative organ perfusion rates in man[a]

Organ	Percent Body weight[b]	Blood flow[c] (ml/min)	Percent cardiac output[b,c]	Blood flow[b,c] (ml/min/100 g)
Well perfused				
Lung	1.2	5000	100	1000
Adrenals	0.02	25	1	550
Kidneys	0.4	1260	23	450
Thyroid	0.04	50	2	400
Liver				
Total	2	1350	25	75
Via portal vein		1050	20	60
Heart	0.4	252	5	70
Intestines	2	1050	20	60
Brain	2	750	15	55
Poorly perfused				
Skin	7	462	9	5
Skeletal muscle	40	840	16	3
Connective tissue	7		1	
Fat	15	95	2	1

[a] The results are for an adult male under resting conditions and are approximate values only.
[b] Adapted from Reference 14.
[c] Adapted from Reference 5.

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Table 4.2 Volumes of body fluids with drugs showing restricted distribution

Fluid	Volume (L)	Percent body weight	Compound[a]
Total body water	41	58	D_2O, antipyrine, ethanol, urea
Extra cellular water	12	17	Na^+, Br^-, tubocurarine, sucrose
Plasma	3	4	Evans Blue, [131]albumin

Adapted from References 14, 33.

a Compounds for which the distribution is restricted to a particular body fluid.

so that relatively low concentrations remain in the plasma after distribution.

The volumes of body fluids and chemicals that distribute in them are given in Table 4.2. However, only rarely do compounds distribute to a single physiologically recognizable volume, and usually some degree of tissue selectivity is observed. Thus, a compound may appear to have dissolved in total body water because the apparent volume of distribution (see below) corresponds to about 60% of body weight, but it may actually show a nonuniform tissue distribution.

Many foreign compounds bind reversibly to plasma proteins, with albumin being of the greatest importance, although acid glycoproteins may be important for certain organic bases (68). Foreign compounds bind at specific sites in a reversible, saturable fashion, and the bound material represents an inactive depot of the chemical. Extensive protein binding lowers the concentration of unbound chemical in the blood, which may increase the concentration gradient and thus the rate of diffusion into blood from the gut (during absorption) or reabsorption from the kidney tubules (during elimination) (56). The dissociation of the chemical-protein complex occurs within milliseconds, and by comparison with tissue perfusion times may be regarded as instantaneous. Thus, for tissues in which the free plasma concentration is lowered rapidly by an active uptake process within the tissue (i.e., liver or kidney), the compound can be effectively stripped from plasma proteins in a single passage. The plasma protein binding of foreign compounds has been reviewed and discussed by several authors (19, 25, 50, 56, 61).

Elimination

There are two main mechanisms by which the circulating levels of a foreign compound may be reduced: metabolism and excretion. *Metabolism* and its toxicological consequences are discussed in an earlier chapter. Certain mathematical implications are discussed later. The principal routes of excretion are via the urine and feces, and in the case of volatile compounds, the expired air.

Excretion via Urine

There are three major processes affecting elimination in the kidney.

Glomerular filtration. The glomerular membrane has pores of 70–80 A. Under the positive hydrostatic conditions in the glomerulus, all molecules smaller than about 20,000 Da are filtered. Thus proteins and protein-bound compounds remain in the plasma, and about 20% of the nonbound chemical is carried with 20% of the plasma water into the glomerular filtrate.

Reabsorption. Because the glomerular filtrate contains many important body constituents (e.g., glucose), there are specific active uptake processes to transport them from urine back into blood. Although not normally substrates for these transport processes, lipid-soluble chemicals diffuse back from the tubule into the blood, especially as the urine becomes more concentrated because of water reabsorption. The pH of the urine is generally lower than that of the plasma, and therefore weak acids are more ionized in plasma, so that pH partitioning (see above) tends to increase the reabsorption of weak acids. The pH of the urine can be altered appreciably by treatment with ammonium chloride (decreases pH) or sodium carbonate (increases pH); the buffered plasma shows little change. It is thus relatively easy to affect the pH partitioning of foreign compounds between tubule contents and plasma, and either increase or decrease the elimination rate. This possibility should be considered when preparing dose solutions, because the use of excess acid or alkali to dissolve the test compound could alter its renal elimination.

Tubular secretion. Foreign compounds may be secreted actively into the renal tubule against a concentration gradient by anion and cation carrier processes. These processes are saturable and of relatively low specificity; many basic or acidic compounds and their metabolites (especially the phase II or conjugation products) are removed by them (26). Organic cation transporters are expressed on the luminal membrane of

the renal tubule and are important in eliminating charged molecules (125). Because the dissociation rate for the chemical-albumin complex is rapid, it is possible for highly protein-bound compounds to be almost completely cleared at a single passage through the kidney.

Excretion via the Gut

The bile is the most important route allowing foreign compounds to move from the general circulation into the gut. The biological aspects of this mechanism have been reviewed (99), and certain pertinent points have emerged. Organic cation transporters on the sinusoidal membrane transfer large polar cations into the hepatocyte and from the hepatocyte in the bile (125). The bile may be regarded as a complementary pathway to the urine, with small molecules being eliminated by the kidney and large molecules in the bile. Thus the bile becomes the principal excretory route for many xenobiotic conjugates. Species differences exist in the molecular weight requirement for significant biliary excretion, which has been estimated as 325±50 Da in the rat, 440±50 Da in the guinea pig, 475±50 Da in the rabbit (43), and about 500 Da in humans. In the rat, small molecules (<350 Da) are not eliminated in the bile and large molecules (>450 Da) are not excreted in the urine, even if the principal excretory mechanism is blocked by ligation of the renal pedicles or bile duct, respectively. Compounds of intermediate molecular weight (350–450 Da) are excreted by both routes, and ligation of one pathway results in increased use of the other (42).

Foreign compounds may also enter the gut by direct diffusion or secretion across the gut wall, elimination in the saliva, pH partitioning of bases into the low pH of the stomach, and elimination in the pancreatic juice. In most cases, these routes are quantitatively of minor importance, although they may play an important role in toxicity by allowing a foreign compound to undergo metabolism by the gastrointestinal flora (30, 72, 74). The toxicological implications of the gut microflora have been reviewed by Scheline (91).

MATHEMATICAL PRINCIPLES

In order to adequately describe the changes in blood or plasma concentrations of foreign compounds, it is necessary to assign a suitable mathematical model that accurately predicts the shape of the plasma concentration-time curve. However, certain aspects are model independent and are considered first, because these considerations are usually constituent parts of the various models. In addition, there has been a marked trend away from multicompartmental mathematical analysis, which offers little apart from mathematical predictability, toward physiologically more relevant model-independent concepts such as clearance (17, 118). Physiologically related parameters such as clearance and bioavailability represent an intermediate stage between mathematical multicompartment models and PBPK models.

Model-Independent Considerations

Biochemical and physiological processes are usually either zero-order or first-order reactions. In zero-order reactions the rate of change in concentration occurs at a fixed amount per time, that is,

$$\frac{dC}{dt} = k$$

where C is concentration, t is time, and k is a constant with units of amount per time, for example, micrograms per minute. In first-order reactions the rate of change in concentration is proportional to the concentration of the chemical available for the reaction, that is,

$$\frac{dC}{dt} = kC$$

where k is a constant that represents a proportional change with time and has units of time^{-1}, for example, min^{-1}.

Most processes (e.g., diffusion, carrier-mediated uptake, metabolism, excretion) are first-order reactions at low concentrations. *Most of the equations given below make this assumption.* Zero-order reactions are particularly important at high concentrations, when enzymes are working at maximum rate and an increase in C cannot result in an increase in rate. This situation produces nonlinear, or saturation, kinetics, which can assume considerable importance in toxicity studies, and is discussed later.

First-order reactions can be described by equations employing exponential functions. In many cases the entry of a foreign compound into the body or into a tissue follows an exponential increase, which may be described mathematically by

$$\text{Uptake} = 1 - e^{-kt} \quad [1]$$

where the uptake is the concentration present at time t divided by the final concentration when all the compound has entered the body or tissue. This equation assumes that there is no elimination process

occurring. The elimination of a compound (by a single mechanism) once it has entered the body or tissue may be described by

$$C=C_0e^{-kt} \quad [2]$$

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where C is the concentration present at time t, and C_0 is the initial concentration. In Eq. (1) and (2), k is the rate constant for that process.

Exponential equations of the type given in Eq. (2) may be solved as

$$\ln C = \ln C_0 - kt$$

or using $\log_{10}$

$$\log C = \log C_0 - \frac{kt}{2.303}$$

which represents an equation of the generalized form

$$y = C + mx$$

where m and C are constants, and x and y are variables. In such cases, a plot of x against y gives a straight line graph with a slope of m and an intercept of C. Thus for toxicokinetics, a graph of $\ln C$ against time gives a slope of $-k$ and an intercept of $\ln C_0$. If such a graph is drawn using log-linear graph paper, the slope must be calculated by taking the $\log_{10}$ of the concentration terms and dividing by the time (see below), and the slope will be $-k/2.303$.

Frequently, the equation necessary to describe the kinetics of a compound in the body requires the use of two exponential rate terms, that is, absorption into a single compartment plus elimination, or elimination from a two-compartment system (see below). In such cases the early time points in the concentration-time curve are influenced by both rates. However, provided the rate constants are sufficiently dissimilar, eventually the influence of the component with the higher rate becomes negligible, whereas the smaller rate constant still affects the concentration. Thus the terminal phase of the concentration-time curve is determined by the process with the smaller rate constant, and the earlier phase by the sum of both processes. This process allows both rate constants to be determined by the procedure known as the method of residuals or stripping (see below).

Tissue Extraction

Removal of a compound from the blood by a tissue is schematized in Figure 4.4. On constant infusion, the rate of entry into the tissue may be regarded as equivalent to a first-order absorption rate (78):

$$\text{Uptake} = 1 - e^{-kt} \quad [1]$$

where uptake=the fractional uptake=$C_t/C_{equilibrium}$.

In *perfusion limited uptake*, the value k is related to the flow rate *(Q)* as follows (Figure 4.4):

$$\text{Fractional uptake} = 1 - e^{-(Q/PV_t)t} \quad [3]$$

(Q/V_t is the volume-adjusted flow rate and P is the par

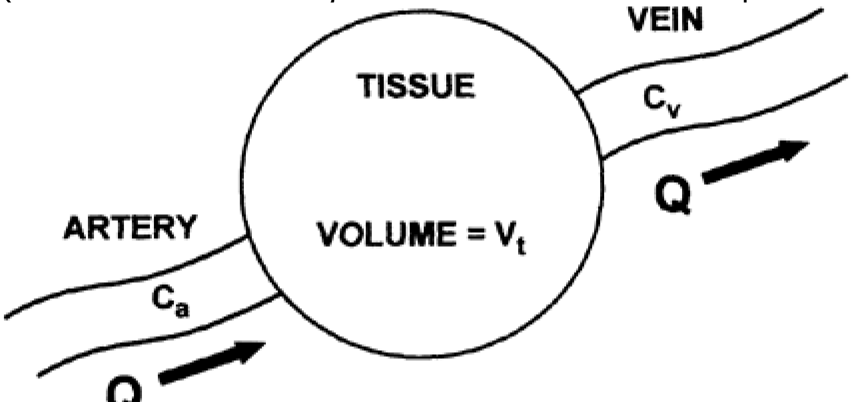

FIG. 4.4. Tissue uptake of foreign compounds. Q is the blood flow, C_a is the arterial concentration, C_v is the venous concentration, C_t is the concentration in tissue, and V_t is the volume of tissue. We define the following parameters: rate of delivery of drug=QC_a, rate of outflow of drug=QC_v, and rate of uptake=QC_a-QC_v. The units for these rates are mass time^{-1} (i.e., μg min^{-1}, etc.). Thus

$$\text{Extraction ratio } (E) = \frac{\text{rate of uptake}}{\text{rate of delivery}} = \frac{QC_a - QC_v}{QC_a} = \frac{C_a - C_v}{C_a}$$

$$\text{Partition ratio } (P) = \frac{\text{Concentration in tissue}}{\text{Concentration in blood supply}} = \frac{C_t}{C_a}$$

These relationships are an essential part of PBPK models.
tition ratio). The uptake half-time may be derived as described below for Eq. (19):

$$t_{1/2}(\text{uptake}) = \frac{0.693}{k} = \frac{0.693PV_t}{Q} = \frac{0.693P}{Q/V_t} \quad [4]$$

For *diffusion-limited uptake,* the value *k* is related to the diffusion rate constant and thus is not readily measurable.

Plasma Protein Binding

The extent of protein binding may be represented by an equilibrium reaction:

$$P^r + C_u \rightleftharpoons C_b$$

where *Pr* is the free protein, *Cu* is the unbound compound, and *Cb* is the compound-protein complex. The equilibrium constant *K* is given by

$$K = \frac{[C_b]}{[P^r][C_u]} = \frac{[\text{product}]}{[\text{reactants}]} \quad [5]$$

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The fraction unbound a is given by

$$\alpha = \frac{C_u}{C_u + C_b} = \frac{C_u}{C_p} \quad [6]$$

where Cp is total plasma concentration. Scatchard plots of r/Cu against r (where is the moles of drug bound per mole of protein and Cu is the free drug concentration in moles) can be used to derive the number of binding sites and their association or affinity constants (25). Normally, for the binding of organic compounds to albumin, two or more binding sites are revealed. This approach is valuable for detailed studies on binding to and displacement from albumin, but it could yield multiple binding sites if applied to a complex protein mixture, such as plasma, containing different proteins at different concentrations.

Because it is the unbound compound in plasma that undergoes equilibration with the unbound compound in tissues (Figure 4.5), adequate information may be obtained from a knowledge of α, or of the percentage bound. Detailed knowledge is not required for pharmacokinetic analysis of the plasma data because the plasma concentrations used to calculate kinetic parameters are usually the total concentrations $(Cu+Cb)$. (When the nonbound concentration is used this has to be specified in the description of the parameters, e.g. clearance [nonprotein bound]; in the absence of such a description it is assumed that the total plasma concentration has been used). Because plasma protein binding is a saturable process (see later), in vitro binding studies should be performed over a range of concentrations.

Clearance

Clearance (CL) is defined as the ratio:

$$\mathrm{CL} = \frac{\text{Rate of elimination}}{\text{Plasma concentration}}$$

and may be regarded as the volume of plasma or blood that is cleared of compound in unit time by the route under consideration. The units are volume $time^{-1}$, for example, usually ml min^{-1} because if rate is μg min^{-1} and plasma concentration is μg ml^{-1} the plasma clearance will be ml min^{-1}.

Renal Clearance

The renal clearance (CLR) is given by

$$\mathrm{CL}_R = \frac{\text{rate of elimination in urine}}{\text{plasma concentration}} = \frac{C_u \times F_u}{C_p} \quad [7]$$

where Cu is the urine concentration, Fu is the urine flow (volume in unit time), and Cp is the plasma concentration at the midpoint of the urine collection period. The con

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$$Cp_b \underset{}{\overset{K_{ap}}{\rightleftarrows}} Cp_u \leftrightarrow Ct_u \overset{K_{at}}{\rightleftarrows} Ct_b$$

Plasma Tissue

FIG. 4.5. Protein binding and tissue distribution. Cp is the plasma concentration, Ct is the tissue concentration, u=unbound, b=bound, Kap is the association constant for plasma protein binding. At equilibrium, $Cpu=Ctu$; and the relative distribution between plasma and tissue is determined by the values of Kap and Kat.

centration in urine is dependent on a number of variables, which are now described.

Glomerular filtration. The rate of filtration is given by

$$\mathrm{GFR} \times Cpu = \mathrm{GFR} \times Cp \times \alpha \quad [8]$$

where GFR is the glomerular filtration rate, Cpu is the unbound concentration in plasma, Cp is the total

plasma concentration, and a is the fraction unbound. Thus compounds binding extensively to plasma proteins show limited clearance by glomerular filtration. The protein binding equilibrium is not disturbed in the glomerulus because after loss of 20% free drug and 20% water, *C*pu is unaltered, whereas the concentrations of both free protein and drug-protein complex increase by 20%, that is,

Before filtration

$$K_{ap} = \frac{[\text{complex}]}{[C_{pu}][P^r]}$$

After filtration

$$K_{ap} = \frac{1.2\,[\text{complex}]}{[C_{pu}]\,1.2[P^r]}$$

where *Kap* is the protein-binding association constant. Thus, the chemical–protein complex does not dissociate in the glomerulus. The complex dissociates to give more free compound when the plasma is diluted by water reabsorbed in the distal parts of the renal tubule. Under such circumstances, about 99% of the plasma water is reabsorbed, so that the concentrations of the protein and complex return to almost their initial levels, whereas the concentration of unbound compound is diluted to about 80% of its former level, that is, after reabsorption:

$$K_{ap} \neq \frac{[\text{complex}]}{0.8[C_{pu}][P^r]}$$

Therefore the complex dissociates to restore the

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equilibrium. The glomerular filtration rate is about 130 ml min^{-1} in men and 120 ml min^{-1} in women, or approximately 2 ml $min^{-1}kg^{-1}$, which is lower than that of the Wistar rat (3.4 ml $min^{-1}kg^{-1}$) (100).

Reabsorption. Reabsorption from the renal tubule back into the blood is variable and dependent on the lipid solubility of the compound, the pH of the urine, and the extent of concentration of the urine (i.e., water reabsorption). Mathematical quantitation is impracticable, but an indication of the extent of reabsorption may be obtained (see below). In certain instances the administered foreign compound may be a substrate for carrier mediated reabsorption, in which case the renal elimination is dose dependent and is greater at high doses when this reuptake is saturated. Such a saturable reuptake process is obviously ideal for maintaining a constant low body load of an essential compound, for example, glucose or riboflavin, which might show adverse effects at high body concentrations.

Tubular secretion. Saturable carrier-mediated processes are present in the proximal part of the tubule. They show a relatively low substrate specificity, and the extent of their involvement for a particular compound is dependent on the affinity between the compound and the carrier protein. The extent of clearance by these active processes may be regarded as analogous to hepatic clearance, which is another active, saturable process. The specificities of the carriers have been studied extensively (26, 116, 125) and structural requirements such as

$$\begin{array}{c} \mathrm{R-\underset{\displaystyle O}{\overset{}{\underset{\|}{C}}}-\underset{\displaystyle H}{\underset{|}{N}}-(CHR')_n-COOH} \end{array}$$

have been proposed. Saturation of secretion causes a decreased elimination at high doses.

All three processes described above can alter, simultaneously and independently, the value of C_u for any given value of C_p. The final renal clearance may be regarded as a composite expression:

$$\text{Renal excretion} = \text{glomerular filtration} - \text{reabsorption} + \text{tubular secretion}$$

$$\text{Rate of excretion} = \text{GFR}\, C_p\alpha - \text{rate of reabsorption} + \text{rate of tubular secretion}$$

The values of GFR, C_p, and α can be determined experimentally. Measurement of inulin clearance (or creatinine clearance in man) determines the GFR, because this compound does not undergo significant reabsorption, tubular secretion, or protein binding; thus for inulin:

$$\text{Rate of renal excretion} = \text{GFR}\, C_p$$

and because

$$CL_R = \frac{\text{rate of excretion}}{C_p}$$

$$CL_R = \text{GFR}$$

The extent of reabsorption and secretion of a compound may be inferred from a comparison of its renal clearance with the value of GFR×α.

$CL_R < \text{GFR} \times \alpha$ Reabsorption must be occurring and is greater than secretion (which may or may not be present).

$CL_R = \text{GFR} \times \alpha$ Reabsorption, which may or may not be present, is negated by an equal rate of secretion.

$CL_R > \text{GFR} \times \alpha$ Tubular secretion must be occurring and is greater than reabsorption (which may or may not be present).

The mathematical implications of the renal elimination process have been the subject of a number of reviews (31, 115).

Hepatic Clearance

The clearance of a compound by the liver may be regarded as dependent on the rate of delivery to the organ (blood flow) and the efficiency of removal from the blood (extraction ratio; see Figure 4.4). Thus

$$CL_H = QE \qquad [9]$$

where CL_H is the hepatic (metabolic) clearance, Q is the hepatic blood flow, and E is the extraction ratio. This simple relationship has been verified experimentally for a number of compounds. However, it is complicated by the finding that the variables Q and E are not independent, because for certain compounds an increase in blood flow decreases the extraction efficiency. This finding led Rowland et al. (87) to propose the following relationship, known as the *perfusion limited model:*

$$CL_H = Q\left(\frac{\alpha \times CL_{int}}{Q + \alpha CL_{int}}\right) \qquad [10]$$

where α is the fraction unbound in plasma, CLint is the intrinsic metabolic clearance by the hepatocytes from the cell water, and *Q* is the blood flow (as plasma).

If the metabolic clearance (CLint) is high, the value in parentheses in Eq. (10), which is equivalent to the term *E* in Eq. (9), approaches unity; under these circumstances the hepatic clearance approximates to the hepatic blood flow and becomes dependent on the blood flow. However, if the metabolic clearance is low, $Q+\alpha$CLint approximates to *Q*, and therefore the extraction ratio *(E)* decreases with an increase in blood flow and the

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hepatic clearance remains relatively constant. These equations adequately explain the effects of changes in perfusion rate on the extraction ratio and clearance of compounds that show a range of extraction ratios. In addition, comparison of the hepatic clearance (calculated by measurement of *Q* and *E*) with "nonrenal" clearance (calculated as plasma clearance minus renal clearance) can indicate the role of extrahepatic tissues in the elimination of the compound.

Further analysis of this equation (see ref. 117 for discussion) indicates that

$$\mathrm{CL_{int}} = \frac{V_{max}}{K_m + C_{pu}} \qquad [11]$$

where V_{max} and K_m are Michaelis-Menton constants for the enzyme metabolizing the foreign compound, and C_{pu} is the hepatic venous concentration of unbound compound. If C_{pu} is low and is much less than the K_m for the enzyme (i.e., well below saturation levels), this term may be ignored and

$$\mathrm{CL_{int}} = \frac{V_{max}}{K_m} = \text{constant}$$

When the value of C_{pu} approaches or exceeds K_m, the substrate concentration is sufficient to saturate the enzyme, and the kinetics are grossly altered and become nonlinear. This situation is a distinct possibility in high-dose toxicity testing and is discussed later in more detail. The concepts given above are important for the use of V_{max} and K_m values in interspecies comparisons. V_{max}/K_m is taken as a measure of enzyme activity; species differences in clearance will relate to differences in this measure of enzyme activity for low clearance compounds, but will relate to liver blood flow for high clearance compounds (irrespective of differences in V_{max}/K_m). This possible source of error in extrapolation across species is avoided when both the enzyme activity and organ blood flows are part of a PBPK model (see below).

Biliary Clearance

The clearance via the bile CL_B is given, by analogy with renal clearance, as

$$\mathrm{CL}_B = \frac{\text{rate of elimination in bile}}{C_p} = \frac{C_B \times F_B}{C_p} \qquad [12]$$

where C_B is the concentration in bile and F_B is the volume of bile in unit time (bile flow).

Plasma Clearance

Plasma clearance (CL) may be defined as

$$\mathrm{CL} = \frac{\text{rate of elimination from plasma}}{C_p}$$

The plasma clearance is the sum of the various contributory clearance processes:

$$\mathrm{CL} = \mathrm{CL}_R + \mathrm{CL_H} + \mathrm{CL}_B + \text{etc.} \qquad [13]$$

Plasma clearance, which is one of the most valuable toxicokinetic constants, is determined from the plasma concentration-time curve and is discussed in detail later. It may be used to derive other model-independent variables, for example, mean residence time, which are given later under Statistical Moment Analysis.

Physiologically Based Pharmacokinetic Models

In recent years models have been developed which are based on the principles discussed above, that is, organ blood flow, tissue extraction, and rates of metabolism and excretion. These models are derived from the physiology of the test animal and are discussed in detail in Chapter 5. PBPK models have been applied successfully to a number of compounds and have been particularly successful for organic solvents, e.g., benzene (108). This approach represents a powerful method, capable of dealing with saturation of metabolism and valuable for the extrapolation of animal data to humans (69, 57, 113). However, its ability to predict concentrations is dependent on the precision of the parameter estimates used and the model chosen (9). Therefore, PBPK modeling should be considered as one of three possible approaches to the analysis and interpretation of toxicokinetic data:

1. Simple physiologically related concepts, such as bioavailability and clearance (this chapter)
2. Compartmental analysis, which gives mathematical precision but does not directly reflect organ concentration (this chapter)
3. PBPK modeling, which allows the prediction of target organ concentrations (Chapter 5)

Compartmented Systems: Modeling

In order to describe plasma concentration-time curves mathematically, an appropriate predictive model

has to be fitted to the data. The correlation between the actual data and the concentration-time curve generated using the model shows the suitability of the model in describing the experimental results. Thus considering the data presented in Figure 4.6 and Table 4.3, it is apparent that the same model cannot describe the properties of both compounds, although in both cases the initial and final plasma measurements were the same. The differences in the plasma concentration-time profiles originate in the number of rates at which the compound may leave and enter the plasma.

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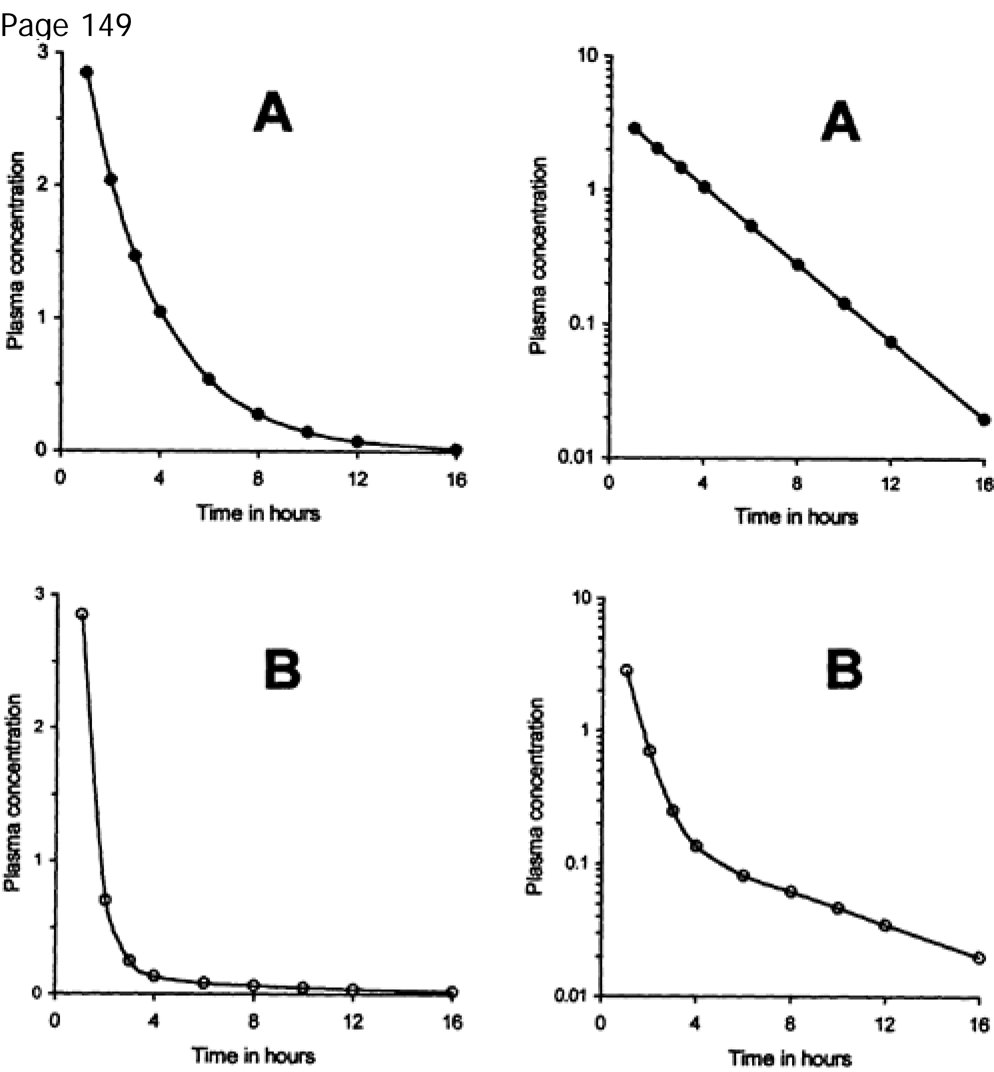
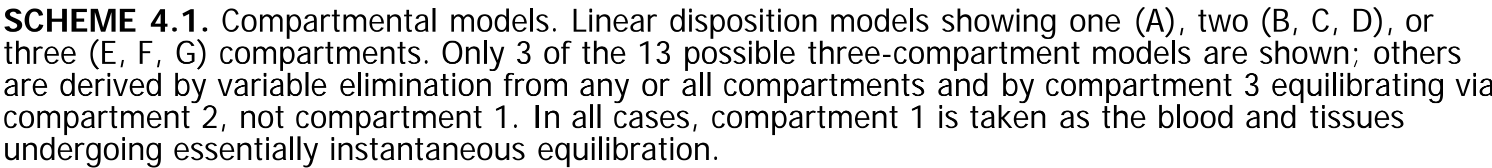


FIG. 4.6. Plasma concentration-time data for two compounds; results are plotted in linear and semilogarithmic forms. The data used to generate the curves are given in Table 4.3.

If the tissues show instantaneous equilibration with plasma, the compound leaves the plasma by a single process (Scheme 4.1A), an elimination process with a simple exponential decrease. Alternatively, the compound may leave the plasma to enter "other tissues" at measurable rates, as well as undergoing elimination from the plasma. Under such circumstances, the "other tissues" may be adequately described mathematically by a second compartment in addition to the plasma (or the central compartment) (Scheme 4.1B). In some cases, two or more additional compartments are required. It is important to realize that these "other tissues" share only one criterion, that is, their associated rate constants, and that biologically diverse tissues may be part of the same compartment. In addition, elimination may occur from compartments other than the central compartment (Schemes 4.1C-G). In most cases the processes of elim

Table 4.3 Data used for Figure 4.6

Time after IV Dosing (h)	Cp (μg ml−1) Compound A	Compound B
1	2.850	2.850
2	2.040	0.705
3	1.470	0.250
4	1.050	0.136
6	0.540	0.082
8	0.280	0.062
10	0.145	0.047
12	0.075	0.035
16	0.020	0.020



SCHEME 4.1. Compartmental models. Linear disposition models showing one (A), two (B, C, D), or three (E, F, G) compartments. Only 3 of the 13 possible three-compartment models are shown; others are derived by variable elimination from any or all compartments and by compartment 3 equilibrating via compartment 2, not compartment 1. In all cases, compartment 1 is taken as the blood and tissues undergoing essentially instantaneous equilibration.

ination and distribution of foreign compounds are by first-order reactions, that is, the rate of the reaction is proportional to the amount of substrate available for the reaction.

Wagner (111) reviewed compartmental models and showed that 17 linear models existed to describe one-, two, and three-compartment systems, that is, models in which the plasma concentration-time curve could be resolved into a number of linear components (see below). However, if the input into the model was noninstantaneous, additional models would be generated. Wagner (111) concluded that there were 760 possible pharmacokinetic models comprising up to three distribution compartments and two input compartments, but that in many cases and for many calculations a knowledge of the best-fit model was not necessary. Because the aim of this chapter is to provide an introduction to toxicokinetics (i.e., samples needed, data handling, and the type of information that can be obtained), only simple models are discussed in detail. Readers are referred to standard texts on pharmacokinetics if the plasma data are not fitted by the simple models discussed here. However, the two models selected show widespread applicability, and an understanding of the principles underlying these simple models is essential, if the data generated by computer analysis of more complex models are to have any meaning. In addition, more complex models contain greater numbers of variables, and blood sampling must be increased to define the rate constants accurately.

Texts recommended for further reading include those by Rescigno and Segre (78), a mathematical approach with few drug illustrations; Gibaldi and Perrier (36), a classic text, which is a mathematical approach that is well explained and illustrated using actual experimental data; Rowland and Tozer (85), a well-written, readable text with many excellent illustrations and study problems at the end of each section; Wagner (112), an approach similar to that of Gibaldi and Perrier but with a useful "biological" introductory chapter and expanded sections on dosage regimen calculations, pharmacological response, and automated pharmacokinetic analysis. Additional useful sources for background reading include Benet et al. (8), a collection of papers from a symposium to honor S.Riegelman; Rowland and Tucker (86), which has useful sections on interspecies scaling and timeand dose-dependent kinetics; and Yacobi et al. (124), which relates to drug development. A particularly valuable book has been published recently by Gabrielsson and Weiner (28), which gives a clear account of different models in relation to the use of WinNonlin, and provides excellent explanations of the basics of data fitting. All volumes provide references, either at the end of each chapter or for each illustration.

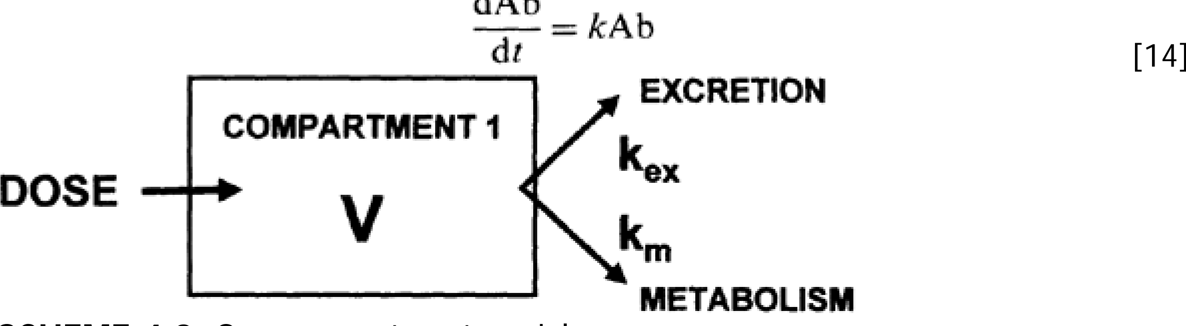
One-Compartment Open Model

Intravenous Bolus Dose

The compound is dissolved in and evenly distributed within a single compartment of volume *V*. Elimination of the compound, by both excretion and metabolism, is by first-order processes, and changes in plasma concentration are reflected in similar and simultaneous decreases in the tissue concentrations, because all tissues represent part of the single compartment (Schemes 4.1A and 4.2). In Scheme 4.2, *V* is the volume of distribution, k_{ex} is the excretion rate constant, and k_m is the metabolism rate constant. The plasma concentration-time curve for a one-compartment system is given

in Figure 4.7, the data are presented in Table 4.4. In mathematical terms, such a system may be described adequately by a simple first-order equation, where the rate of removal of a compound from the body (e.g., in milligrams per hour) is proportional to the body load (e.g., in milligrams):

$$\frac{\mathrm{dAb}}{\mathrm{d}t} = k\mathrm{Ab} \qquad [14]$$



SCHEME 4.2. One-compartment model.

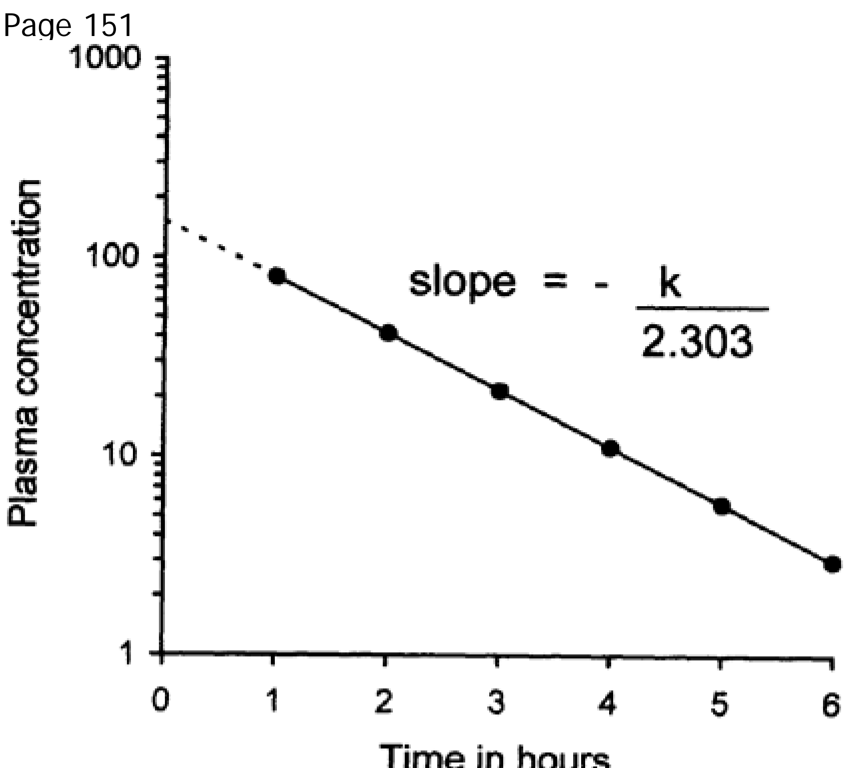


FIG. 4.7. Plasma concentration-time curve after a bolus intravenous dose for a one-compartment system. The data are given in Table 4.4.

Table 4.4 Data used for Figure 4.7*a*

Time (h)	C_p (μg ml^{-1})	ln C_p	
1	80.0	4.382	
2	41.5	3.726	k=0.656 h^{-1}
3	21.5	3.068	$t_{1/2}$=1.06 h
4	11.2	2.416	
5	5.8	1.758	C_{p0}=154.3 μg m1^{-1}
6	3.0	1.099	V=324 ml Kg^{-1}

a The plasma concentrations were obtained after an intravenous bolus dose of 50 mg/kg.

where Ab is the amount of compound in the body, and k is the elimination rate constant (k equals $[k_{ex}+k_m]$). A solution to this equation to give the amount remaining in the body at time t after injection is given by

$$Ab_t = Ab_0 e^{-kt} \quad [15]$$

where Ab_t is the amount of compound at time t, and Ab_0 is the amount at time zero.

Assuming uniform distribution within a single compartment, the concentration in the plasma *(Cp)* may be related to Ab by the *apparent volume of distribution (V)*. This volume may be regarded as the volume of plasma in which the body burden (body load) would have to be dissolved, in order to give the plasma concentration measured. For a chemical that is lipid soluble or that readily binds to tissue components, the plasma concentration represents a small fraction of the total amount in the body, and thus the compound appears to have been dissolved in a large volume of plasma (see below):

$$C_p = \frac{Ab}{V} \quad [16]$$

where C_p is the plasma concentration and V is the apparent volume of distribution. Thus Eq. (15) may be rewritten in its more usual form,

$$C_{pt} = C_{p0} e^{-kt} \quad [17]$$

where C_{pt} is the plasma concentration at time t, and C_{p0} is the concentration at time zero. For such a system, we can define the following parameters.

Apparent volume of distribution. The apparent volume of distribution *(V)* is the apparent volume into which the dose would have been dissolved to give the initial plasma concentration, C_{p0}, that is,

$$V = \frac{\text{Ab}}{C_p} = \frac{\text{dose}}{C_{p0}} \qquad [18]$$

The units are in liters, milliliters, liters per kilogram, or milliliters per kilogram.

Elimination rate constant. The elimination rate constant *(k)* represents the fractional loss of compound from the body per unit time, that is,

$$k = \frac{\text{amount of chemical eliminated in unit time}}{\text{amount of chemical in the body}} = \frac{(\text{dAb}/\text{d}t)}{\text{Ab}}$$

Equation (17) may be rewritten as

either $\ln C_{pt} = \ln C_{p0} - kt$

or $$\log C_{pt} = \log C_{p0} - \frac{kt}{2.303}$$

Thus a graph of ln C_p against time has a slope of $-k$ and an intercept of ln C_{p0}; a graph of log C_p against time has a slope of $-k/2.303$ and an intercept of log C_{p0} (Figure 4.7). The units of k are h^{-1} or min^{-1}. Thus if the elimination rate constant is determined as 0.4 h^{-1}, it means that 40% of the body load is removed each hour. The value of k is the summation of component elimination rate constants (e.g., k_{ex}, k_m).

Elimination half-life. The elimination half-life ($t_{1/2}$) is the time taken for the amount in the body (Ab) or the plasma concentration (Ab/V) to decrease to one-half. Thus after one half-life, C_p in Eq. (17) equals $C_{p0}/2$, that is,

$$\frac{C_{p0}}{2} = C_{p0}e^{-kt_{1/2}} \qquad \text{or} \qquad \frac{1}{2} = e^{-kt_{1/2}}$$

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Therefore,

$$\ln 0.5 = -kt_{1/2} \qquad \text{or} \qquad -0.693 = -kt_{1/2}$$

$$t_{1/2} = \frac{0.693}{k} \qquad [19]$$

where the units are hours or minutes.

Plasma clearance. Plasma clearance (CL) is the amount of chemical eliminated in unit time related to the plasma concentration and may be regarded as the volume of blood that is cleared of chemical in unit time. CL is a constant for first-order reactions. In many respects, this measurement is a better reflection of the inherent capacity of the tissues to eliminate the compound than is the half-life or elimination rate constant:

$$\mathrm{CL} = \frac{\text{rate of elimination}}{\text{plasma concentration}}$$

$$\mathrm{CL} = \frac{(\mathrm{dAb}/\mathrm{d}t)}{C_p} \qquad [20]$$

Substituting from Eq. (14),

$$\mathrm{CL} = \frac{k\mathrm{Ab}}{C_p}$$

The amount in the body at any time (Ab) is given by Eq. (16); therefore

$$\mathrm{CL} = \frac{kC_pV}{C_p} = kV \qquad [21]$$

where the units are in L h^{-1}, L min^{-1}, ml h^{-1}, or ml min^{-1}. Rearranging Eq. (21),

$$k = \frac{\mathrm{CL}}{V}$$

This equation shows clearly that the elimination rate constant *(k)* is derived from two independent variables that can be related to physiological processes: the *clearance*, which reflects the capacity of the organs of elimination to remove the compound from the plasma, and the *apparent volume of distribution*, which reflects the proportion of the total body burden that is circulated to the organs of elimination. Plasma clearance may depend on the rate of the active process in the organs of elimination or on the plasma flow to the principal organ(s) of elimination.

Clearance may also be obtained without knowing the value of *V*. Rearranging Eq. (20),

$$\frac{\mathrm{dAb}}{\mathrm{d}t} = \mathrm{CL} \times C_p$$

or in time d*t*, the amount lost $\mathrm{dAb} = \mathrm{CL} \times C_p \times \mathrm{d}t$. Integrating between time=0 and infinity (∞) the total dose will have been eliminated, so that dAb=Dose,

$$\mathrm{Dose} = \mathrm{CL} \int_0^{\infty} C_p \mathrm{d}t$$

$$\mathrm{Dose} = \mathrm{CL} \times \mathrm{AUC}$$

$$\mathrm{CL} = \mathrm{Dose}/\mathrm{AUC} \qquad [22]$$

where AUC is the area under the plasma concentration-time curve extrapolated to infinity. For Eq. (22) to be valid, the dose has to be fully available to the organs of elimination (i.e. intravenous dosage) and the AUC has to be extrapolated to infinity.

This relationship can also be used to calculate *V;* substituting CL from Eq. (21) into Eq. (22):

$$V = \frac{\mathrm{dose}}{\mathrm{AUC} \times k} \qquad [23]$$

The value of Eqs. (22) and (23) is that both the clearance and the apparent volume of distribution can be derived from infusion or parenteral administration, where the determination of *V* using Eq. (18) is not possible, because the total dose is not present in the central compartment at *t*=0. These equations may

also be applied to oral administration, providing that allowance is made for incomplete absorption of the dose. This method of calculating CL is also applicable to multicompartment linear systems with elimination from the central compartment.

Information Obtainable from Urinary Data

From Eq. (7), we obtain

$$\text{Rate of urinary excretion} = CL_R \times C_p$$

where CL_R is the renal clearance. Thus

$$CL_R \times C_p = k_R \times V \times C_p = k_R \times Ab$$

from Eqs. (21) and (18), where k_R is the renal excretion rate constant. However, Ab at any time=dose $\times e^{-kt}$. Therefore

$$\text{Rate of urinary excretion} = k_R \times \text{dose} \times e^{-kt} \quad [24]$$

or

$$\log(\text{rate of urinary excretion}) = (\log k_R \times \text{dose}) - \frac{kt}{2.303} \quad [25]$$

A plot of rate of urinary excretion (amount excreted per time interval) against time gives a straight line on log-linear graph paper, the slope of which is $-k/2.303$, and the intercept is log k_R dose. It is important to note that the slope of this graph gives the overall elimination rate constant, not the specific urinary elimination rate

constant. In other words, the decrease in the amount appearing in the urine mirrors the overall decrease in the plasma concentration. It is not possible to obtain information regarding other kinetic parameters (such as CL_R or V) without sampling the central (blood) compartment. The value of k_R may be derived from the values of renal clearance

$$CL_R = \frac{C_u \times F_u}{C_p} \quad [7]$$

and the apparent volume of distribution, V, when $CL_R = k_R \times V$.

The above approach is subject to considerable fluctuation in the excretion rate due to factors such as incomplete bladder emptying. To overcome this problem, the rate constant can be derived more reliably from the amount remaining to be excreted, using the *sigma-minus* method. This method is based on the equation below, which is derived from integration of Eq. 24:

$$A_{ex} = \frac{k_R \times \text{dose}}{k}[1 - e^{-kt}] \quad [26]$$

where A_{ex} is the total amount excreted up to time t. At infinite time, $(1-e^{-kt})$ equals unity. Therefore

$$A_{ex}^{\infty} = \frac{k_R \times \text{dose}}{k}$$

where A_{ex}^{∞} is the cumulative total amount excreted in urine up to time infinity. Substituting back into Eq. (26)

$$A_{ex} = A_{ex}^{\infty}[1 - e^{-kt}]$$

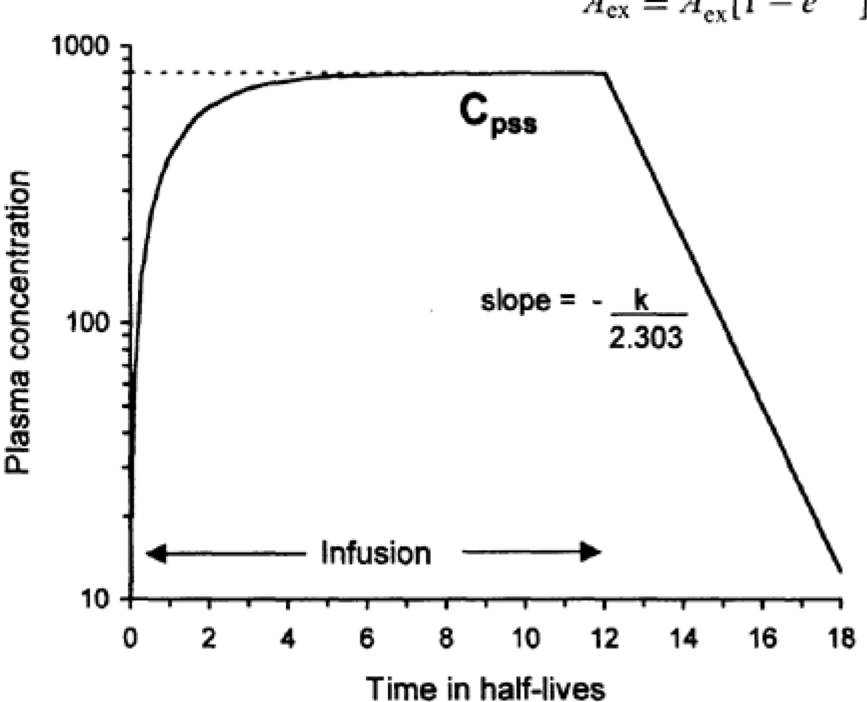

FIG. 4.8. Plasma concentration–time curve for constant intravenous infusion into a single-compartment system. The foreign compound was infused at a constant rate from time =0 to time=12 half-lives when the infusion was stopped.

or

$$A_{ex}^{\infty} - A_{ex} = A_{ex}^{\infty} e^{-kt} \quad [27]$$

The left-hand side of Eq. (27) is equivalent to the amount finally excreted minus the amount excreted up to that time (ΔA_{ex}). Taking logs,

$$\log \Delta A_{ex} = \log A_{ex}^{\infty} - \frac{kt}{2.303} \quad [28]$$

A semilog plot of ΔA_{ex} against time gives a straight line of slope $-k/2.303$. An example of this method is described below under Data Handling.

By analogy with Eq. (22), CL_R may be calculated from the total amount excreted and the plasma AUC

$$CL_R = \frac{A_{ex}}{AUC}$$

where A_{ex} and AUC refer to the same time interval.

Constant Intravenous Infusion

During infusion the plasma concentration (C_p) rises to reach a plateau or steady-state concentration (C_{pss}) at which time the rate of infusion equals the rate of elimination. By analogy with Eq. (1)

$$\frac{C_p}{C_{pss}} = (1 - e^{-kt})$$

or

$$C_p = C_{pss}(1 - e^{-kt}) \quad [29]$$

The various kinetic parameters may be derived from the plasma concentration-time curve for infusion (as given in Figure 4.8).

Decrease at end of infusion. The slope equals $-k$ because on cessation of entry into the single compartment, $C_p = C_{p0} e^{-kt}$. The same slope would be obtained if the infusion was stopped at any stage during the infusion.

Plateau level (C_{pss}). At steady state, the rate of infusion (R) equals the rate of elimination:

$$R = CL \times C_{pss}$$

or

$$CL = \frac{R}{C_{pss}} = V \times k$$

Increase to plateau. Rearranging Eq. (29),

$$C_{pss} - C_p = C_{pss} e^{-kt}$$

Therefore, a plot of ln ($C_{pss} - C_p$) against time gives a straight line with a slope equal to k. The time taken to

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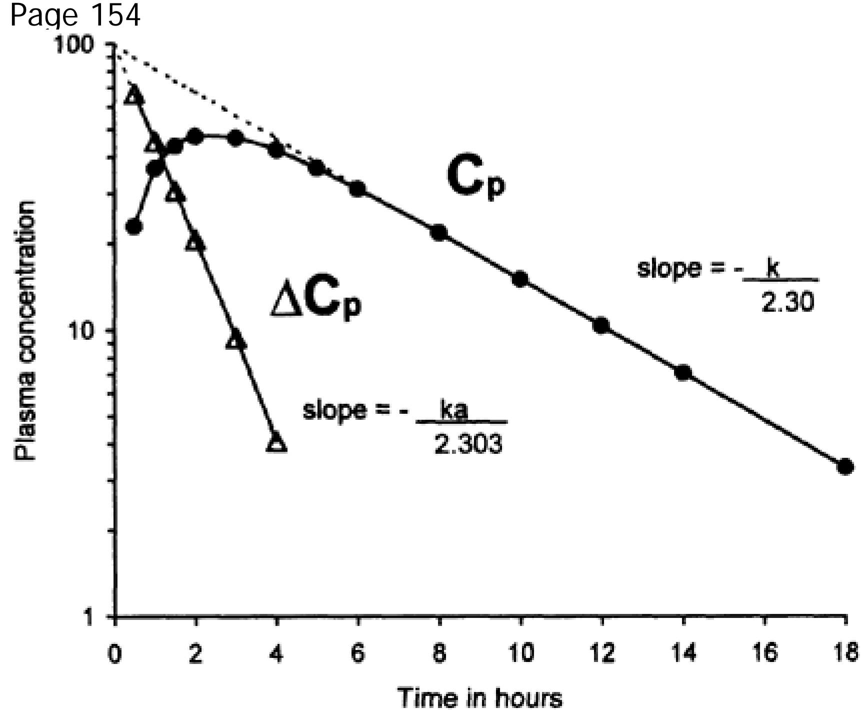

FIG. 4.9. Use of the method of residuals to calculate the absorption rate constant for a one-compartment system. The dose was given at time 0, and plasma levels *(Cp)* were measured at intervals. The linear terminal phase was extrapolated to yield the values corresponding to the measurement times. The difference values (*Cp* extrapolated −*Cp* measured) are plotted (Δ*Cp*) to yield a line of slope −*ka*/2.303 or −*k*/2.303; see text. The data are given in Table 4.5.

reach the plateau is therefore similar to the time taken to eliminate the compound after infusion, or about 97% of the final level within five half-lives.

Area under the curve. Both CL and *V* may be derived using Eqs. (22) and (23).

Oral Administration

Absorption frequently obeys first-order kinetics (80), but may involve a lag time due to delayed gastric emptying. The plasma concentration-time profile may thus resemble Figure 4.9 (see also Table 4.5), and the various pharmacokinetic parameters are related by the equation:

$$C_p = \frac{F \times \text{dose} \times k_a(e^{-kt} - e^{-k_a t})}{V(k_a - k)} \qquad [30]$$

where *F* is the fraction of the dose absorbed and *ka* is the absorption rate constant.

Decrease after peak. The decrease after the peak concentration is determined by the slower of the two processes (absorption or elimination), but it is usually elimination, and the slope is equal to −*k*. (Note that for a polar compound showing slow absorption and rapid elimination, this decrease is equivalent to −*ka*, a situation described by Gibaldi and Perrier (36) as "flip-flop" kinetics [see Figure 4.10]).

Peak plasma concentration. The peak plasma concentration is determined by the relative rates *ka* and *k*. This may be of toxicological importance, especially for acute effects when the extent of toxicity is frequently related to the peak concentration rather than to the area under

Table 4.5 Data used for Figure 4.9

Time (h)	*Cp*	In *Cp*	In *Cp*ex	*Cp*ex	Δ*Cp*	In Δ*Cp*	
0.5	23.0		4.501	90.1	67.1	4.206	
1	36.5		4.406	82.0	45.5	3.818	
1.5	43.9		4.312	74.6	30.7	3.424	
2	47.2		4.218	67.9	20.7	3.030	
3	46.8		4.029	56.2	9.4	2.241	$ka=0.797\ h^{-1}$

4	42.4		3.840	46.5	4.1	1.411
5	36.7		3.652	38.5	1.8	
6	31.1		3.463	31.9	0.8	
8	21.8	3.082				
10	15.0	2.708				
12	10.3	2.332				
14	7.1	1.960	k=0.1887 h−1			
18	3.3	1.194				

Note: The 5- and 6-h points are not included in the residuals analysis, as an error of 3% in the original value of C*p* would translate into an error of 61 and 117%, respectively, for the Δ*Cp* value.
In *Cp*ex=Data generated by linear regression analysis of the terminal phase of In *Cp* against time.
*Cp*ex=Antilogs.
Δ*Cp*=The values *(Cp*ex−*Cp)* used to draw the residuals line.

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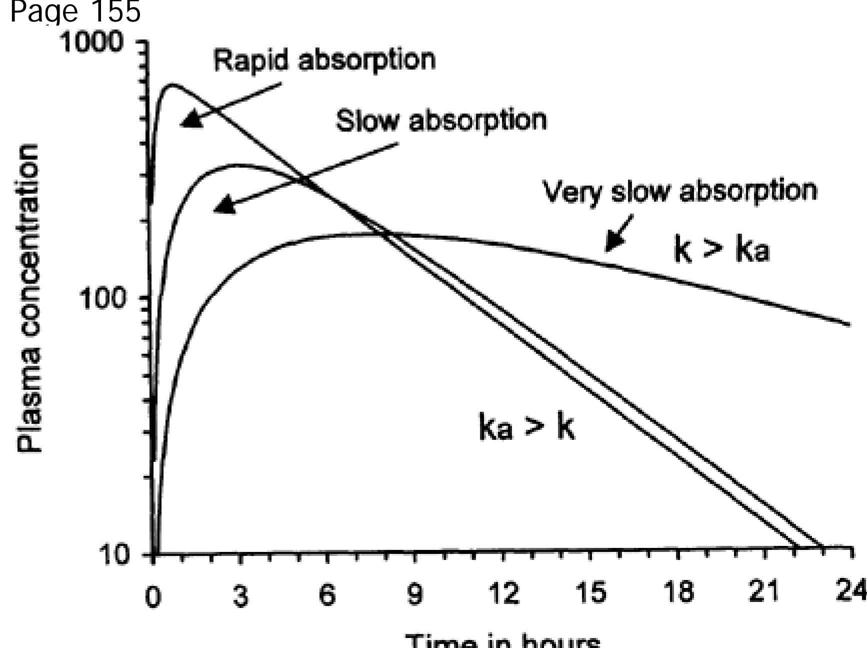


FIG. 4.10. The effect of absorption rate on the shape of the plasma concentration-time curve.

the curve. An increase in the absorption rate may be as important toxicologically therefore as a decrease in elimination rate.

Area under the curve. Both CL and *V* may be derived using Eqs. (22) and (23), providing that the dose used in the calculation is adjusted for the fraction absorbed *(F)*, that is,

$$\mathrm{CL} = \frac{\mathrm{dose} \times F}{\mathrm{AUC}}$$

It is common to see CLoral calculated as (dose/AUC) in the absence of any information on *F*. Such a term is meaningless physiologically; if the value of F is unknown, oral AUC data should be compared as such. Intravenous data are necessary to relate a nonlinear change in AUC at high oral doses to either altered CL or *F*.

The value of *F* may be determined by comparison of oral with intravenous dosing, because CL remains constant.

$$CL = \frac{\mathrm{dose}_o \times F}{\mathrm{AUC}_o} = \frac{\mathrm{dose}_{\mathrm{iv}}}{\mathrm{AUC}_{\mathrm{iv}}}$$

$$F = \frac{\mathrm{dose}_{\mathrm{iv}} \times \mathrm{AUC}_o}{\mathrm{AUC}_{\mathrm{iv}} \times \mathrm{dose}_o} \quad [31]$$

where *o* relates to oral and iv relates to intravenous dosing. These relationships are valid only if the AUC/dose ratio is constant: if not, the value of either *F* or CL must alter with an increase in dose, suggesting saturation of absorption or elimination (see below).

Alternatively, the fraction *F* may be derived from the cumulative urinary excretion:

$$\mathrm{F} = \frac{A^{\infty}_{\mathrm{exo}}}{A^{\infty}_{\mathrm{exiv}}} \times \frac{\mathrm{dose}_{\mathrm{iv}}}{\mathrm{dose}_o} \quad [32]$$

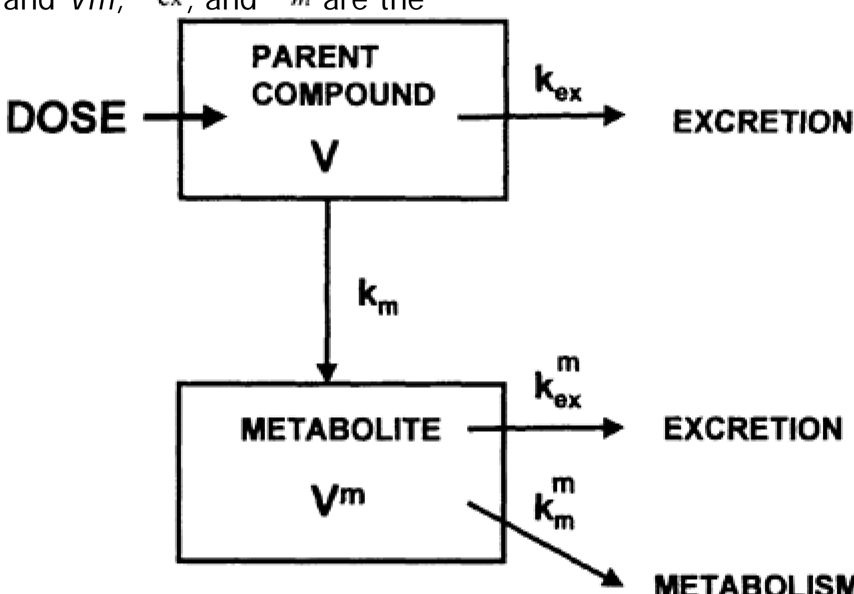
Increase to peak. The increase to peak is determined by the more rapid of the two processes, that is, usually absorption. Measurement of the absorption rate constant must make allowance for the excretion occurring throughout the postdosing period; the method of residuals is used (see Gibaldi and Perrier (36) for the mathematical basis of this method). The method is illustrated and explained in Figure 4.9 and Table 4.5. In cases where absorption is slow, the rate of increase may be determined by the elimination rate constant. Thus the value of *ka* can be assigned to the increase to peak only after demonstration that the value of *k* for the decrease is similar to that seen after intravenous dosing.

Metabolite Kinetics

As discussed elsewhere, the biotransformation of xenobiotics usually results in detoxication but is sometimes associated with the formation of a toxic metabolite. Measurement of the rate of metabolism

in vivo can provide much useful information on detoxication or bioactivation processes. In most cases, the rate of metabolite formation is governed by in vivo enzyme kinetics, which are first-order only over a limited substrate concentration range. Saturation of metabolism is discussed in more detail below, and the following analysis relates to metabolite formation under first-order reaction conditions, when CL depends on enzyme activity rather than liver blood flow.

The measurements that are available for analysis of metabolite kinetics include plasma levels of unchanged drug *(Cp)* and metabolite (C_p^m). The simple system given earlier, for the parent compound (Scheme 4.2) can be extended into Scheme 4.3, where *V, k*ex, and *km* are, respectively, the apparent volume of distribution, excretion rate constant, and metabolism rate constants for the parent compound, and *Vm*, k_{ex}^m, and k_m^m are the



SCHEME 4.3. One-compartment model with metabolite formation.

same parameters for the metabolite. The time course for the metabolite is given by

$$\frac{dM}{dt} = k_m \mathrm{Ab} - k^m M$$

where Ab and *M* are the amount of parent compound and metabolite in the body, respectively, and *km* is the overall elimination rate constant for the metabolite, that is, $k^m = k^m_{ex} + k^m_m$. This equation may be solved to yield

$$C^m_p = \frac{k_m \mathrm{dose}(e^{-k_m t} - e^{-kt})}{V^m(k - k^m)} \quad [33]$$

where C^m_p is the plasma concentration of the metabolite at time=*t*.

In many cases the overall elimination rate of the metabolite *(km)* is greater than the overall elimination rate of the parent compound *(k)* (for example in the case of the formation of a more polar metabolite). In such cases the term $e^{-k^m t}$ approaches zero before *e−kt*, and thus at late time points Eq. (33) may be rewritten and solved omitting e^{-k^m} when it becomes

$$\log C^m_p = \log \frac{k_m \times \mathrm{dose}}{V^m(k^m - k)} - \frac{kt}{2.303} \quad [34]$$

Thus the log plasma concentration of the metabolite-time curve has a terminal slope similar to that of the parent compound (i.e., *k*/2.303) (Figure 4.11). In this case the rate of elimination of the metabolite is limited by the elimination of the parent drug, and the metabolite/drug ratio remains constant during the elimination phase (Figure 4.11).

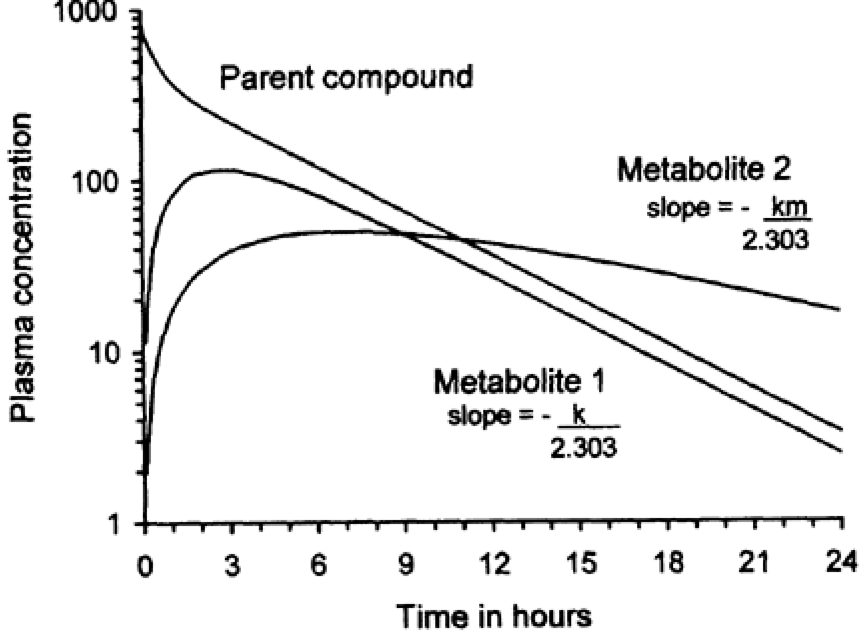


FIG. 4.11. Plasma concentration-time curves for parent compound and metabolites after intravenous dosing. The parent compound was given as an intravenous bolus dose at time=0.

In those cases where the elimination rate of the metabolite *(km)* is less than that of the parent compound *(k)*, the term *e−kt* approaches zero before $e^{-k^m t}$, and thus Eq. (33) may be written

$$\log C^m_p = \log \frac{k_m \times \mathrm{dose}}{V^m(k - k^m)} - \frac{k^m t}{2.303} \quad [35]$$

and a plot of log plasma concentration of the metabolite-time curve has a slope of −*km*/2.303. In this case, the ratio metabolite/drug increases during the elimination phase (Figure 4.11). The latter case is of particular interest to toxicologists because on repeated exposure the concentrations of metabolite at steady state may exceed those of the parent compound.

The overall elimination rate constants may also be derived from urinary metabolite levels as described above for the parent compound, although again the derived rate may be either *k* or *km* and the identity

can be determined only by measuring k and k_m separately after administration of both the parent compound and the metabolite. However, if metabolite kinetics are based solely on urinary excretion data, the formation of more lipid-soluble metabolites may be missed. For example, the active thioether metabolite of sulfinpyrazone is a major circulating metabolite of which negligible amounts are excreted in the urine (102).

Two-Compartment Open Model

Mathematically and physiologically, it is often more appropriate to regard the body as representing a simple two-compartment open system in which the distribution to certain peripheral tissues is not an instantaneous process. In such a system the chemical initially enters a central compartment (the plasma and those tissues for which distribution is instantaneous) and is subsequently distributed to a second, peripheral compartment. Elimination occurs from the central compartment, so that chemical in the peripheral compartment must transfer back to the central compartment in order to be eliminated (Scheme 4.1B or Scheme 4.4). In Scheme 4.4, k_{12} and k_{21} are the rate constants for transfer from compartment 1 to 2 and from 2 to 1, respectively, and k_{10} is the elimination rate from the central compartment.

Intravenous Bolus Dose

After a single intravenous bolus dose into a twocompartment system, the plasma concentration *(Cp)* at time t may be described by

$$Cp = Ae^{-\alpha t} + Be^{-\beta t} \qquad [36]$$

where A and B may be regarded as analogous to Cp_0 for each compartment, and $A+B=Cp_0$; α and β correspond

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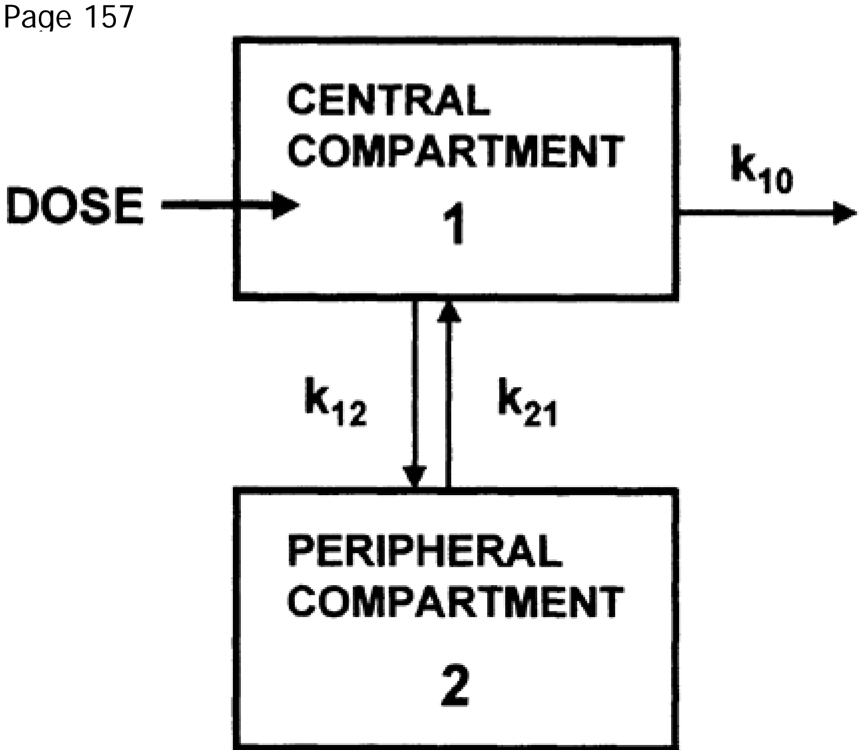

SCHEME 4.4. Two-compartment model.

to hybrid rate constants, each influenced by all the individual distribution, redistribution, and elimination rate constants, that is, $k12$, $k21$, and $k10$ (36). The shape of a typical plasma concentration-time curve following a bolus intravenous dose is given in Figure 4.12 (see also Table 4.6) with the plasma data and the method of derivation of the various constants. As with the determination of absorption rate constants discussed above, the method of residuals or line stripping is used to separate α and β. In the terminal phase, $Ae-\alpha t$ approaches zero, and the data are described by $Cp=Be-\beta t$. For example, using the data in Table 4.6,

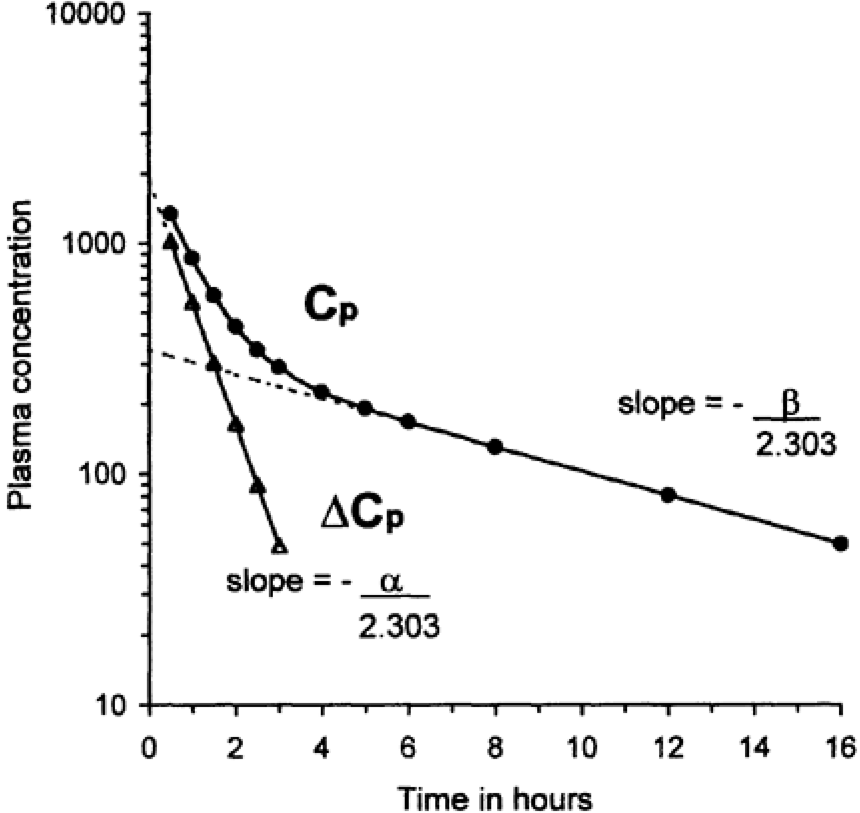

FIG. 4.12. Plasma concentration-time curve for two-compartment system. The data are given in Table 4.6.

when $t=8$ h, $Be^{-\beta t}=346e^{-0.121\times 8}=131$ μg/ml, whereas $Ae^{-\alpha t}=1875e^{-1.214\times 8}=0.1$ μg/ml; therefore, the contribution of the latter term is negligible. The terminal phase after 8 h is therefore extrapolated back to time 0 when the intercept is equal to B and the slope of log Cp against time is $\beta/2.303$. As described in Table 4.6, the values of B and β may also be derived by least-squares linear

Table 4.6 Data used for Figure 4.12

Time (h)	Cp (μg ml^{-1})	ln Cp	ln Cp_{ex}	Cp_{ex}	ΔCp	ln ΔCp	
0.5	1345		5.788	326	1,019	6.927	By linear regression $\alpha=1.214$ h^{-1}; ln $Cp_0=7.537$; ∴ $A=1875$ μg ml^{-1}
1	864		5.727	307	557	6.323	
1.5	593		5.666	289	304	5.717	
2	438		5.606	272	166	5.112	
2.5	346		5.545	256	90	4.500	
3	290		5.485	241	49	3.892	
4	228		5.364	214	15	2.708	
5	193		5.243	189	4	1.386	
6	168	5.122					Terminal phase; by linear regression $\beta=0.1210$ h^{-1}; ln $Cp_0=5.848$; ∴ $B=346$ μg ml^{-1}
8	131	4.879					
12	81	4.395					
16	50	3.911					

ln Cp_{ex}=Data generated by linear regression analysis of the terminal phase data for ln Cp against time.
Cp_{ex}=Antilogs of these extrapolated points; similar values may be obtained from the extrapolated line on the graph.
ΔCp=The values $(Cp-Cp_{ex})$; they may be used to derive the log_{10} residuals line (slope $-\alpha/2.303$) or may be converted to natural logarithms and analyzed by linear regression.

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regression analysis of the terminal phase, after graphical analysis to determine the point at which linearity commences.

At early time points, the difference between the actual *Cp* values and the concentrations derived by backextrapolation of the $Be^{-\beta t}$ line are due to the contribution from $Ae^{-\alpha t}$. The values of *A* and *α* may be similarly derived by calculated linear regression or graphical analysis of the residuals or Δ*Cp* (*Cp*actual−*Cp*extrapolation). In the analysis of the residuals (Table 4.6), the Δ*Cp* values for 4 and 5 h were not included, as these values represent only about 5% or less of the original value of *Cp* and thus are subject to large inaccuracies (up to +100%) owing to the errors inherent in all methods of analysis of foreign compounds in biological fluids.

Thus the plasma concentration-time curve in Figure 4.12 may be represented by the equation:

$$Cp = 1875e^{-1.214t} + 346e^{-0.1210t}$$

The rate constants *α* and *β* are composite rate constants, from which it is possible to derive k_{12}, k_{21}, and k_{10} given in Scheme 4.4 using the following equations (see refs. 36 and 112 for derivations):

$$C_{p0} = A + B$$

$$\alpha + \beta = k_{12} + k_{21} + k_{10}$$

$$V_1 = \frac{\text{dose}}{A + B} \quad [37]$$

where V_1 is the volume of the central compartment, and

$$k_{21} = \frac{A\beta + B\alpha}{A + B} \quad [38]$$

$$k_{10} = \frac{\alpha\beta}{k_{21}} \quad [39]$$

$$k_{12} = \alpha + \beta - k_{21} - k_{10} \quad [40]$$

For the example given in Figure 4.10,

$$k_{21} = \frac{(1875 \times 0.1210) + (346 \times 1.214)}{(1875 + 346)} = 0.291$$

$$k_{10} = \frac{(1.214 \times 0.1210)}{0.291} = 0.505$$

$$k_{12} = 1.214 + 0.1210 - 0.291 - 0.505 = 0.539$$

It is important to note that k_{10} (0.505) and *β* (0.121) do not relate to the same process, because k_{10} refers to the elimination from the central compartment, whereas *β* refers to the overall elimination from the body (and is slower due to transfer out of tissues as well as elimination from the central compartment). The relation between *β* and k_{10} is given by Eq. (40), which may be rewritten as

$$\beta = k_{10} + k_{21} + k_{12} - \alpha$$

which clearly shows that *β* is a hybrid rate constant. It is, however, a valuable constant and can be used to derive the terminal half-life (0.693/*β*).

As with the one-compartment system, an intravenous bolus allows derivation of most pertinent pharmacokinetic parameters:

1. *A, B, α,* and *β* may be derived from plasma data (see above).
2. k_{10}, k_{12}, k_{21}, and V_1 may be derived by manipulation of *α, β,* etc. (see above).
3. *α, β,* k_{10}, k_{12}, and k_{21} may be derived from urine by plotting the excretion rate against time. In this case, the intercept values of excretion rate $(A'+B')$ do not equate to $A+B$, and thus V_1 cannot be deduced. However, k_{10}, k_{12}, and k_{21} can be obtained from Eqs. (38–40) by substitution of *A* and *B* by *A′* and *B′*. The renal elimination rate constant (k_R) is given by

$$k_R = \frac{A' + B'}{\text{dose}}$$

4. *α, β,* k_{10}, k_{12}, and k_{21}, may be derived from urine by the sigma-minus method, where log $(A_{ex}^{\infty} - A_{ex})$ [see Eq. (27)] is plotted against time. Again *α* and *β* may be derived by the method of residuals; k_{10}, k_{12}, and k_{21} can be calculated from from *α* and *β* and the intercepts (*A″* and *B″*) by substitution in Eqs. (38–40). The renal elimination rate constant (k_R) is given by

$$k_R = \frac{A_{\text{ex}}^{\infty}}{\text{dose}} \times k_{10}$$

5. The renal elimination constant, k_R, may be derived also from the renal clearance

$$CL_R = \frac{C_u \times F_u}{C_p}$$

and the value of V_1 as $CL_R = k_R V_1$.

6. The amount in the peripheral compartment may be calculated from the following equation (which is similar to Eq. (30) for absorption into a single compartment):

$$C_2 = \frac{\text{dose} \times k_{12}(e^{-\beta t} - e^{-\alpha t})}{V_2(\alpha - \beta)} \qquad [41]$$

where C_2 and V_2 are respectively the concentrations in, and volume of, the peripheral or deep compartment.

During the terminal phase of the concentration-time curve, $e^{-\alpha t}$ approaches zero and therefore Eq. (41) may be simplified as

$$C_2 = \frac{\text{dose} \times k_{12} \times e^{-\beta t}}{V_2(\alpha - \beta)}$$

Therefore, a graph of log C_2 against time has a slope of $-\beta/2.303$. Thus the terminal rate of decrease in the peripheral compartment of a two-compartment system is identical to the decrease in the central compartment.

In absolute terms, the calculation of C_2 is not particularly valuable, because the peripheral tissues comprising the deep compartment are not homogeneous, and the compound may not show a uniform concentration. Thus C_2 should not be regarded as the effective drug concentration, even if the target organ lies within the deep compartment. Rather, the concentration in the target organ should be measured, from which subsequent concentrations may be calculated using β defined from the central compartment.

A further useful kinetic parameter (V_β), which relates the total amount of chemical in the body to the plasma concentration, is given by the equation

$$V_\beta \times \beta = V_1 \times k_{10} = \frac{\text{dose}}{\text{AUC}} = \text{CL}$$

Just as β is a hybrid term reflecting overall elimination from the body, so V_β is a composite but valuable function.

$$V_\beta = \frac{\text{dose}}{\text{AUC} \times \beta}$$

Intravenous Infusion

The shape of the plasma concentration-time curve on intravenous infusion into a two-compartment open system is similar to that given in Figure 4.8, but with a biphasic increase at the start of the infusion and a biphasic decrease at the end. The kinetic parameters may be derived from the graph similarly to the one-compartment model, as follows.

Increase to plateau. The increase to plateau follows a complex exponential function with 90 and 99% of the steady-state concentration being reached after four and seven half-lives, respectively.

Plateau level (C_{pss}). At steady state, the rate of infusion *(R)* equals the rate of elimination. Therefore

$$\frac{R}{C_{pss}} = \text{CL} = V_1 \times k_{10} = V_\beta \times \beta$$

Decrease after plateau. The decrease after plateau follows the equation:

$$C_p = A^* e^{-\alpha t^*} + B^* e^{-\beta t^*}$$

where A^* and B^* are the intercepts by back extrapolation to the end of the infusion of the α and β slopes (determined as described for Figure 4.12) and t^*=time since cessation of infusion.

In many cases, two-compartment characteristics seen after a bolus dose are obscured in postinfusion data, because much of the distribution phase will have occurred during the infusion, so that the duration of the α phase may be reduced.

Area under the curve. The AUC can be used to derive the plasma clearance using Eq. (22).

Oral Administration

Assuming first-order absorption into compartment 1, the plasma concentration at time t is given by

$$C_p = A^{\ddagger} e^{-\alpha t} + B^{\ddagger} e^{-\beta t} + C^{\ddagger} e^{-k_a t}$$

Graphical analysis by a semilogarithmic plot of log C_p against time may reveal three separate phases, from which α, β, and k_a should be measurable using the method of residuals. However, in practice the value of k_a is frequently similar to a, and compounds that require a two-compartment model after intravenous administration appear to fit first-order absorption into a one-compartment model following oral dosing (18). Thus analysis is not possible without reference to intravenous data to determine which rate constant refers to the absorption rate. An example of linear regression analysis to obtain the three rate constants was given by Wagner (112). An alternative method (deconvolution method) may be used that derives the absorption rate constant by a comparison of plasma concentrations for intravenous and oral administration, and does not require fitting the data to a particular one-, two-, or three-compartment model. This method (36, 112) does, however, require analysis of the plasma concentrations at the same time points after both oral and intravenous dosing. Various methods of

calculating the absorption rate are discussed in Gibaldi and Perrier (36).

The absorption rate is likely to be of greatest importance in acute toxicity studies, whereas the bioavailability *(F)* may be more significant in chronic studies; the latter may be measured using model-independent equations [Eq. (31) or (32)]. However, absorption from the gastrointestinal tract is complex, as it involves physiologically different membranes at differing luminal pH values. Thus the process may involve more than one first-order rate, or a zero-order component, or both; an alternate approach to compartmental analysis and a valuable measure is the mean absorption time (see below).

Metabolite Kinetics

Frequently, metabolites of foreign compounds fit a two-compartment open model, in which case a second

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compartment for the metabolite is in equilibrium with the central metabolite compartment, as well as a second compartment for the parent compound (see Scheme 4.3). The equation describing these four compartments requires four exponential terms, but often the concentration-time curve for the metabolite appears as a bi-exponential decrease. The slow terminal phase of the metabolite is given by either β for the parent compound, or the terminal rate for the metabolite (see earlier); the faster rate is a composite of the other three rate constants.

Multiple Dosing: Chronic Administration

On multiple dosing or continuous intake, the plasma levels increase over a period of four to five half-lives to establish a plateau concentration similar to that seen with intravenous infusion (Figure 4.8). The average plateau level is subject to variations around a mean as material is eliminated between "doses." In oral toxicity studies, these "doses" may represent either repeated single-gavage doses or the feeding habits of the animals if the test compound is incorporated into the diet and fed ad libitum. On cessation of chronic intake, the rate of decrease in blood levels is usually but not always similar to that seen after a single dose (22).

One-compartment Open Model

The time taken to reach plateau plasma levels is four to five times the half-time of the terminal phase of the plasma concentration-time curve. The average plateau level is given (by analogy with intravenous infusion) as

$$C_{p\,\text{mean}} = \frac{\text{dose} \times F}{V \times k \times T} \qquad [42]$$

where F is the fraction absorbed, T is the dose interval, and k is the elimination rate constant. However, it is important to realize that this equation is appropriate only if the terminal phase following oral administration is due to elimination. When the compound exhibits slow absorption and rapid elimination, the decrease in plasma levels is determined by the slower absorption rate *(ka)*. An alternative equation can be derived from the fact that at steady state the rate of input ($F \times \text{dose}/T$) is balanced by the rate of elimination ($Cp\text{mean} \times \text{CL}$); therefore

$$C_{p\,\text{mean}} = \frac{\text{dose} \times F}{T \times \text{CL}}$$

The fluctuations around the mean plateau level depend on the dosing interval in relation to the terminal elimination rate. Thus compounds with a short half-life show much larger fluctuations, as more of the chemical is eliminated between each dose. In the case of compounds with a short half-life (2–3 h), single daily dosing gives plasma levels approaching zero prior to each dose. Interdose fluctuations may be reduced and blunted by slow absorption. The equations relating to these processes were detailed by Gibaldi and Perrier (36) and Wagner (112). In summary, at steady state after repeated intravenous doses, the minima and maxima are given by

$$C_{p\,\text{minimum}} = \frac{\text{dose}}{V}\left(\frac{e^{-kT}}{1-e^{-kT}}\right) \qquad [43]$$

$$C_{p\,\text{maximum}} = \frac{\text{dose}}{V}\left(\frac{1}{1-e^{-kT}}\right) \qquad [44]$$

When absorption from the gut occurs as a first-order process, the fluctuations in the steady-state concentration-time curve can be described by the following equation:

$$C_p = \frac{F \times \text{dose} \times k_a}{V(k_a - k)} \times \left[\left(\frac{1}{1-e^{-kT}}\right)e^{-kt} - \left(\frac{1}{1-e^{-k_aT}}\right)e^{-kt}\right] \qquad [45]$$

where Cp is the concentration at time t and T is the dose interval. The similarity between this equation and Eq. (30) for absorption of a single dose is apparent.

The value of the mean plasma concentration at steady state (Cpmean) may be calculated without knowledge of F, V, ka, or k by measuring the area under the plasma concentration-time curve for a single oral dose, as

$$\text{AUC}_{\text{oral}} = \frac{\text{dose} \times F}{V \times k} = \frac{\text{dose} \times F}{\text{CL}} \qquad [23]$$

where AUCoral is the area under the plasma concentration-time curve between $t=0$ and $t=\infty$ for a single oral dose. Substituting into Eq. (42),

$$C_{\text{mean}} = \frac{\text{AUC}_{\text{oral}}}{T}$$

[46]

It is important to realize, however, that substitution of Eq. (23) into Eq. (42) assumes that the AUC is directly proportional to the dose, that is, that dose-dependent kinetics are absent and that CL does not alter during chronic administration of the compound. The latter possibility may be assessed by comparison of the $\text{AUC}_{0-\infty}$ for a single dose, with the AUC for a dose interval at steady state, that is, AUC_{0-T} for chronic administration,

$$\begin{aligned} \text{CL} &= \frac{\text{dose (single)} \times F}{\text{AUC}_{0-\infty}} \\ &= \frac{\text{dose (chronic)} \times F}{C_{p\,\text{mean}} \times T} \\ &= \frac{\text{dose (chronic)} \times F}{\text{AUC}_{0-T}} \end{aligned}$$

If $\text{AUC}_{0-T}(\text{chronic}) < \text{AUC}_{0-\infty}(\text{single})$, either induction

of metabolism or decreased bioavailability is indicated; conversely if AUC_{0-T}(chronic)>$AUC_{0-\infty}$ (single), then inhibition or saturation of metabolism is suggested.

The extent of accumulation on repeated intake may be measured by the average amount in the body at steady state (Abmean), divided by the amount in the body after a single dose (Ab), that is,

$$\text{extent of accumulation} = \frac{Ab_{\text{mean}}}{Ab} = \frac{Ab_{\text{mean}}}{\text{dose} \times F}$$

The amount in the body at the plateau is given by Eq. (42):

$$Ab_{\text{mean}} = VC_{p\,\text{mean}} = \frac{F \times \text{dose}}{k \times T}$$

Therefore

$$\text{Extent of accumulation} = \frac{1}{k \times T} = \frac{1}{0.693/t_{1/2} \times T} = \frac{1.44 \times t_{1/2}}{T}$$

Two-compartment Open Model

The equations giving the plasma concentration at time *t* at steady state into a two-compartment system with first-order absorption are considerably more complex than those for the one-compartment system. However, the simplified equation [Eq. (42)] applies in the form

$$C_{p\,\text{mean}} = \frac{\text{dose} \times F}{V_1 \times k_{10} \times T} = \frac{\text{dose} \times F}{V_\beta \times \beta \times T}$$

and the value of $C_p$mean may still be derived from Eq. (46):

$$C_{p\,\text{mean}} = \frac{\text{AUC}_{\text{oral}}}{T} = \frac{\text{dose} \times F}{\text{CL} \times T}$$

In addition, the relationship between the AUC between $t=0$ and $t=\infty$ for a single dose and the AUC for a dose interval at steady state applies on the condition that neither CL or *F* changes on chronic intake (see above); a difference between these AUC estimates indicates changes in CL or *F* during chronic treatment.

Statistical Moment Analysis

In recent years both clinical pharmacokinetic and animal toxicokinetic studies have moved away from compartmental analyses, because they involve multiple variables, which require numerous properly timed blood samples to characterize them adequately. Also, curve fitting is dependent on the terminal slope, which is frequently measured using plasma concentrations that approach the limit of detection of the assay method, that is, are the weakest data. In contrast, terms such as clearance are measured from dose and AUC, the latter being determined largely from the highest and most accurately measured concentrations. Such "time-averaged" parameters may be extended to "time-related" parameters by the use of statistical moment theory, which allows assessment of additional useful kinetic parameters such as *mean residence time* (MRT). The plasma concentration-time curve may be regarded as a statistical distribution curve for which the zero and first moments are the AUC and MRT respectively:

$$\text{AUC} = \int_0^\infty C_p \text{d}t$$

$$\text{MRT} = \frac{\text{AUMC}}{\text{AUC}} \quad [48]$$

where AUMC is the area under the first moment of concentration time curve, that is, $\int_0^\infty t \times C_p \text{dt}$.

The AUC and AUMC may be calculated using the trapezoid rule, which is illustrated under Data Handling (see Table 4.17). The AUC from the last data point to infinity can be calculated as $C_p$last/β. The AUMC from the last data point to infinity has to be calculated as

$$\frac{t_{\text{last}} \times C_{p\,\text{last}}}{\beta} + \frac{C_{p\,\text{last}}}{\beta^2}$$

Clearly any inaccuracy in the value of *β* affects the extrapolation of AUMC to infinity more than the extrapolation of AUC. This situation is shown in the data given in Table 4.17, where the extrapolated area is 17% of the AUMC, but only 3% of the AUC.

In the same way that the AUC can be related to CL, *k* and ***V*, *β*** and *Vβ*, etc., so the AUMC can be used to derive additional useful parameters.

Intravenous Administration

Following an intravenous bolus dose, the MRT can be calculated by Eq. (48) as illustrated in Table 4.17. The *apparent volume of distribution at steady state (V*ss*)* may be regarded as the volume of plasma in which the compound appears to be dissolved and that has to be "removed" from the body, that is, the product of clearance (ml min−1) and MRT (min):

$$V_{ss} = \mathrm{CL} \times \mathrm{MRT} = \frac{\mathrm{dose}}{\mathrm{AUC}} \times \frac{\mathrm{AUMC}}{\mathrm{AUC}} = \frac{\mathrm{dose} \times \mathrm{AUMC}}{\mathrm{AUC}^2} \qquad [49]$$

Attempts to separate the MRT, which refers to the whole body, into central and peripheral components (110) may prove to be of value, but are dependent on the data fitting a two-compartment model.

If the compound is too toxic to be given as an instantaneous bolus, the MRT can be calculated from the AUMC determined following an intravenous infusion

using the equation:

$$\mathrm{MRT}_{\mathrm{infusion}} = \mathrm{MRT} + \frac{T}{2} \qquad [50]$$

where $\mathrm{MRT}_{\mathrm{infusion}}$ is calculated from the AUMC and AUC by Eq. (48) from the infusion data, and *T* is the infusion time.

V_{ss} cannot be derived directly from the AUMC and AUC data from infusions, because the AUMC value contains a component due to the infusion time. The following equation therefore applies,

$$V_{ss} = \frac{\text{infused dose} \times \mathrm{AUMC}}{\mathrm{AUC}^2} - \frac{\text{infused dose} \times T}{2 \times \mathrm{AUC}} \qquad [51]$$

In the same way that CL may be related to *V* by the rate constant *k* [Eq. (21)], so it may be related to V_{ss} by the first-order rate constant k_{ss} (7, 36).

$$\mathrm{CL} = k_{ss} V_{ss} = \frac{V_{ss}}{\mathrm{MRT}}$$

Therefore k_{ss} is equivalent to 1/MRT; for a two-compartment system k_{ss} is intermediate between α and β. The half-life derived from k_{ss} ($0.693/k_{ss}$ or $0.693\times\mathrm{MRT}$) is therefore a composite half-life, and may be regarded as the "effective" half-life. This is shown in the data analyzed later (Table 4.17) where the half-life derived from MRT ($0.693\times25=17$ min) is intermediate between that calculated from α ($0.693/0.0705=10$ min) and β ($0.693/0.0240=34$ min).

Oral Administration

A major strength of the statistical moment theory is its ability to derive meaningful data following oral administration, because it is both more reliable and easier to use than most other methods (18) and does not rely on assumptions about a first-order or zero-order process. The most useful parameter is the *mean absorption time* (MAT), which is the difference between the mean residence times following oral and intravenous dosing:

$$\mathrm{MAT} = \mathrm{MRT}_{\mathrm{oral}} - \mathrm{MRT}_{\mathrm{iv}} \qquad [52]$$

The MAT may be used to derive apparent first-order rate constants and half-lives:

$$k_a = \frac{1}{\mathrm{MAT}}$$

$$\text{Absorption } t_{1/2} = 0.693 \times \mathrm{MAT}$$

Alternatively, if absorption appears to be zero order, by analogy with Eq. (50):

$$\mathrm{MAT} = \frac{T_{\mathrm{abs}}}{2}$$

where T_{abs} is the duration of the absorption process.

The measurement of MAT is generally applied to absorption from a solution. If a sparingly soluble compound is given,

$$\mathrm{MRT}_{\mathrm{oral}} = \mathrm{MRT}_{\mathrm{iv}} + \mathrm{MAT} + \mathrm{MDT}$$

where MDT is the mean dissolution time.

The statistical moment theory is therefore a valuable technique for comparisons on the influence of dosage formulations on absorption (18, 79).

Chronic Administration

As discussed previously, the increase to steady state for multicompartment models is complex. AUC data may be used, in the absence of compartmental analysis, to derive the proportion of steady state reached at any time after dosing. The proportion of steady state is equal to the AUC to that time (AUC_{0-t}) calculated as a proportion of the total AUC extrapolated to infinity ($\mathrm{AUC}_{0-\infty}$), that is,

$$\%\ \text{steady state} = 100 \times \frac{\mathrm{AUC}_{0-t}}{\mathrm{AUC}_{0-\infty}}$$

There is, however, a significant complication in the use of statistical moments for the analysis of steady-state data because, unlike AUC data, $\mathrm{AUMC}_{0-\infty}$ for a single dose does not equal AUMC_{0-T} during regular dosing (6). A simple way to overcome this difficulty is to apply a method of residuals to the steady-state plasma concentration prior to the regular dose (C_{min}). Assuming that this level decreases by a single first-order rate (β) determined from the terminal phase of the interdose period (or the terminal phase following a single dose), the contribution of this residue to each subsequent sample can be calculated as $C_{min}e^{-\beta t}$, where *t* is the time of that particular sample after C_{min}. The calculated

residue is then subtracted from the measured value at each time to derive pseudo-single-dose data $(C - C_{min} e^{-\beta t})$ that can be used to calculate AUMC.

Dose-Dependent or Nonlinear Kinetics

Whereas simple diffusion obeys first-order kinetics at all concentrations, many of the other processes fundamental to toxicokinetics involve an interaction between the foreign chemical and a specific site on a protein (examples being active transport across the gut, plasma and tissue protein binding, metabolism, and renal tubular secretion). These processes have a finite capacity for interaction between the chemical and the protein; thus at high concentrations of chemical, all the specific sites on the protein may be occupied. Addition of further chemical cannot result in further interaction between the chemical and protein, and the concentration of free compound increases rapidly. Depending on the nature of the

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protein-chemical interaction, there are a number of possible consequences, which are summarized in Table 4.7. This table represents a considerable simplification because the effect of saturation at one site may affect another protein-chemical interaction. For example, saturation of renal tubular secretion gives increased AUC/dose and elevated plasma concentrations. However, the resultant high concentrations may saturate plasma protein binding, resulting in an increase in free drug and increased glomerular filtration and/or hepatic clearance. Thus the decreased elimination in the renal tubule may be overcome to some extent by increased elimination elsewhere.

Almost all of the processes listed in Table 4.7 may be described by a Michaelis-Menten equation of the type introduced into Eq. (11), that is,

$$-\frac{dC}{dt}=\frac{V_{max}\times C}{K_m+C} \quad [53]$$

where *V*max is the theoretical maximum rate of the reaction and *Km* is the Michaelis constant (which reflects the concentration giving 50% saturation of the protein).

At low concentrations $C \ll K_m$, and *Km*+C approximates to *Km* so that

$$-\frac{dC}{dt}=\frac{V_{max}\times C}{K_m}$$

and *Vmax/Km* is equivalent to the first-order rate constant *k*.

At higher concentrations $C \gg K_m$, and *Km*+C approximates to *C* so that

$$-\frac{dC}{dt}=\frac{V_{max}\times C}{C}=V_{max}$$

and thus the elimination is a zero-order reaction. The shape of the plasma concentration-time curve for a hypothetical compound showing saturation kinetics is given in Figure 4.13, which clearly shows that although low doses are indistinguishable from first-order elimination, the decrease at high plasma concentrations shows zero-order and then first-order reaction components.

It is important to note that the terminal slope and terminal half-life are derived from low plasma concentrations, and do not provide evidence of dose dependence. However, the plasma clearance, which is derived from AUC data and which reflects the capacity of the organs of elimination to remove the chemical from plasma, provides the best evidence of saturation. This is shown clearly by derivation of the appropriate rate constants, etc., for the example given in Figure 4.13 and Table 4.8, which shows a fivefold change in CL; it also illustrates the power of the statistical moment approach, which shows a fourfold increase in MRT. The value of *k* (0.0485) approximates to *Vmax/Km* (0.050).

Table 4.7 Consequences of saturation of chemical-protein interactions

Site	Interaction	Possible consequences of saturation at high dose
Absorption	Active uptake	Reduced plasma levels and AUC after oral but not IV doses.
	First-pass metabolism	Increased plasma levels and AUC after oral but not IV doses.
Distribution	Plasma protein	Increased volume of distribution; increased glomerular filtration; increased hepatic clearance if extraction ratio is low.
	Tissue protein	Decreased volume of distribution; a graph of *Ct/Cp* against *Cp* will be nonlinear.
Metabolism	Metabolizing enzyme (saturation by substrate, depletion of cofactors, product inhibition)	Decreased clearance; AUC/dose ratio increases for parent compound, whereas AUC of metabolite/dose ratio may decrease for both oral and IV doses; enzymes with high *Km* values may handle a larger proportion of the dose.
Excretion	Renal tubular secretion	Decreased renal clearance; AUC/dose ratio increases for oral and IV doses; nonrenal routes of elimination become of more importance; total excretion in urine per dose may decrease depending on the availability of other routes of elimination.
	Renal tubular reabsorption (rare)	Opposite of effects for saturation of renal tubular secretion.
	Biliary excretion	Decreased biliary clearance; decreased enterohepatic recirculation; renal route may become more important; AUC/dose ratio increases for oral and IV doses.

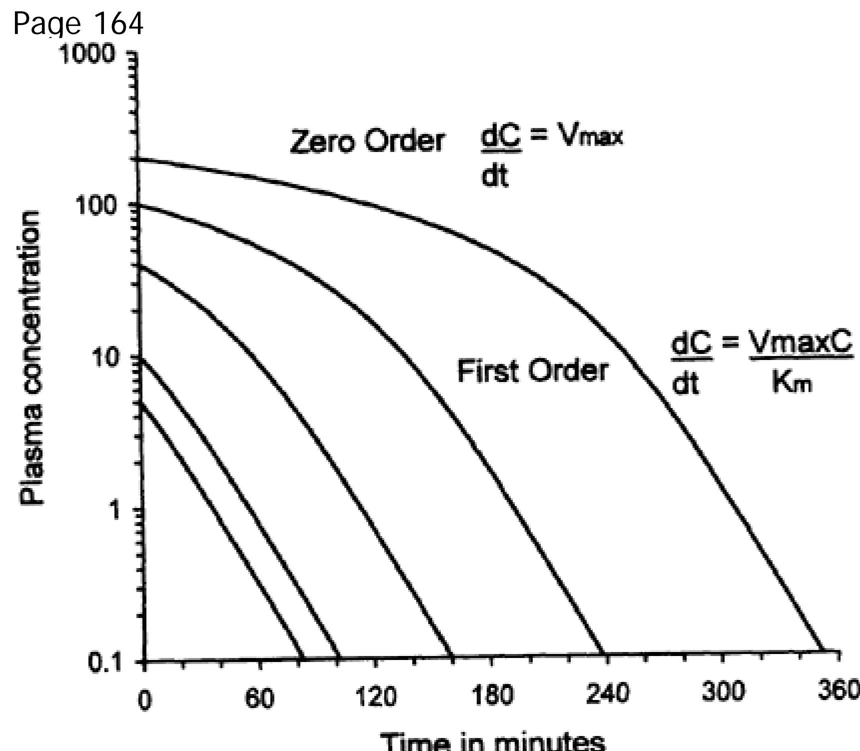

FIG. 4.13. Plasma concentration-time curve for a compound showing saturation kinetics. The data were generated using an apparent V_{max} of 1 μg/min and a K_m of 20 μg/ml for initial concentrations of 5, 10, 40, 100, and 200 μg/ml. Data points were obtained using a derivative of Eq. (53), $V_{max}(t - t_0) = C_{p0} - K_m \ln(C_{p0}/C_p)$.

An increased understanding of saturation kinetics can be obtained by the determination of K_m and V_{max} from in vivo data. The value of K_m which reflects the plasma concentration necessary to give 50% saturation of the active process, is particularly useful for interpreting toxicity dose-response relationships. These constants can be determined following a single intravenous bolus dose using various equations, provided that the elimination is by a single saturable process (see below). The simplest method, applicable to a one-compartment model, is by calculation directly from the plasma concentration-time curve using the equations

$$\ln C_p = \ln C_{p0e} - \frac{V_{max} \times t}{K_m}$$

and

$$K_m = \frac{C_{p0a}}{\ln(C_{p0e}/C_{p0a})}$$

where C_{p0e} is the value of C_p at t=0 derived by back extrapolation of the terminal linear phase, and C_{p0a} is the actual concentration measured at t=0. A plot of ln C_p against time has a terminal "first-order" slope of V_{max}/K_m.

Applying these equations to the data in Figure 4.13 for the highest dose gives values of 0.0486 for the slope, 200 for C_{p0a}, and 2,701,271 for C_{p0e}. Thus

$$K_m = \frac{200}{\ln(2,701,271/200)} = 21\ \mu g\ ml^{-1}$$

and

$$V_{max} = 0.0486 \times 21 = 1.0\ \mu g\ min^{-1}$$

Alternative equations for the calculation of K_m and V_{max} require calculation of the rate of change of concentration from one sample to the next ($\Delta C_p/\Delta t$) as well as the plasma concentration at the midpoint (C_{pm}).

Table 4.8 Pharmacokinetic parameters derived from data showing saturation kinetics: Figure 4.13

Parameter	Curve 1	Curve 2	Curve 3	Curve 4	Curve 5
Dose (mg/kg)	5	10	40	100	200

C_p0 (μg ml^{-1})	5	10	40	100	200
k (min^{-1})[a]	0.0486	0.0486	0.0486	0.0485	0.0486
Half-life (min)[a]	14.3	14.3	14.3	14.3	14.3
AUC (μg ml^{-1} min)[b]	115	254	1614	7020	24,027
AUMC (μg ml^{-1} min^2)[b]	2437	5702	50,682	355,788	2,002,493
CL (ml min^{-1} kg^{-1})[c]	43.5	39.4	24.8	14.2	8.3
MRT (min)[d]	21.2	22.4	31.4	50.7	83.3

The parameters were calculated assuming a one-compartment model with a volume of distribution of 1 L/kg, which is not dose-dependent.

[a]Derived from data between 2.0 and 0.1 μg/ml for each dose.

[b]Calculated by the trapezoid rule with extrapolation to infinity. (See Table 4.17 for a worked example.)

[c]CL=dose/AUC [Eq. (22)].

[d]MRT=AUMC/AUC [Eq. (48)].

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Lineweaver-Burk plot

$$\frac{1}{\Delta C_p/\Delta t}=\frac{K_m}{V_{\max}\times C_{pm}}+\frac{1}{V_{\max}}$$

Therefore a plot of 1/(Δ*Cp*/Δ*t*) against 1/*Cpm* has a slope of *Km/V*max and an intercept of 1/*V*max.

Hanes-Woolf plot

$$\frac{C_{pm}}{\Delta C_p/\Delta t}=\frac{K_m}{V_{\max}}+\frac{C_{pm}}{V_{\max}}$$

Therefore a plot of *Cpm*/(Δ*Cp*/Δ*t*) against *Cpm* has a slope of 1/*V*max and an intercept of *Km/V*max.

Woolf-Augustinsson-Hofstee plot

$$\frac{\Delta C_p}{\Delta t}=V_{\max}-\frac{(\Delta C_p/\Delta t)K_m}{C_{pm}}$$

Therefore a plot of (Δ*Cp*/Δ*t*) against (Δ*Cp*/Δ*t*)/*Cpm* has a slope of −*Km* and an intercept of *V*max.
When the data for the highest dose in Figure 4.13 are analyzed by these techniques (Table 4.9) the following values are obtained (Figure 4.14):

Lineweaver-Burk plot

$$x\text{-intercept}=-\frac{1}{K_m}=-0.0537;\qquad K_m=18.6\,\mu\text{g ml}^{-1}$$

$$y\text{-intercept}=\frac{1}{V_{\max}}=1.04;\qquad V_{\max}=0.96\,\mu\text{g min}^{-1}$$

$$\text{Slope}=\frac{V_{\max}}{K_m}=0.0517;\qquad \frac{0.96}{18.6}=0.0516$$

Hanes-Woolf plot

$$\text{Slope}=\frac{1}{V_{\max}}=0.999;\qquad V_{\max}=1.001\,\mu\text{g min}^{-1}$$

$$\text{Intercept}=\frac{K_m}{V_{\max}}=19.85;\qquad K_m=19.9\,\mu\text{g ml}^{-1}$$

Woolf-Augustinsson-Hofstee plot

Slope=−*Km*=−20.3; Km=20.3 μgml−1

Intercept=*V*max=1.005; *V*max=1.005 μg min−1

The values of *V*max and *Km* may be derived from plateau levels on intravenous infusion, providing that elimination is essentially by a saturable process only, because at

Table 4.9 Calculation of *Km* and *V*max from plasma concentration time data

Time[a]	C_p^a	Δ*Cp*/Δ*t*[b]	C_{pm}^c	1/(Δ*Cp*/Δ*t*)	1/*Cpm*	*Cpm*/(Δ*Cp*/Δ*t*)	(Δ*Cp*/Δ*t*)/*Cpm*
0	200	0.905	188	1.10	0.0053	207.7	0.0048
22.1	180	0.893	168	1.12	0.0060	188.1	0.0053
44.5	160	0.885	147	1.13	0.0068	166.1	0.0060
67.1	140	0.865	127	1.16	0.0079	146.8	0.0068
90.2	120	0.844	107	1.18	0.0093	126.8	0.0079
113.9	100	0.820	87	1.22	0.0115	106.1	0.0094
138.3	80	0.775	68	1.29	0.0147	87.7	0.0114
164.1	60	0.712	49	1.40	0.0204	68.8	0.0145
192.2	40	0.637	34.2	1.57	0.0292	53.7	0.0186
207.9	30	0.549	24.5	1.82	0.0408	44.6	0.0224
226.1	20	0.467	17.7	2.14	0.0565	37.9	0.0264
236.8	15	0.382	12.5	2.62	0.0800	32.7	0.0306
249.9	10	0.265	7.3	3.77	0.1370	27.5	0.0363
268.8	5	0.141	3.3	7.09	0.3030	23.4	0.0427
290.1	2	0.067	1.4	14.93	0.7140	20.9	0.0479
305.0	1	0.035	0.70	28.57	1.4286	20.0	0.0500

319.3	0.5	0.012	0.225	83.33	4.44	18.8	0.0530
351.9	0.1						

*a*Raw data.
*b*Calculated as 200−180/22.1−0=0.905, etc.
*c*Read off the concentration time curve at midpoint of interval.

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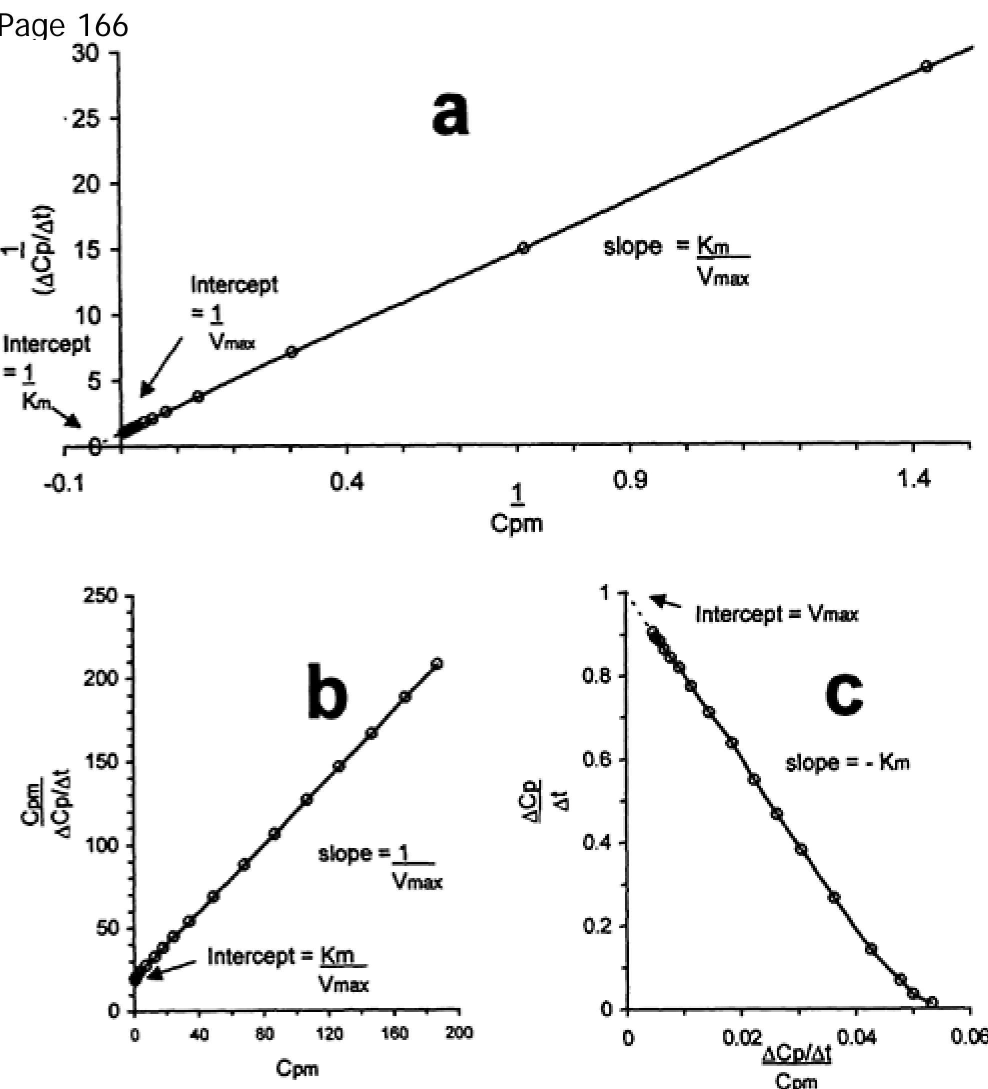

FIG. 4.14. Analysis of the maximum dose given in Figure 4.13 to derive *Km* and *V*max using the data in Table 4.9 and the methods of (a) Lineweaver-Burk, (b) Hanes-Woolf, and (c) Woolf-Augustinssohn-Hofstee.

steady state the rate of input=rate of elimination:

$$R = \frac{V_{\max} \times C_{pss}}{K_m + C_{pss}}$$

where *R* is the rate of infusion, or

$$R = V_{\max} - \left(K_m \times \frac{R}{C_{pss}}\right)$$

Thus a plot of *R* for different rates of infusion against *R*/*Cp*ss gives a straight line with a slope of *Km* and an intercept of *V*max on the *R* axis.

Frequently, the rate of elimination can be described by a combination of saturable and nonsaturable processes

when

$$-\frac{dC}{dt} = \frac{V_{\max} \times C}{(K_m + C)} + k'C$$

where *k′* is the rate constant for the nonsaturable process: *k′* may be replaced by CL′/*V* where CL′ is the clearance by the nonsaturable process; for example for glomerular filtration the value (GFR×*a*/*V*) may be substituted for *k′*.

Of greatest importance for toxicology is the clear demonstration of saturation at high doses, an estimation of the plasma concentration above which first-order kinetics cease to apply, and the plasma

concentrations present in animals showing overt toxicity. Wagner (112) proposed five tests for the establishment of saturation or nonlinear kinetics.

1. Graphs of Cp/dose against time should be superimposable for linear kinetics at different doses. Although considerable scatter is seen, an overall trend to increased or decreased levels at higher doses should be apparent for nonlinear systems.
2. Administer different intravenous doses and estimate $Cp0$ by fitting only the first two or three early time points to the equation $\ln Cp = \ln Cp0 - kt$. Graphs of $Cp/Cp0$ against time should be superimposable if linear kinetics apply.
3. Fit each set of concentration-time data to a linear model and derive the appropriate kinetic parameters (CL, V, k, $k12$, $k21$, $V1$, etc.). A dose-dependent change in a parameter indicates nonlinearity or saturation kinetics (which will invalidate some of the derived parameters, e.g., k).
4. If Michaelis–Menten kinetics apply, the percentage metabolized by that pathway decreases with an increase in dose (provided other elimination routes are available), the value of AUC/dose is not constant, and plots of log Cp or ln Cp against time curve downward, as shown in Figure 4.13.
5. Measure the tissue and unbound plasma concentrations over a range of doses. A graph of tissue concentration against unbound concentration in plasma should be a straight line for a linear tissue extraction. Saturation of tissue binding is shown by the tissue concentration having a smaller increase at higher concentrations.

A consequence of nonlinear kinetics is that the time to reach steady state is also dose dependent. In simple terms, this is because the "effective half-life," which can be calculated by $0.693 \times \text{MRT}$, increases with an increase in dose (e.g., Table 4.8) so that the time to steady state (four to five half-lives) must also increase. This situation should be borne in mind when planning short-term studies. The importance of nonlinear kinetics in toxicology is discussed in greater detail after Data Handling.

PRACTICAL METHODS

General information on techniques may be obtained from the texts by Waynforth (114) and Cocchetto and Bjornsson (20). Waynforth described practical methods ranging from how to hold the animal for injection to such specialist techniques as renal transplantation. The other work, which contains 501 references, is an extensive and invaluable literature review of methods for the collection of body fluids. A number of modifications applicable to other species are given below.

The methods for dosing, blood sampling, urine collection, etc., described below are largely related to the rat, as it is the species most commonly used in toxicological studies.

Administration Techniques

Oral Dosing

Because a number of lipid and metabolic barriers separate the lumen of the gastrointestinal tract from the systemic circulation (Figure 4.2), the plasma levels of test compounds usually increase gradually after oral administration to reach a maximum. Therefore it is possible to give higher doses by this route than by intravenous injection, and this technique is aided by the capacity of the stomach to hold a large volume of liquid. For toxicokinetic studies, the oral route can provide valuable information on elimination and clearance values, provided that the extent of absorption of the parent compound (F) is known. The latter may be determined by measuring either the area under the plasma concentration-time curve or the total amount of the test substance excreted in the urine unchanged after both oral and intravenous administration at low doses. The fraction absorbed is given by Eq. (31), and this value can then be used to calculate the clearance as described in the derivative of Eq. (22). The possibility of saturation of absorption may be determined by plotting the value Cp/dose against time for doses up to and including those producing overt toxicity.

Rats, guinea pigs, and mice may be dosed orally using a syringe fitted with a suitable intubation needle; in rabbits, a polyethylene cannula is passed into the stomach while the jaws are held open by a gag. Certain precautions should be taken to prevent artifacts. For example, it is important that the test chemical is completely dissolved, because if the chemical is given as a suspension, the apparent absorption rate must include a component due to dissolution of the chemical. If this factor is rate limiting, the measured absorption rate reflects the dissolution rate and is not related to the biological availability of the chemical. The ideal vehicle for dissolution is water or a small volume of a water-miscible solvent such as ethanol, propylene glycol (propane-1,2-diol), or dimethylsulfoxide, although for very lipid-soluble compounds, it may be necessary to give the dose in corn oil or as an emulsion. Excess acids or bases should not be used to dissolve the test compound, and the pH of the dose solution should be near pH 7, because pH partitioning in either the gut or the renal tubule could be affected, which could alter the measured absorption or elimination rate constants. If a water-miscible organic solvent is used to dissolve the chemical, water should be added to reduce the dehydrating effect of the solvent within the gut lumen. The volume of water or solvent/water used to dissolve the chemical

should be kept low, because excess quantities may distend the stomach and cause rapid gastric emptying. In addition, large volumes of water may carry the chemical through membrane pores and increase the absorption rate. If dose-dependent absorption is

suspected, the different doses should be given in the same volume of solution. The maximum volume of aqueous solution that can be administered without the possibility of gross interference with absorption is approximately 5–10 ml/kg. Larger volumes may be given, although nonlinear kinetics seen under such circumstances may be due to solvent-induced alteration of intestinal function.

The use of water-immiscible solvents such as corn oil, which are sometimes used for gavage doses, should be avoided if possible, because mobilization from the vehicle may be rate limiting. However, such a vehicle would obviously be appropriate if it was the method of administration used in toxicity studies, because it would give information on the rate of absorption and bioavailability under the conditions of the toxicity study.

The rate of absorption can have a major effect on the toxicokinetic profile, affecting not only the time to maximum concentration and the maximum concentration but also the total amount entering the systemic circulation by saturating hepatic uptake and first-pass metabolism. The hepatotoxicity of oral carbon tetrachloride is markedly higher after a bolus dose compared with gastric infusion (89).

When toxicity studies are performed by mixing the compound into the animals' diet, it is important to measure the concentration-time curve over a 24-h period at steady state, because both the peak concentration and the AUC may be different from data obtained from bolus gavage studies.

Nasal Administration

Methods have been described for assessing absorption from the nasal cavity based on plasma pharmacokinetics following intranasal and intravenous dosing and by in situ perfusion experiments (45, 103). A technique for inhalation with nose-only exposure has been described for studies in guinea pigs (52).

Rectal Administration

Because a number of therapeutic compounds are given in the form of suppositories, an indication of the bioavailability after rectal administration is sometimes required. Normally, toxicity studies and initial drug formulations of such compounds are performed by the oral route, and the rectal formulation comes late in development and marketing. Animal bioavailability studies late in drug development are of limited value because of the differences between laboratory animals and man in intestinal anatomy and microflora of the colon and rectum. However, in cases where an indication of rectal bioavailability is required, the compound may be introduced into the rectum of the rat using an oral dosing needle to prevent tissue damage. To avoid rapid excretion of the unabsorbed dose, anesthetized animals are used and the dose is retained with an inert plug or bung.

Inhalation

As indicated previously, a major problem associated with determining the kinetics of inhalation concerns the measurement of the extent to which the chemical is absorbed across the lung, rather than passed back into the mouth to be swallowed, exhaled in the expired air, or absorbed across the skin. Comparison of the plasma AUC or the total urinary excretion of unchanged compound, after a period of inhalation with the same parameter after a known intravenous dose, can be used to determine the total dose, entering via the lungs plus gut.

A method used successfully by McKenna et al. (59) to obtain kinetic data involved a 6-h exposure to the vapor of [14*C*]vinylidene chloride in rats, after which the animals were transferred to a metabolism cage. The body load at the time of removal was determined by the total recovery of radioactivity in the expired air, excreta, cage washings, and carcass. This method is appropriate for determining the total dose because the nonspecific measurement of 14C includes parent compound and all metabolites. If the parent compound alone is measured, the inhalation data must be compared to intravenous data in order to measure the extent of exposure after inhalation. The approach of McKenna et al. (59) was capable of revealing differences between fasted and fed animals in their capacity to metabolize vinylidene chloride, which correlated well with the toxicity of this compound. The absorption of most compounds given by inhalation is rapid, although certain compounds, for example the highly polar antiasthmatic drug sodium cromoglycate, may show a measurable first-order absorption across the lungs. It should be remembered that the observed absorption rate after instillation of a micronized powder into the trachea may be that of the formulation, not of the chemical moiety itself.

The metabolism rate constants of inhaled 1,1-dichloroethylene were determined by measurement of the rate of removal of the compound from circulating air in a closed chamber system containing the experimental animal (1). The air was recirculated, with oxygen added to maintain the concentration at 19–21%, and the air was sampled at regular intervals and analyzed for unabsorbed 1,1-dichloroethylene by gas-liquid chromatography. The rate of removal showed two phases: (i) a rapid phase proportional to the mass of the animal and the concentration of chemical, (ii) and a slow phase, which represented

metabolism of the compound. The slow phase showed saturation (Michaelis-Menten) kinetics, and rate constants (Km and Vmax) were derived in terms of the concentration of chemical in the chamber. This approach is interesting as the data are obtained by a noninvasive method. Also, the kinetic constants

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are derived in terms of vapor or gas concentrations, which are most appropriate when interpreting inhalation studies in relation to human exposure to volatile agents.

Percutaneous Absorption

The percutaneous route is likely to be of increasing importance in drug formulation in the future. Animals may be used as suitable models; however, there may be major species differences related to the presence of hair follicles and the barrier function of the stratum corneum. An added pharmacokinetic advantage to this route is that the fraction absorbed may be measured as described above for the oral route (i.e., AUC data) and also by analysis of the amount remaining at the site of administration. The dermal absorption of vapors can be assessed in rats using a body-only chamber (58). Shaving the hair from the backs of rats can provide a suitable site for in vivo absorption studies (47), although in vitro data provide a suitable model for extrapolation to humans.

Intravenous Injection

The bolus intravenous dose is the most important single technique for deriving information concerning the kinetics of the distribution and elimination of chemicals. As with oral administration, aqueous or aqueous miscible solvents should be used, although the maximum dosage volume is considerably lower, that is, about 2 ml/kg for aqueous and 1 ml/kg for solvent-aqueous mixtures. Ideally, the solution is made isotonic by the addition of sodium chloride, although in practice dissolution of low doses in isotonic saline is adequate.

There are two important parameters to be considered in such studies. First, the data processing assumes that the material was administered instantaneously at time zero. In practice, a rapid injection may produce considerable toxicity, which a slower injection can prevent. Generally, a"bolus" dose, given over a finite period of up to a few minutes, is regarded as instantaneous provided that the total injection time does not represent more than about 5% of the half-life of the most rapid phase of the plasma concentration-time curve. The other parameter to be considered is the true location of the dose, because in kinetic studies it is important that 100% of the dose is intravenous and none ends up in a perivascular site. The following techniques have proved successful.

Rat. The tail and hind paw veins are convenient for dosing but neither is particularly easy to use or gives 100% intravascular dosing repeatedly and routinely without the necessary expertise. The following technique, although more complex, is preferable because it overcomes the problems experienced with the above routes, and the cannula can be used subsequently for sample collection. The animal is anesthetized with ether, and an incision is made through the skin of the neck to the right of the midline. Blunt dissection is used to separate the thin layer of muscle covering the external jugular vein, which is exposed and cleaned. Thread is passed under the vein at both anterior and posterior ends of the exposed section but is not tied off posteriorly. A small incision is made in the vein, and a length of polyethylene cannula tubing, connected to the dosing syringe, is passed into the vein toward the heart for a distance of about 2 cm. The dose solution is injected and the cannula rinsed with isotonic saline. The tubing may then be removed and the vein tied off, or the cannula tubing can be tied in situ for subsequent sampling, providing that the compound is not adsorbed to the cannula (see below). In anesthetized rats, which will not regain consciousness during the study, an alternative site of injection is the femoral vein, which may be exposed by an incision into the ventral surface of the top of the hind leg.

Guinea pig. A modification of the external jugular method described above may be used for the guinea pig. The external jugular vein of an anesthetized animal is exposed but not cleaned of connective tissue. The dose is injected directly using a fine needle (0.5-mm gauge) bent inward through about 45°, which allows the needle to be positioned without undue stretching or movement of the vein. The vein is clamped, above and below the injection site, as the needle is withdrawn. The vein is subsequently ligated and the incision sutured.

Rabbit. It is relatively easy to administer intravenous doses to rabbits, because the vein running around the periphery of the ear lobe can be readily exposed by shaving with a scalpel blade. This vein is of sufficient size and visibility to give reliable intravenous dosing.

Intravenous Infusion

For intravenous infusion studies, the dose must be given via an indwelling cannula, and the external jugular vein is a suitable site in rats. If the infusion period is prolonged, such that recovery from anesthesia is envisaged, the cannula can be run under the skin from the ventral surface of the neck and exteriorized on the dorsal surface behind the ears. If the cannula is then secured on the dorsal surface, the animal is prevented from damaging it during infusion while being permitted a degree of restricted movement. This method of exteriorization is also valuable as a method of long-term sampling (see

below).

The delivery of compound during intravenous infusion must be at a constant but low rate such that the animal is not subjected to excessive hemodilution. To date, the standard method has been to use a high-quality infusion pump. The development of osmotically driven minipumps (Alzet), which can be implanted into the animal and deliver a constant rate as low as 0.5 *μ*l/h for up to 2 weeks, opened up exciting possibilities. Minipumps allow investigations of steady-state plasma and tissue concentrations associated with toxicity, and the

pharmacokinetics under similar steady-state conditions. Such devices have been used to study the renal clearance of 63Ni under steady-state conditions; interestingly, these studies revealed diurnal fluctuations in the steady-state levels, probably due to metabolic changes that were not suspected from earlier studies (94).

Sampling Techniques

Blood (Plasma and Serum)

When considering the frequency, timing, and duration of blood sampling, it is important that an adequate number of samples are taken to define each section of the plasma concentration-time curve (Figure 4.6). It has been suggested that plasma samples be collected during the first four to five half-lives, during which time 93–97% of the compound will have been eliminated (119). However, it is possible that such a restriction may mask a quantitatively minor distribution component, for example a third compartment, capable of significant accumulation on continuous ingestion as part of a toxicity test. Such a compartment could be perfusion- or diffusion-limited distribution to a tissue in which the tissue affinity was high. If such a tissue was the site of toxicity on chronic administration, a single-dose kinetic investigation restricted to four half-lives would have failed to throw any light on the process involved. However, autoradiography following a radiolabeled dose may reveal such a selective distribution; but it must be remembered that this method cannot differentiate between parent compound and metabolites. Thus, as a general guideline, the plasma concentrations should be measured until the limit of detection of the analytical method is reached. Obviously, if the limit of detection allows analysis over a large number of half-lives, less frequent sampling is required during the slow terminal phase, which can be defined adequately by about four samples. Thus a three-compartment system can be accurately analyzed by about 12 samples provided that they are correctly timed. The corollary to this situation is that a relatively insensitive analytical method is incapable of yielding full pharmacokinetic data. Considering the data in Figure 4.6, if the limit of detection for compounds A and B were 0.1 μg/ml, both would appear to be represented adequately by a one-compartment model, with different values of k. Indeed, under these circumstances the plasma concentration of both A and B would fall from 2.85 to 0.10, a decrease of 97%, equivalent to about five half-lives.

Using the methods described below, it is possible to withdraw a significant fraction of the total blood volume (64 ml/kg in the rat), thereby modifying the perfusion of the organs of elimination and corrupting the derived pharmacokinetic data. This problem can be avoided by taking the smallest samples consistent with accurate analysis and the minimum number of samples necessary to define adequately the various phases (i.e., smaller samples at early time points). As a general rule, individual blood samples should be restricted to a maximum of about 0.5 ml/kg body weight, providing the total number of samples is small (i.e., less than 10). This general rule takes no account of the duration of the experiment, which may indicate either smaller or larger sample sizes.

Rat. Various methods have been used successfully to obtain small serial blood samples from anesthetized and conscious rats.

Tail vein. Whole-blood samples (approximately 70 μl) are obtained on cutting the tail vein with a scalpel blade and collecting the blood into heparinized capillary tubes. This method is widely used and was employed by Sauerhoff et al. (90) in their study of the dose-dependent kinetics of 2,4,5-trichlorophenoxyacetic acid. Care must be taken to wash the tail to avoid contamination of late blood samples by the much higher concentrations present in urine and feces. Washing the tail vein with warm water will often remove the blood clot and reinstigate blood flow.

Toe vasculature. Blood samples (up to 500 μl) may be collected by clipping the toenail into the vascular bed and allowing the blood to run into heparinized capillary tubes (41), but this would be more stressful for the animal than the use of the tail vein.

Cardiac puncture. Multiple cardiac sampling is possible but involves more trauma than the above methods, and anesthesia (ether) is essential. However, provided that a fine (0.5-mm) needle is used, it is possible, with practice, to obtain a number of samples without evidence of extravascular blood loss, although this technique is also more stressful than a simple incision into the tail vein.

Orbital sinus. The animal is anesthetized with ether and held down by gentle pressure with thumb and forefinger behind the head. A heparinized capillary tube is inserted into the orbit at the anterior apex and moved to rupture the sinus membrane in the anterior dorsal region at the back of the orbit. A considerable blood flow is obtained, which stops on removal of the tube and the pressure at the back of the neck.

External jugular vein. Methods have been described in which silicon medical grade cannula tubing (Silastic; 0.05 cm i.d., 0.09 cm o.d.) implanted in the external jugular vein has remained patent for

blood sampling for periods up to 2 months (109). The use of silicon tubing is preferred to polyethylene for such long-term studies because it is more flexible for exteriorization on the dorsal

surface and less apt to cause thrombosis.
The tubing is inserted into the vein by either of two methods. First, a suitable size syringe needle shaft is attached, which is passed into the vein and then pushed back out again about 5 mm lower down. The needle shaft is then removed, and the cannula tubing is gently pulled back until it reenters the vein. Alternatively, the end of the cannula is made into a point by a steep diagonal cut and inserted via a small incision in the vein. A shallow diagonal cut (i.e., almost parallel with the longitudinal axis of the tubing) aids insertion but is more prone to obstruction by the vein wall during sampling. Careful positioning of the end of the cannula tubing by gentle maneuvering may be necessary to achieve optimal sampling, which usually involves passing the cannula toward the heart for a distance of about 2 cm. A similar technique has been described (4) in which the cannula is then exteriorized and secured behind the head of the animal. However, under such circumstances a collar may be necessary to prevent the animal damaging the tubing (20).
Common carotid artery. In anesthetized animals in which the external jugular vein is used for infusion, the ipsilateral carotid artery can be used for sampling. The artery lies deep below the sternohyoid muscle and may be reached using blunt dissection. The artery is a robust structure and can be brought to the surface by curved forceps. Cotton ties are placed anterior (×1) and posterior (×2) to the intended site of incision and tied loosely. The artery is then placed under tension by artery forceps attached to the anterior tie and to the posterior tie nearer the heart so as to prevent blood loss during incision. A small incision is made in the artery, and a length of cannula tubing, attached to a saline-filled syringe, is inserted and passed toward the heart, through the first posterior tie. It is tied firmly, and the tension on the second posterior tie is released. The cannula can now be slid through the second posterior tie, which is then tied securely. The anterior tie is now tightened and the artery forceps removed. Blood may be sampled by removing the syringe when the blood pressure is sufficient to expel the saline. The sampling is stopped by clamping the tubing, replacing the syringe, and passing saline back up the tubing. It is important to keep the artery under tension during insertion of the cannula, or significant blood loss may occur. An alternative method of applying tension is to insert a pair of forceps underneath the artery and allow them to open and stretch the artery.
Other species. For experiments performed under anesthesia, a major vein or artery (e.g., jugular, carotid, femoral) can be cannulated. For multiple sampling under temporary ether anesthesia, cardiac puncture has been used successfully for guinea pigs. On the other hand, the orbital sinus is a more appropriate site for the mouse, and the marginal ear vein can be used for the rabbit without anesthesia.
Urine
A knowledge of the urinary excretion rate is necessary for calculating the overall renal clearance of a compound. The bladder causes variable slowing of the output; for compounds with a short half-life, a method of overcoming sporadic urination is necessary. Calculating results by the sigma-minus method, rather than using excretion rate data, reduces the importance of incomplete bladder emptying and the resultant scatter in the data. For compounds with a half-life of many hours, sufficiently frequent samples may be obtained merely by placing the animals in a metabolism cage, which gives adequate separation of urine and feces, and by encouraging reflex urination (20).
Under anesthesia, the effect of the bladder may be overcome by (a) inserting and tying a cannula into the bladder via the urethra or directly across the bladder wall, and emptying and rinsing the bladder with isotonic saline using a syringe; (b) inserting a cannula via the urethra and allowing the urine to be expelled naturally or with the aid of gentle massage (11); or (c) cannulation of both ureters and collection of the urine without it passing through the bladder (77). The third technique was used by the author to analyze the extent of reabsorption of saccharin from the rat urinary bladder, but was found to be technically difficult, because both ureters had to be cannulated with polyethylene tubing stretched to give a suitable taper, and even slight twisting of the ureter effectively blocked the urine flow. This technique is not recommended for routine investigations. However, these studies did reveal that although the intact urinary bladder was relatively impermeable to saccharin (which is highly ionic), manipulation of the bladder with forceps produced a slight increase in permeability, and bladder cannulation (as described above) produced a marked increase in permeability and reabsorption. Thus any damage or irritation caused by the cannula should be kept to a minimum; if a cannula is inserted across the wall into the apex of the bladder, it is essential that the contents be removed and rinsed at frequent intervals, that is, at least every 15 min. Similarly, any palpation used in method (b) above, should not be excessive, or increased reabsorption from the bladder may occur and cause a decreased apparent renal clearance. In addition, cannulae passed via the urethra, which is particularly suitable for female animals, should be positioned carefully such that

they do not enter too far into the bladder lumen and damage the epithelium.

Renal clearance studies may be performed either after single doses or during infusion at steady state (when the clearance can be related to total clearance and plasma concentration). Insights into the extent of reabsorption and tubular secretion can be obtained by measuring the renal clearance of inulin given simultaneously (1–20 μCi of [14C]inulin/kg or 50–100 μCi of [3H]inulin/kg).

Bile

Bile may be collected from a cannula inserted into the common bile duct such that the tip is located at the point of bifurcation near the hilar region of the liver. The common bile duct is found by making an incision through the midline into the anterior part of the body cavity. Slight tension on the proximal part of the duodenum reveals the bile duct running through the pancreatic tissue. The bile duct is cleaned, a thread is placed loosely around it, and cannula tubing is inserted via a small incision and tied in place. Bile flow is usually 0.5–1.0 ml/h in the rat. Bile may be collected by either placing the animal in a restraining cage and collecting from the exteriorized cannula or passing the tubing into a suitable container (sealed plastic sachet) placed subcutaneously. The test chemical is usually given soon after establishing the cannula, because changes in bile composition occur if the bile salts are not allowed to recirculate. A modified technique avoiding the use of animals still under the stress of surgery has been demonstrated by Light et al. (55). With this technique, the bile cannula was exteriorized and joined to a second cannula, which passed back into the body cavity and entered the duodenum via the greater curvature of the stomach. The animals were then left for 4 days, after which constant feed intake and body weight were observed. This method could be useful for studies with chronic dietary intake of the chemical; however, chronic intake for more than 4 days might be necessary after surgery in order to reestablish steady-state intake via the diet, which may be different from that of normal animals.

In animal species that possess a gallbladder (i.e., guinea pig and rabbit), it is necessary to prevent this organ from delaying elimination by ligation around its base so that bile has to pass directly down the cannula.

DATA HANDLING

The type of information that can be obtained from kinetic studies, and its derivation from raw plasma, and urine data are illustrated by results obtained by the author and colleagues. Saccharin is a nonnutritive sweetener which causes an increased incidence of tumors of the urinary bladder in male rats when fed at high dietary concentrations (more than 3%) for two generations (88) or from birth (93). The possibility of nonlinear kinetics at such high doses was investigated using the Charles River CD-derived rat, the same strain used in the cancer bioassays. The study, which has been published elsewhere (105), investigated the concentrations of saccharin in the tissues of animals fed saccharin-containing diets and used the techniques outlined above to investigate details of the disposition of this compound. Previous studies using [14C]saccharin had shown that it was incompletely absorbed from the gut, and eliminated in the urine and feces without undergoing detectable metabolism (73).

On feeding rats with saccharin-containing diets ad libitum for a period of 22 days, significant nonlinearity was apparent in the concentrations of saccharin in the plasma and tissues, with elevated concentrations at high dietary levels (Figure 4.15). The following studies were

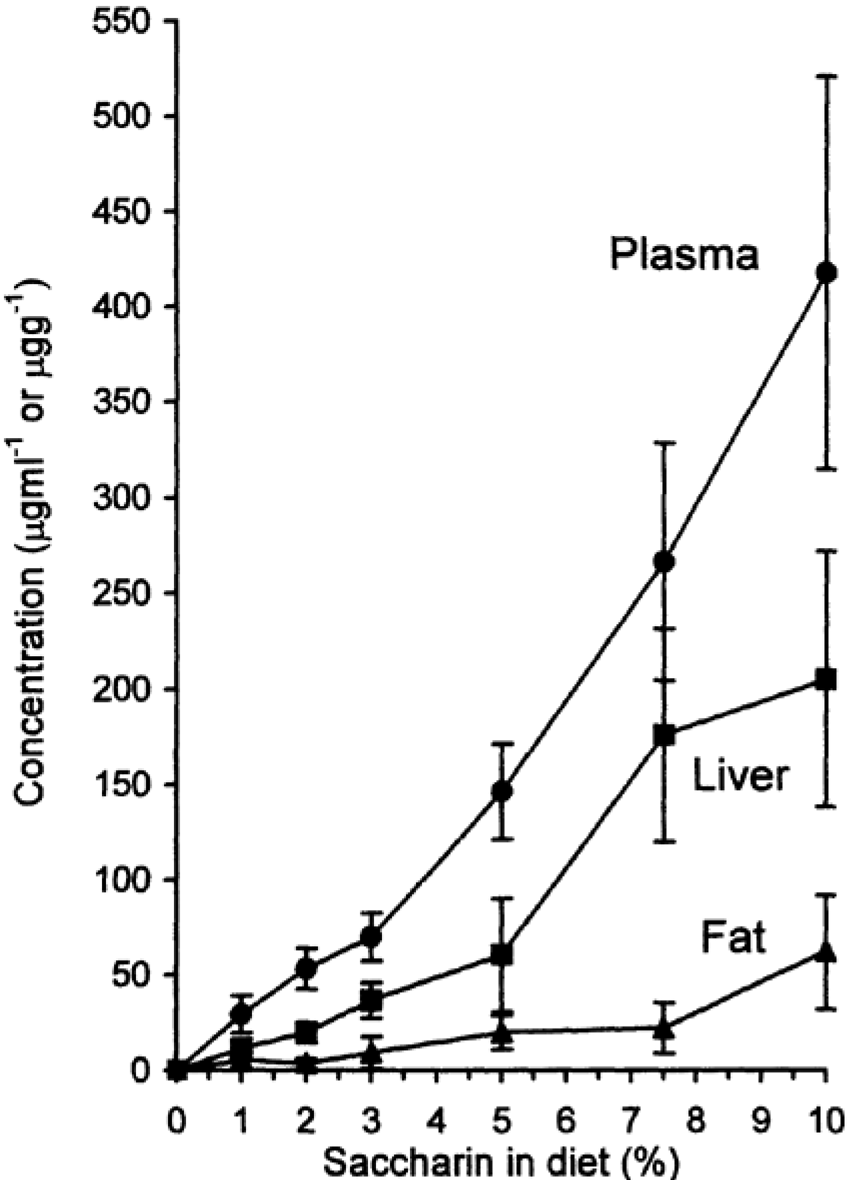

FIG. 4.15. Concentrations of saccharin in the plasma and tissues of rats given saccharin-containing diets. Adult male rats were given saccharin-containing diets ad libitum for 22 days prior to killing at 6 AM. The results are the means with standard deviations represented by vertical bars. From Reference 105.

Table 4.10 Concentrations of saccharin in the plasma and urine of a rat given a single intravenous bolus dose[a]

Plasma		Urine	
Time of sample (min)	Concentration (μg ml^{-1})	Collection period (min)	Amount excreted (μg)
5	184.3	0–5	7518
15	102.0	5–15	6275
30	50.5	15–30	4989
45	24.9	30–45	2580
60	14.1	45–60	1485
75	8.0	60–75	861
90	5.7	75–90	561
105	4.0	90–105	363
120	2.9	105–120	300

[a]The animal (body weight 570 g) was given a single IV bolus dose of saccharin (50 mg/kg) at time 0.

performed in an attempt to investigate the cause of this phenomenon.

Intravenous Bolus Dose: Plasma Analysis

Saccharin is a strong organic acid (p*Ka* about 2) that forms a highly water-soluble sodium salt. 5-[^{3}H]Saccharin was used in these studies, and the concentrations in plasma and urine samples were determined by measuring the total radioactivity present *(because saccharin does not undergo metabolism)*. A range of doses of saccharin were given (1–1000 mg/kg as a single intravenous bolus over a period of 30 s) to anesthetized male rats via a cannula inserted into the jugular vein. The dead volume in the cannula was displaced with saline. Plasma samples were subsequently withdrawn from the same cannula, because saccharin showed no tendency to adhere to the polyethylene tubing used. The bladder was cannulated, emptied, and rinsed at each plasma collection period. The results for an individual animal given 50 mg/kg are presented in Table 4.10.

A graph of plasma concentration against time (Figure 4.16) clearly shows a biphasic decrease, which may be analyzed graphically using the method of residuals as shown in Table 4.11. Alternatively, the β phase can be analyzed by linear regression analysis to yield the extrapolated values, which can then be analyzed by linear regression to give the values of A, B, α, and β as shown in Table 4.11. The data selected for this discussion were for an individual animal that showed a prolonged $\alpha+\beta$ phase. This choice was necessary, as information on the initial $\alpha+\beta$ phase is decreased when urine data are analyzed. For other animals, the $\alpha+\beta$ phase was apparent only during the first 30 min, and linearity occurred between 30–45 and 120 min, so that the corresponding urine data could not be studied adequately by the methods used in Tables 4.13 and 4.14 (see below). Thus, in the animal selected, the data are actually deficient in that the β phase is dependent on only four

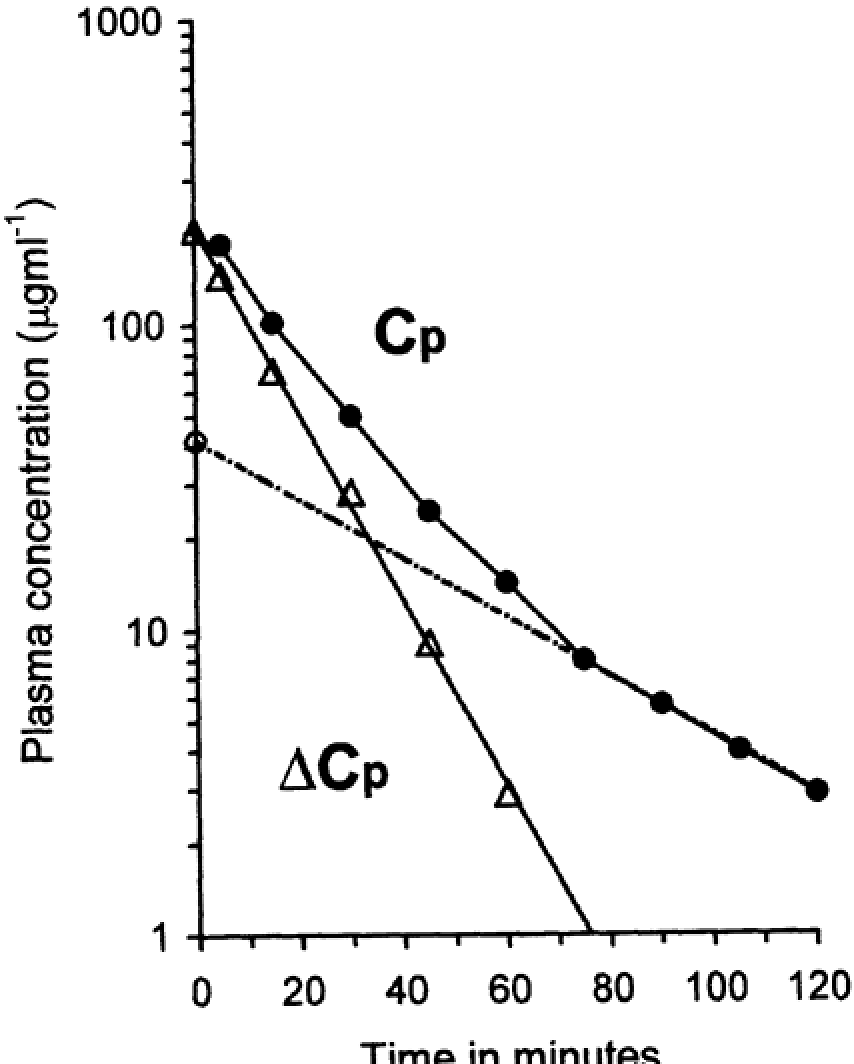

FIG. 4.16. Plasma concentration-time curve after a bolus of saccharin (50 mg/kg IV) into a male rat. The data used are given in Tables 4.10 and 4.11.

Table 4.11 Analysis of plasma data shown in Figure 4.16

Graphical Analysis		Linear regression analysis	
75–120 min			
$\beta = \frac{(\log 8.0 - \log 2.9)}{120 - 75} \times 2.303 = 0.0226\ \text{min.}^{-1}$		β=0.0226 min−1	
B=42 μg ml−1		B=43.7 μg ml−1	
Residuals			
Time (min)	*Conc. (μg ml−1)*	*Time (min)*	*Conc. (μg ml−1)*
5	184.3−37.5=146.8	5	184.3−39.0=145.3
15	102.0−30.0=72.0	15	102.0−31.1=70.9
30	50.5−21.6=28.9	30	50.5−22.1=28.4
45	24.9−15.5=9.4	45	24.9−15.8=9.1
60	14.1−11.1=3.0	60	14.1−11.2=2.9
5–45 min (residuals)			
$\alpha = \frac{(\log 198 - \log 9.4)}{45 - 0} \times 2.303 = 0.0677\ \text{min}^{-1}$		α=0.0683 min−1	
A=198 μg ml−1		A=204.5 μg ml−1	

Analysis using WinNonlin

	All weights equal	Weighted 1/*y*	Weighted 1/*y*2
A (μg ml−1)	148.6	212.4	218.4
B (μg ml−1)	104.9	30.3	19.2
α (min−1)	0.0887	0.0629	0.0585
β (min−1)	0.0335	0.0202	0.0162

(*Note:* 1/*y* weighting is normally used to allow for analytical errors of ±*x*%.)

points. Thus it is apparent that more reliable results would have been obtained had the sampling period been extended for another 30 min in this animal, such that both the *α* and *β* phases were represented by five or six points. This observation emphasizes an important principle, that is, that the more data points measured, the more reliable are the results (provided that it does not involve removal of too much blood).

Intravenous Bolus Dose: Urine Analysis

The urinary excretion data have been recalculated in Table 4.12 in a form suitable for analysis of the excretion rate against time. A graph of excretion rate against time, using the midpoint of the sample collection period (Figure 4.17 and Table 4.13), shows a biphasic decrease similar to that seen in plasma. Because of the scatter in the points of the terminal *β* phase, it is not clear whether the 52.5-min point should be included in the *β* or the *α*+*β* phase. However, from the plasma curve (Figure 4.16), it seems that the *β* phase started after 60 min, and thus the 52.5-min point was not included in the analysis of *β*. The residuals line (Figure 4.17) was analyzed with the omission of the 2.5-min point, because this value did not fit the line clearly shown by other points (possibly due to high initial renal elimination prior to mixing of the compound within the central compartment). The constants *α* and *β* may be derived from the excretion rate or using the sigma-minus method, which is described by Eq. (28). The total amount finally excreted (Table 4.12) is obtained by extrapolation of the cumulative total (column 5, Table 4.12) to infinity, which may be done either graphically or from the excretion rate data, as shown in Table 4.12. The amount remaining to be excreted (ΔAex) is calculated by subtracting the running total from the final total for each time point (column 6, Table 4.12). A graph of ΔAex against time (Figure 4.18)

Table 4.12 Urinary excretion of saccharin after a single bolus dose of 50 mg/kg

Time of collection*a* (min)	Midpoint (min)	Amount excreted (μg)	Excretion rate (μg min−1)	Cumulative total (μg)	A_{ex}^{∞} −cumulative total (ΔAex) (μg)
0–5	2.5	7518	1503.6	7518	18,247
5–15	10.0	6275	627.5	13,793	11,972
15–30	22.5	4989	332.6	18,782	6983
30–45	37.5	2580	172.0	21,362	4403
45–60	52.5	1485	99.0	22,847	2918
60–75	67.5	861	57.4	23,708	2057
75–90	82.5	561	37.4	24,269	1496
90–105	97.5	363	24.2	24,632	1133
105–120	112.5	300	20.0	24,932	833
120–∞	—	833*b*	—	25,765*b*	—

a Raw data from Table 4.10.

b The additional amount excreted from the last data point to infinity can be calculated as the excretion rate (last) divided by the terminal slope. The graph of rate against time (Figure 4.17; Table 4.13) gives a terminal slope of 0.024 min−1; therefore the amount excreted, 120–∞, equals 20.0 µg min−1/0.024 min−1=833 μg.

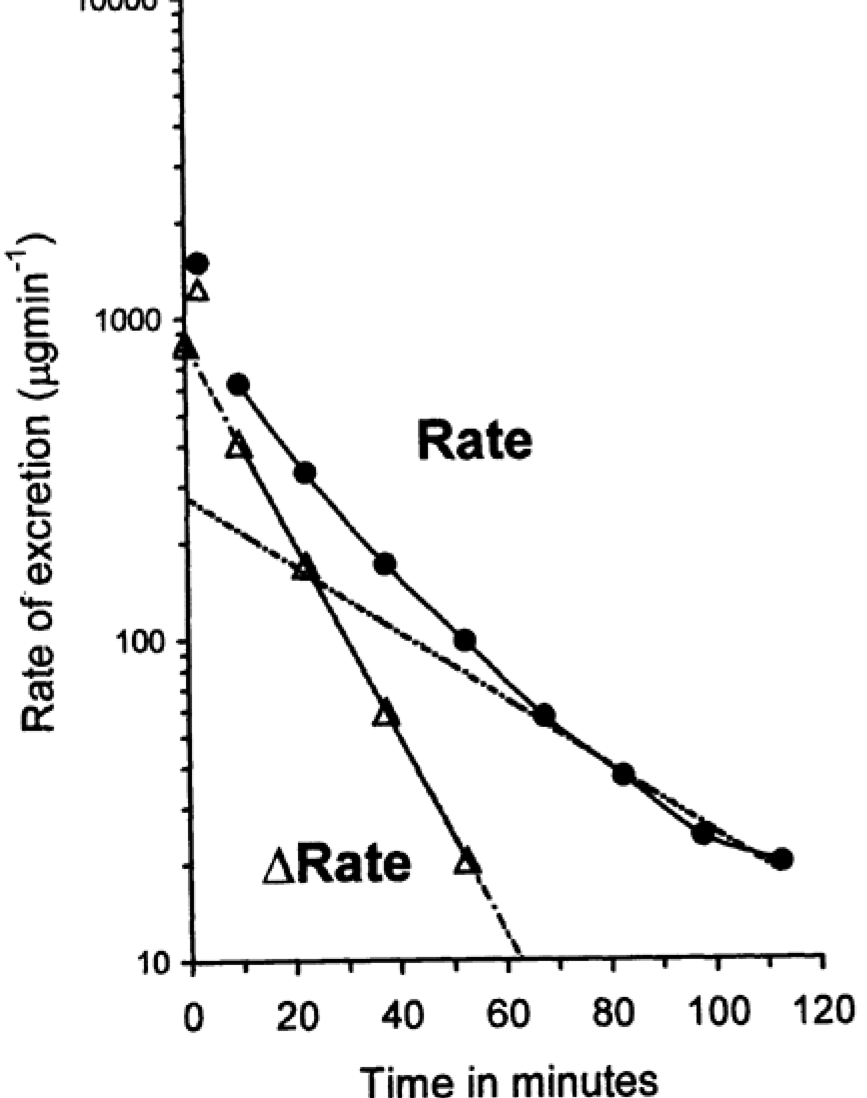

FIG. 4.17. Urinary excretion of saccharin after a single intravenous dose. The results are given as micrograms excreted per minute plotted against the time at the midpoint of the collection period. The data used are given in Table 4.12.

clearly shows a biphasic decrease, although the *β* phase appears to have started slightly earlier, at 60 min. Analysis of this curve by the method of residuals gave values of ***α, β, A″,*** and ***B″*** (Table 4.14).
The renal clearance of the compound can be determined by Eq. (7) using the excretion rate during individual collection periods and the plasma concentrations at the middle of the collection period. These values are given in Table 4.15 and show a clearance of 5.0 ml min^{-1}±10% for seven of the nine time points. The clearance adjusted for body weight was 8.82 ml min^{-1} kg^{-1}. As an alternative to averaging the values derived during the experiment, CL_R may be calculated also from the time-averaged values A_{ex} and AUC (Table 4.15).

Intravenous Bolus Dose: Rate Constants

The values ***α, β, A, B, A′,*** and so on, derived from plasma and urine, are given in Table 4.16. Using the six values for each constant given in the table, it is interesting that the values of *β* (0.0222 ±0.0017 [SD]) and *α* (0.0691 ±0.0023 [SD]) show much less variability (±7.5 and ±3.3%, respectively) than the values of k_{21}, k_{10}, and k_{12} derived by Eqs. (38), (39), and (40) (±12, ±16, and ±19%, respectively).
Another important parameter that can be derived from the raw data is the plasma clearance, which is given by Eq. (22). The AUC may be measured by the trapezoid rule, as shown in Table 4.17. With the linear trapezoid method, the concentrations are joined by straight lines; thus after oral dosing, the area during the increase is underestimated and that after the peak is overestimated, as a result

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Table 4.13 Analysis of excretion rate data shown in Figure 4.17

	Graphical analysis	Linear regression analysis
67.5–112.5 min	$\beta=\frac{(\log 270-\log 20)}{110}\times 2.303=0.0237\ \text{min}^{-1}$	β=0.0240 min−1
	B'=270 μg min−1	B'=211 μg min−1

Residuals

Time (min)	*Rate (μg min−1)*	*Time (min)*	*Rate (μg min−1)*
2.5	1503.6−256=1247.6	2.5	1503.6−260.5=1243.1
10	627.5−215=412.5	10	627.5−217.6=409.9
22.5	332.6−160=172.6	22.5	332.6−161.2=171.4
37.5	172.0−112=60.0	37.5	172.0−112.5=59.5
52.5	99.0−79=20.0	52.5	99.0−78.5=20.5

	Graphical analysis	Linear regression analysis
10–52.5 min (residuals)	$\alpha=\frac{(\log 830-\log 20)}{52.5-0}\times 2.303=0.0710\ \text{min}^{-1}$	α=0.0705 min−1
	A'=830 μg min−1	A'= 833 μg min−1
	(Using the 2.5 min point, α=0.0784 and A'=1138)	

Analysis using WinNonlin

	All weights equal	Weighted 1/*y*	Weighted 1/*y*2
A'(µg min−1)	818	898	936
B' (μg min−1)	273	161	43.4
α (min−1)	0.0680	0.0599	0.0496
β (min−1)	0.0248	0.0198	0.0089

(*Note:* 1/*y* weighting is normally used to allow for analytical errors of ±*x*%.)

the errors tend to cancel. After intravenous dosing, however, the AUC for each segment is overestimated, the total extent of which depends on the number of time points available. Such errors may be minimized by applying the trapezoid rule to log-transformed data. These points are shown in Table 4.17, which also illustrates the derivation of the model-independent parameters MRT and *V*ss. Because the terminal half-life (β phase) from plasma was 30.8 min (0.693/0.0225), the duration of the study was the minimum necessary to adequately define the curve. Ideally, the data collection should have been extended for at least another 30 or 60 min in order to give five half-lives, which was borne out by the fact that the β phase was dependent on only four data points. Because the duration of the experiment was four half-lives, a total of about 94% of the dose should have been eliminated, which is in good agreement with that pre dicted from the urinary data, as the urinary clearance, 8.82 ml min−1 kg−1 (Table 4.15), represented 87% of the plasma clearance, and the total urinary recovery in 2 h (24.9 mg) represented 87% of the dose administered (28.5 mg, or 50 mg/kg). The urinary elimination rate constant *kR* may be calculated by a number of methods using the data obtained, and these figures show good agreement (Table 4.17).

It is clear from these results that low doses of saccharin fit a two-compartment open model with a terminal half-life of about 30 min, a plasma clearance of 10.1 ml min−1 kg−1, and a renal clearance of about 8.8 ml min−1 kg−1. The latter value is considerably higher than the glomerular filtration rate in the rat (3.4 ml min−1 kg−1). Because glomerular filtration removes only the nonprotein-bound compound and saccharin is about 80% protein-bound (105), the clearance due to filtration would be only about

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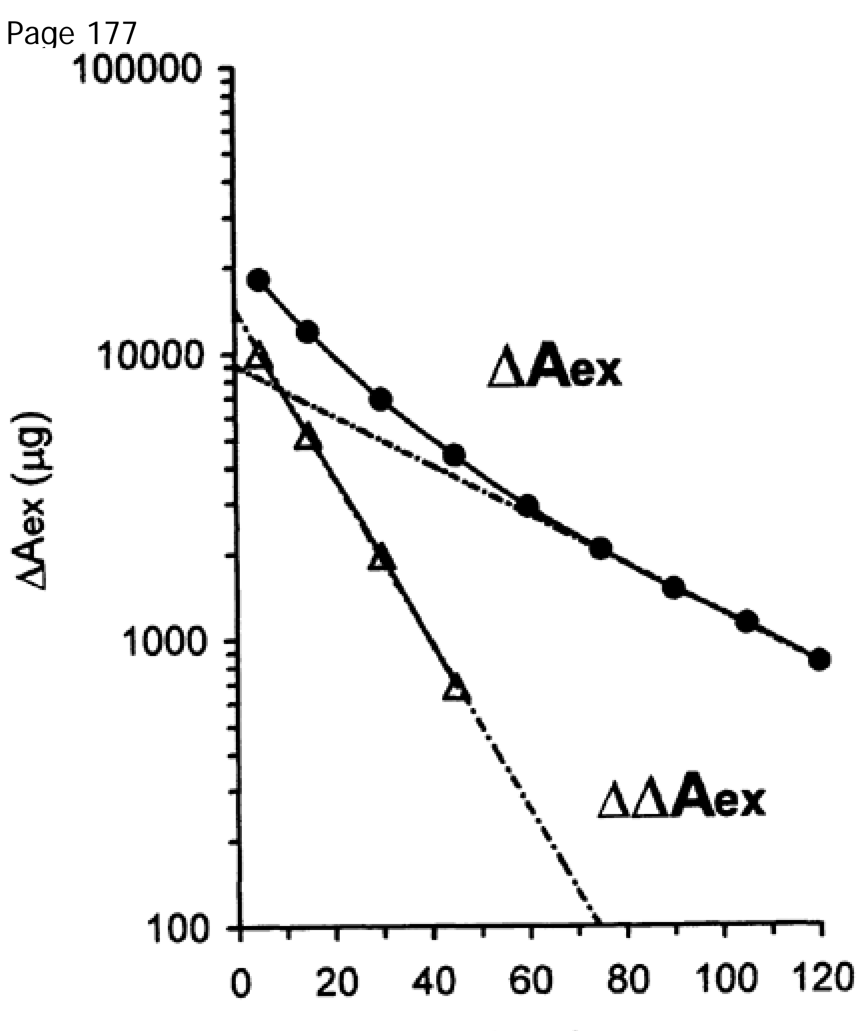

FIG. 4.18. Urinary excretion of saccharin analyzed by the sigma-minus method. The results are the amount remaining to be excreted (Δ*A*ex) plotted against time. The data used are given in Table 4.12.

0.7 ml min−1 kg−1. It is therefore apparent that extensive secretion and negligible reabsorption must be occurring in the renal tubule, and we can conclude that the major route of elimination of saccharin is by renal tubular secretion. Renal tubular secretion is responsible for about 80% [(8.8−0.7)/10.1×100] of total elimination. This finding was confirmed by studies in which the plasma clearance of saccharin was reduced by about 70% by the drug probenecid, which inhibits renal tubular secretion (105).

Because renal tubular secretion is a saturable process, it was possible that the nonlinearity of plasma levels, seen on dietary administration, arose from saturation of this major route of elimination. This possibility was investigated by giving a range of intravenous bolus doses of saccharin (1–1000 mg kg−1) and calculating the plasma clearance. The plasma concentration-time curves for high doses (Figure 4.19) reflected nonlinear kinetics (Figure 4.13) being superimposed on the two-compartment pattern seen at low doses (Figure 4.16). It resulted in high doses appearing to be a simple one-compartment system during the course of the experiment. Obviously, better data would have been obtained if the collection period was extended (105). These data illustrated well that the

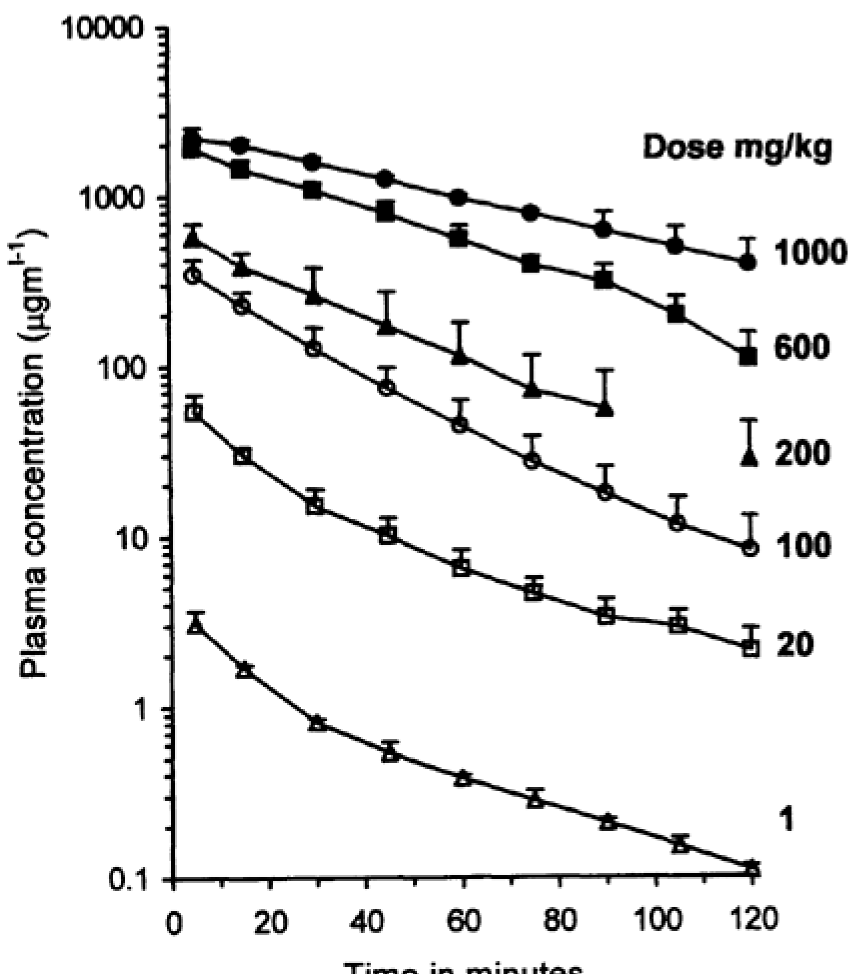

FIG. 4.19. Plasma concentration-time curves for rats given bolus intravenous doses of saccharin. Adult male rats were given [3H]saccharin (1–1000 mg/kg IV) by bolus dose, and plasma levels were measured by liquid scintillation counting. From Reference 105.

terminal half-life, which was derived at low plasma levels, did not show a dose-dependent increase and that the best indication of saturation kinetics was given by plasma clearance, which was decreased at doses of 300 mg kg−1 or more (Figure 4.20). The increased half-life at the highest dose is probably a reflection of the duration of the study rather than a true value.

Intravenous Infusion

Saturation kinetics apparent after single doses of about 300 mg kg−1 (shown by the decreased plasma clearance and altered plasma concentration-time curve) could not be related closely to a particular plasma concentration, because the levels fell from about 500 to 30 μg ml−1 during the 2-h study period. Infusion studies were used to relate altered clearance to a particular plasma level, because at steady state the rate of infusion equals the rate of elimination for a *fixed* plasma concentration. CL can be calculated using Eq. (20),

$$\mathrm{CL} = \frac{\text{rate of infusion}}{C_{pss}}$$

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Table 4.14 Analysis of urinary sigma-minus data shown in Figure 4.18

Graphical analysis		Linear regression analysis	
60–120 min			
$\beta=\frac{(\log 9700-\log 830)}{120}\times 2.303=0.0205\ \text{min}^{-1}$		β=0.0199mm−1	
B''=9700 μg		B''=9115 μg	
Residuals			
Time (min)	*Amount (μg)*	*Time (min)*	*Amount (μg)*
5	18,247−8800=9447	5	18,247−8251=9996
15	11,972−7200=4772	15	11,972−6760=5212
30	6983−5270=1713	30	6983−5013=1970
45	4403−3870=533	45	4403−3717=686
$\alpha=\frac{(\log 14,100-\log 800)}{40}\times 2.303=0.0717\ \text{min}^{-1}$		a=0.0668 min−1	
A'' 14,100 μg		A''=14,159 μg	

(*Note: A″+B″* is equivalent to the total amount excreted; it equals 23,800 μg by graphical and 23, 274 μg by linear regression analysis.)

Analysis using WinNonlin

	All weights equal	Weighted 1/*y*	Weighted 1/*y*2
A'' (μg)	14,669	15,316	15,509
B'' (μg)	8165	7407	7140
a (min−1)	0.0608	0.0581	0.0569
β (min−1)	0.0193	0.0183	0.0180

(*Note:* 1/*y* weighting is normally used to allow for analytical errors of ±*x*%.)

The rats were anesthetized and infused at a rate of 9.6 μl/min with [3H]saccharin solution in isotonic saline via the jugular vein using a Harvard infusion pump. The infusion rate was selected because it approximates 14 ml/day and is therefore not an excessive fluid intake. Each animal was infused at a constant rate, within the range 50-2000 μg min−1, and the plasma was analyzed every 30 min from 90 min onward until three consecutive samples showed the same concentration (C*p*ss). The clearance for each animal was calculated using the equation given above. A graph of clearance against steady-state plasma concentration (Figure 4.21) shows that the clearance was about 8–12 ml min−1 kg−1 at plasma con300 μg ml−1 (a value similar to that seen after an intracentrations below 200 μg ml−1, whereas the clearance decreased to 4–8 ml min−1 kg−1 at concentrations above venous bolus dose of 600 mg kg−1).

Thus, based on these studies, the renal tubular secretion of saccharin appears to be saturated by doses giving plasma concentrations of 200–300 μg ml−1 or more. This value correlates well with the concentration in the plasma of rats fed a 7.5% saccharin diet, and which was associated with elevated levels in the plasma and most organs (Figure 4.15) (105). [Intravenous infusion in dogs was used to relate serum concentrations of minoxidil at steady state to cardiovascular effects and to cardiac toxicity (60).]

Oral Studies

Because saccharin has a short terminal half-life (30 min), it is possible that there are rapid fluctuations in plasma concentrations during chronic dietary administration. This possibility was investigated by studies on the plasma concentration-time curve for animals maintained on a 5% saccharin diet for an extended period. The diuraal variation showed relatively small changes, with a peak at around 6 AM and a minimum

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Table 4.15 Renal clearance of saccharin after an intravenous bolus dose

Time of collection (min)	Midpoint (min)	Excretion rate (μg min−1)	Midpoint plasma concentrationa (μg ml−1)	CL_R^b (ml min−1)
0–5	2.5	1503.6	225	6.68
5–15	10.0	627.5	138	4.55
15–30	22.5	332.6	71	4.68
30–45	37.5	172.0	35.5	4.84
45–60	52.5	99.0	18.8	5.27
60–75	67.5	57.4	10.6	5.42
75–90	82.5	37.4	6.9	5.42
90–105	97.5	24.2	4.8	5.04
105–120	112.5	20.0	3.4	5.88
			Mean value (5–105 min)	5.03

a Data are from Figure 4.16.

b Renal clearance, calculated using Eq. (7). Mean renal clearance=5.03 ml min−1 (per 570 g)=8.82 ml min−1 kg−1. CLR *m*ay be calculated also from *A*ex (Table 4.12) and AUC (Table 4.17) between 0 and 120 min:

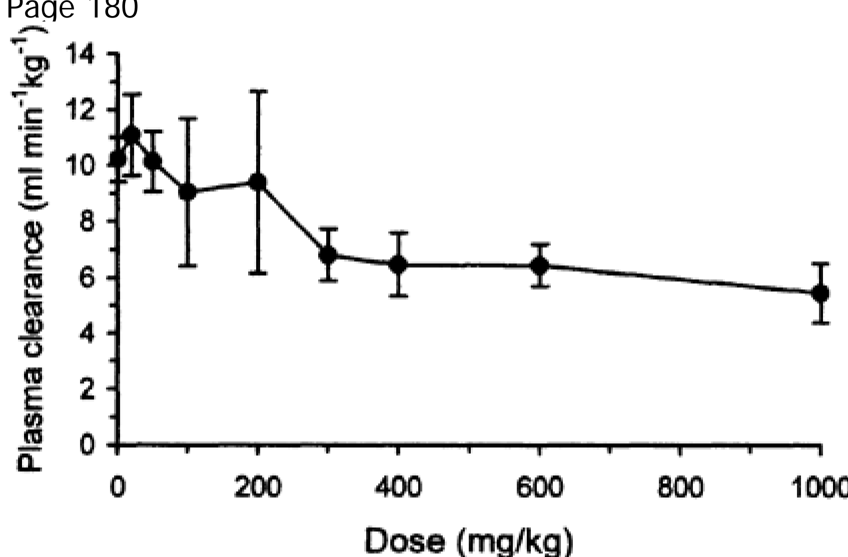
$$CL_R = \frac{A_{ex}}{AUC} = \frac{24,932\,\mu g}{4895\,\mu g\,ml^{-1}\,min} = 5.09\,ml\,min^{-1}$$

at 6 PM. (Figure 4.22). The extent of variation was less than might have been expected from the half-life, and the fact that little saccharin-containmg feed was consumed between 6 AM and 6 PM. It therefore seemed probable that the rate of absorption from the gut was low and blunted any large change in plasma level. The plasma concentration-time curve after oral administration was studied and showed much lower levels than after similar doses given intravenously (Figure 4.23). The peak concentration was at the time of the first sample, followed by a slow and variable decrease. The decrease in concentration was obviously not related to the value of *β*, and thus saccharin is a good example of a compound for which the decrease after oral administration is related to *ka*, and not to *k* or *β* (see Mathematical Principles, above). The decrease was slow and complex, emphasizing that if absorption from the gut is slow, a single rate constant cannot be derived to apply to the many rates occurring simultaneously at different sites within the gastrointestinal tract. The duration of sample collection after oral dosage was inadequate for definition of the ACU0–∞, and thus the bioavailability could not be calculated from these plasma data. The methods for deriving simple absorption rate constants are described above under Mathematical Principles.

In summary, these studies have been used to illustrate the derivation of kinetic data and the insights they can give into the handling of the compound during chronic toxicity studies. The possibility of saturation of one pathway revealing a second pathway of elimination could result in saccharin (which is not metabolized normally) undergoing metabolism in rats fed high-saccharin diets. However, this phenomenon has not been detected for saccharin (104), although Gehring and Young (33) showed that metabolism of 2,4,5-trichlorophenoxyacetic acid was revealed by doses resulting in saturation of renal tubular secretion.

Computation

The use of computer programs to derive kinetic constants from plasma and urinary concentrations can greatly simplify data handling. Because data handling is also optimized, computer usage is the method of choice. There are a number of suitable programs (e.g. BLIN, NONLIN, SIPHAR) available for nonlinear least-squares regression analysis, which is the most appropriate method, and readers are referred to Gibaldi and Perrier (36), Wagner (112), and Gabrielsson and Weiner (28) for further details. The use of such a program would automatically put the best-fit line through the data presented in Figures 4.16–4.18 and would avoid a possibly erroneous decision as to the time at which the a component was exerting an insignificant influence and the line was described by *β* alone. In the analysis of data by computer program, it is common to apply a suitable weight to each data point to ensure the most appropriate fit. The weights that can be applied to the concentration data include (a) all weights equal, which is applicable if the errors in measurement are a constant amount, for example, ±2 μg/ml; (b) weighted by 1/*y*, which is



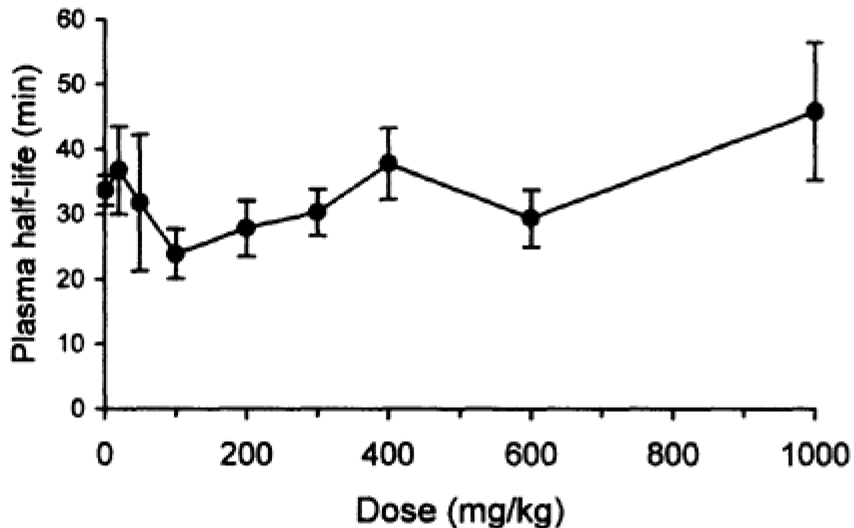


FIG. 4.20. Influence of dose on the plasma clearance and half-life of saccharin in male rats following an intravenous bolus dose. From Reference 105.

applicable if the errors of measurement are a constant proportion, for example ±2%; and (c) weighted by 1/*y*2, which can be used to force the fit through the later time points at the expense of the early higher values. The second option, 1/*y*, closely represents the accuracy of most assay procedures and is used most frequently. It is important with computer fitting of data that some indication of appropriateness of fit is obtained by either a graphical representation or analysis of the deviation between observed and calculated concentrations (error analysis). With the latter approach, a consistent positive or negative deviation is more important than wider but randomly distributed deviations, because it indicates an inadequate fit. Reasons for this situation could be the choice of an inappropriate model to fit to the data or incorrect weighting. Another factor to consider is that although adoption of a more complex model may give a closer fit to the data, the sampling times may be inadequate to provide accurate parameter estimates.

It should be realized that although kinetic constants derived from sophisticated computerized line fitting contain the minimum possible errors due to data handling, any errors in the raw data, due to methodological problems, will still be present. Indeed, the adage "rubbish in, rubbish out" is particularly pertinent to the use of sophisticated data handling to analyze inaccurate or badly designed animal toxicokinetic experiments.

Interpretation of Toxicokinetic Data

There are three principle aims of kinetic studies as applied to toxicology. First, toxicokinetics can provide

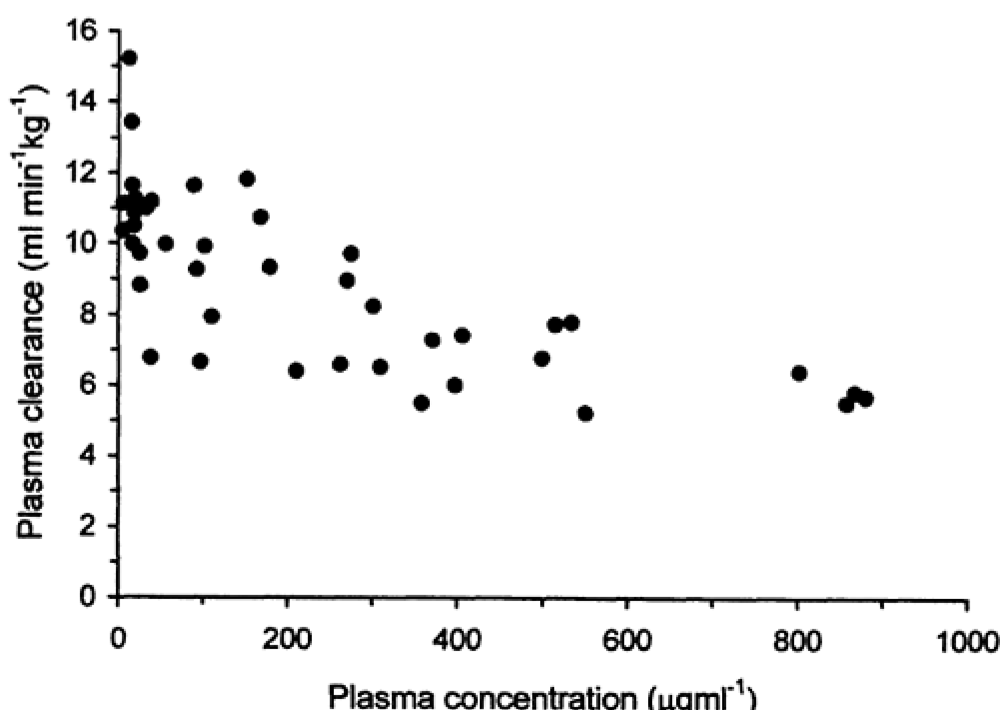


FIG. 4.21. Plasma clearance of saccharin at steady-state plasma concentrations. Adult male rats were given [3H]saccharin in isotonic saline by intravenous infusion (0.0096 ml/min) until a constant plasma 3H concentration was obtained. The plateau saccharin concentration was calculated from the specific activity of the dose solution, and the plasma clearance from the infusion rate and plasma concentration. From Reference 105.

Table 4.16 Summary of kinetic data after intravenous saccharin

	Plasma		Urine (excretion rate)		Urine (sigmus minus)	
Parameter	Graphical	Regression	Graphical	Regression	Graphical	Regression
β (min−1)	0.0224	0.0226	0.0237	0.0240	0.0205	0.0199
B or B' or B''	42	43.7	270	277	9700	9115
α (min−1)	0.0664	0.0683	0.0710	0.0705	0.0717	0.0668
A or A' or A''	198	204.5	830	833	14,100	14,159
V1 (ml kg−1)	208	201				
k21 (min−1)	0.0301	0.0307	0.0353	0.0356	0.0414	0.0383
K10 (min−1)	0.0494	0.0503	0.0477	0.0475	0.0355	0.0347
k12 (min−1)	0.0093	0.0099	0.0117	0.0113	0.0153	0.0137

$$V_1 = \frac{\text{dose (50 mg/kg)}}{A+B}$$

$$k_{21} = \frac{A\beta + B\alpha}{A+B}; \quad \text{or } A', B'; \quad \text{or } A'', B''$$

$$k_{10} = \frac{\alpha\beta}{k_{21}}$$

$k12 = \alpha + \beta - k21 - k10$

The constants were derived by graphical analysis or by least-squares linear regression analysis applied to β phase and residuals.

$$\text{Renal excretion} = \frac{25,765}{50,000 \times 0.57} \times 100 = 90.4\% \text{ of dose } (0.57 = \text{body weight in kg})$$

Renal elimination rate constant (kR)

$$k_R = \frac{A' + B'}{\text{dose}} = \frac{277 + 833}{28,500} = 0.039\,\text{min}^{-1}$$

$$k_R = \frac{A_{ex}^{\infty} \times k_{10}}{\text{dose}} = \frac{25,765 \times 0.050}{28,500} = 0.045\,\text{min}^{-1}$$

$$k_R = \frac{CL_R}{V_1} = \frac{8.82}{201} = 0.044\,\text{min}^{-1}$$

$$\text{MRT} = \frac{\text{AUMC}}{\text{AUC}} = \frac{125{,}072}{5024} = 25\,\text{min}$$

$$V_{ss} = \frac{\text{dose} \times \text{AUMC}}{\text{AUC}^2} = \frac{50{,}000 \times 125{,}072}{5024^2} = 248\,\text{ml kg}^{-1}$$

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Table 4.17 Plasma clearance and renal elimination of saccharin

Plasma clearance: $CL = \frac{\text{dose}}{\text{AUC}}$ Trapezoid rule: AUC = sum of $\frac{(t_2 - t_1)}{2}(C_{p1} + C_{p2})$, etc.

time (min) (t)	*Cp* (μg ml−1)	AUC (μg ml−1 min)	*t*×*Cp* (μg ml−1 min)	AUMC (μg ml−1 min2)
0	242.0*a*	1066	0	2305
5	184.3	1432	922	12,260
15	102.0	1144	1530	22,838
30	50.5	566	1515	19,770
45	24.9	293	1121	14,753
60	14.1	166	846	10,845
75	8.0	103	600	8348
90	5.7	73	513	6998
105	4.0	52	420	5760
120	2.9	129*b*	348	21,195*c*
Total		5024 μg ml−1 min		125,072 μg ml−1 min2

Plasma clearance=50,000 μgkg−1/5024 μml−1 min=9.95 ml min−1 kg−1

CL=*k*10*V*1=208×0.0494=10.27 ml min−1 kg−1 or 201×0.0506=10.17ml min−1 kg−1 (Table 4.16)

Renal clearance (CLR)=8.82 ml min−1 kg−1 (Table 4.15)

*a*By extrapolation of plasma concentration-time curve.

*b*Calculated by *Cp*last/*β*=2.9/0.0225.

*c*Calculated by *t*×*Cp*last/*β*+*Cp*last/*β*2=348/0.0225+2.9/0.000506=15.467+5728.

an understanding of the physiological processes that are involved in the fate of the chemical in the body. Second, the relation between dose and toxicokinetics may be the key to either the establishment of appropriate dose levels for chronic studies (101) or the interpretation of such studies. Third, comparative toxicokinetics may be used to assess potential human risks with a more secure basis by reducing the number of unknown variables involved in the extrapolation from animal to humans (see ref. 76).

A good example of the physiological insights that may be obtained is provided by the data on saccharin discussed above. In summary, these data showed that the sweetener is slowly absorbed from the gut. Thus, during chronic feeding of saccharin diets, there are only slight diurnal fluctuations due to the absorption rate producing "flip-flop" kinetics. The absorbed saccharin has a low volume of distribution so that the concentrations in most tissues are similar to or lower than those in plasma. The urinary bladder tissue is part of the central compartment (105). The sweetener is cleared rapidly from plasma, mostly as a result of renal tubular secretion. This process is saturated at the high dietary intakes that are necessary to produce an increase in bladder tumors. Renal tubular secretion is a general mechanism for the elimination of organic acids, and saturation of renal clearance of saccharin at high dietary concentrations is accompanied by decreased renal clearance of indican, a major urinary metabolite of tryptophan (95). Subsequent studies demonstrated that the slow absorption of saccharin from the gut results in altered metabolism of essential nutrients within the gastrointestinal tract (53, 95, 96). Thus the dietary levels necessary throughout life (≥3%) to increase the incidence of bladder tumors in male rats produce profound perturbations of the physiology and biochemistry of the test animal, although these are not directly related to bladder tumor formation. Neither saturation of renal clearance (73) nor altered excretion of amino acid metabolites (81) has been found in humans following doses equivalent to the highest likely human intake.

The relationship between kinetics, dose, and toxicity is probably the single most important contribution that kinetics can make to the field of toxicity testing. Although a few therapeutic chemicals show nonlinear kinetics at the doses normally given to man (notable examples being salicylates, phenytoin [diphenylhydantoin], and ethanol), the plasma levels of foreign chemicals in humans are usually well below those necessary to saturate any protein-mediated reactions. However, in toxicity testing,

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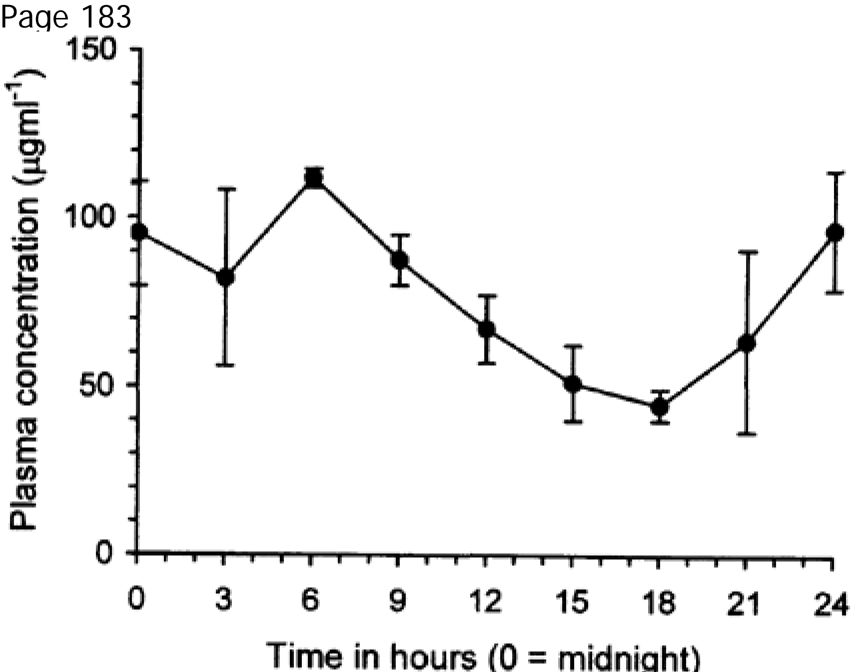

FIG. 4.22. Diurnal variation in the concentration of saccharin in the plasma of rats given a 5% saccharin diet. Adult male rats were given a 5% saccharin (sodium saccharin dihydrate) diet for 66 days ad libitum; plasma samples were collected during a 24-h period and analyzed for saccharin content by HPLC. The results given are the mean concentrations ±*SD* (as sodium saccharin dihydrate) for three animals. From Reference 105.

when the maximum dose tested is designed to show some degree of toxicity, nonlinear kinetics are a distinct possibility and should be fully and carefully investigated. At doses above saturation, the body load of free compound increases steeply with an increase in dose. Under such circumstances, effective tissue concentrations of the chemical will also be considerably higher than predicted by extrapolation from doses showing first-order kinetics. The presence of dose-dependent kinetics may result in an extremely steep dose-response curve for the toxic effect observed. In such circumstances, the nonlinearity in kinetics shown in animal toxicity testing must be taken into account when extrapolating the effects to man.

A possible cause of toxicity associated with large saturating doses of chemicals is that normally minor pathways of metabolism may become of major significance. Thus if a chemical undergoes metabolism by two routes, one with a low *Km* (high affinity) and one with a high *Km* (low affinity), at low doses most chemical in the cell is eliminated by the former route. However, if the levels increase to saturate the high affinity enzyme, any further input will exceed removal and levels will rise such that the low-affinity enzyme will metabolize the excess. (A useful analogy is that of water pouring into a bucket that has two holes in the side at different heights.

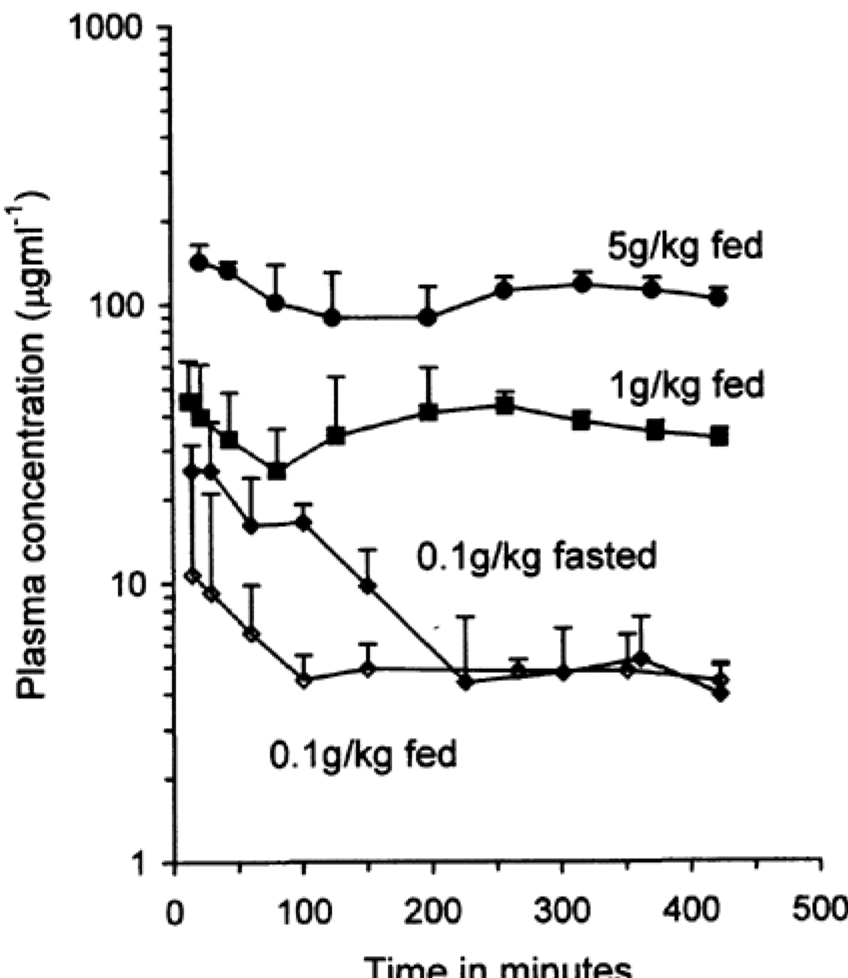

FIG. 4.23. Concentration of saccharin in the plasma of rats given [3H]saccharin orally. Adult male rats were given a single oral dose of [3H]saccharin, and the plasma 3H content was determined by liquid scintillation counting. The saccharin (5, 1, and 0.1 g/kg) was given in aqueous solution (20, 10, and 0.5 ml/kg). The plasma concentration-time curve for 0.1 g/kg was not altered by giving the dose in a large volume (10 ml/kg). The results given are the mean of three animals ±*SD*. From Reference 105.

Little escapes through the upper hole until the rate of input exceeds the rate of removal by the lower hole.)

There have been some notable examples of toxicity occurring largely at saturating doses of foreign chemicals. In a series of papers by Brodie and co-workers (48, 62, 63, 71), it was shown that the hepatotoxicity of paracetamol (acetaminophen) was related to the metabolism of the compound and occurred only at high doses, which were associated with extensive covalent binding of the compound to tissue components. Little binding occurred at low doses or if the metabolism of toxic doses was inhibited by treatment with piperonyl butoxide. The binding at high doses arose from saturation of the capacity of the hepatocytes to protect themselves from the reactive metabolite produced. The protective mechanism was conjugation with glutathione, and saturation of this system at toxic doses was caused by depletion of the available glutathione.

Another example of saturation in toxicology is seen in the studies of Gehring and co-workers on the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (33, 90). This compound showed a higher toxicity in dogs than in rats because the former species exhibits a longer half-life, reduced renal and increased biliary elimination, and the presence of metabolites. In the rat, embryotoxicity was seen with doses of 100 mg kg^{-1}, and nonlinear kinetics were seen at similar doses due to saturation of elimination by renal tubular secretion. Metabolites of 2,4,5-T were detected in the urine of rats given saturating doses (i.e., 100 or 200 mg kg^{-1}). Saturation of the metabolism of 1,4-dioxane has also been shown at doses associated with toxicity (33). Saturation of biliary excretion of FPL 57787 in the dog has been demonstrated at toxic doses of this antiallergy compound (97).

A good example is also provided by studies on the metabolism and toxicokinetics of cyclohexylamine (82, 83). This compound produces testicular toxicity when given chronically to rats, but not to mice (10). Toxicokinetic studies indicated that the plasma clearance was higher in mice than in rats, and that rats, but not mice, showed evidence of nonlinear kinetics at high doses (84). The steady-state concentrations in the plasma and testes during chronic administration confirmed dose-dependent kinetics in the rat (Figure 4.24), which coincided with the dose-response curve for testicular

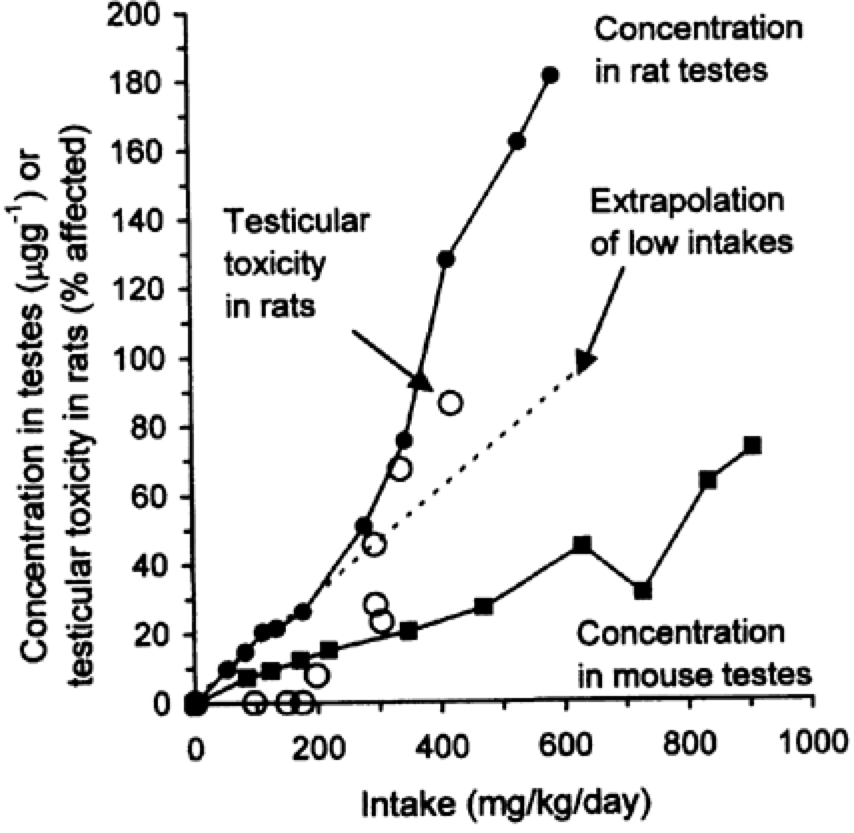

FIG. 4.24. Relation between dose, toxicity, and target organ concentrations of cyclohexylamine during chronic administration; (●) concentration in rat testes; (■) concentration in mouse testes; (○) testicular toxicity in rats. Data from Reference 84.

atrophy in this species (84). These toxicokinetic data thus provide a possible explanation for the steepness of the dose-response curve in the rat and the apparent species difference in sensitivity. Cyclohexylamine is an indirectly acting sympathomimetic amine in rats (12), but it does not increase blood pressure in humans following its formation from cyclamate metabolism (13). The apparent difference in response arises from the different concentration-time profiles when the metabolite is administered orally or when it is formed by the intestinal microflora from cyclamate (13), illustrating further the importance of kinetics in the interpretation of dose-effect relationships.

Finally, toxicokinetic data can be invaluable for the interpretation of animal toxicity with respect to possible human risk. This analysis may be made in the absence or the presence of human pharmacokinetic data. In the absence of human data, extrapolation may be by either physiological or compartmental modeling methods (3). The physiological approach relies on the scale-up between animals and man of such parameters as tissue volume and blood flow (Figure 4.4) and by their relation to body weight. Assuming that the uptake from blood to tissue (extraction ratio) is a function of the

chemical and therefore independent of species, it is possible to derive complex models involving all the major tissues of the body (34). These models may then be scaled up from known animal data to humans based on the known physiological differences. Alternatively and more pragmatically, the plasma kinetics in various species may be fitted by compartmental modeling and then scaled up empirically according to the body mass of the species studied and extrapolated to humans (39, 64).

The most secure comparison is when kinetic data are available in animals and humans, allowing direct analysis of the potential risk. In any such analysis the species difference in basic physiological processes such as cardiac output and relative tissue weight usually result in lower clearances and longer half-lives in humans than in animals (75). Thus comparisons of animals and humans on the basis of plasma levels or AUC values, rather than intake or exposure data expressed per kilogram body weight, result in a decreased apparent safety margin compared with intake data. However, by removing important variables from interspecies comparisons, such an approach provides a more secure basis for the safety assessment (76, 92).

Toxicokinetic data can also be useful in defining the contributions of parent compound and metabolites to toxicity. Retinol (vitamin A) is an animal and human teratogen, which is metabolized to a number of biologically active species such as all-*trans*-retinoic acid, 13-*cis*-retinoic acid, and 9-*cis*-retinoic acid. The principle sources of exposure to retinol (rather than the precursor carotenoids) are from the consumption of animal livers

Table 4.18 The AUC values for retinol and its metabolites in young women after oral doses of 50 mg and 150 mg of retinol given as retinyl palmitate in a supplement or in cooked calf liver

	AUC values in ng ml−1h					
Dose	Retinyl palmitate	Retinol	All-*trans*-retinoic acid	13-*cis*-retinoic acid	All-*trans*-4-oxo-retinoic acid	13-*cis*-4-oxo-retinoic acid
50 mg S	10,400 (5,300)	2070 (1900)	86 (63)	359 (104)	30 (33)	877 (207)
50 mg L	5900 (3,700)	1530 (2490)	6 (5)	243 (70)	11 (15)	492 (145)
150 mg S	18,900 (20,200)	2300 (1180)	170(118)	674 (156)	133 (47)	2385 (563)
150 mg L	14,500 (8,800)	2430 (2090)	23 (11)	596 (131)	120 (84)	1820 (294)

S=Supplement given under fasting conditions.
L=Cooked liver as part of a meal.
The results are the mean (with *SD* in parentheses) for 10 subjects dosed at 4-week intervals (data from ref. 14).

and from vitamin supplements. The evidence for teratogenicity in humans is from case reports of excessive consumption of vitamin supplements; there is little evidence of a risk from liver consumption, despite the fact that this source may give similar or even higher intakes than those causing malformations after excessive supplement consumption. The areas under the plasma concentration-time curve of retinol and its metabolites in young women following oral doses of retinyl palmitate as an oral solution (fasting) and as cooked liver (as part of a meal) (14) are given in Table 4.18. The main difference was the 5–10-fold difference in the AUC of all-*trans*-retinoic acid, which is recognized to be the major teratogenic metabolite. However, 13-*cis*-retinoic acid (isotretoin), also a recognized human teratogen, showed no major difference. Therefore, the key issue for the assessment of human risk was the plasma concentrationresponse relationship for the main metabolites, and whether the teratogenicity of retinol and 13-*cis*-retinoic acid could be explained by their metabolism to all-*trans*-retinoic acid. A subsequent study (107) gave single oral doses of retinol, all-*trans*-retinoic acid and 13-*cis*-retinoic acid to pregnant rats and determined the dose-response relationship (Figure 4.25) and plasma kinetics of the different metabolites for each compound. Both retinol and 13-*cis*-retinoic acid gave measurable levels of all-*trans*-retinoic acid in the plasma. A graph of teratogenic response against the AUC of all-*trans*-retinoic acid for the three compounds (Figure 4.26) shows that whereas the teratogenicity of 13-cis-retinoic acid could be explained on the basis of the circulating all-*trans*-retinoic acid, this was not the case for retinol. Retinol was considerably more potent than would be predicted from the plasma AUC of all-*trans*-retinoic acid after retinol administration, possibly due to local bioactivation within the fetus. Consequently, the

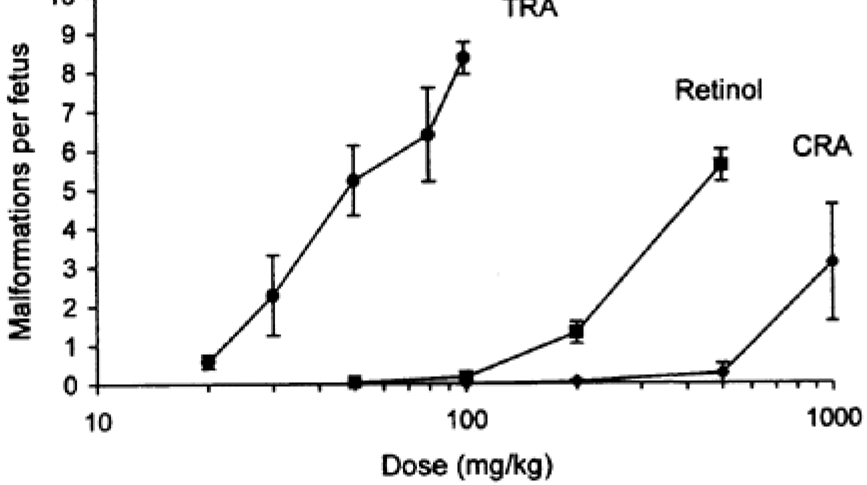

FIG. 4.25. Dose-response relationships for teratogenicity of all-*trans*-retinoic acid (TRA), retinol, and 13-*cis*-retinoic acid (CRA) given as a single oral dose to rats on day 10 of gestation. Data from Reference 107.

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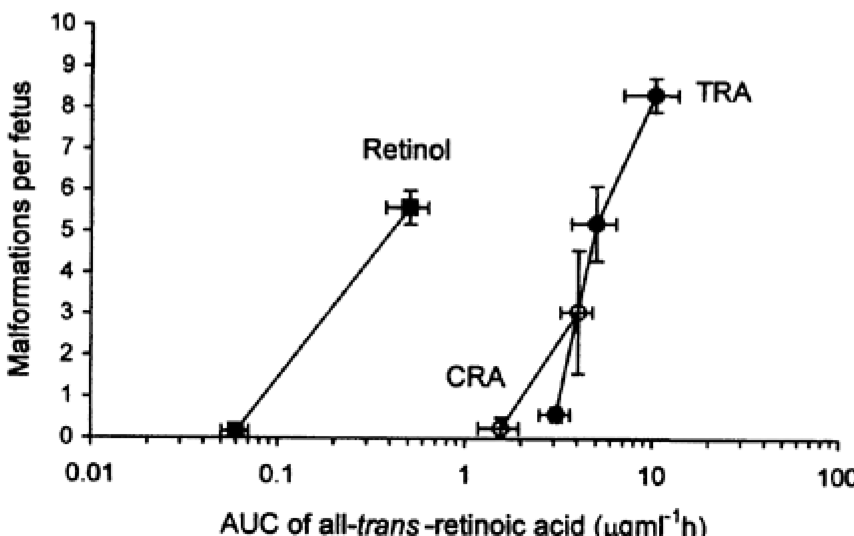

FIG. 4.26. Relationship between AUC of all-*trans*-retinoic acid in maternal plasma and teratogenicity in female rats given all-*trans*-retinoic acid (TRA), retinol, and 13-*cis*-retinoic acid (CRA) on day 10 of gestation. Data from Reference 107.

5–10-fold difference in the AUC of all-*trans*-retinoic acid between liver and supplements (Table 4.18) would not be expected to be translated into a similar difference in teratogenic risk. Using the comparative potency ratios in rats (Figure 4.25) of about 25:3:1 for all-*trans*-retinoic acid, 13-*cis*-retinoic acid, and retinol, and the AUC data in Table 4.18, the doses of 50 and 150 mg as supplements would be expected to be about 2.2 and 1.8 times, respectively, more active than the equivalent doses given in cooked liver. However, this analysis was heavily biased by the AUC and response data for retinol; also, there could be major species differences in local activation of retinol in the fetus and/or vehicle-dependent differences (liver compared with supplements) in the proportions bound to retinol-binding protein (which could alter this conclusion).

The Use and Interpretation of In Vitro Data

In vitro data can provide important qualitative insights into the metabolic fate of the compound, but care must be taken in their quantitative incorporation into risk assessment. There is a wide variety of in vitro systems of increasing cell integrity, that can be used, for example subcellular fractions (such as microsomes), cell homogenates, isolated cells and cell lines, and tissue slices. Each preparation has strengths and weaknesses and these can be exploited to provide useful information (24, 51).

Strengths of In Vitro Systems

Microsomes comprise the smooth endoplasmic reticulum and its associated enzymes, cytochrome P450s and UDP-glucuronyl transferases. The rates of reaction in vitro are determined by the availability of appropriate cofactors, and therefore it is possible to study oxidation by the addition of NADPH in the absence of glucuronidation, which requires UDPGA (and vice versa). This allows the rates of P450-mediated oxidation to be studied directly, because the product of the primary oxidation produced is not lost due to conjugation. An additional major value for such simple systems is that they can be used to generate metabolites for structural analysis.

More complex systems, such as isolated cells and tissue slices, provide a comprehensive picture of the metabolic fate of the compound. All enzyme systems are present, including cytoplasmic and mitochondrial enzymes, and the cell architecture can affect cell uptake and intracellular distribution of the chemical. Perhaps the most integrated in vitro system is the isolated perfused rat liver, which can give excellent correlations with in vivo clearance (23). As a result, these systems can provide infor mation on the relative importance of alternative metabolic pathways.

A major advantage of in vitro systems is that they allow data to be generated on the potential metabolism in humans, without the need for in vivo exposure. This has been particularly valuable for carcinogens, where the generation of in vivo data would be unethical. Identification of the specific isoenzymes of cytochrome P450 involved in metabolism of a compound is important in understanding the potential variability in metabolism within the human population, because of the genetic polymorphism in some of the isoforms, for example CYP2D6. Such information can be generated by in vitro studies in three ways:

1. Comparisons of the rates of metabolism in stored (banked) liver preparations from individuals with characteristic isoenzyme profiles
2. The use of isoenzyme specific inhibitors or inducers (in cell-intact preparations)
3. The use of expression systems in which the DNA for specific isoenzymes is incorporated and expressed by a suitable host, such as a yeast or bacteria (38)

The generation of in vitro data using human tissues allows characterization of species differences both qualitatively and quantitatively by the generation of the appropriate enzyme constants *V*max and *Km*. Such data represent critical components of PBPK models (70) and for the prediction of in vivo clearance (44, 46, 120, 121).

The outline given above should be sufficient to indicate the huge potential for in vitro studies and explain why these methods have been the basis for much of our understanding of pathways of xenobiotic metabolism.

Precautions with In Vitro Systems

A number of limitations need to be remembered when considering in vitro data.

1. Many studies give data on the extent of metabolism at a single high concentration in vitro and therefore represent *V*max, which may be of limited relevance to in vivo concentrations. A full analysis of the enzyme kinetics is necessary to give both *V*max and *Km*.
2. The strengths of simple systems, for example microsomes outlined above, will be weaknesses if the data are overinterpreted in relation to the fate in vivo.
3. Changes in enzyme expression occur in vitro, for example isolated cell lines show a different complement of cytochrome P450 activities to those in the same cells at isolation.

4. Many human data are generated from stored liver samples obtained at postmortem. The in vitro enzyme activity could be affected by both in vivo aspects, such as drugs given in attempts at resuscitation, disease, etc., and ex vivo aspects, such as the period between death and freezing and storage (67).

5. In vitro data may still be misleading, even if all of these aspects are optimum. This occurs when the clearance of drug by an organ is blood flow limited rather than enzyme limited (see earlier). Under these conditions, both interspecies differences and interindividual variability will reflect organ blood flow, rather than *V*max and *Km*. A good example of this is furan (49) for which the rate of oxidation in vitro would greatly exceed delivery via the liver blood flow.

These problems can be avoided if the in vitro data are incorporated into a PBPK model that will allow for organ blood flow, partitioning between blood and tissue, and enzyme kinetics.

The increasing use of in vitro test systems facilitates a quantitative analysis of the dose-toxicity curve and may provide information on mechanisms of action (24, 27). The logical interpretation of such data with respect to human risk requires information on the steady-state concentrations of the active chemical species in the target organ and plasma of the test animal during chronic toxicity testing, combined with knowledge of the toxicokinetics of the chemical in the test animal at toxic doses and in human at the likely exposure level. It must be emphasized that large safety factors have been introduced to protect us from our own ignorance. The increased use of pharmacokinetic data, especially when combined with knowledge of the mechanism of toxicity, will allow the future use of potentially toxic chemicals to be based on scientific principles and understanding (76, 92).

STUDY QUESTIONS

1. A new chemical has been administered to rats and humans by both oral and intravenous routes. Basic toxicokinetic measurements (extrapolated to infinity) are given below.

	Rat	Human
Intravenous		
Dose (mg kg−1)	10	1
AUC (*μ*g ml−1 min)	2000	500
Terminal slope (min−1)	0.0025	0.001
% dose excreted unchanged in urine	1	15
Oral		
Dose (mg kg−1)	100	1
AUC (*μ*g ml−1 min)	8000	490
Terminal slope (min−1)	0.0025	0.001

Calculate appropriate toxicokinetic parameters and suggest biochemical and physiological mechanisms that could explain the species difference.

2. The pharmaceutical company for which you work has synthesized a new antianxiety drug, a basic compound, structurally related to the old drug debrisoquine. The parent drug, the active form, causes enzyme (cytochrome P450) induction and liver enlargement; the hydroxylated metabolite, formed on incubation of the drug with liver microsomes, is inactive. After an oral dose, 40% is excreted in the urine within 24 h as the parent compound, 40% is in urine as an hydroxylated metabolite, and 20% is in feces as the parent drug. After an intravenous dose, 80% is in urine as the parent drug, and 20% is in the urine as the metabolite. What advice would you give the company about the following issues:

a. Is the drug likely to be toxic after oral dosage?

b. Would the oral and intravenous doses associated with toxicity be the same?

c. What are the likely sources of variability in kinetics in young physically healthy adults (20–30 years old)?

d. Would the kinetics be different in the elderly (70–80 years old)?

e. How much would a 50% decrease in liver or kidney function affect the kinetics, and would the toxicity be increased or decreased?

f. How much would a 50% increase in liver or kidney function affect the kinetics, and would the toxicity be increased or decreased?

g. Should the pharmaceutical group develop a slow-release formulation and would this be likely to affect the toxicity?

3. The company you work for has developed a novel opioid for the treatment of intractable pain. The drug is 20 times more potent than morphine in relation to both analgesia and respiratory depression when given to rats by intravenous injection, and binding studies show that it has a high and similar affinity for *μ*-receptors of rats and humans. Initial kinetic studies in humans after a single intravenous

bolus dose of 10 mg gave the following data.

Time after dose (h)	*Plasma concentration (ng/ml)*
0.5	367
1.0	336
2	283
4	200
6	141
10	71
24	6.3

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The area under the plasma concentration-time curve (AUC) extrapolated to infinity was 2310 ng ml^{-1} h. Urine was collected over the period 2–4 h after dosing and contained a total of 1.85 mg of the parent drug and 0.1 mg of an hydroxy metabolite. The plasma concentration of parent drug at 3 h was 238 ng/ml.
After a single oral dose of 10 mg the maximum plasma concentration occurred at 8 h and was only 48 ng/ml; the blood concentration reached 6 ng/ml by 36 hours. The AUC to infinity was 1155 ng ml^{-1} h.
Plot the intravenous data on graph paper. Calculate appropriate pharmacokinetic parameters to describe the elimination rate, clearance, distribution, and absorption of the drug. Describe the probable overall fate of the drug in the body (e.g., routes of elimination).
Your research director needs the following advice:
i. What extra studies/data could support your description of the fate of the drug?
ii. What route(s) of administration should the company use for its first trials of clinical effect for pain relief?
iii. How should the drug be administered to provide relief of chronic pain?

Hints and Clues

Question 1

a. Calculate clearance (per kg body weight)—why is it different? (See b).
b. Use urinary excretion data to think about pathways of elimination.
c. Use clearance and "terminal slope" (k or *β*—we don't know) to calculate the apparent volume of distribution.
d. Use AUC data to calculate bioavailability.
e. Are terminal rates different after oral dosage? What would it mean if they were?
f. What are the likely causes of differences between species? Could clearance and bioavailability be interrelated? If so, how?
g. Would scaling to body surface area affect the calculations and conclusions? If so, how?

Question 2

a. Use urinary excretion data to interpret the potential for exposure (or not) of the liver to the parent compound. (Obviously the dose will affect the response, but is toxicity possible?)
b. Use urinary excretion data to calculate bioavailability. What processes are giving rise to the low bioavailability?
c. Variability in adults: What are the routes of elimination? What is the relevance of debrisoquine?
d. Consider 50% changes in liver in relation to bioavailability and clearance. Then, consider changes in renal function similarly. Will kidney function affect bioavailability?
e. A slow-release formulation is necessary when a drug has a very short half-life (e.g., 3–4 h or less). There is information on the rate of elimination in the question. What can you conclude about half-life? (*Clue:* Could it be 24 h?)

Question 3

a. You can calculate clearance, apparent volume of distribution, and half-life from the intravenous data. But what route is important for elimination? (*Clue*: Use urine data to calculate renal clearance and compare with plasma clearance.)
b. The extra studies should relate to kinetics. (*Clue*: What studies would we normally have before giving the first dose to humans?)
c. What is happening with the oral data? What is the extent of absorption? Why are blood levels at 36 h higher after oral dosage? (*Clue*: You can calculate the concentration at 36 h after IV dosage using the exponential terms derived from the IV data.)
d. Phase 1 studies (initial human studies) are usually by the oral route. Is this likely to produce analgesia or side effects with this compound?
e. Chronic pain relief requires the maintenance of constant concentrations of the analgesic. Which route would be likely to give this profile? If oral dosage could not give effective plasma levels without unacceptable side effects (such as constipation), how could you give the drug parenterally to provide similar constant concentrations?

REFERENCES

1. Andersen, M.E., Gargas, M.L., Jones, R.A., and Jenkins, L.J. (1979): The use of inhalation techniques to assess the kinetic constants of 1,1-dichloroethylene metabolism. *Toxicol. Appl. Pharmacol.*, 47:395–409.
2. Anderson, M.W., Hoel, D.G., and Kaplan, N.L. (1980): A general scheme for the incorporation of pharmacokinetics in low-dose risk estimation for chemical carcinogens: Example—vinyl chloride. *Toxicol.*

Appl. Pharmacol., 55:154–161.
3. Bachmann, K. (1989): Predicting toxicokinetic parameters in humans from kinetic data acquired in three small mammalian species. *J. Appl. Toxicol.*, 9:331–338.
4. Bakar, S.K., and Niazi, S. (1983): Simple reliable method for chronic cannulation of the jugular vein for pharmacokinetic studies in rats. *J. Pharm. Sci.*, 72:1027–1029.
5. Bard, P. (1956): In: *Medical Physiology*,. 10th ed., p. 221. Henry Kimpton, London.
6. Bauer, L.A., and Gibaldi, M. (1983): Computation of mod el-independent pharmacokinetic parameters during multiple dosing. *J. Pharm. Sci.*, 72:978–979.

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7. Benet, L.Z., and Galeazzi, R.L. (1979): Noncompartmental determination of the steady state volume of distribution. *J. Pharm. Sci.*, 68:1071–1074.
8. Benet, L.Z., Levy, G., and Ferraiolo, B.L. (eds.) (1984): *Pharmacokinetics: A Modern View.* Plenum Press, New York.
9. Bois, F.Y., Woodruff, T.J., and Spear, R.C. (1991): Comparison of three physiologically based pharmacokinetic models of benzene disposition. *Toxicol. Appl. Pharmacol.*, 110:79–88.
10. Bopp, B.A., Sonders, R.C., and Kesterson, J.W. (1986): Toxicological aspects of cyclamate and cyclohexylamine. *CRC Crit. Rev. Toxicol.*, 16:213–306.
11. Bourgoignie, J.J., Hwang, K.H., Espinel, C., Klahr, S., and Bricker, N.S. (1972): A natriuretic factor in the serum of patients with chronic uremia. *J. Clin. Invest.*, 51:1514–1527.
12. Buss, N.E., and Renwick, A.G. (1992): Blood pressure changes and sympathetic function in rats given cyclohexylamine by intravenous infusion. *Toxicol. Appl. Pharmacol.*, 115:211–215.
13. Buss, N.E., Renwick, A.G., Donaldson, K.M., and George, C.F. (1992): The metabolism of cylamate to cyclohexylamate and its cardiovascular consequences in human volunteers. *Toxicol. Appl. Pharmacol.*, 115:199–210.
14. Buss, N.E., Tembe, E.A., Prendergast, B.D., Renwick, A.G., and George, C.F. (1994): The teratogenic metabolites of vitamin A in women following supplements and liver. *Human Exp. Toxicol.*, 13:33–43.
15. Butler, T.C. (1971): The distribution of drugs. In: *Fundamentals of Drug Metabolism and Drug Disposition*, edited by B.N.LaDu, H. G.Mandel, and E.L.Way, pp. 44–62. Williams & Wilkins, Baltimore, MD.
16. Caldwell, J., and Varwell Marsh, M. (1982): Metabolism of drugs by the gastrointestinal tract. In: *Presystemic Drug Elimination*, edited by C.F.George, D.G.Shand, and A.G.Renwick, pp. 29–42. Butterworth, Boston.
17. Campbell, D.B., and Ings, R.M.J. (1988): New approaches to the use of pharmacokinetics in toxicology and drug development. *Human Toxicol.*, **7:469–79.**
18. Chan, K.K.H., and Gibaldi, M. (1985): Assessment of drug absorption after oral administration. *J. Pharm. Sci.*, 74:388–393.
19. Clinical implications of drug protein binding (1984): (Levy, R. and Shand, D., Eds). *Clin. Pharmacokinet.*, *9* (Suppl. 1): 1–104.
20. Cocchetto, D.M., and Bjornsson, T.D. (1983): Methods for vascular access and collection of body fluids from the laboratory rat. *J. Pharm. Sci.*, 72:465–492.
21. Cohn, V.H. (1971): Transmembrane movement of drug molecules. In: *Fundamentals of Drug Metabolism and Drug Disposition*, edited by B.N.LaDu, H.G.Mandel, and E.L.Way, pp. 3–43. Williams & Wilkins, Baltimore, MD.
22. Colburn, W.A., and Matthews, H.B. (1979): Pharmacokinetics in the interpretation of chronic toxicity tests: The last-in, first-out phenomenon. *Toxicol. Appl. Pharmacol.*, 48:387–395.
23. Damian, P., and Raabe, O.G. (1996): Toxicokinetic modeling of dose-dependent formate elimination in rats: *In vivo-in vitro* correlations using the perfused rat liver. *Toxicol. Appl. Pharmacol.*, 139:22–32.
24. Davila, J.C., Rodriguez, R.J., Melchert, R.B., and Acosta, D. (1998): Predictive value of *in vitro* model systems in toxicology. *Annu. Rev. Pharmacol. Toxicol.*, 38:63–96.
25. Davison, C. (1971): Protein binding. In: *Fundamentals of Drug Metabolism and Drug Disposition*, edited by B.N.LaDu, H. G.Mandel, and E.L.Way, pp. 63–75. Williams & Wilkins, Baltimore, MD.
26. Despopoulos, A. (1965): A definition of substrate specificity in renal transport of organic anions. *J. Theor. Biol.*, 8:163–192.
27. Flamm, W.G., and Lorentzen, R.J. (1987): The use of in vitro methods in safety evaluation. *In Vitro Toxicol.*, 1:1–3.
28. Gabrielsson, J., and Weiner, D. (1997): *Pharmacokinetic/Pharmacodynamic Data Analysis: Concepts and Applications*, 2nd ed. Swedish Pharmaceutical Society, Swedish Pharmaceutical Press, Sweden.
29. Garattini, S. (1987): Toxic effects of chemicals: Difficulties in extrapolating data from animals to man. *CRC Crit. Rev. Toxicol.*, 16:1–29.
30. Gardner, D.M., and Renwick, A.G. (1978): The reduction of nitrobenzoic acids in the rat. *Xenobiotica*, 8:679–690.
31. Garrett, E.R. (1978): Pharmacokinetics and clearance related to renal processes. *Int. J. Clin. Pharmacol.*, 16:155–172.
32. Gehring, P.J. (1979): Chemobiokinetics and metabolism. In: *Environmental Health Criteria. 6. Principles and Methods for Eval-uating the Toxicity of Chemicals Part 1*, pp. 116–177. WHO, Geneva.
33. Gehring, P.J., and Young, D.J. (1978): Application of pharmacokinetic principles in practice. In:

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_189.html[2010/10/28 18:41:41]

Proceedings of the First International Congress on Toxicology. Toxicology as a Predictive Science, edited by G.L.Plaa and W.A.M.Duncan, pp. 119–141. Academic Press, New York.
34. Gerlowski, L.E., and Jain, R.K. (1983): Physiologically based pharmacokinetic modelling: Principles and application. *Pharm. Sci.*, 72:1103–1127.
35. Gibaldi, M., and Perrier, D. (1974): Route of administration and drug disposition. *Drug Metab. Rev.*, 3:185–199.
36. Gibaldi, M., and Perrier, D. (1982): *Pharmacokinetics*, 2nd ed. Marcel Dekker, New York.
37. Goldstein, A., Aranow, L., and Kalman, S.M. (1974): *Principles of Drug Action: The Basis of Pharmacology*. John Wiley, New York.
38. Gonzalez, F.J., and Korzekwa, K.R. (1995): Cytochrome P450 expression systems. *Annu. Rev. Pharmacol. Toxicol.*, 35:369–390.
39. Grene-Lerouge, N.A.M., Bazin-Redureau, M.I., Debray, M., and Scherrmann, J.M.G. (1996): Interspecies scaling of clearance and volume of distribution for digoxin-specific Fab. *Toxicol. Appl. Pharmacol.*, 138:84–89.
40. Guy, R.H., Hadgraft, J., and Maibach, H.I. (1984): Percutaneous absorption in man: A kinetic approach. *Toxicol. Appl. Pharmacol.*, 78:123–129.
41. Hiles, R.A., and Birch, C.G. (1978): Non-linear metabolism and disposition of 3,4,4′-trichlorocarbanilide in the rat. *Toxicol. Appl. Pharmacol.*, 46:323–337.
42. Hirom, P.C., Millburn, P., and Smith, R.L. (1976): Bile and urine as complementary pathways for the excretion of foreign organic compounds. *Xenobiotica*, 6:55–64.
43. Hirom, P.C., Millburn, P., Smith, R.L., and Williams, R.T. (1972): Species variations in the threshold molecular-weight factor for the biliary excretion of organic anions. *Biochem, J.*, 129:1071–1077.
44. Houston, J.B., and Carlile, D.J. (1997): Prediction of hepatic clearance from microsomes, hepatocytes, and liver slices. *Drug Metab. Rev.*, 29:891–922.
45. Huang, C.H., Kimcera, R., Nassar, R.B., and Hussain, A. (1985): Mechanisms of nasal absorption of drugs. I. Physicochemical parameters influencing the rate of in situ nasal absorption of drugs in rats. *J. Pharm. Sci.*, 74:608–611.
46. Ito, K., Iwatsubo, T., Kanamitsu, S., Nakajima, Y., and Sugiyama, Y. (1998): Quantitative prediction of *in vivo* drug clearance and drug interactions from *in vitro* data on metabolism, together with binding and transport. *Annu. Rev. Pharmacol. Toxicol.*, 38:461–499.
47. Jepson, G.W., and McDougal, J.N. (1997): Physiologically based modeling of nonsteady state dermal absorption of halogenated methanes from an aqueous solution. *Toxicol. Appl. Pharmacol.*, 144:315–324.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_189.html[2010/10/28 18:41:41]

48. Jollow, D.J., Mitchell, J.R., Potter, W.Z., Davis, D.C., Gillette, J.R., and Brodie, B.B. (1973): Acetaminophen-induced necrosis II. Role of covalent binding in vivo. *J. Pharmacol. Exp. Ther.,* 187:195–202.
49. Kedderis, G.L., and Held, S.D. (1996): Prediction of furan pharmacokinetics from hepatocyte studies: Comparison of bioactivation and hepatic dosimetry in rats, mice and humans. *Toxicol. Appl. Pharmacol.,* 140:124–130.
50. Keen, P.M. (1971): Effect of binding to plasma proteins on the distribution, activity, and elimination of drugs. In: *Handbook of Experimental Pharmacology,* Vol. 28, edited by B.B.Brodie and J.R.Gillette, pp. 213–233. Springer-Verlag, New York.
51. Lake, B.G. (1997): *In vitro* methods. In: *Comprehensive Toxicology, vol. 9, Hepatic and Gastrointestinal Toxicology,* edited by R.S.McCuskey and D.L.Earnest, pp. 233–246. Pergamon Press, New York.
52. Langenberg, J.P., Spruit, H.E. T., van der Wiel, H.J., Trap, H.C., Helmich, R.B., Bergers, W.W.A., van Helden, H.P.M., and Benschop, H.P. (1998): Inhalation toxicokinetics of soman stereoisomers in the atropinized guinea pig with nose-only exposure to soman vapour. *Toxicol. Appl. Pharmacol.,* 151:79–87.
53. Lawrie, C.A., Renwick, A.G., and Sims, J. (1985): The urinary excretion of bacterial amino-acid metabolites by rats fed saccharin in the diet. *Food Chem. Toxicol.,* 23:445–450.
54. Lien, E.J., and Tong, G.L. (1973): Physicochemical properties and percutaneous absorption of drugs. *J. Soc. Cosmet. Chem.,* 24:371–384.
55. Light, H.G., Witmer, C., and Vars, H.M. (1959): Interruption of the enterohepatic circulation and its effects on rat bile. *Am. J. Physiol.,* 197:1330–1332.
56. Lindup, W.E. (1975): Drug-albumin binding. *Biochem. Soc. Trans.,* 3:635–640.
57. Mann, S., Droz, P.-O., and Vahter, M. (1996): A physiologically based pharmacokinetic model for arsenic exposure. *Toxicol. Appl. Pharmacol.,* 140:471–486.
58. McDougal, J.N., Jepson, G.W., Clewell, H.J., and Andersen, M. E. (1985): Dermal absorption of dihalomethane vapours. *Toxicol. Appl Pharmacol.,* 79:150–158.
59. McKenna, M.J., Zempel, J.A., Madrid, E.O., and Gehring, P.J. (1978): The pharmacokinetics of [14C]vinylidene chloride in rats following inhalation exposure. *Toxicol. Appl. Pharmacol.,* 45:599–610.
60. Mesfin, G.M., Higgins, M.J., Robinson, F.G., and Zhong, W.-Z. (1996): Relationship between serum concentrations, hemodynamic effects, and cardiovascular lesions in dogs, treated with minoxidil. *Toxicol. Appl. Pharmacol.,* 140:337–344.
61. Meyer, M.C., and Guttman, D.E. (1968): The binding of drugs by plasma proteins. *J. Pharm. Sci.,* 57:895–918.
62. Mitchell, J.R., Jollow, D.J., Potter, W.Z., Davis, D.C., Gillette, J.R., and Brodie, B.B. (1973): Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. *J. Pharmacol. Exp. Ther.,* 187:185–194.
63. Mitchell, J.R., Jollow, D.J., Potter, W.Z., Gillette, J.R., and Brodie, B.B. (1973): Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. *J. Pharmacol. Exp. Ther.,* 187:211–217.
64. Mordenti, J. (1985): Pharmacokinetic scale up: Accurate prediction of human pharmacokinetic profiles from animal data. *J. Pharm. Sci.,* 74:1097–1099.
65. Munro, A.M. (1990): Interspecies comparisons in toxicology: The utility and futility of plasma concentrations of the test compound. *Reg. Toxicol. Pharmacol.,* 12:137–160.
66. Ochsenfahrt, H., and Winne, D. (1972): Solvent drag influence on the intestinal absorption of basic drugs. *Life Sci.,* 11:1115–1122.
67. Olinga, P., Merema, M., Hof, I.H., de Jong, K.P., Slooff, M.J.H., Meijer, D.K.F., and Groothuis, G.M.M. (1997): Effect of human liver source on the functionality of isolated hepatocytes and liver slices. *Drug Metab. Disp.,* 26:5–11.
68. Piafsky, K.M., Borga, O., Odar-Cedelof, I., Johansson, C., and Sjoqvist, F. (1978): Increased plasma protein binding of propranolol and chlorpromazine mediated by disease-induced elevations of plasma *α1* acid glycoprotein. *N. Engl. J. Med.,* 299:1435–1439.
69. Pierce, C.H., Dills, R.L., Morgan, M.S., Nothstein, G.L., Shen, D.D., and Kalman, D.A. (1996): Interindividual differences in 2H8-toluene toxicokinetics assessed by a semi-empirical physiologically based model. *Toxicol. Appl. Pharmacol.,* 139:49–61.
70. Ploemen, J.-P.H.T.M., Wormhoudt, L.W., Haenen, G.R.M. M., Oudshoorn, M.J., Commandeur, J.N.M., Vermeulen, N. P.E., de Waziers, I., Beaune, P.H., Watabe, T., and van Bladeren, P.J. (1997): The use of human *in vitro* metabolic parameters to explore the risk assessment of hazardous compounds: The case of ethylene dibromide. *Toxicol. Appl. Pharmacol.,* 143:56–69.

71. Potter, W.Z., Davis, D.C., Mitchell, J.R., Jollow, D.J., Gillette, J.R., and Brodie, B.B. (1973): Acetaminophen-induced hepatic necrosis. III. Cytochrome P450-mediated covalent binding in vitro. *J. Pharmacol. Exp. Ther.*, 187:203–210.
72. Renwick, A.G. (1982): First pass metabolism within the lumen of the gastrointestinal tract. In: *Presystemic Drug Elimination*, edited by C.F.George, D.G.Shand, and A.G.Renwick, pp. 3–28. Butterworth, Boston.
73. Renwick, A.G. (1985): The disposition of saccharin in animals and man—a review. *Food Chem. Toxicol.*, 23:429–435.
74. Renwick, A.G. (1986): Gut bacteria and the enterohepatic circulation of foreign compounds. In: *Microbial Metabolism in the Digestive Tract*, edited by M.J.Hill, pp. 135–153. CRC Press, Boca Raton, FL.
75. Renwick, A.G. (1991): Safety factors and establishment of acceptable daily intakes. *Food Addit. Contamin.*, 8:135–150.
76. Renwick, A.G. (1993): Data derived safety factors for the evaluation of food additives and environmental contaminants. *Food Addit. Contamin.*, 10:275–305.
77. Renwick, A.G., and Sweatman, T.W. (1979): The absorption of saccharin from the rat urinary bladder. *J. Pharm. Pharmacol.*, 31:650–652.
78. Rescigno, A., and Segre, B. (1966): *Drug and Tracer Kinetics*, Blaisdell, London.
79. Riegelman, S., and Collier, P. (1980): The application of statistical moment theory to the evaluation of in vivo dissolution time and absorption time. *J. Pharmacokinet. Biopharm.*, 8:509–534.
80. Riegelman, S., Loo, J.C. K., and Rowland, M. (1968): New method for calculating the intrinsic absorption rate of drugs. *J. Pharm. Sci.*, 57:918–928.
81. Roberts, A., and Renwick, A.G. (1985): The effect of saccharin on the microbial metabolism of tryptophan in man. *Food Chem. Toxicol.*, 23:451–455.
82. Roberts, A., and Renwick, A.G. (1985): The metabolism of 14C-cyclohexylamine in mice and two strains of rat. *Xenobiotica*, 15:477–483.
83. Roberts, A., and Renwick, A.G. (1988): The fate of cyclohexylamine in rat and mouse in relation to testicular toxicity. *Human. Toxicol.*, 7:229.
84. Roberts, A., and Renwick, A.G. (1989): The pharmacokinetics and tissue concentrations of cyclohexylamine in rats and mice. *Toxicol. Appl Pharmacol.*, 98:230–242.
85. Rowland, M., and Tozer, T.N. (1980): *Clinical Pharmacokinetics: Concepts and Applications.* Lea & Febiger, Philadelphia.

86. Rowland, M., and Tucker, G. (eds.) (1986): *Pharmacokinetics: Theory and Methodology,* Pergamon Press, New York.
87. Rowland, M., Benet, *L.Z.,* and Graham, G.G. (1973): Clearance concepts in pharmacokinetics. *J. Pharmacokinet. Biopharm*., 1:123–136.
88. *Saccharin: Technical Assessment of Risks and Benefits* (1978): Institute of Medicine, National Research Council-National Academy of Science, Washington, DC.
89. Sanzgiri, U.Y., Kim., H.J., Muralidhara, S., Dallas, C.E., and Bruckner, J.V. (1995): Effect of route and pattern of exposure on the pharmacokinetics and acute hepatoxicity of carbon tetrachloride. *Toxicol. Appl. Pharmacol.,* 134:148–154.
90. Sauerhoff, M.W., Braun, W.H., Blau, G.E., and Gehring, P.J. (1976): The dose-dependent pharmacokinetic profile of 2,4,5-trichlorophenoxyacetic acid following intravenous administration to rats. *Toxicol. Appl. Pharmacol*., 36:491–501.
91. Scheline, R.R. (1973): Metabolism of foreign compounds by gastrointestinal microorganisms. *Pharmacol. Rev*., 25:451–523.
92. Scheuplein, R.J., Shoaf, S.E., and Brown, R.N. (1990): Role of pharmacokinetics in safety evaluation and regulatory decisions. *Annu. Rev. Pharmacol. Toxicol*., 30:197–218.
93. Schoenig, G.P., Goldenthal, E.I., Geil, R.G., Frith, C.H., Richter, W.R., and Carlborg, F.W. (1985): Evaluation of the dose response and in utero exposure to saccharin in the rat. *Food Chem. Toxicol*., 23:475–490.
94. Shen, S.K., Williams, S., Onkelinx, C., and Sunderman, F.W. (1979): Use of implanted minipumps to study the effects of chelating drugs on renal 63Ni clearance in rats. *Toxicol. Appl. Pharmacol.,* 51:209–217.
95. Sims, J., and Renwick, A.G. (1983): The effects of saccharin on the metabolism of dietary tryptophan to indole, a known cocarcinogen for the urinary bladder of the rat. *Toxicol. Appl. Pharmacol*., 67:132–151.
96. Sims, J., and Renwick, A.G. (1985): The microbial metabolism of tryptophan in rats fed a diet containing 7.5% saccharin in a two-generation protocol. *Food Chem. Toxicol*., 23:437–444.
97. Smith, D.A. (1979): Differences in toxicity due to species variation in the metabolism of an oral antiallergy agent. *Br. J. Pharmacol*., 66:422P-423P.
98. Smith, J.W. (1965): Observations on the flora of the alimentary tract of animals and factors affecting its composition. *J. Pathol. Bacteriol*., 89:95–122.
99. Smith, R.L. (1973): *The Excretory Function of Bile. The Elimination of Drugs and Toxic Substances in Bile*. Chapman & Hall, London.
100. Solomon, S. (1977): Developmental changes in nephron number, proximal tubular length and superficial glomerular filtration rate of rats. *J. Physiol. (Lond.)*, 272:573–589.
101. Spurling, N.W., and Carey, P.F. (1992): Dose selection for toxicity studies: A protocol for determining the maximum repeatable dose. *Human Exp. Toxicol*., 11:449–457.
102. Strong, H.A., Renwick, A.G., and George, C.F. (1984): The site of reduction of sulphinpyrazone in the rabbit. *Xenobiotica*, 14:815–826.
103. Su, K.S.E., Campanale, K.M., and Gries, C.L. (1984): Nasal drug delivery system of a quaternary ammonium compound: Clofilium tosylate. *J. Pharm. Sci*., 73:1251–1254.
104. Sweatman, T.W., and Renwick, A.G. (1979): Saccharin metabolism and tumorigenicity. *Science*, 205:1019–1020.
105. Sweatman, T.W., and Renwick, A.G. (1980): The tissue distribution and pharmacokinetics of saccharin in the rat. *Toxicol. Appl. Pharmacol*., 55:18–31.
106. Taylor, A.E., and Gaar, K.A. (1970): Estimation of equivalent pore radii of pulmonary capillary and alveolar membranes. *Am. J. Physiol*., 218:1133.
107. Tembe, E.A., Honeywell, R., Buss, N.E., and Renwick, A.G. (1996): All-trans-retinoic acid in maternal plasma and teratogenicity in rats and rabbits. *Toxicol. Appl. Pharmacol*., 141:456–172.
108. Travis, C.C., Quillen, J.L., and Arms, A.D. (1990): Pharmacokinetics of benzene. *Toxicol. Appl. Pharmacol*., 102:400–420.
109. Upton, R.A. (1975): Simple and reliable method for serial sampling of blood from rats. *J. Pharm. Sci*., 61:112–114.
110. Veng-Pedersen, P., and Gillespie, W. (1985): The mean residence time of drugs in the systemic circulation. *J. Pharm. Sci*., 74:791–792.
111. Wagner, J.G. (1975): Do you need a pharmacokinetic model and, if so, which one? *J. Pharmacokinet Biopharm*., 3:457–478.

112. Wagner, J.G. (1975): *Fundamentals of Clinical Pharmacokinetics.* Drug Intelligence Publications, Hamilton, Illinois.
113. Wang, X., Santostefano, M.J., Evans, M.V., Richardson, V.M., Diliberto, J.J., and Birnbaum, L.S. (1997): Determination of parameters responsible for pharmacokinetic behavior of TCDD in female Sprague-Dawley rats. *Toxicol. Appl. Pharmacol.,* 147:151–168.
114. Waynforth, H.B. (1980): *Experimental and Surgical Technique in the Rat.* Academic Press, New York.
115. Weiner, I.M. (1967): Mechanisms of drug absorption and excretion: The renal excretion of drugs and related compounds. *Annu. Rev. Pharmacol.,* 7:39–56.
116. Weiner, I.M. (1971): Excretion of drugs by the kidney. In: *Handbook of Experimental Pharmacology,* vol. 28, edited by B.B. Brodie and J.R.Gillette, pp. 329–353. Springer-Verlag, New York.
117. Wilkinson, G.R. (1976): Pharmacokinetics in disease states modifying body perfusion. In: *The Effect of Disease States on Drug Pharmacokinetics*, edited by L.Z.Benet, pp. 13–32. American Pharmaceutical Association, Academy of Pharmaceutical Sciences, Washington, DC.
118. Wilkinson, G.R. (1987): Clearance approaches in pharmacology. *Pharmacol. Rev.,* 39:1–47.
119. Withey, J.R. (1978): Pharmacokinetic principles. In: *Proceedings of the First International Congress on Toxicology. Toxicology as a Predictive Science*, edited by G.L.Plaa and W.A.M.Duncan, pp. 97–117. Academic Press, New York.
120. Worboys, P.D., Bradbury, A., and Houston, J.B. (1994): Kinetics of drug metabolism in rat liver slices. Rates of oxidation of ethoxycoumarin and tolbutamide, examples of high- and low-clearance compounds. *Drug Metab. Disp.,* 23:393–397.
121. Worboys, P.D., Bradbury, A., and Houston, J.B. (1996): Kinetics of drug metabolism in rat liver slices. II. Comparison of clearance by liver slices and freshly isolated hepatocytes. *Drug Metab. Disp.,* 24:676–681.
122. World Health Organization (1986): Principles of toxicokinetic studies. In: *Environmental Health Criteria*, Vol. 57. WHO, Geneva.
123. World Health Organization (1987): Principles for the safety assessment of food additives and contaminants in food. In: *Environmental Health Criteria,* Vol. 70. WHO, Geneva.
124. Yacobi, A., Skelly, J.P., and Batra, V.K. (eds.) (1989): *Toxicokinetics and New Drug Development.* Pergamon Press, New York.
125. Zhang, L., Brett, C.M., and Giacomini, K.M. (1998): Role of organic cation transporters in drug absorption and elimination. *Annu. Rev. Pharmacol. Toxicol.,* 38:431–460.

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Chapter **5**

Physiologically Based Pharmacokinetic Modeling in Toxicology

Kannan Krishnan and Melvin E.Andersen

Principles and Methods of Toxicology,
Fourth Edition, edited by A.Wallace Hayes.
Taylor & Francis, Philadelphia © 2001.

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Pharmacokinetic modeling deals with the development of mathematical descriptions of the time-course of chemical concentration in biota. The temporal change in the concentration of a chemical in blood and tissues of an exposed organism is the net result of its absorption, distribution, metabolism, and excretion. Two commonly used compartmental pharmacokinetic models are:

(a) data-based, and
(b) physiologically based.

The data-based pharmacokinetic models correspond to mathematical equations that describe the available data on the temporal change in the blood or tissue concentration of a chemical in the animal species of interest (see the chapter by Renwick). This procedure considers the organism as a single homogeneous compartment or as a multi-compartmental system with elimination occurring in specific compartments of the model (106, 276). The number, behavior, and volume of these hypothetical compartments are estimated by the type of equation chosen to describe the data, and not necessarily by the physiological characteristics of the organism in which the blood/tissue concentration data were acquired.

These data-based pharmacokinetic models can be used for interpolation, but they should not be used for extrapolation outside the range of doses, exposure routes, and species used to generate data for constructing these models. To use the data-based models to describe the pharmacokinetic behavior of a chemical administered at various doses by different routes, extensive animal experimentation would be required to generate similar blood-time course data under respective conditions. Even within the same species of animal, the time-dependent nature of critical biological determinants of disposition (e.g., tissue glutathione depletion and resynthesis) cannot easily be included or evaluated with the data-based pharmacokinetic modeling approach. Further, due to the lack of actual anatomical, physiological, and biochemical realism, these data-based compartmental models cannot easily be used in interspecies

extrapolation, particularly to predict pharmacokinetic behavior of chemicals in humans. These various extrapolations, which are essential for the conduct of dose-response assessment of chemicals, can be performed more confidently with a physiologically based pharmacokinetic modeling approach. This chapter presents the basic

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principles and methods of physiologically based pharmacokinetic (PBPK) modeling as applied to the study of toxicologically important chemicals.

PBPK modeling refers to the development of mathematical descriptions of the uptake and disposition of chemicals based on quantitative interrelations among the critical biological determinants of these processes. These determinants include partition coefficients, rates of biochemical reactions, and physiological characteristics of the animal species. The biological and mechanistic bases of the PBPK models enable them to be used, with limited animal experimentation, for extrapolation of the kinetic behavior of chemicals from high dose to low dose, from one exposure route to another, and from test animal species to people.

The development of PBPK models for volatile and gaseous anesthetics dates back to the research work of Haggard (112) who mathematically described the uptake of inhaled diethyl ether from a physiological perspective. Further developments in PBPK modeling with vapors were contributed by Kety (136) and Riggs (233), who provided mathematical descriptions of the kinetics of chemicals in the body based on parameters such as blood flow rates, tissue volumes, and chemical partitioning into tissues, and by Mapleson (178), who developed PBPK models for inert gases utilizing an electric analog. This electric analog approach was expanded by Fiserova-Bergerova (80) for describing the pharmacokinetic behavior of metabolized vapors and gases relying on numerical integration of mass balance equations. In the pharmaceutics area, PBPK modeling traces back to Teorell's pioneering work in the 1930s (258, 259). Beginning in the early 1960s, scientists trained in chemical engineering also developed PBPK models of various drugs, particularly antineoplastic agents such as methotrexate, 5-fluorouracil, and cisplatin (18, 42, 77). Subsequently, the PBPK modeling approach has found extensive application in toxicology, particularly for conducting various extrapolations essential for the dose-response assessment of chemicals.

The development of PBPK models is initiated in four interconnected steps: model representation, model parameterization, model simulation, and model validation (Figure 5.1). *Model representation* involves the development of conceptual and mathematical descriptions of the relevant compartments of the animal as well as the exposure and metabolic pathways of the chemical. *Model parameterization* involves obtaining independent measures of the mechanistic determinants, such as physiological, physicochemical, and biochemical parameters, which are included in one or more of the PBPK model equations. *Model simulation* involves the prediction of the uptake and disposition of a chemical for defined exposure scenarios, using a numerical integration algorithm, a software program, and a computer. The

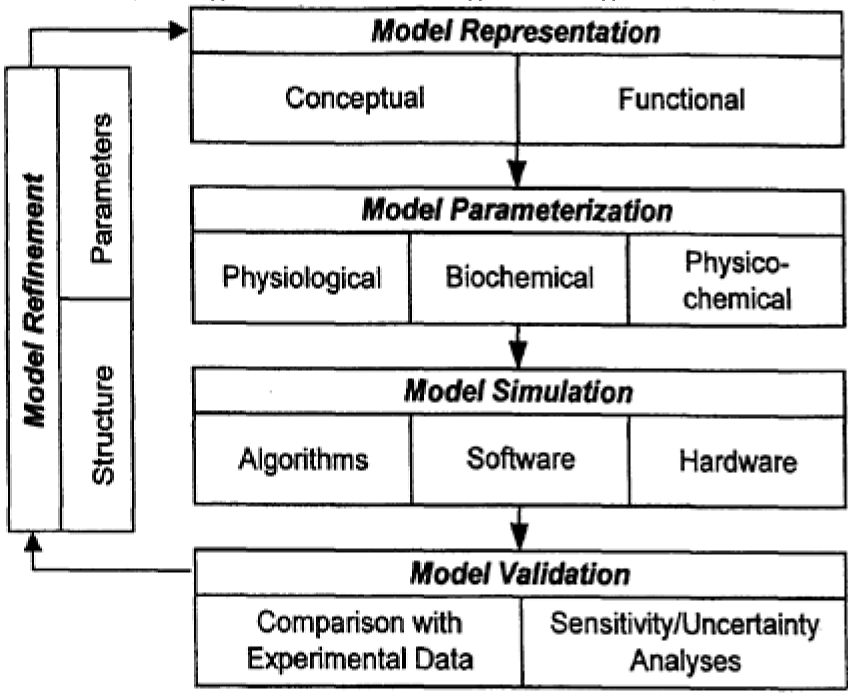

FIG. 5.1. Schematic of the steps involved in the development of physiologically based pharmacokinetic models.

model validation step involves the comparison of the a priori predictions of the PBPK model with experimental data to refute, validate, or refine the model description. PBPK models after appropriate

testing or refinement and validation can be used to conduct extrapolations of the pharmacokinetic behavior of chemicals from one exposure route/scenario to another, from high dose to low dose, and from one species to another.

The PBPK model development for a chemical is preceded by the definition of the problem, which, in toxicology, may often be related to the apparently complex nature of toxicity. Examples of such apparently complex toxic responses include non-linearity in dose-response, sex/species differences in tissue response, differential response of tissues to chemical exposure, qualitatively and/or quantitatively different responses for the same cumulative dose administered by different routes/scenarios, and so on. In these instances, PBPK modeling studies can be utilized to evaluate the pharmacokinetic basis of the apparent complex nature of toxicity induced by the chemical. One of the values of PBPK modeling, in fact, is that accurate description of target tissue dose often resolves behavior that appears complex at the administered dose level.

The problem identification step is followed by the specification of the goal(s) of the PBPK modeling effort. At this stage, the integrated model development process begins with the model representation step.

MODEL REPRESENTATION

Model representation refers to the development of conceptual (i.e., diagrammatic) and mathematical des

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criptions of the relationships among system elements as they relate to system response of interest (e.g., tissue dose).

Conceptual Representation

This step involves the diagrammatic representation of the relevant anatomical and physiological features of the organism, and the uptake and disposition pathways of the chemical. The organism is represented as a network of compartments, each of which is physically, physiologically, and biochemically characterized. The pathways of uptake and disposition of chemicals are indicated by adding arrows to the appropriate compartments in the conceptual representation of the PBPK model. The conceptual representation of the PBPK model for a chemical requires an understanding of the anatomical and physiological characteristics of the test animal species, and the pathways of uptake and disposition of the chemical under study such that both the animal and the chemical can be represented adequately.

Representing the Animal

The diagrammatic representation of the organism (e.g., rat) should correspond to the real system; in other words, it should clearly show how the relevant individual compartments are placed and interconnected in the test organism (Figure 5.2).

In representing the animal system, the organism as a whole in terms of its body weight (e.g., 250 g for a rat) should be accounted for, so the mass balance of chemicals can be accurately maintained. More precisely, maintaining the mass balance in PBPK models requires that the total blood flow (i.e., cardiac output) in the model be equal to the sum of the flows to the tissue compartments of the model. In other words, the tissues that receive the chemical via blood flow need only be represented in the model. Further, it is not necessary to represent these tissues as individual compartments, and they may be lumped together as long as the total flow in the model is accounted for. The simplest conceptual model may consider the organism as a one-compartment system, whereas there is virtually no limit to the number of compartments in larger systems with more detailed representation of events at the cellular/molecular levels.

In addition, the necessity for representing a particular tissue as a separate compartment in a PBPK model is determined by whether its

(a) chemical,
(b) biochemical,
(c) physiological, and/or
(d) anatomical characteristics contribute significantly to the uptake and disposition of the chemical being modeled.

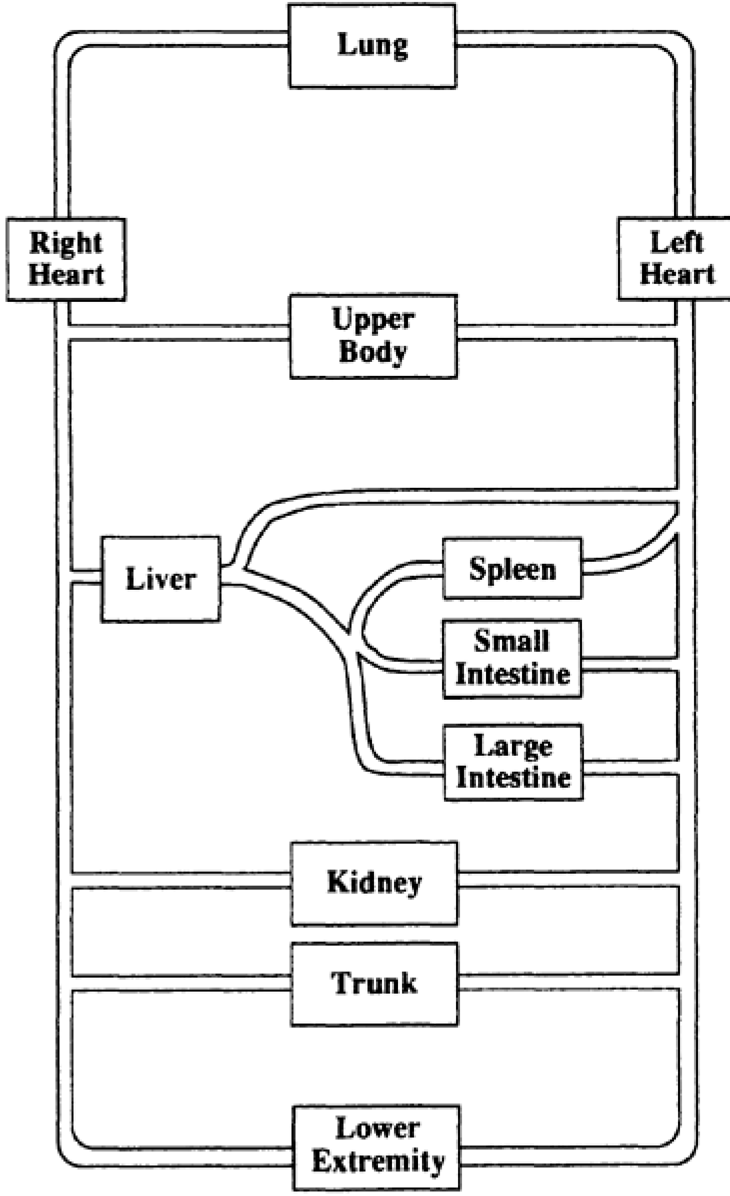

FIG. 5.2 Flow diagram for mammals. Adapted with permission from Reference 17.

The chemical composition of tissues in the present context refers primarily to the water and non-polar lipid contents. These tissue constituents are non-reactive but account for the differential solubility of a chemical among various tissues. In this respect, the adipose tissue is represented as a separate compartment in many PBPK models (Figure 5.3; fat) because of its ability to sequester lipophilic chemicals during exposure and release them after the cessation of exposure. In the case of a hydrophilic chemical, adipose tissue may be combined with the rest of the body since it may not show any particular kinetic behavior that is unique and different from the rest of the body. If other chemical components of the tissue (e.g., chloride levels or pH) are critical determinants of disposition, then individual or groups of tissue compartments defined with this kind of information should be included in the model as necessary.

The biochemical characteristics, in the present context, refer primarily to the binding and metabolizing capacities of the tissues. These properties account primarily for the removal of chemicals from the circulating blood by mechanisms other than chemical partitioning. For

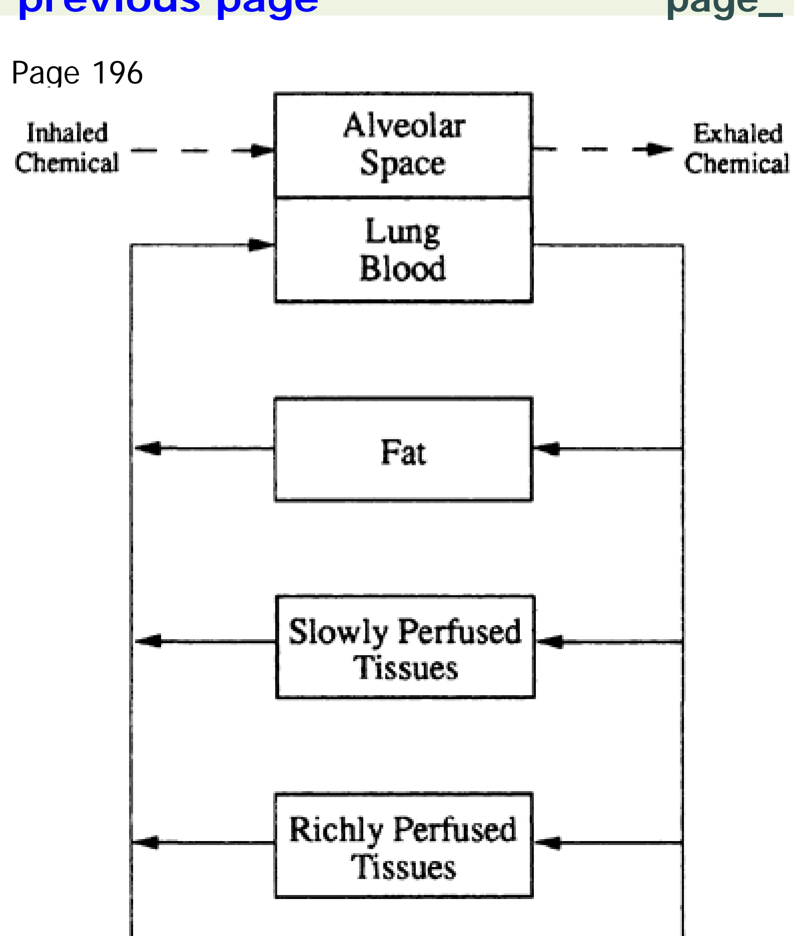

FIG. 5.3. Conceptual representation of a physiologically based pharmacokinetic model for styrene. Adapted with permission from Reference 223.

example, liver is often represented as a compartment in the PBPK models because of its central role in the metabolism of many organic chemicals. Representation of other tissues as separate compartments may be required according to the extent of expression of specific enzyme activities of relevance to the metabolism of the chemical being modeled (e.g., P450 and glutathione S-transferase in lung or kidney, epoxide hydrolase in testis, or myeloperoxidase in bone marrow).

The physiological characteristics refer to breathing rate, cardiac output, glomerular filtration rate, tissue blood flow rates, and so on. These characteristics essentially determine the biodisposition of chemicals. The tissue compartments possessing these properties (i.e., lung, heart, and kidney) or a quantitative description of these physiological processes should be included in the PBPK model. Respiration, urinary excretion, and blood circulation often are represented as quantitative descriptions of the processes themselves. Depending on the proposed use of the model, the tissues involved may be represented individually and characterized for particular aspects. For example, lung is represented as both an uptake and a metabolizing tissue in PBPK models for cer

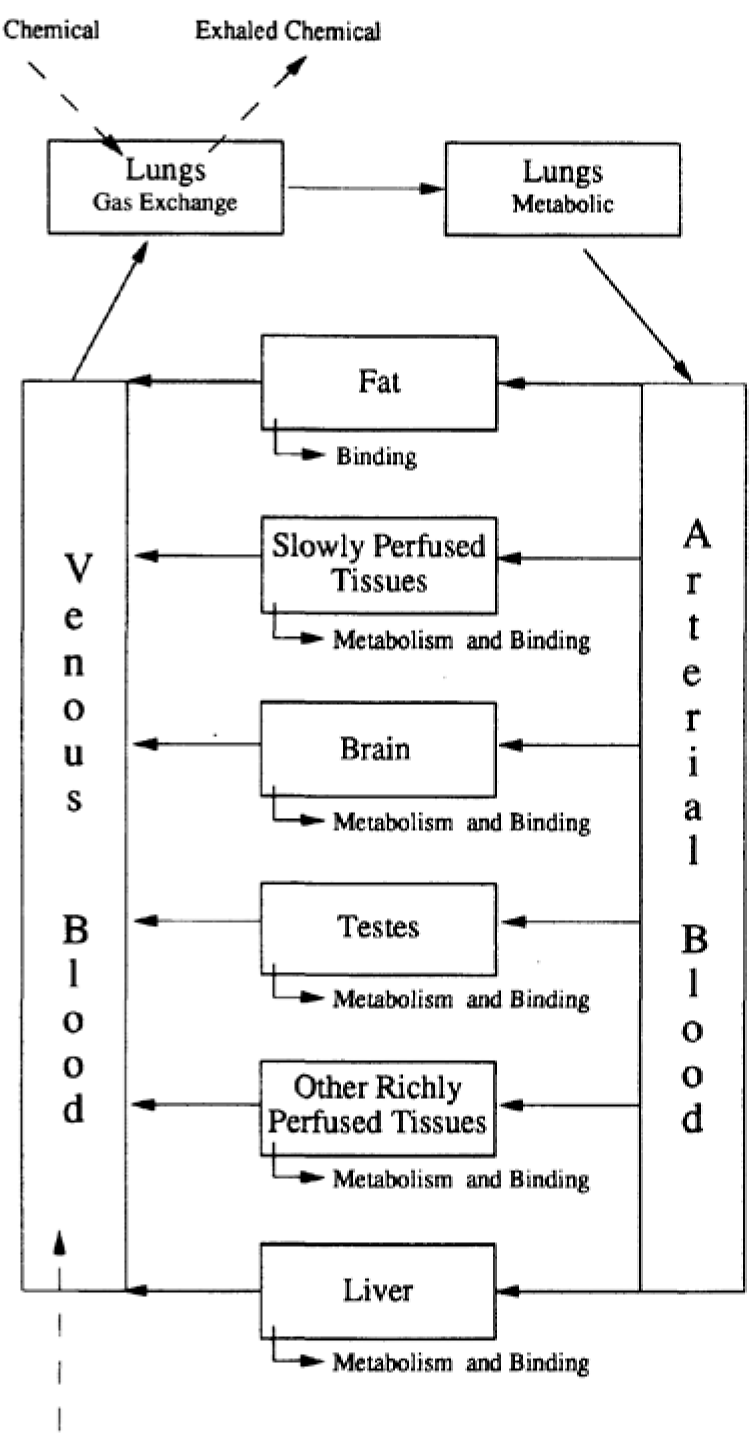

FIG. 5.4. Conceptual representation of a physiologically based pharmacokinetic model for ethylene oxide. Adapted with permission from Reference 151.

tain volatile organics (Figure 5.4), whereas it is not characterized separately in the case of nonvolatile organics which are neither eliminated by exhalation nor metabolized significantly by this tissue (Figure 5.5).

The anatomical location of certain tissues makes them particularly important for the uptake and elimination of chemicals. Lung, skin, and gastrointestinal tract serve as portals of entry for chemicals. According to their relevance and relative importance to the pharmacokinetics of a chemical, they should be included in PBPK descriptions. Target organs are included as separate compartments.

In principle, if the characteristic time constants of tissue disposition (i.e., the product of partition coefficient

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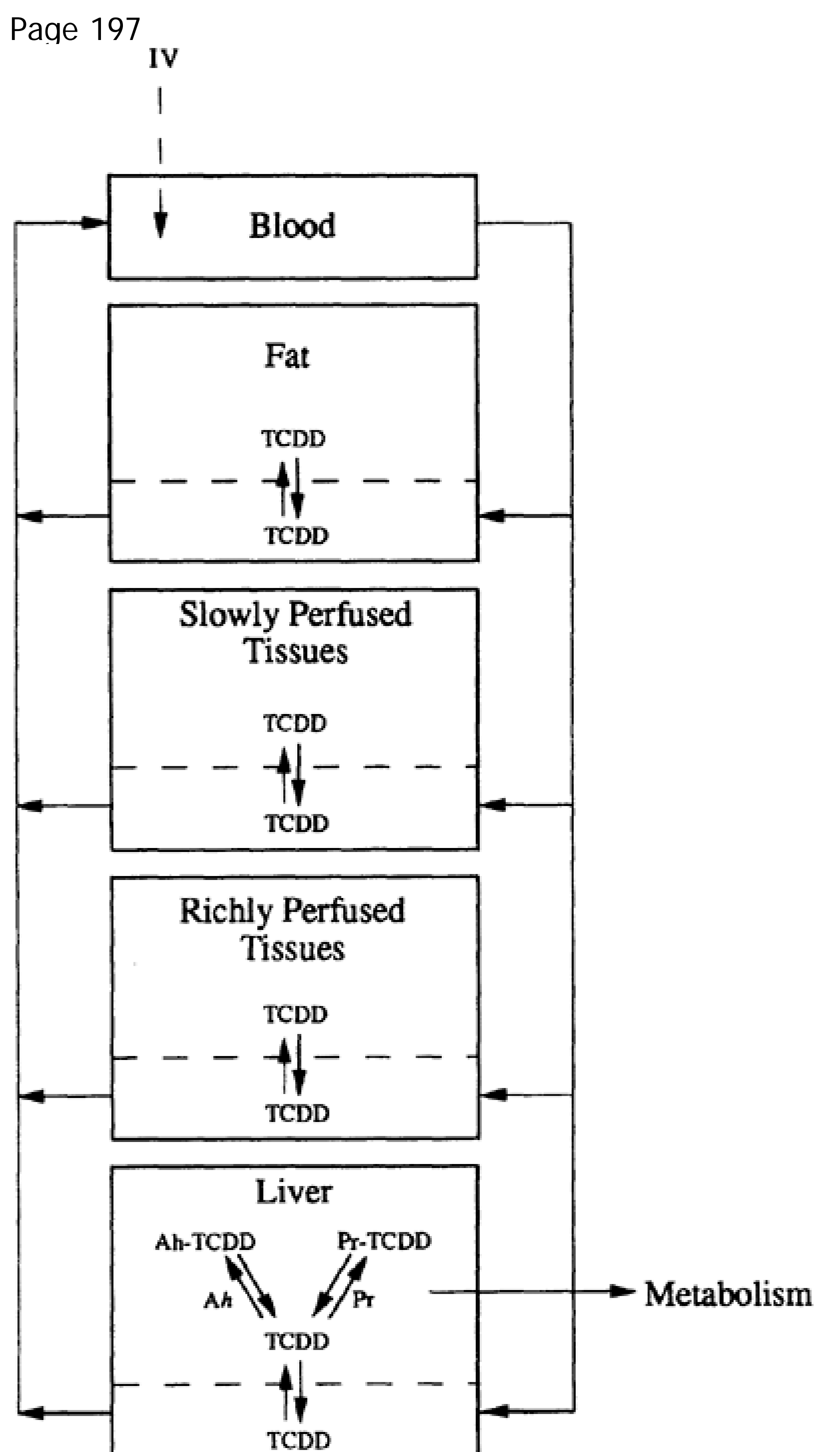

FIG. 5.5. Conceptual representation of a physiologically based pharmacokinetic model for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Pr, protein; Ah, Ah receptor. Adapted with permission from Reference 9.

and volume divided by blood flow rate) are similar for various tissues, they can be lumped together to form a single tissue group. In other words, when the critical determinants of pharmacokinetics do not vary quantitatively among several tissues, the time course of the chemical concentration in these tissues will be similar. That is why fat depots such as perirenal, epididymal, and omental fat are frequently grouped and represented as a single "fat" compartment (Figures 5.3–5.5). If necessary, a fat compartment may be subdivided into two or more groups according to the perfusion rates (e.g., inner and subcutaneous adipose tissues). Another example of this kind involves tissues such as adrenal, kidney, thyroid, brain, lung, heart, testis, and hepatoportal system, which often are pooled into one compartment and referred to as "richly perfused tissues" (Figures 5.3 and 5.5). When the contents of

relevant metabolizing enzymes quantitatively differ among the richly perfused tissues, the individual organs are represented as separate compartments even though the blood flow rate and solubility characteristics are somewhat similar (Figure 5.4). Tissues with poor blood perfusion characteristics (muscle, skin) are frequently grouped as "slowly perfused tissues." Other groupings of tissues based on perfusion characteristics can also be defined, but this has not been done routinely.

Since the skeletal and structural components of the body have only a negligible perfusion and do not play a significant role in the disposition of many organic chemicals, they have not been included in the PBPK model descriptions for these chemicals. When describing certain metals and metalloids stored in bone, inclusion of this compartment is essential.

In PBPK models for organic and inorganic chemicals that are not stored to a significant extent in the skeletal/structural components of the body, approximately 91% of the body weight is represented by the tissue compartments included in the models (100% body weight minus 9% skeletal/structural component weight). In other words, 91% of the body weight is fractionated into the four, five, or nine compartments included in the models presented in Figures 5.3–5.5. In some models, blood is not described as a separate compartment (Figure 5.3 vs. Figure 5.4) even though blood concentrations are calculated. When blood is not described as a separate compartment, its total volume is apportioned among the tissues implicitly or explicitly.

Although PBPK models are mechanistically based and more detailed than the classical data-based pharmacokinetic models, they still represent a significant simplification of the true complexities of the biological systems. Model complexity and the number of compartments should not be equated with accuracy and usefulness of the model description; oftentimes model complexity yields a multitude of parameters to be estimated and greater uncertainty of the model description. Parsimony in PBPK modeling, on the other hand, refers to the choice of a model structure that has minimal but necessary elements that together adequately describe the pharmacokinetics of a chemical.

Representing the Chemical

The pathways of uptake, distribution, metabolism, and excretion of the chemical should also be conceptually represented in the PBPK model. The pathways of uptake are indicated by adding arrows to appropriate model compartments for representing the port of entry for each exposure route of interest (Figures 5.3–5.5). The interconnections among the individual tissue com

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partments of the conceptual model serve to represent the working hypothesis of the researcher regarding the distribution of the chemical in the organism. With respect to binding and metabolism, the specific pathways in each of the relevant tissue compartments should be identified/hypothesized so changes in parent chemical concentration and the formation and/or tissue distribution of the metabolite(s) can be followed. Even though there is not much uncertainty regarding the mechanistic basis of the absorption and distribution of chemicals, there are larger uncertainties regarding the importance, basis, and magnitude of specific aspects of binding to tissue macromolecules, metabolic pathways, and extra-hepatic metabolism. In such cases, the extent of metabolism or binding by a particular pathway may be included in the model structure and verified by specific model-directed experiments.

Once the tissue compartments of the animal and the pathways of uptake and disposition of a chemical are identified/hypothesized and conceptually represented, mathematical descriptions of pharmacokinetic processes are developed.

Mathematical Representation

This phase of the PBPK modeling process involves the development of mathematical representations of
(a) the quantitative interrelationships among the mechanistic determinants of the functions of each tissue compartment, and
(b) the interrelationships among the individual tissues.

The functional representation of a PBPK model requires a rudimentary knowledge of calculus. A brief review of basic mathematics and differential calculus required for pharmacokinetic modeling has been provided by O'Flaherty (192).

Prior to the development of mathematical descriptions of the various functions of the tissue compartments, it is essential to characterize them physicochemically, physiologically, and biochemically. The symbols and abbreviations that refer to these characteristics, in the mathematical representations of the PBPK models described in this chapter, are provided in Table 5.1.

In PBPK modeling, each tissue compartment is described with a mass balance differential equation (MBDE) that consists of a series of clearance terms as follows:

$$\frac{dA_t}{dt} = Cl_u C_a - Cl_e C_{vt} - Cl_m C_a - Cl_f C_a \qquad (1)$$

Dimensionally, the units for each of the clearance terms is flow per time, that is L/h or ml/min.

Table 5.1 Symbols and abbreviations used in the mathematical representations of PBPK models described in this chapter

Symbol or abbreviation	Description	
A	Amount (mg)	
	Subscripts representing the chemical	
	o	orally absorbed
	bm	bound to macromolecules
	bo	rest of the body
	i	liver
	met	metabolized
	sk	skin
	stom	remaining in the stomach
C	Concentration (mg/liter or mmol/liter)	
	Subscripts representing the chemical	
	a	arterial blood
	air	air contacting skin
	alv	end-alveolar air
	b	blood
	bo	rest of the body
	cm	cellular matrix
	f	fat
	inh	inhaled air
	1	liver
	r	richly perfused tissues
	s	slowly perfused tissues
	sk	skin

	t	tissue "t"
	tb	tissue blood
	v	mixed venous blood
	vbo	venous blood leaving the rest of the body
	vf	venous blood leaving fat
	vl	venous blood leaving liver
	vr	venous blood leaving richly perfused tissues
	vs	venous blood leaving slowly perfused tissues
	vsk	venous blood leaving skin
	vt	venous blood leaving tissue "t"
	Subscripts representing tissue components	
	cf	cofactor in tissue "t"
	hb	hemoglobin in blood
	prot	microsomal protein
Cl	Clearance (liters hr−1)	
	Subscripts	
	e	efflux clearance
	f	functional clearance
	int	intrinsic clearance
	m	metabolic clearance
	u	uptake clearance
D0	Oral dose of chemical (mg)	
E	Hepatic extraction ratio	
F	Tissue content (fraction of tissue weight)	
	Subscripts	
	lb	lipid in blood
	lt	lipid in tissue "t"
	nb	neutral lipid in blood
	nt	neutral lipid in tissue "t"
	nleb	neutral lipid equivalent in blood
	nlet	neutral lipid equivalent in tissue "t"
	pb	phospholipid in blood
	pt	phospholipid in tissue "t"
	wb	water in blood
	wt	water in tissue "t"
	web	water equivalent in blood
	wet	water equivalent in tissue "t"
Ftiss	Volume fraction of the tissue (g tissue/g body weight)	
k	Transfer constant (liters hr−1)	
K	Binding affinity constants or metabolic rate constants	
	Subscripts representing affinity	
	m	Michaelis–Menten affinity constant (mg/liter)

Symbol or abbreviation	Description
	Subscripts representing reaction rates
	f first-order metabolism (hr^{-1})
	o oral absorption (hr^{-1})
	p skin permeability constant (cm hr^{-1})
	s second-order metabolism (liters mg^{-1} hr^{-1})
K0	zero-order infusion constant (mg hr^{-1})
n	Number of binding sites/protein molecule
P	Partition coefficient
	Subscripts
	b blood:air
	l:a lipid:air
	o:a n-octanol:air
	o:w n-octanol:water
	s:a skin:air
	s:b skin:blood
	t tissue:blood
	t:a tissue:air
	w:a water:air
PAt	Permeation area cross product for tissue "t" (liters hr^{-1})
Q	Flow rate (liters hr^{-1}) *Subscripts*
	bo blood flow to rest of body
	c cardiac blood flow
	f blood flow to fat
	1 blood flow to liver
	p alveolar ventilation
	r blood flow to richly perfused tissues
	s blood flow to slowly perfused tissues
	sk blood flow to skin
	t blood flow to tissue "t"
S	Exposed skin surface area (cm^2)
S	Elapsed time (hr)
ss	Steady-state
	Apparent transport maximum of the carrier system (mg hr^{-1})
	Volume (liters)
	Subscripts
	alv alveolar
	b blood
	cm cellular matrix in a tissue
	t tissue "t"
	tb tissue blood
Vmax	Maximal velocity of enzymatic reaction (mg hr^{-1})

Of the various clearance processes described above, the basic process that applies to all PBPK model compartments is chemical uptake, that is, inter- and intra-tissue transfers of chemicals. The uptake of a chemical by a tissue from the blood is described according to Fick's law of simple diffusion, which states that the flux of a chemical is proportional to its concentration gradient:

$$\text{Flux} = \frac{dC_t}{dt} = k\Delta C \quad (2)$$

For high molecular weight compounds, diffusion is often the rate-limiting process; therefore, their uptake through the tissue sub-compartments (Figure 5.6) must be considered. This requires that tissue blood and cellular matrix be described separately.

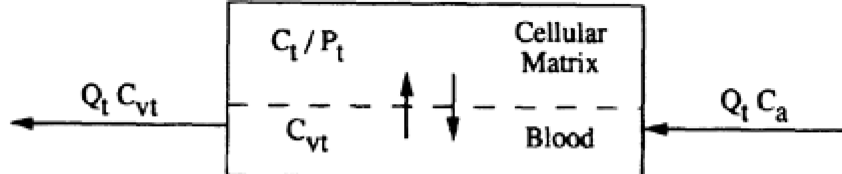

FIG. 5.6. Schematic of a tissue compartment. Qt is tissue blood flow rate, Ca is arterial blood concentration, Cvt is the concentration of the chemical in the venous blood leaving tissue, Ct is the tissue concentration, and Pt is the tissue-blood partition coefficient.

The rate of change in the amount of chemical in cellular matrix is equal to the product of the diffusion rate constant and the net flux from tissue blood:

$$V_{cm}\frac{dC_{cm}}{dt} = PA_t\left(C_{vt} - \frac{C_t}{P_t}\right) \tag{3}$$

The rate of change in the tissue blood sub-compartment equals the sum of the net retention from blood flow plus the net flux from cellular matrix:

$$V_{tb}\frac{dC_{tb}}{dt} = Q_t(C_a - C_{vt}) + PA_t\left(\frac{C_t}{P_t} - C_{vt}\right) \tag{4}$$

If the diffusion of a chemical from tissue blood to cellular matrix is slow with respect to total tissue blood flow, both equations are necessary. On the other hand, if tissue blood flow (i.e., perfusion) is slow with respect to diffusion, tissues are described as homogeneous, well-mixed compartments such that the rate of change in the amount of chemical in the tissue is described with a single equation for the whole tissue mass [cellular matrix plus tissue blood, i.e., Eq. (3) plus Eq. (4)] as follows:

$$V_t\frac{dC_t}{dt} = Q_t(C_a - C_{vt}) \tag{5}$$

In the perfusion-limited tissue descriptions, the transfer constant is the rate of blood flow to the compartment and the effluent venous blood concentration *(Cvt)* is in equilibrium with the tissue concentration *(Ct)* as specified by the tissue-blood partition coefficient *(Pt)* such that *Cvt=Ct/Pt*.

When a tissue included in a PBPK model contributes to the clearance of the chemical by metabolism and macromolecular binding, the MBDE for that tissue becomes:

$$V_t\frac{dC_t}{dt} = [Q_t(C_a - C_{vt})] - \frac{dA_{met}}{dt} - \frac{dA_{bm}}{dt} \tag{6}$$

Rate of change in the amount of the chemical in the tissue=(blood flow×arteriovenous concentration difference)—rate of loss due to metabolism—rate of loss due to macromolecular binding.

The rate of the amount of chemical consumed by macromolecular binding process has been calculated either as a second order reaction or using equations based on reversible equilibrium relationships. The rate of the amount metabolized can be described as a first order, second order, or a saturable process as follows:

$$\frac{dA_{met}}{dt} = K_f C_{vt} V_t \quad (7)$$

$$\frac{dA_{met}}{dt} = K_s C_{vt} V_t C_{cf} \quad (8)$$

$$\frac{dA_{met}}{dt} = \frac{V_{\max} C_{vt}}{K_m + C_{vt}} \quad (9)$$

Conjugation reactions are traditionally described as a second order process (Equation 5.8) with respect to the concentration of the cofactor and the chemical (70, 151). Alternatively, descriptions based on a ping-pong mechanism have also been used successfully (50).

The saturable [Eq. (9)] or first-order [Eq. (7)] metabolism descriptions presented above use the venous blood concentration, and appear to describe metabolism independent of the blood flow limitations. Once this equation becomes a part of Eq. (6), the blood flow limitation is accounted for. Alternatively, metabolism can be described using the following equation:

$$\frac{dA_{met}}{dt} = Q_l \times E \times C_a \quad (10)$$

Since $Ql \times E$=hepatic clearance (L/h), the above equation represents the classical way of calculating the amount of chemical metabolized from knowledge of hepatic clearance and arterial blood concentration. Since $Cv1 = Ca\,(1-E)$, and $Vmax/Km$ or $Clint = Q \times E/(1-E)$, Eqs. (9) and (10) are mathematically equivalent (219).

In several PBPK models, the rate of metabolism has been calculated using Eq. (9) which uses the venous blood concentrations of chemicals (i.e., $Cv1$). This is equivalent to the venous equilibration model for hepatic metabolism. Other types of physiological descriptions of liver metabolism include the parallel tube model and the distributed sinusoidal perfusion model (234, 235). The parallel tube model describes the flow of substrate through the sinusoids lined with enzymes, by considering them to be functionally homogeneous. On the contrary, however, the distributed sinusoidal model accounts for the functional heterogeneity among sinusoids by including statistical distributions of enzyme contents and sinusoidal blood flow (234). Although the distributed sinusoidal perfusion model is physiologically more realistic than the venous equilibration model, the latter simpler model may often be sufficient. Even though the predictions of tissue dose might vary in cases where the metabolizing organ alone is considered in isolation (237), the difference in predictions between these models might not be significant when considering the whole-body clearance of chemicals. A recent effort has produced a geometric multicompartmental description for liver, which can be used to simulate regional protein induction (2). The decision to use a multicompartmental liver depends on the objective and intended use of the model.

In perfusion-limited PBPK descriptions, the tissues that exhibit no significant capacity to bind or metabolize chemicals are described with the form of Eq. (5), and those tissues that exhibit significant binding and metabolic capacity are described as per Eq. (6). Thus, the basic form of equation representing chemical flux is the same for all tissues. In the case of metabolizing and eliminating tissues, however, additional terms are included to represent chemical loss due to specific biochemical processes. All tissue compartments receive the chemical via systemic arterial blood and lose the chemical via venous blood (Figures 5.3–5.5). The venous effluents of the various tissue compartments combine to yield a mixed venous concentration.

In PBPK models for volatile chemicals without a specific venous blood compartment, the mixed venous blood concentration has been calculated as follows (223):

$$C_v = \frac{\sum_t^n Q_t C_{vt}}{Q_c} \quad (11)$$

The above equation represents the steady-state solution of the MBDE for venous blood:

$$V_b(dC_b/dt) = \sum^{n} Q_t C_{vt} - C_v Q_c \quad (12)$$

The chemical in venous blood, on reaching the pulmon ary compartment, may be exhaled or retained and further introduced into systemic arterial blood along with new chemical that is inhaled during the passage via the lungs. In PBPK models for some volatile chemicals, arterial blood has not been represented as a separate compartment and described with a MBDE, but, instead, described with the steady-state solution of the MBDE for the combined lung tissue-alveolar air compartments (223) as follows:

$$C_a = \frac{Q_p C_{inh} + Q_c C_v}{Q_c + \left(\frac{Q_p}{P_b}\right)} \quad (13)$$

The above algebraic expression is derived from the following mass conservation equation for lung, which specifies that the loss of chemical from the air is balanced

by an identical gain of the chemical in the pulmonary blood:

$$Qp(Cinh-Calv)=Qc(Ca-Cv) \quad (14)$$

Since the lung equilibrates vapor between alveolar air and blood, $Calv=Ca/Pb$. This relationship assumes rapid equilibrium of the chemical across the alveolar walls, no significant metabolism by the lung tissue, and negligible storage capacity in the lungs. Pulmonary metabolism, in addition to uptake, can be included by describing both functions of the lung (151). The concentration of the chemical appearing in the systemic arterial blood is then affected by the pulmonary first-pass effects associated with metabolic processes (6, 151). The types of mathematical descriptions used for calculating the rate of uptake following oral, iv, and dermal administrations are presented elsewhere (see below).

It is not essential to have both (arterial and venous) blood compartments or the lung compartment to provide venous-to-arterial interconnections for the tissues. The linkage can be done as well without a lung or a separate blood compartment (223). In these cases, the blood volume is not specified explicitly in the model but distributed implicitly among the tissues (223). However, in such descriptions it is not possible to calculate the amount of chemical in the blood compartment. Therefore, it is important to formulate the questions to be answered with the model during the "problem identification" step so a desirable model structure can be chosen.

PBPK models are based on various assumptions. Some may be reasonable, others might be questionable. Their appropriateness and accuracy should be verified experimentally. The experimentally obtained mechanistic data can then be used to accept, replace, or modify the assumptions/empirical descriptions included in the model. Some of the more general assumptions that apply to many PBPK models are (232):

(a) the mixing of the chemical in the effluent blood from the tissues is instantaneous and complete;
(b) blood flow is unidirectional, constant, and non-pulsatile; and
(c) the flow of chemicals through the blood is smaller than the blood flow, with the former not adding appreciably to the total flow.

In basic PBPK models, certain processes are considered together and described in simple mathematical terms; however, simplified terms can later be replaced with specific mechanistic details as relevant information becomes available. The level of mechanistic detail in the model description conforms to the intended use of the model. PBPK models, then, are of varying complexities according to the particular model's intended purpose (2). A list of toxicologically important chemicals for which PBPK models have been developed in one or more species is provided in Table 5.2. The following paragraphs provide examples of prototypical representations employed in PBPK models for diverse groups of chemicals.

Examples of Model Representations

Organic Chemicals

Volatile organics, lipophilic. The tissue uptake of low molecular weight, non-polar, volatile organic chemicals is a perfusion-limited process, whereas the inhalational uptake may either be blood flow- or ventilation-limited (223). The basic mathematical representation that is applicable to many members of this category is provided in Figure 5.7. In this example, the model consists of four tissue compartments—liver, fat, richly perfused tissue group, and slowly perfused tissue group—similar to the conceptual representation shown in Figure 5.3. Here the chemical input to the model results from the inspiration of the chemical in the inhaled air at a flow rate equal to the alveolar ventilation rate. The chemical in alveolar air is assumed to equilibrate very rapidly with arterial blood so that the concentration of chemical in arterial blood and in alveolar air leaving the lungs maintains a constant ratio specified by the blood-air partition coefficient (PC). Arterial blood, flowing at a rate equal to the cardiac output, is apportioned among liver, fat, richly perfused tissue, and slowly perfused tissue. Venous blood leaving each tissue compartment mixes simultaneously to yield the chemical concentration in the mixed venous blood returning to the lungs at a flow rate equal to cardiac output. In this example, tissue uptake of the chemical is assumed to occur rapidly and metabolism is assumed to occur only in the liver by a single saturable process. It is entirely possible that extrahepatic metabolism is important and that there is a need to estimate the total amount of a chemical in the blood (151). In such cases, the metabolic capacity of each tissue included in the model is characterized, and blood is included as a separate compartment and described explicitly (151).

Volatile organics, hydrophilic. In the models presented above, pulmonary uptake is represented by assuming that all the chemicals disappearing from the inspired air appear in the arterial blood and that the chemicals in alveolar air and arterial blood are in instantaneous equilibrium. In these descriptions, the conducting airways (i.e., nasal passages, larynx, trachea, bronchi, and bronchioles) are considered inert tubes that carry the chemical to the pulmonary region, where diffusion occurs. There is mounting

evidence that this kind of a simple, continuous ventilation equilibration model is

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Table 5.2

Environmental chemicals for which PBPK models have been developed in different mammalian species

Chemicals	Species[a]	References
Organic chemicals		
Acetone	H	154, 126
Acrylic acid	R, H	88
Acrylonitrile & cyanoethylene oxide	R	96, 134
Aldicarb	H	202
Benzene	R, M, H	20, 21, 60, 83, 182, 239, 248, 266
Benzo(a)pyrene	R	237
Benzoic acid	R	175
Bromochloromethane	R	95, 99, 181
Bromodichloromethane	R	166
Bromotrifluoromethane	H	271
Butadiene (1,3-) and metabolites	R, M, H	23, 50, 113, 129, 130, 143, 159, 255
Butanol (2-)	R	66
Butanol (tertiary)	R	24
Butoxyacetic acid (2-)	R, H	44
Butoxyethanol (2-)	R, M, H	44, 46, 125, 160, 247
Carbon tetrachloride	R	95, 98, 201, 245, 264
Chlordecone	R	27
Chlorobenzene	H	153
Chloroethane	R	98
Chloroform	R, M, H	45, 98, 104, 227, 250, 251
Chloromethane	R	98
Chloropentafluorobenzene	R, M, H, Mk	40, 48
Cyclohexane	H	207
Dibromomethane	R	95, 181
Dichloroethane (1,1-)	R	98
Dichloroethane (1,2-)	R, M	98
Dichloroethane (1,2-)	R	70
Dichloroethylene (1,1-)	R	69, 98, 99
Dichloroethylene (*cis* 1,2-)	R	98
Dichloroethylene (*trans* 1,2-)	R	15, 98
Dichlorophenoxyacetic acid (2,4-)	R, Rb	139, 140
Dieldrin	R, H	169
Diethylether	R, H	95, 126
Difluoromethane	R	71, 99
Diisopropylfluorophosphate	R, M	103
Dioxane (1,4-)	R, M, H	161, 229
Dioxin, chlorinated & brominated	R, M, H, Mk	9, 135, 158, 162–164, 238, 240, 277
Ethyl acrylic acid	R	89
Ethylene dibromide	R, H	210
Ethylene dichloride	R	70
Ethylene oxide	R	151
Furans	R, M, H	133, 141
Chlorofluorohydrocarbons	R, M, H, Ha	14, 99, 171–173, 271, 273
Heptafluoropropane	H	271
Hexachlorobenzene	H	90, 282
Hexachloroethane	R	94
Hexanedione (2,5-)	H	205, 206
Isopropene	R, M, H	79

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Chemicals	Species[a]	References
Lindane	R	61
Methanol	R, M, H, Mk	117, 278
Methoxyacetic acid (2-)	M	36, 261
Methoxyethanol (2-)	M	36
Methyl chloroform	R, M, H	55, 93, 95, 157, 172, 230
Methyl *tertiary*-butyl ether	R	24
Methylene chloride	R, H	6, 8, 12, 98, 99, 181, 251
Methylethylketone	H	165
m-xylene	H, R	132, 156, 257
Naphtalene & naphtalene oxide	R, M	256
n-Hexane	H	205
Nicotine	R	211
Parathion	M	253
PCBs and PBBs	R, M, H, D, Mk	11, 174, 268, 269
p-chlorobenzotrifluoride	R, H	142
Pentachloroethane	R	94
Pentafluoroethane	H	271
Phthalate (diethylhexyl-)	R	137
Polychlorotrifluoroethylene	R	272
Pyrene	R	111
Styrene	R, M, H	8, 209, 223
Tetrachloroethane (1,1,1,2-)	R	94
Tetrachloroethane (1,1,2,2-)	R	94
Tetrachloroethylene	R, M, H, D	28, 29, 52, 53, 98, 144, 225, 229, 279
Tetrahydrofuran	H	68
Toluene	R, H	62, 156, 208, 257
Trichloroacetic acid	R, M, H	1, 82, 86, 87, 108
Trichloroethane (1,1,2-)	R	94, 93
Trichloroethylene and its metabolites	R, M, H	1, 15, 19, 54, 82, 85–87, 98, 108, 144, 157, 252
Trichloropropane (1,2,3-)	R	274
Trifluoroethane	R	172
Trifluoroiodomethane	H	271
Trimethylbenzene (1,2,4-)	H	124
Vinyl acetate	R	212
Vinyl chloride	R	15, 98
Vinyl fluoride	R	31
Inorganic chemicals		
Arsenic	R, H, M, Rb, Ha	176, 177, 283
Carbon dioxide	H	126
Carbon monoxide	R, H	5
Chromium	R	194
Fluoride	R, H	224
Lead	R	56, 193
Mercury, organic & inorganic	R, H, Mk	76, 102, 107
Nickel	R	183
Ozone	R, H, GP	185, 196
Zinc	R	123

[a]Rat (R), mice (M), human (H), monkey (Mk), dog (D), rabbit (Rb), hamster (Ha), guineapig (GP).

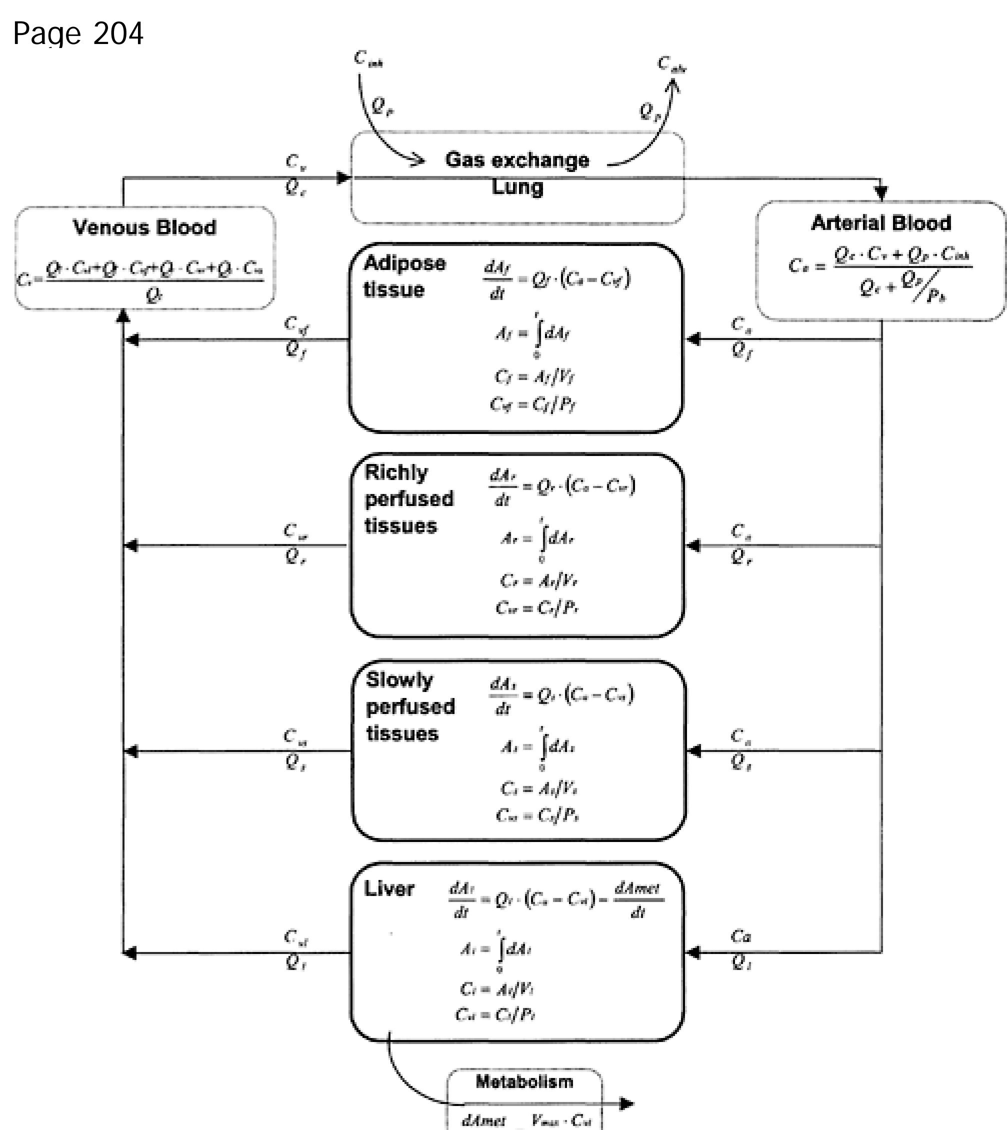

FIG. 5.7. A schematic of the PBPK model for styrene. In this model, the rat is represented as a four-compartment system interconnected by systemic circulation. The input for the system is the product of the inhaled concentration of styrene times the alveolar ventilation rate. The resulting arterial blood concentration is in turn provided as input to the tissue compartments, the effluent venous blood concentrations of which are provided as input for the calculation of mixed venous concentration. All abbreviations are defined in Table 5.1.

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not predictive of either total respiratory uptake or regional uptake of highly soluble polar solvents (126). With these chemicals there are complex relationships between uptake and the blood-air partition coefficient. Further, several studies have shown that the total respiratory uptake is less than 100 percent as predicted by the continuous ventilation equilibration model (126). It has been suggested that the reduced pulmonary uptake of polar solvents is due to their adsorption and/or dissolution in the surface of the respiratory epithelium during inhalation, and their desorption during exhalation (126). This adsorption-desorption mechanism is a consequence of both the aqueous solubility of the chemicals and the cyclic nature of respiratory exchange.

PBPK models for polar solvents, then, include a description of the adsorption of vapors during inhalation and desorption during exhalation in addition to accounting for the anatophysiological characteristics of the respiratory tract, blood flow rates, and partition coefficients of the chemical (105, 126). The PBPK model for polar solvents developed by Johanson (126) consists of nine serially connected central compartments, each one corresponding to an anatomical level of the respiratory tree. The first central compartment corresponds to the trachea and the last compartment to the alveolar region. Each of the central compartments corresponds to the airway and the outermost layer of mucus lining the airway wall. Radial diffusion of solvent from the outermost layer and deeper portions of the airway wall is accounted for by linking a peripheral compartment with each of the first eight compartments. The central compartment of the ninth and final region corresponds to the pulmonary or gas exchange region of the respiratory tract (respiratory bronchioles, alveolar ducts, and alveoli), the volume of which increases during inhalation and decreases during exhalation. The peripheral compartment of this ninth region represents the rest of the body, where immediate equilibrium between alveolar air and arterial blood is assumed. Either a single compartmental or a multi-compartmental physiological description can be used to account for chemical disposition in the body.

Nonvolatile organic chemicals, uncharged. PBPK models for nonvolatile organics describe chemical uptake as a diffusion-limited or a perfusion-limited process, and accommodate descriptions for chemical input via oral, dermal, intraperitoneal, and intravenous routes. These models typically consist of the following compartments: liver, fat, slowly perfused tissues, richly perfused tissues, and blood. Evidently, lung tissue or a description for pulmonary uptake is not included if its contribution to the overall kinetic behavior of the chemical is negligible (9). When there is evidence to the contrary, and, also, when there is evidence for pulmonary metabolism, lungs should be separated from the richly perfused tissues group and described as a separate compartment. In the case of diffusion-limited uptake description, each tissue compartment has a specified cellular matrix volume and tissue blood volume. The movement of a chemical from tissue blood into cellular matrix is described as being proportional to the permeation coefficient-surface area cross-product *(PAt)* for the tissue (t). Tissue uptake is diffusion-limited when $PAt<Qt$. Binding to tissue macromolecules and other clearance processes are described as appropriate (9, 135, 240).

Nonvolatile organic chemicals, charged species. The distribution of water-soluble, charged species, such as weak acids and weak bases, is determined primarily by the *pKa* of the compound and pH of the body fluids. The tissue uptake of these chemicals can be defined by the conventional flow-limited exchange between plasma/ blood and tissues, with partitioning being determined by the *pKa* of the chemical and the pH of the body fluids in accordance with the Henderson–Hasselbach equation (195). The elimination of these chemicals is mainly via urine, and therefore time-course information on this process may be necessary to adequately describe the kinetics of excretion.

Inorganic Chemicals

Gases and vapors. For inorganic gases that do not interact with walls of the conducting airways, simple dosimetry descriptions similar to those discussed in the preceding sections have been employed (5, 126). For reactive inorganic gases, however, simulation models should incorporate critical elements of local absorption in the lower respiration tract and reactions with the biological constituents (185, 196). Typically, these dosimetry models use quantitative information on the physiology of the lower respiratory tract, i.e., ventilation parameters and varying airway dimensions during breathing cycle. Lung dimensions are taken into account by making use of the airway models, in which airways of the lower respiratory tract are represented by a sequence of sets of right circular cylinders. All cylinders in series corresponding to a particular generation are of the same size. The upper respiratory tract (if used) consists of pre-tracheal generations or sequential segments. For each generation, the simulation model requires the specification of the number of airways or segments and their diameters and lengths. Additionally, for the pulmonary region, the alveolar volume and the surface area for each generation are needed. Similarly, the surface area of each upper respiratory segment is included.

The processes of transport and chemical reactions are described with a series of partial differential equations. In the liquid lining, tissue, and blood compartments, where only the processes of molecular diffusion and chemical reactions are considered, the form of the equation is the same. In the lumen of the airway and

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in the alveolar air spaces, axial convection, axial dispersion, the loss of chemical to the liquid lining, and lung expansion and contraction are taken into account. With quantitative information on the boundary conditions, initial conditions, and the physical, chemical, and biological parameters, these equations are solved to simulate dose and dose patterns. In this case, computational requirements are greater than those associated with the simpler PBPK models discussed above. More recently, a hybrid computational fluid dynamics and PBPK model has been constructed to estimate the regional tissue dose of organic acids in rodent and human nasal cavity (88).

Metals. The functional description used in PBPK models for metals is basically similar to that for organics; however, the common assumptions of flow-limited tissue uptake and linear partitioning into tissues may not be applicable for some metals (191). Further, the systemic uptake of metals may be mediated by ion channels or carrier-mediated transport mechanisms, and metabolism may be limited to oxidation state transitions and alkylation/dealkylation reactions. In the PBPK models for metals, the uptake has been described as a diffusionlimited process in some tissues and as a flow-limited process in others, to obtain adequate fitting of the model to experimental data (123). Clearance associated with binding to subcellular proteins occurs to a greater extent in the case of metals. Another important phenomenon associated with certain metals is their storage in bone. The metals for which PBPK models have been developed include arsenic, nickel, lead, chromium, zinc, and mercury (Table 5.2).

MODEL PARAMETERIZATION

Model parameterization refers to obtaining independent measures of the mechanistic determinants, namely, physiological parameters, physicochemical parameters, and biochemical rate constants, which are included in one or more of the PBPK model equations.

Physiological Parameters

Physiological parameters included in most PBPK models include alveolar ventilation rate, cardiac output, tissue blood flow rates, and tissue volumes. Additional parameters (e.g., tissue DNA levels, hematocrit) may be required in certain cases. The physiological parameters can generally be measured directly in the animal species of interest (33, 67, 120, 236, 244). For example, breathing rates can be measured with the use of a spirometer, plethysmograph, pneumotachograph, hotwire anemometer, or nonbreathing valves (179). Cardiac output has been determined from dye dilution curves using oximeters (65). Representative data from compilations of reference physiological parameters for laboratory animals and humans (13, 26, 57) are presented in Tables 5.3–5.5.

Physicochemical Parameters

The physicochemical parameters required for PBPK models refer primarily to the partition coefficients (PCs), which represent the relative distribution of a chemical between two phases at equilibrium. Partitioning between

Table 5.3 Reference physiological parameters for mice, rats, and humans[a]

Physiological parameters	Mouse	Rat	Human
Body weight (BW) (kg)	0.025	0.25	70.0
Tissue volume (fraction of BW)			
Liver	0.055	0.04	0.026
Fat	0.10	0.07	0.190
Richly perfused	0.05	0.05	0.05
Slowly perfused	0.70	0.75	0.62
Cardiac output (Q_c) (liters/min)	0.017	0.083	6.20
Tissue perfusion (fraction of Q_c)			
Liver	0.25	0.25	0.26
Fat	0.09	0.09	0.05
Richly perfused	0.51	0.51	0.44
Slowly perfused	0.15	0.15	0.25
Minute volume (liters/min)	0.037	0.174	7.50
Alveolar ventilation (liters/min)	0.025	0.117	5.00

[a]Reproduced with permission from Reference 265.

Table 5.4 Range of plausible values of the volume and perfusion of selected tissues in mice (26)

Tissue	Volume (% body weight)		Regional blood flow (% cardiac output)	
	Mean	Range	Mean	Range
Adipose	7.0	5–14[a]		
Brain	1.7	1.35–2.03	3.3	3.1–3.5
Heart	0.5	0.4–0.6	6.6	5.9–7.2

Kidneys	1.7	1.35–1.88	9.1	7.0–11.1
Liver	5.5	4.19–7.98	16.1	
Lungs	0.7	0.66–0.86	0.5	
Muscle	38.4	35.8–39.9	15.9	12.2–19.6
Skin	16.5	15.9–20.8	5.8	3.3–8.3

[a]Varies proportionately with body weight.

Table 5.5

Range of plausible values of the volume and perfusion of selected tissues in the rat (26)

Tissue	Volume (% body weight)		Regional blood flow (% cardiac output)	
	Mean	Range	Mean	Range
Adipose	7.0	4.6–12.0 *a*	7.0	
Brain	0.6	0.38–0.83	2.0	1.5–2.6
Heart	0.3	0.27–0.40	5.1	4.5–5.1
Kidneys	0.7	0.49–0.91	14.1	9.5–19.0
Liver	3.4	2.14–5.16	18.3	13.1–22.1
Lungs	0.5	0.37–0.61	2.1	1.1–17.8
Muscle	40.4	35.4–45.5	27.8	
Skin	19.0	15.8–23.6	5.8	

*a*Varies proportionately with body weight.

two media (e.g., blood and air) as described by Henry's law for gases is a balance of the solubility of a chemical in the two media. PCs are represented as the ratio of the concentration of a chemical in the two media (e.g., blood:air, tissue:blood) at equilibrium. These physicochemical parameters are necessary to describe the tissue distribution of most uncharged xenobiotics as well as the pulmonary uptake of volatile organic chemicals. Several in vitro, in vivo, and animal replacement methods are available for estimating the PCs of chemicals.

In Vitro Methods

Vial equilibration. This in vitro method invol ves comparison of the equilibrium concentration of a chemical in the headspace of test vials containing tissues with empty/reference vials (81, 97, 100, 127, 242). The experimental procedure for determining the tissue: air and blood: air PCs of volatile organic chemicals by vial equilibration is given below:

1. Prepare a batch of 16 glass vials (volume ~25 ml) stoppered with Teflon septa. Transfer a measured quantity of raw ground tissue (e.g., 200 mg) or blood (e.g., 200 *µ*l) into even-numbered vials. The odd-numbered empty vials are used as reference vials. All vials are placed in a shaker-incubator at 37°C.
2. Aerate the vials after a 5-min initial equilibration at 37°C, and cap them again.
3. Remove a predetermined volume of air (e.g., 0.5 or 1.0 ml) from each vial individually with a gas-tight syringe inserted through the septa, and replace it with an equal volume of air containing a known quantity of the chemical.
4. Draw a sample of headspace atmosphere (e.g., 1.0 ml) from one set of eight individual vials (four reference+four sample) at either of the sampling time points (e.g., 1 h and 2 hr).
5. The tissue:air (or blood:air) PCs is calculated as follows (97):

$$P_{t:a} = \frac{(C_{ref} V_{ref}) - [C_{sam}(V_{ref} - V_{sam})]}{C_{sam} V_{sam}} \quad (15)$$

where C_{ref}=chemical concentration in the headspace of reference vial, C_{sam}=chemical concentration in the headspace of sample vial, V_{ref}= volume of the reference vial, and V_{sam}=volume of sample.

If the $P_{t:a}$ values obtained at both time points are not significantly different from each other, they can be averaged and standard deviations calculated. If the $P_{t:a}$ values obtained at the later time point are significantly different from those obtained at the earlier time point, they are not considered to represent a true measure of solubility. An increase in $P_{t:a}$ values with incubation time indicates chemical reactions in the aqueous phase, and/or the non-attainment of equilibrium during the time points chosen for sampling. In such cases, additional experiments have been conducted to generate a time course of the distribution ratio to estimate $P_{t:a}$ by extrapolating to time 0 (151). Metabolic inhibitors may also be added in excess before introducing the chemical. The latter approach has been shown to eliminate chemical uptake by tissues due to specific and known reactions (e.g., glutathione conjugation) (89, 103). On the other hand, if the calculated $P_{t:a}$ value decreases with incubation time, then deterioration of the tissue sample during incubation is likely; in such cases, headspace sampling for the determination of PCs should be conducted at earlier time points.

The tissue and blood samples do not have to be used as raw preparations in these experiments. Especially for chemicals with low solubility, the tissue and blood samples can be prepared as a 1:2 or 1:3 homogenate in saline (0.9% NaCl). The corresponding reference vials will contain saline alone. The $P_{t:a}$ value in this case is obtained using the following formula (97):

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$$P_{t:a} = [C_{ref}(V_{ref} - V_{liq})] \frac{-[C_{sam}(V_{ref} - V_{liq} - V_{sam})] + [(C_{ref} - C_{sam})(V_{liq}P_{liq})]}{C_{sam}V_{sam}} \quad (16)$$

where *Vliq*=volume of the diluent liquid and *Pliq*=diluent liquid-air PC.
PCs for nonvolatile chemicals have been determined in vitro using equilibrium dialysis or ultrafiltration techniques.

Equilibrium dialysis. In this technique, the cell cavities are separated with dialysis membrane of desired molecular weight specifications. The experimental procedure consists of dialyzing the tissue homogenate or whole blood prepared in a buffer (e.g., Tris HCl, 0.1 M, pH 7.4) against the same buffer in a metabolic shaking bath (118, 167, 254). These experiments are conducted for several different initial concentrations of the chemical (radiolabeled+cold). Preliminary studies using different dialysis durations should be conducted to determine the time needed to attain equilibrium. At the end of the incubation, radioactivity in both the tissue and buffer is determined separately by liquid scintillation counting. The sum of the buffer and tissue radioactivity should account for all the radioactivity initially added to the dialysis cells. The unbound fraction of radioactive chemical in the tissue is determined by dividing the chemical concentration in buffer by the concentration in the tissue homogenate (167, 168). The P*t* values obtained from these experiments will be accurate only if the ratio of the bound to free form of chemical is constant over a wide range of concentrations.

Ultrafiltration. The ultrafiltration assembly consists of an ultrafiltration device, and a semipermeable membrane. In this approach, the tissue homogenates are spiked with a known amount of a chemical (radiolabeled+cold) and allowed to equilibrate for a predetermined time. Following equilibration, they are transferred into the reservoir portion of the ultracentrifugation device, placed in an angle rotor, and spun in a superspeed centrifuge. The concentrations of the chemical in the tissue homogenate and the buffer are determined and solved for the concentration of the chemical in the tissue (167).

The time and speed of ultracentrifugation should be determined for particular cases so that a desired volume of the ultrafiltrate is collected. Untreated tissues should be used to generate a protein-free ultrafiltrate for measuring the nonspecific binding onto the surface of the ultrafiltration device.

In Vivo Methods

Methods for estimating *Pt* values based on the analysis of data on blood and tissue concentrations of parent chemical after a single-bolus dose, or at steady-state condition following constant intravenous infusion or administration by other routes, have been published (35, 91, 92, 155). *Pt* values have also been estimated from the slope of best-fit straight line with a unit slope drawn through the log-log plot of tissue concentration vs. blood concentration of the parent chemical for each tissue (59).

The steady-state approach will work only if the chemical is not removed by active binding/metabolic processes in one or more tissues. In this case where there is active tissue metabolism, the estimated *Pt* values tend to underestimate the true *Pt* values. Estimates of *Pt* for these tissues can be obtained if the amount of chemical consumed by the metabolic process is accounted for. Thus, for metabolizing tissues, the partition coefficients are determined after adjusting for clearance (35).

A potential problem associated with the determination of the PCs relates to the presence of residual blood in the tissues. The contamination of tissues with blood in the tissue vasculature might introduce errors in the estimated PCs. The importance of this problem has been investigated and the means of correcting these errors proposed (138).

Animal Replacement Approaches

Tissue: air partition coefficients. The partitioning of a chemical between two matrices can be predicted if its solubility and binding in each of the matrices can be estimated with reasonable accuracy. Using this basic premise, mechanistic animal replacement approaches for predicting tissue: air, blood: air, and tissue: blood PCs have been developed. Accordingly, the tissue: air PCs of low-molecular-weight VOCs, for which macromolecular binding is negligible, have been calculated as follows (74, 200):

$$Pt{:}a = (Pl{:}a \times Flt) + (Pw{:}a \times Fwt) \qquad (17)$$

In the above equation, $Pl{:}a \times Flt$ represents the partitioning of a chemical between the tissue lipids and air, and $Pw{:}a \times Fwt$ represents the partitioning between tissue aqueous phase and air. Po:a has been used as a predictor of *Pl*:a, and *Pw:a* as a surrogate of chemical partitioning between tissue water and air (74, 200). However, "tissue lipids" is too generic to represent the differential lipophilicity characteristics of neutral lipids (e.g., triglyceride) and polar lipids (e.g., phospholipid). Therefore, the partitioning of a chemical into neutral lipids and polar lipids may have to be considered separately. The physicochemical properties of phospholipids are dependent on the presence of a hydrophobic (e.g., glyceride) and hydrophilic (e.g., phosphomonoester) groups. Therefore, the use of *Po:a* or *Pw:a* alone cannot adequately predict tissue phospholipid:air PCs. The partitioning of a chemical between tissue polar lipids (i.e., phospholipids) and air can be calculated as a fractional additive function of their partitioning into neutral lipids ($0.3 \times Po{:}a$) and water ($0.7 \times Pw{:}a$). This approximation of chemical partitioning into tissue phospholipids is based on the assumption that the lipophilicity-hydrophilicity characteristics of tissue phospholipids is similar to that of commercial lecithin (220). Based on this working hypothesis, Poulin and Krishnan (216, 217) have proposed the following equation to predict

Pt:a of volatile organic chemicals, con-

sidering separately the partitioning of chemicals into neutral lipid and polar lipid portions:

$$P_{t:a} = (P_{o:a} \times F_{nt}) + (P_{o:a} \times 0.3F_{pt}) + (P_{w:a} \times 0.7F_{pt}) + P_{w:a} \times F_{wt} \quad (18)$$

The above equation can be rewritten as:

$$[Pt{:}a = Po{:}a(Fnt + 0.3Fpt)] + [Pw{:}a(Fwt + 0.7Fpt)] \quad (19)$$

In Eqs. (18) and (19), the partitioning of a chemical between tissue neutral lipids and air is assumed to correspond directly to *Po:a*, while the partitioning between tissue water and air is assumed to correspond to *Pw:a*. Accordingly, *Pt:a* can be calculated with knowledge of tissue composition data (*Fnt*, *Fpt*, *Fwt*), and physicochemical properties of chemicals (*Po:a* and *Pw:a*). Compilations of species-specific tissue composition data (72, 216, 217, 220), *Po:a* and *Pw:a* values of several VOCs at 37°C are available in the literature (97, 200). To facilitate the use of *Po:w* values instead of *Po:a* values which are not readily available in the literature, Equation 19 can be rewritten as follows:

$$Pt{:}a = [Po{:}wPw{:}a(Fnt + 0.3Fpt)] + [Pw{:}a(Fwt + 0.7Fpt)] \quad (20)$$

Eq. (20) has been used to predict rat and human *Pt:a* (liver, muscle, fat) of several alkanes, haloalkanes, and aromatic hydrocarbons (204, 216–219, 221). In general, the predicted *Pt:a* values were within a factor of two of the experimentally determined PCs. For chemicals such as alcohols, acetate esters, and ketones, the values of rat and human fat: air calculated using Eq. (20) differed substantially from the experimental data (214, 216, 217). These results have been explained by the choice of the surrogate of biotic lipid used in Eq. (20). n-Octanol, being an alcohol, would appear to solubilize other alcohols to a greater extent than biotic neutral lipids. Based on its hydrophilicity-lipophilicity characteristics and its fatty acid composition, vegetable oil has been suggested as an acceptable alternative to n-octanol as a surrogate of biotic neutral lipids, especially for hydrophilic organics (214). Then, to predict *Pt:a* of hydrophilic VOCs, especially for fatty tissues, *Po:w* in Eq. (16) should represent vegetable oil: water PCs. However, in the case of relatively lipophilic VOCs (log $Po{:}w > 1.25$), there is little difference between the n-octanol: water PCs and vegetable oil: water PCs. Therefore, either one of these PCs can be used as the biotic lipid surrogate for solving Eq. (20) to predict tissue: air PCs of these chemicals (217).

Blood: air partition coefficients. Based on Eq. (20), Poulin and Krishnan (215, 217) proposed the following equation for predicting *Pb* of VOCs that do not bind sig nificantly to blood proteins:

$$Pb = [Po{:}wPw{:}a(Fnlep)] + [Pw{:}a(Fweb)] \quad (21)$$

where *Fnlep*=neutral lipid equivalents, calculated as the sum of neutral lipids plus 0.3× phospholipid content, and Fweb=water equivalents, calculated as the sum of tissue water content plus 0.7× phospholipid content.

Accordingly, *Pb* of VOCs can be calculated with the knowledge of blood composition data, *Po:w* and *Pw:a*. The data on lipid and water levels in rat and human blood are available in the literature (215) and so are the numerical values of *Po:w* and *Pw:a* at 37°C for several VOCs (97, 127, 200) The predictions of rat *Pb* obtained using Eq. (21) are adequate for relatively hydrophilic organics (e.g., alcohols, ketones, acetate esters), but are not the case for relatively lipophilic organic chemicals (e.g., alkanes, haloalkanes, aromatic hydrocarbons). *Pb* of a chemical is a composite number that potentially represents two processes occurring in the blood, namely, solubility and binding. While chemical solubility is likely to be determined by the neutral lipid, phospholipid, and water contents in blood, the binding would appear to be associated with plasma proteins and/or hemoglobin. For alcohols, acetate esters, and ketones, rat and human *Pb* appear to be adequately predicted solubility-based algorithms [i.e., Eq. (21)] (215, 217). For more lipophilic VOCs (e.g., alkanes, haloalkanes, aromatic hydrocarbons), however, the rat *Pb* calculated using Eq. (21) were lower (60–80%) than the experimental data (215, 217). The fact that the rat *Pb* of lipophilic VOCs are under-predicted could be explained by the potential binding of these substances to blood proteins (215), a phenomenon not considered in Eq. (21). At the present time, there does not exist a validated animal replacement algorithm for predicting association constants for blood protein binding of organic chemicals.

Tissue:blood partition coefficients. Tissue:blood PCs of VOCs, for which macromolecular binding in tissue and blood is negligible, can be estimated from n-octanol:water PCs or vegetable:water PCs (*Po:w*) using the following general equation (64, 221):

$$P_t = \frac{(P_{o:w} \times F_{nlet}) + F_{wet}}{(P_{o:w} \times F_{nleb}) + F_{web}} \quad (22)$$

The numerator and denominator of Eq. (22) correspond to Eqs. (20) and (21) divided by *Pw:a*. In the

case of VOCs then, *Pt* values can be obtained by dividing *Pt:a* by *Pb*. The predictions of Eq. (22) will therefore be identical to the ratio of the predictions obtained using Eqs. (20) and (21) (214, 217, 220). In the above equation, the neutral lipid equivalent has in some cases been considered to be equivalent to total lipid content (74). The sum of *Fnlet*+*Fwet* is not equal to 1 in most cases due to the presence of other tissue components such as

Table 5.6

Neutral lipid and water equivalent of major tissues in human and rat blood (221)

	Water equivalent		Neutral lipid equivalent	
Tissues	*Rat*	*Human*	*Rat*	*Human*
Blood	0.8423	0.8217	0.0020	0.0040
Fat	0.1215	0.1514	0.8536	0.7986
Liver	0.7176	0.7400	0.0425	0.0473
Muscle	0.7471	0.7573	0.0117	0.0378

proteins. This aspect should be appropriately considered while using the tissue composition data for calculating PCs of chemicals.

When the tissue:air, blood:air, and tissue:blood PCs of unionized organic chemicals are not known, Eqs. (20), (21), and (22) can be used to provide first-cut estimates. Since the tissue and blood composition data can be estimated experimentally or obtained from literature (Table 5.6), only the numerical values of the physicochemical properties are needed for each new chemical. The *Po:w* and *Pw:a* of chemicals can be predicted from molecular structure information (217). An example of the prediction of tissue:blood PC from molecular structure information of 1,1,1-trichloroethane is presented in Figure 5.8. Semi-empirical methods relating

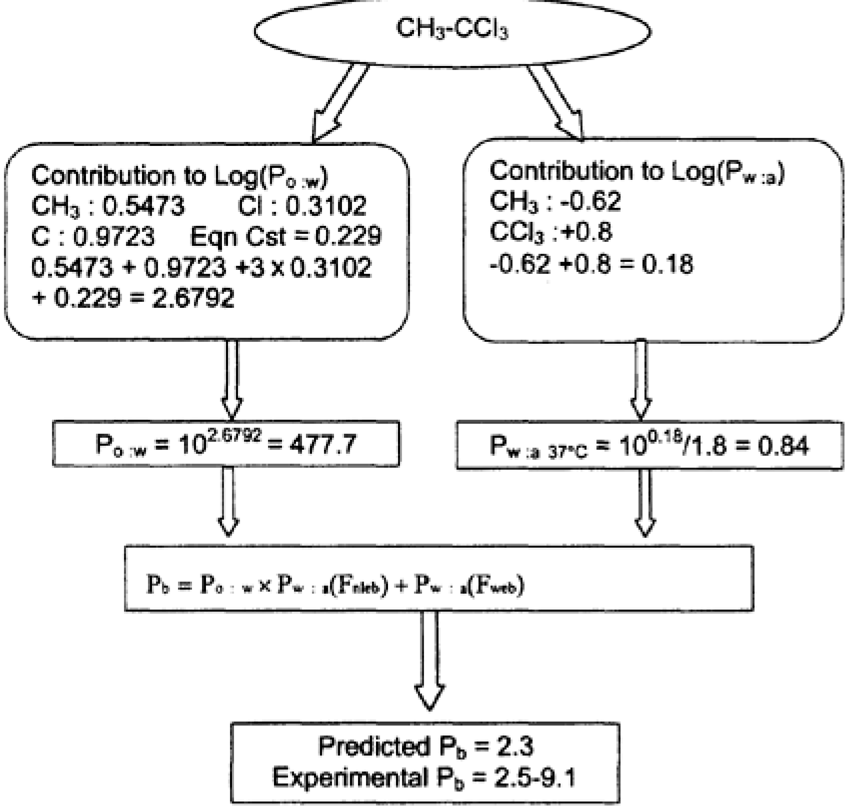

FIG. 5.8. Prediction of the human blood: air partition coefficient (Pb) of 1,1,1-trichloroethane from knowledge of its molecular structure, according to Reference 217.

molecular structure information to tissue:blood and blood:air PCs of chemicals may also be used as appropriate (64, 198).

Biochemical Parameters

Biochemical parameters such as the rates of absorption, biotransformation, macromolecular binding, and excretion can be determined by conducting time-course analysis in vivo or in vitro. One strategy for accurate estimation of specific biochemical parameters in vivo is to conduct experiments under conditions where pharmacokinetic behavior of a chemical is related to one or two dominant factors and thereby derive estimates of these parameters.

Thus, the rate constant for dermal absorption of VOCs has been determined by conducting body-only exposure of animals covered with a latex face mask. The total amount of the chemical absorbed through the skin during exposure is calculated by analyzing the blood-time course data collected during the exposure with a PBPK model that has all parameters except *Kp* defined. The value of *Kp* is estimated by

fitting PBPK model simulations to the blood-time course concentration data obtained experimentally (180, 181).

The skin permeability constant can also be determined in vitro using excised skin tissue. In these experiments, the test material is placed on excised skin in a vehicle and its appearance in the bathing medium determined (3). A plot of concentration in the bath vs. skin has a time lag, a period of increasing slope, and a final phase of constant slope (Figure 5.9). The plot of rate of uptake—the first derivative of this curve—gives the maximum uptake rate. The permeability uptake rate *Kp* (cm/hr) is calculated by

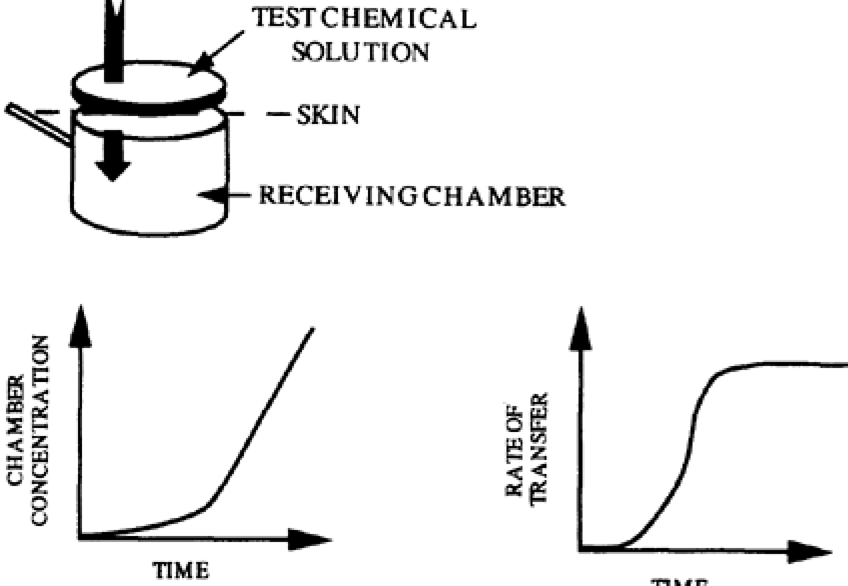

FIG. 5.9. Schematic representation of in vitro approaches to estimate skin permeability constants. Redrawn with permission from Reference 3.

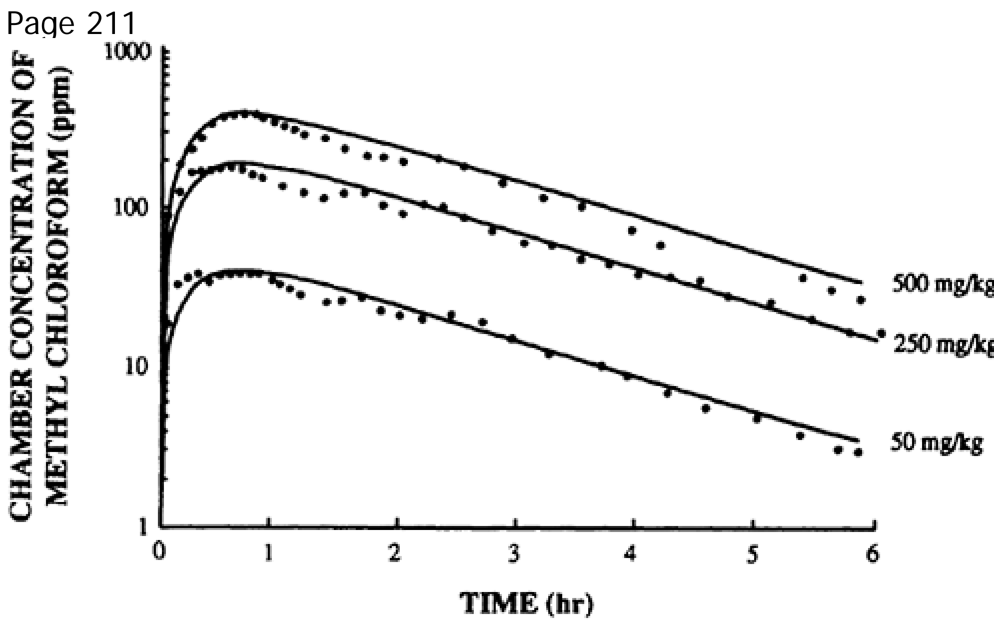

FIG. 5.10. Exhaled breath chamber time course for groups of four mice following oral dose of 50, 250, or 500 mg 1,1,1-trichloroethane/kg body weight. PBPK model simulations (solid lines) obtained using an oral absorption rate constant of 0.72 h^{-1} are compared with the experimental data (closed circles). Redrawn with permission from Reference 93.

dividing the uptake rate (mg/cm2/hr) with applied concentration (mg/cm).

The rate constant for gastrointestinal absorption of volatile organics has been estimated by analyzing the time course of exhalation of the parent chemical after oral administration, with a PBPK model that had all parameters except *Ko* defined (Figure 5.10) (93). The estimation of the rate of gastrointestinal absorption for hydrophilic chemicals (*Pb*>90) has been performed by determining the blood concentration following oral dosing and analyzing the data with a PBPK model that defined all parameters except *Ko* (87).

The rate constants for metabolism can be determined in vivo or in vitro. Two innovative noninvasive methods have been devised for the estimation of the in vivo metabolic rate constants of VOCs. These are

(a) the closed chamber or gas uptake method, and
(b) the exhaled breath chamber method.

The closed chamber or gas uptake method uses a desiccator-type chamber with a recirculating atmosphere for exposing groups of animals to volatile chemicals (7, 78). This approach involves periodically monitoring the chamber concentration of a chemical during exposures. The rate of change in the chamber concentration of the chemical both in the absence and in the presence of animals is determined for various starting concentrations. The net difference in the rates determined in these two sets of experiments represents the loss of chemical due to uptake and metabolism by the animals. The rate of spontaneous loss of chemicals in the empty chamber, corresponding to degradation and/or adsorption to chamber surface, should not exceed ~2 percent per hour. Otherwise the decline in the chamber concentration may not be sensitive enough to enable the determination of metabolic rate constants. When animals are placed in the gas uptake chamber, the rate of decline in chamber concentration of the chemical increases, the magnitude being proportional to the rate of metabolism once the chemical has equilibrated within the organism.

The data analysis is conducted with a PBPK model that has all parameters defined except the metabolic rate constants (95). Initially, the PBPK model for closed chamber exposures is run for several starting concentrations with the metabolic rate constants set to 0 (i.e., Vmax=0, *Kf*=0). The model simulations obtained at this stage reflect chemical uptake by the organism in the absence of tissue metabolism. Then, by setting the *V*max, *Km*, and *Kf* to some numerical values, it can be seen that the model simulations correspond to experimentally observed decline in chamber chemical concentrations. By optimization to get the best fit to the set of gas uptake curves, the values of metabolic rate constants are estimated. A single set of *Km* and *V*max obtained in these in vivo experiments has been considered to represent the role of a single isoenzyme, or the average of the sum of the activities of multiple enzymes. This method has been used successfully to obtain metabolic rate constants of VOCs that are biotransformed by a single first-order process, a saturable process, or a combination of both (6, 98).

It is important to monitor the oxygen concentration, humidity level, and chamber pressure during gas uptake studies. Any change in the respiratory rate should be investigated so that modeling of gas uptake exposure provides reliable estimates of metabolic rates (51, 128). Control gas uptake runs, during which naive animals are placed in the chamber without any added chemical, are necessary to ensure the absence of interfering chromatographic peaks of exhaled endogeneous chemicals.

Since the gas uptake studies involve whole-body exposures, there is a possibility for adsorption to fur and also dermal uptake. If dermal absorption is

important, or if the PBPK model cannot satisfactorily simulate the chamber decline data with only pulmonary uptake description, then rate constants for dermal absorption process should be determined independently and included in the model. To determine whether or not adsorption to fur has contributed significantly to chemical uptake during whole-body exposures, the animals, after termination, should be placed in a clean chamber and the time course of the appearance of the chemical determined (94).

The gas uptake method is not particularly well suited for use with those organic chemicals that
(a) have low vapor pressure,
(b) exhibit high chamber loss rates, or
(c) are highly soluble in blood and tissue.

For example, in the case of a chemical with blood-air PC greater than 60, the equilibration phase may become prolonged, thus occupying most of the gas uptake curves. The behavior in these gas uptake curves is restricted largely to tissue uptake. In such cases, the metabolic rate constants have been assessed using an exhaled breath chamber (93, 94).

The exhaled breath method involves placing an animal, previously exposed to a chemical, in a flow-through type of chamber and collecting samples of the chamber effluent for chromatographic analysis. Several exhaled breath samples are taken at periodic intervals during the experiment. The time intervals are chosen based on the appearance of the decay phase or whenever transitions in the elimination behavior are expected or observed. These curves are then analyzed with a PBPK model in which all parameters except metabolic rate constants are defined. The metabolic rate constants are estimated by obtaining an optimal fit of the PBPK model simulations to the exhaled breath curves.

Metabolic rate constants also have been determined by measuring the production of a stable metabolite resulting from the conversion of the parent compound in vivo (99). For a particular metabolite to provide a quantitative measure of metabolism of the parent chemical, it is ideal if it is produced in the first step of metabolism and is resistant to further biotransformation. Only very few metabolites exhibit these attributes. An example of this kind is bromide ion resulting from the initial metabolism of dibromomethane. This metabolite is distributed almost exclusively in the extracellular fluid spaces and is excreted slowly in the urine. In this case, the metabolic rate constants were estimated by fitting the simulations of a PBPK model that accounted for the formation, distribution, and excretion of bromide to experimental data on plasma bromide levels (99).

The blood time-course data obtained after intravenous administration also have been used to determine the rate constants of metabolism (229). Accordingly, the blood or tissue time course of the parent chemical is obtained following its intravenous administration over a dose range. The experimental data are then analyzed with a PBPK model that has all parameters defined except metabolic rate constants. A single combination of metabolic rate constants (i.e., *V*max, *Km*, and/or *Kf*) that best describes the data is obtained by fitting the model simulations to the set of blood time-course curves (Figure 5.11). Similarly, pharmacokinetic data obtained following other modes of administration can be used to determine the metabolic rate constants, provided the rate constant of absorption for the particular exposure pathway has been obtained independently.

In the above methods, we know or hypothesize that the decline in the blood/chamber level of parent chemical is determined by the magnitude of the metabolic rate constants, and that metabolism occurs via a single first-order, second-order, or saturable process, or via a combination of any two processes in a particular tissue. In the case of chemicals that are metabolized by more than two competing metabolic pathways to varying extents in several tissues, the gas uptake, exhaled breath chamber, or intravenous pharmacokinetic studies alone cannot be used to determine the rate constants for each of the multiple pathways occurring in several tissues. The use of these methods in this case will yield one set of rate constants that represent the overall metabolic clearance of the chemical (i.e., the sum total of metabolism via all metabolic pathways). Alternatively, if the rate constants for

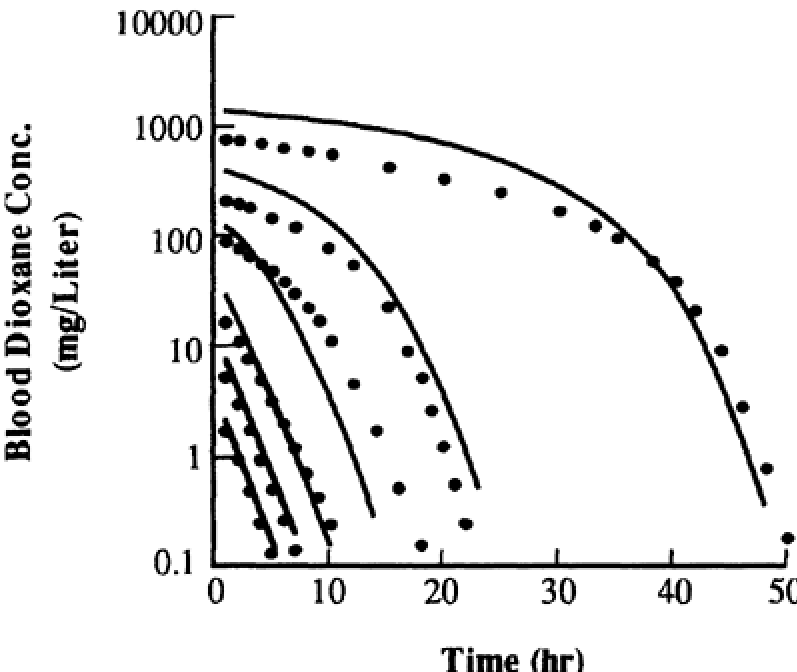

FIG. 5.11. Comparison of PBPK model simulations (solid lines) with the experimental data (symbols) of blood concentrations of 1,4-dioxane following intravenous dosing (3, 10, 30, 100, 300, and 1000 mg/kg). Model simulations were obtained using metabolic rate constants estimated by statistical optimization of the model fit to the experimental data. Redrawn with permission from Reference 229.

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all metabolic pathways except one have been determined independently, then an intravenous dosing or a gas uptake study can be conducted to obtain the rate constants for this pathway (151). The rate constants for individual metabolic pathways can potentially be obtained from in vitro studies.

Subcellular fractions, postmitochondrial preparations, isolated cells, tissue slices, and isolated perfused organs are all potentially useful as in vitro systems for the estimation of metabolic rate constants (58, 60, 62, 63, 103, 116, 121, 167, 170, 186-188, 228, 231, 241). The relevance of rate constants determined in vitro to the intact animal is not clear in all cases. However, several studies using microsomes, post-mitochondrial fractions preparations, or hepatocytes to determine metabolic rate constants for direct incorporation into PBPK models have successfully described the kinetics of volatile and nonvolatile organic chemicals (150). The *Km* obtained in in vitro studies has been used directly [or scaled to reflect the in vitro/in vivo ratios in a test species (231)], but the *V*max obtained in vitro has been scaled to the whole organism based on the mass recovery of the particular fraction. For example, the *V*max for the intact animal has been estimated from the *V*max obtained using rat liver microsomes as follows:

$$V\text{max (in vivo)} = V\text{max (in vitro)} \times 60 \times Cprot \times Vt \qquad (23)$$

where *V*max(in vivo) is expressed in mg/hr/kg, *V*max(in vitro) is expressed in mg/min/mg protein, 60 is the factor for converting the per-minute rate to per-hour rate, *Cprot* is the concentration of protein in the microsomal sample (mg protein/g tissue), and *Vt* refers to the volume of tissue (g).

Care must be taken to check the validity of the various in vitro systems to adequately predict the kinetics of chemicals in vivo (50). For example, the clearance of dichloromethane (DCM) by oxidative metabolism estimated in vitro using rat liver microsomes is lower than the actual clearance estimated by in vivo methods. The examination of the in vitro- and in vivo- derived rate constants for oxidative metabolism revealed that the *V*max agreed well, but there are differences of up to four orders of magnitude in the *Km* values between the two approaches. Use of these in vitro *Km* values in a PBPK model would underestimate severely the amount of DCM metabolized via the oxidative pathway at low exposure concentrations (5). Such a description would have the oxidative pathway competing less efficiently with the glutathione (GSH) conjugation pathway, thus over-predicting the metabolite production via the GSH pathway. Products from the GSH pathway have been correlated with tumor outcome and this parameter misspecification would lead to substantial errors in assessing the carcinogenic risk associated with low-level DCM exposures (5).

The identification of in vitro systems for determining metabolic rate constants that give values consistent with those operative in vivo is crucial for eventually predicting human dosimetry. Recent studies indicate that freshly isolated hepatocytes are a better system that provides rate constants comparables to the in vivo estimates (e.g., 133). Other studies have succeeded in conducting extrapolations between in vitro metabolic systems, based on an understanding of biochemical principles at the quantitative level (e.g., Lipscomb et al. (170)). Our ability to predict metabolic rates from one in vitro system to another is a significant step forward in predicting the rate constants for the in vivo system.

Mechanistic animal-replacement approaches for predicting the numerical values of *E, V*max, *Km,* or *Clint* of chemicals are not available yet. However, semi-empirical approaches relating the molecular structure information to PBPK model parameters such as PCs and metabolic rate constants have been developed (198, 199, 281). A major limitation of these approaches is that experimental data need to be collected before developing equations that consistently describe the relationship between molecular structure and PBPK model parameters. Truly predictive approaches can be developed only as our understanding of the biochemical processes improve. At the present, however, the hepatic extraction can be assumed to be complete or negligible in PBPK models in order to generate simulations. Accordingly, the numerical value of E in Eq. (10) should be set to 0 or 1 during the model simulations. The region encompassed by the simulated lines obtained with $E=0$ and $E=1$ will naturally contain the experimental data for that particular exposure scenario (e.g., Figure 5.12).

The rate constants of chemical reaction with hemoglobin and tissue proteins determined in vitro or in vivo, have been incorporated into the PBPK model to make predictions of these phenomena in vivo (89, 151). Attempts also have been made to include receptor binding and DNA binding properties of chemicals into a PBPK modeling framework based on in vitro-derived data (77, 260).

Some PBPK models, in which perfusion-limited tissue descriptions are used, may predict greater tissue uptake of chemicals than that which actually is observed. In such cases, tissue uptake is described as a diffusion-limited process and the mass transfer coefficient for each tissue is required. PAt is estimated by fitting model simulations to experimental data on tissue concentration of the parent chemical by varying the numerical value of the PAt term (9, 59) (Figure 5.13).

Once the mathematical representation of a PBPK model is prepared and its parameters estimated, the model can be used to simulate the kinetic behavior of a chemical in the test species.

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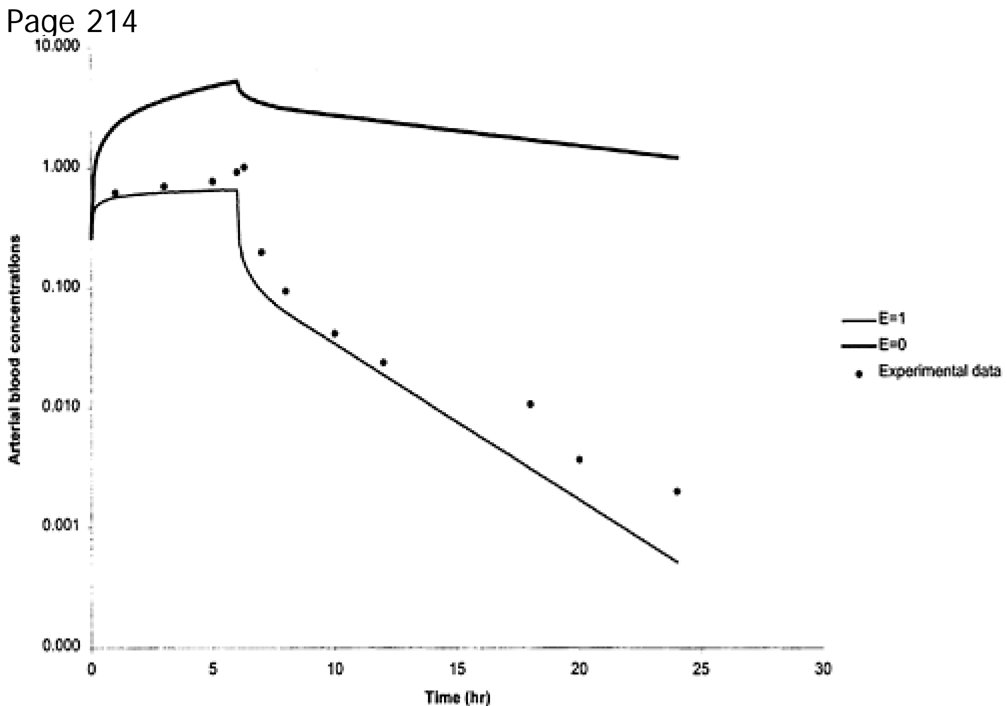


FIG. 5.12. Comparison of experimental data (closed circles) on arterial blood concentration with the envelope simulated by rat PBPK model for a 6-h exposure to 80 ppm styrene (solid lines). The upper line corresponds to the simulation obtained when the hepatic extraction was set to zero, and the lower line represents simulation obtained when the hepatic extraction ratio was set to 1. The experimental data were obtained from Reference 223.

MODEL SIMULATION

Simulation is the system behavior predicted by solving the differential equations representing the quantitative interrelations among the various model parameters. In the context of PBPK modeling, simulation refers to the prediction of the kinetic profiles of chemicals in blood and tissues by solving the set of MBDEs. Typically, simulation is chosen when:

(a) the real system does not exist,
(b) the real system exists but experimentation is expensive,
(c) a forecasting model is required to analyze events occurring over long periods of time in a compressed format, and
(d) the model does not have practical analytical solutions (e.g., stochastic models, nonlinear systems) (232).

Since PBPK models often contain differential equations and describe nonlinear processes, it is usually impractical to obtain analytical solutions and, therefore, a numerical simulation approach is adopted. For conducting computer simulations, the mathematical equations should be written in such a way as to facilitate their solution by a fixed, step-by-step procedure (i.e., algorithm).

Algorithms

Algorithms for simulation are chosen based primarily on the following criteria:

(a) thoroughness of the theoretical basis of the algorithm,
(b) self-starting capability,
(c) automatic control of step size and method order,

(d) ability to deal with both stiff and nonstiff problems and to detect stiffness automatically, and, finally, (e) proof that the algorithm works for test problems of the same kind as the one(s) under consideration (232).

Some of the commonly used algorithms include those of Euler and Gear, Runge-Kutta routines, and predictor-corrector methods. The general principle underlying these algorithms used for solving first-order ordinary differential equations can be represented in simple terms as follows:

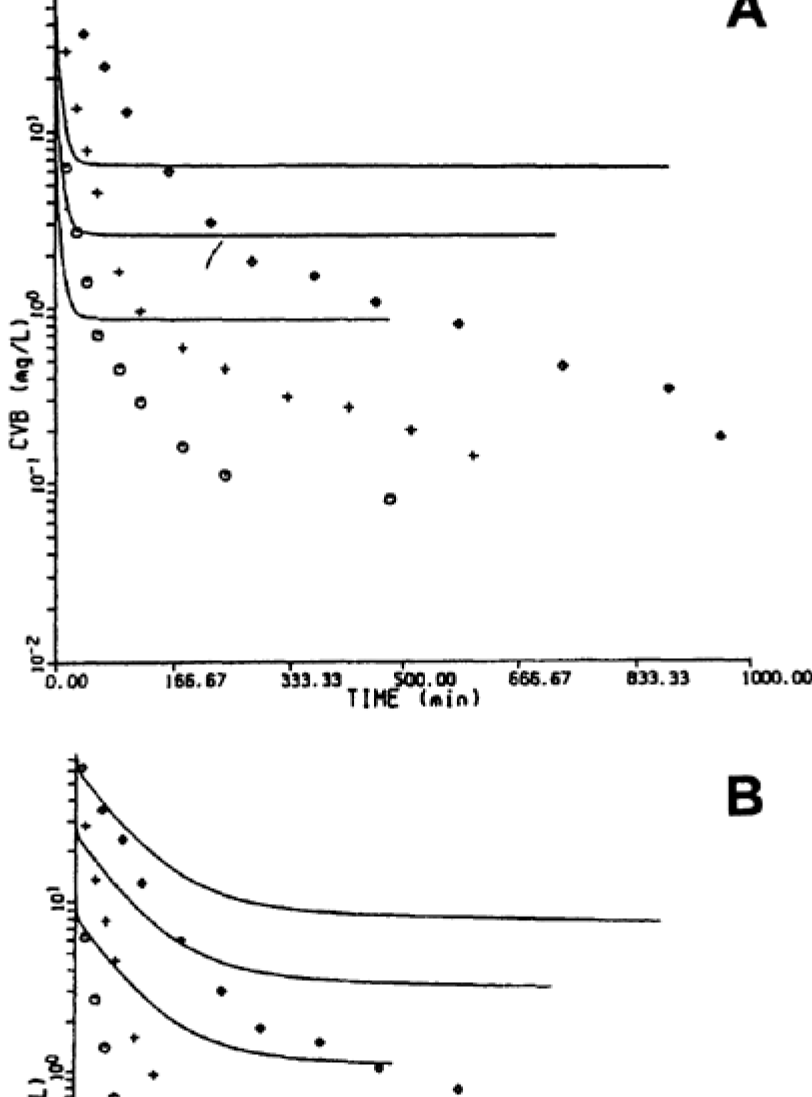
$$\text{New value} = \text{Old value} + (\text{slope} \times dt) \quad (24)$$

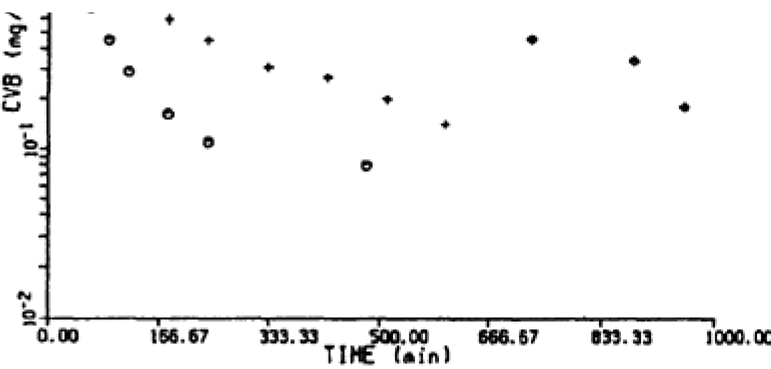
For a tissue compartment in PBPK model,

$$A_{t,1} = A_{t,0} + (dA_t/dt \times dt) \quad (25)$$

where dt is the integration interval or the predetermined length of time for which solution is obtained. Using the above algorithm, the numerical solution will approach the reality as long as the dt is prohibitively short. The specific form of the numerical solution represented in Eq. (25) is referred to as the Euler algorithm. In this method, the error arises from the negligence of the second- and other higher-order derivatives of the Taylor expansion series (110). In other words, the error associated with the Euler method is proportional to dt^2. Therefore, with the use of smaller dt values, the error associated with this first-order integration method can be minimized.

Some of the algorithms (e.g., Gear) and not others (e.g., Euler) can deal with stiff systems (101, 110). The stiffness in models is reflected by the ratio (generally of several orders of magnitude) of the largest to the smallest time constant in the model. The stiffness requires that variable integration intervals be used as simulation progresses due to the compartments with the smallest time constants attaining steady-state condition gradually. The fact that the integration interval is not changed with the progression of simulation time, in accordance with the





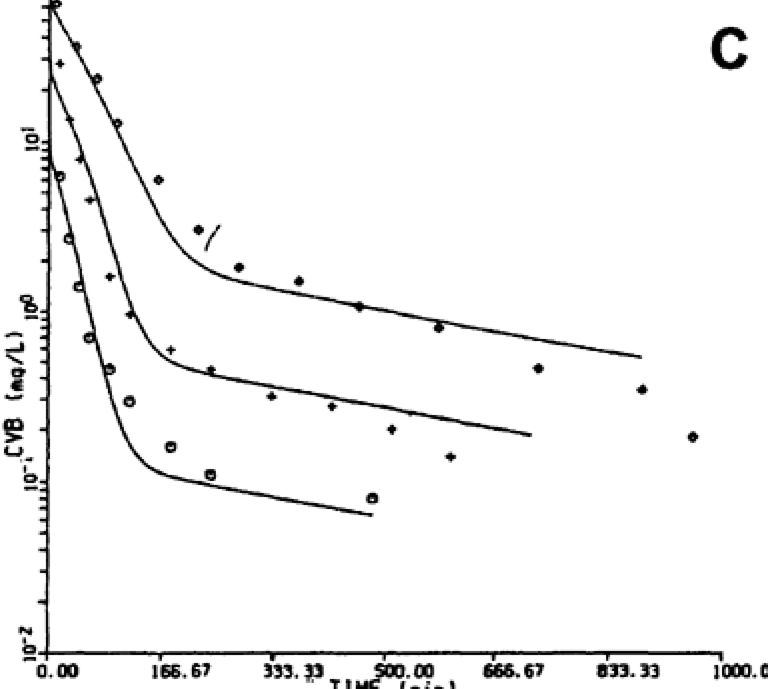


FIG. 5.13. Comparison of the experimental data (symbols) on the venous blood concentration of unchanged pyrene in rats with PBPK model simulations (solid lines). The simulations were obtained with a model that described uptake as a perfusion-limited process in all tissues (A), or as a diffusion-limited process in adipose tissues and slowly perfused tissues (B). With the additional description of metabolism in appropriate tissues, the PBPK model simulations correspond to the experimental data (C). The dose levels were 2, 6, 15 mg/kg (iv). Reproduced with permission from Reference 111.

existing stiffness state, represents only a disadvantage of the Euler algorithm taking a longer time to complete a given simulation. This is particularly because at steady-state conditions (at which $dAt/dt=0$) larger integration intervals can be used, thus saving time without losing the accuracy of the simulation. The adequacy of the Euler algorithm for numerical integration of differential equations used in PBPK models has been demonstrated (110, 131).

Software

The PBPK model equations, along with the integration algorithms, can be written and solved using programming languages, simulation software, or spreadsheets. In the first two cases, the style of computational representation of a model is determined by the grammatical and precedence rules of the programming language (FORTRAN, BASIC, etc.) or simulation language to be used. Simulation languages are computer programming packages that are general in nature but may have special features for modeling certain types of systems. Examples of simulation languages that possess features particularly useful for PBPK modeling are listed in Table 5.7. When selecting a particular simulation language for modeling, it is important to ensure that it

(a) provides a convenient means for initializing the status of the model (e.g., generating random numbers in case of stochastic models),

(b) permits the introduction of changes in both the status and temporal structure of the model as simulation time evolves (i.e., scheduling the occurrence of events),

Table 5.7

Examples of softwares used in PBPK modeling

Software	Source	Some references
ACSL	Pharsight Corporation, Mountain View, CA Aegis Research Corporation, Huntsville, AL	52, 172, 223
D02EBF	NAG Library	190
Excel	Microsoft	110, 131
Matlab	MATLAB Manual, University of Manchester Regional Computer Center, NAT 657	222, 275
ScoP	Simulation Resources, Inc., Berrien Springs, MI	145, 224
Simusolv	Dow Chemical, Midland, MI Mitchell and Gauthier Associates, Concord, MA	29, 139, 273
STELLA	High Performance Systems, Inc., Hanover, NH	209

(c) provides simple methods by which model results and statistical summaries can be obtained,

(d) allows considerable flexibility in conducting sensitivity and other types of model analyses, and

(e) contains error detection facilities (232).

The choice of a particular simulation software is up to the individual as long as the software package provides the framework for creating and solving the type of model equations under consideration. Several commercially available simulation or programming software packages can be used for conducting PBPK model simulations (184). These programs/ packages are easily accessed and understood by individuals who are familiar with the techniques of programming or mechanics of simulation. The models constructed and solved using simulation packages appear like a "black box" to the analyst, who gets to see the end results but not the temporal evolution of solutions to the complex mathematical formulations constituting the basis of the end results. The "internal mechanics" of computer simulation in such cases can be visualized if the user can reconstruct the way in which the simulation software

(a) solves each equation in the model, and

(b) takes the output of one equation and provides it as input to other equations of the model.

This can be accomplished using spreadsheet programs such as Lotus 1–2-3, QuattroPro, and Microsoft Excel. The limitations of the spreadsheet approach relate to

(a) the number of cells to be selected in the spreadsheet, and

(b) the runtime required to solve complex PBPK models.

Therefore, this approach is recommended for individuals who do not have sufficient knowledge of numerical integration algorithms and simulation software. Once a beginner understands how the PBPK models work using the spreadsheets-based methodology, he or she can move on to using advanced techniques and specialized simulation languages offering flexibility, speed, and additional features (sensitivity analysis, optimization routines, etc).

Hardware

The equations and algorithms constituting PBPK models can be solved using programming languages, simulation software, or spreadsheet programs on many types of computers—mainframes, workstations, microcomputers, or minicomputers. In general, for running large models, dedicated workstations and

multiuser mainframe computers offer the lowest execution times, thus providing overall time savings. For a PBPK modeler working with small models and simple descriptions, the

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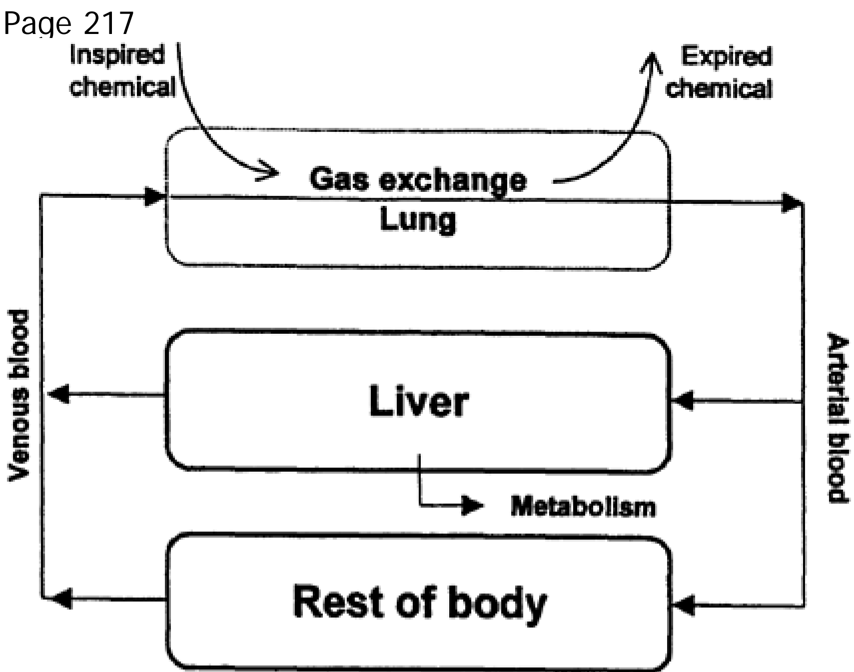

FIG. 5.14. Conceptual representation of a two-compartmental PBPK model for styrene in the rat.

Macintosh or IBM PC-based modeling packages are sufficient (114, 131, 145, 223). The processing speed, hard disk space, and run time memory of computers marketed currently are quite adequate for PBPK modeling. The methodological approach of PBPK modeling using spreadsheets is presented in the following section.

PBPK Modeling Using Spreadsheets

Consider a simple, physiologically based model with compartments interconnected by systemic circulation for simulating the pharmacokinetics of inhaled styrene in the rat (Figure 5.14). The parameters and equations of this hypothetical PBPK model should be entered in spreadsheets to obtain simulations of styrene pharmacokinetics in the rat. The numerical values for each of the PBPK model parameters should be entered into a specific cell in the spreadsheet and identified appropriately. For example, the numerical value contained in cell C5 is referred to as *Qp* (Table 5.8). Since the alveolar ventilation is referred to as *Qp* in this example, whenever *Qp* is typed in any other cell of the spreadsheet, the numerical value found in cell C5 will be imported automatically.

Table 5.9 lists the manner in which the model equations are written in the spreadsheets. In addition to the equations for computing blood concentrations, four equations per compartment are written. These correspond to the tracking of:

(a) the rate of change in the amount of chemical in tissue,
(b) the amount of chemical in tissue,
(c) the concentration of chemical in tissue, and
(d) the concentration of chemical in venous blood leaving tissue.

The calculation of the amount of chemical in tissue is based on the Euler algorithm. If intended, other integration algorithms can be used. In the various equations (Table 5.9), the model parameters are referred to using the appropriate abbreviations (e.g., *Qp*, *Qc*, *Vl*) and the variables are referred to with the use of relative reference expressions. The relative reference expression

Table 5.8

List of parameters for the two-compartment PBPK model, their numerical values, and location in Excel spreadsheet.

Parameters	Abbreviation*a*	Numeric valuesb	Place of cell*c*
Cardiac output	Qc	5.64 L/hr	C4
Alveolar ventilation rate	Qp	4.5 L/hr	C5
Hepatic blood flow	Ql	2.11 L/hr	C6
Blood flow in rest of body	Qbo	0.261 L/hr	C8
Liver volume	Vl	0.012 L	D6
Volume of rest of body	Vbo	0.027 L	D8
Liver: blood partition coefficient	Pl	2.7	E6
Rest of body: blood partition coefficient	Pbo	50	E8

Blood: air partition coefficient	Pb	40	F7
Maximal velocity of metabolism	Vmax	3.6 mg/hr	G6
Michaelis-Menten affinity constant	Km	0.36 mg/L	H6

*a*The various model parameters are referred to, with the use of these abbreviations, in the spreadsheet.
*b*All parameters estimates were based on Reference 223.
*c*The cell locations provided here correspond to the column and row coordinates, respectively, that is, the alphabetical letters denote the columns and the Arabic numerals correspond to the rows of the spreadsheet.

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Table 5.9

Equations used in the calculation of tissue, arterial and venous blood concentrations of styrene, and their expression in EXCEL spreadsheet.

Compartment	Equations*a*	Expression in EXCEL*b*
Arterial blood	$C_{a,n}=\frac{Q_c\times C_{v,n-1}+Q_p\times C_{inh,n}}{Q_c+Q_p/P_b}$	D36=((Qc*M35)+(Qp*C36))/(Qc+(Qp/Pb))
Liver	$dA_l/dt_n=Q_l\times(C_{a,n}-C_{vl,n-1})-\frac{V_{\max}\times C_{vl,n-1}}{K_m+C_{vl,n-1}}$	E36=(Ql*(D36-H35))–(Vmax*H35/(Km+H35))
	$A_{l,n}=dA_l/dt_{,n}\times t+A_{l,n-1}$	F36=E36*t+F35
	$C_{l,n}=\frac{A_{l,n}}{V_l}$	G36=F36/Vl
	$C_{vl,n}=\frac{C_{l,n}}{P_l}$	H36=G36/Pl
Rest of body	$dA_{bo}/dt_n=Q_{bo}\times(C_{a,n}-C_{vbo,n-1})$	I36=Qbo*(D36–L35)
	$A_{bo,n}=dA_{bo}/dt_n\times t+A_{bo,n-1}$	J36=I36*t+J35
	$C_{bo,n}=\frac{A_{bo,n}}{V_{bo}}$	K36=J36/Vbo
	$C_{vbo,n}=\frac{C_{bo,n}}{P_{bo}}$	L36=K36/Pbo
Venous blood	$Cv_n=\frac{Q_l\times C_{vl,n}+Q_{bo}\times C_{vbo,n}}{Q_c}$	M36=((Q1*H36)+(Qbo*L36))/Qc

*a*All abbreviations and symbols used in the equations, except n and n–1, are defined in Table 5.1. Subscripts n and n–1 refer to the current and previous simulation times. The difference between n and n–1 in the styrene example was 0.005 hr.

*b*The components of these equations refer either to absolute references (in the case of constant input parameters, as defined in Table 5.8) or to relative references (in the case of state variables).

involves referring to a cell according to its location relative to another cell where the calculation is being carried out. This option is particularly useful when the output of an equation contained in one cell is to be provided as input for an equation contained in another (e.g., adjacent) cell. Thus, the use of relative reference expressions in spreadsheets may be useful to facilitate loop-type calculations essential for advancing the state of a system during simulations.

Table 5.10 presents a part of the spreadsheet depicting how equations presented in Table 5.9 are actually entered into spreadsheets. Accordingly, in the spreadsheet, the descriptions of the two tissue compartments occupy eight columns (columns E–L, in Table 5.10 and Figure 5.15) and the calculation/representation of the simulation time, exposure concentration, arterial concentration, and venous blood concentration occupy one column each (columns B, C, D, and M, respectively). The mixed venous blood concentration resulting from that of the venous blood exiting the two tissue compartments described in a particular row is calculated in the same row (i.e., cell M35 in Table 5.10 and Figure 5.15). This *Cv* then is used along with *Cinh* (i.e., cell C36) to calculate *Ca* in the subsequent row (e.g., cell D36). In this structure then, according to the schematics shown in Figures 5.14 and 5.15, all model equations are interconnected by specifying the proven/hypothetical input-output connections among them.

Once

(a) the numerical values of model parameters are provided,
(b) the equations in the first and subsequent rows of the spreadsheet are entered,
(c) the time interval for integration is specified, and
(d) the required number of cells are chosen, the simulation begins.

One has only to repeat the calculations shown in row 36 of Figure 5.15 for each time interval of integration until the end of the desired duration of simulation. In the present example, the time interval of integration was fixed at 0.005 h. Each line in the Excel spreadsheet then rep-

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Table 5.10 A portion of the spreadsheet depicting the entry of model equations

	B	C	D	E	F	G	H	I	J	K	L	M
33				Liver				Body				
34	Time (hr)	Cinh (mg/L)	Ca	dAl/dt	Al	Cl	Cvl	dAbo/dt	Abo	Cbo	Cvbo	Cv
35	=t	=Cinh	=[(Qc*0) + (Qp*C36)]/(Qc + (Qp/Pb))	=(Ql*(D36-0)) − (Vmax*0/(Km + 0))	=E36*t + 0	=F36/Vl	=G36/Pl	=Qbo*(D36-0)	=I36*t + 0	=J36/Vbo	=K36/Pbo	=[(Ql*H36) + (Qbo*L36)]/Qc
36	=t + B35	=Cinh	=[(Qc*M35) + (Qp*C36)]/(Qc + (Qp/Pb))	=(Ql*(D36-H35)) − (Vmax*H35/(Km + H35))	=E36*t + F35	=F36/Vl	+G36/Pl	=Qbo*(D36-L35)	=I36*t + J35	=J36/Vbo	=K36/Pbo	=[(Ql*H36) + (Qbo*L36)]/Qc

The row and column coordinates are designated by an Arabic numeral and an alphabetical letter, respectively. The equations found in rows 35 and 36 correspond to calculations at the first and second integration intervals. For continuing the simulation, the set of equations in row 36 should be copied onto desired number of subsequent rows. In this table, column B represents the state of the system which advances during each time interval (t). Column C contains the exposure concentration at any given time during the simulation, and columns D and M represent calculations of arterial and venous blood concentrations of the chemical. In between, four columns per compartment (e.g., liver: columns E–H, rest of the body: columns I–L) are devoted for calculation of

(1) the rate of change in the amount of chemical in tissue,
(2) amount of chemical in tissue,
(3) concentrations of chemical in tissue, and
(4) concentrations of chemical in venous blood leaving tissue. All abbreviations are defined in Table 5.8.

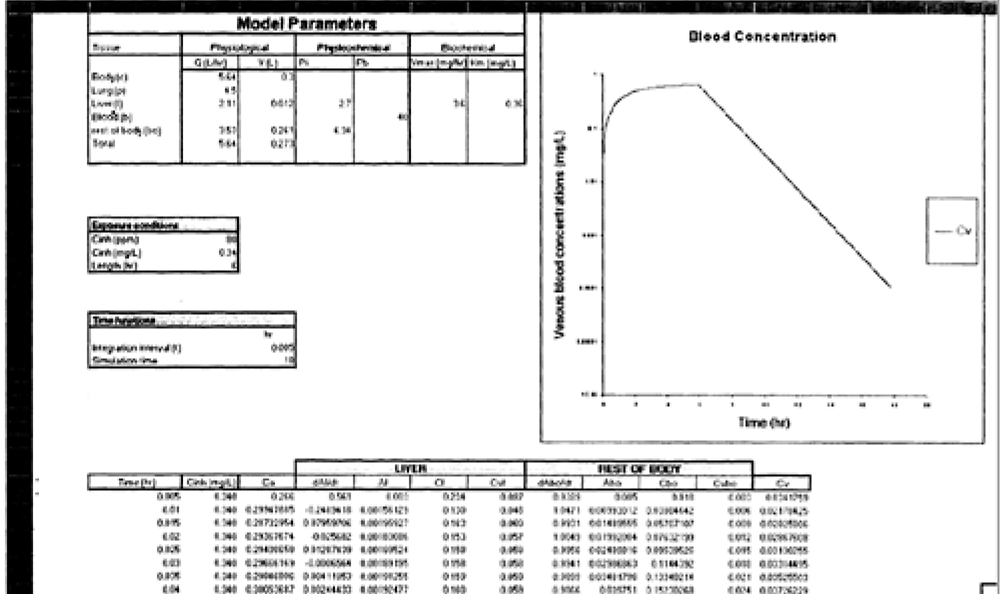

FIG. 5.15. Printout of a computer screen depicting an EXCEL spreadsheet with
(1) the plot of a two-compartment PBPK model simulation of venous blood concentrations of styrene during and following a 6-h exposure of rats to 80 ppm of this chemical,
(2) the numerical values of the PBPK model parameters, and
(3) a portion of the raw numbers corresponding to the rate of change in the amount of styrene in tissue, amount in tissue, concentration in tissue, and venous blood concentration leaving the tissues, generated during simulations between time 0.005 and 18 h.

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resents calculations characterizing the state of the system at every 0.005 h. In the present example, simulations were conducted for 24 h using 0.005 h as the integration interval (Figure 5.15). Therefore, in total, 4800(=24/0.005) lines were used up for conducting PBPK simulations. The solution to the set of PBPK model equations is generated every time the numerical values in cells corresponding to model parameters are changed, since these cells are specified in one or more equations appearing in the spreadsheet (Figure 5.15). Figure 5.16 presents simulations of the pharmacokinetics of styrene obtained using the parameters and equations for a four-compartment model developed by Ramsey and Andersen (223).

MODEL VALIDATION

Model validation refers to the evaluation of the adequacy of the conceptual and mathematical representations of the system under study in specific use conditions. Since PBPK models are only simplified representations of the actual systems, only those system variables that the investigator hypothesizes to be critical determinants are described in detail. The purpose of the validation process is then to determine whether all major determinants/processes that are essential for describing the system behavior have been adequately identified and characterized. When model predictions and experimental data agree, there is a higher level of confidence regarding the adequacy of the model and its use in decision-making (i.e., risk assessment). The approaches used for testing the adequacy of PBPK models can be classified into three categories:

(a) inspection approach,
(b) discrepancy measures, and
(c) statistical tests.

Inspection Approach

The testing of the degree of concordance between PBPK model simulations and experimental data to date has been conducted by eye balling or a visual inspection approach. This approach involves visual comparison of the plots of simulated data (usually continuous and represented by solid lines) with experimental values (usually discrete and represented by symbols) against a common independent variable (usually time). The rationale behind this approach is that the greater the commonality between the simulated and experimental data, the greater will be our confidence in the model. Instead of simply visualizing the degree of concordance between experimental data and simulation results, the residuals (i.e., difference between experimental and simulated data) can be examined. The residual analysis can be applied either to the whole model as it is or, in some other cases (as in diagnostic checkup during model building), to some estimated parameters of a tentatively ascertained theoretical model, so as to pinpoint inadequacies (122). The residuals should be random if the model is adequate. Time plots of residuals as well as the plots of residuals with respect to various controllable variables can detect possible model inadequacies, which can shed light on how to improve the model (122).

The inspection approach to model validation continues to be used pending the validation of statistical tests and discrepancy measure tests appropriate for application to PBPK models.

Statistical Tests

Statistical comparison of the model output with experimental observations is not as easy as it might appear. None of the classical tests (*t*, Mann-Whitney, two-sample *X*2, two-sample Kolmogrov, etc.) to determine whether the underlying distributions of the two data sets are similar is applicable, since the output processes of almost all real-world systems and simulations are non-stationary and autocorrelated. Furthermore, there is a question of whether the use of statistical hypothesis tests is even appropriate. Since the model is only an approximation of the actual system, a null hypothesis that the system and model are the same is clearly false. The more appropriate question is to ask whether or not the differences between the system and the model are significant enough to affect conclusions derived from the model. In this regard, Haddad et al. (109) screened various statistical procedures (correlation, regression, confidence interval approach, lack fit *F* test, univariate analysis of variance, and multivariate analysis of variance) for their potential usefulness in testing the degree of agreement of PBPK model simulations and experimental data obtained in intact animals. According to these authors, the multivariate analysis of variance represents the most appropriate test, with the variance for the simulation data permitting. Alternatively, lack of fit *F* test represents a useful way of evaluating the adequacy of simulation models. Particularly, this simple procedure permits the consideration of multiple data sets (e.g., data for several endpoints collected at various time intervals) in conducting such an evaluation of model validity.

The *F* statistic in model fitting may be defined as lack of fit

$$\frac{\text{Mean square}}{\text{Pure error mean square}}$$

This ratio is compared to the critical value of *F* at the required degree of confidence and the corresponding

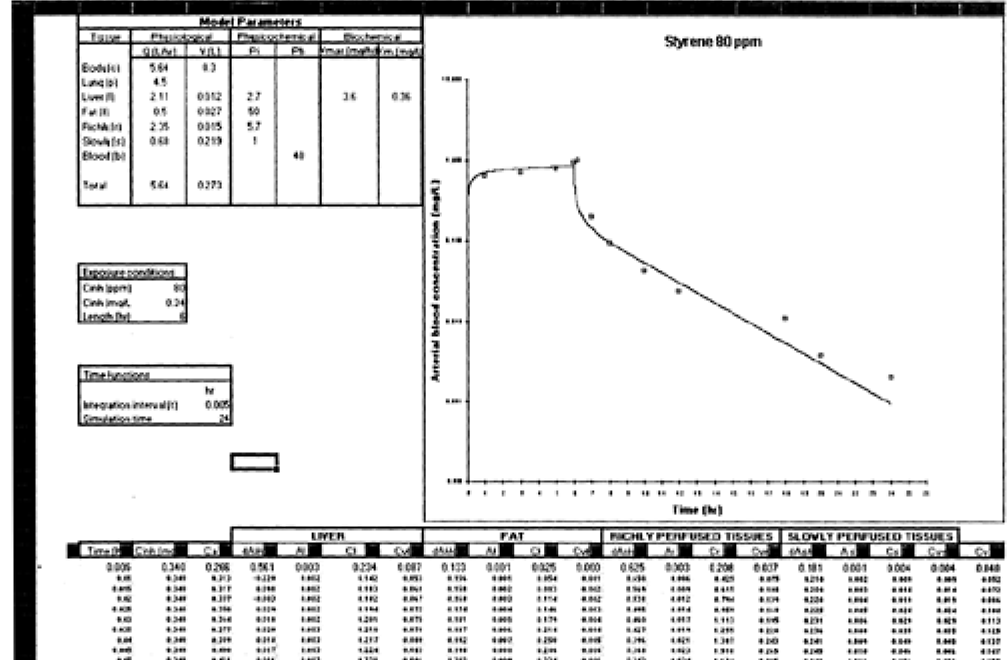
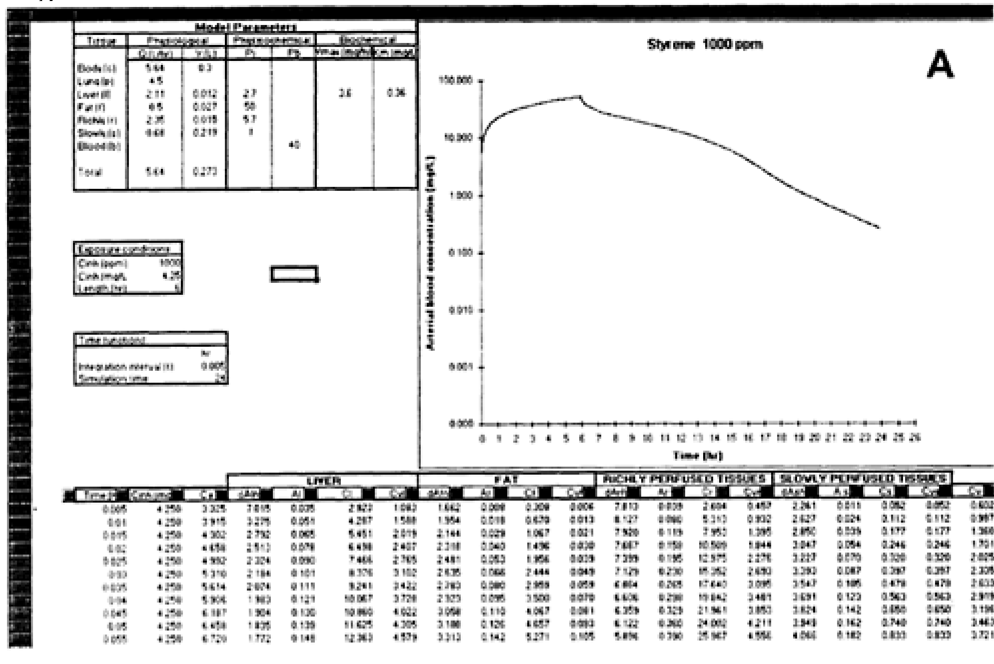


FIG. 5.16. Printout of a computer screen depicting an EXCEL spreadsheet with
(1) the plot of experimental data (symbols) and a four-compartment PBPK model simulations (solid line) of arterial blood concentrations of styrene during and following a 6-h exposure of rats to 80 ppm of this chemical,
(2) the numerical values of the PBPK model parameters, and
(3) a portion of the raw numbers corresponding to the rate of change in the amount of styrene in tissue, amount in tissue, concentration in tissue, and venous blood concentration leaving the tissues, generated during simulations between time 0.005 and 24 h.
Experimental data were obtained with permission from Reference 223.



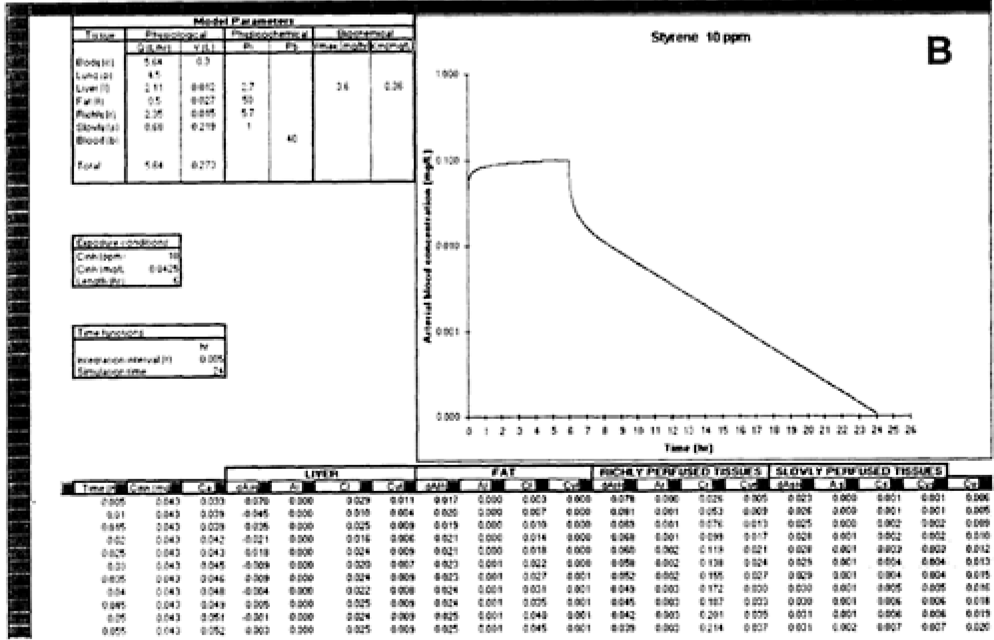


FIG. 5.17. Printout of computer screens depicting EXCEL spreadsheets with the PBPK modeling results obtained in rats exposed to
(A) 1000 ppm and
(B) 10 ppm of styrene.
Each of the two panels contain
(1) a plot of PBPK model simulation of arterial blood concentrations of styrene during and following a 6-h exposure,
(2) the numerical values of the PBPK model parameters, and
(3) a section of the raw numbers corresponding to the rate of change in the amount of styrene in tissue, amount in tissue, concentration in tissue, and venous blood concentration leaving the tissues, generated during simulations between time 0.005 and 24 h.
Note that only the exposure concentration (cell C19), and no other input parameter, differs between the two simulation exercises presented here.

degrees of freedom. If the above ratio is greater than F_{crit}, then the model is considered inadequate (122).

Discrepancy Measures

The application of appropriate statistical tests provides a means of evaluating whether or not model simulations are significantly different from experimental values. Regardless of the outcome of such statistical analyses, it is often necessary and useful to be able to represent, in a quantitative manner, the extent to which the model simulations differ from experimental data. In this context, Krishnan et al. (148) have developed a simple index, referred to as the PBPK index, to represent the degree of closeness or discrepancy between model predictions and experimental data used during the model validation phase.

This approach involved the calculation of the root mean square of the error (representing the difference between the individual simulated and experimental values for each sampling point in a time course curve), and division by the root mean square of the experimental values. The resulting numerical values of discrepancy measures for several data sets (each corresponding to an endpoint) obtained in a single experimental study are then combined on the basis of a weighting proportional to the number of data points contained in the data set. Such consolidated discrepancy indices obtained from several experiments (e.g., exposure scenarios, doses, routes, and species) are averaged to get an overall discrepancy index. The application of this kind of a "quantitative" method, which has not been done routinely yet, may help remove the ambiguity in communicating the degree of concordance or discrepancy between PBPK model simulations and experimental data. The routine calculation of this kind of index for PBPK models at the end of the model development phase should result in tagging each model with an index value, and such an open declaration of the face value of PBPK models might be appreciated by the end users.

MODEL REFINEMENT

Since errors or difficulties relating to computational representations can be solved easily, model failure during the validation stage would primarily reflect incorrect presentation of the system (i.e., conceptual representation), or failure to include specific mechanistic determinants or biochemical processes (i.e., mathematical representation). Further experimentation in such cases has resulted in significant improvement in the biological understanding of the system under study (39, 111).

The use of a discrepancy measure test or statistical test to show that a priori predictions of a particular end point are in agreement with the experimental data does not provide sufficient proof of the validity of the assumptions and model-building approaches used. These approaches are only useful in providing a quantitative measure of differences, and do not provide any information on either the model robustness or the reliability of the model structure. In this context, it is important to verify the influence of variability, uncertainty, and sensitivity associated with model parameters.

PBPK models contain a number of parameters; the uncertainty associated with the values of these parameters influences the predictions of tissue dose. Whereas uncertainty represents our imprecise knowledge about the actual value of a parameter, variability reflects the range of values for a parameter expected among individuals in a given population. On the other hand, sensitivity analysis refers to the evaluation of the effect of changes in the value of a particular parameter on tissue dose estimates provided by a PBPK model. Sensitivity is expressed as the magnitude of change in the endpoint of interest (e.g., tissue dose) as a function of change in the value of a particular model parameter. Sensitivity analysis involves determination of the response of the system to defined changes in the parameter values to identify the most critical model parameters. This approach does not provide an indication of the likelihood of a particular output or range of outputs. Monte Carlo methods are designed to provide an estimate of this probability, the idea being to make repeated computations using inputs selected at random that have the same statistical properties expected of each input parameter. After performing a large number of such computations, a statistical distribution of the output can be generated. If the output is sensitive to certain aspects of the model, then those aspects must be modeled carefully. Sensitivity and uncertainty analyses with PBPK models have been conducted with respect to specific endpoints, related either to pharmacokinetic behavior or to cancer risk estimates (21, 22, 41, 75, 114, 115, 119, 146, 213, 262, 263). The greatest potential for uncertainty/ sensitivity analysis is perhaps in improving experimental design and resource allocation in risk assessment-oriented research.

The validated PBPK models can be applied to predict the behavior of chemicals administered by various exposure routes, at differing doses, and in several animal species. This is particularly important because human health risk assessment is based on responses seen in animal toxicity studies in which the test

chemical is administered at high doses by routes often different from anticipated human exposures. With advances in mathematical modeling and molecular biology fronts, the tissue dose of chemicals in an individual can be simulated by accounting for the individual-specific information, including data on polymorphism of enzymes (73).

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MODEL APPLICATIONS

The principal application of PBPK models is to predict the target tissue dose of the toxic parent chemical or its reactive metabolite. Using the tissue dose of the toxic moiety of a chemical in risk assessment calculations provides a better basis of relating to the observed toxic effects than the external or exposure concentrations of the parent chemical (8, 16). Because PBPK models facilitate the prediction of target tissue dose for various exposure scenarios, routes, doses, and species, they can help reduce the uncertainty associated with the conventional extrapolation approaches.

High-Dose to Low-Dose Extrapolation

High-dose-low-dose extrapolation of tissue dose is accomplished with PBPK models by accounting for the nonlinear kinetic behavior of chemicals (39). The description of metabolism in these cases frequently involves the use of a Michaelis-Menten equation. Nonlinearity arising from mechanisms other than saturable metabolism, such as enzyme induction, enzyme inactivation, and depletion of glutathione reserves, also has been described with PBPK models (39, 70, 151). An example of high-dose-low-dose extrapolation is presented in Figure 5.17. Panel A of this figure shows the blood kinetic profile of styrene in rats exposed for 6 h to 1000 ppm styrene, whereas Panel B depicts the concentration vs. time course profile following a 6-h inhalation exposure to 10 ppm styrene. For conducting high-dose to low-dose simulation, in this particular example, the numerical value of the exposure concentration (indicated in cell C19) alone was changed. With the change in the numerical value of this input parameter, all calculations in the spreadsheet are carried out automatically, thus providing the simulations corresponding to the exposure concentration specified.

Route-to-Route Extrapolation

The extrapolation of the kinetic behavior of a chemical from one exposure route to another can be performed by adding appropriate equations to represent each exposure pathway. For simulating the intravenous administration of a chemical, a single input *(K0)* representing the dose administered to the animal can be included in the equation for mixed venous concentration:

$$Cv=(Qf\times Cvf+Ql\times Cvl+Qr\times Cvr+Qs\times Cvs+K0)/Qc \qquad (26)$$

More accurate description of intravenous administration includes the specification of time of infusion to calculate the rate of entry of the chemical. The parameters (iv dose, infusion time, and iv rate) essential for calculating the dose received intravenously should be included and linked to the rest of the equations constituting the PBPK model. Once the equations describing the route-specific entry of chemicals into systemic circulation are included in the model, it is possible to conduct extrapolations of kinetics. This approach is illustrated in Figure 5.18 for inhalation → iv extrapolation of the kinetics of styrene in rats. For simulating the inhalation pharmacokinetics, the iv dose is set to zero, while for simulating styrene kinetics following iv administration Cinh is set to 0 (Figure 5.18).

Similarly, for simulating oral absorption of a chemical, appropriate equations should be additionally included to describe process(es) occurring in the stomach compartment. At time zero, there is no chemical in the stomach. Appearance of the chemical in the stomach, and thereafter in the liver, will depend on the rate constant of absorption. For the chemical, whose oral absorption is first order, certain parameters (i.e., oral dose [mg/kg], rate of absorption [h−1)], and equations are added to basic inhalation model depicted in Figure 5.7:

$$\frac{dA_o}{dt} = K_o A_{stom} \qquad (27)$$

$$A_{stom} = D_o - \int_0^t \frac{dA_o}{dt} \qquad (28)$$

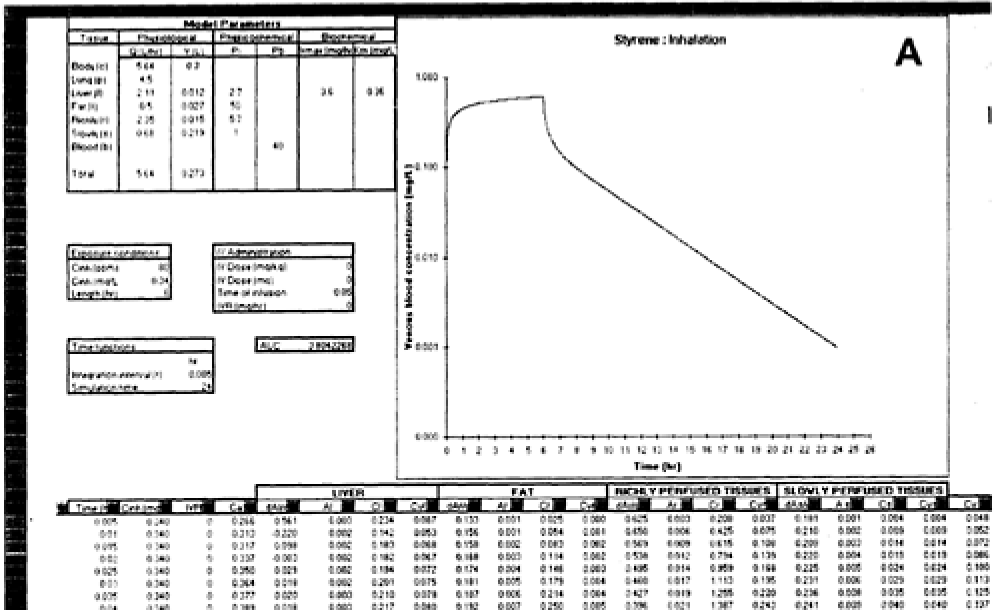
$$\frac{dA_l}{dt} = Q_l(C_a - C_{vl}) - \frac{dA_{met}}{dt} + K_o A_{stom} \qquad (29)$$

The above approach requires that parameters to define oral dose in mg/kg, and *Do* in mg and rate of oral absorption *(Ko)* be included along with mass balance equations for the stomach compartment and modifications to the liver reflecting the input from the stomach. More complicated descriptions may be required in certain cases depending on the vehicle of administration, extent of intestinal retention, and other factors (129, 251).

For simulating dermal absorption of chemicals, it is essential to introduce a skin compartment in the model and write the mass balance differential equation (MBDE) for that compartment. The chemical uptake by the skin may be described in the same way as the other tissues. To this MBDE, however, an

additional term to account for the absorption of chemical present in the environment should be added as follows (181):

$$\frac{dA_{sk}}{dt} = Q_{sk}(C_a - C_{vsk}) + K_p \times S[C_{air} - (C_{sk}/P_{s:a})] \quad (30)$$



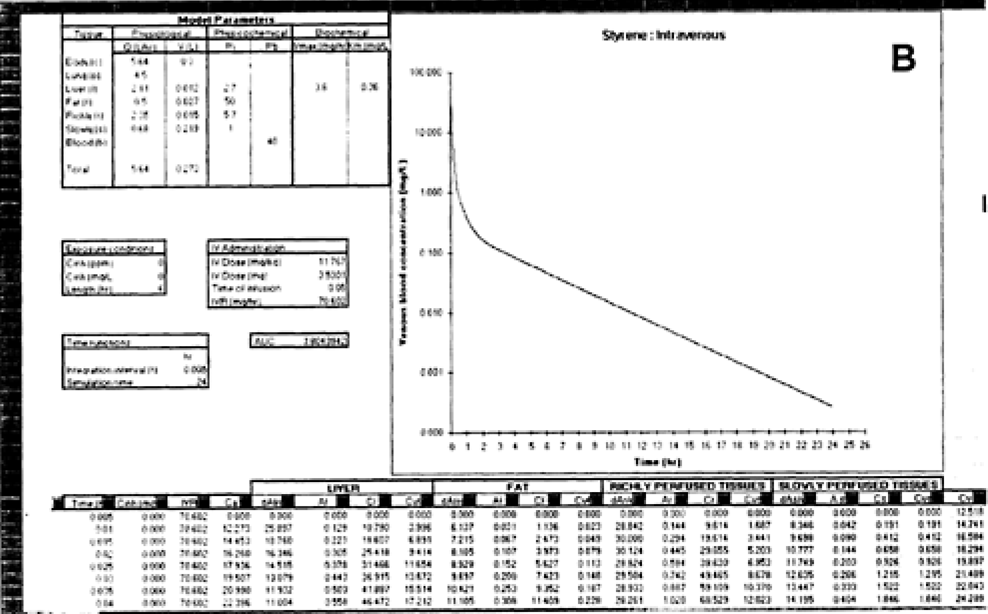


FIG. 5.18. Printout of computer screens depicting EXCEL spreadsheets with the PBPK modeling results obtained in rats exposed to styrene
(A) by inhalation (80 ppm, 6 h) or
(B) by intravenous administration (11.767 mg/kg). Each of the two panels contains
(1) a plot of PBPK model simulation of venous blood concentrations of styrene during and following exposure,
(2) the numerical values of the PBPK model parameters, and
(3) a section of the raw numbers corresponding to the rate of change in the amount of styrene in tissue, amount in tissue, concentration in tissue, and venous blood concentration leaving the tissues, generated during simulations between time 0.005 and 24 h.
Note that only the parameters related to the exposure route (Cinh, iv dose) differ between the two simulation exercises presented here.

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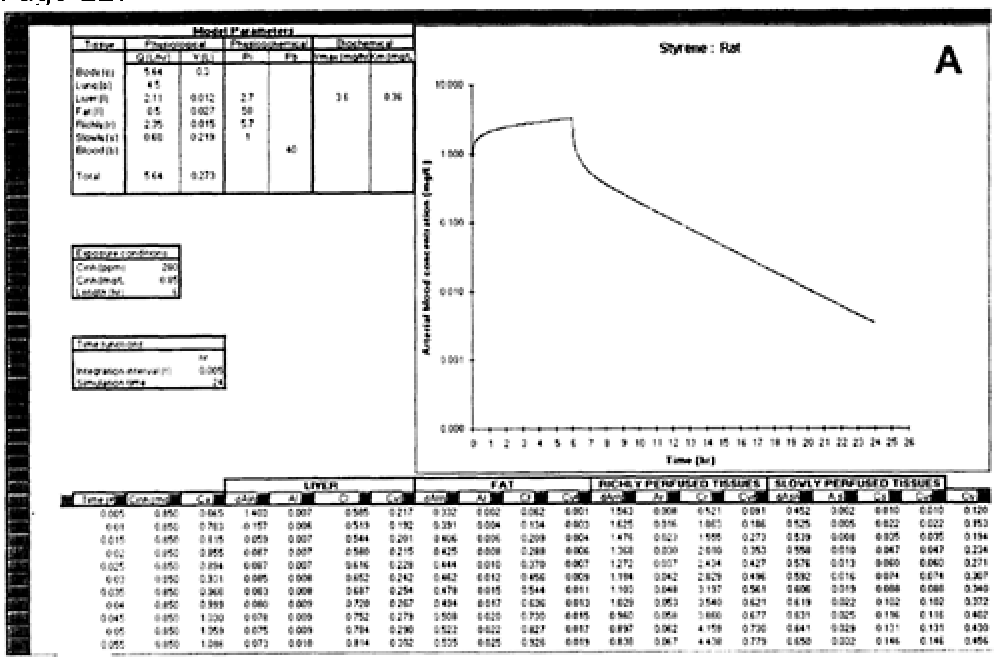

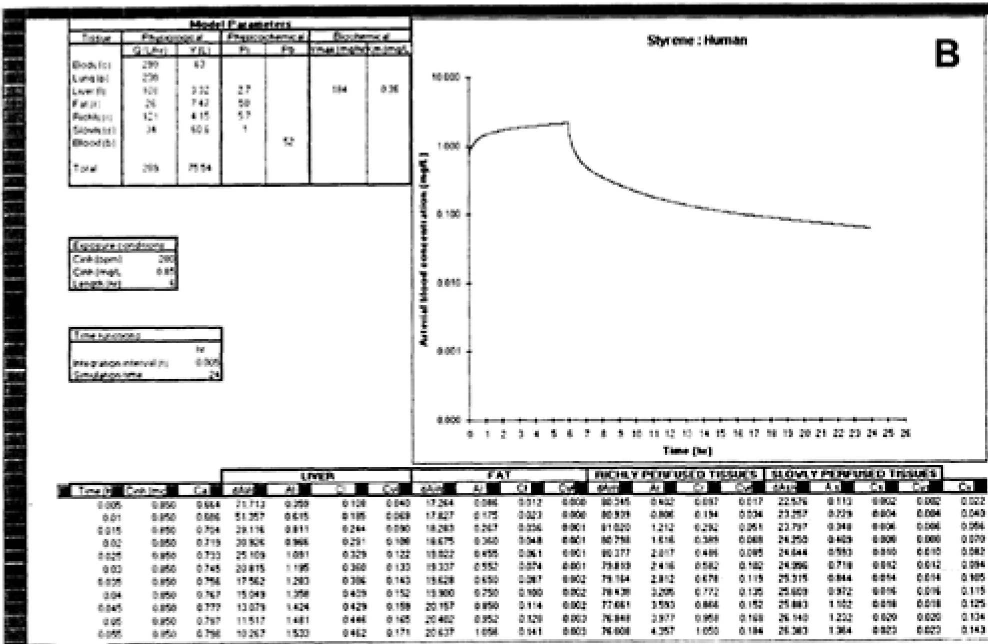

FIG. 5.19. Printout of computer screens depicting EXCEL spreadsheets with the PBPK modeling results obtained in
(A) rats and
(B) humans exposed to 200 ppm of styrene.
Each of the two panels contains
(1) a plot of PBPK model simulation of arterial blood concentrations of styrene during and following a 6-h exposure,
(2) the numerical values of the PBPK model parameters, and
(3) a section of the raw numbers corresponding to the rate of change in the amount of styrene in tissue, amount in tissue, concentration in tissue, and venous blood concentration leaving the tissues, generated during simulations between time 0.005 and 24 h.
Note that the numerical values of all species-specific parameters are different between the two simulation exercises presented here.

Table 5.11

Algebraic expressions for calculated blood and tissue concentrations under steady-state conditions

Parameter	Algebraic expression
Concentration in arterial blood[a,b]	$C_{a,ss} = \frac{C_{inh}}{1/P_b + Q_{lc}E}$
Concentration in venous blood[b]	$C_{v,ss} = C_{a,ss}(1 - Q_{1c}E)$
Concentration in non-metabolizing tissues	$C_{t,ss} = C_{a,ss}P_t$
Concentration in metabolizing tissues (e.g., liver)b	$C_{l,ss} = C_{a,ss}(1 - E) \times P_l$

[a] If $Q_p \neq Q_c$, $C_{a,ss} = \frac{Q_p C_i}{Q_p/P_b + Q_l E}$

[b] $E = \frac{V_{max}/K_m}{(V_{max}/K_m) + Q_l}$

Exposure Scenario Extrapolation

PBPK models also have been used to predict the kinetic behavior and tissue dosimetry of chemicals during unusual exposure scenarios (39). The ability to predict tissue dose of toxic moieties of chemicals during short-duration exposures to higher concentrations or during variable-exposure concentrations is a real challenge. Such extrapolations can be conducted with PBPK models by introducing a mathematical function that is consistent with the temporal change in the exposure concentration. In the spreadsheet depicted in Figure 5.19, all one has to do is change the numerical value corresponding to exposure concentration (Column C) at specific time points as required. This capacity of the PBPK models has been used to determine the adjusted threshold limit values (TLVs) during extended hours of work (25, 156). Further, quantitative changes in pharmacokinetics of chemicals due to environmental, pathological, and physiological alterations can be predicted by perturbation of the appropriate model parameter(s), for example physical activity/workload (156, 243), pregnancy and lactation (82, 91, 149, 246), or co-exposure to chemicals (147). During continued exposures, a steady-state is attained. Steady-state is a condition during which the rate of change in concentration of parent chemicals in tissues is equal to zero. The steady-state concentrations predicted using PBPK models can also be obtained using simple algebraic equations (Table 5.11) (203).

Interspecies Extrapolation

For conducting interspecies extrapolation of pharmacokinetic behavior of a chemical, quantitative estimates of species differences in the model parameter values (i.e., partition coefficients [PCs], physiological parameters, and metabolic rate constants) should be obtained.

The tissue-air PCs of chemicals appear to be relatively constant across species, while blood-air PCs show some species-dependent variability (92, 97). Therefore, the tissue-blood PCs, for species (e.g., humans) to which the pharmacokinetic data are to be scaled, have been calculated by dividing the rodent tissue-air PCs by the appropriate (e.g., human) blood-air partition values (223). The tissue: air and blood: air PCs for volatile organic chemicals (VOCs) may also be predicted using appropriate data on the content of lipids and water in human tissues and blood (Table 5.6; Figure 5.8) (217, 220).

Even though the physiological parameters vary coherently across species, the kinetic constants for metabolizing enzymes do not necessarily follow any type of readily predictable pattern, making the interspecies extrapolation of xenobiotic metabolism difficult. It is therefore preferable to obtain the metabolic rate constants for xenobiotics in the species of interest. In vivo approaches for determining metabolic rate constants are not always feasible for application in humans. The alternative is to obtain such data under in vitro conditions while the default position is to scale the metabolic rate constants obtained in vivo in rodents or in vitro using rodent and human tissue fractions. In the case of chemicals exhibiting high affinity (low *Km*) for metabolizing enzymes, V max has been scaled to approximately the 3/4th power of body weight, keeping the *Km* species-invariant. This approach may be useful as a crude approximation but should be used only when other direct measurements of metabolic parameters are not available or feasible. An example of rat-human extrapolation of the kinetics of styrene using a PBPK model is presented in Figure 5.19. In this example, the model was validated using human data for styrene. Whenever the human data

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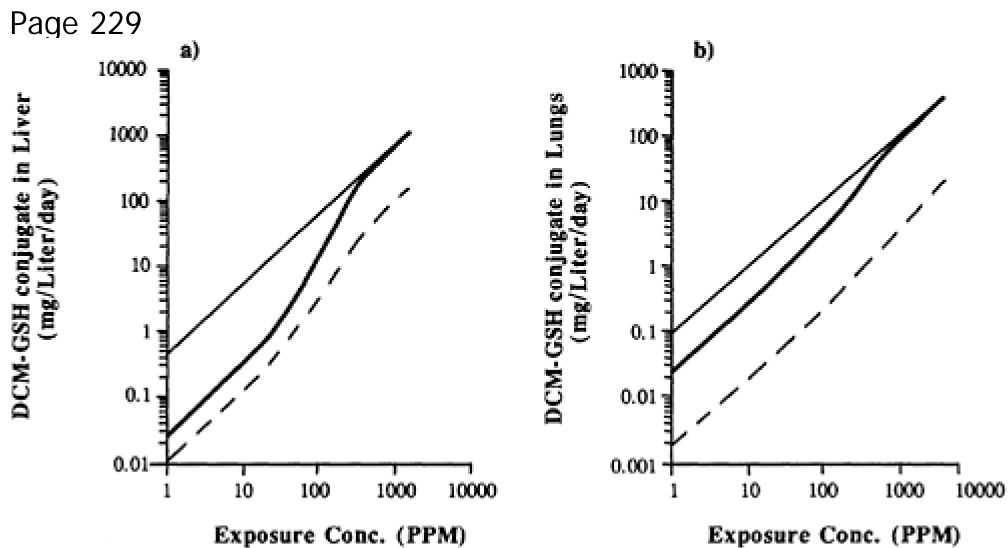

FIG. 5.20. Quantitative relationship between (a) liver or (b) lung dose of DCM-GSH conjugate and external exposure concentration of DCM determined with a PBPK model for B6C3F1 mice (heavy solid line) and humans (dashed line). The lighter solid line depicts linear back-extrapolation to 1 ppm. Redrawn with permission from Reference 10.

for a particular chemical are not available for validation purposes, a corollary approach permitting the use of human data on similar chemicals may be attempted (280).

Example of PBPK Model Application

The usefulness of PBPK models in reducing the uncertainties associated with high-dose-low-dose, route-route, and interspecies extrapolations has been demonstrated during the risk assessment process for a number of chemicals. This aspect was initially demonstrated with dichloromethane (DCM) (5). DCM caused liver and lung tumors in mice exposed to 2000 or 4000 ppm, 6 hr/day, for their lifetimes (189). DCM is metabolized by two processes:

(a) oxidation leading to the production of highly reactive formyl chloride, as well as carbon monoxide and small amounts of carbon dioxide, and

(b) GSH conjugation, yielding chloromethylglutathione (a reactive intermediate) and carbon dioxide (152).

Either of the reactive metabolites resulting from the oxidation or GSH conjugation could be involved in the mutagenic changes leading to cancer. In the PBPK model for DCM, these metabolic pathways were described according to their different kinetic characteristics. DCM metabolism in lung and liver was described with a saturable term for oxidation and with a linear term for reaction with GSH (5).

The PBPK model for DCM developed for the mouse by integrating quantitative information on physiological characteristics, partition coefficients, and metabolic rate constants, described adequately the disposition of DCM. The mouse DCM model then was used to calculate the tissue dose of metabolites and parent chemical arising from exposure scenarios comparable to those of the NTP bioassay studies (Table 5.12). The relationship of tissue dose of metabolic and parent chemical to the observed tumor incidence then was examined. Since the parent chemical is unreactive, it is unlikely to be directly involved in the tumorigenicity. Hence, the relationship between the tissue exposure to its metabolites and tumor incidence was examined (Table 5.12). Although the dose surrogate based on oxidative pathway did not vary between DCM exposure concentrations of 2000 and 4000 ppm, the flux through the GSH pathway did correspond well with the degree of DCM-induced cancer at these exposure concentrations. These observations are consistent with a role for the metabolite(s) arising from the GSH conjugation pathway in DCM-induced lung and liver cancer. The GSH conjugation of DCM, mediated by glutathione *S*-transferase enzymes, yields

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Table 5.12

Tumor incidence and calculated tissue dose of dichloromethane (DCM) metabolites following inhalation exposures of 0, 2000, or 4000 ppm DCM in female mice

DCM exposure concentration	Tumor incidence (%)	Tissue dose (amount in mg/liter tissue/day)	
		GSH pathway	Oxidative pathway
	A. Liver		
0	6	—	—
2000	33	851	3575
4000	83	1800	3701
	B. Lung		
0	6	—	—
2000	63	123	1531
4000	85	256	1583

From Reference 6 with permission.

formaldehyde as a metabolite. Casanova et al. (32) reported DNA–formaldehyde-protein crosslinks resulting from DCM exposure, further strengthening the case for GSH conjugation as the pathway leading to potentially carcinogenic metabolites. Therefore, high-doselow-dose extrapolation and interspecies extrapolation of DCM-induced cancer risk were conducted with the tissue dose of the GSH pathway metabolite predicted by the PBPK model.

High-Dose-Low-Dose Extrapolation

The model prediction of the target tissue dose of the DCM-GSH conjugate resulting from 6-h inhalation exposures to 1–4000 ppm of DCM is presented in Figure 5.20. The estimation of target tissue dose of DCM–GSH conjugate by linear back-extrapolation gives rise to a 21-fold higher estimate than that obtained by the PBPK modeling approach. This discrepancy arises from the nonlinear behavior of DCM metabolism at high exposure concentrations (Figure 5.21). At exposure concentrations exceeding 300 ppm, the cytochrome P450-mediated oxidation pathway is saturated, giving rise to a corresponding disproportionate increase in the flux through the GSH conjugation pathway.

Interspecies Extrapolation

The interspecies extrapolation of DCM disposition behavior was possible because the critical biological determinants were first identified in the test species, the mouse. Thus, the physiological parameters in the mouse PBPK model were scaled allometrically, the metabolic parameters were determined experimentally, and

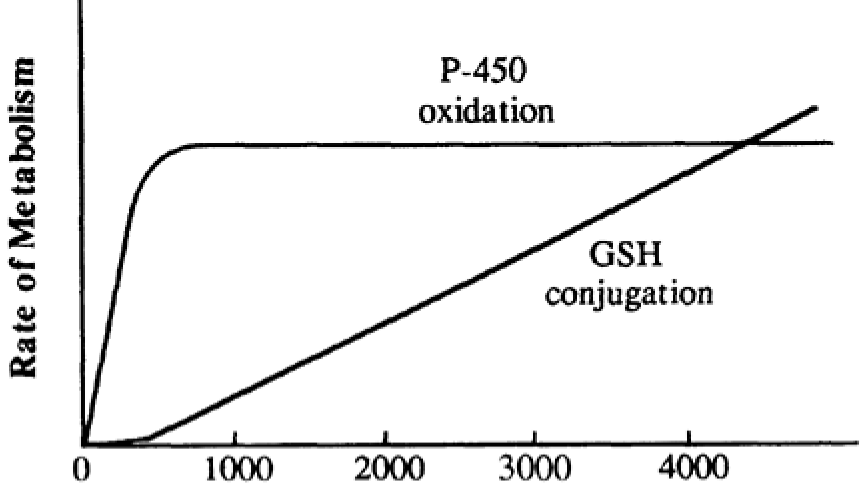

FIG. 5.21. Representation of the rate of metabolism of dichloromethane (DCM) at various inhaled concentrations. DCM is metabolized by P450-mediated oxidation and by conjugation with GSH. These two metabolic pathways have different characteristics. At low inhaled concentrations, oxidation is the preferred manner of metabolism, but at higher concentrations GSH conjugation becomes more favored. From Reference 10, with permission.

the tissue–blood PCs were assumed to be species-invariant. The PBPK model adequately simulated the blood levels of DCM observed in humans after a 6-h inhalation exposure to 100 or 350 ppm DCM. The target tissue-dose for humans was estimated to be some 2.7 times lower than that for the mouse. Considered together, the human tissue dose of DCM-GSH conjugate for a 6-h exposure to 1 ppm DCM is expected to be some 57 times lower than that expected by linear extrapolation of its behavior at high exposure concentrations, such as the ones used in the mouse cancer bioassay.

The cancer risk assessment for DCM, then, was conducted using the linearized multistage (LMS) model to relate tissue dose of DCM-GSH metabolite (rather than DCM exposure concentration) to the tumor incidence rates observed at high exposure concentrations in the mouse. In assessing the tumorigenic risks associated with human exposure to this chemical, it was assumed that humans are as sensitive as the most sensitive test species. Therefore, equal target tissue doses are expected to produce similar tumor incidence regardless of the species. This PBPK model-based DCM risk assessment predicted human low-dose risk about 100- to 200-fold less than that predicted by the U.S. Environmental Protection Agency (EPA) using standard default assumptions (249). With further refinement of the model with the estimation of the metabolic rate constants for humans in vitro (231), the PBPK model-based approach using tissue dose of the DCM-GSH conjugate predicted a cancer risk of 3.7×10^{-8} for a lifetime inhalation exposure of 1 $\mu g/m^3$.

Table 5.13

Examples of chemicals for which risk assessment has been performed using PBPK models

Chemical	References
Benzene	47
Chloroform	227
1,4-Dioxane	161, 229
Methylene chloride	213
Tetrachloroethylene	30, 34, 267
Trichloroethylene	19, 38, 49, 84
Vinyl chloride	38, 226

This risk estimate was still lower, by more than two orders of magnitude, than that calculated by the EPA using the standard default assumptions and exposure concentrations of DCM (270).

Following the DCM example, there have been several reports of the use of PBPK models for enhancing the scientific and mechanistic basis of cancer and non-cancer risk assessments of environmental agents (37, 197), (Table 5.13) The use of PBPK models in quantitative risk assessment does not always result in the estimation of risk lower than that derived from the conventional approach adopted by regulatory agencies. For example, if the test chemical is a direct-acting agent, the PBPK approach actually could predict greater risk to humans than conventional methods because enzyme-mediated metabolic clearance (detoxification) is expected to be lower in larger species. Similarly, if the toxicity of a chemical is mediated by reactive intermediates resulting from a saturable metabolic process, then the high-dose-low-dose extrapolation conducted with the PBPK modeling approach would predict a risk greater than that predicted by the linear extrapolation procedure.

CONCLUDING REMARKS

Modeling, in general terms, involves mathematical description of the interrelationships among critical parameters that determine the behavior of the system under study. Mathematical models can be constructed to fit experimental data by adjusting one or more model parameters, or by deriving an equation that describes the data. The latter approach reflects the methodological basis of conventional pharmacokinetic models. This approach might well be sufficient to describe the kinetics of prescription drugs and other pharmaceutical products, since these substances are often tested in humans in the dose range of prescription. Such empirical models are not sufficient in the case of environmental contaminants for which human health risk assessments need to be performed based on data obtained in animal studies conducted by administering high doses of chemicals by routes often different from anticipated human exposures. In this respect, mechanistically based PBPK models are useful for conducting the required extrapolations. So, the kind of modeling approach—physiological or nonphysiological—that is required/sufficient to describe the kinetics of a chemical depends on the intended use. If extrapolation to untested scenarios and design of useful mechanistic toxicology studies is the goal, then the choice will be PBPK modeling. However, the ability to conduct extrapolations with PBPK models will be compromised if the methods employed for developing and validating the models are inappropriate.

For example, PBPK models should not be constructed by assembling sets of equations, in which the parameters are not interpretable in terms of physicochemical, biochemical, or physiological properties. That sort of an approach will compromise the very basis of PBPK modeling, that is, the mechanistic basis. In other words, the mathematical equations employed in PBPK modeling should show clearly the interrelationships among the critical biological determinants. One should be able to dissect each equation into subsections, each of which describes a particular phenomenon (e.g., tissue uptake, metabolism). Further, the dissociation of each term should provide parameters that are biologically meaningful (e.g., breathing rate, tissue volume). If the mathematical descriptions employed in the model do not satisfy this basic criterion (i.e., use biologically relevant parameters), then it should not be considered a "true" physiologically based model, and the appropriateness of the use of such models in conducting extrapolations is questionable. The guiding principles of modeling in toxicology and the characteristics of a good modeling paper are summarized elsewhere (4).

A related problem is circularity in PBPK modeling. This aspect refers to the practice of using sets of experimental data to construct the model and then using the model to simulate the same data. Even though such an approach provides a framework to integrate various data sets, it does not instill confidence in using the model to predict the kinetic behavior of chemicals at untested exposure scenarios. Genuine PBPK modeling efforts should ensure that

(a) the assumptions on which the model is based are appropriate,

(b) the coding of model equations is errorless,
(c) the model parameter values are accurate, and
(d) the model is adequately tested/validated.

The model is as good as the input parameters. Therefore, accurate parameterization is fundamentally important for constructing useful PBPK models.

Methodological aspects of some of the important and widely used techniques for model parameterization were provided in the earlier sections of this chapter. A number of prototypical descriptions have also been provided to serve as examples for developing PBPK models for other chemicals of interest. These prototypical structures and descriptions may not be directly applicable to a chemical of interest to the researcher. The model structure and the phenomena to be represented in a particular model depend on the chemical whose kinetics are being modeled. Each chemical may possess some unique properties, thus presenting some very different problems and requiring the modification of existing model structures and functional representations. This might lead to the development of totally new descriptions. Thus, PBPK modeling is as much an art as a science. The creativity of the researcher is as much implied in the formulation of these models as the experimental techniques to obtain parameter estimates, such that novel model structures and descriptions will evolve continually in this field.

Parameter identifiability and model overspecification are problems inherent in these PBPK models or in any other multiparameter model. Direct measurement of model parameters by experimental methods, independent of analysis of tissue time-course curves, is the preferred approach. Nonetheless, limited numbers of parameters often will still have to be estimated by analysis of time-course data by curve-fitting techniques, under well-defined experimental conditions where the curves are particularly sensitive to the parameter(s) of interest.

The motivation for the use of PBPK models in toxicology research is to uncover the biological determinants of tissue dosimetry. These models are part of a systematic approach to studying how chemicals gain entry into, are distributed within, and are eliminated from the body. A major advantage of PBPK models is their use in designing critical mechanistic toxicological studies. With respect to the design of studies, PBPK and other biologically based models provide an opportunity to evaluate the various plausible hypotheses by computer simulation. We can ask questions of an "if-then" nature. For example, if the model structure is correct and the rate of a reaction or another process is varied, what is the expected impact on tissue dosimetry? The PBPK model can be used to generate quantitative predictions of the expected experimental outcome based on the most attractive hypothesis of the experimentalists, and this then can be verified experimentally. In this case, the model serves as a tool in designing experimental studies to enable efficient resource utilization and maintenance of the focus on human health risk assessment endpoints.

For example, examining the PBPK model-based risk assessment approach for DCM presented in this chapter, the following questions arise: Is GSH conjugation really the key determinant in DCM tumorigenesis? Are all the important biological determinants of uptake, metabolism, and disposition of DCM included in the model description? These are essentially biological, research-oriented questions with answers that rely on the state of knowledge of mechanisms of toxicokinetics and carcinogenicity of this chemical. Further, in the DCM example presented in this chapter, the low-dose extrapolation of the tissue response was conducted with the LMS modeling approach. The improvement over the conventional methodology is that here the independent variable specified in the LMS model—dose—is not administered dose or inhaled concentration; it is the tissue dose of the toxic moiety estimated with the PBPK model. The uncertainties associated with the low-dose extrapolation using the LMS model can be addressed with the use of biologically based response models (43).

Unlike the mandated mathematical models used in conventional risk assessment, the biologically based dosimetry and response models are versatile and, often, but not always, difficult to validate. In contrast to the "mandated" models, which are useful only for generating a risk number, the biologically based models allow integration of various observations, identification of critical data gaps, and estimation of risk numbers, along with attendant appreciation of areas of significant biological uncertainty.

QUESTIONS

1. Calculate the fat: blood partition coefficient for a chemical with a *Po:w* of 152.
2. Develop a conceptual representation for PBPK modeling of human exposure to airborne n-octane (*Po:w*=151356, *Pw:a*=0.00762).
3. Calculate the alveolar ventilation rate *(Qp)* for a human weighing 64 kg, knowing that the body weight-normalized *Qp* for mammals is 15 L/hr/kg.
4. The *V*max and *Km* of pyrene determined in vitro using rat liver post-mitochondrial fractions were 5.935×10^{-4} μmol/min per mg protein and 27.73 *μ*mol/L, respectively. Convert these potentially useful in vitro values for incorporation into in vivo model (protein concentration=88 mg protein/g liver, and liver weight=10 g).
5. Using the rat PBPK model for styrene presented in Figure 5.16 of this chapter, determine the external exposure concentration of styrene corresponding to an area under the curve for liver concentration of

150 mg/L/h (for the parent chemical). Set the exposure duration and the length of simulation to 24 hr.

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REFERENCES

1. Abbas, R., and Fisher, J.W. (1997): A physiologically based pharmacokinetic model for trichloroethylene and its metabolites, chloral hydrate, trichloroacetate, dichloroacetate, trichloroethanol, and trichloroethanol glucuronide in B6C3F1 mice. *Toxicol. Appl. Pharmacol.*, 147:15–30.
2. Andersen, M.E., Eklund, C.R., Mills, J.J., Barton, H.A., and Birnbaum, L.S. (1997): A multicompartment geometric model of the liver in relation to regional induction of cytochrome P450s. *Toxicol. Appl. Pharmacol.*, 144:135–144.
3. Andersen, M.E., and Keller, W.C. (1984): Toxicokinetic principles in relation to percutaneous absorption and cutaneous toxicity. In: *Cutaneous Toxicity,* edited by V.A.Drill and P.Lazar, pp. 9–27, Raven Press, New York.
4. Andersen, M.E., Clewell, H.J., III, and Frederick, C.B. (1995): Contemporary issues in toxicology. Applying simulation modeling in toxicology and risk assessment—A short perspective. *Toxicol. Appl. Pharmacol.,* 133:181–187.
5. Andersen, M.E., Clewell, H.J., III, and Gargas, M.L. (1991): Physiologically-based pharmacokinetic modeling with dichloromethane, its metabolite carbon monoxide and blood carboxyhemoglobin in rats and humans. *Toxicol. Appl. Pharmacol.,* 108:14–27.
6. Andersen, M.E., Clewell, H.J., III, Gargas, M.L., Smith, F.A., and Reitz, R.H. (1987): Physiologically-based pharmacokinetics and risk assessment process for methylene chloride. *Toxicol. Appl. Pharmacol.*, 87:185–205.
7. Andersen, M.E., Gargas, M.L., Jones, R.A., and Jenkins, L.J. (1980): Determination of the kinetic constants for metabolism of inhaled toxicants in vivo by gas uptake measurements. *Toxicol. Appl. Pharmacol.,* 54:116.
8. Andersen, M.E., MacNaughton, M.G., Clewell, H.J., III, and Paustenbach, D.J. (1987): Adjusting exposure limits for long and short exposure period using a physiological pharmacokinetic model. *Am. Ind. Hyg. Ass. J.,* 48:335–343.
9. Andersen, M.E., Mills, J.J., and Gargas, M.L. (1993): Modeling receptor-mediated processes with dioxin: Implications for pharmacokinetics and risk assessment. *Risk Anal.*, 13:25–36.
10. Andersen, M.E., and Krishnan, K. (1995): Relating in vitro to in vivo exposures with physiologically based tissue dosimetry and tissue response models. In: *Animal Test Alternatives: Refinement, Reduction, Replacement*, edited by H.Salem, pp. 2–25. Marcel Dekker, Inc., New York.
11. Anderson, M.W., Eling, T.E., Lutz, R.L., and Mattews, H.B. (1977): The construction of a pharmacokinetic model for the disposition of PCBs in the rat. *Clin. Pharmacol. Ther.*, 22:765–773.
12. Angelo, M.J., and Pritchard, A.B. (1987): Route to route extrapolation of dichloromethane exposures using a physiological pharmacokinetic model. *Drinking Water and Health*, 8:254–264.
13. Arms, A.D., and Travis, C.C. (1988): *Reference Physiological Parameters in Pharmacokinetic Modeling.* Office of Health and Environmental Assessment, U.S. Environmental Protection Agency (EPA), Washington, D.C. NTIS PB 88–196019.
14. Auton, M.J., and Woollen, B.H. (1991): A physiologically based mathematical model for the human inhalation pharmacokinetics of l,l,2-trichloro-l,2,2-trifluoroethane. *Int. Arch. Occup. Environ. Health*, 63:133–138.
15. Barton, H.A., Creech, J.R., Godin, S., Randall, G.M., and Seckel, C.S., (1995): Chloroethylene mixtures: Pharmacokinetic modeling and in vitro metabolism of vinyl chloride, trichloroethylene, and trans-l, 2-dichloroethylene in rat. *Toxicol. Appl. Pharmacol.*, 130:237–247.
16. Benignus, V.A., Boyes, W.K., and Bushnell, P.J. (1998): A dosimetric analysis of behavioral effects of acute toluene exposure in rats and humans. *Toxicol. Sci.*, 43:186–195.
17. Bischoff, K.B. (1987): Physiogically-based pharmacokinetic modeling. *Drinking Water and Health,* 8:36–64.
18. Bischoff, K.B., Dedrick, R.L., Zakharo, D.S., and Longstreth, J. A. (1971): Methotrexate pharmacokinetics. *J. Pharm. Sci.,* 60:1128–1133.
19. Bogen, K.T., and Gold, L.S. (1997): Trichloroethylene cancer risk: Simplified calculation of PBPK-based MCLs for cytotoxic end points. *Regul. Toxicol. Pharmacol.*, 25:26–43.
20. Bois, F.Y., Smith, M.T., and Spear, R.C. (1991): Mechanism of benzene carcinogenesis. Application of a physiological model of benzene pharmacokinetics and metabolism. *Toxicol. Lett.*, 56:283–298.
21. Bois, F.Y., Woodruff, T.J., and Spear, R.C. (1991): Comparison of three physiologically-based pharmacokinetic models for benzene disposition. *Toxicol. Appl. Pharmacol.*, 110:79–88.
22. Bois, F.Y., Zeise, L., and Tozer, T.N. (1990): Precision and sensitivity of pharmacokinetic models for cancer risk assessment. Tetrachloroethylene in mice, rats and humans. *Toxicol. Appl. Pharmacol.*,

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_233.html[2010/10/28 18:42:07]

102:300–315.
23. Bond, J.A., Himmelstein, M.W., Seaton, M., Boogaard, P., and Medinsky, M.A. (1996): Metabolism of butadiene by mice, rats, and humans: A comparison of physiologically based toxicokinetic model predictions and experimental data. *Toxicology*, 113:48–54.
24. Borghoff, S.J., Murphy, J.E., and Medinsky, M.A. (1996): Development of a physiologically based pharmacokinetic mode for methyl tertiary-butyl ether and tertiary-butanol in male Fischer-344 rats. *Fundam. Appl. Toxicol.*, 30:264–275.
25. Brodeur, J., Laparé, S., Krishnan, K., Tardif, R., and Goyal, R. (1990): Le problème de 1′ajustement des valeurs limites d′exposition pour des horaires de travail non-conventionnels: Utilité de la modelisation pharmacocinétique a base physiologique. *Travail et Santé*, 6:811–16.
26. Brown, R.P., Delp, M.D., Lindstedt, S.L., Rhomberg, L.R., and Belisle, R.P. (1997): Physiological parameter values for physiologically based pharmacokinetic models. *Toxicol. Ind. Health*, 13:407–484.
27. Bungay, P.M., Dedrick, M.L., and Mattews, H.B. (1981): Enteric transport of chlordecone in the rat. *J. Pharmacokin. Biopharm.*, 9:309–341.
28. Byczkowski, J.Z., Kinkead, E.R., Leahy, H.F., Randall, G.M., and Fisher, J.W. (1994): Computer simulation of the lactational transfer of tetrachloroethylene in rats using a physiologically based model. *Toxicol. Appl. Pharmacol.*, 125:228–236.
29. Byczkowski, J.Z., and Fisher, J.W. (1994): Lactational transfer of tetrachloroethylene in rats. *Risk Anal.*, 14:339–349.
30. Byczkowski, J.Z., and Fisher, J.W. (1995): A computer program linking physiologically based pharmacokinetic model with cancer risk assessment for breast-fed infants. *Comput. Methods Programs Biomed.*, 46:155–163.
31. Cantoreggi, S., and Keller, D.A. (1997): Pharmacokinetics and metabolism of vinyl fluoride in vivo and in vitro. *Toxicol. Appl. Pharmacol.*, 143:130–139.
32. Casanova, M., d′Heck, H., and Deyo, D.F. (1992): Dichloromethane (methylene chloride): Metabolism to formaldehyde and formation of DNA-protein crosslinks in B6C3F1 mice and Syrian golden hamsters. *Toxicol. Appl. Pharmacol.*, 114:162–165.
33. Caster, W.O., Poncelet, J., Simon, A.B., and Armstrong, W.B. (1956): Tissue weights of the rat. I. Normal values determined by dissection and chemical methods. *Proc. Soc. Exp. Biol. Med.*, 91:122–126.
34. Chen, C.W., and Blancato, J.N. (1987): Role of pharmacokinetic modeling in risk assessment: Perchloroethylene as an example.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_233.html[2010/10/28 18:42:07]

In: *Pharmacokinetics in Risk Assessment. Drinking Water and Health*, 8:369–385.
35. Chen, H.S.G., and Gross, J.F. (1979): Estimation of tissue to plasma partition coefficients used in physiological pharmacokinetic models. *J. Pharmacokin. Biopharm.*, 7:117–125.
36. Clarke, D.O., Elswick, B.A., Welsch, F., and Conolly, R.B. (1993): Pharmacokinetics of 2-methoxyethanol and 2-methoxyacetic acid in the pregnant mouse: A physiologically-based mathematical model. *Toxicol. Appl. Pharmacol.*, 121:239–252.
37. Clewell H.J., Gentry, P.R., and Gearhart, J.M. (1997): Investigation of the potential impact of benchmark dose and pharmacokinetic modeling in noncancer risk assessment. J. *Toxicol. Environ. Health*, 52:475–515.
38. Clewell H.J., Gentry, P.R., Gearhart, J.M., Allen, B.C., and Andersen, M.E. (1995): Considering pharmacokinetic and mechanistic information in cancer risk assessments for environmental contaminants—examples with vinyl chloride and trichloroethylene. *Chemosphere*, 31:2561–2578.
39. Clewell, H.J. III, and Andersen, M.E. (1987): Dose, species and route extrapolation using physiologically-based pharmacokinetic models. *Drinking Water and Health*, 8:159–182.
40. Clewell, H.J. III, and Jarnot, B.M. (1994): Incorporation of pharmacokinetics in noncancer risk assessment: Example with chloropentafluorobenzene. *Risk Anal.*, 14:265–276.
41. Cohn, M.S. (1987): Sensitivity analysis in pharmacokinetic modeling. *Drinking Water and Health*, 8:265–272.
42. Collins, J.M., Dedrick, R.L., Flessner, M.F., and Guarino, A.M. (1982): Concentration dependent disappearance of fluorouracil from peritoneal fluid in the rat: Experimental observations and distributed modeling. *J. Pharm. Sci.*, 71:735–738.
43. Conolly, R.B., and Andersen, M.E. (1991): Biologically based pharmacodynamic models: Tools for toxicological research and risk assessment. *Annu. Rev. Toxicol. Pharmacol.*, 31:503–523.
44. Corley, R.A., Bormett, G.A., and Ghanayem, B.I. (1994): Physiologically based pharmacokinetics of 2-butoxyethanol and its major metabolite, 2-butoxyacetic acid, in rats and humans. *Toxicol. Appl. Pharmacol.*, 129:61–79.
45. Corley, R.A., Mandrela, A.L., and Smith, F.A. (1990): Development of a physiologically-based pharmacokinetic model for chloroform. *Toxicol. Appl. Pharmacol.*, 103:512–527.
46. Corley, R.A., Markham, D.A., Banks, C., Delorme, P., Masterman, A., and Houle, J.M. (1997): Physiologically based pharmacokinetics and the dermal absorption of 2-butoxyethanol vapor by humans. *Fundam. Appl. Pharmacol.*, 39:120–130.
47. Cox. L.A. (1996): Reassessing benzene risks using internal doses and Monte Carlo uncertainty analysis. *Environ. Health Perspect.*, 104 (suppl.6):1413–1429.
48. Crank, W.D., and Vinegar, A. (1992): A physiologically-based pharmacokinetic model for chloropentafluorobenzene in primates to be used in the evaluation of protective equipment against toxic gases. *Toxicol. Ind. Health*, 8:21–35.
49. Cronin, W.J., Oswald, E.J., Shelley, M.L., Fisher, J.W., and Flemming, C.D. (1995): A trichlororthylene risk assessment using a Monte Carlo Analysis of parameter uncertainty in conjunction with physiologically-based pharmacokinetic modeling. *Risk Anal.*, 15:555–565.
50. Csanady, G.A., Kreuzer, P.E., Baur, C., and Filser, J.G. (1996): A physiological toxicokinetic model for 1,3-butadiene in rodents and man: Blood concentrations of 1,3-butadiene, its metabolically formed epoxides, and of haemoglobin adducts-relevance of glutathione depletion. *Toxicology*, 113:300–305.
51. Dallas, C.E., Bruckner, J.V., Megden, J.L., and Weir, F.W. (1986): A method for direct measurement of systemic uptake and elimination of volatile organics in small mammals. *J. Pharmacol. Meth.*, 16:239–250.
52. Dallas, C.E., Chen, X.M., Muralidhara, S., Varkonyl, P., Tackett, L., and Bruckner, J.V. (1995): Physiologically based pharmacokinetic model useful in prediction of the influence of species, dose, and exposure route on perchloroethylene pharmacokinetics. *J. Toxicol. Environ. Health*, 44:301–317.
53. Dallas, C.E., Chen, X.M, O'Barr, K., Muralidhara, S., Varkonyl, P., and Bruckner, J.V. (1994): Development of a physiologically based pharmacokinetic model for perchloroethylene using tissue concentration-time data. *Toxicol. Appl. Pharmacol.*, 128:50–59.
54. Dallas, C.E., Gallo, J.M., Ramanthan, R., Muralidhara, S., and Bruckner, J.V. (1991): Physiological pharmacokinetic modeling of inhaled trichloroethylene in rats. *Toxicol. Appl. Pharmacol.*, 110:303–314.
55. Dallas, C.E., Ramanthan, R., Muralidhara, S., Gallo, G.M., and Bruckner, J.V. (1989): The uptake and elimination of 1,1,1-trichloroethane during the following inhalation exposures in rats. *Toxicol. Appl. Pharmacol.*, 98:385–397.
56. Dalley, J.W., Gupta, P.K., and Hung, C.T. (1990): A physiological pharmacokinetic model describing

the disposition of lead in the absence and presence of 1-ascorbic acid in rats. *Toxicol. Lett.*, 50:337–348.
57. Davies, B., and Morris, T. (1993): Physiological parameters in laboratory animals and humans. *Pharm. Res.*, 10:1093–1095.
58. Dedrick, R.L., Forrester, D.D., and Ho, D.H.W. (1972): In vitro-in vivo correlation of drug metabolism: Deamination of l-β-D-arabinosyl cytosine. *Biochem. Pharmacol.*, 21:1–16.
59. Dedrick, R.L., Zaharko, D.S., and Lutz, R.J. (1973): Transport and binding of methotrexate in vivo. *J. Pharm. Sci.*, 62:882–890.
60. DeJongh, J., and Blaauboer, B.J. (1996): In vitro-based and in vivo-based simulations of benzene uptake and metabolism in rats. *ATLA*, 24:179–190.
61. DeJongh, J., and Blaauboer, B.J. (1997): Simulation of lindane kinetics in rats. *Toxicology*, 122:1–9.
62. DeJongh, J., and Blaauboer, B.J. (1996): Simulation of toluene kinetics in the rat by a physiologically based pharmacokinetic model with application of biotransformation parameters derived independently in vitro and in vivo. *Fundam. Appl. Toxicol.*, 32:260–268.
63. DeJongh, J., and Blaauboer, B.J. (1997): Evaluation of in vitro-based simulations of toluene uptake and metabolism in rats. *Toxicol. in vitro*, 11:485–489.
64. DeJongh, J., Verhaar, H.J.M., and Hermens, J.L.M. (1997): A quantitative property-property relationship (QPPR) approach to estimate in vitro tissue-blood partition coefficients of organic chemical sin rats and humans. *Arch. Toxicol.*, 72:17–25.
65. Delp, M.D., Manning, R.O., Bruckner, J.V., and Armstrong, R. B. (1991): Distribution of cardiac output during diurnal changes of activity in rats. *Am. J. Physiol.*, 261:H1487–1493.
66. Dietz, K.F., Rodriguez-Giaxola, M., Traiger, G.J., Stella, V.J., and Himmelstein, K.J. (1981): Pharmacokinetics of 2-butanol and its metabolites in the rat. *J. Pharmacokin. Biopharm.*, 9:553–573.
67. Domenech, R.J., Hoffman, J.E., Noble, M.M., Saunder, K.B., Hensen, J.R., and Subijanto, S. (1969): Total and regional coronary blood flow measured by radioactive microsphere in conscious and anesthetized dogs. *Circul. Res.*, 25:581–596.
68. Droz, P.O., Berode, M., and Jang, J.Y. (1999): Biological monitoring of tetrahydrofuran: Contribution of a physiologically based pharmacokinetic model. *Am. Ind. Hyg. Ass. J.*, 60:243–248.
69. D'Souza, R.W., and Andersen, M.E. (1988): Physiologically-based pharmacokinetic model for vinylidine chloride. *Toxicol. Appl. Pharmacol.*, 95:230–240.
70. D'Souza, R.W., Francis, W.R., and Andersen, M.W. (1988): Physiological model for tissue glutathione depletion and decreased

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_234.html[2010/10/28 18:42:08]

resynthesis after ethylene dichloride exposures. *J. Pharmacol. Exp. Ther.*, 245:563–568.
71. Ellis, M.K., Trebilcock, R., Naylor, J.L., Tseung, K., Collins, M. A., Hext, P.M., and Green, T. (1996): The inhalation toxicology, genetic toxicology, and metabolism of difluoromethane in the rat. *Fundam. Appl. Toxicol.*, 31:243–251.
72. El-Masri, H.A., and Portier, C.J. (1998): Physiologically-based pharmacokinetics model of primidone and its metabolites phenobarbital and phenylethylmalonamide in humans, rats and mice. *Drug Metab. Dispos.*, 26:585–594.
73. El-Masri, H.A., Bell, D.A., and Portier, C.J. (1999). Effects of glutathione transferase theta polymorphism on the risk estimates of dichloromethane to humans. *Toxicol. Appl. Pharmacol.*, 158:221–230.
74. Falk, A., Gullstrand, E., Löf, A., and Wigaeus-Hjelm, E. (1990): Liquid/air partition coefficients of four terpenes. *Br. J. Ind. Med.*, 47:62–64.
75. Farrar, D., Allen, B., Crump, K., and Shipp, A. (1989): Evaluation of uncertainty in input parameters to pharmacokinetic models and the resulting uncertainty in output. *Toxicol. Lett.*, 49:371–385.
76. Farris, F.F., Dedrick, R.L., Allen, P.V., and Smith, J.C. (1993): Physiological model for the pharmacokinetics of methylmercury in the growing rat. *Toxicol. Appl. Pharmacol.*, 119:74–90.
77. Farris, F.F., Dedrick, R.L., and King, F.G. (1988): Cisplatin pharmacokinetics: Applications of a physiological model. *Toxicol. Lett.*, 43:117–137.
78. Filser, J.G., and Bolt, H.M. (1979): Pharmacokinetics of halogenated ethylenes in rats. *Arch. Toxicol.*, 42:123–136.
79. Filser, J.G., Csanady, G.A., Denk, B., Hartmann, M., Kauffman, A., Kessler, W., Kreuzer, P.E., Putz, C., Shen, J.H., and Stei, P. (1996): Toxicokinetics of isopropene in rodents and humans. *Toxicology*, 113:278–287.
80. Fiserova-Bergerova, V. (1975): Mathematical modeling of inhalation exposure. *J. Combust. Toxicol.*, 32:201–210.
81. Fiserova-Bergerova, V., and Diaz, M.L. (1986): Determination and prediction of tissue-gas partition coefficients. *Int. Arch. Occup. Environ. Health*, 58:75–87.
82. Fisher, J.W., Whittaker, T.A., Taylor, D.H., Clewell, H.J., and Andersen, M.E. (1990): Physiologically-based pharmacokinetic modeling of the lactating rat and nursing pup: A multiroute exposure model for trichloroethylene and its metabolite, trichloroacetic acid. *Toxicol. Appl. Pharmacol.*, 102:497–513.
83. Fisher, J., Mahle, D., Bankston, L., Greene, R., and Gearhart, J. (1997): Lactational transfer of volatile chemicals in breast milk. *Ind. Hyg. Assoc. J.*, 58:425–431.
84. Fisher, J.W., and Allen, B.C. (1993): Evaluating the risk of liver cancer in humans exposed to trichloroethylene using physiological models. *Risk Anal.*, 13:87–95.
85. Fisher, J.W., Gargas, M.L., Jepson, G.W., Allen, B., and Andersen, M.E. (1991): Physiologically based pharmacokinetic modeling with trichloroethylene and its metabolite, trichloroacetic acid in the rat and mouse. *Toxicol. Appl. Pharmacol.*, 109:183–195.
86. Fisher, J.W., Mahle, D., and Abbas, R. (1998): A human physiologically based pharmacokinetic model for trichloroethylene and its metabolites, trichloroacetic acid and free trichloroethanol. *Toxicol. Appl. Pharmacol.*, 152:339–359.
87. Fisher, J.W., Whittaker, T.A., Taylor, D.H., Clewell, H.J., and Andersen, M.E. (1989): Physiologically-based pharmacokinetic modeling of the pregnant rat: Multiroute exposure model for trichloroethylene and trichloroacetic acid. *Toxicol. Appl. Pharmacol.*, 99:395–414.
88. Frederick, C.B., Bush, M.L., Lomax, L.M., Black, K.A., Finch, L., Kimbell, J.S., Morgan, K.T., Subramaniam, R.P., Morris, J.B., and Ultman, J.S. (1998): Application of a hybrid computational fluid dynamics and physiologically based inhalation model for interspecies dosimetry extrapolation of acidic vapors in the upper airways. *Toxicol. Appl. Pharmacol.*, 152:211–231.
89. Frederick, C.B., Potter, D.W., Chang-Mateu, M.L, and Andersen, M.E. (1992): A physiologically-based pharmacokinetic and pharmacodynamic model to describe the oral dosing of rats with ethyl acrylate and its implications for risk assessment. *Toxicol. Appl. Pharmacol.*, 114:246–260.
90. Freeman, R.A., Rozman, K.K., and Wilson, A.G. E. (1989): Physiological pharmacokinetic model of hexachlorobenzene in the rat. *Health Phys.*, 57:139–147.
91. Gabrielsson, J.L., Paalkow, L.K., and Nordstrom, L. (1987): A physiologically-based pharmacokinetic model for theophylline disposition in the pregnant and nonpregnant rat. *J. Pharmacokin. Biopharm.*, 12:149–165.
92. Gallo, J.M., Lam, F.C., and Perrier, D.G. (1987): Area method for the estimation of partition coefficients for physiological pharmacokinetic models. *J. Pharmacokin. Biopharm.*, 15:271–280.

93. Gargas, M.L. (1990): An exhaled breath chamber system for assessing rates of metabolism and rates of gastrointestinal absorption with volatile chemicals. *J. Am. Coll. Toxicol.,* 9:447–453.
94. Gargas, M.L., and Andersen, M.E. (1989): Determinating the kinetic constants of chlorinated ethane metabolism in the rat from rates of exhalation. *Toxicol. Appl. Pharmacol.,* 99:344–353.
95. Gargas, M.L., Andersen, M.E., and Clewell, H.J. (1986): A physiologically-based simulation approach for determining metabolic rate constants from gas uptake data. *Toxicol. Appl. Pharmacol.,* 86:341–352.
96. Gargas, M.L., Andersen, M.E., Teo, S.K., Batra, R., Fennell, T. R., and Kedderis, G.L. (1995): A physiologically based dosimetry description of acrylonitrile and cyanoethylene oxide in the rat. *Toxicol. Appl. Pharmacol.,* 134:185–194.
97. Gargas, M.L., Burgess, R.J., Voisard, D.E., Cason, G.H., and Andersen, M.E. (1989): Partition coefficients of low molecular weight volatile chemicals in various liquids and tissues. *Toxicol. Appl. Pharmacol.,* 98:87–99.
98. Gargas, M.L., Clewell, H.J., and Andersen, M.E. (1986): Gas uptake inhalation techniques and the rates of metabolism of chloromethanes, chloroethanes and chloroethylenes in the rat. *Inhal. Toxicol.,* 2:319.
99. Gargas, M.L., Clewell, H.J., and Andersen, M.E. (1986): Metabolism of inhaled dihalomethanes in vivo: Differentiation of kinetic constants for two independent pathways. *Toxicol. Appl. Pharmacol.,* 87:211–223.
100. Gargas, M.L., Seybold, P.G., and Andersen, M.E. (1988): Modeling the tissue solubilities and metabolic rate constants of halogenated methanes, ethanes and ethylenes. *Toxicol. Lett.,* 43:235–256.
101. Gear, C.W. (1971): *Numerical Initial Value Problems in Ordinary Differential Equalions.* Prentice-Hall, Englewoods Cliffs, New Jersey.
102. Gearhart, J.M, Clewell, H.J.I., Crump, K.S., Shipp, A.M., and Silvers, A. (1995): Pharmacokinetic dose estimates of mercury in children and dose-response curves of perfomance tests in a large epidemiological study. *Water Air Soil Pollut.,* 80:49–58.
103. Gearheart, J.M., Jepson, G.W., Clewell, H.J., Andersen, M.E., and Conolly, R.B. (1990): A physiologically-based model for the in vivo inhibition of acetylcholinesterase by diisopropylfluorophosphate. *Toxicol. Appl. Pharmacol.,* 106:295–310.
104. Georgopoulos, P.G., Roy, A., and Gallo, M.A. (1994): Reconstruction of short-term multi-route exposure to volatile organic compounds using physiologically based pharmacokinetic models. *J. Expos. Anal. Environ. Epidemiol.,* 4:309–328.
105. Gerde, P., and Dahl, A.R. (1991): A model for the uptake of inhaled vapors in the nose of the dog during cyclic breathing. *Toxicol. Appl. Pharmacol.,* 109:276–288.

106. Gibaldi, M., and Perrier, D. (1982): *Pharmacokinetics.* Marcel Dekker, New York.
107. Gray, D.G. (1995): A physiologically based pharmacokinetic model for methyl mercury in the pregnant rat and fetus. *Toxicol. Appl. Pharmacol.,* 132:91–102.
108. Greenberg, M.S., Burton, G.A., and Fisher, J.W. (1999): Physiologically based pharmacokinetic modeling of inhaled trichloroethylene and its oxidative metabolites in B6C3F1 mice. *Toxicol. Appl. Pharmacol.,* 154:264–278.
109. Haddad, S., Gad, S.C., Tardif, R., and Krishnan, K. (1995): Statistical approaches for the validation of physiologically-based pharmacokinetic (PBPK) models. *Toxicologist*, 15 (258).
110. Haddad, S., Pelekis, M., and Krishnan, K. (1996): A methodology for solving physiologically based pharmacokinetic models without the use of simulation softwares. *Toxicol. Lett.*, 85:113–126.
111. Haddad, S., Withey, J., Lapare, S., Law, F., and Krishnan, K. (1998): Physiologically-based pharmacokinetic modeling of pyrene in the rat. *Environ. Toxicol. Pharmacol.,* 5:245–255.
112. Haggard, H.W. (1924): The absorption, distribution and elimination of ethyl ether. Analysis of the mechanism of the absorption and elimination of such a gas or vapor as ethyl ether. *J. Biol. Chem.*, 59:753–770.
113. Hallenbeck, W.H. (1992). Cancer risk assessment for the inhalation 1,3-butadiene using PBPK modeling. *Bull. Environ. Contam. Toxicol.,* 49:66–70.
114. Hattis, D., White, P., Marmorstein, L., and Koch, P. (1990): Uncertainties in pharmacokinetics modeling for perchloroethylene. I. Comparison of model structure, parameters, and predictions for low dose metabolic rates for models by different authors. *Risk Anal.*, 10:449–458.
115. Hetrick, D.M., Jarabek, A.M., and Travis, C.C. (1991): Sensitivity analysis for physiologically-based pharmacokinetic models. *J. Pharmacokin. Biopharm.*, 19:1–20.
116. Hilderbrand, R.L., Andersen, M.E., and Jensen, L.J. (1981): Prediction of in vivo kinetic constants for metabolism of inhaled vapors from kinetic constants measured in vitro. *Fundam. Appl. Toxicol.*, 1:403–409.
117. Horton, V.L., Higuchi, M.A., and Rickert, D.E. (1992): Physiologically based pharmacokinetic model for methanol in rats, monkeys and humans. *Toxicol. Appl. Pharmacol.*, 117:26–36.
118. Igari, Y., Sugiyama, Y., Sawada, Y., Iga, Y., and Hanano, M. (1983): Prediction of diazepam disposition in rat and man by a physiologically-based pharmacokinetic model. *J. Pharmacokin. Biopharm.*, 11:577–593.
119. Iman, R., and Helton, J. (1988): An investigation of uncertainty and sensitivity analysis techniques for computer models. *Risk Ana*l., 8:71–90.
120. International Commission on Radiation Protection (1975): *Report of the task group on reference man.* ICRP Publication No. 23. Pergamon Press, New York.
121. Iwatsubo, T., Suzuki, H., and Sugiyama, Y. (1997). Prediction of species differences (rats, dogs, humans) in the in vivo metabolic clearance of YM796 by the liver from in vitro data. *J. Pharmacol. Exp. Ther.*, 283:462–469.
122. Iyengar, S., and Rao, M.S. (1983): Statistical techniques in modeling of complex systems: Single versus multiresponse models. *IEEE Trans. Syst. Man. Cybernet.*, 13:175–189.
123. Jain, R.K., Gerlowski, L.E., Weissbrod, J.M., Wang, J., and Pierson, R.N. (1982): Kinetics of uptake, distribution and excretion of zinc in rats. *Ann. Biomed. Eng.*, 9:347–361.
124. Järnberg, J., and Johanson, G. (1999): Physiologically based modeling of 1,2,4-trimethylbenzene inhalation toxicokinetics. *Toxicol. Appl. Pharmacol.*, 155:203–214.
125. Johanson, G. (1986): Physiologically-based pharmacokinetic modeling of inhaled 2-butoxyethanol in man. *Toxicol. Lett.*, 34:23–31.
126. Johanson, G. (1991): Modeling of respiratory exchange of polar solvents. *Ann. Occup. Hyg.*, 35:323–339.
127. Johanson, G., and Dynesius, B. (1988): Liquid:air partition coefficients for six commonly used glycol ethers. *Br. J. Ind. Med.*, 45:561–564.
128. Johanson, G., and Filser, J.G. (1992): Experimental data from closed chamber gas uptake studies in rodents suggest lower uptake rate of chemical than calculated from literature values on alveolar ventilation. *Arch. Toxicol.*, 66:291–295.
129. Johanson, G., and Filser, J.G. (1993): A physiologically based pharmacokinetic model for butadiene and its metabolite butadiene monoepoxide in rat and mouse and its significance for risk extrapolation. *Arch. Toxicol.*, 67:151–163.
130. Johanson, G., and Filser, J.G. (1996): PBPK model for butadiene metabolism to epoxides: Quantitative species differences in metabolism. *Toxicology*, 113:40–47.

131. Johanson, G., and Naslund, P.H. (1988): Spreadsheet programming: A new approach in physiologically-based modeling of solvent toxicokinetics. *Toxicol. Lett.*, 41:115–127.
132. Kaneko, T., Endoh, K., and Sato, A. (1991): Biological monitoring of exposure to organic solvent vapors. I. A physiological simulation model of m-xylene pharmacokinetics in man. *Yamanashi Med. J.*, 6:127–135.
133. Kedderis, G.L., and Held, S.D. (1996): Prediction of furan pharmacokinetics from hepatocyte studies: Comparison of bioactivation and hepatic dosimetry in rats, mice, and humans. *Toxicol. Appl. Pharmacol.*, 140:124–130.
134. Kedderis, G.L., Teo, S.K., Batra, R., Held, S.D., and Gargas, M. L. (1996): Refinement and verification of the physiologically based dosimetry description for acrylonitrile in rats. *Toxicol. Appl. Pharmacol.*, 140:422–435.
135. Kedderis, L.B., Mills, J.J., Andersen, M.E., and Birnbaum, L.S. (1993): A physiologically-based pharmacokinetic model of 2,3,7,8-tetrabromo dibenzo-p-dioxin (TBDD) in the rat: Tissue distribution and CYPIA induction. *Toxicol. Appl. Pharmacol.*, 121:87–98.
136. Kety, S.S. (1951): The theory and application of the exchange of inert gas at the lungs. *Pharmacol. Rev.*, 3:1–41.
137. Keys, D.A., Wallance, D.G., Kepler, T.B., and Conolly, R.B. (1999): Quantitative evaluation of alternative mechanisms of blood and testes disposition of di-(2-ethylhexyl)phthalate and mono(2-ethylhexyl)phthalate in rats. *Toxicol. Sci.*, 49:172–185.
138. Khor, S.P., and Mayersohn, M. (1991): Potential error in the measurement of tissue to blood distribution coefficients in physiological pharmacokinetic modeling: Residual tissue blood. I. Theoretical considerations. *Drug Metab. Disp.*, 19:478–485.
139. Kim, C.S., Gargas, M.L., and Andersen, M.E. (1994): Pharmacokinetic modeling of 2,4-dichlorophenoxyacetic acid (2,4-D) in rat and in rabbit brain following single dose administration. *Toxicol. Lett.*, 74:189–201.
140. Kim, C.S., Slikker, W., Ninienda, Z., Gargas, M.L., and Andersen, M.E. (1995): Development of a physiologically based pharmacokinetic model for 2,4-dichlorophenoxyacetic acid dosimetry in discrete areas of the rabbit brain. *Neurotoxicol. Teratol.*, 17:111–120.
141. King, F.G., Dedrick, R.L., Collins, J.M., Mattews, H.B., and Birnbaum, L.G. (1983): Physiological model for the pharmacokinetics of 2,3,7,8-tetra-chloro dibenzofuran in several species. *Toxicol. Appl. Pharmacol.*, 67:390–400.
142. Knaak, J.B., and Smith, L.W. (1998): In vitro hepatic metabolism of PCBTF: Development of Vmax and *Km* values and partition

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_236.html[2010/10/28 18:42:09]

coefficients and their use in an inhalation PBPK model. *Inhal. Toxicol.*, 10:65–85.
143. Kohn, M.C., and Melnick, R.L. (1993): Species differences in pharmacokinetics and clearance of 1,3-butadiene metabolites: A mechanistic model indicates predominantly physiological, not biochemical control. *Carcinogenesis*, 14:619–628.
144. Koizumi, A. (1989): Potential of physiological pharmacokinetics to amalgamate kinetic data of trichloroethylene and tetrachloroethylene obtained in rats and man. *Br. J. Ind. Med.*, 46:239–249.
145. Kootsey, J.M., Kohn, M.C., Feezor, M.D., Mitchell, G.R., and Fletcher, P.R. (1986): SCoP: An interactive simulation control program for micro-and minicomputers. *Bull. Math. Biol.*, 48:427–441.
146. Krewski, D., Wang, Y., Bartlett, S., and Krishnan, K. (1995): Uncertainty, variability, and sensitivity analysis in physiological pharmacokinetic models. *J. Biopharm. Statist.*, 5:245–271.
147. Krishnan, K., Andersen, M.E., Clewell, H.J., III, and Yang, R.S. H. (1994): Physiologically-based pharmacokinetic modeling of chemical mixtures. In: *Toxicology of Chemical Mixtures*, edited by R.S.A.Yang, pp. 399–437. Academic Press, New York.
148. Krishnan, K., Pelekis, M.L., and Haddad, S. (1995): A simple index for describing the discrepancy between PBPK model simulations and experimental data. *J. Toxicol. Ind. Health*, 11:413–421.
149. Krishnan, K., and Andersen, M.E. (1998): Physiologically based pharmacokinetic models in risk assessment of developmental neurotoxicants. In: *Handbook Developmental Neurotoxicology*, pp. 709–725.
150. Krishnan, K., Gargas, M.L., and Andersen, M.E. (1993): In vitro toxicology and risk assessment. *Altern. Meth. Toxicol.*, 9:185–203.
151. Krishnan, K., Gargas, M.L., Fennell, T.R., and Andersen, M.E. (1992): A physiologically-based description of ethylene oxide dosimetry in the rat. *Toxicol. Ind. Health*, 8:121–140.
152. Kubic, V.L., Anders, M.W., Engel, R.R., Barlow, C.H., and Caughey, W.S. (1974): Metabolism of dihalomethanes to carbon monoxide. *Drug Metab. Dispos.*, 2:53–57.
153. Kumagai, S., and Matsunaga, I. (1995): Effect of variation of exposure to airborne chlorobenzene on internal exposure and concentrations of urinary metabolite. *Occup. Environ. Med.*, 52:65–70.
154. Kumagai, S., and Matsunaga, I. (1995): Physiologically based pharmacokinetic model for acetone. *Occup. Environ. Med.*, 52:344–352.
155. Lam, G., Chen, M.L., and Chiou, W.L. (1982): Determination of tissue: blood partition coefficients in physiologically-based pharmacokinetic models. *J. Pharm. Sci.*, 71:454–456.
156. Lapare, S., Tardif, R., and Brodeur, J, (1993): Effect of various exposure scenarios on the biological monitoring of organic solvents. I. Toluene and xylene. *Int. Arch. Occup. Environ. Health*, 64:569–580.
157. Lapare, S., Tardif, R., and Brodeur, J. (1995): Effect of various exposure scenarios on the biological monitoring of organic solvents in alveolar air. II. 1,1,1-trichloroethane and trichloroethylene. *Int. Arch. Occup. Environ. Health*, 67:375–394.
158. Lawrence, G.S., and Gobas, F.A.P.C. (1997): A pharmacokinetic analysis of interspecies extrapolation in dioxin risk assessment. *Chemosphere*, 35:427–452.
159. Leavens, T.L., Moss, D.R., and Bond, J.A. (1996): Dynamic inhalation system for individual whole-body exposure of mice to volatile organic chemicals. *Inhal. Toxicol.*, 8:655–677.
160. Lee, K.M., Dill, J.A., Chou, B.J., and Roycroft, J.H. (1998): Physiologically based pharmacokinetic model for chronic inhalation of 2-butoxyethanol. *Toxicol. Appl. Pharmacol.*, 153:211–226.
161. Leung, H.W., and Paustenbach, D.J. (1990): Cancer risk assessment for dioxane based upon a physiologically-based pharmacokinetic modeling approach. *Toxicol. Lett.*, 51:147–162.
162. Leung, H.W., Ku, R.H., Paustenbach, D.J., and Andersen, M.E. (1988): A physiologically-based pharmacokinetic model for 2,3,7,8-tetrachloro dibenzo-p-dioxin in C57BL/6J and DBA/2J mice. *Toxicol. Lett.*, 42:15–28.
163. Leung, H.W., Paustenbach, D.J., Murray, F.J., and Andersen, M.E. (1990): A physiologically-based pharmacokinetic description and enzyme-inducing properties of 2,3,7,8-tetrachlorodibenzo-p-dioxin in the rat. *Toxicol. Appl. Pharmacol.*, 103:399–410.
164. Leung, H.W., Poland, A.P., Paustenbach, D.J., and Andersen, M. E. (1990): Dose-dependent pharmacokinetics of (125-I)-2-iodo-3, 7,8-trichlorodibenzo-p-dioxin in mice: Analysis with a physiological modeling approach. *Toxicol. Appl. Pharmacol.*, 103:411–419.
165. Liira, J., Johanson, G., and Riihimaki, V. (1990): Dose-dependent kinetics of inhaled methylethylketone in man. *Toxicol. Lett.*, 50:195–201.
166. Lilly, P.D., Andersen, M.E., Ross, T.M., and Pegram, R.A. (1998): A physiologically based pharmacokinetic description of the oral uptake, tissue dosimetry, and rates of metabolism of bromodichloromethane in the male rat. *Toxicol. Appl. Pharmacol.*, 150:205–217.

167. Lin, J.H., Sugiyama, Y., Awazu, S., and Hanano, M. (1982): In vitro and in vivo evaluation of the tissue to blood partition coefficients for physiological pharmacokinetic models. *J. Pharmacokin. Biopharm.,* 10:637–647.
168. Lin, J.H., Sugiyama, Y., Awazu, S., and Hanano, M. (1982): Physiological pharmacokinetics of ethoxybenzamine based on biochemical data obtained in vitro as well as on physiological data. *J. Pharmacokin. Biopharm.,* 10:649–661.
169. Lindstrom, F.T., Gillette, J.W., and Rodecap, S.E. (1974): Distribution of HEOD (dieldrin) in mammals. I. Preliminary model. *Arch. Environ. Contam. Toxicol.,* 2:9–42.
170. Lipscomb, J.C., Fisher, J.W., Confer, P.D., and Byczkowski, J.Z. (1998): In vitro to in vivo extrapolation for trichloroethylene metabolism in humans. *Toxicol. Appl. Pharmacol.* 152:376–387.
171. Loizou, G.D., and Anders, M.W. (1995): Gas-uptake pharmacokinetics and metabolism of 2-chloro-1,1,1,2-tetrafluoroethane (HCFC-124) in the rat, mouse, and hamster. *Drug Metab. Dispos.,* 23:875–880.
172. Loizou, G.D., Eldirdiri, N.I., and King, L.J. (1996): Physiologically based pharmacokinetics of uptake by inhalation of a series of 1,1,1-trihaloethanes: Correlation with various physicochemical parameters. *Inhal. Toxicol.,* 8:1–19.
173. Loizou, G.D., Urban, G., Dekant, W., and Anders, M.W. (1994): Gas-uptake pharmacokinetics of 2,2-dichloro-1,1,1-trifluoro-ethane (HCFC-123). *Drug Metab. Dispos.,* 22:511–517.
174. Lutz, R.J., Dedrick, R.L., Tuey, D., Sipes, I.G., Andersen, M. W., and Mattews, H.B. (1984): Comparison of the pharmacokinetics of several polychlorinated biphenyls in the mouse, rat, dog, and monkey by means of a physiological pharmacokinetic model. *Drug Metab. Dispos.,* 12:527–535.
175. Macpherson, S.E., Barton, C.N., and Bronaugh R.L. (1996): Use of in vitro skin penetration data and a physiologically based model to predict in vivo blood levels of benzoic acid. *Toxicol. Appl Pharmacol.,* 140:436–443.
176. Mann, S., Droz, P.O., and Vahter, M. (1996): A physiologically based pharmacokinetic model for arsenic exposure. I. Development in hamsters and rabbits. *Toxicol. Appl. Pharmacol.,* 137:8–22.
177. Mann, S., Droz, P.O., and Vahter, M. (1996): A physiologically based pharmacokinetic model for arsenic exposure. II. Validation and application in humans. *Toxicol. Appl. Pharmacol.,* 140:471–486.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_237.html[2010/10/28 18:42:10]

178. Mapleson, W.W. (1963): An electric analog for uptake and elimination in man. *J. Appl. Physiol.*, 18:197–204.
179. Mauderly, J.L. (1990): Measurment of respiration and respiratory responses during inhalation exposures. *J. Am. Coll. Toxicol.*, 9:397–406.
180. McDougal, J.N., Jepson, G.W., Clewell, H.J., and Andersen, M. E. (1985): Dermal absorption of dihalomethane vapors. *Toxicol. Appl. Pharmacol.*, 79:150–158.
181. McDougal, J.N., Jepson, G.W., Clewell, H.J., MacNaughton, M. G., and Andersen, M.E. (1986): A physiological pharmacokinetic model for dermal absorption of vapors in the rat. *Toxicol. Appl. Pharmacol.*, 85:286–294.
182. Medinsky, M.A., Sabourin, P.J., Lucier, G., Birnbaum, L.S., and Henderson, R.F. (1989): A physiological model for simulation of benzene by rats and mice. *Toxicol. Appl. Pharmacol.*, 99:193–206.
183. Menzel, D.B. (1988): Planning and using PBPK models: An integrated inhalation and distribution model for nickel. *Toxicol. Lett.*, 43:67–83.
184. Menzel, D.B., Wolpert, R.L., Boger, J.R., and Kootsey, J.M. (1987): Resources available for simulation in toxicology: Specialized computers, generalized software and communication networks. *Drinking Water and Health*, 8:229–254.
185. Miller, F.J., Overton, J.H., Jaskot, R.H., and Menzel, D.B. (1985): A model for the regional uptake of gaseous pollutants in the lung. I. The sensitivity of the uptake of ozone in the human lung to lower respiratory tract secretions and exercise. *Toxicol. Appl. Pharmacol.*, 79:11–27.
186. Mortensen, B., Lokken, T., Zahlsen, K., and Nilsen, O.G. (1997): Comparison and in vivo relevance of two different in vitro headspace metabolic systems: Liver S9 and liver slices. *Pharmacol. Toxicol.*, 81:35–41.
187. Mortensen, B., and Nilsen, O.G. (1988): Allometric species comparison of toluene and n-hexane metabolism: Prediction of hepatic clearance in man from experiments with rodent liver S9 in headspace vial equilibration system. *Pharmacol. Toxicol.*, 82:183–188.
188. Nakajima, T., and Sato, A. (1979): Enhanced activity of liver drug-metabolizing enzymes for aromatic and chlorinated hydrocarbons following food deprivation. *Toxicol. Appl. Pharmacol.*, 50:549–556.
189. National Toxicology Program (1985): NTP Technical Report on the Toxicology and Carcinogenesis Studies of Dichloromethane in Fisher-344 Rats and B6C3F1 Mice (inhalation studies). NTP TR No. 306.
190. Nestorov, I.A., Aarons, L.J., and Rowland, M. (1997): Physiologically based pharmacokinetic modeling of a homologous series of barbiturates in the rat: A sensitivity analysis. *J. Pharmacokin. Biopharm.*, 25:413–447.
191. O'Flaherty, E. (1998): Physiologically based models of metal kinetics. *Crit. Rev. Toxicol.*, pp. 271–317.
192. O'Flaherty, E.J. (1981): *Toxicant and Drugs: Kinetics and Dynamics.* John Wiley, New York.
193. O'Flaherty, E.J. (1991): Physiologically-based models for boneseeking elements. II. Kinetics of lead disposition in rats. *Toxicol. Appl Pharmacol.*, 111:313–331.
194. O'Flaherty, E.J. (1993): A pharmacokinetic model for chromium. *Toxicol. Lett.*, 68:145–158.
195. O'Flaherty, E.J., Scott, W., Schreiner, C., and Beliles, R.P. (1992): A physiologically-based kinetic model of rat and mouse gestation: Disposition of a weak acid. *Toxicol. Appl. Pharmacol.*, 112:245–256.
196. Overton, J.H., Graham, R.C., and Miller, F.J. (1987): Mathematical modeling of ozone absorption in the lower respiratory tract. *Drinking Water and Health*, 8:302–311.
197. Page, N.P., Singh, D.V., Farland, W., Goodman, J.I., Conolly, R. B., Andersen, M.E., Clewell, H.J., Frederick, C.B., Yamasaki, H., and Lucier, G. (1997): Implementation of EPA revised cancer assessment guidelines: Incorporation of mechanistic and pharmacokinetic data. *Fundam. Appl. Toxicol.*, 37:16–36.
198. Parham, F.M., Kohn, M.C., Matthews, H.B., DeRosa, C., and Portier, C.J. (1997): Using structural information to create physiologically based pharmacokinetic models for all polychlorinated biphenyls. I. Tissue: blood partition coefficients. *Toxicol. Appl. Pharmacol.*, 144:340–347.
199. Parham, F.M., and Portier, C.J. (1998): Using structural information to create physiologically based pharmacokinetic models for all polychlorinated biphenyls. II. Rates of metabolism. *Toxicol. Appl. Pharmacol.*, 151:110–116.
200. Paterson, S., and MacKay, D. (1989): Correlation of tissue, blood, and air partition coefficients of volatile organic chemicals. *Br. J. Ind. Med.*, 46:321–328.
201. Paustenbach, D., Andersen, M.E., Clewell, H.J., and Gargas, M. L. (1988): A physiologically-based pharmacokinetic model for inhaled carbon tetrachloride in the rat. *Toxicol. Appl. Pharmacol.*, 96:191–211.

202. Pelekis, M., and Krishnan, K. (1999): Physiologically based modeling of the pharmacokinetics and pharmacodynamics of aldicarb in humans. In: *Proceeding of the 1999 Medical Science Simulation Conference,* edited by J.G.Anderson, and M.Katzper, pp. 124–128. Society for Computer Simulation International, San Diego, California.
203. Pelekis, M., Krewski, D., and Krishnan, K. (1997): Physiologically based algebraic expressions for predicting steady-state toxicokinetics of inhaled vapors. *Toxicol. Meth.,* 7:205–225.
204. Pelekis, M., Poulin, P., and Krishnan, K. (1995): An approach for incorporating tissue composition data into physiologically based pharmacokinetic models. *Toxicol. Ind. Health,* 11:511–522.
205. Perbellini, L., Mozzo, P., Brugnone, F., and Zedde, A. (1986): Physiologicomathematical model for studying human exposure to organic solvents: Kinetics of blood/tissue n-hexane concentrations and of 2,5-hexanedione in urine. *Br. J. Ind. Med.,* 43:760–768.
206. Perbellini, L., Mozzo, P., Olivata, D., and Brugnone, F. (1990): Dynamic biological exposure indices for n-hexane and 2,5-hexanedione, suggested by a physiologically-based pharmacokinetic model. *Am. Ind. Hyg. Ass. J.,* 51:356–362.
207. Perico, A., Cassinelli, C., Brugnone, F., Bavazzano, P., and Perbellini, L. (1999): Biological monitoring of occupational exposure to cyclohexane by urinary 1,2-and 1,4-cyclohexanediol determination. *Int. Arch. Occup. Environ. Health,* 72:115–120.
208. Pierce, C.H., Dills, R.L., Morgan, M.S., Nothstein, G.L., Shen, D.D., and Kalman, D.A. (1996): Interindividual differences in 2H8-toluene toxicokinetics assessed by a semi-empirical physiologically based model. *Toxicol. Appl. Pharmacol.,* 139:49–61.
209. Pierce, C.H., Becker, C.E., Tozer, T.N., Owen, D.J., and So, Y. (1998): Modeling the acute neurotoxicity of styrene. *J. Occup. Environ. Med.,* 40:230–240.
210. Ploemen, J.P.H.T.M., Wormhoudt, L.W., Haenen, G.R. M.M., Oudhoorn, M.J., Commandeur, J.N.M., Vermeulen, N.P.E., De Wazier, I., Beaune, P.H., Watabe, T., and van Bladeren, P.J. (1997): The use of human in vitro metabolic parameters to explore the risk assessment of hazardous compounds: The case of ethylene dibromide. *Toxicol. Appl. Pharmacol.,* 143:56–69.
211. Plowchalk, D.R., Andersen, M.E., and Bethizy, J.D. (1992): A physiologically-based pharmacokinetic model for nicotine disposition in the Sprague-Dawley rat. *Toxicol. Appl. Pharmacol.,* 116:177–188.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_238.html[2010/10/28 18:42:10]

212. Plowchalk, D.R., Andersen, M.E., and Bogdanffy, M.S. (1997): Physiologically based modeling of vinyl acetate uptake, metabolism, and intracellular pH changes in the rat nasal cavity. *Toxicol. Appl. Pharmacol.,* 142:386–400.
213. Portier, C.J., and Kaplan, N.L. (1989): Variability of safe estimated when using complicated models of carcinogenic processes. A dose study: Methylene chloride. *Fundam. Appl. Toxicol.,* 13:533–544.
214. Poulin, P., and Krishnan, K. (1995): An algorithm for predicting tissue blood partition coefficients of organic chemicals from n-octanol: water partition coefficient data. *J. Toxicol. Environ. Health,* 46:117–129.
215. Poulin, P., and Krishnan, K. (1996): A mechanistic algorithm for predicting blood:air partition coefficients of organic chemicals with the consideration of reversible binding in hemoglobin. *Toxicol. Appl. Pharmacol.,* 136:131–137.
216. Poulin, P., and Krishnan, K. (1996): A tissue composition-based algorithm for predicting tissue: air partition coefficients of organic chemicals. *Toxicol. Appl. Pharmacol.,* 136:126–130.
217. Poulin, P., and Krishnan, K. (1996): Molecular structure-based prediction of the partition coefficients of organic chemicals for physiological pharmacokinetic models. *Toxicol. Meth.,* 6:117–137.
218. Poulin, P., and Krishnan, K. (1998): A quantitative structuretoxicokinetic relationship model for highly metabolised chemicals. *ATLA,* 26:45–59.
219. Poulin, P., and Krishnan, K. (1999): Molecular structure-based prediction of the toxicokinetics of inhaled vapors in humans. *Int. J. Toxicol.,* 18:7–18.
220. Poulin, P., and Krishnan, K. (1995): A biologically-based algorithm for predicting human tissue: blood partition coefficients of organic chemicals. *Human Exp. Toxicol.,* 14:273–280.
221. Poulin, P., Beliveau, M., and Krishnan, K. (1999): Mechanistic animal replacement approaches for predicting pharmacokinetics of organic chemicals. In: *Toxicity Assessment Alternatives: Methods, Issues, Opportunities,* edited by H.Salem, and S.A. Katz, pp. 115–139. Humana Press Inc., Totowa, New Jersey.
222. Ramchandani, V.A., Bolane, J., Li, T.-K., and O'Connor, S. (1999): A physiologically-based pharmacokinetic (PBPK) model for alcohol facilitates rapid BrAC clamping. *Alcohol Clin. Exp. Res.,* 23:617–623.
223. Ramsey, J.C., and Andersen, M.E. (1984): A physiologically-based description of the inhalation pharmacokinetics of styrene in rats and humans. *Toxicol. Appl. Pharmacol.,* 73:159–175.
224. Rao, H.V., Beliles, R.P., Whitford, G.M., and Turners, C.H. (1995): A physiologically based pharmacokinetic model for fluoride uptake by bone. *Regul. Toxicol. Pharmacol.,* 22:30–42.
225. Reitz, R.H., Gargas, M.L., Mendrala, A.L., and Schumann, A. M. (1996): In vivo and in vitro studies of perchloroethylene metabolism for physiologically based pharmacokinetic modeling in rats, mice, and humans. *Toxicol. Appl. Pharmacol.,* 136:289–306.
226. Reitz, R.H., Gargas, M.L., Andersen, M.E., Provan, W.M., and Green, T.L. (1996): Predicting cancer risk from vinyl chloride exposure with a physiologically based pharmacokinetic model. *Toxicol. Appl. Pharmacol.,* 137:253–267.
227. Reitz, R.H., Mandrela, A.L., Corley, R.A., Quast, J.F., Gargas, M.L., Andersen, M.E., Staats, D.E., and Conolly, R.B. (1990): Estimating the risk of liver cancer associated with human exposures to chloroform using physiologically-based pharmacokinetic modeling. *Toxicol. Appl. Pharmacol.,* 105:443–459.
228. Reitz, R.H., Mandrela, A.L., and Guengerich, F.P. (1989): In vitro metabolism of methylene chloride in human and animal tissues: Use in physiologically-based pharmacokinetic models. *Toxicol. Appl. Pharmacol.,* 97:230–246.
229. Reitz, R.H., McCroskey, P.S., Park, C.N., Andersen, M.E., and Gargas, M.L. (1990): Development of a physiologically-based pharmacokinetic model for risk assessment with 1,4-dioxane. *Toxicol. Appl. Pharmacol.,* 105:37–54.
230. Reitz, R.H., McDougal, J.N., Himmelstein, M.W., Nolan, R.J., and Schumann, A.M. (1988): Physiologically-based pharmacokinetic modeling with methyl chloroform: Implications for interspecies, high-low dose and dose-route extrapolations. *Toxicol. Appl. Pharmacol.,* 95:185–199.
231. Reitz, R.H., Mendrala, A.L., Park, C.N., Andersen, M.E., and Guengerich, F.P. (1988): Incorporation of in vitro enzyme data into the physiologically-based pharmacokinetic (PBPK) model for methylene chloride: Implications for risk assessment. *Toxicol. Lett.,* 43:97–116.
232. Rideout, V.C. (1991): *Mathematical and Computer Modeling of Physiological Systems.* Prentice-Hall, New York.
233. Riggs, D.S. (1970): *The Mathematical Approach to Physiological Problems: A Critical Treatise.* MIT Press, Cambridge, Massachusetts.

234. Robinson, P.J. (1991): Effect of microcirculatory heterogeneity in the determination of pharmacokinetic parameters: Implications for risk assessment. *Drug Metab. Rev.*, 23:43–64.
235. Robinson, P.J. (1992): Physiologically-based liver modeling and risk assessment. *Risk Anal.*, 12:139–148.
236. Ross, R., Leger, L., Guardo, R., de Guise, J., and Pike, B.G. (1991): Adipose tissue volumes measured by magnetic resonance imaging and computerized tomography in rats. *J. Appl. Physiol.*, 70:2164–2172.
237. Roth, R.A., and Vinegar, A. (1990): Action by the lungs on circulating xenobiotic agents with a case study of physiologically-based pharmacokinetic modeling of benzo(a)pyrene disposition. *Pharmacol. Therap.*, 48:143–155.
238. Roth, W.L., Ernst, S., Weber, L.W. D., Kerecsen, L., and Rozman, K.K. (1994): A pharmacodynamically responsive model for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) transfer between liver and fat at low and high doses. *Toxicol. Appl. Pharmacol.*, 127:151–162.
239. Roy, A., and Georgopoulos, P.G. (1998): Reconstructing week-long exposures to volatile organic compounds using physiologically based pharmacokinetic models. *J. Expos. Anal. Environ. Epidemiol.*, 8:407–422.
240. Santostefano, M.J., Wang, X., Richardson, V.M., Ross, D.G., DeVito, M.J., and Birnbaum, L.S. (1998): A pharmacodynamic analysis of TCDD-induced cytochrome P450 gene expression in multiple tissues: Dose- and time-dependent effects. *Toxicol. Appl. Pharmacol.*, 151:294–310.
241. Sato, A., and Nakajima, T. (1979): A vial equilibration method to evaluate the drug metabolizing enzyme activity for volatile hydrocarbons. *Toxicol. Appl. Pharmacol.*, 47:41–46.
242. Sato, A., and Nakajima, T. (1979): Partition coefficients of some aromatic hydrocarbons and ketones in water, blood and oil. *Br. J. Ind. Med.*, 36:231–234.
243. Sato, A., Endoh, K., Kaneko, T., and Johanson, G. (1991): A simulation study of physiological factors affecting pharmacokinetic behavior of organic solvent vapors. *Br. J. Ind. Med.*, 48:342–347.
244. Schoeffner, D.J., Warren, D.A., Muralidhara, S., Bruckner, J.V., and Simmons, J.E. (1999): Organ weights and fat volume in rats as a function of strain and age. *J. Toxicol. Environ. Health*, Part A, 56:449–462.
245. Semino, G., Lilly, P., and Andersen, M.E. (1997): A pharmacokinetic model describing pulsatile uptake of orally-administered carbon tetrachloride. *Toxicology*, 117:25–33.
246. Shelley, M.L., Andersen, M.E., and Fisher, J.W. (1989): A risk assessment approach for nursing infants exposed to volatile

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organics through the mothers occupational inhalation exposure. *Appl. Ind. Hyg.*, 4:21–26.
247. Shyr, L.J., Sabourin, P.J., Medinsky, M.A., Birnbaum, L.S., and Henderson, R.F. (1993): Physiologically based modeling of 2-butoxyethanol disposition in rats following different routes of exposure. *Environ. Res.*, 63:202–218.
248. Sinclair, G.C., Gray, C.N., and Sherwood R.J. (1999): Structure and validation of a pharmacokinetic model for benzene. *Am. Ind. Hyg. Ass. J.*, 60:249–258.
249. Singh, D.V., Spitzer, H.L., and White, P.D. (1987): *Addendum to the Health Risk Assessment for Dichloromethane. Updated Carcinogenicity Assessment for Dichloromethane.* EPA 600/8–82/004F.
250. Smith, A.E., Gray, G.M., and Evans, J.S. (1995): The ability of predicted internal dose measures to reconcile tumor bioassay data for chloroform. *Regul. Toxicol. Pharmacol.*, 21:339–351.
251. Staats, D.A., Fisher, J.W., and Conolly, R.B. (1991): Gastrointestinal absorption of xenobiotics on physiologically-based pharmacokinetic models. A two-compartmental description. *Drug Metab. Dispos.*, 19:144–149.
252. Stenner, R.D., Merdink, J.L., Fisher, J.W., and Bunge, A.L. (1998): Physiologically-based pharmacokinetic model for trichloroethylene considering enterohepatic recirculation of major metabolites. *Risk Anal.*, 18:261–269.
253. Sultatos, L.G. (1990): A physiologically-based pharmacokinetic model for parathion based on chemical specific parameters determined in vitro. *J. Am. Coll. Toxicol.*, 9:611–617.
254. Sultatos, L.G., Kim, B., and Woods, L. (1990): Evaluation of estimations in vitro of tissue: blood distribution coefficients for organothiophosphate insecticides. *Toxicol. Appl. Pharmacol.*, 103:52–55.
255. Sweeney, L.M., Schlosser, P.M., Medinsky, M.A., and Bond, J. A. (1997): Physiologically based phamacokinetic modeling of 1,3-butadiene, l,2-epoxy-3-butene, and l,2:3,4-diepoxybutane toxicokinetics in mice and rats. *Carcinogenesis*, 18:611–625.
256. Sweeney, L.M., Shuler, M.L., Quick, D., and Babish, J.G. (1996): A preliminary physiologically based pharmacokinetic model for naphthalene and naphthalene oxide in mice and rats. *Ann. Biomed. Eng.*, 24:305–320.
257. Tardif, R., Lapare, S., Krishnan, K., and Brodeur, J. (1992): Physiologically-based modeling of the toxicokinetic interaction between m-xylene and toluene in the rat. *Toxicol. Appl. Pharmacol.*, 120:266–273.
258. Teorell, T. (1937): Kinetics of distribution of substances administered to the body. I. The extravascular modes of administration. *Arch. Int. Pharmacodyn.*, 57:205–225.
259. Teorell, T. (1937): Kinetics of distribution of substances administered to the body. II. The intravascular modes of administration. *Arch. Int. Pharmacodyn.*, 57:226–240.
260. Terasaki, T., Iga, T., Sugiyama, Y., Sawada, Y., and Hanano, M. (1984): Nuclear binding as a determinant of tissue distribution of adriomycin, daunomycin, adriamycinol, daunorubicinol and actinomycin *D.J. Pharmacodyn.*, 7:269–277.
261. Terry, K.K., Elswick, B.A., Welsch, F., and Conolly, R.B. (1995): Development of a physiologically based pharmacokinetic model describing 2-methoxyacetic acid disposition in the pregnant mouse. *Toxicol. Appl. Pharmacol.*, 132:103–114.
262. Thomas, R.S., Bigelow, P.L., Keefe, T.J., and Yang, R.S.H. (1996): Variability in biological exposure indices using physiologically based pharmacokinetic modeling and Monte Carlo simulation. *Am. Ind. Hyg. Ass. J.*, 57:23–32.
263. Thomas, R.S., Lytle, W.E., Keefe, T.J., Constan, A.A., and Yang, R.S.H. (1996): Incorporating Monte Carlo simulation into physiologically based pharmacokinetic models using advanced continuous simulation language (ACSL): A computational method. *Fundam. Appl. Pharmacol.*, 31:19–28.
264. Thrall, K.D., and Kenny, D.V. (1996): Evaluation of a carbon tetrachloride physiologically based pharmacokinetic model using real-time breath-analysis monitoring. *Inhal. Toxicol.*, 8:251–261.
265. Travis, C.C., and Hattemer-Frey, H.A. (1991): Physiological pharmacokinetic models. In: *Statistics in Toxicology*, edited by D.Krewski and C.Franklin, p. 170. Gordon and Breach, New York.
266. Travis, C.C., Quillen, J.L., and Arms, A.D. (1990): Pharmacokinetics of benzene. *Toxicol. Appl. Pharmacol.*, 102:400–420.
267. Travis, C.C., White, R.K., and Arms, A.D. (1989): A physiologically based pharmacokinetic approach for assessing the cancer risk of tetrachloroethylene. In: *The Risk Assessment of Environmental and Human Health Hazards. A Textbook of Case studies*, edited by D.J.Paustenbach, pp. 769–796. Wiley-Interscience Publications, New York.
268. Tuey, D.B., and Mattews, D.H. (1980): Distribution and excretion of 2,2′,4′,4′,5,5′-hexabromobiphenyls in rats and man: Pharmacokinetic model predictions. *Toxicol. Appl. Pharmacol.*,

53:420–431.
269. Tuey, D.B., and Mattews, D.H. (1980): Use of a physiological compartmental model for the rat to describe the pharmacokinetics of several chlorinated biphenyls in the mouse. *Drug Metab. Dispos.*, 8:397–403.
270. U.S. EPA (1987): *Update to the Health Risk Assessment Document and Addendum for Dichloromethane: Pharmacokinetics, Mechanism of Action and Epidemiology.* EPA 600/8–87/030A.
271. Vinegar, A., and Jepson, G.W. (1996): Cardiac sensitization thresholds of halon replacement chemicals predicted in humans by physiologically based pharmacokinetic modeling. *Risk Anal.*, 16:571–579.
272. Vinegar, A., Seckel, C.S., Pollard, D.L., Kinkead, E.R., Conolly, R.B., and Andersen, M.E. (1992): Polychlorotrifluoroethylene oligomer pharmacokinetics in F-344 rats: Development of a physiologically-based model. *Fundam. Appl. Toxicol.*, 18:504–514.
273. Vinegar, A., William, R.J., Fisher, J.W., and McDougal, J.N. (1994): Dose-dependent metabolism of 2,2-dichloro-1,1,1-tri-fluoroethane: A physiologically based pharmacokinetic model in the male Fisher 344 rat. *Toxicol. Appl. Pharmacol.*, 129:103–113.
274. Volp, R.F., Sipes, I.G., Falcoz, C., Carter, D.E., and Gross, J.F. (1984): Disposition of 1,2,3-trichloropropane in the Fischer-344 rats: Conventional and physiological pharmacokinetics. *Toxicol. Appl. Pharmacol.*, 75:8–17.
275. Wada, D.R., Stanski, D.R., and Ebling, W.F. (1995): A PC-based graphical simulator for physiological pharmacokinetic models. *Comput. Methods Programs Biomed.*, 46:245–255.
276. Wagner, J.G. (1975): *Fundamentals of Clinical Pharmacokinetics.* Drug International, Hamilton, Illinois.
277. Wang, X., Santostefano, M.J., Evans, M.V., Richardson, V.M., Diliberto, J.J., and Birnbaum, L.S. (1997): Determination of parameters responsible for pharmacokinetic behavior of TCDD in female Sprague-Dawley rats. *Toxicol. Appl. Pharmacol.*, 147:151–168.
278. Ward, K.W., Blumenthal, G.M., Welsch, F., and Pollack, G.M. (1997): Development of a physiologically based pharmacokinetic model to describe the disposition of methanol in pregnant rats and mice. *Toxicol. Appl. Pharmacol.*, 145:311–322.
279. Ward, R.C., Travis, C.C., Hetrick, D.M., Andersen, M.E., and Gargas, M.L. (1988): Pharmacokinetics of tetrachloroethylene. *Toxicol. Appl Pharmacol.*, 93:108–117.
280. Williams, R.J., Vinegar, A., McDougal, J.N., Jarabek, A.M., and Fisher, J.W. (1996): Rat to human extrapolation of HCFC-123 kinetics deduced from halothane kinetics—A corollary approach

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to physiologically based pharmacokinetic modelling. *Fundam. Appl. Pharmacol.*, 30:55–66.
281. Yamaguchi, T., Yabuki, M., Saito, S., Watanabe, T., Nishimura, H., Isobe, N., Shono, F., and Matsuo, M. (1996): Research to develop a predicting system of mammalian subacute toxicity (3) Construction of a predictive toxicokinetics model. *Chemosphere*, 33:2441–2468.
282. Yesair, D.W., Feder, P.I., and Chin, A.E. (1986): Development, evaluation and use of a pharmacokinetic model for hexachlorobenzene. In: *Hexachlorobenzene: Proceedings of an International Symposium*, edited by C.R.Morris and J.R.P.Cabral, pp. 297–318. Oxford University Press, New York.
283. Yu, D. (1999): A physiologically based pharmacokinetic model of inorganic arsenic. *Regul. Toxicol. Pharmacol.*, 29:128–141.

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Chapter **6**

The Toxicological Assessment of Pharmaceutical and Biotechnology Products

Michael A.Dorato and Mary Jo Vodicnik

Principles and Methods of Toxicology,
Fourth Edition, edited by A.Wallace Hayes.
Taylor & Francis, Philadelphia © 2001.

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GENERAL OVERVIEW OF DRUG DEVELOPMENT

The World Health Organization (WHO) Scientific Group has defined a drug as "…any substance or product that is used or intended to be used to modify or explore physiological systems or pathological states for the benefit of the recipient" (152). The drug discovery process covers a wide range of therapeutic areas and treatment regimens, and is a risky, multifaceted, expensive undertaking. The goal is to develop a new product with therapeutic benefits (efficacy) and few side effects (toxicity) (3). The drug development process for a new chemical entity (NCE) starts at the chemist's bench with its isolation; moves through efficacy pharmacology testing using various in vivo and in vitro models; then proceeds through an abbreviated toxicology profile, including pharmacological profiling (the determination of pharmacological effects other than the desired therapeutic effect), based on the proposed clinical plan for first human dose (FHD).

The principal aim of nonclinical safety testing is to understand the toxicity of the candidate drug well enough to make a judgment that it is safe to initiate clinical trials (89). Provided the efficacy pharmacology and initial toxicology profiles are acceptable, clinical safety, pharmacokinetic, and pharmacodynamic studies are initiated. As the human clinical trials progress, the drug candidate moves through nonclinical subchronic studies, chronic, and developmental toxicology studies, and oncogenic evaluations. Zbinden (209) has provided a summary of the biological parameters that should be evaluated for new drug candidates (Table 6.1).

The technical risks in new drug development programs are enormous. The risk of failure related to one or more of these aspects has been reviewed by Chien (26), where it is reported that <0.02 percent of NCEs result in marketed drug products, and even fewer, 0.002%, return a profit to support continued drug research (Figure 6.1). Very little of what enters the drug development pipeline ever enters the marketplace (58). It has been estimated that the cost of developing a NCE ranges from $0.5 billion to $1.2 billion.

By its nature, a drug must modify a biological process, that is, alter or adjust a physiological system in some way (43). Toxicology is a critical part of both earlyand late-phase drug development. The role of toxicology in that process has been extensively reviewed (79, 55, 59). The initial purpose of toxicology testing programs is to identify the circumstances—for example, dose, treatment duration, route—under which a NCE produces potentially harmful effects (44). A general approach to developing a toxicity profile for a pharmaceutical agent is given in Figure 6.2, and will be discussed more extensively below. During the discovery process, toxicologists employ rapid, quantitative screening methods, focusing on a limited spectrum of toxicity, to help identify potential drug candidates with the best safety profile. These early

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Table 6.1

Biological properties of chemicals that should be considered in safety evaluations[a]

Acute Toxicity
Cumulative Toxicity
Absorption from Various Routes
Elimination t1/2 and Accumulation in Deep Compartments
Penetration of Barriers
Milk Excretion
Teratogenicity
Mutagenicity
Carcinogenicity
Sensitization
Local Irritation

*a*From Reference 209.

screening procedures, however, are only a prelude to the required comprehensive safety assessments expected from the toxicologist. Regulatory requirements, termed Good Laboratory Practices (GLPs), dictate many aspects of the toxicology study protocol, and must be followed closely for all definitive (those that support human studies) toxicology studies (64). The early toxicology studies, conducted in the discovery phase, are not required to be in full compliance with the GLPs.

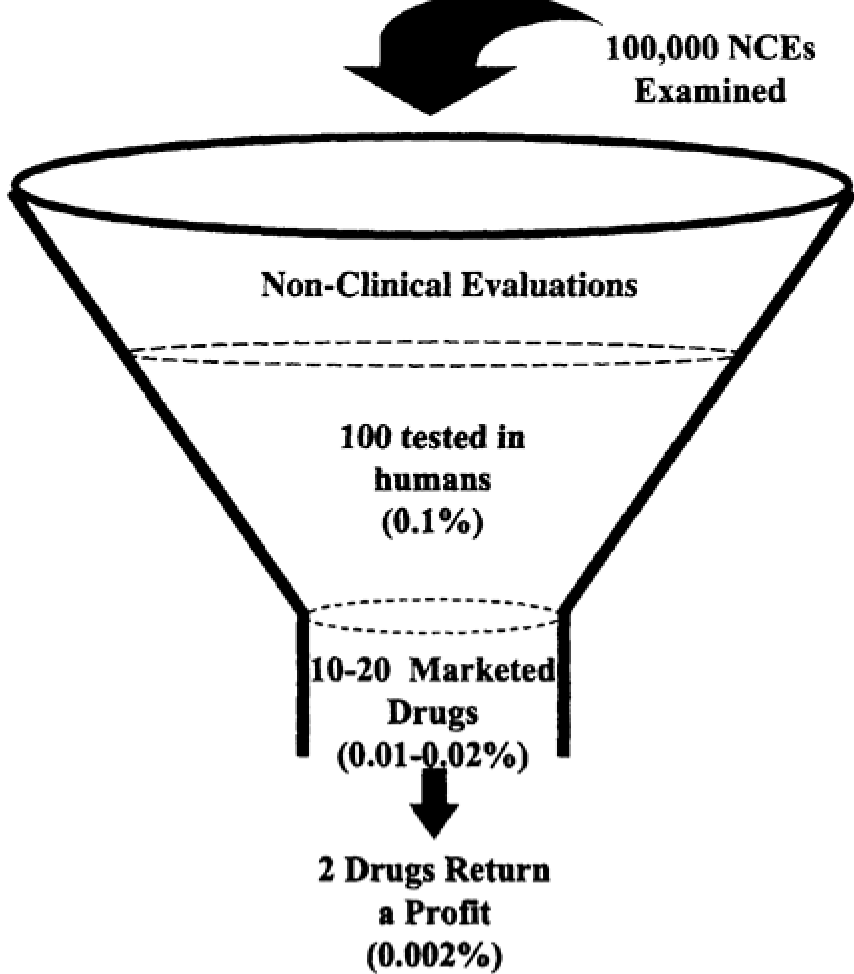

FIG. 6.1. Attrition rate of new drug candidates. Drawn from information presented in Reference 28.

Prior to initiating clinical trials, physicians need an estimate of the extent of toxicity produced by the drug candidate in relevant animal models. Obviously, prior to FHD, the determination of the relevance of animal models, that is, metabolism relative to humans, is limited to in vitro evaluations using human and animal tissue preparations. On the basis of the clinicians' needs, the toxicology profile of a NCE is characterized by a number of questions (124):

• What dose/exposure produces toxic effects in animals?

- What dose/exposure does not produce toxic effects in animals?
- Were the animals relevant models for predicting human toxicity?
- What were the signs and duration of toxic responses?
- Did effects differ following single or multiple dosing?
- Were the toxic responses reversible?
- What were the target organs or systems?
- Was the toxicity expected for this chemical class?
- Are toxic metabolites produced?
- Was accommodation to the toxic effects observed?

The answers to these questions form the basis of the toxicology profile supporting initial and continued clinical trials.

The major objectives of toxicological evaluation change according to the stage of the development process (208). The early stages of development focus on toxicological screening (Table 6.2). Definitive toxicology studies are very time-consuming and costly. Thus, relatively inexpensive, short-term screening procedures are used to eliminate the most toxic compounds (205). Inherent to these initial approaches to evaluate potential drug toxicity are a number of imperfections—the affected systems may not be routinely examined; the assay procedures are inadequate or improperly timed relative to the onset of the toxic response; target organ exposure is insufficient; there is an inability to identify and measure adverse effect (lack of functional evaluations); there is an inability to predict metabolic, anatomical, and physiological differences between species; and, the test animals may not express human-specific responses (212). There is no simple answer to the often asked question: "What toxicity profile would cause a company to stop development of a new drug candidate?" (100). However, the demonstrated toxicity of other compounds in the class, if available, and the gravity of the disease state under study often provide guidance as to what might be an acceptable safety profile for a NCE. Varied opinions on the occurrence of drug toxicity in the human population have been reported (31, 115).

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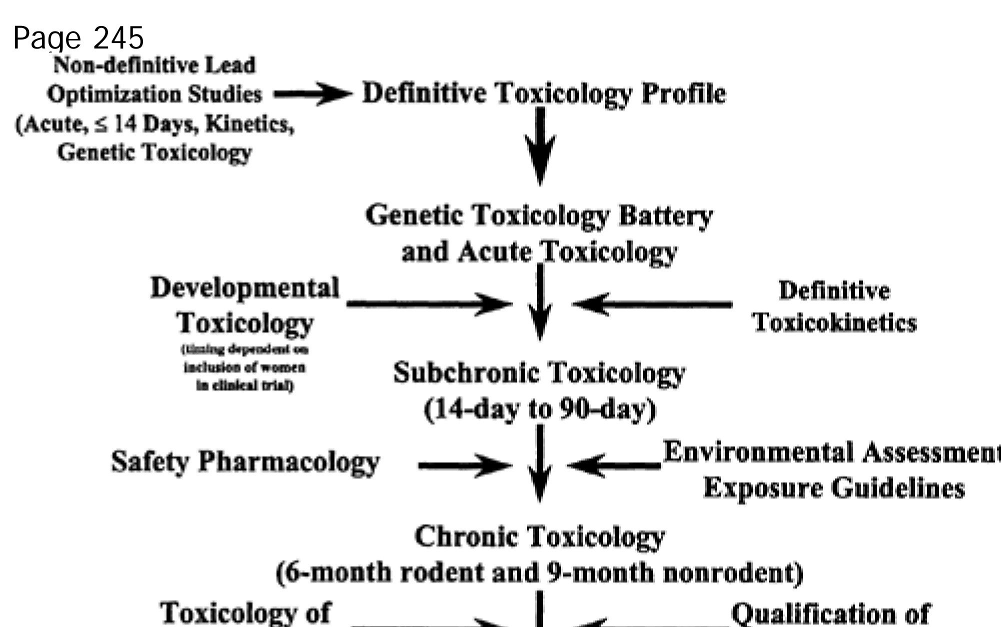

FIG. 6.2. General approach to developing a toxicity profile for pharmaceutical agents.

Although the magnitude of adverse responses seems small—1 per 10,000 patients demonstrates untoward adverse reactions (115)—focus must be placed on identification of all potentially adverse effects (31).

In addition to the drug substance, the delivery system may also require nonclinical evaluation because it may alter pharmacodynamic (action of the drug on the body) and pharmacokinetic (action of the body on the drug) relationships. The regulatory requirements for known and novel drug delivery systems have been reviewed by Weissinger (192).

Commercially advantageous forms of genetic manipulation date back to antiquity, that is, inbreeding, cross fertilization, and so on. The introduction of modern recombinant DNA (rDNA) technology has had a major impact on life science research, and has allowed for the large-scale production of protein pharmacological agents that would have been very difficult to produce by normal chemical synthetic means.

A New Biological Entity (NBE) is defined as a complex, high molecular weight material, which cannot be fully characterized by standard chemical analysis, and which

Table 6.2

Purpose of toxicology evaluations of new drugs*a*

Phase	Principle Activity	Purpose
Discovery	Identification of Candidates	Toxicological Screening
Before FHD	Safety and Principle Target Organs	Regulatory Prerequisites for Human Exposure
During Clinical Trial	Toxicological Spectrum	Cumulative Effects and Mechanisms
Pre-Marketing	Complete Routine Test Program	Regulatory Requirements
Post-Marketing	Identify Special Risks Due to Population or Use Circumstances	Improve Utility and Safety

*a*From Reference 208.

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may require immunological, biochemical, or bioassay techniques to measure the quantity present and to assess activity (46). The development, utility, and relative safety of human insulin (83), human growth hormone (133), and interferon (139) have fueled the current interest in the production of biologically active peptides. As the interests of pharmacologists in biotechnology research expand, the difficulties of producing a comprehensive set of safety guidelines increase.

As NBEs were introduced, starting with insulin, regulatory concepts were not in place to address the problems presented by this new technology (210). The regulatory issues relating to recombinant products are formidable, and the possibility that each biotechnology product might require customized safety testing has been given serious consideration (40, 163, 171). Clearly, the immunological response to foreign proteins may compromise the utility of using traditional animal models in the safety assessment of these agents. Other major regulatory issues include the assurance that recombinant production methods do not result in addition of contaminants, and the demonstration to regulators that biosynthetic products are identical to natural substances (81). In the biosynthetic human insulin approval process, meetings between regulatory agencies and industry scientists to review the manufacturing process, molecular biology, and purification of the hormone, as well as clinical trial programs, were critical in facilitating eventual approvals. Industry and regulatory agency representatives agreed that the chemistry of a NBE should prove its identity (188). The identity and purity of the rDNA insulin, therefore, received much attention (26). Anticipation of problems, and communication of concerns, was the key to the rapid New Drug Application (NDA) approval for biosynthetic human insulin (5.5 months). The U.S. Food and Drug Administration (FDA) has strongly recommended that it be involved early in the nonclinical and clinical development plan to facilitate the approval process for both NCEs and NBEs (87). The U.S. biotechnology policy states "...the same physical and biological laws govern the response of organisms modified by modern molecular and cellular methods and those produced by classical methods. ... No conceptual distinction exists between genetic modification of plants and microorganisms by classical methods or by molecular techniques that modify DNA and transfer genes" (183). Thus, it would not be expected that NBEs, per se, pose an unusual risk to human health and the environment (183). The toxicologist should be aware, however, that compounds made via rDNA techniques are not necessarily identical to the natural material, as might be assumed (198). Dayan (45) suggested that the toxicology profile for a NBE should be defined in terms of chemical identity of the material, extent of prior knowledge, and intended use. The U.S. Pharmaceutical Research and Manufacturers Association (PhRMA) has recommended that nonclinical toxicological evaluations of NBEs should be decided on a case-by-case basis (171), and regulatory and industry representatives attending the first International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) also supported this position (111). The established toxicology information will guide the clinical trial and address possible hazards in the workplace, where humans are exposed to the compound, and its precursors and contaminants that are also contained in the bulk material to be tested, during the chemical or biological synthetic process.

The role of the toxicologist is usually less routine and requires more innovation in study design when dealing with NBEs than when dealing with NCEs (46). However, as is the case with NCEs, the principal goals of the toxicological evaluation of recombinant products are: to detect major toxicity; to identify lesser toxicity; to determine the dose relationship of toxic effects and their duration in order to guide the clinical dose schedule; and, to investigate the mechanisms of action related to the toxic response. There are three main areas of concern relative to the toxicity of NBEs: toxicity per se, exaggerated pharmacodynamic effects (anticipated toxicity based on the pharmacological mechanism of action), and allergic reactions (210) (Figure 6.3). Intrinsic toxicity has been defined as undesirable effects having no obvious relationship to the molecule's pharmacodynamic properties. Pharmacodynamic toxicity is defined as an exaggerated pharmacological response, e.g., hypoglycemic shock from insulin. Immunotoxicity has been related to hypersensitivity, cell transformation, and production of neutralizing antibodies. The loss of a recombinant therapeutic agent's biological activity through production of neutralizing antibodies or the development of immune complex disease in experimental animals are factors that must be given individual attention (171). It has been suggested that animal models of immunotoxicity are of limited usefulness because no animal model may be fully suitable for predicting the toxicity of highly species-specific proteins. Friedmann (78) has indicated, however, that the lack of hypersensitivity reactions in response to small peptides in animal experiments may be viewed as an indication of their acceptability in humans. Graham (88) emphasized the use of a case-by-case approach to toxicological evaluations of NBEs based on their similarity to natural human proteins, immune response in animal models, and production of neutralizing antibodies in nonclinical and clinical studies.

The unique regulatory approval of recombinant insulin most likely resulted in unrealistic expectations in the biotechnology industry regarding the rapidity of review

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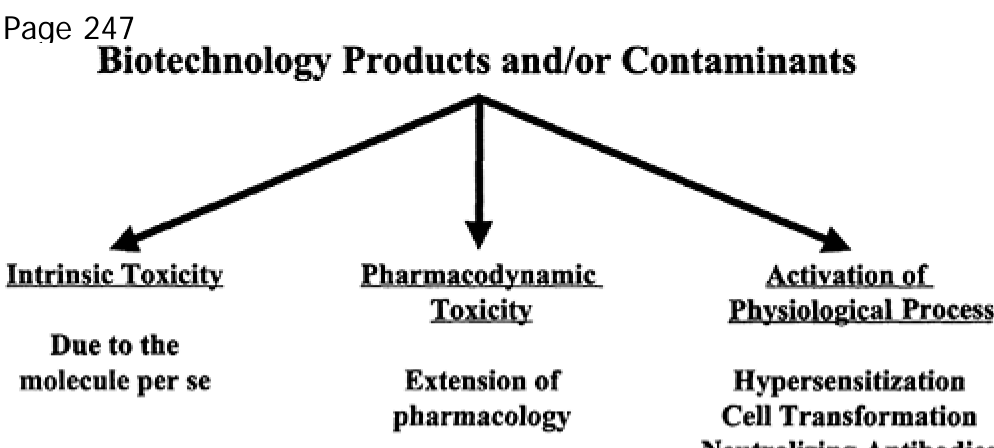

FIG. 6.3. Main areas of concern with response to biotechnology products. Redrawn, with permission, from Reference 210, pp. 143–159.

of NBE applications (121). Two factors facilitate the regulatory approval of NBEs. As is true in the development requirements for all NCEs, the first factor is therapeutic importance, and the FDA has established a "fast-track" rapid approval procedure for NBEs that target unsatisfied indications. The second factor is the relationship of the NBE to an established drug. It appears likely that new therapeutic agents derived from biotechnology will have to satisfy all the usual demands of regulatory agencies (121). The possibility that subtle changes in chemical structure may exist, and may thus influence pharmacokinetics, pharmacodynamics, and/or immunogenicity, is used to support this regulatory position (80).

Questions of safety are not only properly asked about the NBE per se, but about contaminants or residues resulting from the manufacturing and/or purification processes, or antigenic variation or reversion to the wild type of a living organism (46). Worker exposure in the production process may be of concern due to relatively high-level, long-term exposure to various end products of the biotechnology process—live and dead microorganisms and mammalian cells and their derivatives (198). This leads to the area where traditional scientific approaches and techniques do not provide a satisfactory toxicological profile, for example, transfer of an immortalization factor from a mammalian cell; allergic reactions (198).

RELEVANCE OF ANIMAL MODELS IN TOXICOLOGICAL ASSESSMENT

The suitability of experimental animal data for assessing risk to humans is an important contemporary issue in toxicology. Animals and humans have much in common anatomically, physiologically, and biochemi cally (202). There are two main guiding principles of experimental toxicology: that effects produced in animals, when properly qualified, are applicable to humans; and, that exposure of experimental animals to high doses of a test compound is necessary and valid in determining human hazard (117). Although it is generally agreed that animal assays are not as predictive of human effects as would be desired, they are more predictive than generally thought (93, 156). It has been reported that animal assays are predictive of human toxicity in all but 10 percent of comparisons (130). It must be recognized, however, that major differences in response to chemical agents can exist both within and between species (101). The most serious differences between laboratory animal studies and human clinical trials are related to anatomical and physiological "species differences," such as metabolism and genetics (hypersensitivity responses), and experimental design, including quantity, route, and duration of drug administration (122). Humans can be as much as 50 times more sensitive on a mg/kg basis than experimental animals (122).

Regulatory agencies and research-based pharmaceutical companies consider laboratory animal toxicology studies a critical part of the assessment of new drug candidates (74, 128). Confidence in the validity of experimental toxicology is based on the large inventory of chemically induced lesions that occur both in animals and humans (215). It may be incorrect to assume that what is demonstrated in animal toxicology studies will occur in human clinical trials, but until it is shown that the toxicity expressed is not relevant to humans, that assumption must be made (8). Also, until our knowledge base expands, animal data must be extrapolated to the human situation using a conservative approach, that is use of relatively high doses, assumption that humans are more sensitive than the most sensitive species, and so on (16).

The ultimate goals of the toxicology assessments are to characterize toxicity in animal models to identify potential problems in short- and long-term clinical studies, identify the circumstances under which toxicity occurs, evaluate the extent to which the data warrant extrapolation to humans, recommend safe levels of exposure, and contribute to the decision whether to test the new drug candidate in humans (8, 44, 129, 185, 179). It has been recognized that qualitative extrapolation of drug toxicity from animals to humans is more reliable than estimation of the magnitude of dose producing a similar effect in animals and humans; the pharmacodynamics of an agent are more predictable than its pharmacokinetics (144). Complicating the ability to extrapolate data from animals to humans are the excessive doses sometimes used, and often required, in animal studies. As a result, adverse effects are described that may be the result of frank intoxication of the animal and that are irrelevant in humans. Zbinden (203) has reported that the ability of animal toxicity studies to predict potential human toxicity is related to the mechanism of drug action. Within limitations, animals and humans respond in ways similar enough, from a pharmacodynamic perspective, for animal toxicity evaluations to serve as useful predictors of human toxicity (41, 96, 125, 129, 172). However, toxicological evaluations in animals can predict toxic responses in humans only if the response is not unique to humans (74). Those compounds that are toxic to humans but relatively nontoxic to animals—for example thalidomide—are of greatest concern. The extrapolation of animal data to humans is likely to become even more complicated as molecular biology techniques continue to allow the more sophisticated characterization of specific human therapeutic targets (human enzymes, receptors) and the ultimate development of drugs specific for these targets. Table 6.3 lists common undesirable drug effects seen in human studies; 76 percent of the findings are predictable from animal studies. Predictability is enhanced for those adverse effects that can be directly related to the compounds pharmacological mechanism of action. Adverse responses commonly referred to as dose- and time-related are relatively well predicted from animal studies. It is more difficult to extrapolate effects that are not doseor time-related (207).

There is a small element of toxicity that cannot be predicted until large-scale clinical studies are conducted (96). This may be the result of a very low incidence of occurrence, or idiosyncratic responses in a small subset of the patient population. However, considering the increased use of pharmaceutical agents and the relative infrequency of major incidence of human toxicity, the initial laboratory studies are clearly serving a valuable function (8). A large majority of human drug exposures are free of toxicity, and in good accordance with the results of animal toxicity studies (207). The use of adequate test systems is critical to the predictive ability of animal toxicity evaluations. Cahn (20) reported that the cardiac effects of calcium antagonists—ectopic beats, ventricular tachycardia, and ventricular fibrillation —were seen in humans, but were not described in long-term animal studies. However, these effects were demonstrable in animals using appropriate functional evaluations not always included in routine toxicological testing. Oftentimes, toxicologically important end points, such as cardiac, pulmonary, or renal function, are not

Table 6.3

Common untoward reactions to drugs*a*

Clinical Side Effect	Predictable from Animal Studies (Y/N)	Clinical Side Effect	Predictable from Animal Studies (Y/N)	Clinical Side Effect	Predictable from Animal Studies (Y/N)
Drowsiness	Y	Hypertension	Y	Anorexia	Y
Nausea	N	Insomnia	Y	Depression	Y
Dizziness	N	Fatigue	N	Increased Appetite	Y
Sedation	Y	Constipation	Y	Tremor	Y
Dry Mouth	Y	Tinnitus	N	Perspiration	Y
Nervousness	Y	Weight Gain	Y	Dermatitis	Y
Epigastric Distress	N	Hypotension	Y	Increased Energy	Y
Headache	N	Dryness of Nasopharynx	Y	Vertigo	N
Vomiting	Y	Heartburn	N	Palpitation	Y
Weakness	Y	Diarrhea	Y	Blurred Vision	Y
Nasal Stuffiness	Y	Skin Rash	Y	Lethargy	Y

*a*From References 145, 204, and 206.

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taken into consideration in the design of "routine" toxicology studies. The above example emphasizes the importance of using an adequate test system to evaluate the toxicity of new drug candidates. The toxicologist is challenged to consider potential adverse effects related to the pharmacodynamics of the test compound in the design of appropriate safety studies (172).

As with standard pharmaceuticals, no animal model is fully appropriate to evaluate the toxicity of highly species-specific proteins in humans (88). Animal testing for biotechnology products is limited to the species showing the same pharmacological response as humans, without showing signs of immunity (45, 80). This is only feasible when proteins are highly conserved across species. The production of neutralizing antibodies will limit the study duration and, thus, support for clinical trials. Administration of a highly specific human protein to laboratory animals for a sufficient duration to produce immune complex disease will do nothing to reveal effects anticipated in clinical trials. Antigenicity of the test material can be a major complicating factor because the potential allergic etiology of all lesions developing in animals treated with human proteins must be considered (214). Alternatively, nonclinical toxicology studies of biotechnology products may be less predictive of allergic responses that may occur in humans following chronic therapy (198). The appropriate laboratory species for biotechnology product testing should demonstrate similar pharmacodynamics and adverse responses relative to humans. If an animal model demonstrating similar pharmacological response to humans cannot be selected, species selection based on toxicity likely to be representative of effects expected in humans may be acceptable (191). The FDA does not currently require the study of recombinant proteins in primate models, but these animals demonstrate many similarities to humans at the molecular level, and often turn out to be the most appropriate species for toxicity testing (88).

TOXICOKINETICS

Species differences in expression of toxicity (Figure 6.4) due to differences in pharmacokinetics (metabolic processes and physicochemical characteristics of the compound that affect its absorption, disposition, metabolism, and elimination related to the expression of pharmacological endpoints) or toxicokinetics (related to toxicological endpoints), are important factors in toxicology studies (47, 152). Toxicokinetic analyses have become common components of nonclinical toxicology profiles of NCEs and NBEs. Toxicokinetic analyses provide information on dose proportionality, potential dose accumulation, and sex and species differences in drug candidate distribution and elimination (42). A major purpose

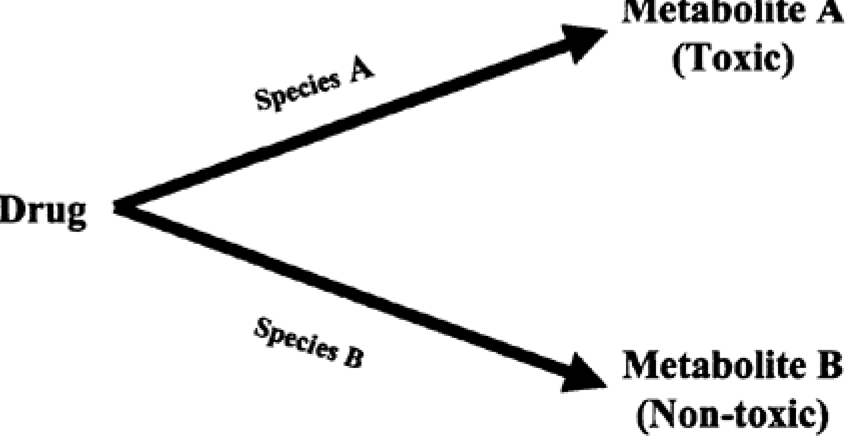

FIG. 6.4. Hypothetical drug metabolism pathways in two species, demonstrating the importance of metabolic data when interpreting toxic responses.

of kinetic studies is to evaluate the level of parent drug or metabolite in various body fluids, tissues, and/or excreta and to use these data to determine suitability of animal data for human hazard assessment (30, 43, 150, 164). Another purpose is to determine the levels of the compound, and its potentially active metabolites, that result in toxicity.

To fully interpret the results and applicability of toxicology studies, the metabolic profile and kinetics of the drug candidate in animal models and in humans must be compared (143). Wherever possible, species that handle the chemical in a manner similar to humans should be used in toxicology studies (190). Rapid detoxification in animals can cause the toxicologist to miss signs of potential human toxicity (207). Alternatively, signs of toxicity in experimental animals may be the result of a metabolic conversion that is only slightly present in humans. For example, the increased liver weights seen with ketotifen (Zaditen, an antianaphylactic agent) in dogs were due to an N-oxide metabolite which is produced in insignificant amounts in humans (172). Toxic responses in humans can be predicted from animal studies

more accurately if the absorption, distribution, metabolism, and excretion of the test chemical are compared (10).

The determination of metabolic and pharmacokinetic profiles in animals is of great value in the design of extended clinical trials, once these parameters have also been characterized in initial limited human testing. Specifically, interspecies variation in response that may preclude extrapolation of animal toxicity data to humans could be explained by toxicokinetics (57). Qualitative metabolic profiles are remarkably similar among the four or five laboratory animals commonly used in toxicology studies and humans (23). Quantitative differences in drug metabolism parameters among species, however, are apparently the rule. It may be unreasonable to expect that, from the few animal species used in toxicological

evaluations, one will be found that handles a test chemical exactly as humans do (207). In a review of the comparative metabolism of 32 compounds, Caldwell (22) has reported that the rhesus monkey is a good model for humans approximately 73 percent of the time; the rat, approximately 29 percent of the time; and the dog, approximately 32 percent of the time. The rhesus monkey was a poor model for humans in only 4 percent of cases. In actual practice, however, the rat is the more frequently used species in long-term toxicology evaluations (23).

Toxicokinetic evaluations have a great deal of relevance in explaining species differences in toxicology profiles (56). The kinetic parameters that are pertinent to an understanding of toxic responses across species are absorption (extent, rate, and relationship to route of administration); distribution (blood and tissue levels of parent compound and metabolites, accumulation in various body compartments); metabolism (species differences, activation); and elimination (route, rate), better known as ADME (164). These processes are interdependent and occur simultaneously (132). Association of drug toxicity with parent compound or metabolite, or the association of enzyme induction with the late accumulation of a novel metabolite, can be characterized by kinetic evaluations (114).

Toxicokinetic data provide another important contribution to toxicology evaluations, that is, assessment of nonlinear kinetics, which frequently occur at the high dose levels used in toxicity testing (151). In this situation, the relationship of dose to internal concentration is not representative of the relationship at the lower, unsaturated, linear dose levels that occur in humans. It is very important that toxicity be evaluated in conjunction with kinetics over a dose range wide enough to define whether a transition from linear to nonlinear kinetics occurs (151). This kinetic information can be used to establish a more realistic upper dose level for a toxicity study, rather than the maximum tolerated dose (MTD), which may far exceed the metabolic capabilities of the animal, and thus result in irrelevant toxicity. If the kinetic evaluations are not conducted simultaneously with the toxicology study, they should be conducted at doses encompassing those actually used in the toxicity evaluation, as well as those which produce the desired pharmacological effect (185). This assessment of systemic exposure to parent drug and metabolites allows a more realistic expression of the difference between the doses that result in efficacy and toxicity (margin of safety) than comparing the doses administered on a mg/kg basis.

The use of in vitro techniques allows the toxicologist to establish some level of comfort for comparative metabolic similarities between humans and the common species employed in toxicity testing. In vitro techniques have several advantages: rapid assessment of permeability and metabolism; study of molecular mechanisms of toxicity; drug targeting; early studies comparing human and animal systems; and, minimization of animal usage (5). The in vitro models do not provide the entire metabolic picture, but they are useful in illuminating parts of a very complex process. Dedrick and Bischoff (52) have stated that it should be possible to use in vitro comparative metabolic information to provide a basis for predicting drug behavior in any mammalian species, providing anatomy and physiology are known.

Several in vitro methods are available that are applied to human and animal liver samples: liver subcellular fractions (14), that is, postmitochondrial supernatant or microsomes; specific forms of cytochromes P450, that is, species-specific metabolism and effects of enzyme induction/inhibition (62, 95); hepatocytes (162); and, liver slices (15, 165). Subcellular fractions are easy to prepare and cryopreserve, but may be altered in the preservation process (148). Hepatocytes allow for the study of integrated hepatocellular metabolism, express many of the functional activities of the intact liver, and may provide a good compromise between cell extracts and isolated organs (52, 62). Liver slices have the additional advantage of maintaining the structural heterogeneity of the liver, and are not exposed to the potential hepatocyte membrane damaging effects of collagenase used during their preparation. Currently, the most commonly used in vitro preparations are subcellular fractions, hepatocytes, and liver slices (75), and the early assessment of metabolic pathways in cultured human and animal hepatocytes and liver slices is widely practiced (215).

The correlation of the in vitro techniques with in vivo metabolic profiles has been demonstrated with hepatocyte suspensions from rat, rabbit, dog, squirrel monkey, and human livers (90). In general, the metabolic profile from the hepatocytes corresponded to the profile of urinary metabolites previously established for each species (90). As a further positive comparison, the metabolism of caffeine by human liver microsomes, slices, or hepatocyte cultures compared very well with its in vivo metabolism (12). An understanding of the mechanism of species-specific acetaminophen toxicity in rat, rabbit, dog, and cynomolgus monkey was provided by studies in primary hepatocyte cultures (166). The applicability of this in vitro approach has been further demonstrated for diazepam and ketotifen (27, 123). Wrighton and Stevens (200) have compared and contrasted specific human P450s, involved in the metabolism of a very large number of endogenous and exogenous lipophilic compounds, with P450s of several

experimental animal species. The evaluation of the metabolism of drug candidates by individual P450 isozymes from animals and humans may provide valuable information relative to both species differences and potential idiosyncratic responses in the human population. These in vitro test systems generally provide a more rapid, less expensive means of evaluating

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toxicity and mechanisms of toxicity, across species during early drug development (165, 178).
The use of these in vitro systems may also improve the selection of appropriate species for subsequent in vivo testing. Importantly, how the drug is metabolized relative to other drugs that may be administered simultaneously, may provide critical guidance in the design of subsequent clinical studies to address potential drug-drug interaction issues. Many animal and human subcellular, cellular, and tissue preparations that can aid the toxicologist in selecting species for toxicity testing or extrapolating animal data to humans are now commercially available.
Toxicity studies are designed to produce a higher level of exposure in the experimental animals than anticipated in the human clinical trial. Generally, small animals (rats and mice) have more rapid basal metabolic rates, shorter life spans, and more rapid drug metabolic activity than humans (23). Thus, small animals usually require a higher dose of the drug, administered more frequently, to mimic the human clinical exposure (136). Campbell and Ings (23) have provided an example showing that, all else being equal, rats would have to be dosed at 5 mg/kg/day to achieve similar systemic exposure as humans receiving 1 mg/kg/day. It should be realized, therefore, that a dose multiple comparison based on a mg/kg relationship is often an inappropriate measure of safety. It is now generally accepted that exposure (plasma level) provides a better estimate of safety margin than administered dose. Issues related to tissue accumulation, measures of exposure, and safety margins are discussed below.
The assessment of systemic exposure in toxicity studies has been addressed by the ICH and the objectives of toxicokinetic evaluation and specific recommendations are contained in ICH Topic S3A (107).

TOXICOLOGY GUIDELINES

Drug Development Time Lines

Development time has become an important focus for the pharmaceutical industry. The available data indicate a four fold increase in drug development time between the 1960s and the 1980s. From the early 1980s to 1996, however, mean drug development times have been relatively constant at about 10–12 years, with a very wide variability (177). The pharmaceutical industry has committed to increasing drug discovery/development efficiency leading to a halving of the mean drug development time.
NBEs generally have had a shorter development time than NCEs. The NBEs have a mean development time of approximately 6–9 years. The NBEs registered to date have generally been well-characterized natural molecules and the shorter development time is probably related to a better understanding of their actions in humans. The introduction of analogs of natural proteins, some designed to be used at supra-physiological levels, will probably lead to an increase in development time for NBEs.

Regulatory Guidelines for Toxicity Testing

In this section, the toxicology support packages for the registration of NCEs and NBEs are reviewed from slightly different perspectives. More detailed information on the specific studies conducted, their results, and interpretation are included for the classical agents (omeprazole and zidovudine [AZT]) since the majority of compounds currently in development would fall into this category and thus require similar testing strategies. Omeprazole was selected for discussion because it has a comprehensive toxicology package and represents an example of where additional mechanistic studies were critical in the approval process. Zidovudine is discussed due to its proposed use to treat life-threatening disease where no adequate therapy was available. The rapid approval of AZT, in spite of significant toxicology findings and an abbreviated toxicology support package, demonstrates the inherent flexibility in the approval system even with regard to NCEs.
The discussion of specific human NBEs (gonadotropin-releasing hormone analogs, interferon, human insulin) is presented from a more philosophical perspective. Since these agents are naturally occurring, and since the major limiting toxicity in animal studies (immunogenicity) is not applicable to clinical trials, the design of the toxicology package posed special issues that were considered on a case-by-case basis. Furthermore, the toxicological profile was anticipated based on extensive clinical experience with less specific agents (animal-derived insulins) and/or a broad understanding of the hormones' physiological functions. Thus, the following discussion of the NBEs poses questions, concerns, and general guidelines to be considered in the development of these agents.

Acute, Subchronic, and Chronic Testing

Toxicity testing can be considered to be composed of several major types (44). Acute (single-dose), subchronic (multiple-dose, less than 6 months duration), and chronic (multiple-dose, greater than or equal to 6 months duration), studies are intended to elucidate the target organs for toxicity and demonstrate dose-response relationships. They are useful for determining the mechanism of toxic action

and often provide important information for dose selection in other study types. A variety of endpoints are routinely evaluated in subchronic

and chronic studies, including body weight, feed consumption, hematology, clinical chemistry, urinalysis, and gross and histological pathology of numerous tissues. A list of common parameters assessed in subchronic/chronic studies is presented in Table 6.4. However, the toxicologist is continually challenged to modify study design to address the anticipated actions of the compound under investigation. This may result in the addition of certain parameters or tissues to be evaluated, or a more comprehensive analysis of tissues (i.e., electron microscopic evaluation, immunohistochemistry). Furthermore, previous studies, or knowledge of the toxicity of other agents in the therapeutic class or those that have a similar structure, may recommend alternative assessments, such as the determination of the propensity of the agent to induce hepatic microsomal enzymes, cause phospholipid accumulation, or result in peroxisome proliferation.

An assessment of bioavailability and pharmacokinetics is often an important endpoint of subchronic and chronic studies. As discussed previously, these data are critical to extrapolate toxicity findings to humans. Often, the kinetic profile of the compound is determined early and late in the study so that the potential for drug accumulation can be revealed. Alternatively, drug levels may be lower toward study termination, or the metabolite profile may differ, due to the induction of drug metabolizing enzymes. Tissues may also be collected for drug analysis, so that levels in affected tissues can be related to the extent of the histopathological findings. Finally, important dose-response relationships can be established, relative to both parent compound and metabolites, that may be critical in the interpretation of toxicity data.

Acute (single dose followed by a 2-week observation period) and subchronic (usually 2-week or 1-month studies) testing is required prior to FHD. One-month studies in one rodent (usually rat or mouse) and one nonrodent (usually dog or primate) species usually will support 1 to 2 weeks of dosing in humans. Where possible, the animal studies should be carried out using the same route of administration anticipated for use in patients. As an aside, it should be acknowledged that clinical studies may initially be conducted by the intravenous route, regardless of the desired ultimate route of administration, particularly for those molecules that are anticipated to show efficacy rapidly, in order to demonstrate the proof of concept of a new pharmacological mechanism. Drug developers might thus avoid the time and expense associated with formulation development and maximization of the desired properties of the chemical if it has been demonstrated that the molecule/ mechanism is ineffective. These clinical trials require the support of intravenous toxicology assessments. In these circumstances, it is extremely important to evaluate the risk associated with the potential for demonstrating toxicity in the intravenous study that may be irrelevant to the ultimate route of administration.

Phases II and III, efficacy testing in patients, are supported by longer-term studies. Depending on the proposed duration of human exposure, toxicity studies to support Phases II/III may be of 3, 6, and/or 9/12 months duration. Two or more subchronic/chronic studies may be conducted simultaneously (i.e., 3-month and 6-month studies may be initiated at the same time) so that patients can be placed on the trial earlier (upon completion of the 3-month study), and maintained on the trial longer (supported by the 6-month study) if the human efficacy and safety data support continued therapy. A potential problem with this approach is that dose selection for the more extended study may be found to be inadequate (doses either too low or too high) based on the findings of the shorter test.

There has been much discussion surrounding the utility of 1-year studies. The FDA has been a strong proponent of the 1-year study approach, while Japan and the European Union (EU) have suggested that 1-year studies reveal little new information beyond that gained from 6-month studies. These data have been reviewed by Lumley et al. (127), who suggest that for 154 compounds in which short-(≤6 months) and long- (>6 months) term animal data are available, tests lasting longer than 6 months (excluding carcinogenicity studies) have not provided new substantive safety information. They also point out that although new findings became evident after 6 months of treatment in 9 out of 75 cases, the data did not influence the decision whether to continue the development of the compound.

Based on the present ICH position, it is generally accepted that 6-month rodent and 9-month nonrodent multiple-dose studies are acceptable for a tripartite development plan (108). Even so, it is strongly recommended that the sponsor have a written commitment from the appropriate reviewing division of the FDA on the acceptability of the 9-month versus the 1-year nonrodent chronic toxicity study.

Additional Toxicology Studies to Support Clinical Trials

Other tests conducted prior to initial clinical trials include mutagenicity studies and pharmacological assessments. A variety of mutagenicity studies currently are employed that assess various types of DNA damage in vitro and in vivo in an attempt to predict the oncogenic potential of the compound under investigation. In pharmacological screening, the ability of the compound to produce toxicities or "side

effects" based on its pharmacological mechanism of action is assessed. For example, an agent that is shown to bind to β-receptors in vitro might be anticipated to influence cardiac function in subsequent toxicity and clinical testing. Both of these study types are often employed as a very early screen in the

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Table 6.4

Parameters that might typically be assessed in a subchronic/chronic toxicology study

Live-phase
Body weight
Feed consumption
Efficiency of food utilization (g body weight gained/100 g feed consumed)
Clinical observations
Ophthalmology
Electrocardiogram (large animal)
Physical examination

Hematology	
Erythrocyte count	Total leukocyte count
Hemoglobin	Leukocyte differential
Packed cell volume	Thrombocyte count
Mean corpuscular volume	Activated partial thromboplastin time
Mean corpuscular hemoglobin	Prothrombin time
Mean corpuscular hemoglobin concentration	M:E ration (bone marrow smears)

Clinical chemistry	
Glucose	Inorganic phosphorus
Blood urea nitrogen	Sodium
Creatinine	Potassium
Total bilirubin	Chloride
Alkaline phosphatase	Cholesterol
Alanine transaminase	Triglycerides
Aspartate transaminase	Total protein
Gamma glutamyltransferase	Albumin
Creatinine phosphokinase	Globulin
Calcium	Albumin/globulin ratio

Urinalysis	
Color	Glucose
Clarity	Occult blood
Specific gravity	Ketones
pH	Bilirubin
Protein	Urobilinogen

Organ weights	
Kidneys	Prostate
Liver	Adrenals
Heart	Thyroids (with parathyroids)
Ovaries	Brain
Testes	

Histopathology		
Kidney	Stomach	Skin
Urinary bladder	Duodenum	Skeletal muscle
Liver	Jejunum	Bone
Gallbladder	Ileum	Bone marrow
Heart	Cecum	Adrenal
Aorta	Colon	Thyroid
Trachea	Rectum	Parathyroid
Lung	Ovary	Pituitary
Spleen	Uterus	Cerebrum
Lymph node	Cervix	Cerebellum
Thymus	Vagina	Brain stem
Salivary gland	Testis	Spinal cord
Pancreas	Epididymis	Sciatic nerve
Tongue	Prostate	Eye
Esophagus	Mammary gland	Harderian gland
Other		

Blood levels of parent compound/metabolites
Hepatic microsomal enzyme activity/cytochromes P450 content
Hepatic oxidation
Tissue phospholipid phosphorus concentration

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evaluation of potential drug candidates to select one of a group of structurally related compounds that would be least likely to result in carcinogenicity, and most likely to demonstrate the specific desired pharmacological activity. The types and utility of these studies are further described below.

Finally, special studies might be conducted prior to initial clinical testing to address specific issues, for example, irritation testing of an agent proposed for topical use in the patient population.

Reproductive and Developmental Toxicity Studies

Since the thalidomide incident, there has appropriately been a great deal of concern relative to predictive testing for developmental toxicity, as well as, fertility effects in both males and females. Although regulations have differed substantially among countries, worldwide harmonized guidelines for reproductive toxicity testing have now been established (109, 110). The ultimate goal of reproductive and developmental toxicity studies is to assess reproductive risk to adults as well as to the developing individual at all stages from conception to sexual maturity. Traditionally, animal studies have been conducted in three "segments"—in adults, treatment pre-mating through mating in the male, and pre-mating through either implantation or lactation in the female (Segment I); in pregnant animals, treatment during organogenesis (Segment II or teratology studies); and in pregnant/lactating animals, treatment from the completion of organogenesis through lactation (Segment III—perinatal and postnatal study). Although guidelines addressing treatment regimens have been rather similar throughout the world, the required endpoints measured, in both the adult and developing organism, have varied widely, and this is an area where much duplicative testing has occurred to support worldwide registration.

The harmonized ICH guidelines (109, 110) stress the need for flexibility in testing for reproductive and developmental toxicity, and challenge the toxicologist to "custom design" a combination of studies that will reveal potential effects on all the parameters considered in the classical Segments I, II, and III studies. For treated adults, these include development and maturation of gametes, mating behavior, fertilization, implantation, parturition, and lactation. In the developing organism, where the maternal animal may be exposed to the drug candidate from prior to mating through lactation, assessments of early embryonic development, major organ formation, fetal development and growth, postnatal development and growth, including behavioral assessments, and attainment of full reproductive function are required. These evaluations might be carried out as one comprehensive study with interim assessments, or they might be segmented into several treatment components. Thus, the new guidelines have not diminished the extent of evaluation, but allow flexibility in study design based on what is already known of the compound under investigation. The harmonized guideline suggests a three-study design that is likely to provide all the developmental toxicity data necessary to support product registration, assuming no untoward toxicity (Figure 6.7). Should toxicity be demonstrated, further mechanistic studies would be conducted to clarify effects and determine whether the responsible mechanism(s) would be applicable to humans. Clearly, the results from previous subchronic/chronic studies (i.e., was there evidence for an effect on spermatogenesis upon histopathological examination of the testes) are critical in the design of an appropriate reproduction package.

Women of child bearing potential are generally first recruited into Phase II trials, and several countries require that efficacy be demonstrated in male patients prior to recruitment of women of child bearing potential into trials, regardless of the outcomes of the nonclinical reproductive studies. However, the FDA has encouraged the inclusion of women of child bearing potential in early clinical trials, especially in the case of drugs intended to treat life-threatening conditions or in the study of disease states that more commonly afflict women. Typically, prior to the inclusion of such women into clinical trials, studies are conducted to evaluate effects on organogenesis (Segment II) in two species (usually the rabbit and the rodent species that has been selected for the subchronic and chronic studies). Female fertility assessments are also usually undertaken prior to longer-term treatment or addition of significant numbers of patients. Early studies in men are supported by histological evaluation of the testes at the conclusion of the subchronic and chronic studies, and specific animal studies to examine drug effects on male fertility are not required until Phase III. Unless there are concerns regarding a specific chemical class or mechanism of action, the more sophisticated analyses of perinatal and postnatal development and behavior (Segment III studies) are often conducted in conjunction with the Phase III clinical trials. Frequently, as is the case with other toxicity evaluations, special studies may be conducted to determine the mechanism(s) of observed reproductive effects in an effort to assess whether these findings are meaningful to humans, and whether use of the compound should be restricted depending on the reproductive status of the patient (i.e., the drug should not be administered to pregnant women).

Finally, the inclusion of female patients in clinical trials may be allowed following a more limited assessment of reproductive/developmental parameters if the compound under development is shown to

be efficacious, and/or thought to provide distinct advantages over available therapies, in the treatment of life-threatening disease.

Carcinogenicity Studies
Among the final toxicity studies to be conducted to support the registration of chronic-use therapeutics are the carcinogenicity bioassays. These lifetime studies are usually conducted in two rodent species (normally rat and mouse). Selection of the top dose for the carcinogenicity bioassays has been an intensely debated topic of discussion in the international harmonization process. The international consensus has based the selection of the top dose for carcinogenicity bioassay on any one of the following (104):
1. Maximum tolerated dose (MTD) (see discussion below).
2. Area under the plasma concentration: time curve (AUC): ratio of 25-fold (rodent:human):applies when there is no genotoxicity.
3. Saturation of absorption.
4. Dose-limiting pharmacodynamics: that is, hypotension, hypoglycemia, decreased blood clotting time.
5. The use of a limit dose (1500 mg/kg): applies when the maximum recommended human dose is **<500 mg/day, and the AUC (rodent:human) is ≥10.**
The middle and low doses should also be selected to provide additional information to use in the risk assessment. The consideration of dose linearity, saturation of metabolic pathways, margin of safety, pharmacodynamics, specific animal physiology, threshold effects, and progression of toxic effects should be included in the selection of the middle and low doses for carcinogenicity evaluations.
The use of an MTD as the top dose in a carcinogenicity study is a subject of some controversy. The MTD has been classically defined as the dose that causes no more than a 10 percent decrease in body weight, and does not produce mortality, clinical signs of toxicity, or pathological lesions that would be predicted to shorten the natural life span of an experimental animal for any reason other than the induction of neoplasms (167). The MTD is suggested to produce a level of toxicity indicative of sufficient chemical challenge to define chronic toxic manifestations (99). Many regulatory bodies default to the use of the MTD as the maximum dose in the rodent bioassays. A major concern with the MTD approach is that metabolic saturation, as discussed above, may occur at high doses, leading to abnormal metabolism (138), or in the case of inhaled therapeutics, abnormal clearance (137). Chemicals administered at the MTD in animal bioassays tend to induce mitogenesis as a result of cell death due to frank intoxication, with the target tissues differing among species and sexes (86). This stimulation of cell proliferation, a natural recovery process in response to severe toxicological insult that does not normally occur at reasonable multiples of human exposure levels, can account for the carcinogenic response of nongenotoxic compounds (32). Thus, the fact that a chemical is a carcinogen at MTD levels in rodents may provide little meaningful information relative to low-dose risk assessment in humans (2). MTDs for chronic toxicity studies are usually estimated based on the results of subchronic toxicity studies. However, since compound distribution and disposition may be affected by dose and/or duration of treatment, this may be a very crude estimate (138, 140).
The choice of an MTD is a critical aspect of chronic toxicity evaluations (173). Cell proliferation indices in subchronic toxicology studies may provide a useful estimation of an appropriate MTD by determining the highest dose that does not result in the phenomenon (19). The use of kinetic parameters (Cmax, AUC related to dose), would better predict the dose at which saturation (nonlinearity) might occur, and therefore provide a better estimate of the MTD in a particular species. Also, changes in urinary metabolite profile, in relation to dose, may be a good way of indicating metabolic overload, and aid in more accurately selecting upper dose levels in toxicology studies (199).
The utility of using two rodent species has also been an active area for discussion. The ICH has indicated that the rat would be preferable to the mouse for the conduct of carcinogenicity studies (103). The rat seems to have been given a preference because background mechanistic data are usually available for rats (not mice), studies of metabolic disposition are more often carried out in rats than in mice, and mouse carcinogenicity studies are dominated by liver tumors of questionable relevance to humans. A review of the European Regulatory Database has concluded that studies in the mouse add little to the ability to detect carcinogenic risk from pharmaceuticals (184, 34); but it found, however that carcinogenicity studies in two rodent species are necessary to identify transspecies tumorigens. NCEs active across species are considered to pose a relatively greater risk to humans than NCEs positive in only one specie. The conduct of a study using an alternative in vivo carcinogenicity model along with a standard bioassay in one specie was considered to be an acceptable alternative for assessing carcinogenic potential (34).
The use of alternative models for carcinogenicity assessment meet the desire of the FDA to have an assessment in two species, and provides the advantages of using fewer animals, being of shorter

duration, and being capable of improving the accuracy of the rodent bioassay (33). A number of transgenic animal models are currently being evaluated as alternatives to the two-year bioassay (84, 33, 4). It is early, yet, however, to evaluate the contribution of alternative models of carcinogenic potential to the risk assessment process, and it would be advisable

to discuss the selection of alternative models with the appropriate regulatory agency prior to study initiation.
Within the FDA, it is highly recommended that protocols, dose justification documents, and supporting data be submitted to the Carcinogenicity Assessment Committee (CAC) for evaluation prior to the initiation of carcinogenicity studies. The CAC provides consultation on study designs, assures consistency and quality in the analysis and interpretation of animal carcinogenicity studies, and monitors scientific developments to ensure that scientific standards of design and interpretation are upheld.
Since 1982, there has been a rise in the number of NBEs presented for registration. Even so, this represents a relatively small number of molecules. Due in part to this lack of practical experience, the safety programs for the NBEs have been designed on a case-by-case basis. The ICH has provided two guidelines that address carcinogenicity studies with products of biotechnology (103, 111). There is general acknowledgment that carcinogenicity studies are not appropriate for biotechnology products given essentially as replacement therapy, at physiological levels, especially when clinical experience exists (e.g., insulin, calcitonin, pituitary derived growth hormone) (111, 103, 94). Product-specific assessment of carcinogenic potential may be needed depending on clinical dosing regimen, patient population, and/or biological activity of the product. For products that have the potential to induce cell proliferation (e.g., growth factors), an in vitro evaluation of receptor expression, in cells relevant to the patient population, may be conducted. If these data indicate a need for further evaluation of carcinogenic potential, two-year studies in a single rodent species should be considered.
Long-term carcinogenicity evaluations with endogenous peptides and proteins, or their analogs, are generally indicated when (103):
1. there are significant differences in biological effects to the natural substances.
2. modifications lead to significant changes in structure compared to the natural substance.
3. therapeutic exposure levels exceed those that normally occur in the systemic circulation or in tissues.
A specific example of studies recommended for the analog of a naturally occurring decapeptide, gonadotropin-releasing hormone (GnRH), is presented below (149).
Regulatory guidelines exist as to when and why carcinogenicity studies should be conducted with naturally occurring substances and their analogs. There are opinions from industry, academia, and regulatory agencies on the propriety of conducting these studies (94). For each NBE under development, the existing opinions and guidance must be considered, a reasonable plan to establish safety must be developed, and a discussion held with the appropriate regulatory agency to test the plan. Safety evaluation of NBEs is still very much a case-by-case consideration. The pharmaceutical industry must be careful not to over-interpret the position that carcinogenicity studies are not usually appropriate for biotechnology products.

New Chemical Entities

The extent and types of safety testing of synthetic organic pharmaceutical agents in animal models depend on a variety of factors, including the potential duration of treatment of patients (e.g., short-term—antibiotics; chronic—antihypertensives), route of administration, pharmacological mechanism of action, proposed patient population, and clinical experience with other agents in that therapeutic class. Furthermore, the design of animal toxicity studies that occur later in development must carefully consider the results of previous tests in animals and humans relative to bioavailability, unanticipated toxic responses, and relevance of species selected.
Generally, toxicity testing in animals can be considered in three phases—testing to support FHD (single- and multiple-dose, Phase I), testing to support longer-term and broader efficacy studies (weeks to months, Phase II), and testing to support final registration and, if appropriate, chronic treatment (Phase III) (Figure 6.5). Although the great majority of testing is performed prior to registration, special studies may be requested by regulatory agencies during the review and approval processes. Following widespread clinical use of a new agent, further testing may be appropriate to examine potential mechanisms of action for unanticipated side effects that become evident in the increasing patient population or sub-populations. These may occur due to genetic differences, environmental factors, age, patient history, existence of other diseases/pathologies, and drug interactions. Other tests may be considered if new formulations of the drug are developed, if the drug is suggested for new indications, or if it will be used in patient populations that were not anticipated during initial development (e.g., pediatrics).
The primary purposes of initial clinical testing (support for FHD) are to determine the toxicity and pharmacokinetics (and oral bioavailability, if appropriate) of the drug candidate in humans following one or several doses. Usually, the drug is administered to humans at doses below the anticipated efficacious

dose, and doses are escalated until a satisfactory multiple over the anticipated efficacious dose is achieved or toxicity becomes evident. Unless the drug candidate has known, serious toxicity, as is the case with many oncolytics, it is usually first tested

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	Early Discovery Nonclinical Testing	Phase I	Phase II	Phase III	FDA Review	Phase IV
Years	3.5-6.5	1-1.5	2	3-3.5	2.5-1.5	Post-marketing Testing required by FDA (could be related to line extension strategy)
Test Population	Nonclinical Laboratory Studies	<100 Healthy Volunteers	< 300 Patients	< 3000 Patients	Data Review	
Purpose	Efficacy and Safety	Kinetics Dose Range Safety	Efficacy Safety	Confirm Efficacy Long-term Safety		
Success Rate	100,000 NCEs	100 NCEs Enter Trials (0.1%)			10-20 Approved (0.01-0.02%)	

Short term studies ≤ 2 weeks, 1 or 2 species, in vitro alternatives Detect serious adverse effects

File IND

Genotox., Safety pharmacology, definitive studies ≤ 1 month, 2 species, Safety evaluation in support of FHD

Definitive studies ≤ 6 months (9 months to 1 year nonrodent), support extended clinical trials, Carcinogenicity studies or alternatives

Fertility and teratology studies conducted before inclusion of women of child bearing potential (WCBP) in clinical trials

File NDA

Carcinogenicty studies or other studies dictated by clinical experience

FIG. 6.5. Schematic of the drug development and approval process in the United States. Similar processes are employed for worldwide pharmaceutical testing. From Reference 85.

in a limited male, non-patient population. As indicated above, the FDA supports the early inclusion of women in clinical trials for new therapies, especially those to be used in the treatment of life-threatening diseases (65, 66). Because of this interest, studies of developmental toxicity, which usually occur later, may be moved to a much earlier point in the drug development process (142).

When designing animal studies to support FHD, a major consideration in dose selection must be the anticipated "margin of safety" between animals and humans. Ideally, doses in animal studies should provide exposure to the compound well in excess of what is anticipated at the highest doses to be tested in humans. As discussed previously, a comparison of these doses on a mg/kg basis is no longer considered to provide adequate information in this respect due to potential species differences in absorption and rates and routes of metabolism. Thus, a good estimate of the pharmacokinetic behavior of the agent in animals is an important goal of nonclinical testing. There is no firm guideline regarding what should be considered an adequate margin of safety. However, a "smaller" margin between the potentially efficacious dose and a toxic dose is tolerated for those compounds under development for life-threatening diseases, particularly if they are expected to offer a distinct therapeutic advantage over other agents currently marketed in the class.

Based on the activities of the ICH, the designs and goals of clinical trials are similar throughout the world. Figure 6.6 shows the recommendations for the duration of animal tests relative to proposed human exposure to NCEs. It is possible to discuss the nonclinical and clinical programs with regulatory bodies, and, depending on the characteristics and proposed use of the chemical, modify these recommendations.

The duration of animal tests necessary to support a specific duration of clinical trials for NBEs is much more flexible (111). Short-term clinical trials for life-threatening conditions may be supported by 2-week nonclinical toxicology studies. Subchronic clinical trials

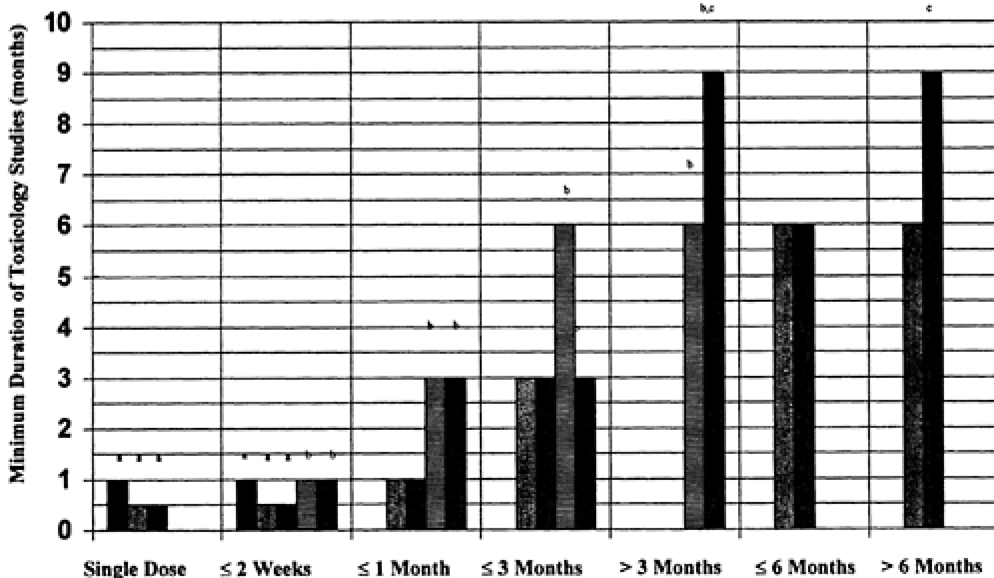


FIG. 6.6. International guidelines for the duration of animal toxicology studies necessary to support clinical trials of various duration. Assessment of reversibility may be necessary in three-month or six-month toxicology studies. Carcinogenicity studies are not needed in advance of clinical trials, unless there is a cause for concern. They may be conducted post-approval for some indications. From Reference 102.

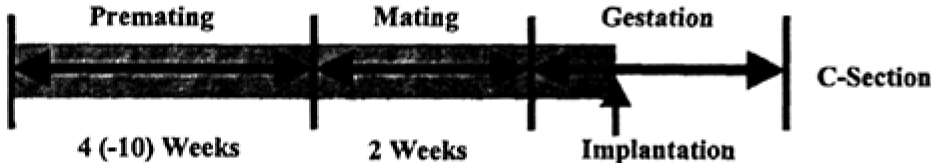
a. In the United States and the European Union (EU), two-week rodent and nonrodent studies are the minimum duration. In: Japan, four-week rodent and two-week nonrodent are needed. In the United States, single-dose toxicity with extended examinations can support single-dose CT.

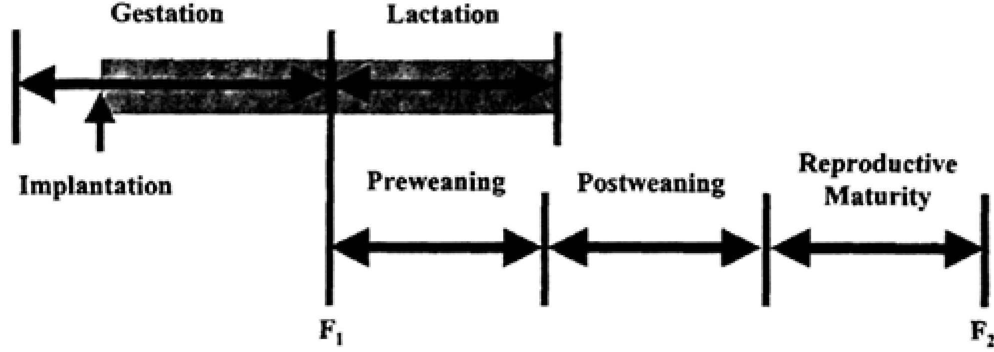
b. Studies in support of Phase III trials in EU, and marketing in all regions.

c. Nine-month nonrodent study is an international consensus, EU and Japan favor six-month, United States favors 12-month. Check with regulators before initiating study.

can be supported with toxicology studies of 1–3 months. Clinical trials to support long-term, chronic therapy, can be supported with 6-month toxicology studies.

Regulators throughout the world have recognized that resources could be used more efficiently, and efficacious and safe drugs could be made available more rapidly, if guidelines for nonclinical clinical testing and registration were comparable across countries. The ICH has developed a comprehensive set of safety guidelines to harmonize the regulatory requirements of the EU, United States, and Japan. The ICH Expert Working Groups (EWG) have considered appropriate guidelines for all of the various types of toxicity tests, including acute and subchronic testing, chronic and carcinogenicity testing, reproduction and developmental toxicity studies, and mutagenicity testing. The required duration of animal studies to support human exposure has also been addressed. There has been a good deal of collaboration between regulatory agencies and pharmaceutical companies in the development of the safety guidelines. Dorato and Buckley (59) have provided a more complete discussion of the role of the ICH process in drug development. The ICH guidelines addressing the various nonclinical studies required to support clinical trials and registrations in the three major regions (EU, United States, Japan) are shown in Table 6.5. Selected Internet websites providing information on design of toxicology studies to support clinical trials are shown in Table 6.6.





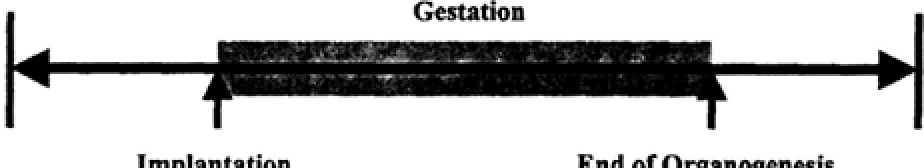


FIG. 6.7. The three-study design proposed for the assessment of reproductive and developmental toxicity for a standard pharmaceutical. From Reference 110.

Specific Agents

Omeprazole (Prilosec)

Omeprazole (Figure 6.8) is a substituted benzimidazole that is a potent inhibitor of H^+/K^+ ATPase ("proton pump") at the secretory surface of the gastric parietal cell, thereby inhibiting gastric acid secretion (155, 189). Omeprazole is indicated for the short-term (4- to

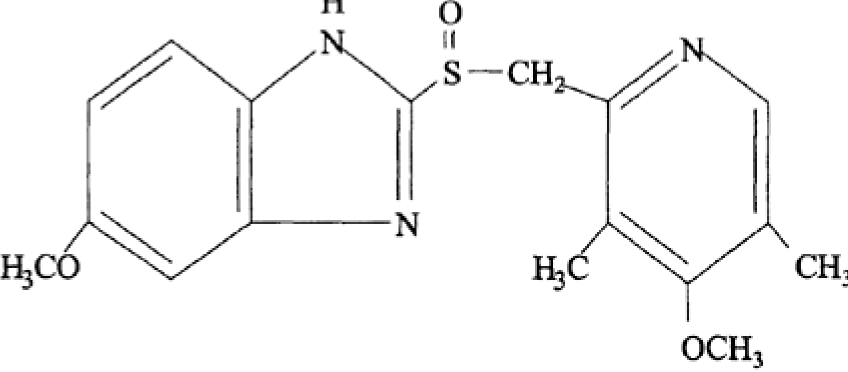


FIG. 6.8. Structure of omeprazole.

12-week) treatment of active duodenal and gastric ulcer, gastroesophageal reflux disease (GERD), severe erosive esophagitis, and the maintenance of healing of erosive esophagitis; and the long-term treatment of pathological hypersecretory conditions such as the Zollinger-Ellison syndrome. It is also

approved for use, with clarithromycin, for treatment of *H. pylori* infections. The recommended dosage for the short-term indications is 20–40 mg daily (approximately 0.4–0.8 mg/kg in a 50 kg individual). For the long-term indications, the recommended initial dose is 60 mg daily; however, doses up to 120 mg three times daily have been administered (146). Table 6.7 lists the toxicology studies that were submitted to the FDA (68) to support the U.S. registration of omeprazole. The content of the toxicology package suggests that the intravenous route may have also been a considered route for therapy.
The results of acute, subchronic, and chronic studies suggested that the toxicology profile of omeprazole was generally unremarkable (24, 61, 68, 97). The acute

Table 6.5
ICH guidelines for the conduct of nonclinical studies

Single- and Repeat-Dose Toxicity Studies
Topic S4 Single-Dose and Repeat-Dose Toxicity Tests (STEP 5)
- LD50 determination should be abandoned
- Reduction in duration of longest-term repeat-dose toxicity study in rodents from 12 to 6 months

Topic S4A Repeat-Dose Toxicity Tests in Nonrodents (STEP 3)
- Reduction of duration of repeat-dose toxicity studies in nonrodents from 12 months to 9 months

Carcinogenicity Studies
Topic S1A Need for Carcinogenicity Studies of Pharmaceuticals (STEP 5)
- Defines circumstances requiring carcinogenicity studies, taking into account known risks, indications, and duration of exposure

Topic S1B Testing for Carcinogenicity in Pharmaceuticals (STEP 5)
- Need for studies in two species
- Alternatives to two-year rodent bioassay

Topic S1C & S1CR Dose Selection for Carcinogenicity Studies in Pharmaceuticals (STEP 5)
- Criteria for selection of high dose

Genotoxicity Studies
Topic S2A Genotoxicity: Specific Aspects of Regulatory Tests (STEP 5)
- Specific guidance for in vitro and in vivo tests pluss glossary of terms

Topic S2B Genotoxicity: Standard Battery Tests (STEP 5)
- Identification of a standard set of assays
- Extent of confirmatory experimentation

Reproductive Toxicology
Topic S5A Detection of Toxicity to Reproduction from Medicinal Products (STEP 5)
- Specific guidance for testing reproductive toxicity

Topic S5B Reproductive Toxicology: Male Fertility Studies (STEP 5)
- Recommendation for pre-mating treatment during and observations

Toxicokinetics and Pharmacokinetics
Topic S3A Toxicokinetics: Guidance on the Assessment of Systemic Exposure in Toxicity studies (STEP 5)
- Integration of kinetic information into toxicity testing

Topic S3B Pharmacokinetics: Guidance for Repeat-Dose Tissue Distribution Studies (STEP 5)
- Need for tissue distribution studies, when appropriate data cannot be derived from other sources

Biotechnology Products
Topic S6 Safety Studies for Biotechnology Products (STEP 5)
- Nonclinical safety studies, use of animal models of disease and other alternative methods, need for genotoxicity and carcinogenicity studies, impact of antibody formation

Multidisciplinary Safety/Efficacy Studies
Topic M3 Timing of Nonclinical Studies in Relation to Clinical Trials (STEP 5)
- Principles for development of nonclinical testing strategies
- Addresses full range of studies to support clinical trials for NCEs

toxicity of the compound in rats and mice was low as demonstrated by oral LD50s (the dose that kills 50% of the animals tested) generally in excess of 4 g/kg. Multiple-dose studies in rats were conducted at doses up to 414 mg/kg/day for 3 months, and up to 138 mg/kg/day for 6 months. There were no consistent effects on body weight or food consumption reported in those studies. Treatment-related findings that occurred at high doses in these studies included decreases in several erythrocytic parameters, and decreases in plasma glucose and triiodothyronine. The latter finding was ascribed to a reduction in the peripheral conversion of thyroxine to triiodothyronine. Increased liver and kidney weights were observed in both studies, as well as in the 24-month rat oncogenicity study. Elevated kidney weights were correlated with an apparent exacerbation of the progress of chronic nephropathy that normally occurs in aging Sprague–Dawley rats.

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Table 6.6

Selected internet websites providing information on the design and expectations on nonclinical toxicology studies

1. http://www.fda.gov/cder/guidance
   Access to guidance documents representing the agency's current thinking on a particular subject relating to the drug development process. Includes access to adopted and draft ICH guidelines.
2. http://www.eudra.org/humandocs/humans/swp.htm
   Safety Working Party (SWP) documents covering aspects of safety evaluation in Europe. Includes access to adopted and draft ICH guidelines.
3. http://www.ich.org
   The process and the adopted and draft safety guidelines of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH).
4. http://www.eudra.org
   The European Agency for the Evaluation of Medicinal Products (EMEA).
5. http://www.cmr.org
   The Centre for Medicines Research International (CMR) is a not-for-profit organization funded by the worldwide research-based pharmaceutical industry to provide unique data and expert analysis to address technical, medical, economic, regulatory, and policy issues in the discovery, development, and safe use of medicines.

Dogs were treated with omeprazole for 3 months at doses up to 138 mg/kg/day or 12 months at doses up to 28 mg/kg/day. Clinical chemistry findings were generally unremarkable, although as observed in the rat, some decreases in hematology parameters and plasma triiodothyronine were noted.

The most significant nonclinical finding in both rats and dogs was a reversible gastric mucosal cell hyperplasia with increases in mucosal thickness and folding. In the 6-month study in rats, omeprazole induced a dose-related eosinophilia of the zymogen granules of the pepsinogen-secreting chief cells, with slight atrophy of these cells occurring at the high dose. Slight chief cell atrophy was also observed in dogs given the high dose of omeprazole for 3 or 12 months. To characterize these gastric changes more rigorously, a rather extensive reversibility study was conducted in rats in which animals were treated with either 0 or 138 mg omeprazole/kg/day for 14 days, and 1, 3, or 6 months. Other groups of animals were treated with that dose of omeprazole for 3 or 6 months, followed by recovery periods of 14 days, and 1, 3, or 6 months. This study demonstrated the time dependency and complete reversibility of the gastric lesions in rats. A 3-month recovery period following 3 months of treatment in dogs showed that the slight chief cell atrophy observed at 3 months was reversible, and a 4-month recovery period following 12 months of treatment in dogs demonstrated the reversibility of mucosal hyperplasia and chief cell atrophy, although increased mucosal folding was still evident.

No teratological findings were observed in the rat at omeprazole doses up to 138 mg/kg/day administered on days 6–15 of pregnancy. The two highest doses tested in rabbits (approximately 70 and 140 mg/kg), administered during days 6–18 of pregnancy, resulted in maternal toxicity as evidenced by anorexia and reduced water intake. Signs were sufficiently severe that treatment of animals at the high dose was discontinued on day 14. Fetal mortality was increased in conjunction with maternal toxicity, but fetal development was unaffected by maternal omeprazole treatment. The major finding of the fertility and perinatal and postnatal studies was a decrease in weight gain of pups of maternal animals given the high dose of 138 mg/kg/day during late pregnancy and lactation. This correlated with a decrease in maternal body weight and food consumption during late lactation. Whether the decrease in pup weight gain was the result of the decrease in maternal feed consumption, or whether it may have been a direct effect on offspring via the breast milk transfer of the compound is not known, and whether nursing or drug therapy ought to be discontinued in the nursing woman, for this and other pharmacological agents, should be considered.

In the mouse oncogenicity study, animals were treated with up to 138 mg omeprazole/kg/day for 18 months. A decrease in survival was noted at the high dose, but no neoplasia was observed in any organ. Different results, however, were obtained in the rat oncogenicity study, in which animals were treated with 13.8, 43, or 138 mg omeprazole/kg/day for 24 months. Enterochromaffin-like (ECL) cell hyperplasia, progressing to ECL cell carcinoids, occurred in dose-related fashion in these animals, with males being affected at doses of 43 and 138 mg/kg/day, and females being affected at all dose

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levels. These positive findings resulted in the temporary suspension of the clinical trial program. The carcinoids were characterized as "end-of-life" tumors, since the first was discovered at 82 weeks of treatment in an animal that had died prematurely. Carcinoid tumors were not identified as the cause of death in any animals, and no metastases were found. A 2-year study was repeated in females in an attempt to define a dose at which ECL cell carcinoids did not occur. However, carcinoid formation again occurred in a dose-related fashion, including at the lowest dose tested (1.7 mg/kg/day).

A major question that must be addressed following positive results in a carcinogenicity bioassay is whether tumorigenesis was the direct result of chemical insult, or whether it can be related to the pharmacological mechanism of action of the compound. Furthermore, whether the model is appropriate for extrapolation of these findings to humans requires evaluation. For example, at this late stage in the development of a compound, sufficient pharmacokinetic data should be available in both the animal species tested and humans to determine whether a finding might be restricted to a species that metabolizes the compound quite differently from humans. If this is the case, further mechanistic studies can be designed to support or refute the applicability of the findings.

A number of in vivo and in vitro mutagenicity studies were conducted with omeprazole (Table 6.7). An initial mouse micronucleus test with omeprazole administered to animals at a high dose of 5000 mg/kg produced equivocal results, with slight increases in the mean numbers of micronucleated cells compared to controls (approximately 2-fold, compared to 30-fold following a 0.4 mg/kg dose of the positive control, mitomycin C). It was noted that the dose of 5000 mg/kg was not well tolerated. A second mouse micronucleus test was conducted, using a maximum dose of approximately 800 mg/kg, which did not show evidence of mutagenic potential. All other mutagenicity tests conducted produced negative results, suggesting that the tumorigenesis observed in the 2-year rat studies was not the result of a genotoxic action of omeprazole or its metabolites.

Mechanistic studies in dogs and rats, combined with correlative data from clinical trials, ultimately provided the information to support the safe use and registration of omeprazole. At the doses selected for the toxicity studies, the sustained decrease in luminal pH of the stomach resulting from the inhibition of gastric acid secretion by omeprazole caused a substantial increase in the release of gastrin into the blood. In fed rats, gastrin levels in plasma normally range from 150–200 pg/ml. Omeprazole administered at doses of 13.8–138 mg/kg/ day increased plasma gastrin concentrations to 1000–3000 pg/ml. Gastrin has a trophic effect on the gastric mucosa and results in the hyperplasia of several

Table 6.7

Summary of toxicology studies conducted to support the registration of omeprazole in the United States

Acute toxicology
Oral study in mice
IV study in mice
Oral study in rats
IV study in rats
Oral study in dogs
Subchronic toxicology
2-Week iv study in rats
1-Month iv study in rats
1-Month iv study in dogs
3-Month oral study in rats
3-Month oral study in mice
3-Month oral study in dogs
3-Month oral study in dogs with 3-month recovery
Chronic toxicology
6-Month oral study in rats
3- and 6-Month oral studies in rats with 2-week to 6-month recovery
2-Year study in female rats to examine gastrin-dependent variables
1-Year oral study in dogs with 4-month recovery
5-Year oral study in dogs (ongoing at time of application)
Genetic toxicology
Ames *Salmonella* test with/without metabolic activation
Mouse lymphoma forward mutation assay
Mouse micronucleus test
Mouse chromosome aberrations

Rat liver DNA damage assay
Reproductive and developmental toxicology
Segment I oral fertility in rats
Segment II oral teratology in rats
Segment II oral teratology in rabbits
Segment III oral perinatal and postnatal in rats
Segment III extended oral perinatal and postnatal in rats
Carcinogenicity studies
78-Week oral study in mice
104-Week oral study in rats
104-Week oral study in female rats

cell types, including ECL cells, and consequent mucosal thickening. These data suggest that omeprazole does not inherently cause ECL cell hyperplasia and resulting carcinoid formation. Indeed, in antrectomized dogs, where the major source of gastrin is surgically removed, high doses of omeprazole for 1 year resulted in neither hypergastrinemia nor mucosal hyperplasia (61). Similarly, antrectomy in rats prevents the hypergastrinemia and ECL cell hyperplasia associated with omeprazole treatment (120).

The course of development of omeprazole demonstrates the importance of conducting mechanistic studies to elucidate the significance of findings in animal safety studies, and whether the effects can be meaningfully extrapolated to humans. A close collaboration between the toxicologist and clinician during advancing human trials is critical to resolve questions related to human safety. The extensive clinical experience with omeprazole has confirmed its safe and effective use

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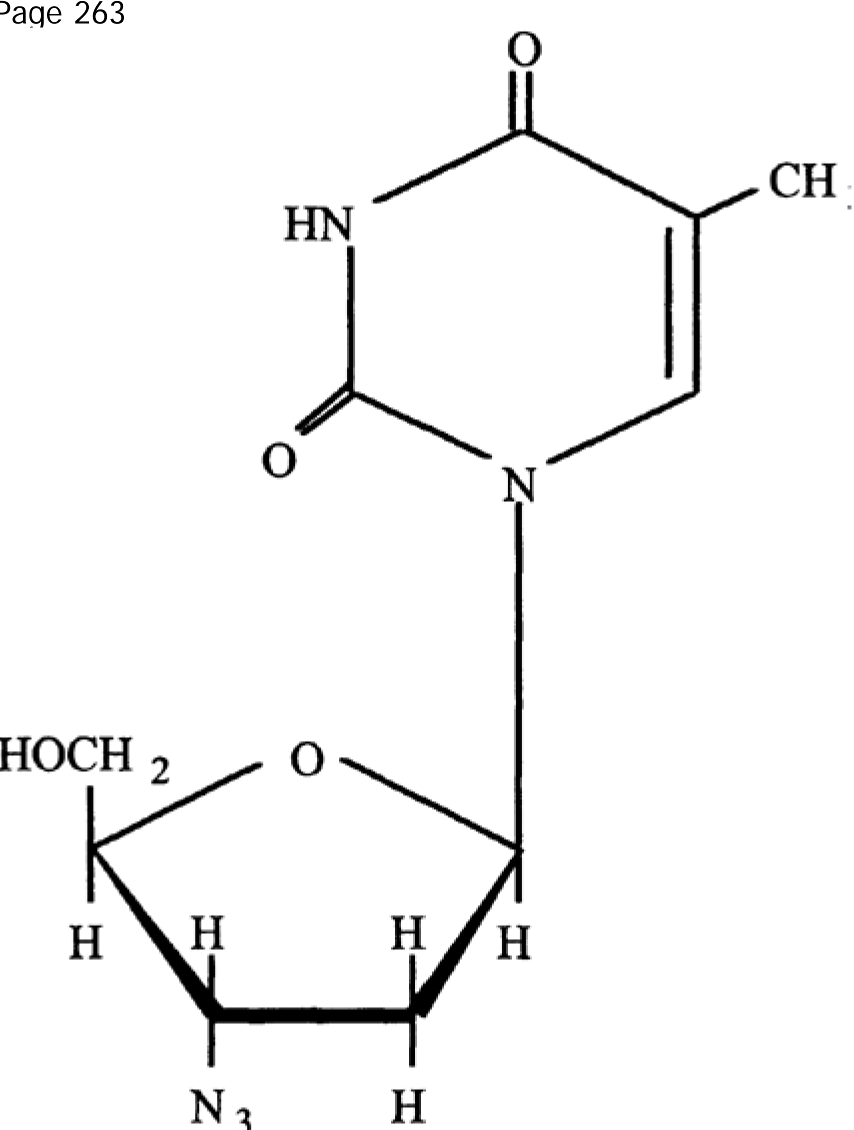

FIG. 6.9. Structure of zidovudine.

for treatment of the described indications (146, 11). In a group of patients who required continuous treatment with 40 mg of omeprazole for up to 4 years, there was no evidence for dysplastic or neoplastic changes (39). Over 12,000 endoscopic biopsies further support the clinical safety of omeprazole relative to its potential for causing hyperplastic changes.

Zidovudine (Retrovir/AZT)

Zidovudine (azidothymidine, AZT, Figure 6.9) inhibits viral RNA-dependent DNA polymerase (reverse transcriptase) and, thus, viral replication. Furthermore, as a thymidine analog, zidovudine becomes incorporated into growing strands of DNA by viral reverse transcriptase, and inhibits the further addition of nucleotides. It is intended for use in the management of adult patients with HIV infection when antiretroviral therapy is warranted (147). It is also indicated for the prevention of maternal–fetal HIV transmission during gestation and labor, and in the neonate after birth. The recommended dose for adults is 600 mg/day in divided doses in combination with other antiretroviral agents and 500 mg/day (100 mg every 4 hours while awake) or 600 mg/day in divided doses for monotherapy. Zidovudine is also available for intravenous infusion in

Table 6.8
Summary of toxicology studies submitted for initial FDA review to support the registration of zidovudine in the United States

Acute toxicology
  IV study in mice
  IV study in rats
Subchronic toxicology

2-Week iv study in dogs with 2-week recovery
2-Week oral study in rats
2-Week oral study in dogs
2-Week oral study in monkeys
1-Month iv study in rats with 2-week recovery
3-Month oral study in rats with 2-week recovery
3-Month oral study in monkeys with 6-week recovery
6-Month oral study in rats with 2-month recovery
Reproductive and developmental toxicology
Segment II oral teratology in rats
Segment II oral teratology in rabbits
Genetic toxicology
Mouse lymphoma assay
Ames *Salmonella* test with/without metabolic activation
Cell transformation assay
In vivo cytogenetic study in rats
In vitro cytogenetic study in human lymphocytes
Summary of toxicology studies planned or in progress at the time of initial FDA review of zidovudine
6-Month oral study in monkeys
Segment I oral reproduction/fertility study in rats
Segment III oral perinatal and postnatal study in rats
Segment II oral teratology study in rabbits
1-Year oral study in rats
1-Year oral study in monkeys
Oral carcinogenicity study in rats
Oral carcinogenicity study in mice

patients with advanced disease and for use in women during labor and delivery. In spite of the intended long-term use of the compound, it was approved with a minimal toxicology package due to the serious nature of the disease and lack of alternative efficacious therapies.

The studies listed in the upper portion of Table 6.8 were either submitted as part of the original NDA in December 1986, or as amendments to the application shortly thereafter (69). At the time of the initial pharmacology–toxicology review, a variety of chronic toxicity studies were still underway or planned. The FDA commentary indicated that

"nonclinical toxicity data submitted in support of the application include results of studies in rats, dogs and cynomolgus monkeys. FDA guidelines would have prescribed more extensive nonclinical testing than that reported thus far. However, the urgency for developing an anti-AIDS drug has been so great that clinical testing has preceded the usual/customary nonclinical testing. For example, while data from a 6-month *clinical* study are available, results for the supporting 6-month *nonclinical* toxicity studies have not yet been submitted" (emphasis added).

An approvable letter issued by the FDA in March 1987, less than four months following submission of the NDA, stipulated the timing for the conduct of these outstanding studies. Comprehensive reviews of the acute, subchronic, chronic, genetic, carcinogenic, reproductive, and developmental toxicity studies have been published (6, 7, 91).

Zidovudine demonstrated relatively low acute toxicity with intravenous MLDs (median lethal doses) of greater than 750 mg/kg in rats and mice. The most consistent findings in the subchronic/chronic studies with rats, dogs, and monkeys were effects on hematological parameters. In rats given two divided doses of zidovudine at approximately 50, 150, or 500 mg/kg/day orally for 3 or 6 months, there were reversible decreases in red blood cell counts and hemoglobin concentration primarily in the mid- and high-dose groups. The severity of these effects appeared to progress slightly between 3 and 6 months of treatment. No remarkable histopathology was noted in these studies. A subsequent 1-year study, submitted well following the initial approval of zidovudine, revealed a similar toxicity profile to that observed in the 3- and 6month studies. The severity of anemia did not progress between 6 and 12 months of exposure, and effects were again reversible following discontinuation of treatment.

Dogs were more sensitive to zidovudine treatment. In a 2-week study in which animals were administered 125-500 mg/kg/day orally in divided doses, decreases in erythroid values, and leukopenia and thrombocytopenia were observed at all dose levels. Cytostatic effects were observed in the small intestine at the high dose, and were also evidenced by slight to moderate non-dose-related lymphoid depletion, and mild to marked dose-related bone marrow hypocellularity at all dose levels. No cytostatic effects were observed at similar or higher doses in either rats or monkeys. Studies revealed that zidovudine was metabolized almost identically in monkeys and humans, and as a result, the continued nonclinical development of the drug was conducted in the monkey, rather than the dog. However, the species differences in metabolism were not of sufficient magnitude to account for the much greater sensitivity of the dog to zidovudine, and the design of subsequent nonclinical and clinical studies continued to respect the significant findings in this species.

Monkeys responded to a 2-week treatment at divided doses of 125–500 mg/kg/day with a slight reduction in hemoglobin concentrations in one animal at the low dose, and in both monkeys given the high dose. In a 3-month monkey study, at divided doses of 35–300 mg/kg/day, dose-related decreases in erythron parameters were noted as early as day 15 of treatment and progressed to live-phase termination. Platelet counts were also increased. Values returned to normal during the 6-week recovery phase of the study. Subsequent (post-approval) 6- and 12-month studies were conducted in the monkey. In addition to the findings in the 3-month study, bone marrow cytology revealed changes consistent with the hematology findings, and marginal decreases in white blood cell counts were observed at the 300 mg/kg/day dose. All findings were again reversible.

Teratology studies were also carried out in rats and rabbits prior to approval of zidovudine. Divided doses up to 500 mg/kg/day resulted in no evidence for teratogenicity in either species, but non-treatment-related low fertility rates and mortalities in the rabbit study prompted the FDA to request that a second study be initiated prior to drug approval. Effects were limited to an increase in fetal resorptions and an associated decrease in fetal body weights at the maternally toxic high dose. The potential use of zidovudine in pregnant women to inhibit transplacental HIV transmission prompted additional in vitro and in vivo reproductive and developmental toxicity studies following the initial approval of the compound. These subsequent studies demonstrated that zidovudine is embryotoxic in rats at doses that are not overtly maternally toxic. Postnatal survival, growth, and development of offspring from zidovudine-treated rats were unaffected following several treatment regimens. In general, exposure levels associated with the effects observed in the reproductive and developmental toxicity studies were significantly higher than those observed clinically.

No evidence for mutagenicity by zidovudine was observed in the Ames Salmonella study either with or without mammalian metabolic activation. The compound was weakly mutagenic in the mouse lymphoma assay without metabolic transformation at concentrations of 4000 and 5000 μg/ml; it was also weakly mutagenic with metabolic activation at concentrations greater than or equal to 1000 μg/ml. A positive response was obtained in the mammalian cell transformation assay at concentrations of 0.5 μg/ml or greater. In an in vitro cytogenetic assay in human lymphocytes, zidovudine caused structural chromosomal abnormalities at concentrations equal to or greater than 3 μg/ml. However, in an in vivo rat assay, no chromosomal abnormalities were noted following the intravenous administration of doses up to 300 mg/kg (plasma levels over 400 μg/ml). Subsequent in vivo micronucleus studies in mice and rats revealed dose-related increases in micronucleated erythrocytes, reflecting chromosome breakage or mitotic spindle damage.

Carcinogenicity studies in mice were initiated using single daily doses of 30, 60, or 120 mg/kg. These doses were reduced to 20, 30, or 40 mg/kg at 3 months of treatment due to treatment-related anemia. Rats were dosed with 80, 220, or 600 mg/kg/day, with the high dose being reduced to 450, then 300 mg/kg. As expected, hematological changes were observed, but there were no deaths or morbidities that were considered

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treatment-related in either study. In the mouse study, one benign vaginal neoplasm occurred at 30 mg/kg, and five malignant and two benign neoplasms occurred at 40 mg/kg. Two vaginal neoplasms occurred at the high dose of the rat study. In both cases, the tumors were late-occurring and non-metastasizing. An eloquent argument has been put forth suggesting that these vaginal tumors result from high local exposure to zidovudine due to the retrograde flow of urine containing high levels of the excreted compound into the vagina. An additional lifetime mouse study to support this hypothesis was conducted in which animals were administered zidovudine intravaginally. Thirteen vaginal squamous cell carcinomas occurred in animals receiving the highest concentration in that study, supporting the contention that systemic exposure to the drug was unlikely to be responsible for the neoplasia observed in the oral studies.

A variety of adverse reactions have been documented in patients receiving zidovudine. Due to the wide range of symptoms associated with the opportunistic infections seen in AIDS patients, it is difficult to assess which adverse reactions are clearly the result of zidovudine therapy. However, the animal safety studies were highly predictive of the major hematological toxicities of zidovudine described in humans—granulocytopenia and severe anemia.

Similar to what was described previously under the development of omeprazole, additional mechanistic studies were conducted with zidovudine to explain toxicity findings, even though the drug was intended for the treatment of a fatal disease.

Although the toxicology support package for zidovudine ultimately responded to existing guidelines for registration of a chronic-use pharmaceutical in the United States, its development history demonstrates that the approval system allows considerable flexibility in cases where the market for a life-threatening disease is clearly not satisfied (i.e., antivirals, antifungals, oncolytics). This type of development strategy can only occur with close collaboration between the submitter and the regulatory agency, and after careful consideration of the risk-benefit assessments.

New Biological Entities

The development of highly purified species-specific protein pharmaceutical agents, made possible through advances in rDNA technology, presents a significant challenge to toxicologists. The major question presented is, “What nonclinical toxicology evaluations should be conducted to insure safety in human clinical trials?” The major issue is the testing of these specific proteins in nonhomologous animal species, where the possibility of immunogenicity, not applicable to the clinical trial, exists (174). As there are no universally accepted methods/procedures for the nonclinical evaluation of new biological entities (NBEs), decisions of appropriate nonclinical study design are made on a case-by-case basis. The general consensus is that nonclinical toxicity evaluations with species-specific proteins reveal little more than enhanced pharmacodynamic activity rather than predicting the potential for adverse effects. Furthermore, the toxicity observed in animal studies may be the result of an immunological response to the foreign protein. Toxicology studies with NBEs should demonstrate that the product has no adverse effects other than those specifically related to pharmacodynamics, and that safety for the expected clinical dose range, rather than exaggerated toxicity (i.e., MTD), should be demonstrated (9, 145, 174). Regulatory agencies have placed great emphasis on chemical characterization of the NBE as a means of establishing that it is identical to the naturally occurring protein (manufacturing contaminant issues aside). Establishing this identity has allowed for appropriate modification of toxicology requirements and abbreviation of the toxicology support package. However, NBEs are being developed that contain amino acid sequences that have been purposefully manipulated to differ from the naturally occurring protein in order, for example, to result in a prolonged duration of action over the naturally occurring agent. These molecules may require a more comprehensive toxicology package, such as established for NCEs (see above).

Safety testing of NBEs can be presented in three categories (Table 6.9). The reasonably clear-cut time sequence of nonclinical and clinical studies established with NCEs often is not feasible with NBEs. The interactions between toxicologists and clinicians are important in addressing suspected adverse reactions in the clinical trials (9). Nonclinical toxicology evaluations of NBEs should be designed according to the risks anticipated from the type of product, the contaminant profile, and the intended clinical use (9). There will continue to be major questions and differences of opinion relative to

Table 6.9

Safety testing of biotechnology products*a*

Category	Requirements
1	Identity, purity, pharmacology, safety pharmacology
2	Category 1 plus: Detailed pharmacological activity (human, animal), relationship of plasma

	concentration and antibody titer (human, animal, in vitro) tolerance, selected toxicological testing
3	Categories 1 and 2 plus: Studies guided by indication, studies guided by duration of treatment

[a]From Reference 9.

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the evolution of nonclinical toxicology testing strategies of NBEs. The major questions will arise concerning appropriate species (174, 216), the need to conduct genetic toxicology studies (9, 174), and the conduct of reproductive toxicology assessments (174).

As examples of NBEs, we have chosen to discuss toxicology support for the registration of gonadotropin-releasing hormone (GnRH) analogs, interferon, and human insulin. The development of interferon has provided a great deal of guidance for nonclinical toxicity testing of NBEs. The pharmacological effects of insulin are well known from extensive clinical experience. This experience has aided the relatively rapid approval of rDNA insulin products, and has allowed the chemical characterization of test material to play a major role in supporting a more limited toxicology profile. Human insulin, therefore, provides an example of a NBE that was approved rapidly, in approximately five months (121).

Gonadotropin-Releasing Hormone (GnRH) Analogs

GnRH analogs are either agonists or antagonists of the receptor for the naturally occurring hypothalamic decapeptide GnRH. The chemical modifications either increase the biological activity and duration of action or affect the solubility, potency, and kinetics of the molecule. GnRH analogs were first introduced for the treatment of cancer (e.g., prostatic carcinoma) and their toxicological assessment was less complete than usually recommended for new drugs. Since their first introduction, the use of GnRH analogs has expanded into treatment of non-life-threatening conditions, and they now are expected to have to undergo the same rigorous toxicology evaluation as other new drugs (149). In the case of GnRH analogs, the FDA has allowed the multi-dose toxicity studies to be conducted at a multiple of human exposure (30- to 50-fold) rather than at doses that define the toxic limits of the compound. Due to the chronic nature of therapy, and chemical dissimilarity with native GnRH, the FDA has recommended that both rat and mouse carcinogenicity studies be conducted. As is the case with the multiple-dose toxicity studies, the FDA has allowed the MTD not to be used, but required, instead, that some multiple of the human clinical exposure be used to set the top dose in the carcinogenicity studies (e.g., 15- to 50-fold). The full toxicity profile recommended for GnRH analogs includes: single-dose acute toxicity (rodent and nonrodent), repeat-dose toxicity studies through 6 months in rodents and 9 months in nonrodents, genetic toxicology, developmental toxicology, and carcinogenicity (149).

Interferon

Interferons (IFNs) are classified as IFN-α (leukocyte), -*β*(fibroblast), or *γ* (immune) (Figure 6.10). IFN-α con sists of a family of at least 14 highly homologous species. The amino acid sequence homology of the IFN-α subtypes has been reported to be 52 to 75 percent (168, 169, 181). The biological activities of IFNs include antiviral, anticellular, and immunomodulatory activities (181). The properties of interferons and their potential uses have been reviewed by Bocci (13).

The adverse clinical experiences reported with the use of IFNs include: fever, chill/rigor, headache, tremor, nausea, vomiting, myalgia, anxiety, fatigue, malaise, anorexia, confusion, local inflammation, cardiovascular toxicity, hepatotoxicity, and abnormal EEGs (71, 160, 161, 169, 187). The most commonly reported adverse effects are fever, fatigue, and leukopenia (169). The effects that cause the most distress in clinical subjects are those related to central nervous system (CNS) depression (187). The toxicity seen with very pure and single clone IFN preparations is almost identical to that reported for the less pure, more heterogeneous preparations of IFNs. The responses reported, particularly the influenza-like syndrome, therefore, are likely intrinsically related to IFNs, and not to a contaminant or impurity (169, 187).

The species specificity of highly purified human IFNs implies that classical animal (nonhomologous) efficacy and toxicity models are not applicable in evaluation of these materials. Nonclinical safety testing of IFNs has not identified an appropriate animal model (72, 98, 153, 157, 170, 213), supporting the recommendation that the routine safety tests applied to NCEs should not be applied haphazardly (174) to NBEs. Yet, given the traditional significance and predictive nature of nonclinical toxicology evaluations, and acknowledging the lack of generally accepted and validated nonclinical animal models for the testing of these entities, drug regulatory agencies have published safety testing guidelines that place NBEs on a level with conventional drugs relative to the comprehensive requirements for animal safety studies (77, 112). A representative example of these guidelines/requirements is given in Table 6.10.

Interferon-α2a (Roferon-A,) is a commercially available NBE identical to one of the 15 subtypes of human leukocyte IFN (182). At the time clinical trials were initiated with this drug, considerable clinical data were available from studies with other leukocyte IFNs to indicate the types of adverse reactions, described above, that might be expected (169, 187). The species specificity of recombinant IFN-α2a has led to production of neutralizing antibodies in rodent and nonrodent species (182). This has impaired

the ability of toxicology studies to detect the expected adverse clinical signs in common toxicology species.

Acute, single-dose toxicology studies (Table 6.11) were conducted in a variety of species in an attempt to disclose any unexpected acute toxicity related to the clinical dos-

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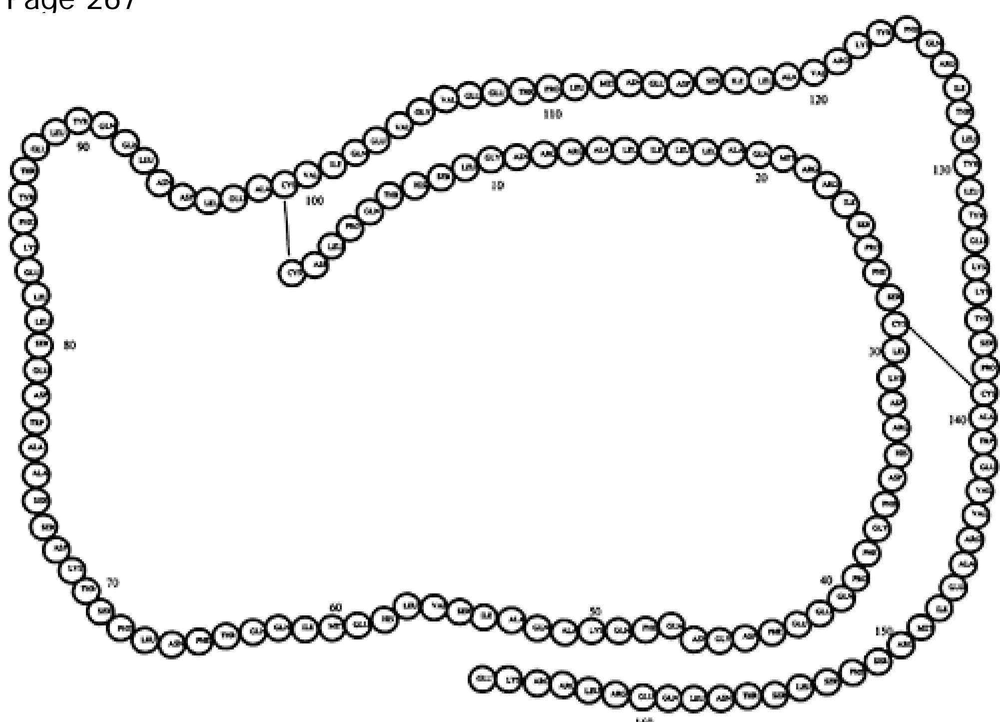

FIG. 6.10. Consensus sequence of human leukocyte interferons. Redrawn with permission from Reference 169.

Table 6.10

Recommendations for Interferon testing by the French Ministry of Social Affairs[a]

Toxicological Test	Recommendation
Acute	2 species, both sexes in 1 species, 2 routes, 2-week observation
Subchronic	2 species, rodent and primate, 3 months daily injection
Reproduction	Segments I, II, III
Mutagenicity	In vivo and in vitro clastogenesis
Carcinogenicity	Not required
Pyrogenicity	Rabbit
Safety Pharmacology	In vivo cardiopulmonary, isolated organs; Neurobehavioral studies
Cell culture	Cytostatic and cytotoxic effects

[a]From Reference 210.

age form (excipients, active ingredients). No mortalities were noted in the species tested. The LD50 of IFN-α2a was determined to be >22.8×106 units/kg iv. These studies were conducted at multiples of a single clinical dose (3×106 units/kg), ranging from 10- to 167-fold. Multiple-dose toxicology studies were conducted over a range of 5 to 26 weeks at 3- to 78-fold the weekly clinical dose (9×106 units/kg) (Table 6.12). A low frequency of treatment-related adverse findings was reported: slight weight loss in rats; a slight, reversible, increase in platelets and total leukocytes in mice; a slight decrease in hemoglobin and hematocrit in squirrel monkeys; dose-dependent anorexia and weight loss in *M. mulatta;* and transient anorexia in *M. fasicularis*. In studies longer than 2 weeks, neutralizing antibodies developed in rabbits, guinea pigs, and *M. fasicularis* (182). These results were expected, and may have affected the signs of toxicity. Reproductive studies carried out in *M. mulatta* indicated that a dose-dependent increase in abortion was related to the administration of IFN-α2a.

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Table 6.11

Acute toxicology studies conducted with Interferon-α2a

Species	Route[a]	Dose (units × 106/kg)	Clinical Multiple[b]
Mouse	iv	30, 250	≤83×
	im	30, 500	≤167×
	sc	30	10×
Rat	iv and im	30, 100	≤33×
	sc	30	10×
Rabbit	iv and im	100	33×
Ferret	im and sc	30	10×

[a]iv=intravenous; im=intramuscular; sc=subcutaneous
[b]Recommended clinical dose=3×106 units/kg im or sc, there times weekly.

Table 6.12

Multiple dose toxicology studies conducted with Interferon-α2a

Species	Route[a]	Duration (weeks)	Dose (units×106/kg)	Clinical Multiple[b] (per week)
Mouse	im	5	0, 1.4, 2.8, 5.7	≤4×
Rat	im and iv	5	0, 1, 10, 100	≤78×
Rat	im	26	0, 7.5, 15, 30	≤23×
S. sciureus	im	2	0, 2.5	2×
M. mulatta	im	4	0, 2.5, 10, 25	≤19×
M. fasicularis	im	13	0, 2, 10	≤3×

[a]im=intramuscular; iv=intravenous
[b]Recommended clinical dose=3×106 units/kg three times weekly (9×106 units/kg/week)

Insulin

The nonclinical toxicity of biosynthetic human insulin (BHI) (Figure 6.11) was evaluated in an unconventional way. The use of graded increments of dose representing multiples of the projected clinical exposure was not feasible due to the pharmacological effect of hypoglycemia caused by the insulin molecule. Since the pharmacological effects of insulin were well known, a primary goal of the toxicology evaluations was to determine whether BHI contained potentially toxic contaminants/impurities (i.e., *E. coli* proteins, endotoxins, etc.) that are introduced as a result of the synthetic process. Toxicology studies on BHI were conducted simultaneously with purified porcine pancreatic insulin (PPI) as a positive control, at doses previously established to produce hypoglycemia but not mortality. The doses selected for toxicology studies were varied according to species sensitivity, route of administration, and duration of treatment. In acute, single-dose toxicity studies (Table 6.13), the minimal lethal dose of BHI to rats and mice was >10 units/kg sc. Dogs given single doses of two units BHI/kg sc, showed the expected hypoglycemia but no toxicity. No toxic effects were seen in either rats or dogs given BHI sc or iv for one month (Table 6.14). Chronic toxicity, reproductive toxicity, and carcinogenicity studies were not conducted due to the extensive clinical experience with insulin, and the extensive chemical analysis of the NBE, establishing its identical nature with natural human insulin. BHI was negative in a genetic toxicology screen composed of bacterial mutation, DNA repair, and sister chromatid exchange evaluations. BHI was also not pyrogenic. Overall, BHI did not induce any effects different from those induced by PPI, and all effects seen were extensions of known insulin pharmacology. Investigations demonstrating the virtual absence of endogenous *E. coli* proteins, and the absence of antigenic response in rats

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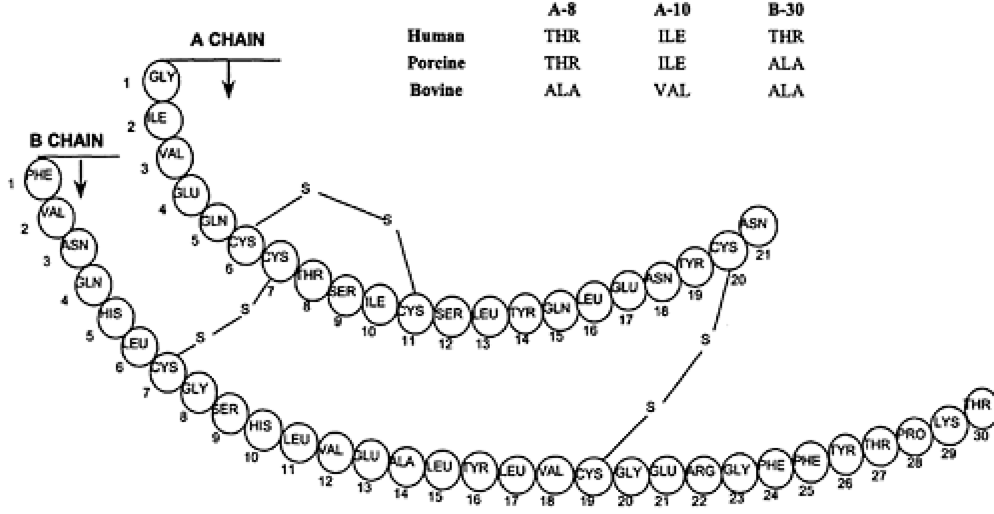

FIG. 6.11. Sequence of biosynthetic human insulin (BHI).

Table 6.13

Acute toxicology studies conducted with biosynthetic human insulin

Species	Route[a]	Dose (units/kg)	Clinical Multiple[b]
Mouse	sc	10	40×
Rat	sc	10	40×
Dog	sc	2	8×
Monkey	iv	0.1	—

[a]sc=subcutaneous; iv=intravenous

[b]Anticipated clinical dose=0.24 units/kg/day, sc.

Table 6.14

Multiple dose toxicology studies conducted with biosynthetic human insulin

Species	Route[a]	Duration (weeks)	Dose (units/kg)	Clinical Multiple[b]
Rat	sc	4	2.4	10×
Dog	sc	4	2.0	8×
Dog	iv	4	0.1	—

[a]sc=subcutaneous; iv=intravenous

[b]Anticipated clinical dose=0.24 units/kg/day, sc.

and guinea pigs sensitized with *E. coli* polypeptides, further addressed the safety of the rDNA-derived human insulin product.

Special Issues

Immunotoxicology

Immunotoxicology can be defined as the discipline concerned with the study of adverse effects on the immune system as a result of exposure to xenobiotics (51). It is not the purpose of this section to review in detail the specific evaluations conducted to define immunotoxicity (48, 51, 179, 201), but, rather, to discuss the use of these evaluations in a hazard assessment tier approach. Adverse responses of the immune system are known to occur secondary to malnutrition, radiation exposure, neonatal thymectomy, and exposure to certain drugs and chemicals (54, 186). Historically, few chemicals have been shown to be immunosuppressive in toxicology evaluations, probably because the lymphoid organs and the immune system, in general, have been poorly examined. It would be desirable, therefore, to establish an effective tier approach to detect immunotoxicity in standard subchronic and chronic toxicology studies, and also to evaluate the functional nature of the changes observed as a result of drug exposure. It is presumed that a functional change detected in the immune system is predictive of adverse health effects (201). It must be remembered that a critical function must be depressed beyond a defined,

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minimal point (reserve capacity) to indicate a health risk (131). The tier approach is encouraged because it more carefully directs the use of resources, and a single immune function assay may not comprehensively characterize the myriad of potential toxic effects on the immune system (51).
Specific immune function tests for increasing the capability of toxicology studies to reveal effects on lymphoid tissue, and to evaluate more fully the risk of chemical exposure by determining the functional significance of the responses observed, have been reviewed (48). It is known that acute and chronic effects of drug exposure on the immune system can result in three principal undesirable effects: immunosuppression or enhancement, autoimmunity, and allergic reactions (131). Immunosuppression has also been related to an increased incidence of neoplasia, though the relationship between immunosuppression and carcinogenesis is not a direct one (54). An early consensus meeting held by the National Institute for Environmental Health Sciences (1979) resulted in the development of a list of relevant immunological parameters for evaluating chemically induced immunotoxicity. This immunology screening panel has been reviewed (48), and includes: pathotoxicology, hematology, host resistance, radiometric delayed hypersensitivity, lymphoproliferation, humoral immunity, and evaluation of bone marrow progenitor cells.
Some of the first guidelines for immunotoxicology testing were those developed by the EU in the late 1970s. The focus of these guidelines was to evaluate the potential risk of chemical exposure by evaluating the functional significance of any histopathological or hematological effects seen on lymphoid organs in routine toxicity studies (141). The intention was to pursue the significance of these effects with specific function tests as necessary. It is known that immunotoxicity following drug exposure may take the form of changes in lymphoid tissue organ weights or histology, or changes in bone marrow or peripheral leukocytes (51). Norbury (141), however, pointed out that the evaluation of drug effects on the immune system is related to immune responsiveness, and is not simply a single point examination of lymphoid tissue using histopathology and hematology. Histopathological changes are generally not believed to be sensitive indicators of drug-induced immunotoxicity, are seen only at fairly high dose levels, and do not necessarily equate with functional immune alterations (51, 141, 201). The route and time of exposure relative to the maturational development of the immune system are important considerations in designing an immunotoxicity protocol (25, 49).
The application of nonspecific immunotherapy for bacterial and viral diseases has led to an increased level of importance in the determination of immunotoxic effects. The standard acute, subchronic, and chronic/oncogenic studies that form the basis of toxicological evaluations should be complemented with specific evaluations useful in determining functional effects on the immune system, especially if the agent in question is a known immune modulator. Immune system function results from a balance of the activities of various cellular components and their soluble factors (154), and an alteration in any factor could result in an imbalance of the entire system (63). The effects of immunomodulating agents, therefore, could result in either enhancement (i.e., hypersensitivity, autoimmunity) or suppression (i.e., decreased host resistance) (Figure 6.12).
Several tier approaches to immunotoxicity testing have been proposed (50, 131). For one example, the National Toxicology Program (NTP) has proposed an immunotoxicology testing strategy including a limited number of functional and host resistance assays (50). The first tier (Tier 1) of the two-tier approach consists of a screen which represents a limited effort that includes the assessment of cell-mediated immunity, humoral immunity, and immunopathology. Tier 1 provides little information on the specificity of an observed immune defect, or its relevance to the host; however, it can detect an immune alteration resulting from drug exposure (131). Tier 2 represents an in-depth evaluation, initiated only if functional changes are seen in Tier 1 at otherwise nontoxic doses (131). The in-depth immune function and host resistance evaluations provide information on the mechanism(s) of the immunotoxicity and aid risk assessment. Luster et al. (131) have reported that no compound evaluated to date has been found to produce an effect in Tier 2 without demonstrating some effect in Tier 1. The NTP procedure for detection of immune alterations following chemical or drug exposure in rodents is shown in Table 6.15. The concept of performing func-

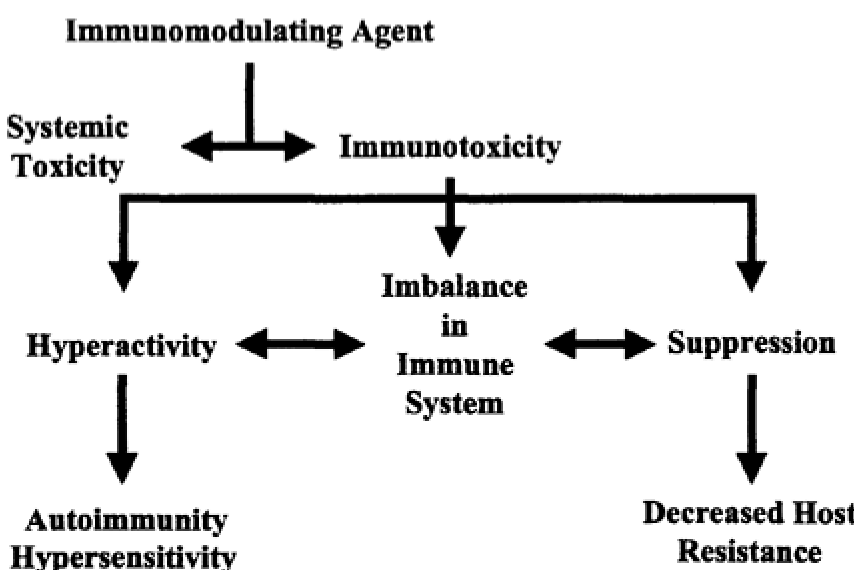

FIG. 6.12. Potential toxic responses of immunomodulating agents. Redrawn, with permission, from Reference 63.

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Table 6.15
NTP immunotoxicology procedure[a]

Tier 1		Tier 2	
Immunopathology	Hematology (complete and differential blood count)	Immunopathology	Quantitation of Splenic B and T Cells
	Organ Weights (spleen, thymus, kidney, liver)	Humoral-Mediated Immunity	IgG response to sheep RBCs
	Body Weight	Cell-Mediated Immunity	Delayed Hypersensitivity
	Cellularity (spleen)	Nonspecific Immunity	Macrophage Function
	Histology (spleen, thymus, lymph node)	Host Resistance	Syngenic Tumor Cells (tumor incidence)
Humoral-Mediated Immunity	Plaque-Forming Cells		Listeria monocytogenes (mortality)
Cell-Mediated Immunity	Lymphocyte Blastogenesis to Mitogens		Influenza (mortality)
Nonspecific Immunity	Natural Killer Cell Activity		*Plasmodium yoelii* (parasitemia)

[a]From Reference 131.

tional tests is critical to defining potential mechanisms of the toxic response and their applicability to humans. An international collaborative effort has focused on the evaluation of limited pathology or enhanced pathology evaluations to better understand potential immunotoxicity (176). The enhanced pathology approach—for example, weight determination, examination of additional lymphoid organs, and grading of changes in the principal compartments of lymphoid tissue—was determined to provide an advantage in revealing effects on the immune system.

The direction for pharmaceutical development is to include tests of potential immune system involvement in the traditional toxicological evaluations for subchronic and chronic toxicity. Due to the sensitivity of the immune system to toxicants that could adversely affect the critical balance of the various immune factors, and the adverse health effects that could ensue, it is extremely important to define any potential interaction of a new drug and immune system function (201).

As can be anticipated from the previous discussion, immunogenicity is a major scientific issue relative to the development of biotechnology products. Concern has been raised over the comparison of the recombinant protein and the naturally occurring protein, since animal models are thought to be inadequate to assess the chemically subtle, but potentially immunologically significant, differences in the human response to these molecules. It has been assumed that a recombinant protein, designed for human use, would produce a number of adverse effects, including the production of neutralizing antibodies, in experimental animals. It has now become clear, through chronic exposures in nonclinical studies, that some small molecular weight human proteins are not immunogenic in animals, or are only weakly so. They have also been observed not to produce neutralizing antibodies. In the case where antibodies to human proteins have been detected in nonclinical studies, they do not necessarily cause expected immunopathology or neutralization activity. The rhesus monkey has been shown to predict the relative immunogenicity of several recombinant proteins in humans (216), and may serve as a good model. A further question is, Should all recombinant DNA products be routinely screened in animals prior to their introduction into humans? The major reason for conducting immunotoxicity evaluations in experimental animals is to detect those compounds which could induce anaphylaxis or anaphylactoid reactions in humans (193). New molecules, previously minimally tested in animals, such as enkephalins, would have a greater potential risk than well-known molecules, such as insulin. An approach to testing recombinant proteins, as well as NCEs, for immunogenicity/antigenicity has been suggested based on the extent of the clinical database and the existing regulatory requirements (Figure 6.13). Although studies in animals seem well justified for poorly characterized chemicals, it remains an open question whether or not regulatory agencies will accept an existing extensive clinical database as justification for not performing immunogenicity evaluations. Again, the chemical characterization of the recombinant product relative to

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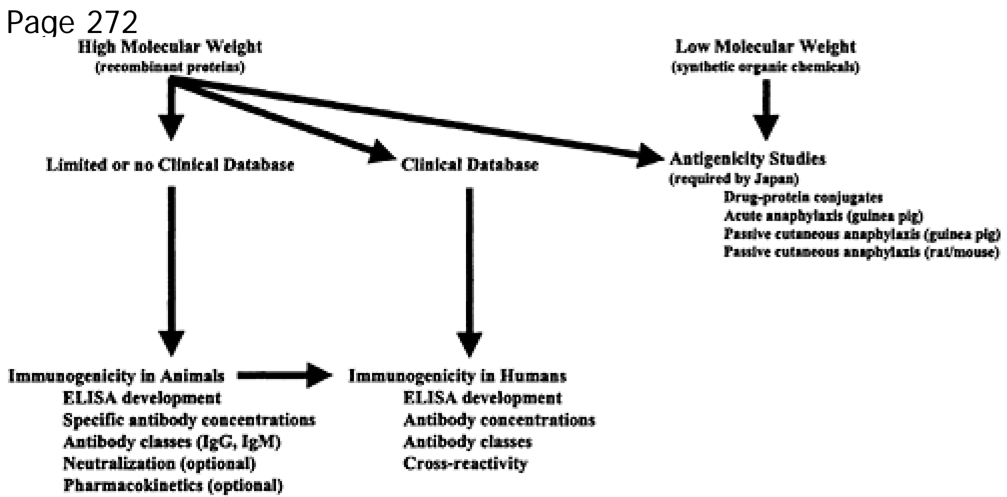

FIG. 6.13. Proposed approach for addressing immunogenicity and antigenicity issues with established and novel biotechnology products and NCEs. From Reference 193.

the natural material will have some bearing on this debate.

Genetic Toxicology

Genotoxicity has been defined as the ability of either a chemical or physical agent to damage DNA, resulting in a mutation (29). An important element of toxicology is the early identification of potentially hazardous substances. Since the actions of toxins are ultimately exerted at the cellular level, isolated cell systems represent an important model for identifying toxic effects. In vitro assays allow a greater control over xenobiotic metabolism (i.e., addition of enzymes or inhibitors), and facilitate mechanistic studies that could not be performed in vivo (113). In vitro tests generally provide a reasonable approximation of the potential for an agent to have an effect on genetic material; in vivo procedures provide a better test for the potential for genetic alterations to occur in the intact organism (119). The short-term in vitro tests for genotoxicity, potentially predictive of in vivo carcinogenicity, are among the most important techniques available for the rapid determination of potential severe undesirable effects of compounds selected for development. They are also useful in the prioritization of compounds to be studied in the more extended and expensive in vivo toxicology studies.

A number of assays are available to evaluate the potential for genotoxicity. The majority opinion is in favor of a battery approach to identify potential genotoxic activity since different assays assess different types of genetic damage (116, 158). The ICH has published a guideline on how to conduct genotoxicity tests, and a guideline on the recommended standard genotoxicity testing battery for evaluations of pharmaceuticals (105, 106). The ICH test battery includes:

1. gene mutation in bacteria: detect relevant genetic changes and the majority of genotoxic rodent carcinogens.
2. in vitro mammalian cell chromosomal aberration, or in vitro mouse lymphoma tk assay: detect either gross chromosomal damage or detection of gene mutation and clastogenic effects.
3. In vivo chromosomal damage in rodent hematopoietic cells: allows evaluation of additional relevant factors, for example, absorption, distribution, metabolism, and excretion.

This battery may be expanded when appropriate, such as when compounds with structural alerts are negative in the three standard tests (106).

Excellent reviews of methods to study genotoxic potential, and issues concerning nongenotoxic, yet carcinogenic, chemicals are available (17, 18, 194, 195). In vivo exposure assays have the advantage of an intact metabolic system to effectively assay those compounds which must be activated (metabolized to a reactive entity) to achieve an effect. The in vitro assays may be conducted with or without the addition of a postmitochondrial supernatant from livers of rats treated with polychlorinated biphenyls to maximally induce drug metabolizing enzyme activities, and, thus, enhance the detection of indirect-acting agents.

In vitro genotoxicity assays are often used in the early drug discovery process aimed at selecting drug candidates for further development. The definitive in vitro genotoxicity evaluations of mutation and chromosomal damage are generally submitted prior to FHD. The complete battery of recommended tests should be submitted prior to Phase II clinical development.

The conduct of genotoxicity screens on NBEs has been an area of much discussion. The ICH has recognized that the standard genotoxicity testing battery may not be applicable to NBEs (111). Despite this, many pharmaceutical companies conduct genotoxicity evaluations on NBEs primarily to evaluate process impurities, to meet perceived regulatory expectations, or to meet specific regulatory agency requests (94).

Safety Pharmacology

Safety pharmacology involves establishing the pharmacological profile of new drug candidates by evaluating the pharmacodynamics related to the therapeutic indication and by evaluating the pharmacodynamics on other organ systems not related to the therapeutic indication (37). These studies are usually conducted at doses well below those used to establish the toxicology profile. It is not necessary, or even desirable, to produce frank toxicity to establish a valid pharmacological profile. Pharmacological profiling was initially focused on guiding the synthetic chemist in the discovery of new pharmacologically active chemicals, rather than on the detection of adverse drug effects in humans (204). In reviewing the common adverse drug findings in humans, Zbinden (204) pointed out that some responses are easily detected in both humans and experimental animals, including sedation, anorexia, body weight changes, tremor, and tachycardia; and some responses are only detectable in humans, such as tinnitus, vertigo, nausea, and headache. In any event, when one considers the nature of the functional disturbances encountered in both nonclinical and clinical testing, it becomes evident that pharmacological profiling is critical to the safety evaluation of potential therapeutic agents (196, 197). Every chemical that enters the body has the potential for creating effects that may or may not be related to its pharmacological activity. Antihistamines, for example, produce sedation, related to their pharmacological effects, and anticholinergic responses, which are not (196). Pharmacological profiling can help identify the potential incidence of effects unrelated to the known pharmacological activity.

The Japanese Ministry of Health and Welfare (MHW) has published Guidelines for General Pharmacology Studies (60). These studies are designed to characterize effects and potency, and to determine mechanism. The guidelines include studies to determine effects on: general activity and behavior; the CNS; auto nomic nervous system and smooth muscle; respiratory and cardiovascular systems; gastrointestinal tract; and, renal excretion.

A proposal has been made to the ICH for establishment of an EWG to harmonize the safety pharmacology requirements for submission in the EU, United States, and Japan. The EU and Japan have completed similar draft guidelines for safety pharmacology (37). The proposed approach is based on a flexible strategy, including a basic evaluation and a complementary evaluation of safety pharmacology parameters. It has been suggested that the basic evaluation include:

1. Central nervous system: behavioral changes, motor activity, reflex responses, and cardiovascular potential.
2. Cardiovascular system: heart rate, ECG, contraction force, resistance, flow rate, and blood pressure.
3. Respiratory system: frequency, tidal volume, resistance, blood gases.

The following have been proposed for the complementary evaluation:

4. Renal system: volume, pH, fluid/electrolyte balance, protein, and glomerular filtration rate.
5. Immune system.
6. Gastrointestinal system: gastric secretion (pH), ulcerogenic potential, and gastrointestinal transit time.

In addition, the EU Committee for Proprietary Medicinal Products (CPMP) has introduced a guideline to address the potential for QT-interval prolongation by non-cardiovascular medicinal products (36).

Measure of Exposure

The relationship of administered dose to toxicological response is not always a simple correlation. Traditionally, the administered dose (mg/kg) has been the most commonly used expression to compare toxicological responses between species. The value of the administered dose term as the most appropriate comparator with toxicological response, however, has been questioned in scientific and regulatory circles. It is becoming increasingly well recognized that both beneficial and toxic effects of therapeutic agents are dependent on the quantity of material reaching the target site (134, 211, 179). Knowledge of the magnitude of drug exposure is necessary in substantiating efficacy and drug safety evaluations. Furthermore, exposure data have provided important information relative to the interpretation of unanticipated toxicity (143). Toxicologists, therefore, must make an effort to monitor

concentrations of parent compound and/or major active metabolites in the blood and, when possible, the tissues. Measurement of plasma concentrations in toxicology studies provides much

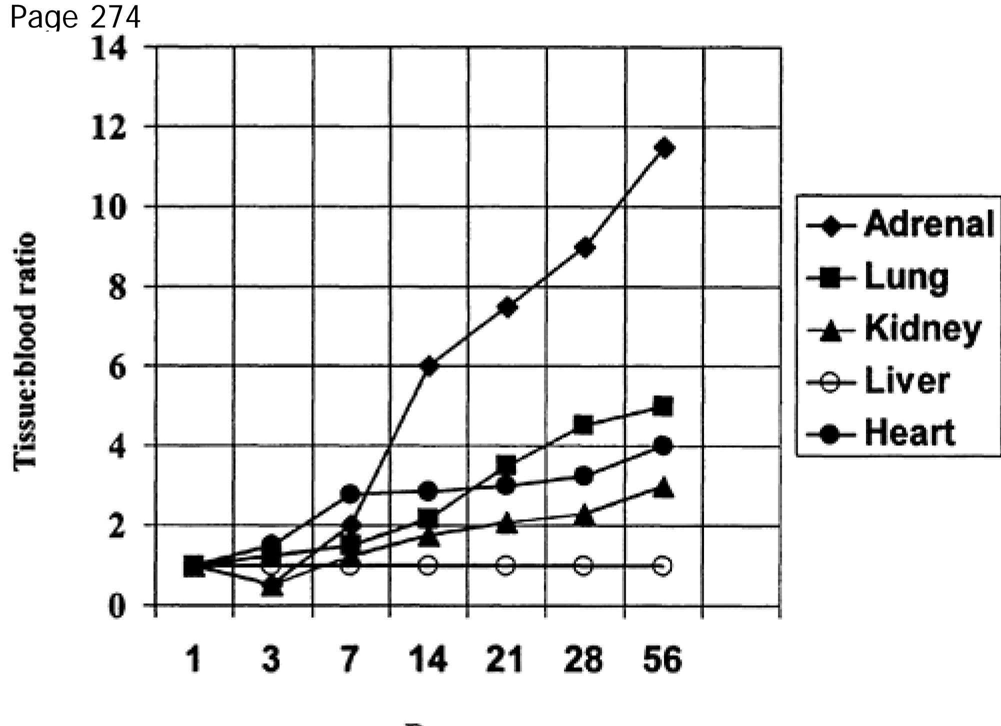

FIG. 6.14. Tissue to blood ratio, over time, in various tissues following single daily doses of chlorphentermine. Redrawn with permission from Reference 126.

needed proof of absorption and exposure. It also, provides a more appropriate dose comparator than administered dose when responses between species are being evaluated. For example, because of differences in metabolism, bioavailability, and so on, rats may have much lower exposure (plasma level) for a given administered (mg/kg) dose than humans receiving the same administered dose. Therefore, the use of multiples of the administered human dose on a mg/kg basis in the experimental animal species may result in an actual exposure that is unpredictable, and differs substantially (either greater or less), from that achieved in patients. The potential of expressing toxicity that is poorly correlated with exposure to a clinical dose, therefore, increases. Setting clinical dose multiples for toxicology studies on the basis of exposure is much more appropriate. Measures of exposure are also useful in establishing nonlinearity in kinetics, which, as described above, is important in explaining toxic responses seen in particular species (134). It seems more rational to establish an upper dose in toxicology studies based on linearity of kinetics rather than at the MTD, since it is often the case that the MTD falls in the range of nonlinear kinetics, saturating normal metabolic processes. Thus, an animal treated at the MTD may be exposed to much higher levels of parent drug, or toxic metabolite(s), than would be observed at meaningful multiples of the clinical dose.

The relationship of administered dose to delivered dose remains a central issue in the interpretation of toxicology data. The measurement of plasma concentrations of parent compound and metabolites represents a partial resolution of this problem. There are limitations, however, in using plasma concentration as a relevant measure of exposure for those compounds that are tissue-sequestered (135). Although many therapeutic agents achieve tissue levels proportional to plasma concentration, some continue to accumulate in tissue with continued dosing (77). In studies of phentermine and chlorphentermine, it was found that the blood to tissue ratios of phentermine remained constant over the entire period of treatment, while those for chlorphentermine significantly increased with time in most of the tissues studied (Figure 6.14) (126). In addition to becoming tightly bound to tissue, chlorphentermine also induced new binding sites during treatment. The difference in behavior between the two closely related chemicals was related to the increased amphiphilic nature of chlorphentermine (126).

Extrapolation of toxicological test results across species is based on the assumption that mammalian species will respond to toxicants in a similar fashion. The convention in toxicology has been to express dose on the basis of mg/kg body weight. Whether body weight is the most appropriate scaling factor for toxicity data has been the subject of much discussion. It had been previously observed that the toxicity of a variety of anticancer drugs was best predicted across species if the dose was expressed on the

basis of body surface area rather than body weight (76). Others, however, have

Table 6.16

Dose by weight and surface area (1 mg/kg)*a*

Species	Body Weight (g)	Surface Area (cm2)	Total Dose (mg/animal)	Administered Dose (mg/cm2)	Multiple of Human Dose
Mouse	20	45	.02	.00044	0.10
Rat	200	325	.20	.00061	0.16
Monkey	4000	2980	4.00	.00134	0.34
Dog	12000	5770	12.00	.00207	0.53
Human	70000	18000	70.00	.00389	1.00

*a*From References 23, 118.

reported that although heat loss appears to be related to body surface area, basal metabolic rate, an important aspect of toxicity evaluation, is more closely related to body weight (82). The use of surface area for the purpose of dose extrapolation is a more conservative approach than the use of body weight (21, 21, 82, 118) (Table 6.16). Assuming that the average adult mouse (0.02 kg) and the normal adult human (70 kg), are given the same administered dose on a body weight basis, the adult human will receive 3500 times the total dose received by the mouse. The same dose, given on a body surface area basis to mice and men, would represent only a 400-fold difference. Although there is support for using surface area as the most appropriate dose extrapolation factor, there is an increasing body of data that suggest the use of body weight to be the most accurate factor (1). Target organ specificity may be a deciding factor in the selection of the most appropriate allometric relationship (that is, antihypertensives vs. oncolytics), The appropriate scaling factor can probably only be determined on a case-by-case basis using data from a variety of species.

Clinical Trials in Pediatric Populations

The FDA has found that most products indicated for treatment of diseases that occur in both adults and children have little clinical trial support for pediatric use. As a result, a new regulation requiring pediatric studies for certain NCEs and NBEs has been proposed (67). The EU Committee for Proprietary Medicinal Products (CPMP) has also concluded that specific age-dependent differences in pharmacokinetics, pharmacodynamics, growth process and development, and specific pathology require that therapeutic agents be tested in the target age group (35). The ICH has recommended that pediatric clinical trials be supported by repeated dose toxicity studies of an appropriate duration, all developmental toxicity studies, and the full battery of genotoxicity tests. These studies should be concluded before the pediatric clinical trials begin (102). Due to the potentially long duration of treatment, carcinogenicity studies must be considered prior to the initiation of long-tenn pediatric clinical trials. The performance of nonclinical studies in juvenile animals may also be necessary if previous toxicology evaluations and human safety data are not sufficient or raise a cause for concern.

Nonclinical Evaluation of Anticancer Drugs

The development of drugs for life-threatening diseases, such as cancer and AIDS, requires a modification of the approach established for the safe development of NCEs or NBEs (see above). The treatment of cancer usually includes the use of potent agents designed to halt cell replication. The therapeutic index for these agents is often small. Due to the life-threatening nature of the disease, a greater risk of drug toxicity and a shorter nonclinical testing strategy is generally accepted for therapeutic agents in this class, since serious drug effects are often less threatening than the targeted disease. However, due to the greater intrinsic toxicity of the agents in this class, the early clinical trials are often conducted in patients rather than normal volunteers, as with other therapeutic agents.

The history of anticancer drug development has been extensively reviewed (92, 53, 180). The basic approach for development of anticancer drugs includes:

1. Establishment of a safe clinical trial entry dose.
2. Determination of potential dose limiting target organ toxicity.
3. Evaluation of reversibility of effects.
4. Determination of MTD in animals.
5. Determination of dose schedule toxicity.

The use of nonclinical studies has been successful in accurate predictions of the MTD in humans and a safe starting dose for clinical trials (92).

The CPMP has provided a specific note for guidance on the nonclinical evaluation of anticancer agents (38). Safety pharmacology is generally required prior to Phase I studies, as are the determination of Cmax (maximum plasma concentration of the drug) and AUC at the animal MTD. Other kinetic parameters are expected to be determined prior to Phase II/III testing. Determination of

the single-dose MTD in rodents and the approximate MTD in nonrodents, using a relevant route of exposure, is also expected prior to Phase I. Repeated-dose toxicity studies in two rodent species are expected prior to Phase I clinical studies. Longer-term repeat-dose studies, in a rodent and nonrodent species, equal in duration to the clinical trial but <6 months, are expected prior to Phase II/III. Genotoxicity testing is not necessary prior to Phase I/II, but the genotoxicity battery is expected to be conducted prior to Phase II/III. Since cytotoxic anticancer agents are known to have an adverse effect on reproduction, developmental toxicity studies are not required but are encouraged.

The development of anticancer drugs and other therapies for life-threatening diseases has unique characteristics based on the life-threatening nature of the disease and the inherent toxicity of the therapeutic agents.

Alternative Methods for Carcinogenicity Determination

The testing for carcinogenic potential has relied primarily on the rodent bioassay. Recently, through the ICH process, the rat has been identified as either the most acceptable or most relevant model for the two-year bioassay. In addition to the rat bioassay, an alternative short-term method of carcinogenicity evaluation is recommended (ICH S1b). These approaches may include studies in transgenic mice—for example, p53+/− heterozygous gene deficient mouse or Tg/AC mouse—or use of a neonatal rodent tumorigenicity model.

To evaluate and verify the available alternative models, the International Life Sciences Institute (ILSI) has initiated a collaborative effort among industry, academic, and government laboratories to study chosen chemicals in the alternative models and evaluate the results in light of the known bioassay data. Currently, there is insufficient information to guide us in the choice of suitable alternative models for carcinogenicity evaluation.

Once validated, the proposed transgenic animal models may be used as follows (33):

1. To confirm results in equivocal 2-year rodent bioassays.
2. To set priorities for 2-year carcinogenicity bio assays.
3. As an alternative to the mouse 2-year bioassay, in conjunction with the rat 2-year bioassay.
4. To assess carcinogenic potential of new genotoxic contaminants/degredants in a drug product after 2-year bioassays are completed.

In addition, the use of transgenic animals may support weight-of-evidence decisions, is relatively short-term, and is generally less expensive than the 2-year rodent bioassay.

Several transgenic mouse models are available to complement the rat 2-year bioassay. The Tg/AC mouse and the p53+/− heterozygous-gene-deficient mouse are taken as examples. It must be remembered that these models are not fully validated.

The Tg/AC mouse model (175) presents an animal model of initiated skin as a target for tumorigenesis. The Tg/AC line may be able to differentiate carcinogens from noncarcinogens, but may not be able to differentiate genotoxic carcinogens from those that cause only tumor promotion activity. This model may only be useful in combination with other transgenic animal models (159).

The p53+/− heterozygous-gene-deficient mouse model is based on rendering mice heterozygous for the p53 tumor suppressor gene (84). These animals are at elevated risk for tumor development. The model has been proposed to best approximate humans at risk for heritable forms of cancer. This model may be able to detect genotoxic carcinogens in a 6-month period.

The neonatal mouse assay has been available longer than the transgenic animal models. The detailed protocol for this 1-year study has been reviewed previously (73). Neonates are treated with the test compound on days 8 and 15 of age, and then observed to 1 year of age. At that time the animals are evaluated for tumor production. This assay is sensitive to direct-acting carcinogens, primarily those that work through formation of covalently bound DNA adducts.

The assessment of carcinogenicity in the drug development process is at a crossroad. The rodent bioassay has been used for over 25 years, and has provided useful data, although it is not a perfect system and is one that has received much criticism. The investigation of alternatives to the standard bioassay in two rodent species has been encouraged by ICH. Conducting a 2-year study in the rat, and an alternative study in the mouse, may provide an acceptable transition. There is, however, much work to be done before the alternative models are validated and fully useful in carcinogenicity risk assessments. The alternative assays are relative newcomers, full of promise but short on experience. There should not be undue enthusiasm about their ability to dramatically improve our carcinogenicity risk assessment process.

Development of Stereoisomeric Drugs

The development of individual enantiomers and racemates requires specific considerations and

interactions with regulatory agencies, especially the FDA. In 1992, the FDA, developed a specific policy statement to deal with development of enantiomers and racemates (70). It is recognized that the properties of enantiomers may be similar and desirable. For example, both enantiomers of dobutamine have positive inotropic properties. It may also occur that the isomers may have desirable but different properties, for example, the d-isomer of propoxyphene is an analgesic, the s-isomer is an

antitussive. It is also recognized that toxicity may be linked to one member of a stereoisomeric pair, for example, granulocytopenia is ascribed to the d-isomer of the antiparkinsonian drug levodopa.
The FDA position is that the establishment of a relatively benign nonclinical toxicological profile for the racemate should support the clinical development of the enantiomer without a separate toxicological evaluation. Since the FDA policy statement (1992) leaves some room for interpretation of "benign toxicology profile," it is highly advisable to engage in a conversation with the agency before proceeding with development.
The FDA makes specific recommendations for pharmacokinetic and toxicology profiles. The potential for interconversion of the isomers must be monitored in nonclinical studies and compared to the clinical profile. It is sufficient to carry out toxicity studies with the racemate unless toxicity unrelated to the pharmacology of the drug occurs at relatively low multiples of the clinical exposure. In that case, the drug developer should investigate the individual enantiomers to examine whether the pharmacological and toxicological effects can be segregated.
The development of a single stereoisomer after the racemate had been evaluated in nonclinical studies requires abbreviated toxicology studies:
1. 3-month toxicology studies of the single enantiomer in rodent and nonrodent species.
2. Segment II (teratology) developmental toxicology study of the single enantiomer in the most sensitive species.
3. A positive control group consisting of the racemate. If there are no differences between the toxicological profile of the single enantiomer and the racemate, no further toxicology studies would be needed.

CONCLUSION

The next decade promises to be another exciting one for the industrial toxicologist. The physician and patient continue to demand more efficacious and safer medications more quickly. Basic research efforts in biochemistry, physiology, and pharmacology have allowed the more precise characterization of receptors, and the normal and perturbed sequelae of receptor binding, which continues to stimulate the development of more specific, potent modulators of cellular functions. The etiologies of human diseases are becoming better understood, thanks to the technological ability to elucidate their characteristics at the molecular level. This has resulted in the potential to therapeutically modify the disease process at its origin—the human genome. These molecular approaches have already resulted in the development of agents that are highly species-specific, and the use of these techniques to elucidate normal and pathological cellular function will only continue to escalate. It is unlikely that the classical tools of toxicology will be adequate to ensure the human safety of the highly specific potential therapeutics forthcoming from these sophisticated technologies. The development of NBEs has already challenged the established norms of safety assessment.
Consider the example of an agent that has shown selectivity for modifying the activity of a human-specific enzyme critical to a pathological process. Although traditional animal studies are likely to be predictive of toxicity that is unrelated to the pharmacology of the compound, they will not be useful for the prediction of adverse findings relative to the action of the drug at the enzyme which will occur only in humans. Thus, not only may data generated from classical animal studies be inadequate to predict toxic responses in humans, but the information may be irrelevant or misleading. A major concern is whether modification of this enzyme activity in the only responsive species, humans, might result in unanticipated, severe toxicity. How can this best be predicted prior to the initiation of clinical trials?
The future direction of discovery research suggests that industrial and regulatory toxicologists will need to collaborate more closely in the design of the toxicology studies to support registration, and ultimately, these may have to be considered on a case-by-case basis. Indeed, the current guidelines resulting from global harmonization efforts repeatedly emphasize the need for defending the scientific rationale supporting the design of proposed toxicological assessments. Although these guidelines are viewed as much more flexible than the country-specific regulations of the past, they also place a greater burden on toxicologists relative to defending the relevance of their studies. The era of "checking the tox box" has, thankfully, come to a close.
Another challenge on the horizon concerns the need to improve the efficiency of the drug development process without compromising its quality. Currently, the drug development and approval processes are taking longer, and costing more than ever before. More stringent regulatory requirements have resulted in the conduct of more studies (in both animals and humans), taking more time and costing more money. These costs are passed on to the patient, who ultimately must also compensate for the resultant decreased market life, due to patent length restrictions, of the registered product. Furthermore, the use

of large numbers of experimental animals remains a concern from both the ethical and financial points of view. One approach to solving these dilemmas is to ensure that the toxicological studies conducted meet the needs of the regulatory agency, the physician, and, ultimately, the patient. As suggested earlier, this can be most

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efficiently accomplished by early and routine interactions between the industry and these customers, especially in cases where the agent under development represents a unique therapeutic approach.

Finally, the major role of the toxicologist as a mechanistic scientist will continue to be enhanced. For the reasons discussed above, the interpretation of toxicology data will become increasingly more sophisticated, requiring a broad knowledge base in a variety of other scientific disciplines. Elucidation of the mechanisms responsible for observed toxicities would improve the ability to achieve the traditional, ultimate purpose of the discipline of toxicology—the appropriate extrapolation of these data to humans. Achievement of this goal will surely become more challenging, but also more exciting, in the future.

ACKNOWLEDGMENTS

The authors gratefully acknowledge and appreciate the efforts of Ms. Arlene Adkins in the preparation of this document.

QUESTIONS

1. What are the major objectives of toxicity testing relative to the development of pharmaceuticals?
2. In what major ways would a toxicology submission package differ for a drug intended to be prescribed chronically (e.g., for essential hypertension) as opposed to one that will be used acutely (e.g., an antibiotic)?
3. In what situations would the conduct of oncogenicity studies most likely be encouraged? For NCEs? For NBEs?
4. Why is the use of the maximum tolerated dose (MTD) as the high dose in oncogenicity studies controversial?
5. What are the different phases of clinical testing and what is the purpose of each phase?
6. Why is exposure in animal models considered a better parameter than administered dose when extrapolating toxicity data to humans?
7. Under what conditions might a compound with significant animal toxicity be considered for regulatory approval?
8. What are some of the special considerations necessary in designing the toxicology package for NBEs?

REFERENCES

1. Allen, B.C., Crump, K.S., and Shipp, A.M. (1988): Correlation between carcinogenic potency of chemicals in animals and humans. *Risk Anal.*, 8:531–561.
2. Ames, B.N., and Gold, L.S. (1990): Too many rodent carcinogens: Mitogenesis increases mutagenesis. *Science*, 249:970–971.
3. Ankier, S.I., and Warrington, S.J. (1989): Research and development of new medicines. *J. Internat. Med. Res.*, 17:407–416.
4. Ashby, J. (1996): Alternatives to the 2-species bioassay for the identification of potential human carcinogens. *Human Exper. Toxicol.*, 15(3): 183–202.
5. Audus, K.L., Bartel, R.L., Hidalgo, I.J., and Borchardt, R.T. (1990): The use of cultured epithelial and endothelial cells for drug transport and metabolism studies. *Pharm. Res.*, 7(5):435–451.
6. Ayers, K.M., Clive, D., Tucker, W.E., Jr., Hajian, G., and De Miranda, P. (1996): Nonclinical toxicology studies with zidovudine: Genetic toxicity tests and carcinogenicity bioassays in mice and rats. *Fundam. Appl. Toxicol.*, 32:148–158.
7. Ayers, K.M., Tucker, W.E., Jr., Hajian, G., and De Miranda, P. (1996): Nonclinical toxicology studies with zidovudine: Acute, subacute, and chronic toxicity in rodents, dogs, and monkeys. *Fundam. Appl. Toxicol.*, 32:129–139.
8. Baker, S.B. deC., and Davey, D.G. (1970): The predictive value for man of toxicological tests of drugs in laboratory animals. *Br. Med. Bull.*, 26(3):208–211.
9. Bass, R., and Scheibner, E. (1987): Toxicological evaluation of biotechnology products: A regulatory viewpoint. *Arch. Toxicol.*, (Suppl. 11):182–190.
10. Beret, L.Z. (1984): Pharmacokinetics: Basic principles and its use as a tool in drug metabolism. In: *Drug Metabolism and Drug Toxicity*, J.R.Mitchell, and M.G.Horning, pp. 199–211. Raven Press, New York.
11. Berlin, R.G. (1991): Omeprazole: Gastrin and gastric data (August 1991). *Digest. Dis. Sci.*, 36:1501–1502.
12. Berthou, F., Ratanasavanh, D., Riche, C., Picart, D., Voirin, T., and Guillouzo, A. (1989): Comparison of caffeine metabolism by slices, microsomes and hepatocyte cultures from adult human liver. *Xenobiotica*, 19(4):401–417.
13. Bocci, V. (1992): Physicochemical and biological properties of interferons and their potential uses in drug delivery systems. *Crit. Rev. Therapeut. Drug Carrier Syst.*, 9(2):91–133.

14. Boobis, A.R., and Davies, D.S. (1984): Human cytochromes P450. *Xenobiotica*, 14:151–185.
15. Brendel, K., Fisher, R.L., Krumdieck, C.L., and Gandolfi, A.J. (1990): Precision-cut rat liver slices in dynamic organ culture for structure-toxicity studies. *J. Am. Coll. Toxicol.*, 9(6):621–627.
16. Brent, R.L. (1980): The prediction of human diseases from laboratory and animal tests for teratogenicity, carcinogenicity, and mutagenicity. In: *Controversies in Therapeutics*, L.Lasagna, pp. 131–150. W.B.Saunders Co., Philadelphia.
17. Brusick, D. (1989): Genetic toxicology. In: *Principles and Methods of Toxicology*, 2nd ed., edited by A.W.Hayes, pp. 407–434. Raven Press, New York.
18. Butterworth, B. (1989): Nongenotoxic carcinogens in the regulatory environment. *Reg. Toxicol. Pharmacol.*, 9:244–256.
19. Butterworth, B.E., Goldsworthy, T.L., Popp, J.A., and McClellan, R.O. (1991): The rodent cancer test: An assay under siege. *CIIT Activities*, 11(9):l-6.
20. Cahn, J. (1983): Forecasting of cardiac side effects: Vincamine and calcium antagonists, a comparative study in animals and man. In: *Current Problems in Drug Toxicology*, edited by G.Zbinden, J. Y.Detaille, and G.Mazue, pp. 90–94. John Libbey Eurotext, London.
21. Calabrese, E.J., Beck, B.D., and Chappell, W.R. (1992): Does the animal-to-human uncertainty factor incorporate interspecies differences in surface area? *Reg. Toxicol. Pharmacol.*, 15:172–179.
22. Caldwell, J. (1981): The current status of attempts to predict species differences in drug metabolism. *Drug Metab. Rev.*, 12(2):221–237.

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23. Campbell, D.B., and Ings, R.M. (1988): New approaches to the use of pharmacokinetics in toxicology and drug development. *Hum. Toxicol.,* 7:469–479.
24. Carlsson, E., Larsson, H., Mattson, H., Ryberg, B., and Sundell, *G.* (1986): Pharmacology and toxicology of omeprazole—with special reference to the effects on the gastric mucosa. *Scand. J. Gastroent.,* (Suppl. 118):31–38.
25. Chan, P.K., O'Hara, G.P., and Hayes, A.W. (1981): Principles and methods for acute and subchronic toxicity. In: *Principles and Methods of Toxicology,* edited by A.W.Hayes, pp. 1–51. Raven Press, New York.
26. Chance, R.E., Kroeff, E.P., and Hoffman, J.A. (1981): Chemical, physical and biological properties of recombinant human insulin. In: *Insulins, Growth Hormone, and Recombinant DNA Technology.* J.L.Gueriguian, pp. 71–84. Raven Press, New York.
27. Chenery, R.J., Ayrton, A., Oldham, H.G., Standring, P., Norman, S.J., Seddon, T., and Kirby, R. (1987): Diazepam metabolism in cultured hepatocytes from rat, rabbit, dog, guinea pig and man. *Drug Metab. Dispo.,* 15:312–318.
28. Chien, R.E., ed. (1979): *Issues in Pharmaceutical Economics.* Lexington Books, Lexington, Massachusetts.
29. Choy, W.N. (1996): Principles of genetic toxicology. *Drug Chem. Toxicol.,* 19(3):149–160.
30. Clark, B., and Smith, D.A. (1984): Pharmacokinetics and toxicity testing. *Crit. Res. Toxicol.,* 12(4):343–385.
31. Cluff, L.E. (1980): Is drug toxicity a problem of great magnitude? Yes! In: *Controversie in Therapeutics,* edited by L. Lasagna, pp. 44–50. W.B. Saunders Co., Philadelphia.
32. Cohen, S., and Ellwein, L.B. (1990): Cell proliferation in carcinogenesis. *Science,* 249:1007–1011.
33. Contrera, J.F. (1998): Transgenic animals: Refining the two-year rodent carcinogenicity study. *Lab. Animal,* 27(2):30–33.
34. Contrera, J.F., Jacobs, A.C. and DeGeorge, J.J. (1997): Carcinogenicity testing and the evaluation of regulatory requirements for pharmaceuticals. *Reg. Toxicol. Pharmacol.,* 25:130–145.
35. CPMP (1997): Note for guidance on clinical investigation of medicinal products in children. London, 17 March, CPMP/EWP/ 462/95.
36. CPMP (1997): Points to consider: The assessment of the potential for QT interval prolongation by non-cardiovascular medicinal products. London, 17 December, CPMP/986/96.
37. CPMP (1998): Note for guidance on safety pharmacology studies in medicinal product development (draft of preliminary consultation). CPMP/SWP7872/98.
38. CPMP (1998): Note for guidance on the preclinical evaluation of anticancer medicinal products. CPMP/SWP/997/96. London, 23 July.
39. Creutzfeldt, W., and Lamberts, R. (1991): Is hypergastrinaemia dangerous to man? *Scand. J. Gastroenterol.,(Suppl.* 180): 179–191.
40. D'Agnolo, G. (1983): The control of drugs obtained by recombinant DNA and other biotechnologies. In: *Current Problems in Drug Toxicology,* edited by G.Zbinden, J.Y.Detaille, and G.Mazue, John Libbey Eurotext (London), pp. 241–247.
41. D'Aguanno, W. (1973): Drug toxicity evaluation—Pre-clinical aspects. FDA Introduction to Total Drug Quality. DHEW Publication No. (FDA) 74–3006, pp. 35–40.
42. Dahlem, A.M., Allerheiligen, S.R., and Vodicnik, M.J. (1995). Concomitant toxicokinetics: Techniques for and interpretation of exposure data obtained during the conduct of toxicology studies. *Toxicol. Pathol.,* 23(2):170–178.
43. Davey, D.G. (1964): The study of the toxicity of a potential drug—basic principles. In: *Proceedings of the European Society for the Study of Drug Toxicity,* Vol. III, pp. 2–13. Excerptamedica Foundation, New York.
44. Dayan, A.D. (1981): The troubled toxicologist. *TIPS,* 2(11):1–4.
45. Dayan, A.D. (1986): Preclinical safety studies on genetically engineered medicine for man. *BIBRA J.,* 5(3): 12–15.
46. Dayan, A.D. (1988): Risk assessment of biotechnology products. *Hum. Toxicol.,* 7(1):50–52.
47. De Schaepdryver, A.F. (1978): Toxicology: General and special toxicity testing: A situation paper. In: *The Scientific Basis of Official Regulation of Drug Research and Development: Proceedings of a Satellite Symposium of the 7th International Congress of Pharmacology* pp. 25–27. Heymans Foundation, Ghent, Belgium.
48. Dean, J.H., Cornacoff, J.B., Rosenthal, G.J., and Luster, M.I. (1989): Immune System: Evaluation of injury. In: *Principles and Methods of Toxicology,* 2nd ed., edited by A.W.Hayes, pp. 741–760. Raven

Press, New York.
49. Dean, J.H., Luster, M.I., and Boorman, G.A. (1982): Methods and approaches for assessing immunotoxicity: An overview. *Environ. Health Perspect.,* 43:27–29.
50. Dean, J.H., Luster, M.I., Boorman, G.A., and Laver, L.D. (1982): Procedures available to examine the immunotoxicity of chemicals and drugs. *Pharmacol. Rev.,* 34:137–148.
51. Dean, J.H., and Vos, J.G. (1986): An introduction to immunotoxicology assessment. In: *Immunotoxicology of Drugs and Chemicals,* edited by J.Descotes, Elsevier Science, New York.
52. Dedrich, R., and Bischoff, K.B. (1980): Species similarities in pharmacokinetics. *Fed. Proc.,* 39:54–59.
53. DeGeorge, J.J., Ahn, C.-H., Andrews, P.A.Brower, M.E., Giorgio, D.W., Goheer, M.A., Lee-Han, D.Y., McGuinn, W. D., Schmidt, W., Sun, C.J., and Tripathi, S.C. (1998). Regulatory considerations for preclinical development of anticancer drugs. *Cancer Chemother. Pharmacol.,* 41:173–185.
54. Descotes, G., Mazue, G., and Richey, P. (1982): Drug immunotoxicological approaches with some selected medical products: Cyclophosphamide, methylprednisolone, betamethasone, cefoxitine, minor tranquillizers. *Toxicol. Lett.,* 13:129–138.
55. Diener, R.M. (1997): Safety assessment of pharmaceuticals. In: *Comprehensive Toxicology, Vol. 2, Toxicology Testing and Evaluation,* edited by I.G.Sipes, C.A.McQueen, and J.Gandolfi, pp. 269–290. Elsevier Science Ltd., New York.
56. Dieterle, W., and Faigle, J.W. (1981): Species differences in the disposition and metabolism of sulfinpyrazone. *Xenobiotica,* 11:559–568.
57. Dietz, F.K., Ramsey, J.C., and Watanabe, P.G. (1983). Relevance of experimental studies to human risk. *Environ. Health Perspect.,* 52:9–14.
58. DiMasi, J.A. (1994): Risks, regulation, rewards in new drug development in the United States. *Reg. Toxicol. Pharmacol.,* 19:228–235.
59. Dorato, M.A., and Buckley, L.A. (1998): Toxicology in the drug development process. In: *Current Protocols in Pharmacology,* edited by S.J.Enna, M.Williams, J.W., Ferkany, T.Kenakin, R.D.Porsolt and J.P.Sullivan, J.Wiley and Sons, New York.
60. Drug Registration Requirements in Japan, 4th ed. (1991). Yakuji Nippo Ltd., Tokyo, pp. 69–73.
61. Ekman, L., Hansson, E., Havu, N., Carlsson, E., and Lundberg, C. (1985): Toxicological studies on omeprazole. *Scand. J. Gastroent.,* (Suppl. 108):53–69.
62. Fabre, G., Combalbert, J., Berger, Y., and Cano, J.-P. (1990): Human hepatocyte as a key in vitro model to improve preclinical drug development. *Eur. J. Drug Metab. Pharmacokinetics,* 15(2):165–171.
63. Falchetti, R., Silvestri, S., Battaglia, A., and Caprino, L. (1983): Toxicological evaluation of immunomodulating drugs. In: *Current Problems in Drug Toxicology,* edited by G.Zbinden, J.Detaille, and G.Mazue, pp. 248–263. John Libbey Eurotext, London.

64. FDA (1987): Good laboratory practice for nonclinical laboratory studies. Final Rule, 21CFR58.
65. FDA (1993): Guideline for the study and evaluation of gender differences in the clinical evaluation of drugs. U.S. DHHS *Federal Register notice*, July 22, 58FR39406.
66. FDA (1997): Investigational new drug applications; proposed amendment to clinical hold regulations for products intended for life-threatening diseases. U.S. DHHS *Federal Register* notice. Sept 24, 62FR499446.
67. FDA (1997): Regulations requiring manufacturers to assess the safety and effectiveness of new drugs and biological products in pediatric patients. U.S. DHHS *Federal Register* notice, July 24, 21CFR201,312,314,601.
68. FDA Summary Basis of Approval for omeprazole. (1990).
69. FDA Summary Basis of Approval for zidovudine. (1989).
70. FDA (1992): FDA's Policy Statement for the Development of New Stereoisomeric Drugs. Corrections made Jan 3, 1997. http://www.fda.gov/cder/guidance/stereo.htm.
71. Fent, K., and Zbinden, G. (1987): Toxicity of interferon and interleukin. *TIPS*, 8:100–105.
72. Finter, N.B., Woodrouffe, J., and Priestman, T.J. (1982): Monkeys are insensitive to pyrogenic effects of human alpha-interferons. *Nature* (London), 298:301.
73. Flammang, J.J., VonTungeln, L.S., Kadlubar, F.F., and Fu, P.P. (1997): Neonatal mouse assay for tumorigenicity: Alternative to the chronic rodent bioassay. *Reg. Toxicol. Pharmacol.*, 26:230–240.
74. Fletcher, A.P. (1978): Drug safety tests and subsequent clinical experience. *J. Royal Soc. Med.*, 71:693–696.
75. Frazier, J.M., Tyson, C.A., McCarthy, C. McCormick, J.J., Meyer, D., Powis, G., and Ducat, L. (1989): Potential use of human tissues for toxicity research and testing. *Toxicol. Appl. Pharmacol.*, 97:387–397.
76. Freireich, E.J., Gehan, E.A., Rall, D.P.Schmidt, L.H., and Skipper, H.E. (1966): Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man. *Cancer Chemotherap.*, 50:219–244.
77. French Ministry of Social Affairs (1984): Recommendation concernant le protocole toxicologigue des interferons pour 1'obtention d'une autorisation de mise sur le marche: Direction de la Pharmacie et du Medicament, Sous-Direction des Affaires Techniques et Scientifigues, Paris.
78. Friedmann, N. (1985): Thymopentin: Safety overview. *Sur. Immunol. Res.*, 4(Suppl.1):139–148.
79. Gad, S.C., and Chengelis, C.P. (1995): Human health products: Drugs and medicinal devices. In: *Regulatory Toxicology*, edited by C.P.Chengelis, J.F.Holson, and S.C.Gad, pp. 9–49. Raven Press, New York.
80. Galbraith, W.M. (1987): Safety evaluation of biotechnology-derived products. In: *Preclinical Safety of Biotechnology Products Intended for Human Use*, edited by C.Graham, pp. 3–14. Alan R.Liss, Inc., New York.
81. Galloway, J.A., and Chance, R.E. (1984): *Human insulin* rDNA: From rDNA through the FDA. In *Proceedings of the Second World Conference on Clinical Pharmacology and Therapeutics*, edited by L.Lemberger, M.M.Reidenberg, pp. 503–520. ASPET, Bethesda, Maryland.
82. Goddard, M.J., and Krewski, D. (1992): Interspecies extrapolation of toxicity data. *Risk Analysis*, 12(2):315–317.
83. Goeddel, D.V., Kleid, D.G., Boliva, F., Heyneker, M.L., Yansura, D.G., Crea, R., Mirose, T., Kaszewski, A., Itakura, K., and Riggs, A.D. (1979): Expression in *Escherichia coli* of chemically synthesized genes for human insulin. *Proc. Natl Acad. Sci. USA*, 76:106–110.
84. Goldsworthy, T.L., Recio, L., Brown, K., Donehower, L.A., Mirsalis, J.C., Tennant, R.W., and Purchase, I.F.H. (1994): Transgenic animals in toxicology. *Fund. Appl. Toxicol.*, 22:8–19.
85. Gordon, C.V., and Wierenga, D.E. (1992): The drug development and approval process. *New Drug Approvals (PMA)*, January.
86. Gori, G.B. (1991): Are animal tests relevant in cancer risk assessment? A persistent issue becomes uncomfortable. *Reg. Toxicol. Pharmacol.*, 13:225–227.
87. Goyan, J. (1981): Introduction. In: *Insulins, Growth Hormone and Recombinant DNA Technology*, edited by J.L.Gueriguian, p. xviii. Raven Press, New York.
88. Graham, C.E. (1987): Overview: The industry position. In: *Preclinical Safety of Biotechnology Products Intended for Human Use*, edited by C.E.Graham, pp. 183–187. Alan R.Liss, Inc., New York.
89. Grahame-Smith, D.G. (1982): Preclinical toxicological testing and safeguards in clinical trials. *Eur. J. Clin. Pharmacol.*, 22:1–6.
90. Green, C.E., LeValley, S.E., and Tyson, C.A. (1986): Comparison of amphetamine metabolism using isolated hepatocytes from five species including human. *J. Pharmacol. Exp. Ther.*, 237:931–936.

91. Greene, J.A., Ayers, K.M., Tucker, W.E., Jr., and De Miranda, P. (1996): Nonclinical toxicology studies with zidovudine: Reproductive toxicity studies in rats and rabbits. *Fundam. Appl. Toxicol.*, 32:140–147.
92. Greishaber, C.K. (1991): Prediction of human toxicity of new antineoplastic drugs from studies in animals. In: *The Toxicity of Anticancer Drugs,* edited by G.Powis, and M.P.Hacker, pp. 10–26 Pergamon Press, New York.
93. Griffin, P.J. (1986): Predictive value of animal toxicity studies. In *Long-Term Animal Studies: Their Predictive Value for Man,* edited by S.R.Walker, and A.D.Dayan, pp. 107–116. MTP Press, Lancaster, England.
94. Griffiths, S.A., Ashton, G.A., McAuslane, J.A.N., and Lumley C.E. (1998): Non-clinical safety evaluation of products of biotechnology: Industrial strategies. CMR International report, pp. 5–6.
95. Guengerich, F.P. (1989): Characterization of human microsomal P450 enzymes. *Annu. Rev. Pharmacol. Toxicol.*, 29:241–264.
96. Hanley, T., Udall, V., and Weatherall, M. (1970): An industrial view of current practice in predicting drug toxicity. *Br. Med. Bull.*, 26(3): 203–207.
97. Hansson, E., Havu, N., and Carlsson, E. (1986): Toxicology studies with omeprazole. *Scand. J. Guastroenterol. Suppl.*, 118:89–91.
98. Harada, Y. (1987): Problems presented by animal toxicity studies. In: *Preclinical Safety of Biotechnology Products Intended for Human Use.* edited by C.E., Graham, pp. 127–142. Alan R.Liss, Inc., New York.
99. Haseman, J.K. (1985): Issues in carcinogenicity testing: dose selection. *Fundam. Appl. Toxicol.*, 5:66–78.
100. Hayes, A.H., Jr. (1990): Safety considerations in product development. *Drug Safety*, 5(Suppl. l):24–26.
101. Homburger, F. (1987): The necessity of animal studies in routine toxicology. *Comments Toxicol.*, l(5):245–255.
102. ICH Topic M3, STEP 5 (1997): Non-clinical safety studies for the conduct of human clinical trials for pharmaceuticals. ICH Harmonized Tripartite Guideline.
103. ICH Topic S1A, STEP 5 (1995): Need for carcinogenicity studies of pharmaceuticals. ICH Harmonized Tripartite Guideline.
104. ICH Topic S1C(R), STEP 5. (1995): Dose selection for carcinogenicity studies in pharmaceuticals. ICH Harmonized Tripartite Guideline.
105. ICH Topic S2A, STEP 5. (1996): Genotoxicity: Specific aspects of regulatory tests. ICH Harmonised Tripartite Guideline.

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106. ICH Topic S2B, STEP 5. (1997): Genotoxicity: Standard battery of tests. ICH Harmonized Tripartite Guideline.
107. ICH Topic S3A, STEP 5. (1995): Toxicokinetics: Guidance on the assessment of systemic exposure in toxicity studies. ICH Harmonized Tripartite Guideline.
108. ICH Topic S4A, STEP 3 (1999): Draft guideline for: Duration of chronic toxicity testing. ICH Harmonized Tripartite Guideline.
109. ICH Topic S5A, STEP 5. (1996): Detection of toxicity to reproduction from medicinal products. ICH Harmonized Tripartite Guideline.
110. ICH Topic S5B, STEP 5 (1996): Reproductive toxicity: Male fertility studies. ICH Harmonized Tripartite Guideline.
111. ICH Topic S6, STEP 5 (1997): Preclinical safety evaluation of biotechnology-derived pharmaceuticals. ICH Harmonized Tripartite Guideline.
112. Japanese Ministry of Health and Welfare (1984): Notification on application data for rDNA drugs. Notification No. 243, Pharmaceutical Affairs Bureau.
113. Jenssen, D., and Romet, L. (1990): Studies of metabolism mediated mutagenicity in vitro. *Altern. Lab. Animals,* 18:243–250.
114. Jollow, D.J., Roberts, S., Price, V., Longacre, S., and Smith, C. (1982): Pharmacokinetic considerations in toxicity testing. *Drug. Metab. Rev.*, 13(6):983–1007.
115. Karch, F.E. (1980): Is drug toxicity a problem of great magnitude? Probably not. In: *Controversy in Therapeutics*, edited by L. Lasagna, pp. 51–57. W.B.Saunders Co., Philadelphia.
116. Kier, L.D. (1985): Use of the Ames Test in toxicology. *Reg. Toxicol. Pharmacol.*, 5:59–64.
117. Klaassen, C.D., and Doull, J. (1980): Evaluation of safety: Toxicologic evaluation. In: *Toxicology: The Basic Science of Poisons*, 2nd ed., edited by J.Doull, C.D.Klaassen, and M. O.Amdur, pp. 11–27. Macmillan, New York.
118. Klaassen, C.D., and Eaton, D.L. (1991): Principles of Toxicology. In: *Toxicology: The Basic Science of Poisons*, 4th ed., edited by M. O.Amdur, J.Doull, and C.D.Klaassen, pp. 12–49. Pergamon Press, New York.
119. Kluwe, W.M. (1995): The complementary roles of in vitro and in vivo tests in genetic toxicology assessment. *Reg. Toxicol. Pharmacol.*, 22:268–272.
120 Larsson, H., Carlsson, E., Mattsson, H., Lundell, L., Sundler, F., Sundell, G., Wallmark, B., Watanabe, T., and Hakanson, R. (1986): Plasma gastrin and gastric enterochromaffin-like cell activation and proliferation: Studies with omeprazole and ranitidine in intact and antrectomized rats. *Gastroenterology*, 90:391–399.
121. Lasagna, L. (1986): Clinical testing of products prepared by biotechnology. *Reg. Toxicol. Pharmacol.*, 6:385–390.
122. Lasagna, L. (1987): Predicting human drug safety from animal studies: Current issues. *J. Toxicol. Sci.*, 12:439–450.
123. Le Bigot, J.F., Begue, J.M., Kiechel, J.R., and Guillouzo, A. (1987): Species differences in metabolism of ketotifen in rat, rabbit and man: Demonstration of similar pathways in vivo and in cultured hepatocytes. *Life Sci.*, 40:883–891.
124. Lemberger, L. (1987): Early clinical evaluation in man: The buck stops here. *Xenobiotica*, 17(3):267–273.
125. Litchfield, J.T. (1961): Forecasting drug effects in man from studies in laboratory animals. *J. Am. Med. Assoc.*, 177:104–108.
126. Lullman, H., Rossen, E., and Seiler, K.-V. (1973): The pharmacokinetics of phentermine and chlorphentermine in chronically treated rats. *J. Pharm. Pharmacol.*, 25:239–243.
127. Lumley, C.E., Parkinson, C., and Walker, S.R. (1992): An international appraisal of the minimum duration of chronic animal toxicity studies. *Hum. Exptl. Toxicol.*, 11:155–162.
128. Lumley, C.E., and Walker, S.R. (1985): A toxicology databank based on animal safety evaluation studies of pharmaceutical compounds. *Hum. Toxicol.*, 4:447–460.
129. Lumley, C.E., and Walker, S.R. (1985): The value of chronic animal toxicology studies of pharmaceutical compounds: A retrospective analysis. *Fundam. Appl. Toxicol.*, 5:1007–1024.
130. Lumley, C.E., and Walker, S.R. (1988): Investigation of the relationship between animal and clinical data. Abstr. 29 th Congress *Eur. Soc. Toxicol.* p. 188.
131. Luster, M.I., Munson, A.E., Thomas, P.T., Holsapple, M.P., Fenters, J.D., White, Jr., K.L., Laver, L.D., Germolee, D. R., Rosenthal, G.J., and Dean, J.H. (1988): Development of a testing battery to assess chemical-induced immunotoxicity: National Toxicology Program's Guidelines for immunotoxicity

evaluation in mice. *Fundam. Appl. Toxicol.,* 10:2–19.
132. Malmfors, T. (1981): Toxicology as science. *TIPS,* 2(1):1.
133. Martial, J.A., Hallewell, R.A., Baxter, J.D., and Goddman, H. M. (1979): Human growth hormone: Complementary DNA cloning and expression in bacteria. *Science,* 205:602–607.
134. Monro, A. (1992): What is an appropriate measure of exposure when testing drugs for carcinogenicity in rodents? *Toxicol. Appl. Pharmacol.,* 112:171–181.
135. Monro, A.M. (1990): Interspecies comparisons in toxicology: The utility and futility of plasma concentrations of the test substance. *Regul. Toxicol. Pharmacol.,* 12(2): 137–160.
136. Mordenti, J. (1986): Man versus beast: Pharmacokinetic ceiling in mammals. *J. Pharm. Sci.,* 75(11): 1028–1038.
137. Morrow, P.E. (1992): Dust overloading of the lungs: Update and appraisal. *Toxicol. Appl. Pharmacol.,* 113:1–12.
138. Munro, I.C. (1977): Considerations in chronic toxicity testing: The chemical, the dose, the design. *J. Environ. Pathol. Toxicol.,* 1:183–197.
139. Nagata, S., Taira, M., Mall, A., Johnsrud, L., Streuli, M., Ecsodi, J., Bell, W., Cantell, K., and Weissman, C. (1980): Synthesis in *E. coli* of a polypeptide with human leukocyte interferon activity. *Nature* (London), 284:316–320.
140. National Toxicology Program (NTP). (1984): Report of the NTP Ad Hoc Panel on Chemical Carcinogenesis Testing and Evaluation, USDHHS publication.
141. Norbury, K.C. (1982): Immunotoxicology in the pharmaceutical industry. *Environ. Health Perspect.,* 43:53–59.
142. Parkinson, C., Thomas, K.E., and Lumley, C.E. (1997): Reproductive toxicity testing of pharmaceutical compounds to support the inclusion of women in clinical trials. *Human Exper. Toxicol.,* 16:239–246.
143. Peck, C.C., Barr, W.H., Benet, L.Z., Collins, J., Desjardins, R.E., Furst, D.E., Harter, J.G., Levy, G., Ludden, T., Rodman, J.H., Sonathanan, L., Schentag, J.J., Shah, V.P., Sheiner, L.B., Skelly, J.P., Stanski, D.R., Temple, R.J., Viswanathan, C.T., Weissinger, J., and Yacobi, A. (1992): Opportunities for integration of pharmacokinetics, pharmacodynamics, and toxicokinetics in rational drug development. *J. Pharm. Sci.,* 81(6):605–610.
144. Peck, H.M. (1968): An appraisal of drug safety evaluation in animals and the extrapolation of results to man. In: *Importance of Fundamental Principles in Drug Evaluation.* edited by D.E., Tedeschi, and R.E., Tedeschi, pp. 450–471, Raven Press, New York.
145. Petricciani, J.C. (1983): An overview of safety and regulatory aspects on new biotechnology. *Reg. Toxicol. Pharmacol.,* 3:428–433.
146. *Physicians' Desk Reference* (1999): Medical Economics Data, Montvale, New Jersey, pp. 584–587.
147. *Physicians' Desk Reference* (1999): Medical Economics Data, Montvale, New Jersey, pp. 1202–1210.
148. Powis, G., Jardine, I., Van Dyke, R., Weinshilbaum, R., Moore, D., Wilke, T., Rhodes, W., Nelson, R., Benson, L., and Szumlanski, C. (1988): Foreign compound metabolism studies with

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_281.html[2010/10/28 18:42:35]

human liver obtained as surgical waste: Relation to donor characteristics and effects of tissue storage. *Drug Metab. Dispo.*, 16:582–589.
149. Raheja, K.L., and Jordan, A. (1994): FDA recommendations for preclinical testing of gonadotropin-releasing hormone (GnRH) analogues. *Reg. Toxicol. Pharmacol.*, 19:168–175.
150. Rahmani, R., Richard, B., Fabre, G., and Cano, J.-P. (1988): Extrapolation of preclinical pharmacokinetic data to therapeutic drug use. *Xenobiotica*, 18(Suppl. l):71–88.
151. Ramsey, J.C. (1982): Nonlinear pharmacokinetics relative to toxicity and use of toxicological data. *Drug Metab. Rev.*, 13(15):779–797.
152. Report of a WHO Scientific Group (1966): Principles for pre-clinical testing of drug safety. *Wld. Hlth. Org. Techn. Rep. Ser.*, 341:3–22.
153. Ronneberger, H., and Hilfenhaus, J. (1983): Toxicity studies with human fibroblast interferon. *Arch. Toxicol.*, (Suppl. 6):391–394.
154. Rumjanek, V.M., Hanson, J.M., and Morley, J. (1982): Lymphokines and monokines. In: *Immunopharmacology*, edited by P.Sirois, and M.Pleszczymski, pp. 267–285. Elsevier Press, Amsterdam.
155. Sachs, G., Carlsson, E., Lindberg, P., and Wallmark, B. (1988): Gastric H,K-ATPase as therapeutic target. *Ann. Rev. Pharmacol. Toxicol.*, 28:269–284.
156. Schein, P.S., Davis, R.D., Carter, S., Newman, R.R., and Rall, D. P. (1970): The evaluation of anti-cancer drugs in dogs and monkeys for the prediction of qualitative toxicities in man. *Clin. Pharmacol. Ther.*, 11:3–40.
157. Schellebens, H., de Reus, A., and von den Meide, P.H. (1984): The chimpanzee as a model to test side effects of human interferons. *J. Med. Primatol.*, 13:235–245.
158. Schreiner, C.A. (1983): Application of short-term tests to safety testing of industrial chemicals. *Ann. NY Acad. Sci.*, 407:367–373.
159. Schwetz, B., and Gaylor, D. (1997): New directions for predicting carcinogenesis. *Mol. Carc.*, 20:275–279.
160. Scott, G.M. (1982): Interferon: Pharmacokinetics and toxicity. *Phil. Trans. R. Soc. Lond.* B299:91–107.
161. Scott, G.M. (1983): The toxic effects of interferon in man. *J. Interferon Res.*, 5:85–114.
162. Seddon, T., Michele, I., and Chenery, R.J. (1989): Comparative drug metabolism of diazepam in hepatocytes isolated from man, rat, monkey and dog. *Biochem. Pharmacol.*, 38:1657–1665.
163. Segre, G. (1983): New toxicological problems and proposed solutions: An introduction. In: *Current Problems in Drug Toxicology*, edited by G.Zbinden, J.Y.Detaille, and G.Mazue, pp. 239–240. John Libbey Eurotext, London.
164. Singhvi, S.M., Keim, G.R., and Migdalaf, B.H. (1985): Application of pharmacokinetics in drug safety evaluation. *Reg. Toxicol. Pharmacol.*, 5:3–17.
165. Sipes, I.G., Fisher, R.L., Smith, P.F., Stine, E.R., Gandolfi, A.J., and Brendel, K. (1987): A dynamic liver culture system: A tool for studying chemical biotransformation and toxicity. *Arch. Toxicol.*, 11:20–23.
166. Smolarek, T.A., Higgins, C.V., and Amacher, D.E. (1990): Metabolism and cytotoxicity of acetaminophen in hepatocyte cultures from rat, rabbit, dog and monkey. *Drug Metab. Disp.*, 18(5):659–663.
167. Sontag, J.M., Page, N.P., and Safiotti, V. (1976): Guidelines for carcinogen bioassays in small rodents. DHHS pub. (NIH) 76–801. National Cancer Institute, Bethesda, Maryland.
168. Stebbing, N. (1984): Pharmacological assessment of interferons for clinical use. In: *Proceedings of the Second World Conference on Clinical Pharmacology and Therapeutics*, edited by L.Lemberger, and M.M.Reidenberg, pp. 521–534. *Am. Soc. Pharmacol. Exp. Therp.*, Bethesda, Maryland.
169. Stebbing, N., and Weck, P.K. (1984): Preclinical assessment of biological properties of recombinant DNA derived human interferons. In: *Recombinant DNA Products: Insulin, Interferon and Growth Hormone*, edited by A.P.Bollon, pp. 75–114. CRC Press, Inc., Boca Raton, Florida.
170. Stebbing, N., Weck, P.K., Fenno, J.T., Estell, D.A., and Rinderknecht, E. (1983): Antiviral effects of bacteria derived human leukocyte interferons against encephalomyocarditis virus infection of squirrel monkeys. *Arch. Virol.*, 76:365–372.
171. Stoll, R.E. (1987): The preclinical development of biotechnology-derived pharmaceuticals: The PMA perspective. In: *Preclinical Safety of Biotechnology Products Intended for Human Use*. edited by C.E.Graham, pp. 169–171. Alan R.Liss, Inc., New York.
172. Suter, K.E. (1983): Relevance of standard toxicological tests. Comparison of the experimental and clinical data of six pharmaceutical preparations. In: *Current Problems in Drug Toxicology*, edited by

G.Zbinden, J.Y.Detaille, and G.Mazue, pp. 77–89. John Libbey Eurotext, London.
173. Swenberg, J.A. (1995): Bioassay design and MTD setting: Old methods and new approaches. *Reg. Toxicol. Pharmacol.,* 21:44–51.
174. Teelmann, K., Hohbach, C., Lehmann, H., and the International Working Group (1986): Preclinical safety testing of species-specific proteins produced with recombinant DNA techniques. *Arch. Toxicol.,* 59:195–200.
175. Tennant, R.W., Spalding, J., and French, J.F. (1996): Evaulation of transgenic mouse bioassays for identifying carcinogens and noncarcinogens. *Mutation Res.,* 365:119–127.
176. The ICICIS Group Investigators. (1998): Report of validation study of assessment of direct immunotoxicity in the rat. *Toxicol.,* 125:183–201.
177. Spence, C., ed. (1997): The Pharmaceutical R&D Compendium: CMR International/SCRIP's Complete Guide to Trends in R&D. CMR International and PJB Publishers Ltd., Surrey, United Kingdom.
178. Thenot, J.P., Durand, A., and Morselli, P.L. (1990): In vitro techniques for metabolism studies during drug development. *Acta Pharm. Jugosl.,* 40:395–408.
179. Thomas, P.T. (1990): Approaches used to assess chemically induced impairment of host resistance and immune function. *Toxic Substan. J.,* 10:241–278.
180. Tomaszewski, J.E., and Smith, A.C. (1997): Safety testing of antitumor agents. In: *Comprehensive Toxicology, Vol. 2, Toxicity Testing and Evaluation,* edited by P.D.Williams and G.H. Hottendorf, pp. 299–309. Elsevier Science Ltd., New York.
181. Trotta, P.P. (1986): Preclinical biology of alpha interferons. *Seminars in Oncol*, 13(3):3–12.
182. Trown, P.W., Wills, R.J., and Kamm, J.J. (1986): The preclinical development of Roferon-A. *Cancer*, 57(8):1648–1656.
183. U.S. Biotechnology Policy. (1992): *Nature,* 356:1–2.
184. Van Oosterhoot, J.P.J., Vanderhann, J.W., DeWaal, E.J., Olejiniczak, K., Hilgenfeld, M., Schmidt, V., and Bass, R. (1997): The utility of two rodent species in carcinogenic risk assessment of pharmaceuticals in Europe. *Reg. Toxicol. Pharmacol.,* 25:6–17.
185. Voisin, E.M., Ruthsatz, M., Collins, J., and Hoyle, P.C. (1990): Extrapolation of animal toxicity to humans: Interspecies comparisons in drug development. *Reg. Toxicol. Pharmacol.,* 12:107–116.
186. Vos, J.G. (1977): Immune suppression as related to toxicology. *CRC Crit. Rev. Toxicol.,* 5:67–101.
187. Wagstaff, J., Chadwick, G.Howell, A., Thatcher, N., Scarffe, J. H., and Crowther, D. (1984): A phase I toxicity study of human rDNA interferon in patients with solid tumors. *Cancer Chemother. Pharmacol.,* 13:100–105.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_282.html[2010/10/28 18:42:36]

188. Waife, S.O., and Lasagna, L. (1985): From DNA to NDA—The impact of recombinant DNA technology on new drug development. *Reg. Toxicol. Pharmacol.*, 5:212–224.
189. Wallmark, B. (1986): Mechanism of action of omeprazole. *Scand. J. Gastroent.*, (Suppl. 118):11–16.
190. Weil, C.S. (1972): Guidelines for experiments to predict the degree of safety of a material for man. *Toxicol. Appl. Pharmacol.*, 21:194–199.
191. Weissinger, J. (1989): Nonclinical pharmacologic and toxicologic considerations for evaluating biologic products. *Reg. Toxicol. Pharmacol.*, 10:255–263.
192. Weissinger, J. (1990): Pharmacology and toxicology of novel drug delivery systems: Regulatory issues. *Drug Safety*, 5(Suppl 1):107–113.
193. Wierda, D. (1992): Personal communication. Biochemical Toxicology, Eli Lilly and Company, Greenfield, Indiana.
194. Williams, G.M., Dunkel, V.C., Ray, V.A., eds. (1983): Cellular systems for toxicity testing. *Ann. NY Acad. Sci.*, V. 407.
195. Williams, G.M., and Weisburger, J.H. (1991): Chemical carcinogenesis. In: *Toxicology: The Basic Science of Poisons*, 4th ed., edited by M.O.Amdur, J.Doull, and C.D.Klaassen, pp. 127–200. Pergamon Press, New York.
196. Williams, P. (1990): The role of pharmacological profiling in safety assessment. *Reg. Toxicol. Pharmacol.*, 12(3):238–252.
197. Williams, P.D., Calligaro, D.O., Colbert, W.E., Helton, D.R., Shetler, T., Turk, J.A., and Jordan, W.H. (1991): General pharmacology of a new potent 5-hydroxytryptamine antagonist. *Arzneim. Forsch.*, 41(1): 189–195.
198. Wilson, A.B. (1987): The toxicology of the end products from biotechnology processes. *Arch. Toxicol.*, (Suppl. II):194–199.
199. Wolf, F.J. (1980): Effect of overloading pathways on toxicity. *J. Environ. Pathol. Toxicol.*, 3:113–134.
200. Wrighton, S.A., and Stevens, J.C. (1992): The human hepatic cytochromes P450 involved in drug metabolism. *CRC Crit. Rev. Toxicol.*, 22(1): 1–21.
201. Yoshida, S., Golub, M.S., and Gershwin, M.E. (1989): Immunological aspects of toxicology: Premises not promises. *Reg. Toxicol. Pharmacol.*, 9:56–80.
202. Zapp, J.A., Jr. (1977): Extrapolation of animal studies to the human situation. *J. Toxicol. Environ. Health*, 2:1425–1433.
203. Zbinden, G. (1964): The problem of the toxicologic examination of drugs in animals and their safety in man. *Clin. Pharmacol. Ther.*, 5:537–545.
204. Zbinden, G. (1966): The significance of pharmacologic screening tests in the preclinical safety evaluation of new drugs. *J. New Drugs*, 6:1–7.
205. Zbinden, G. (1976): A look at the world from inside the toxicologist's cage. *Eur. J. Clin. Pharmacol.*, 9:333–338.
206. Zbinden, G. (1978): Application of basic concepts to research in toxicology. *Pharmacol. Rev.*, 30(4):605–616.
207. Zbinden, G. (1980): Predictive value of pre-clinical drug safety evaluation. In: *Proceedings of the First World Conference on Clinical Pharmacology and Therapeutics* (London), edited by P.Turner, C.Padghan, and A.Hedges, p. 9–14. Macmillan, London.
208. Zbinden, G. (1982): Current trends in safety testing and toxicological research. *Naturwissenschaften*, 69:255–259.
209. Zbinden, G. (1986): Acute toxicity testing, public responsibility and scientific challenges. *Cell Biol. Toxicol.*, 2(3):325–335.
210. Zbinden, G. (1987): Biotechnology products intended for human use, toxicological targets and research strategies. In: *Preclinical Safety of Biotechnology Products Intended for Human Use*, edited by C.E.Graham, pp. 143–159. Alan R.Liss, Inc., New York.
211. Zbinden, G. (1988): Biopharmaceutical studies, a key to better toxicology. *Xenobiotica*, 18(1):9–14.
212. Zbinden, G. (1989): Improvement of predictability of subchronic and chronic toxicity studies. *J. Toxicol. Sci.*, 14(Suppl. 3):3–21.
213. Zbinden, G. (1990): Effects of recombinant human alpha-interferon in a rodent cardiotoxicity model. *Toxicol. Lett.*, 50:25–35.
214. Zbinden, G. (1990): Safety evaluation of biotechnology products. *Drug Safety*, 5(Suppl. 1):58–64.
215. Zbinden, G. (1991): Predictive value of animal studies in toxicology. *Reg. Toxicol. Pharmacol.*,

14:167–177.
216. Zwickl, C.M., Cocke, K.S., Tamura, R.N., Holzhausen, L.M., Brophy, G.T., Bick, P.H., and Wierda, D. (1991): Comparison of the immunogenicity of recombinant and pituitary human growth hormone in rhesus monkeys. *Fundam. Appl. Toxicol.*, 16:275–287.

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Chapter **7**

Statistics For Toxicologists

Shayne C.Gad

Principles and Methods of Toxicology, Fourth Edition, edited by A.Wallace Hayes.
Taylor & Francis, Philadelphia © 2001.

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Over the years that have passed since the writing of the third edition of this chapter, the rate of change in toxicology, statistics, and in the interface between these two disciplines has continued to accelerate. The author is hopeful that this complete revision adequately reflects these changes.

PHILOSOPHY AND GENERAL PRINCIPLES

This chapter has been written for both practicing and student toxicologists as both a basic text and a practical guide to the common statistical problems encountered in toxicology and the methodologies that are available to solve them. The chapter has been enriched by the inclusion of discussions of why a particular procedure or interpretation is recommended, by the clear enumeration of the assumptions that are necessary for a procedure to be valid, and by worked-through examples and problems drawn from the actual practice of toxicology.

Since 1960, the field of toxicology has become increasingly complex and controversial in both its theory and practice. Much of this change is due to the evolution of the field. As in all other sciences, toxicology started as a descriptive science. Living organisms, be they human or otherwise, were dosed with or exposed to chemical or physical agents, and the adverse effects which followed were observed, but as a sufficient body of descriptive data was accumulated, it became possible to infer and study underlying mechanisms of action to determine in a broader sense why adverse effects occurred. Toxicology has thus entered a later state of development, the mechanistic stage, where active contributions to the field encompass both descriptive and mechanistic studies.

Studies continue to be designed and executed to generate increased amounts of data. The resulting problems of data analysis have then become more complex and toxicology has drawn more deeply from the well of available statistical techniques. Statistics have also been very active and growing during the last 35 years, to some extent, at least, because of the parallel growth of toxicology. These simultaneous changes have led to an increasing complexity of data and, unfortunately, to the introduction of numerous confounding factors that severely limit the utility of the resulting data in all too many cases.

A major difficulty is that there is a very real need to understand the biological realities and implications of a problem, as well as to understand the peculiarities of toxicological data before procedures are selected and employed for analysis. These characteristics include the following:

(1) The need to work with a relatively small sample set of data collected from the members of a population (laboratory animals, cultured cells, bacterial cultures) that are not actually the population of interest (i.e., humans or a target animal population).

(2) Dealing frequently with data resulting from a sample that was censored on a basis other than by the investigator's design. By censoring, of course, we mean that not all data points were col lected as might be desired. This censoring could be the result of either a biological factor (the test animal being dead or too debilitated to manipulate) or a logistic factor (equipment being inoperative or a tissue being missed in necropsy).

(3) The conditions under which our experiments are conducted are extremely varied. In pharmacology (the closest cousin to at least classical toxicology), the possible conditions of interaction of a chemical or physical agent with a person are limited to a small range of doses via a single route over a short course of treatment to a defined patient population. In toxicology, however, all of these variables (dose, route, time span, subject population) are determined by the investigator.

(4) The time frames available to solve our problems are limited by practical and economic factors. This frequently means that there is not time to repeat a critical study if the first attempt fails, so a true iterative approach is not possible.

The training of most toxicologists in statistics remains limited to a single introductory course that concentrates on some theoretical basics. As a result, the armamentarium of statistical techniques of most toxicologists is limited and the tools that usually are used (t-tests, chi-square, analysis of variance, linear regression) are neither fully developed nor well understood. It is hoped that this chapter will help change this situation.

As a point of departure, it is essential that any analysis of study results be interpreted by a professional who firmly understands three concepts: the difference between biological significance and statistical significance, the nature and value of different types of data, and causality.

For the first concept, we should consider the four possible combinations of these two different types of significance for which we find the relationship shown below.

		STATISTICAL SIGNIFICANCE	
		NO	YES
BIOLOGICAL	NO	CASE I	CASE II
SIGNIFICANCE	YES	CASE III	CASE IV

Cases I and IV give us no problems, as the answers are the same statistically and biologically, but cases II and III present problems. In case II (the false-positive) we have a circumstance where there is a statistical significance in the measured difference between treated and control groups, but there is no

true biological significance to the finding. This is not an uncommon happening (e.g., in the case of clinical chemistry parameters). This is called type I error by statisticians, and the probability

Table 7.1

Approximate total sample sizes for comparisons using the t-test and equal group sizes

	β=0.1		β=0.2	
Δ/σ	α=0.05	α=0.10	α=0.05	α=0.10
0.25	672	548	502	396
0.50	168	138	126	98
0.75	75	62	56	44
1.00	42	34	32	24
1.25	28	22	20	16
1.50	18	16	14	12

Δ is the difference in the treatment group means and σ is the standard deviation.

of this occurring is called the a (alpha) level. In case III (the false-negative) we have no statistical significance, but the differences between groups are biologically/ toxicologically significant. This is called type II error by statisticians, and the probability of such an error happening by random chance is called the β (beta) level. An example of a type II error is when a few of a very rare tumor type are seen in treated animals. For both type I and II errors, numerical analysis, no matter how well done, is no substitute for professional judgment. One must have a feeling for the different types of data and for the value or relative merit of each. Note that the two error types interact, and in determining sample size we need to specify both α and β levels. Table 7.1 demonstrates this interaction in the case of the t-test. There are many reasons that biological and statistical significance are not identical and often are multiple, but certainly a central reason is causality. Through our consideration of statistics, we should keep in mind that just because a treatment and a change in an observed organism are seemingly or actually associated with each other does not "prove" that the former caused the latter. Though this fact is now widely appreciated for correlation (e.g., the fact that the number of storks' nests found each year in England is correlated with the number of human births that year does not mean that storks bring babies), it is just as true in the general case of significance. Timely establishment and proof that treatment causes an effect requires an understanding of the underlying mechanism and proof of its validity. At the same time, it is important that we realize that not finding a good correlation or suitable significance associated with a treatment and an effect likewise does not prove that the two are not associated (that a treatment does not cause an effect). At best, it gives us a certain level of confidence that under the conditions of the current test, these items are not associated.

These points, along with other common pitfalls and shortcomings associated with the method, will be discussed in greater detail in the Assumptions section for each method. To help in better understanding the sections to come, terms frequently used in discussion throughout this chapter should first be considered. These are presented in Table 7.2.

Each measurement we make—each individual piece of experimental information we gather—is called a datum; however, we gather and analyze multiple pieces at one time, with the resulting collection being called data. Data are collected on the basis of their association with a treatment (intended or otherwise) as an effect (a property) that is measured in the experimental subjects of a study, such as body weights. These identifiers (i.e., treatment and effect) are termed *variables*. Treatment variables (those that the researcher or nature control and that can be directly controlled) are termed *independent,* whereas effect variables (such as weight, life span, and number of neoplasms) are termed dependent variables (their outcome is believed to dependent on the "treatment" being studied).

All of the possible measures of a given set of variables in all of the possible subjects that exist are termed the population for those variables. Such a population of variables cannot be truly measured (e.g., one would have to obtain, treat, and measure the weights of all the Fischer-344 rats that were, are, or ever will be). Instead, we deal with a representative group—a sample. If our sample of data is appropriately collected and of sufficient size, it serves to provide good estimates of the characteristics of the parent population from which it was drawn.

Two terms refer to the quality and reproducibility of our measurements of variables. The first, accuracy, is an expression of the closeness of a measured or computed value to its actual or "true" value in nature. The second, precision, reflects the closeness or reproducibility of a series of repeated measurements of the same quantity.

If we arrange all of our measurements of a particular variable in order as a point on an axis marked as to the values of that variable, and if our sample were large enough, the pattern of distribution of the data in the sample would begin to become apparent. This pattern is a representation of the frequency distribution of a given population of data (i.e., of the incidence of different measurements, their central

tendency, and dispersion).

The most common frequency distribution—and one we will talk about throughout this chapter—is the normal (or Gaussian) distribution. The normal distribution is such that two thirds of all values are within one standard deviation of the mean (or average value for the entire population), and 95% are within 1.96 standard

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Table 7.2 Some frequently used terms and their general meanings (171)

Term	Meaning
95% confidence interval	A range of values above, below, or above and below the sample mean, median, mode, etc., has a 95% chance of containing the true value of the population (mean, median, mode). Also called the fiducial limit equivalent to the Pc 0.05.
Bias	Systemic error as opposed to a sampling error. For example, selection bias may occur when each member of the population does not have an equal chance of being selected for the sample.
Degrees of freedom	The number of independent deviations, usually abbreviated *df*.
Independent variables	Also known as predictors or explanatory variables.
P-value	Another name for significance level; usually 0.05 or 0.01
Power	The effect of the experimental conditions on the dependent variable relative to sampling fluctuation. When the effect is maximized, the experiment is more powerful. Power can also be defined as the probability that there will not be a type II error (1-Beta). Conventionally, power should be at least .07.
Random	Each individual member of the population has the same chance of being selected for the sample.
Robust	Having inferences or conclusions little effected by departure from assumptions.
Sensitivity	The number of subjects experiencing each experimental condition divided by the variance of scores in the sample.
Significance level	The probability that a difference has been erroneously declared to be significant, typically 0.05 and 0.01 corresponding to 5% and 1% chance of error, respectively.
Type I error (false-positives)	Concluding that there is an effect when there really is not an effect. Its probability is the alpha level.
Type II error (false-negatives)	Concluding there is not an effect when there really is an effect. Its probability is the beta level.

deviations of the mean. The mathematical equation describing the normal distribution is

$$y = \frac{1}{\sigma\sqrt{2\pi}} e^{-(x-\mu)^2/2\sigma^2}$$

where μ is the mean and σ is the standard deviation. Other common frequency distributions include the Poisson and chi square.

In all areas of biological research, optimal design and appropriate interpretation of experiments require that the researcher understand both the biological and technological underpinnings of the system being studied and of the data being generated. From the point of view of the statistician, it is vitally important that the experimenter both knows and is able to communicate the nature of the data and understands its limitations. One classification of data types is presented in Table 7.3.

The nature of the data collected is determined by three considerations. These are the biological source of the data (the system being studied), the instrumentation and techniques being used to make measurements, and the design of the experiment. The researcher has some degree of control over each of these, with least control over the biological system (he/she normally has a choice of only one of several models to study) and most control over the design of the experiment or study. Such choices, in fact, dictate the type of data generated by a study.

Statistical methods are based on specific assumptions. Parametric statistics—those that are most familiar to the majority of scientists—have more stringent underlying assumptions than do nonparametric statistics. Among the underlying assumptions for many parametric

Table 7.3 Types of variables (data) and examples of each type

Classified by		Type	Example
Scale	Continuous	Scalar	Body weight
		Ranked	Severity of a lesion
	Discontinuous	Scalar	Weeks until the first observation of a tumor in a carcinogenicity study
		Ranked	Clinical observations in animals
		Attribute	Eye colors in fruit flies
		Quantal	Dead/alive or present/absent
Frequency distribution		Normal	Body weights
		Bimodal	Some clinical chemistry parameters
		Others	Measures of time to incapacitation

statistical methods (such as the analysis of variance) is that the data are continuous. The nature of the data associated with a variable (as described previously) imparts a "value" to that data, the value being the power of the statistical tests that can be employed.

Continuous variables are those that can at least theoretically assume any of an infinite number of values between any two fixed points (such as measurements of body weight between 2.0 and 3.0 kg). Limitations on our ability to measure a specific parameter constrain the extent to which the real-world situation approaches the theoretical, but many of the variables studied in toxicology are, in fact, continuous. Examples of these are lengths, weights, concentrations, temperatures, periods of time, and percentages. For these continuous variables, we may describe the character of a sample with measures of central tendency and dispersion that we are most familiar with—the mean, denoted by the symbol μ and also called the arithmetic average, and the standard deviation SD, which is denoted by the symbol σ and is calculated as being equal to

$$\sqrt{\frac{\sum X^2 - \frac{(\sum X)^2}{N}}{N-1}},$$

where X is the individual datum and N is the total number of data in the group.

Contrasted with these continuous data, however, we have discontinuous (or discrete) data that can only assume certain fixed numerical values with no possible intermediate values (such as counts of 5 and 6 dead animals respectively). In these cases our choice of statistical tools or tests is, as we will find later, more limited.

Probability

Probability is simply the frequency with which, in a sufficiently large sample, a particular event will occur or a particular value will be found. Hypothesis testing, for example, generally is structured so that the likelihood of a treatment group being the same as a control (the so called "null hypothesis") can be assessed as being less than a selected low level (very frequently 5%), which implies that we are 1.0-α (i.e., 1.0–0.05, or 95%) sure that the groups are *not* equivalent.

Functions of Statistics

Statistical methods may serve to do any combination of three possible tasks. The one we are most familiar with is hypothesis testing (i.e., determining if two [or more] groups of data differ from each other at a predetermined level of confidence). A second function is the construction and use of models that may be used to predict future outcomes of chemical-biological interactions. This is most commonly seen in linear regression or in the derivation of some form of correlation coefficient. Model fitting allows us to relate one variable (typically a treatment or "independent" variable) to another. The third function, reduction of dimensionality, continues to be less commonly used than the first two. This final category includes methods for reducing the number of variables in a system while only minimally reducing the amount of information, therefore making a problem easier to visualize and to understand. Examples of such techniques are factor analysis and cluster analysis. A subset of this last function, discussed later under descriptive statistics, is the reduction of raw data to single expressions of central tendency and variability (such as the mean and standard deviation). There also is a special subset of statistical techniques that is part of both the second and third functions of statistics. This is data transformation, which includes such things as the conversion of numbers to log or probit values.

This chapter is primarily designed to address the first of the three functions of statistical methods that we pre

sented (hypothesis testing). The second function, modeling (especially in the form of risk assessment), is becoming increasingly important as the science continues to evolve from the descriptive phase to a mechanistic phase. Likewise, because the interrelation of multiple factors is becoming a real concern, a discussion of reduction of dimensionality has been included.

Descriptive Statistics

Descriptive statistics are used to summarize the general nature of a data set. As such, the parameters describing any single group of data have two components. One of these describes the location of the data, whereas the other gives a measure of the dispersion of the data in and about this location. Often overlooked is the fact that the choice of which parameters are used to give these pieces of information implies a particular type of distribution for the data.

Most commonly, location is described by giveing the (arithmetic) mean and dispersion by giving the standard deviation (SD) or the standard error of the mean (SEM). The calculation of the first two of these has already been described. If we again denote the total number of data in a group as N, then the SEM would be calculated as

$$\mathrm{SEM} = \frac{\mathrm{SD}}{\sqrt{N}}.$$

The SD and the SEM are related to each other but yet are quite different. To compare these two, let us first demonstrate their calculation from the same set of 15 observations:

Data Points (Xi): 1, 2, 3, 4, 4, 5, 5, 5, 6, 6, 6, 7, 7, 8, 9
Sum(Σ)=78.

Squares (X_i^2): 1, 4, 9, 16, 16, 25, 25, 25, 36, 36, 36, 49, 49, 64, 81
Sum=472

The standard deviation can then be calculated as

$$\mathrm{SD} = \frac{\sqrt{472 - \frac{(78)^2}{15}}}{15 - 1} = \frac{\sqrt{472 - \frac{(6084)}{15}}}{14}$$

$$= \frac{\sqrt{472 - 405.6}}{14} = \sqrt{4.7428571} = 2.1778$$

with a mean $(\bar{x})$ of $\frac{78}{15} = 5.2$ for the data group. The SEM for the same set of data, however, is

$$\mathrm{SEM} = \frac{2.1778}{\sqrt{15}} = \frac{2.1778}{3.8730} = 0.562303.$$

The SEM is quite a bit smaller than the SD, making it very attractive to use in reporting data. This size difference is because the SEM actually is an estimate of the error (or variability) involved in measuring the means of samples and not an estimate of the error (or variability) involved in measuring the data from which means are calculated. This is implied by the *Central Limited Theorem,* which tells us three major things:

• The distribution of sample means will be approximately normal regardless of the distribution of values in the original population from which the samples were drawn.
• The mean value of the collection.
• The standard deviation of the collection of all possible means of samples of a given size, called the SEM, depends on both the standard deviation of the original population and the size of the sample.

The SEM should be used only when the uncertainty of the estimate of the mean is of concern, which is almost never the case in toxicology. Rather, we are concerned with an estimate of the variability of the population, for which the standard deviation is appropriate.

The use of the mean with either the SD or SEM implies, however, that we have reason to believe that the sample of data being summarized are from a population that is at least approximately normally distributed. If this is not the case, then we should rather use a set of statistical descriptions that do not require a normal distribution. These are the median, for location, and semiquartile distance, for a measure of dispersion. These somewhat less familiar parameters are characterized as follows.

Median

When all of the numbers in a group are arranged in a ranked order (i.e., from smallest to largest), the

median is the middle value. If there is an odd number of values in a group, then the middle value is obvious (in the case of 13 values, for example, the seventh largest is the median). When the number of values in the samples is even, the median is calculated as the midpoint between the (N/2)th and the ([N/2]+1)th number. For example, in the series of numbers 7, 12, 13, 19, the median value would be the midpoint between 12 and 13, which is 12.5.

Semiquartile Distance

When all the data in a group are ranked, a quartile of the data contains one ordered quarter of the values. Typically, we are most interested in the borders of the middle two quartiles Q1 and Q3, which together represent the semiquartile distance and which contain the median as their center. Given that there are N values in an ordered group of data, the upper limit of the jth quartile (Qj)

may be computed as being equal to the [(jN÷l)/4th] value. Once we have used this formula to calculate the upper limits of Q1 and Q3, we can then compute the semiquartile distance (which is also called the quartile deviation, and as such is abbreviated as the QD) with the formula QD=(Q3–Q1)/2.

For example, for the 15 value data set 1, 2, 3, 4, 4, 5, 5, 5, 6, 6, 6, 7, 7, 8, 9, we can calculate the upper limits of Q1 and Q3 as

$$Q_1 = \frac{1(15+1)}{4} = \frac{16}{4} = 4,$$

$$Q_3 = \frac{3(15=1)}{4} = \frac{48}{4} = 12.$$

The 4th and 12th values in this data set are 4 and 7, respectively. The semiquartile distance can then be calculated as

$$\mathrm{QD} = \frac{7-4}{2} = 1.5.$$

One final sample parameter that sees some use in toxicology (primarily in inhalation studies) is the geometric mean, denoted by the term $\bar{X}_g$. This is calculated as

$$\overline{X}_g = (X_1.X_2 \ldots X_N)^{1/N}$$

and has the attractive feature that it does not give excessive weight to extreme values (or "outliers"), such as the mass of a single very large particle in a dust sample. In effect, it "folds" extreme values in toward the center of the distribution, decreasing the sensitivity of the parameter to the undue influence of the outlier. This is particularly important in the case of aerosol samples, where a few very large particles would cause the arithmetic mean of particle diameters to present a misleading picture of the nature of the "average" particle.

There are times when it is desired to describe the relative variability of one or more sets of data. The most common way of doing this is to compute the coefficient of variation (CV), which is calculated simply as the ratio of the SD to the mean, or

$$CV = \frac{SD}{\overline{X}}$$

A CV of 0.2 or 20% thus means that the standard deviation is 20% of the mean. In toxicology the CV is frequently between 20% and 50% and may at times exceed 100%.

Outliers and Rounding of Numbers

These two considerations in the handling of numerical data can be, on occasion, of major concern to the toxicologist because of their pivotal nature in cases of borderline significance. Outliers should also be of concern for other reasons, however. On the principle that one should always have a plan to deal with all reasonably likely contingencies in advance of their happening, early decisions should be made to select a policy for handling both outliers and the rounding of numbers.

Outliers

Outliers are extreme (high or low) values that are widely divergent from the main body of a group of data and from what is our common experience. They may arise from an instrument (such as a balance) being faulty, the apparently natural urge of some animals to frustrate research, or they may be indicative of a "real" value. Outlying values can be detected by visual inspection of a data, use of a scattergram (described later), or (if the data set is small enough, which is usually the case in toxicology) by a large increase in the parameter estimating the dispersion of data, such as the SD.

When we can solidly tie one of the above error-producing processes (such as a balance being faulty) to an outlier, we can safely delete it from consideration, but if we cannot solidly tie such a cause to an outlier (even if we have strong suspicions), we have a much more complicated problem, for then such a value may be one of several other things. It could be the result of a particular parameter that is the grounds for the entire study (i.e., the very "effect" that we are looking for) or it could be because of the collection of legitimate effects which constitute sample error. As will be discussed later (under exploratory data analysis), and is now more widely appreciated, outliers can be an indication of a biologically significant effect that is not yet statistically significant. Variance inflation can be the result of such outliers and can be used to detect them. Outliers, in fact, by increasing the variability within a small sample set, decrease the sensitivity of our statistical tests and actually preclude our having a statistically significant result (12).

Alternatively, the outlier may be the result of, for example, an unobserved technician error and may change the decisions made from a set of data. In this case we want to reject the data point—to exclude it from consideration with the rest of the data, but how can one identify these legitimate statistical rejection cases?

There are a wide variety of techniques for data rejection. Their proper use depends on one's having an understanding of the nature of the distribution of the data. For normally distributed data with a single extreme value, a simple method such as Chauvenet's criterion (107) may legitimately be employed. This states that if the probability of a value deviating from the mean is greater than 1/2 N, one should consider that there are adequate grounds for its rejections. This approach is demonstrated below.

USE OF CHAUVENET'S CRITERION

Having collected 20 values as a data set, we find they include the following values: 1, 6, 7, 8, 8, 9, 9, 9, 10, 10, 10, 10, 10, 11, 11, 11, 12, 12, 13 and 14. Was the lowest value (1) erroneous and should it be rejected as a outlier? Some simple calculations are performed, as

Mean (X)=9.55,

Standard deviation (SD)=2.80,

$$\text{Chauvenet's Criterion Value} = \tfrac{1}{2}\text{N} = \frac{20}{2} = 10.$$

So we would reject the value of "1" if its probability of occurrence were less than 10%. Going to a table of Z scores, we see that 10% of the values in a normal distribution are beyond ±1.645 SDs of the mean. Multiplying this by the SD for the sample, we get (1.645)(2.80)=4.606. This means we would reject values beyond this range from the mean—that is, less than (9.55–4.606)=4.944 or greater than (9.55+4.606)=14.156. We therefore reject the value of "1."

One should note that as the sample size gets bigger, the rejection zone for Chauvenet's Criterion will also increase, as N of 20 is about as large as this method is useful for.

A second relatively straightforward approach, for use when the data are normally distributed but contain several extreme values is to winsorize the data. Though there are a number of variations to this approach, the simplest (called the *G-1 method*) calls for replacing the highest and lowest values in a set of data. In a group of data consisting of the values 54, 22, 18, 15, 14, 13, 11, and 4, we would replace 54 with a second 22, and 4 with a replicate 11. This would give us a group consisting of 22, 22, 18, 15, 14, 13, 11, and 11, which we would then treat as our original data. Winsorizing should not be performed, however, if the extreme values constitute more than a small minority of the entire data set.

Another approach is to use Dixon's Test (42) to determine if extreme values should be rejected. In Dixon's test, the set of observations is first ordered according to their magnitude (as we did earlier for the data set used to demonstrate Chauvenet's Criterion, though there this step was simply to make the case clearer). The ratio of the difference of an extreme value from one of its nearest neighbor values in the range of values in the sample is then calculated using a formula that varies with sample size. This ratio is then compared to a table value, and if found to be equal or greater, is considered to be an outlier at the $p \leq 0.05$ level. The formula for the ratio varies with sample size and according to whether it is the smallest or largest value that is suspect.

If we have more information as to the nature of the data or the type of analysis to be performed, there are even better techniques to handle outliers. Extensive discussions of these may be found elsewhere (10, 12, 76, 137).

Rounding Off

When the number of digits in a number is to be reduced (due to limitations of space or to reflect the extent of significance of a number), we must carry out the process of rounding off a number. Failure to have a rule for performing this operation can lead to both confusion and embarrassment for a facility (during such times as study audits). One common rule follows.

A digit to be rounded is not changed if it is followed by a digit less than 5; the digits following it are simply dropped off ("truncated"). If the number is followed by a digit greater than 5 or by a 5 followed by other nonzero digits, it is increased to the next highest number. When the digit to be rounded is followed by 5 alone or by 5 followed by zeros, it is unchanged if it is even but increased by one if it is odd. Examples of this rule, in effect, are (in a case where we must reduce to 3 digits)

137.4 becomes 137

137.6 becomes 138

138.52 becomes 139

137.5 becomes 138

and 138.5 becomes 138

The rationale behind this procedure is that over a period of time the results should even out—as many digits that increased are also decreased.

Sampling

Sampling—the selection of which individual data points will be collected, whether in the form of selecting which animals to collect blood from or to remove a portion of a diet mix from for analysis—is an essential step upon which all other efforts toward a good experiment or study are based. There are three assumptions about sampling that are common to most of the statistical analysis techniques that are used in toxicology. These are that the sample is collected without bias, that each member of a sample is collected independently of the others, and that members of a sample are collected with

replacements. Precluding bias, both intentional and unintentional, means that at the time of selection of a sample to measure, each portion of the population from which that selection is to be made has an equal chance of being selected. Ways of precluding bias are discussed in detail in the section on experimental design.

Independence means that the selection of any portion of the sample is not affected by and does not affect the selection or measurement of any other portion.

Finally, sampling with replacement means that, in theory, after each portion is selected and measured, it is returned to the total sample pool and thus has the opportunity to be selected again. This is a corollary of the assumption of independence. Violation of this

assumption (which is almost always the case in toxicology and all of the life sciences) does not have serious consequences if the total pool from which samples are taken is sufficiently large (say 20 or greater) so that the chance of reselecting that portion is small anyway.

There are four major types of sampling methods—random, stratified, systematic, and cluster. Random is by far the most commonly employed method in toxicology. It stresses the fulfillment of the assumption of avoiding bias. When the entire pool of possibilities is mixed or randomized (procedures for randomization are presented in a later section), then the members of the group are selected in the order that they are drawn from the pool.

Stratified sampling is performed by first dividing the entire pool into subsets or strata and then random sampling from each strata. This method is employed when the total pool contains subsets that are distinctly different but the data within each subset are similar. An example is a large batch of a powdered pesticide in which it is desired to determine the nature of the particle size distribution. Larger pieces or particles are on the top, whereas progressively smaller particles have settled lower in the container; at the very bottom, the material has been packed and compressed into aggregates. To determine a timely representative answer, proportionally sized subsets from each layer or strata should be selected, mixed, and randomly sampled. This method is used more commonly in diet studies.

In systematic sampling, a sample is taken at set intervals (such as every fifth container of reagent or taking a sample of water from a fixed sample point in a flowing stream every hour). This is most commonly employed in quality assurance or (in the clinical chemistry lab) in quality control.

In cluster sampling, the pool is already divided into numerous separate groups (such as bottles of tablets), and we select small sets of groups (such as several bottles of tablets) and then select a few members from each set. This results in a cluster of measures. Again, this is a method most commonly used in quality control or in environmental studies when the effort and expense of physically collecting even a small number of data points is significant.

In classical toxicology studies, sampling arises in a practical sense in a limited number of situations. The most common of these are as follows:

(1) Selecting a subset of animals or test systems from a study to make some measurement (which either destroys or stresses the measured system or is expensive) at an interval during a study. This may include such cases as doing interim necropsies in a chronic study or collecting and analyzing blood samples from some animals during a subchronic study.

(2) Analyzing inhalation chamber atmospheres to characterize aerosol distributions with a new generation system.

(3) Analyzing diet in which test material has been incorporated.

(4) Performing quality control on an analytical chemistry operation by having duplicate analyses performed on some materials.

(5) Selecting data to audit for quality assurance purposes.

EXPERIMENTAL DESIGN

Toxicological experiments generally have two purposes. The first is to determine whether or not an agent results in an effect on a biological system. The second, never far behind, is to find how much of an effect is present. It has become increasingly desirable that the results and conclusions of studies aimed at assessing the effects of environmental agents be as clear and unequivocal as possible. It is essential that every experiment and study yield as much information as possible and that the results of each study have the greatest possible chance of answering the questions it was conducted to address. The statistical aspects of such efforts, so far as they are aimed at structuring experiments to maximize the possibilities of success, are called *experimental design*.

We have now become accustomed to developing exhaustively detailed protocols for an experiment or study prior to its conduct. A priori selection of statistical methodology (as opposed to the post hoc approach) is as significant a portion of the process of protocol development and experimental design as any other and can measurably enhance the value of the experiment or study. Prior selection of statistical methodologies is essential for proper design of other portions of a protocol such as the number of animals per group or the sampling intervals for body weight. Implied in such a selection is the notion that the toxicologist has both an in-depth knowledge of the area of investigation and an understanding of the general principles of experimental design, as the analysis of any set of data is dictated to a large extent by the manner in which the data are obtained.

The four basic statistical principles of experimental design are replication, randomization, concurrent ("local") control, and balance. In abbreviated form, these may be summarized as follows.

Replication: Any treatment must be applied to more than one experimental unit (animal, plate of cells,

litter of offspring, etc.). This provides more accuracy in the measurement of a response than can be obtained from a single observation, as underlying experimental errors tend to cancel each other out. It also supplies an estimate

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of the experimental error derived from the variability among each of the measurements taken (or "replicates"). In practice, this means that an experiment should have enough experimental units in each treatment group (i.e., large enough "N") so that reasonably sensitive statistical analysis of data can be performed. The estimation of sample size is addressed in detail later in this chapter.

Randomization: This is practiced to ensure that every treatment has its fair share of extreme high and extreme low values. It also serves to allow the toxicologist to proceed as if the assumption of "independence" is valid (i.e., there is not avoidable [known] systematic bias in how one obtains data).

Concurrent Control: Comparisons between treatments should be made to the maximum extent possible between experimental units from the same closely defined population. Therefore, animals used as a "control" group should come from the same source, lot, age, etc. as test group animals. Except for the treatment being evaluated, test and control animals should be maintained and handled in exactly the same manner.

Balance: If the effect of several different factors is being evaluated simultaneously, the experiment should be laid out in such a way that the contributions of the different factors can be separately distinguished and estimated. There are several ways of accomplishing this using one of several different forms of design, as will be discussed later.

Types of Experimental Design

There are four basic experimental design types used in toxicology: the randomized block, latin square, factorial design, and nested design. Other designs that are used are really combinations of these and are rarely employed in toxicology. Before examining these four basic types, however, we must first examine the basic concept of blocking.

Blocking is, simply put, the arrangement or sorting of the members of a population (such as all of an available group of test animals) into groups based on certain characteristics that may (but are not sure to) alter an experimental outcome. Such characteristics, which may cause a treatment to give a differential effect, include genetic background, age, sex, overall activity levels, and so on. The process of blocking then acts (or attempts to act) so that each experimental group (or block) is assigned its fair share of the members of each of these subgroups.

We should now recall that randomization is aimed at spreading out the effect of undetectable or unsuspected characteristics in a population of animals or some portion of this population. The merging of the two concepts or randomization and blocking leads to the first basic experimental design, the randomized block. This type of design requires that each treatment group have at least one member of each recognized group (such as age), with the exact members of each block being assigned in an unbiased (or random) fashion.

The second type of experimental design assumes that we can characterize treatments (whether intended or otherwise) as belonging clearly to separate sets. In the simplest case, these categories are arranged into two sets that may be thought of as rows (for, say, source litter of test animal, with the first litter as row 1, the next as row 2, etc.), and the secondary set of categories may be thought of as columns (for, say, our ages of test animals, with 6–8 weeks as column 1, 8–10 weeks as column 2, and so on). Experimental units are then assigned so that each major treatment (control, low dose, intermediate dose, etc.) appears once and only once in each row and each column. If we denote our test groups as A (control), B (low), C (intermediate), and D (high), such an assignment would appear as below:

		Age			
Source Litter		6–8 Weeks	8–10 Weeks	10–12 Weeks	12–14 Weeks
1	:	A	B	C	D
2	:	B	C	D	A
3	:	C	D	A	B
4	:	D	A	B	C

The third type of experimental design is the factorial design in which there are two or more clearly understood treatments, such as exposure level to test chemical, animal age, or temperature. The classical approach to this situation (and to that described under the latin square) is to hold all but one of the treatments constant and at any one time to vary just that one factor. Instead, in the factorial design, all levels of a given factor are combined with all levels of every other factor in the experiment. When a change in one factor produces a different change in the response variable at one level of a factor than at other levels of this factor, there is an interaction between these two factors that can then be analyzed as an interaction effect.

The last of the major varieties of experimental design are the nested designs, where the levels of one factor are nested within (or are subsamples of) another factor. That is, each subfactor is evaluated only

within the limits of its single larger factor.

Censoring

A second concept and its understanding are essential to the design of experiments in toxicology, that of censoring.

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Censoring is the exclusion of measurements from certain experimental units, or indeed of the experimental units themselves, from consideration in data analysis or inclusion in the experiment at all. Censoring may occur either prior to initiation of an experiment (where, in modern toxicology, this is almost always a planned procedure), during the course of an experiment (when they are almost universally unplanned, resulting from events such as the death of animals being tested), or after the conclusion of an experiment (when data usually are excluded because of being identified as some form of outlier).

In practice, a priori censoring in toxicology studies occurs in the assignment of experimental units (such as animals) to test groups. The most familiar example is in the practice of assignment of test animals to acute, subchronic, and chronic studies, where the results of otherwise random assignments are evaluated for body weights of the assigned members. If the mean weights are found not to be comparable by some preestablished criterion (such as a 90% probability of difference by analysis of variance), then members are reassigned (censored) to achieve comparability in terms of starting body weights. Such a procedure of animal assignment to groups is known as a *censored randomization*.

Sample Size

The first precise or calculable aspect of experimental design encountered is determining sufficient test and control group sizes to allow one to have an adequate level of confidence in the results of a study (i.e., in the ability of the study design with the statistical tests used to detect a true difference—or effect —when it is present). The statistical test contributes a level of power to such a detection. Remember that the power of a statistical test is the probability that a test results in rejection of a hypothesis, say $H0$ (the null hypothesis) when some other hypothesis, say H, is valid. This is termed the power of the test "with respect to the (alternative) hypothesis H."

If there is a set of possible alternative hypotheses, the power, regarded as a function of H, is termed the *power function* of the test. When the alternatives are indexed by a single parameter θ, simple graphical presentation is possible. If the parameter is a vector θ, one can visualize a *power surface*.

If the power function is denoted by $\beta(\theta)$ and $H0$ specifies $\theta=\theta0$, then the value of β (II)—the probability of rejecting $H0$ when it is in fact valid—is the significance level. A test's power is greatest when the probability of a type II error is the least. Specified powers can be calculated for tests in any specific or general situation.

Some general rules to keep in mind are

• The more stringent the significance level, the greater the necessary sample size. More subjects are needed for a 1% level test than for a 5% level test.

• Two-tailed tests require larger sample sizes than one-tailed tests. Assessing two directions at the same time requires a greater investment.

• The smaller the critical effect being measured, the larger the necessary sample size. Subtle effects require greater efforts.

• Any difference can be significant if the sample size is large enough.

• The larger the power required, the larger the necessary sample size. Greater protection from failure requires greater effort. The smaller the sample size, the smaller the power (i.e., the greater the chance of failure).

• The requirements and means of calculating necessary sample size depends on the desired (or practical) comparative sizes of test and control groups.

The necessary sample size (N) can be calculated, for example, for equalised test and control groups using the formula

$$N = \frac{(t_1 + t_2)^2}{d^2} S,$$

where t1 is the one-tailed t value with N-l degrees of freedom corresponding to the desired level of confidence, t2 is the one-tailed t value with N-l degrees of freedom corresponding to the probability that the sample size will be adequate to achieve the desired precision, d is the population standard deviation and S is the sample standard deviation, derived typically from historical data and calculated as

$$S = \sqrt{\frac{1}{N-1}\sum(V_1 - V_2)^2}.$$

Determination of the necessary sample size is demonstrated in Example 1.

Example 1

In a subchronic dermal study in rabbits, the principal point of concern is the extent to which the

compound causes oxidative damage to erythrocytes. To quantitate this, the laboratory will be measuring the numbers of reticulocytes in the blood. What then would be an adequate sample size to allow the question at hand to be addressed with reasonable certitude?
To do this, we use the one-tailed t value for an infinite number of degrees of freedom at the 95% confidence level (i.e., $p \leq 0.05$). Going to a set of t tables, we find this

number to be 1.645. From prior experience, we know that the usual values for reticulocytes in rabbit blood are from 0.5 to 1.9×106/ml. The acceptable range of variation, 0, is therefore equal to the span of this range, or 1.4. Likewise, examining the control data from previous rabbit studies, we find our sample standard deviation to be 0.825. When we insert all of these numbers into the equation (presented above) for sample size, we can calculate the required sample size (N) to be

$$= \frac{(1.645 + 1.645)^2}{(1.4)^2}$$

$$= \frac{10.824}{1.96}(0.825)$$

$$= 4.556.$$

In other words, in this case where there is little natural variability, measuring the reticulocyte counts of groups of only five animals each should be sufficient. There are many formulas for calculating sample sizes. Readers are referred to Gad (62) for a more detailed presentation.

Toxicology Experimental Design

There are a number of aspects of experimental design that are specific to the practice of toxicology. Before we look at a suggestion for step-by-step development of experimental designs, these aspects should first be considered as follows:

1. Frequently, the data gathered from specific measurements of animal characteristics are such that there is wide variability in the data. Often, such wide variability is not present in a control or low-dose group, but in an intermediate dosage group, variance inflation may occur (i.e., there may be a large SD associated with the measurements from this intermediate group). In the face of such a set of data, the conclusion that there is no biological effect based on a finding of no statistically significant effect might well be erroneous.
2. In designing experiments, a toxicologist should keep in mind the potential effect of involuntary censoring on sample size. In other words, though the study described in Example 1 might start with five rabbits per group, this provides no margin should any die before the study is ended and blood samples are collected and analyzed. Including just enough experimental units per group frequently leaves too few at the end of a study to allow meaningful statistical analysis, and allowances should be made accordingly in establishing group sizes.
3. It is certainly possible to pool the data from several identical toxicological studies. For example, after first having performed an acute inhalation study where only three treatment group animals survived to the point at which a critical measure (such as analysis of blood samples) was performed, we would not have enough data to perform a meaningful statistical analysis. We could then repeat the protocol with new control and treatment group animals from the same source. At the end, after assuring ourselves that the two sets of data are comparable, we could combine (or pool) the data from survivors of the second study with those from the first. The costs of this approach, however, would then be both a greater degree of effort expended (than if we had performed a single study with larger groups) and increased variability in the pooled samples (decreasing the power of our statistical methods).
4. Another frequently overlooked design option in toxicology is the use of an unbalanced design (i.e., of different group sizes for differential levels of treatment). There is no requirement that each group in a study (control, low dose, intermediate dose, high dose) have an equal number of experimental units assigned to it. Indeed, there frequently are good reasons to assign more experimental units to one group than to others, and, as we shall see later in this chapter, all of the major statistical methodologies have provisions to adjust for such inequalities, within certain limits. The two most common uses of the unbalanced design have larger groups assigned to either the highest dose to compensate for losses due to possible deaths during the study, or to the lowest dose to give more sensitivity in detecting effects at levels close to an effect threshold (or more confidence to the assertion that no effect exists).
5. We frequently are confronted with the situation where an undesired variable is influencing our experimental results in a nonrandom fashion. Such a variable is called a confounding variable. Its presence, as discussed earlier, makes the clear attribution and analysis of effects at best difficult and at worst impossible. Sometimes such confounding variables are the result of conscious design or management decisions, such as the use of different instruments, personnel, facilities, or procedures for different test groups within the same study. Occasionally, however, such confounding variables are the

result of unintentional factors or actions, called a *lurking variable*. Examples of such variables almost always are the result of standard operating procedures being violated—water not being connected to a rack of animals over a

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weekend, a set of racks not being cleaned as frequently as others, or a contaminated batch of feed being used.

6. Finally, some thought must be given to the clear definition of what is meant by experimental unit and concurrent control. The experimental unit in toxicology encompasses a wide variety of possibilities. It may be cells, plates of microorganisms, individual animals, litters of animals, etc. The importance of clearly defining the experiment unit is that the number of such units per group is the "N," which is used in statistical calculations or analyses and critically affects such calculations.

The experimental unit is the unit that receives treatments and yields a response that is measured and becomes a datum. What this means in practice is that, for example, in reproduction or teratology studies, where we treat the parental generation females and then determine results by counting or evaluating offspring, the experimental unit is still the parent. Therefore, the number of litters, not the number of offspring, is the N (162).

A true concurrent control is one that is identical in every manner with the treatment groups except for the treatment being evaluated. This means that all manipulations, including gavaging with equivalent volumes of vehicle or exposing to equivalent rates of air exchanges in an inhalation chamber, should be duplicated in control groups just as they occur in treatment groups.

The goal of the four principles of experimental design is statistical efficiency and the economizing of resources. The single most important initial step in achieving such an outcome is to clearly define the objective of the study—get a clear statement of what questions are being asked. For the reader who would like to further explore experimental design, there are a number of more detailed texts available that include more extensive treatments of the statistical aspects of experimental design (28, 39, 54, 85, 99, 108).

GENERALIZED METHODOLOGY SELECTION

One approach for the selection of appropriate techniques to employ in a particular situation is to use a decision-tree method. Figure 7.1 is a decision tree that leads to the choice of one of three other trees to assist in technique selection, with each of the subsequent trees addressing one of the three functions of statistics that was defined earlier in this chapter. Figure 7.2 is for the selection of hypothesis-testing procedures, Figure 7.3 for modeling procedures, and Figure 7.4 for reduction of dimensionality procedures. For the vast majority of situations, these trees will guide the user into the choice of the proper technique. The tests and terms in these trees will be explained subsequently.

Computational Devices

The range, scope, and availability of aids for the calculation of mathematical techniques in general and for statistical techniques in particular have increased at an almost geometric rate since the mid-1970s. There is no longer any reason to use paper and pencil to perform such calculations; the capabilities of electronic systems are sufficiently developed at each level (as discussed later), and the cost, compared to labor savings, are minimal.

There are now three tiers of computational support available for statistical analysis (though it may be argued that the middle two tiers are becoming indistinguishable), and this chapter will attempt an overview of the major systems available within these tiers and the general

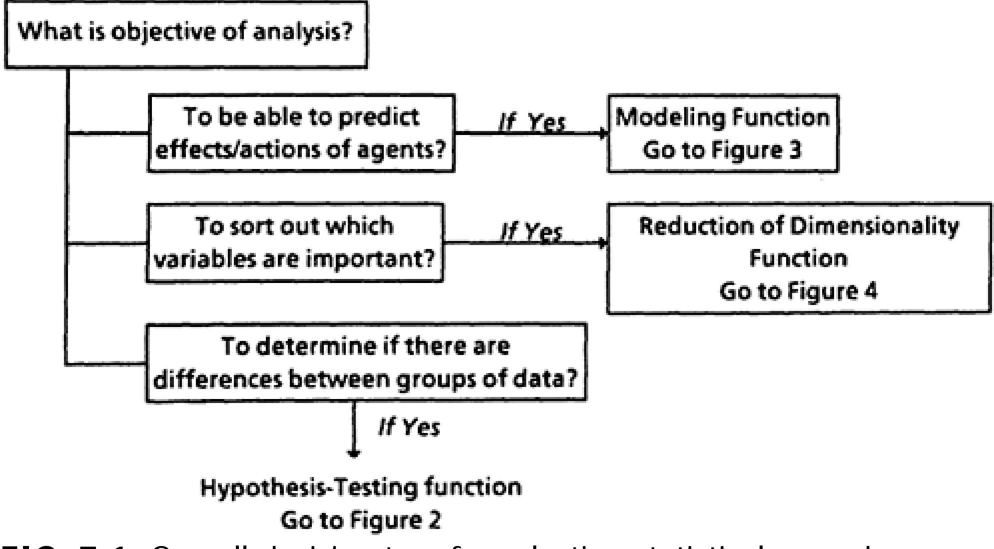

FIG. 7.1. Overall decision tree for selecting statistical procedures.

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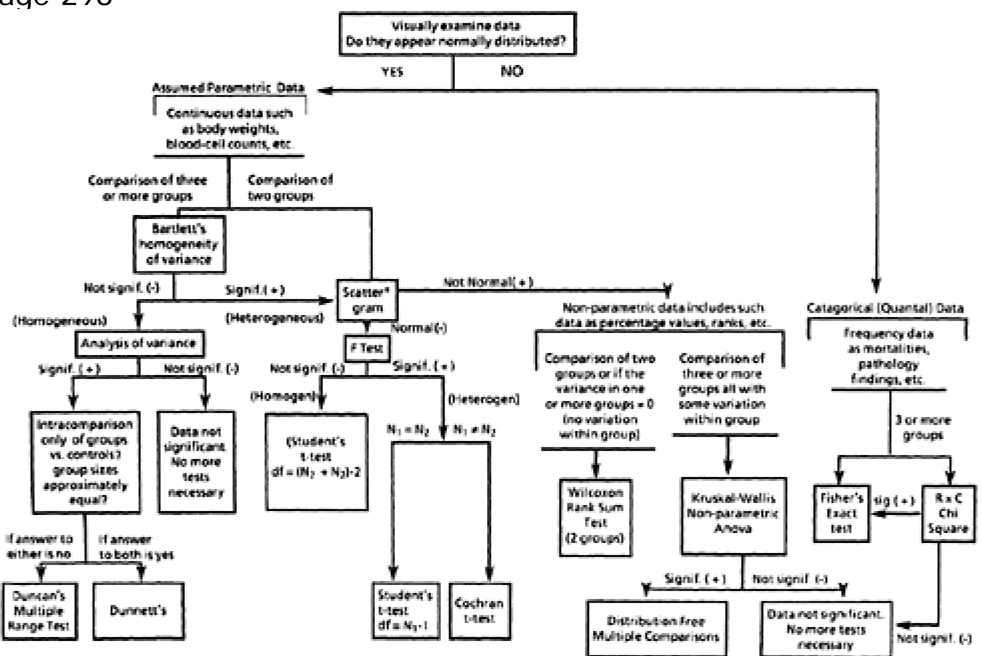

*If plot does not clearly demonstrate lack of normality exact tests may be employed.
– If continuous data, Kalmogorov–Smirnov test.
– If disconyinuous data, Chi-Square Goodness of Fit test may be used.

FIG. 7.2. Decision tree for selecting hypothesis-testing procedures.

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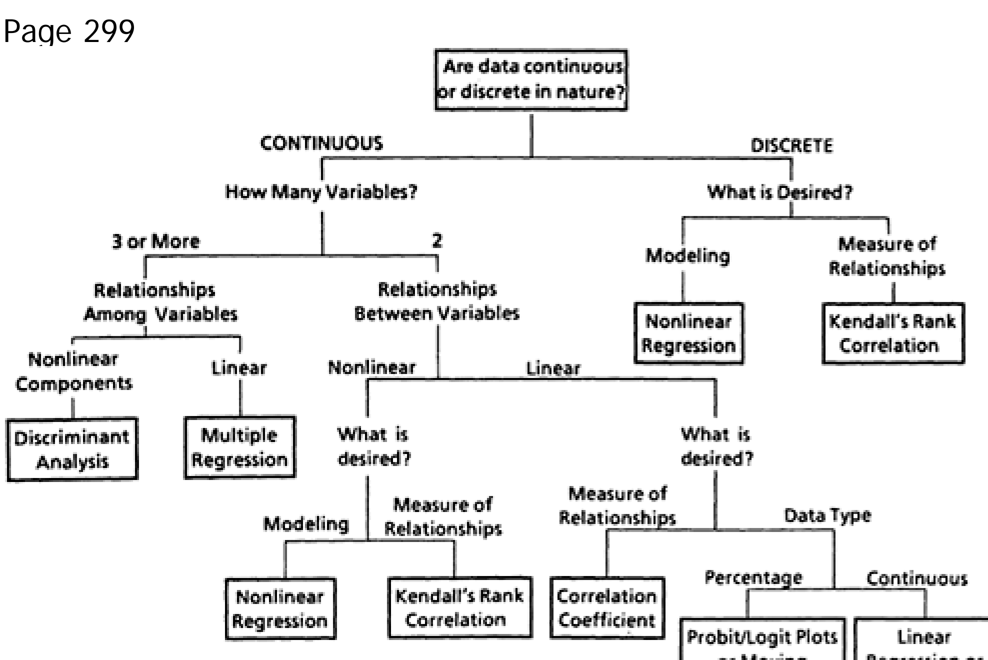

FIG. 7.3. Decision tree for selecting modeling procedures.

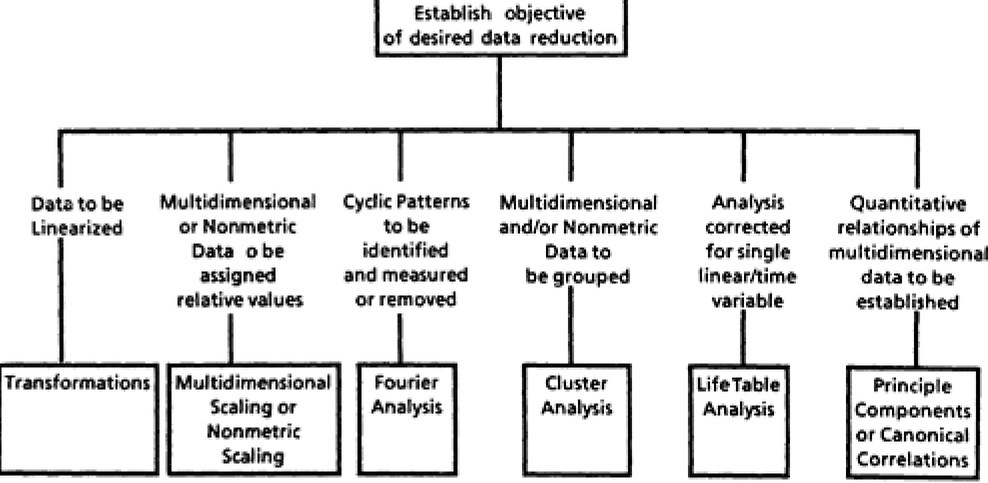

FIG. 7.4. Decision tree for selection of reduction of dimensionality procedures.

characteristics and limitations of each. The three tiers range from programmable calculators (which represent Tier I and include such devices as the Texas Instruments TI-83 and the Hewlett-Packard HP-41) to complete statistical packages available on mainframe computers (the Tier III systems, which include such packages as Statistical Analysis System [SAS], Statistical Package for Social Sciences [SPSS], and Minitab). As a general rule, as one goes from the systems in Tier 1 to those in Tier II, the cost, power, and capabilities of the systems increase, whereas ease of use ("user friendliness") and flexibility decrease.

There are conventions associated with each system or instrument in these tiers, and these conventions should be known because they can affect both results and the ways in which results are reported. The first two conventions, which apply to all three tiers, have to do with the length of numbers. The first is that there is a specific number of digits that a machine (or software system) will accept as input, handle in calculations, and report as a result (either on a screen or as a printout). The most common such limit is 13 digits (i.e., a number such as 12325.67890123). If, in the course of performing calculations, a longer string of digits is produced (e.g., by dividing our example number of four, which should produce the actual number 3086.4197253075), a 14-digit number, a 13-digit system would then handle the last (‘extra") digit in one of two ways: it would either truncate it (i.e., just drop off the last digit) or it would round it by some rule (rounding was discussed earlier). Truncation, particularly on the result of a long series of calculations (as is often the case in statistics) is more likely to produce erroneous results. Rounding is much less likely to produce errors, but knowledge of the rounding method used can be helpful.

The second convention that applies to all systems is that there is also a limit to how long a series of digits any system will report out. There are different ways in which systems report out, and there are different ways in which systems report longer digit series. The first, less common, is truncation; the second is rounding combined with presentation of the results as an exponential. Exponentials are a series of digits followed by an expression of the appropriate powers of 10. Examples of such exponential expressions are

(a) 1.234567×1016

(b) 1.234567×10−16

(c) 1.234567 E 16

(d) 1.234567 E−16

(a) and (c) are the same number expressed two different ways, as are (b) and (d).

Tier I Systems (Programmable and Preprogrammed Statistical Calculators)

The two major Tier I systems (described more fully below) can readily perform all of the univariate (i.e., two variable) procedures described in this book. Data sets may also be stored, and by the clever coding of programs and data sets of extreme length may be processed, but longer programs or longer data sets run extremely slowly. Accordingly, use of these instruments is limited, in practice, to univariate procedures and small data sets.

The two Tier I systems are the Texas Instruments TI-83 and the Hewlett-Packard HP-41. These differ mainly in that the HP programs and data entry systems are in reverse Polish notation (RPN) form, whereas the TI has a straightforward entry form. There are printers available for both units to provide hard copy. Both have unique but simple programming languages.

Tier II Systems (Microcomputers and Software)

At levels above Tier I there are additional conventions as to the way systems operate and handle data. Additionally, there are a larger number of options for both machines and software systems because only a few common programming languages are involved.

The first common convention that must be understood and considered is that the systems actually perform a statistical operation in one of two modes: batch or interactive. In batch mode, the entire desired sequence of analysis is specified, and then the entire set of data is processed in accordance with this specified sequence. The drawback to this approach is that if a result early in the sequence indicates that an alternative latter set of procedures should be performed, there is no opportunity to change to these alternative (as opposed to the originally specified) procedures. In this interactive mode, procedures are specified (and results calculated) one step at a time, usually by the use of a series of menus.

The second convention concerns the structure of commonly used software systems. They are almost always divided into three separate parts or modules:

• A database manager, which allows the person using the system to store data in (and recall it from) a desired format, to perform a transformation(s) or arithmetic manipulation(s), and to transfer the data elsewhere.

• The actual analysis of the data module. Such modules also allow one to tailor reports to one’s specifications.

• A graphics module, which presents (on a display screen) and prints out in any of a wide range of graphics and charts. The range may be limited to line, bar, and pie charts or may extend to contour, cluster, and more exotic plots.

There are now two major microcomputer operating systems that support extensive statistical packages:

the Macintosh and IBM/clone (usually some form of Windows operating system). Each of these have virtually unlimited operating and storage memories, and printers and plotters are available. "Zip" drives, which significantly expand working memory, are also available for both.

Before reviewing software packages, several considerations should be presented. First, systems perform in one of two modes, either as libraries of programs, each of which can be selected to perform an individual procedure, or as an integrated system, where a single loading of the file allows access to each and every procedure. In the first mode, each step in an analysis (such as Bartlett's test, analysis of variance, and Duncan's mul

tiple range test) requires loading a separate file and then executing the procedure. Second, software may load from CD-ROMs or Zip drive media. Third, the available range of transformations should be carefully considered. At least a small number (log, reciprocal, probit, addition, subtraction, multiplication, division, and absolute value) is essential. If unusual data sets are to be handled or exploratory data analysis (discussed later) performed regularly, a more extensive set is required. Fourth, all of the packages listed in Table 7.4 have at least a basic set of capabilities. They can each perform, besides database management and basic transformation and graphic functions, the following simple tests:

- Analysis of Variance (ANOVA)
- 2×2 Chi Square
- Linear Regressions
- Student's t-Test

Table 7.4 presents an overview of 12 widely available commercially statistical packages for microcomputers that the author is familiar with; there are at least 120 additional packages available. Woodward et al. (168) presents an overview of many of these. For each package, the following information is presented:

TITLE—the name of the package. OPERATING SYSTEMS—which systems the package will operate on
EDA—does the system perform exploratory data analysis?
GRAPHICS—how extensive are the graphic functions that the package performs?
SPREADSHEET IMPORT/EXPORT—can the system accept data-give output to popular spreadsheets such as EXCEL and LOTUS?
MENU (M) OR COMMAND (C) DRIVEN—how does the user primarily interface with the system?

Tier III Systems (Main Frame Programs)

The Tier III system programs are all large commercial software packages that run on large computer systems on a time-sharing basis. By definition, this means that these programs operate in a batch mode and use a unique (for each package) code language. Four of these libraries are briefly described below.

PACKAGE	REFERENCE	DESCRIPTION
SPSS	113	With manipulation, SPSS will perform all the procedures described in this book and the full range of graphics.
BMD	41	Has generally wider capabilities than SPSS (which are constantly being added to) and easily manipulated.
SAS	127	Widely available. Easier to format and very strong on data summarization. Has its own higherlevel programming language.
MINITAB	125	Easiest to use and least expensive of these six, but does not have the full range of capabilities.

The difficulty with the recently achieved, wide availability of automated analysis systems is that it has become increasingly easy to perform the wrong tests on the wrong data and from there to proceed to the wrong conclusions. This serves to make at least a basic understanding of the procedures and discipline of statistics a vital necessity for the research toxicologist.

Methods for Data Examination and Preparation

The data from toxicology studies should always be examined before any formal analysis. Such examinations should be directed to determining if the data are suitable for analysis, and if so, what form the analysis should take (see Figure 7.2). If the data as collected are not suitable for analysis or if they are only suitable for low-powered analytical techniques, one may wish to use one of many forms of data transformation to change the data characteristics so that they are more amenable to analysis.

The above two objectives, data examination and preparation, are the primary focus of this chapter. For data examination, two major techniques are presented—the scattergram and Bartlett's test. Likewise, for data preparation (with the issues of rounding and outliers having been addressed in a previous chapter), two techniques are presented—randomization (including a test for randomness in a sample of data) and transformation. Exploratory data analysis (EDA) is presented and briefly reviewed later. This is a broad collection of techniques and approaches to "probe" data (i.e., to both examine and to perform some initial, flexible analysis of the data).

Scattergram

Two of the major points to be made throughout this chapter are (a) the use of the appropriate statistical tests and (b) the effects of small sample sizes (as is often the case in toxicology) on our selection of statistical techniques. Frequently, simple examination of the nature and distribution of data collected from a study can also suggest patterns and results that were unanticipated

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Table 7.4 Popular microcomputer statistical packages

Title	Operating Systems	EDA Performed	Graphics	Spreadsheet Import/Export	Menu(M) or Command(C) Driven
BMD8	Windows	No	Largely	Limited	C
DATA DESK	Mac Windows	Yes	Complete	Full	M
E CHIP	Windows	No	Largely	Limited	M
JMP	Windows Mac	Yes	Largely	Largely	M
NCSS	Windows	Yes	Complete	Full	M
SAS/STAT	MSDOS Windows	Yes	Complete	Full	C
SPSS/PC	MSDOS Windows	No	Complete	Full	C
STAT/MOST	Windows	Yes	Complete	Full	M
STATISTICA	Windows Mac	Yes	Complete	Full	M
STATVIEW	Mac Windows	Yes	Complete	Full	M
STATXALT	Windows	Yes	Complete	Full	M
SYSTAT	Windows	No	Complete	Full	C

and for which the use of additional or alternative statistical methodology is warranted. It was these three points that caused the author to consider a section on scattergrams and their use essential for toxicologists.

Bartlett's test may be used to determine if the values in groups of data are homogeneous. If they are, this (along with the knowledge that they are from a continuous distribution) demonstrates that parametric methods are applicable, but, if the values in the (continuous data) groups fail Bartlett's tests (i.e., are heterogeneous), we cannot be secure in our belief that parametric methods are appropriate until we gain some confidence that the values are normally distributed. With large groups of data, we can compute parameters of the population (kurtosis and skewness, in particular), and from these parameters determine if the population is normal (with a certain level of confidence). If our concern is especially marked, we can use a chi-square goodness-of-fit test for normality, but when each group of data consists of 25 or fewer values, these measures or tests (kurtosis, skewness, chi-square goodness-of-fit) are not accurate indicators of normality. Instead, in these cases we should prepare a scattergram of the data and then evaluate the scattergram to estimate if the data are normally distributed. This procedure consists of developing a histogram of the data and then examining the histogram to get a visual appreciation of the location and distribution of the data.

The abscissa (or horizontal scale) should be in the same scale as the values and should be divided so that the entire range of observed values is covered by the scale of the abscissa. Across such a scale we then simply enter symbols of each of our values. Example 2 shows such a plot.

Example 2

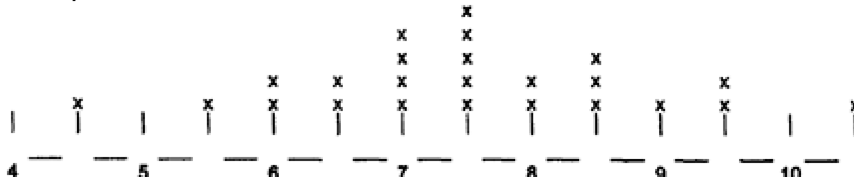
Suppose we have the two data sets below:

Group 1:4.5, 5.4, 5.9, 6.0, 6.4, 6.5, 6.9, 7.0, 7.1, 7.0, 7.4, 7.5, 7.5, 7.5, 7.6., 8.0, 8.1, 8.4, 8.5, 8.6, 9.0, 9.4, 9.5, and 10.4.

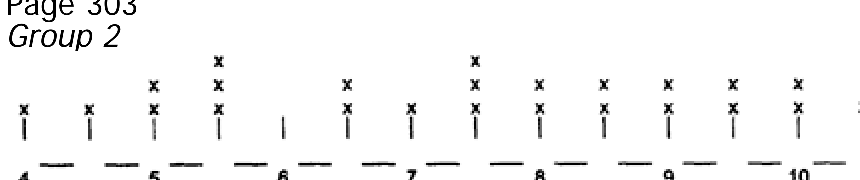
Group 2:4.0,4.5, 5.0, 5.1, 5.4, 5.5, 5.6, 6.5, 6.5, 7.0, 7.4, 7.5, 7.5, 8.0, 8.1, 8.5, 8.5, 9.0, 9.1, 9.5, 9.5, 10.1, 10.0, and 10.4.

Both of these groups contain 24 values and cover the same range. From them we can prepare the following scattergrams.

Group 1



Group 2



Group 1 can be seen to approximate a normal distribution (bell-shaped curve); we can proceed to perform the appropriate parametric tests with such data, but group 2 clearly does not appear to be normally distributed. In this case, the appropriate nonparametric technique must be used.

Example 2 is a traditional and rather limited form of scatterplot, but such plots can reveal significant information about the amount and types of association between the two variables, the existence and nature of outliers, the clustering of data, and a number of other two-dimensional factors (7, 23). Current technology allows us to add significantly more graphical information to scatterplots by means of graphic symbols (letters, faces, or different shapes, such as squares, colors, etc.) for the plotted data points. One relatively simple example of this approach is shown in Figure 7.5, where the simple case of dose (in a dermal study), dermal irritation, and white blood cell count are presented. This graph quite clearly suggests that as dose (variable x) is increased, dermal irritation (variable y) also increases, and as irritation becomes more severe, white blood cell count (variable z), an indicator of immune system involvement suggesting infection or persistent inflammation, also increases. There is no direct association of variables x and z, however.

Cleveland and McGill (25) presented an excellent, detailed overview of the expanded capabilities of the scatterplot, and the interested reader should refer to that article. Cleveland expanded this to a book (26). Tufte (145) has also expanded on this.

Bartlett's Test for Homogeneity of Variance

Bartlett's test (138) is used to compare the variances (values reflecting the degree of variability in data sets) among three or more groups of data, where the data in the groups are continuous sets (such as body weights, organ weights, red blood cells counts, or diet consumption measurements). It is expected that such data will be suitable for parametric methods (normality of data is assumed), and Bartlett's is frequently used as a test for the assumption of equivalent variances.

Bartlett's is based on the calculation of the corrected $\chi 2$ (chi-square) value by the formula

$$\chi^2_{corr} = 2.3026 \frac{\sum df\left(\log_{10}\left[\frac{\sum[df(S^2)]}{\sum df}\right]\right) - \sum[df(\log_{10} S^2)]}{1 + \frac{1}{3(K-1)}\left[\sum \frac{1}{df} - \frac{1}{\sum df}\right]},$$

$$\text{where } S^2 = \text{variance} = \frac{n\sum x^2 - \frac{(\sum x)^2}{n}}{n-1},$$

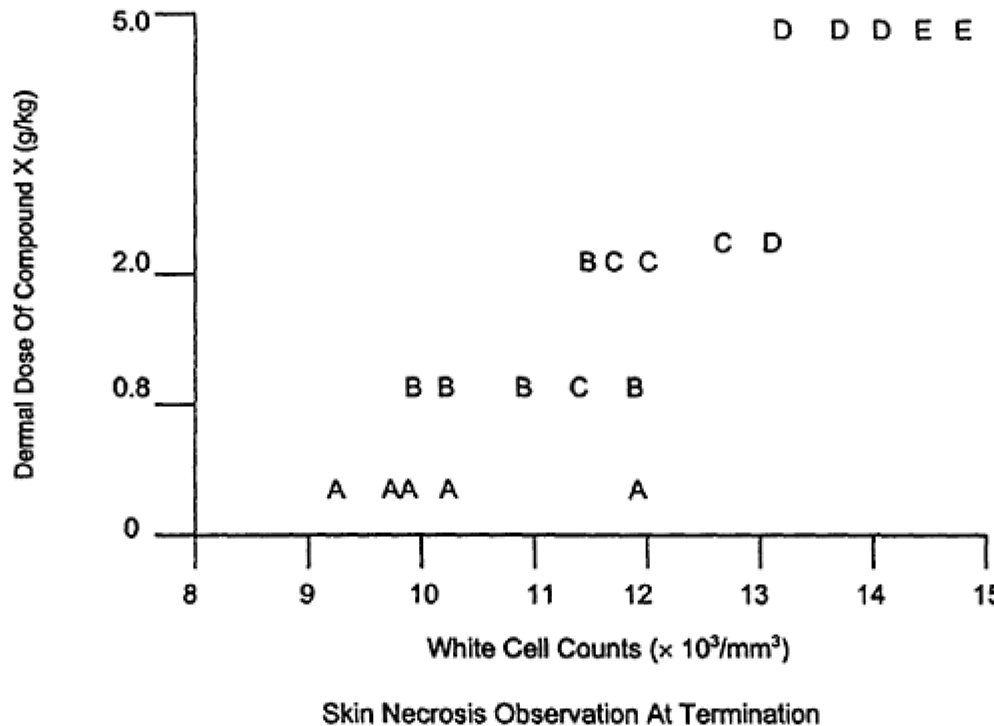


FIG. 7.5. Exploratory data analysis.

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X=individual datum within each group;
n=number of data within each group;
K=number of groups being compared;
d*f*=degrees of freedom for each group=(N−1).
The corrected $\chi 2$ value yielded by the above calculations is compared to the values listed in the chi square table according to the numbers of degrees of freedom (such as found in ref. 137, pp. 470–471). If the calculated value is smaller than the table value at the selected p level (traditionally 0.05), the groups are accepted to be homogeneous and the use of ANOVA is assumed proper. If the calculated $\chi 2$ is greater than the table value, the groups are heterogeneous and other tests (as indicated in Figure 7.2, the decision tree) are necessary. This is demonstrated in Example 3.

Example 3

If the monocytes in a sample of rat blood taken in the course of an inhalation study were counted, the results might appear as follows:

400 ppm		200 ppm		0 ppm	
(X1)	(X1)2	(X2)	(X2)2	(X3)	(X3)2
9	81	5	25	7	49
5	25	5	25	6	36
5	25	4	16	5	25
4	16	6	36	7	49
		7	49		
$\Sigma X_1 = 23$	$\Sigma X_1^2 = 147$	$\Sigma X_2 = 27$	$\Sigma X_2^2 = 151$	$\Sigma X_3 = 25$	$\Sigma X_3^2 = 159$

$${}^{2}_{1} = \frac{\frac{4(147) - (23)^2}{4}}{4-1} = 4.9167,$$

$${}^{2}_{2} = \frac{\frac{5(157) - (27)^2}{5}}{5-1} = 1.3000,$$

$${}^{2}_{3} = \frac{\frac{4(159) - (25)^2}{4}}{4-1} = 0.9167.$$

In continuing the calculations, it is helpful to set up a table as follows:

Concentration	n	d*f*=(N−l)	S2	(d*f*)(S2)	log S2
400 ppm	4	3	4.9167	14.7501	0.6917
200 ppm	5	4	1.3000	5.2000	0.1139
0 ppm	4	3	0.9167	2.7501	−0.0378
Sums (Σ)	13	10		22.7002	

Concentration	(d*f*)(log S2)	$\frac{1}{df}$
400 ppm	2.0751	0.3333
200 ppm	0.4556	0.2500
0 ppm	−0.1134	0.3333
Sums (Σ)	2.4173	0.9166

Now we substitute into our original formula for corrected $\chi 2$

$$\chi^2 = 2.3026\frac{10\left[\log_{10}\left(\frac{22.7002}{10}\right)\right] - 2.4173}{\frac{1}{3(3-1)}\left(0.9166 - \frac{1}{10}\right)}$$

$$= 2.3026\frac{10(0.3560) - 2.4173}{1 + 0.1667(0.8166)}$$

$$= 2.32.$$

The table value for two degrees of freedom at the 0.05 level is 5.99. As our calculated value is less than this, the corrected $\chi 2$ is not significant and the variances are accepted as homogeneous. We may thus use parametric methods (such as ANOVA) for further comparisons.

ASSUMPTIONS AND LIMITATIONS

1. Bartlett's test does not test for normality, but rather homogeneity of variance (also called equality of variances or homoscedasticity).
2. Homoscedasticity is an important assumption for Student's t-test, analysis of variance, and analysis of covariance.
3. The F-test (covered in the next chapter) is actually a test for the two-sample (i.e., control and one test group) case of homoscedasticity. Bartlett's is designed for three or more samples.
4. Bartlett's is very sensitive to departures from normality. As a result, a finding of a significant chi-square value in Bartlett's may indicate non-normality rather than heteroscedasticity. Such a finding can be brought about by outliers, and the sensitivity to such erroneous findings is extreme with small sample sizes.

Statistical Goodness-of-Fit Tests

A goodness-of-fit test is a statistical procedure for comparing individual measurements to a specified type of statistical distribution. For example, a normal distribution is completely specified by its arithmetic mean and variance (the square of the SD). The null hypothesis, that the data represent a sample from a single normal distribution, can be tested by a statistical goodness-of-fit test. Various goodness-of-fit tests have been devised to determine if the data deviate significantly from a specified distribution. If a significant departure occurs, it indicates only that the specified distribution can be rejected with some assurance. This does not necessarily mean that the true distribution contains two or more subpopulations. The true distribution may be a single distribution

based on a different mathematical relationship (e.g., log-normal). In the latter case, logarithms of the measurement would not be expected to exhibit by a goodness-of-fit test a statistically significant departure from a log-normal distribution.

Everitt and Hand (51) recommended using a sample of 200 or more to conduct a valid analysis of mixtures of populations. Even the maximum likelihood method, the best available method, should be used with extreme caution, or not at all, when separation between the means of the subpopulations is less than 3 SD and sample sizes are less than 300. None of the available methods conclusively establish bimodality, which may occur when separation between the two means (modes) exceeds 2 SD. Conversely, inflections in probits or separations in histograms *less than* 2 SD apart may arise from genetic differences in test subjects.

Mendal et al. (106) compared eight tests of normality to detect a mixture consisting of two normally distributed components with different means but equal variances. Fisher's skewness statistic was preferable when one component comprised less than 15% of the total distribution. When the two components comprised more nearly equal proportions (35%-65%) of the total distribution, the Engelman and Hartigan test (50) was preferable. For other mixing proportions, the maximum likelihood ratio test was best. Thus, the maximum likelihood ratio test appears to perform very well, with only small loss from optimality, even when it is not the best procedure.

The method of *maximum likelihood* provides estimators that usually are quite satisfactory. They have the desirable properties of being consistent, asymptotically normal, and asymptotically efficient for large samples under quite general conditions. Often they are biased, but the bias is frequently removable by a simple adjustment (Examples 4 and 5). Other methods of obtaining estimators are also available, but the maximum likelihood method is the most frequently used.

Maximum likelihood estimators also have another desirable property: *invariance*. Let us denote the maximum likelihood estimator of the parameter θ by $\hat{\sigma}$ Then, if *f(θ)* is a single-valued function of *θ,* the maximum likelihood estimator of *f*(*θ*) is $f(\hat{\sigma})$. Thus, for example, $\hat{\sigma} = (\hat{\sigma}^2)^{1/2}$.

The principle of maximum likelihood tells us that we should use as our estimate that value which maximizes the likelihood of the observed event. The following examples demonstrate the technique.

Example 4

Derive the maximum likelihood estimator *p* of the binomial probability *p* for a coin-tossing experiment in which a coin is tossed *n* times and *r* heads are obtained.

We know that the likelihood of the observed event is

$$L = \binom{n}{r} p^r (1-p)^{n-r}.$$

According to the principle of maximum likelihood, we choose the value of *p* which maximizes *L*. This value also maximizes

$$\ln L = \ln\binom{n}{r} + r \ln p + (n-r)\ln(1-p).$$

At the maximum, the derivative with respect to *p* must be zero; that is,

$$\frac{\partial}{\partial \rho} \ln L = r/p - (n-r)/\ln(1-p) = 0$$

and

$$\hat{p} = r/n.$$

Example 5

A sample of *n* observations x1,..., xn is drawn from a normal population. Derive the maximum likelihood estimators $\hat{\mu}$ and $\hat{\sigma}_2$ for the mean and variance of the population. The likelihood of the observed event is

$$L = \left[\frac{1}{\sigma(2\pi)\frac{1}{2}} \exp\left\{ -\frac{1}{2} \left(\frac{x_1 - \mu}{\sigma} \right)^2 \right\} \right]$$
$$\times \Lambda \left[\frac{1}{\sigma(2\pi)\frac{1}{2}} \exp\left\{ -\frac{1}{2} \left(\frac{x_n - \mu}{\sigma} \right)^2 \right\} \right].$$

According to the principle of maximum likelihood, we choose those values of μ and $\sigma2$ which maximize L. The same values maximize In L. So we equate the partial derivatives of In L with respect to $\hat{\mu}$ and $\hat{\sigma}^2$ to zero and find that

$$\hat{\mu} = \bar{x}-$$

and

$$\hat{\sigma}^2 = \frac{1}{n} \sum_{i=1}^{n} (x_i - \bar{x})^2.$$

The latter estimator is slightly biased, but the bias can be removed by multiplying by $n/(n-1)$ and using the estimator

$$\hat{\sigma}^2 \frac{1}{n-1} \sum_{i=1}^{n} (x_i - \bar{x})^2.$$

These maximum likelihood methods can be used to obtain *point estimates* of a parameter, but we must remember that a point estimator is a random variable distributed in some way around the true value of the parameter. The true parameter value may be higher or lower than our estimate. It is often useful, therefore, to obtain an interval within which we are reasonably confident the true value will lie, and the generally accepted method is to construct what are known as *confidence limits*.

The following procedure will yield upper and lower 95% confidence limits with the property that when we say that these limits include the true value of the parameter, 95% of all such statements will be true and 5% will be incorrect:

1. Choose a (test) statistic involving the unknown parameter and no other unknown parameter.
2. Place the appropriate sample values in the statistic.
3. Obtain an equation for the unknown parameter by equating the test statistic to the upper 2.5% point of the relevant distribution.
4. The solution of the equation gives one limit.
5. Repeat the process with the lower 2.5% point to obtain the other limit.

One can also construct 95% confidence intervals using unequal tails (e.g., using the upper 2% point and the lower 3% point). We usually want our confidence interval to be as short as possible, however, and with a symmetric distribution such as the normal or *t*, this is achieved using equal tails. The same procedure very nearly minimizes the confidence interval with other nonsymmetric distributions (e.g., chi-square) and has the advantage of avoiding rather tedious computation.

When the appropriate statistic involves the square of the unknown parameter, both limits are obtained by equating the statistic to the upper 5% point of the relevant distribution. The use of two tails in this situation would result in a pair of nonintersecting intervals. When two or more parameters are involved, it is possible to construct a region within which we are reasonably confident the true parameter values will lie. Such regions are referred to as confidence regions. The implied interval for p1 does not form a 95% confidence interval, however, nor is it true than an 85.7375% confidence region for p1, p2, and p3 can be obtained by considering the intersection of the three separate 95% confidence intervals because the statistics used to obtain the individual confidence intervals are not independent. This problem is obvious with a multiparameter distribution, such as the multinomial, but it even occurs with the normal distribution because the statistic that we use to obtain a confidence interval for the mean and the statistic that we use to obtain a confidence interval for the variance are not independent. The problem is not likely to be of great concern unless a large number of parameters is involved, as illustrated in Example 6.

Example 6

A sample of nine is drawn from a normal population with unknown mean and variance. The sample mean is 4.2 and the sample variance is 1.69. Obtain a 95% confidence interval for the mean μ. The confidence limits are obtained from the equations

$$\sqrt{9}(4.2-\mu)/(1.69)^{1/2} = 2.306;$$

$$\sqrt{9}(4.2-\mu)/(1.69)^{1/2} = -2.306.$$

From these we determine that the 95% confidence interval is from 3.2 to 5.2.

Randomization

Randomization is the act of assigning a number of items (plates of bacteria or test animals, for example) to groups in such a manner that there is an equal chance for any one item to end up in any one group. This is a control against any possible bias in assignment of subjects to test groups. A variation on this is censored randomization, which ensures that the groups are equivalent in some aspect after the assignment process is complete. The most common example of a censored randomization is one in which it is ensured that the body weights of test animals in each group are not significantly different from those in the other groups. This is done by analyzing group weights both for homogeneity of variance and by analysis of variance after animal assignment, then re-randomizing if there is a significant difference at some nominal level, such as $p \leq 0.10$. The process is repeated until there is no significant difference.

There are several methods for actually performing the randomization process. The three most commonly used are card assignment, use of a random number table, and use of a computerized algorithm.

For the card-based method, individual identification numbers for items (plates or animals, for example) are placed on separate index cards. These cards are then shuffled, placed one at a time in succession into piles corresponding to the required test groups. The results are a random group assignment.

The random number table method requires only that one have unique numbers assigned to test subjects and access to a random number table. One simply sets up a table with a column for each group to which subjects are to be assigned. We start from the head of any one column of numbers in the random table (each time the table is used, a new starting point should be used). If our test subjects number less than 100, we use only the last two digits in each random number in the table. If they

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number more than 99 but less than 1000, we use only the last three digits. To generate group assignments, we read down a column one number at a time. As we come across digits that correspond to a subject number, we assign that subject to a group (enter its identifying number in a column), proceeding to assign subjects to groups from left to right filling one row at a time. After a number is assigned to an animal, any duplication of its unique number is ignored. We use as many successive columns of random numbers as we may need to complete the process.

The third (and now most common) method is to use a random number generator that is built into a calculator or computer program. Procedures for generating these are generally documented in user manuals.

One is also occasionally required to evaluate whether a series of numbers (such as an assignment of animals to test groups) is random. This requires the use of a randomization test, of which there are a large variety. The chi-square test, described later, can be used to evaluate the goodness-of-fit to a random assignment. If the result is not critical, a simple sign test will work. For the sign test, we first determine the middle value in the numbers being checked for randomness. We then go through a list of the numbers assigned to each group, scoring each as a "+" (greater than our middle number) or "–" (less than our middle number). The number of pluses and minuses in each group should be approximately equal. This is demonstrated in Example 7.

Example 7

In auditing a study performed at a contract lab, we wish to ensure that their assignment of animals to test groups was random. Thirty-three animals numbered 1 to 33 were assigned to groups of 11 animals each. Using the middle value in this series (17) as our check point, we assign signs as below.

Control		Test Group A		Test Group B	
Animal Number	Sign	Animal Number	Sign	Animal Number	Sign
17	0	18	+	11	−
14	−	1	−	2	−
7	−	12	−	22	+
26	+	9	−	28	+
21	+	5	−	19	+
15	−	20	+	3	−
16	−	33	+	29	+
6	−	27	+	10	−
25	+	8	−	23	+
32	+	24	+	30	+
4	−	31	+	13	−
Sum of signs	−2		+1		+1

Note that 17 is scored as zero, ensuring (as a check on results) that the sum of the sums of the three columns would be zero. The results in this case clearly demonstrate that there is no systematic bias in animal number assignments.

Transformations

If our initial inspection of a data set reveals it to have an unusual or undesired set of characteristics (or to lack a desired set of characteristics), we have a choice of three courses of action. We may proceed to select a method or test appropriate to this new set of conditions, or abandon the entire exercise, or transform the variable(s) under consideration in such a manner that the resulting transformed variates (X′ and Y′, for example, as opposed to the original variates X and Y) meet the assumptions or have the characteristics that are desired.

The key to all this is that scale of measurement of most (if not all) variables is arbitrary. Although we are most familiar with a linear scale of measurement, there is nothing that makes this the "correct" scale on its own, as opposed to a logarithmic scale (familiar logarithmic measurements are that of pH values, or earthquake intensity (Richter scale)). Transforming a set of data (converting X to X′) is really as simple as changing a scale of measurement.

There are at least four good reasons to transform data:

(1) To normalize the data, making them suitable for analysis by our most common parametric techniques, such as analysis of variance (ANOVA). A simple test of whether a selected transformation will yield a distribution of data which satisfies the underlying assumptions for ANOVA is to plot the cumulative distribution of samples on probability paper (i.e., a commercially available paper that has the probability function scale as one axis). One can then alter the scale of the second axis (i.e., the axis other than the one that is on a probability scale) from linear to any other (logarithmic, reciprocal, square

root, etc.) and see if a previously curved line indicating a skewed distribution becomes linear to indicate normality. The slope of the transformed line gives us an estimate of the SD, and if the slopes of the lines of several samples or groups of data are similar, we accordingly know that the variance of the different groups are homogeneous.

(2) To linearize the relationship between a paired set of data, such as dose and response. This is the most common use in toxicology for transformations and is demonstrated in the section under probit/logit plots.

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Table 7.5 Common Data Transformations

Transformation	How Calculated[a]	Example of Use
Arithmetic	$x' = \frac{x}{y}$ or $x' = x + c$	Organ weight/body weight
Reciprocals	$x' = \frac{1}{x}$	Linearizing data, particularly rate phenomena
Arcsine (also called Angular)	$x' = \text{arcsine} \sqrt{x}$	Normalizing dominant lethal and mutation rate data
Logarithmic	x′=log ×	pH values
Probability (Probit)	x′=probability X	Percentage responding
Square roots	$x' = \sqrt{x}$	Surface area of animal from body weights
Box cox	x′=(xv−1)v: for v≠0 x′=In x: for v=0	A family of transforms for use when one has no prior knowledge of the appropriate transformation to use
Rank transformations	Depends on nature of samples	As a bridge between parametric and nonparametric statistics (174)

a x and y are original variables, x′ and y′ transformed values. "C" stands for a constant.

Note: Plotting a double reciprocal (that is, $\frac{1}{x}$ versus $\frac{1}{y}$ will linearize almost any data set. So will plotting the log transforms of a set of variables.

(3) To adjust data for the influence of another variable. This is an alternative in some situations to the more complicated process of analysis of covariance. A ready example of this usage is the calculation of organ weight to body weight ratios in in vivo toxicity studies, with the resulting ratios serving as the raw data for an analysis of variance performed to identify possible target organs. This use is discussed in detail later in this chapter.

(4) Finally, to make the relationships between variables clearer by removing or adjusting for interactions with third, fourth, etc. Uncontrolled variables that influence the pair of variables of interest. This case is discussed in detail under time series analysis.

Common transformations are presented in Table 7.5.

Exploratory Data Analysis

Over the past 20 years, an entirely new approach has been developed to get the most information out of the increasingly larger and more complex data sets that scientists are faced with. This approach involves the use of a very diverse set of fairly simple techniques that comprise exploratory data analysis (EDA). As expounded by Tukey (148), there are four major ingredients to EDA:

Displays: These visually reveal the behavior of the data and suggest a framework for analysis. The scatterplot (presented earlier) is an example of this approach.

Residuals: These are what remain of a set of data after a fitted model (such as a linear regression) or some similar level of analysis has been removed.

Re-expressions: These involved questions of what scale would serve to best simplify and improve the analysis of the data. Simple transformations, such as those presented earlier in this chapter, are used to simplify data behavior (e.g., linearizing or normalizing) and clarify analysis.

Resistance: This is a matter of decreasing the sensitivity of analysis and summary of data to misbehavior so that the occurrence of a few outliers, for example, will not complicate or invalidate the methods used to analyze the data. For example, in summarizing the location of a set of data, the median (but not the arithmetic means) is highly resistant.

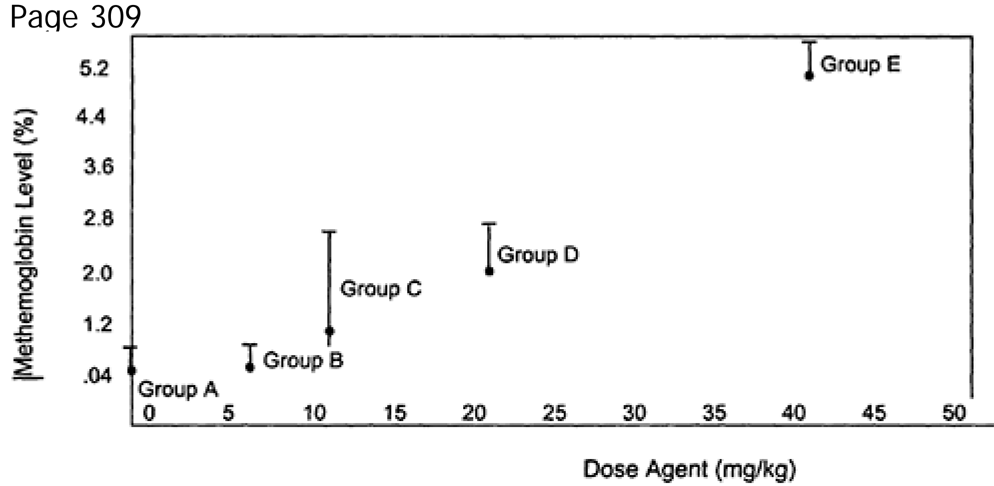

FIG. 7.6. Variance inflation.

These four ingredients are used in a process falling into two broad phases: an exploratory phase and a confirmatory phase. The exploratory phase isolates patterns in and features of the data and reveals them, allowing an inspection of the data before any firm choice of actual hypothesis testing or modeling methods has been made.

Confirmatory analysis allows evaluation of the reproducibility of the patterns or effects. Its role is close to that of classical hypothesis testing, but also often includes steps such as (a) incorporating information from an analysis of another closely related set of data, and (b) validating a result by assembling and analyzing additional data. These techniques are, in general, beyond the scope of this text; however, Velleman and Hoaglin (149) and Hoaglin et al. (86) present a clear overview of the more important methods, along with codes for their execution on a microcomputer (they have also now been incorporated into Minitab). A short examination of a single case of the use of these methods, however, is in order.

In the field of toxicology it has long been recognized that no population—animal or human—is completely uniform in its response to any particular toxicant. Rather, a population is composed of a (presumably normal) distribution of individuals, some resistant to intoxication (hyporesponders), the bulk that respond close to a central value (such as an LD50), and some that are very sensitive to intoxication (hyperresponders). Taking advantage of this population distribution can, in fact, result in additional statistical techniques. The sensitivity of techniques such as ANOVA is reduced markedly by the occurrence of outliers (extreme high or low values, including hyper- and hyporesponders), which, in fact, serve to markedly inflate the variance (standard deviation) associated with a sample. Such variance inflation is particularly common in small groups that are exposed or dosed at just over or under a threshold level, causing a small number of individuals in the sample (who are more sensitive than the other members) to respond markedly. Such a situation is displayed in Figure 7.6, which plots the mean and standard deviations of methaemoglobin levels in a series of groups of animals exposed to successively higher levels of a hemolytic agent.

Though the mean level of methaemoglobin in group C is more than double that of the control group (A), no hypothesis test will show this difference to be significant because it has such a large SD associated with it. Yet this "inflated" variance exists because a single individual has such a marked response. The occurrence of the inflation is certainly an indicator that the data need to be examined closely. Indeed, all tabular data in toxicology should be visually inspected for both trend and variance inflation.

A concept related (but not identical) to resistance and exploratory data analysis is that of robustness. Robustness generally implies insensitivity to departures from assumptions surrounding an underlying model, such as normality.

In summarizing the location of data, the median, though highly resistant, is not extremely robust, but the mean is both nonresistant and nonrobust.

HYPOTHESIS TESTING OF CATEGORICAL AND RANKED DATA

Categorical (or contingency table) presentations of data can contain any single type of data, but generally the contents are collected and arranged so that they can be classified as belonging to treatment and control groups, with the members of each of these groups then classified as belonging to one of two or more response

categories (such as tumor/no tumor or normal/ hyperplastic/neoplastic). For these cases, two forms of analysis are presented—Fisher's Exact Test (for the 2×2 contingency table) and the R×C chi-square test (for large tables). It should be noted, however, that there are versions of both of these tests that permit the analysis of any size of contingency table.

The analysis of rank data—what is generally called nonparametric statistical analysis—is an exact parallel of the more traditional (and familiar) parametric methods. There are methods for the single comparison case (just as Student's t-test is used) and for the multiple comparison case (just as ANOVA is used) with appropriate post hoc tests for exact identification of the significance with a set of groups. Four tests are presented for evaluating statistical significance in rank data—the Wilcoxon rank-sum test, distribution-free multiple comparisons, Mann-Witney U Test, and the Kruskal-Wallis nonparametric analysis of variance. For each of these tests, tables of distribution values for the evaluations of results can be found in any of a number of reference volumes (62).

It should be clearly understood that for data that do not fulfill the necessary assumptions for parametric analysis, these nonparametric methods are either as powerful, or, in fact, more powerful than the equivalent parametric test.

Fisher's Exact Test

Fisher's exact test should be used to compare two sets of discontinuous, quantal (all or none) data. Small sets of such data can be checked by contingency data tables, such as those of Finney et al. (56). Larger sets, however, require computation, including frequency data, such as incidences of mortality or certain histopathological findings, etc. Thus, the data can be expressed as ratios. These data do not fit on a continuous scale of measurement but usually involve numbers of responses classified as either negative or positive (i.e., a contingency table situation) (138).

The analysis is started by setting up a 2×2 contingency table to summarize the numbers of "positive" and "negative" responses as well as the totals of these responses as follows:

	"Positive"	"Negative"	Total
Group I	A	B	A+B
Group II	C	D	C+D
Totals	A+C	B+D	A+B+C+ D=Ntotal

Using the above set of symbols, the formula for P appears as follows:

$$P = \frac{(A+B)!(C+D)!(A+C)!(B+D)!^{*}}{N!A!B!C!D!}$$

The exact test produces a probability (P) that is the sum of the above calculation repeated for each possible arrangement of the numbers in the above cells (i.e., A, B, C, and D) showing an association equal to or stronger than that between the two variables.

The P resulting from these computations will be the exact one- or two-tailed probability, depending on which of these two approaches is being employed. This value tells us if the groups differ significantly (with a probability less than, say, 0.05) and the degree of significance. This is demonstrated in Example 8.

Example 8

The pathology reports from 35 control and 20 treated rats show that 2 control and 5 treated animals have tumors of the spleen. Setting this up as a contingency table, we see:

	Tumor-bearing	No tumors	Total
Control	2	33	35
Treated	5	15	20
Total	7	48	55

The probability for the worst case on this calculates as

$$prob_1 = \frac{(35)! \bullet (20)! \bullet (7)! \bullet (48)!}{(55)! \bullet (2)! \bullet (5)! \bullet (33)! \bullet (15)!} = 0.046.$$

Similarly, the probabilities for the secondary may be computed as prob2=0.007 and the tertiary as prob3=0.000, respectively. The exact one-tailed p-value is p=0.046+0.007+0.000=0.053. Because this is greater than 0.05, we would not reject the hypothesis of equal proportions at a significance level of 0.05 (this is close to 0.05, however, and may give the researcher encouragement to conduct a larger study).

ASSUMPTIONS AND LIMITATIONS

1. Tables are available that provide individual exact probabilities for small sample size contingency tables

(see ref. 171, pp. 518–542).
2. Fisher's exact must be used in preference to the chi-square test when there are small cell sizes.
3. The probability resulting from a two-tailed test is exactly double that of a one-tailed test from the same data.
4. Ghent (69) has developed and proposed a good (though, if performed by hand, laborious) method extending the calculation of exact probabilities to 2×3, 3×3,and R×C contingency tables.
5. Fisher's probabilities are not necessary symmetric. Although some analysts will double the one-tailed p-value to obtain the two-tailed result, this method usually is overly conservative.
* A! is A factorial. For 4!, as an example, this would be (4) (3) (2) (1)=24.

2×2 Chi Square

Though Fisher's Exact Test is preferable for analysis of most 2×2 contingency tables in toxicology, the chi-square test is still widely used and is preferable in a few unusual situations (particularly if cell sizes are large yet only limited computational support is available). The formula is simply:

$$\chi^2 = \frac{(0_1 - E_1)^2}{E_1} + \frac{(0_2 - E_2)^2}{E_2}$$

$$= \sum \frac{(0_i - E_i)^2}{E_i},$$

where 0 are observed numbers (or counts) and E are expected numbers. The common practice in toxicology is for the observed figures to be test or treatment group counts. The expected figure is calculated as

$$E = \frac{\text{(column total)(row total)}}{\text{grand total}}$$

for each box or cell in a contingency table. Example 9 illustrates this.

Example 9

In an subacute toxicity study, there were 25 animals in the control group and 25 animals in the treatment group. Seven days after dosing, control and 13 treatment animals were observed to be exhibiting fine muscle tremors; all other animals had no tremors. Do significantly more treatment animals have tremors?

	Tremors		No tremors		Σ
	Observed	(Expected)	Observed	(Expected)	
Control	5	(8.5)	20	(16.5)	25
Treated	12	(8.5)	13	(16.5)	25
Σ	17		33		50

$$\chi^2 = \frac{(5-8.5)^2}{8.5} + \frac{(12-8.5)^2}{8.5} + \frac{(20-16.5)^2}{16.5} + \frac{(13-16.5)^2}{16.5}$$

$$= \frac{12.25}{8.5} + \frac{12.25}{8.5} + \frac{12.25}{16.5} + \frac{12.25}{16.5}$$

$$= 1.441 + 1.441 + 0.762 + 0.742$$

$$= 4.366.$$

Our degrees of freedom are (R−1)(C−1)=(2−1) (2−1)=1. Looking at a chi-square table for one degree of freedom we see that this is greater than the test statistic at 0.05 (3.84) but less than that of 0.01 (6.64) so that 0.05>p>0.01.

ASSUMPTIONS AND LIMITATIONS

Assumptions:

(1) Data are univariate and categorical.
(2) Data are from a multinomial population.
(3) Data are collected by random, independent sampling.
(4) Groups being compared are of approximately same size, particularly for small group sizes.

When to use:

(1) When the data are of a categorical (or frequency) nature.
(2) When the data fit the assumptions above.
(3) To test goodness-to-fit to a known form of distribution.
(4) When cell sizes are large.

When not to use:

(1) When the data are continuous rather than categorical.
(2) When sample sizes are small and very unequal.
(3) When sample sizes are too small (e.g., when total N is less than 50 or if any expected value is less

than 5).
(4) For any 2×2 comparison (user Fisher's Exact test instead).

R×C Chi Square

The R×C chi-square test can be used to analyze dis-continuous (frequency) data as in the Fisher's exact or 2×2 chi-square tests; however, in the R×C test (R=row, C=column) we wish to compare three or more sets of data. An example would be comparison of the incidence of tumors among mice on three or more oral dosage levels. We can consider the data as "positive" (tumors) or "negative" (no tumors). The expected frequency for any box is equal to (row total)(column total)/(Ntotal).
As in the Fisher's exact test, the initial step is setting up a table (this time an R×C contingency table). This table would appear as follows:

	Positive	Negative	Total
Group I	$A1$	$B1$	$A1+B1=N1$
Group II	$A2$	$B2$	$A2+B2=N2$
	↓	↓	
Group R	AR	BR	$AR+BR=NR$
Totals	NA	NB	Ntotal

Using these symbols, the formula for chi-square ($\chi 2$) is

$$\chi^2 = \frac{N_{tot}^2}{N_A N_B N_K}\left(\frac{A_1^2}{N_1} + \frac{A_2^2}{N_2} + \ldots \frac{A_K^2}{N_K} - \frac{N_A^2}{N_{tot}}\right).$$

The resulting $\chi 2$ value is compared to table values (138, pp. 470–471) according to the number of degrees of freedom, which is equal to (R−1)(C−1). If $\chi 2$ is smaller than the table value at the 0.05 probability level, the groups are not significantly different. If the calculated $\chi 2$ is larger, there is some difference among the groups, and 2×R chi square or Fisher's exact tests will have to be compared to determine which group(s) differ from which other group(s). Example 10 demonstrates this.

Example 10

The R×C square can be used to analyze tumor incidence data gathered during a mouse-feeding study as follows:

Dosage (mg/kg)	No. of mice with tumors	No. of mice without tumors	Total no. of mice
2.00	19	16	35
1.00	13	24	37
0.50	17	20	37
0.25	22	12	34
0.00	20	23	43
Totals	91	95	186

$$\chi^2 = \frac{(186)^2}{91(95)}\left[\frac{19^2}{35}+\frac{13^2}{37}+\frac{17^2}{37}+\frac{22^2}{34}+\frac{20^2}{43}-\frac{91^2}{186}\right]$$

$$= (4.00)(1.71)$$

$$= 6.84$$

The smallest expected frequency would be (91)(34)/ 186+16.6, well above 5.0. The number of degrees of freedom is (5−1)(2−1)=4. The chi-square table value for four degrees of freedom is 9.49 at the 0.05 probability level. Therefore, there is no significant association between tumor incidence and dose or concentration.

ASSUMPTIONS AND LIMITATIONS

(1) Based on data being organized in a table (such as below) so that there are *cells* (below, A, B, C, and D are cells):

		Columns (C)		
		Control	Treated	Total
Rows (R)	No Effect	A	B	A+B
	Effect	C	D	C+D
Total		A+C	B+D	A+B+C+D

(2) None of the “expected” frequency values should be less than 5.0.

(3) Chi-square test is always one tailed.

(4) Without the use of some form of correction, the test becomes less accurate as the differences between group sizes increases.

(5) The results from each additional column (group) is approximately additive. Due to this characteristic, chi-square can be readily used for evaluating any R×C combination.

(6) The results of the chi-square calculation must be a positive number.

(7) Test is weak with either small sample sizes or when the expected frequency in any cell is less than 5 (this latter limitation can be overcome by “pooling”—combining cells.).

(8) Test results are independent of order of cells, unlike Kolmogorov-Smir nov.

(9) Can be used to test the probability of validity of any distribution.

$$\frac{(N)(N+1)}{2},$$

where N is the total number of data in both groups. The result should be equal to the sum of the sum of ranks for both groups.

The sum of rank values are compared to table values (14, pp. 409–413) to determine the degree of significant differences, if any. These tables include two limits (an upper and a lower) that are dependent on the probability level. If the amount of data is the same in both groups (N1=N2), then the lesser sum of ranks (smaller N) is compared to the table limits to find the degree of significance. Normally the comparison of the two groups ends here and the degree of significant difference can be reported. This is demonstrated in Example 11.

Example 11

If we recorded the approximate times of death (in hours) of rats dosed with 5.0 g/kg (Group B) or 2.5 g/kg (Group A) or a given material, we might obtain the following results:

Hours to Death (Group A)	Hours to Death (Group B)

4	3	7	4
6	6	3	3
7	1	6	1
7	7	7	2
2	7	2	5
5	4	5	4
7	6		
7	3		

With n1=16, n2=12 and n=28, the ranked value of the responses are as shown in parenthesis below:

(Group A)				(Group B)			
4	(11.5)	3	(7.5)	7	(24.5)	4	(11.5)
6	(18.5)	6	(18.5)	3	7.5	3	(7.5)
7	(24.5)	1	(1.5)	6	(18.5)	1	(1.5)
7	(24.5)	7	(24.5)	7	(24.5)	2	(4)
2	(4)	7	(24.5)	2	(4)	5	(15)
5	(15)	4	(11.4)	5	(15)	4	(11.5)
7	(24.5)	6	(18.5)				
7	(24.5)	3	(7.5)				

Sums R1=(261), R2=(145).

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As a chech, $R_1 + R_2 = 406, \ \frac{(28)(29)}{2} = 406 = R_1 + R_2.$ The test's statistic, based on a significance level of μ=0.05 in a normal approximation, becomes

Null hypothesis: H0: $\theta1=\theta2$.

Alt. hypothesis: HA: $\theta1 \neq \theta2$.

$$\text{Test statistic: } Z = \frac{|R_1 - \mu_{R_1}| - 0.5}{\sigma_{R_1}}.$$

$$\frac{(261 - 232) - 0.5}{\sqrt{448.38}} = 1.346.$$

Decision rule: reject H0 if |Z|>1.96.

Conclusion: Because 1.346 is not >1.96, we do not reject H0, concluding that there is insufficient evidence of a difference between the two doses in terms of time of death.

Distribution-Free Multiple Comparison

The distribution-free multiple comparison test should be used to compare three or more groups of nonparametric data. These groups are then analyzed two at a time for any significant differences (87, pp. 124–129). The test can be used for data similar to those compared by the rank-sum test. We often employ this test for reproduction and mutagenicity studies (such as comparing survival rates of offspring of rats fed various amounts of test materials in the diet).

As shown in Example 12, two values must be calculated for each pair of groups: the difference in mean ranks and the probability level value against which the difference will be compared. To determine the difference in mean ranks we must first arrange the data within each of the groups in order of increasing values. Then we must assign rank values, beginning with the smallest overall figure. Note that this ranking is similar to that in the Wilcoxon test except that it applies to more than two groups.

The ranks are then added for each of the groups. As a check, the sum of these should equal

$$\frac{N_{tot}(N_{tot}+1)}{2},$$

where Ntot is the total number of figures from all groups. Next we can find the mean rank (R) for each group by dividing the sum of ranks by the numbers in the data (N) in the group. These mean ranks are then taken in those pairs that we want to compare (usually each test group versus the control) and the differences are found (|R1−R2|). This value is expressed as an absolute figure (i.e., it is always a positive number).

The second value for each pair of groups (the probability value) is calculated from the expression

$$z[a/K(K-1)]\sqrt{\frac{N_{total}(N_{total}+1)}{12}}\sqrt{\frac{1}{N_1}\frac{1}{N_2}},$$

where a is the level of significance for the comparison (usually 0.05, 0.01, 0.001, etc.), K is the total number of groups, and Z is a figure obtained from a normal probability table and determining the corresponding "Z-score".

The result of the probability value calculation for each pair of groups is compared to the corresponding mean difference |R1−R2|. If |R1−R2| is smaller, there is no significant difference between the groups; if it is larger, the groups are different and |R1−R2| must be compared to the calculated probability values for a=0.01 and a=0.001 to find the degree of significance.

Example 12

Consider the following set of data (ranked in increasing order), which could represent the proportion of rats surviving given periods of time during diet inclusion of a test chemical at four dosage levels (survival index):

I		II		III		IV	
5.0 mg/kg		2.5 mg/kg		1.25 mg/kg		0.0 mg/kg	
% Value	Rank	% Value	Rank	% Value	Rank	% Value	Rank
40	2.0	40	2.0	50	5.5	60	9.0
40	2.0	50	5.5	50	5.5	60	9.0
50	5.5	80	12.0	60	9.0	80	12.0

100	17.5	80	12.0	100	17.5	90	14.0
		100	17.5	100	17.5	100	17.5
						100	17.5
Sum of ranks	27.0		49.0		55.0		79.0

$N_I = 4, \quad N_{II} = 5, \quad N_{III} = 5, \quad N_{IV} = 6.$

$N_{tot} = 20$

$$\text{Check sums of} = 210, \frac{20(21)}{2} = 210.$$

$$\text{Mean ranks (R):} \quad R_1 = \frac{27.0}{4} = 6.75, \quad R_2 = \frac{49.0}{5} = 9.80,$$

$$R_3 = \frac{55.0}{5} = 11.00, \quad R_4 = \frac{79.0}{6} = 13.17.$$

Comparison Groups	|R1−R2|	Probability test values
5.0 versus 0.0	6.42	$(0.05/4(3)) = Z_{0.00417} = 2.637$ $\sqrt{\frac{(20)(21)}{12}}\sqrt{\frac{1}{4}+\frac{1}{6}} = 10.07$
2.5 versus 0.0	3.37	$(0.05/4(3)) = Z_{0.00417} = 2.637$ $\sqrt{\frac{(20)(21)}{12}}\sqrt{\frac{1}{5}+\frac{1}{6}} = 9.45$
1.25 versus 0.0	2.17	$(0.05/4(3)) = Z_{0.00417} = 2.637$ $\sqrt{\frac{(20)(21)}{12}}\sqrt{\frac{1}{5}+\frac{1}{6}} = 9.45$

Because each of the |R1−R2| values is smaller than the corresponding probability calculation, the pairs of groups compared are not different at the 0.05 level of significance.

ASSUMPTIONS AND LIMITATIONS

1. As with the Wilcoxon Rank-Sum, too many tied ranks inflate the false-positive.
2. Generally, this test should be used as a post hoc comparison after Kruskal-Wallis.

Mann-Whitney U Test

This is a nonparametric test in which the data in each group are first ordered from lowest to highest values, and then the entire set (both control and treated values) is ranked, with the average rank being assigned to tied values (i.e., if two values tie for 12th rank—and therefore would be ranked 12th and 13th—both would be assigned the average rank of 12.5). The ranks are then summed for each group and U is determined according to

$$U_t = n_c n_t + \frac{n_t(n_t+1)}{2} - R_t,$$

$$U_c = n_c n_t + \frac{n_c(n_c+1)}{2} - R_c,$$

where nc, nt=sample size for control and treated groups and Rc, Rt=sum of ranks for the control and treated groups. For the level of significance for a comparison of the two groups, the larger value of Uc or Ut is used. This is compared to critical values as found in tables in Reference 137.

As demonstrated in Example 13, the Mann-Whitney U test is employed for the count data, but which test should be employed for the percentage variables should be decided on the same grounds as described later in a discussion of statistical requirements for reproduction studies.

Example 13

In a 2-week study, the levels of serum cholesterol in treatment and control animals are successfully measured and assigned ranks as follows:

	Treatment		Control	
	Value	Rank	Value	Rank
	10	1	19	4
	18	3	28	13
	26	10.5	29	14.5
	31	16	26	10.5
	15	2	35	19
	24	8	23	7
	22	6	29	14.5
	33	17	34	18
	21	5	38	20
	25	9	27	12
SUM OF RANKS		77.5		132.5

The critical value for one tailed $p \leq 0.05$ is $U \geq 73$. We then calculate

$$U_t = (10)(10) + \frac{10(10+1)}{2} - 77.5$$

$$= 100 + \frac{110}{2} - 77.5 = 77.5$$

$$U_c = (10)(10) + \frac{10(10+1)}{2} - 132.5 = 22.5.$$

As 77.5 is greater than 73, these groups are significantly different at the 0.05 level.

ASSUMPTIONS AND LIMITATIONS

1. It does not matter whether the observations are ranked from smallest to largest, or vice versa.
2. This test should not be used for paired observations.
3. The test statistics from a Mann-Whitney are linearly related to those of Wilcoxon. The two tests will always yield the same result. The Mann-Whitney is presented here for historical completeness, as it has been much favored in reproductive and developmental toxicology studies; however, it should be noted that the authors do not include it in the decision tree for method selection (Figure 7.2).

Kruskal-Wallis Nonparametric ANOVA

The Kruskal-Wallis nonparametric one-way analysis of variance should be the initial analysis performed when we have three or more groups of data that are by nature nonparametric (either not a normally distributed population, or of a discontinuous nature, or all the groups being analyzed are not from the same population) but not a categorical (or quantal) nature. Commonly these will be either rank-type evaluation data (such as behavioral toxicity observation scores) or reproduction study data. The analysis is initiated (119, pp. 170–173) by ranking all the observations from the combined groups to be analyzed. Ties are given the average rank of the tied values.

The sum of ranks of each group (r1, r2,...rk) is computed by adding all the rank values for each group. The test value H is then computed as

$$H = \frac{12}{n(n+1)} \sum r_1^2/n_1 + r_2^2/n_2 + \cdots + r_k^2/n_k) - 3(n+1),$$

where n1, n2,...nk are the number of observations in each group. The test statistic is then compared with a table of H values. If the calculated value of H is greater than the table value for the appropriate number of observations in each group, there is significant difference between the groups, but further testing (using the distribution-free multiple comparisons method) is necessary to determine where the difference lies (as demonstrated in Example 14).

Example 14

As part of a neurobehavioral toxicology study, righting reflex values (whole numbers ranging from 0 to 10) were determined for each of five rats in each of three groups. The values observed, and their ranks, are as follows:

	Control group		5 mg/kg group		10 mg/kg group	
	Reflex Score	Rank	Reflex Score	Rank	Reflex Score	Rank
	0	2	1	5	4	11
	0	2	2	7.5	4	11
	0	2	2	7.5	5	13
	1	5	3	9	8	14.5
	1	5	4	11	8	14.5
Sums of of ranks (r)		16		40		64

From these the H value is calculated as

From these the H value is calculated as

$$H = \frac{12}{15(15+1)} \left[\frac{16^2}{5} + \frac{40^2}{5} + \frac{64^2}{5} \right] - 3(15+1)$$

$$= \frac{12}{240} \left[\frac{(256 + 1600 + 4096)}{5} \right] - 48$$

$$= \frac{1}{20}(1190.4) - 48$$

$$= 59.52 - 48$$

$$= 11.52$$

Consulting a table of values for H, we find that for the case where we have three groups of five observations each, the test values are 4.56 (for p=0.10), 5.78 (for p=0.05), and 7.98 (for p=0.01). As our calculated H is greater than the p=0.01 test value, we have determined that there is a significant difference between the groups at the level of p<0.01 and would now have to continue to a multiple comparisons test to determine where the difference is.

ASSUMPTIONS AND LIMITATIONS

1. The test statistic H is used for both small and large samples.
2. When we find a significant difference, we do not know which groups are different. It is not correct to then perform a Mann-Whitney U test on all possible combinations. Rather, a multiple comparison method must be used, such as the distribution-free multiple comparisons.
3. Data must be independent for the test to be valid.
4. Too many tied ranks will decrease the power of this test and also lead to increased false-positive levels.
5. When k=2, the Kruskal-Wallis chi-square value has 1 d.f. This test is identical to the normal approximation used for the Wilcoxon Rank-Sum Test. As noted in previous sections, a chi-square with 1 d.f. can be represented by the square of a standardized normal random variable. In the case of k=2, the h-statistic is the square of the Wilcoxon Rank-Sum Z-test (without the continuity correction).

6. The effect of adjusting for tied ranks is to slightly increase the value of the test statistic, h. Therefore, omission of this adjustment results in a more conservative test.

Log-Rank Test

The log-rank test is a statistical methodology for comparing the distribution of time until the occurrence of the event in independent groups. In toxicology, the most common event of interest is death or occurrence of a tumor, but it could just as well be liver failure, neurotoxicity, or any other event that occurs only once in an individual. The elapsed time from initial treatment or observation until the *event* is the *event time,* often referred to as "survival time," even when the event is not "death."

The log-rank test provides a method for comparing "risk-adjusted" event rates, useful when test subjects in a study are subject to varying degrees of opportunity to experience the event. Such situations arise frequently in toxicology studies due to the finite duration of the study, early termination of the animal, or interruption of treatment before the event occurs.

Examples where use of the log-rank test might be appropriate include comparing survival times in carcinogenity bioassay animals that are given a new treatment with those in the control group or comparing times to liver failure for several dose levels of a new nonsteroidal anti-inflammatory drug (NSAID) where the animals are treated for 10 weeks or until cured, whichever comes first.

If every animal were followed until the event occurrence, the event times could be compared between two groups using the Wilcoxon Rank-Sum Test, however, some animals may die or complete the study before the event occurs. In such cases, the actual time of the event is unknown since the event does not occur while under study observation. The event times for these animals are based on the last known time of study observation and are called "censored" observations because

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they represent the lower-bound of the true, unknown event times. The Wilcoxon Rank-Sum Test can be highly biased in the presence of the censored data.

The null hypothesis tested by the Log-Rank Test is that of equal event time distributions among groups. Equality of the distributions of event times implies similar event rates among groups not only for the clinical trial as a whole, but also for any arbitrary time point during the trial. Rejection of the null hypothesis indicates that the event rates differ among groups at one or more time points during the study.

The idea behind the log-rank test for comparison of two life tables is simple. If there were no difference between the groups, the total deaths occurring at any time should split between the two groups at that time. So if the numbers at risk in the first and second groups in (say) the sixth month were 70 and 30, respectively, and 10 deaths occurred in that month, we would expect

$$10 \times \frac{70}{70+30} = 7$$

of these deaths to have occurred in the first group, and

$$10 \times \frac{30}{70+30} = 3$$

of the deaths to have occurred in the second group.

A similar calculation can be made at each time of death (in either group). By adding together for the first group the results of all such calculations, we obtain a single number, called the extent of exposure (E1), which represents the "expected" number of deaths in that group if the two groups had the distribution of survival time. An extent of exposure (E2) can be obtained for the second group in the same way. Let O1 and O2 denote the actual total numbers of deaths in the two groups. A useful arithmetic check is that the total number of deaths O1+O2 must equal the sum E1+E2 of the extents of exposure.

The discrepancy between the O's and E's can be measured by the quantity

$$\chi^2 = \frac{(|O_1 - E_1| - 1/2)^2}{E_1} + \frac{(|O_2 - E_2| - 1/2)^2}{E_2}.$$

For rather obscure reasons, X2 is known as the *Log-Rank statistic*. An approximate significance test of the null hypothesis of identical distributions of survival time in the two groups is obtained by referring X2 to a chi-square distribution on 1 degree of freedom. This is demonstrated in Example 15.

Example 15

In a study of the effectiveness of a new monoclonal antibody to treat a specific cancer, the times to re occurrence of the cancer in treated animals in weeks were as follows:

Control Group			Treatment Group		
1	5	11	6	10	22
1	5	12	6	11	23
2	8	12	6	13	25
2	8	15	6	16	32
3	8	17	7	17	32
4	8	22	9	19	34
4	11	23	10	20	35

The table opposite presents the calculations for the log-rank test applied to these times. A chi-square value of 13.6 is significant at the $p<0.001$ level.

Illustration:

$$t = 23, 2 \times \frac{6}{7} = 1.7143, 2 \times \frac{1}{7} = 0.2857.$$

Test of significance:

$$x^2 = \frac{\left(|O_1 - E_1| - \frac{1}{2}\right)^2}{E_1} + \frac{\left(|O_2 - E_2| - \frac{1}{2}\right)^2}{E_2}$$

$$= \frac{\left(|9 - 19.2| - \frac{1}{2}\right)^2}{19.2} + \frac{\left(|21 - 10.8| - \frac{1}{2}\right)^2}{10.8} = 13.6.$$

Estimate of relative risk'.

$$\theta = \frac{(O_1/E_1)}{(O_2/E_2)}$$

$$\hat{\theta} = \frac{9/19.2}{21/10.8} = 0.24.$$

The log-rank test as presented by Peto et al. (118) uses the product-limit life-table calculations rather than the actuarial estimators shown above. The distinction is unlikely to be of practical importance unless the grouping intervals are very coarse.

Peto and Pike (118) suggest that the approximation in treating the null distribution of $\chi2$ as a chi-square is conservative, so it will tend to understate the degree of statistical significance. In the formula for $\chi2$ we have used the continuity correction of subtracting 1/2 from $|O1-E1|$ and $|O2-E2|$ before squaring. This is recommended by Peto et al. (118) when, as in nonrandomized studies, the permutational argument does not apply. Peto et al. (118) gives further details of the log-rank test and its extension to comparisons of more than two treatment groups and to tests that control for categorical confounding factors.

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Log-Rank Calculation for Tumor Data

Time,	At Risk			Relapses			Extent of Exposure		
t	*T*	*C*	Total	*T*	*C*	Total	*T*	*C*	Total
1	21	21	42	0	2	2	1.0000	1.0000	2
2	21	19	40	0	2	2	1.0500	0.9500	2
3	21	17	38	0	1	1	0.5526	0.4474	1
4	21	16	37	0	2	2	1.1351	0.8649	2
5	21	14	35	0	2	2	1.2000	0.8000	2
6	20.5	12	32.5	3	0	3	1.8923	1.1077	3
7	17	12	29	1	0	1	0.5862	0.4138	1
8	16	12	28	0	4	4	2.2857	1.7143	4
10	14.5	8	22.5	1	0	1	0.6444	0.3556	1
11	12.5	8	20.5	0	2	2	1.2295	0.7705	2
12	12	6	18	0	2	2	1.3333	0.6667	2
13	12	4	16	1	0	1	0.7500	0.2500	1
15	11	4	15	0	1	1	0.7333	0.2667	1
16	11	3	14	1	0	1	0.7857	0.2143	1
17	9.5	3	12.5	0	1	1	0.7600	0.2400	1
22	7	2	9	1	1	2	1.5556	0.4444	2
23	6	1	7	1	1	2	1.7143	0.2857	2
	Total			9	21	30	19.2080	10.7920	30
					(O1)	*(O2)*	*(E1)*	*(E2)*	

ASSUMPTIONS AND LIMITATIONS

(1) The endpoint of concern is or is defined so that it is "right censored"—once it happens, it does not reoccur. Examples are death or a minimum or maximum value of an enzyme or physiologic function (such as respiration rate).

(2) The method makes no assumptions on distribution.

(3) Many variations of the Log-Rank Test for comparing survival distributions exist. The most common variant has the form

$$\chi^2 = \frac{(O_1 - E_1)^2}{E_1} + \frac{(O_2 - E_2)^2}{E_2},$$

where Oi and Ei are computed for each group, as in the formulas given previously. This statistic also has an approximate chi-square distribution with 1 degree of freedom under H0.

A continuity correction can also be used to reduce the numerators by one half before squaring. Use of such a correction leads to even further conservatism and may be omitted when sample sizes are moderate or large.

(4) The Wilcoxon Rank-Sum Test could be used to analyze the event times in the absence of censoring. A "Generalized Wilcoxon" Test, sometimes called the Gehan Test, based on an approximate chi-square distribution, has been developed for use in the presence of censored observations. Both the Log-Rank and the Generalized Wilcoxon Tests are nonparametric tests and require no assumptions regarding the distribution of event times. When the event rate is greater early in the trial than toward the end, the Generalized Wilcoxon Test is the more appropriate test because it gives greater weight to the earlier differences.

(5) Survival and failure times often follow the exponential distribution. If such a model can be assumed, a more powerful alternative to the Log-Rank Test is the Likelihood Ratio Test. This parametric test assumes that event probabilities are constant over time. That is, the chance that a patient becomes event-positive at time t given that he is event-negative up to time t does not depend on t. A plot of the negative log of the event times' distribution showing a linear trend through the origin is consistent with exponential event times.

(6) Life tables can be constructed to provide estimates of the event time distributions. Estimates commonly used are known as the Kaplan-Meier estimates.

HYPOTHESIS TESTING: UNIVARIATE PARAMETRIC TESTS

Univariate case* data from normally distributed populations generally have a higher information value associated with them, but the traditional hypothesis testing techniques generally are neither resistant nor robust. All the data analyzed by these methods are also, effectively, continuous (i.e., at least for practical purposes, the data may be represented by any number and each such data number has a

measurable relationship to other data numbers).

Student's t-Test (Unpaired t-Test)

Pairs of groups of continuous, randomly distributed data are compared via this test. We can use this test

*That is, where each datum is defined by one treatment and one effect variable.

to compare three or more groups of data, but they must be intercompared by examination of two groups taken at a time and are preferentially compared by analysis of variance (ANOVA). Usually this means comparison of a test group versus a control group, although two test groups may be compared as well. To determine which of the three types of t-tests described in this chapter should be employed, the F-test usually is performed first. This will tell us if the variances of the data are approximately equal, which is a requirement for the use of the parametric methods. If the F-test indicates homogeneous variances and the numbers of data within the groups (N) are equal, then the Student's t-test is the appropriate procedure (138). If the F is significant (the data are heterogeneous) and the two groups have equal numbers of data, the modified Student's t-test is applicable (42).

The value of t for Student's t-test is calculated using the formula

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sum D_1^2 + \sum D_2^2} \sqrt{\frac{N_1 N_2}{N_1 + N_2}(N_1 + N_2 - 2)},$$

where the value of $\Sigma D^2 = [N \Sigma X^2 - (\Sigma X)^2]/N$.

The value of t obtained from the above calculations is compared to the values in a t-distribution table according to the appropriate number of degrees of freedom (df). If the F value is not significant (i.e., variances are homogeneous), the $df = N_1 + N_2 - 2$. If the F was significant and $N_1 = N_2$, then the $df = N - 1$. Although this case indicates a nonrandom distribution, the modified t-test is still valid. If the calculated value is larger than the table value at $p = 0.05$, it may then be compared to the appropriate other table values in order of decreasing probability to determine the degree of significance between the two groups. Example 16 demonstrates this methodology.

Example 16

Suppose we wish to compare two groups (a test and control group) of dog weights following inhalation of a vapor. First, we would test for homogeneity of variance using the F-test. Assuming that this test gave negative (homogeneous) results, we would perform the t-test as follows:

Dog	Test weight (X_1 in kg)	X_1^2	Control weight (X_2 in kg)	X_2^2
1	8.3	68.89	8.4	70.56
2	8.8	77.44	10.2	104.04
3	9.3	86.49	9.6	92.16
4	9.3	86.49	9.4	88.36
Sums	$\Sigma X_1 = 35.7$	ΣX_1^2	$\Sigma X_2 = 37.6$	ΣX_2^2
Means	8.92		9.40	

The difference in means $= 9.40 - 8.92 = 0.48$.

$$\sum D_1^2 = \frac{4(319.31) - (35.7)^2}{4} = \frac{2.75}{4} = 0.6875,$$

$$\sum D_2^2 = \frac{4(355.12) - (37.6)^2}{4} = \frac{6.72}{4} = 1.6800,$$

$$t = \frac{0.48}{\sqrt{0.6875 + 1.6800}} \sqrt{\frac{4(4)}{4 + 4}(4 + 4 - 2)} = 1.08.$$

The table value for t at the 0.05 probability level for (4+4−2), or six degrees of freedom, is 2.447. Therefore, the dog weights are not significantly different at $p = 0.05$.

ASSUMPTIONS AND LIMITATIONS

1. The test assumes that the data are univariate, continuous, and normally distributed.
2. Data are collected by randomly sampling.
3. The test should be used when the assumptions in 1 and 2 are met and there are only two groups to be compared.
4. Do not use when the data are ranked, when the data are not approximately normally distributed, or when there are more than two groups to be compared. Do not use for paired observations.
5. This is the most commonly misused test method, except in those few cases where one is truly only comparing two groups of data and the group sizes are roughly equivalent. Not valid for multiple comparisons (because of resulting additive errors) or where group sizes are very unequal.
6. Test is robust for moderate departures from normality and, when N_1 and N_2 are approximately equal,

robust for moderate departures from homogeneity of variances.
7. The main difference between the Z-test and the t-test is that the Z-statistic is based on a known standard deviation, *a*, whereas the t-statistic uses the sample standard deviation, s, as an estimate of *a*. With the assumption of normally distributed data, the variance σ^2 is more closely estimated by the sample variance s^2 as n gets large. It can be shown that the t-test is equivalent to the Z-test for infinite degrees of freedom. In practice, a "large" sample usually is considered $n \geq 30$.

Cochran t-test

The Cochran test should be used to compare two groups of continuous data when the variances (as indicated by the F-test) are heterogeneous and the numbers of data within the groups are not equal ($N_1 \neq N_2$). This is the situation, for example, when the data, though expected to be randomly distributed, were found not to be (28, pp. 100–102).

Two t values are calculated for this test, the "observed" t (t_{obs}) and the "expected" t (t′) The observed t is obtained by

$$t_{obs} = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{W_1 + W_2}}$$

where W=SEM^2 (standard error of the mean squared),
$=S^2/N$,

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where *S* (variance) can be calculated from

$$S = \frac{\dfrac{N\sum X^2 - \left(\sum X\right)^2}{N}}{N-1}$$

The value for *t′* is obtained from

$$t' = \frac{t'_1 W_1 + t'_2 W_2}{W_1 + W_2}$$

where t'_1 and t'_2 are values for the two groups taken from the t-distribution table corresponding to *N*–1 degrees of freedom (for each group) at the 0.05 probability level (or such level as one may select). The calculated t_{obs} is compared to the calculated t′ value (or values, if t′ values were prepared for more than one probability level). If t_{obs} is smaller than a t′, the groups are not considered to be significantly different at that probability level. This procedure is shown in Example 17.

Example 17

If we wished to compare the red blood cell count (RBC) of rats receiving a test material in their diet with the RBCs of control rats, we might obtain the following results:

Test RBC (X_1)	X_1^2	Control RBC (X_2)	X_2^2
8.23	67.73	7.22	52.13
8.59	73.79	7.55	57.00
7.51	56.40	7.53	56.70
6.60	46.56	7.32	53.58
6.67	44.49		
$\sum X_1 = 37.60$	$\sum X_1^2$ 285.97	$\sum X_2 = 29.62$	$\sum X_2^2$ 219.41

$$\bar{X}_1 = \frac{37.60}{5} = 7.52, \quad W_1 = \frac{0.804}{5} = 0.1608,$$

$$\bar{X}_2 = \frac{29.62}{4} = 7.40, \quad W_2 = \frac{0.025}{4} = 0.0062,$$

(note that S^2 values of 0.804 and 0.025 are calculated in Example 17)

$$t_{obs} = \frac{7.52 - 7.40}{\sqrt{0.1608 + 0.0062}} = 0.29.$$

From the t-distribution table we use t1=2.776 (d*f*=4) and t2=3.182 (d*f*=3) for the 0.05 level of significance; there is no statistical difference at p=0.05 between the two groups.

ASSUMPTIONS AND LIMITATIONS

1. The test assumes that the data are univariate, continuous, normally distributed, and that group sizes are unequal.
2. The test is robust for moderate departures from normality and very robust for departures from equality of variances.

F-Test

This is a test of the homogeneity of variances between two groups of data (138). It is used in two separate cases. The first is when Bartlett's indicates heterogeneity of variances among three or more groups (i.e., it is used to determine which pairs of groups are heterogeneous). Second, the F-test is the initial step in comparing two groups of continuous data that we would expect to be parametric (two groups not usually being compared using ANOVA), the results indicating whether the data are from the same population and whether subsequent parametric comparisons would be valid.

The F is calculated by dividing the larger variance (S_1^2) by the smaller one (S_2^2). S2 is calculated as

$$S^2 = \frac{\dfrac{N\sum X^2 - \left(\sum X\right)^2}{N}}{N-1},$$

where *N* is the number of data in the group and X represents the individual values within the group. Frequently, S2 values may be obtained from ANOVA calculations. Use of this is demonstrated in Example 18.

The calculated F value is compared to the appropriate number in an F value table for the appropriate degrees of freedom (N–1) in the numerator (along the top of the table) and in the denominator (along the side of the table). If the calculated value is smaller, it is not significant and the variances are considered homogeneous (and the Student's t-test would be appropriate for further comparison). If the calculated F value is greater, F is significant and the variances are heterogeneous (and the next test would be modified Student's t-test if N1=N2 or the Cochran t-test if N1≠N2; see Figure 7.2 to review the decision tree).

Example 18

Using the RBC comparison from Example 17 (with N1=5, N2=4), the following results were determined:

$$\text{Variance for } X_1 = S_1^2 = \frac{\dfrac{5(285.97) - (37.60)^2}{5}}{5-1}$$
$$= 0.804,$$

$$\text{Variance for } X_2 = S_3^2 = \frac{\frac{4(219.41)-(29.62)^2}{4}}{4-1}$$

$$= 0.025,$$

$$F = \frac{0.804}{0.025} = 32.16.$$

From a table for F values, for 4 (numerator) versus 3 (denominator) df, we read the limit of 9.12 at the 0.05 level. As our calculated value is larger (and, therefore, significant), the variances are heterogeneous and the Cochran t-test would be appropriate for comparison of the two groups of data.

ASSUMPTIONS AND LIMITATIONS

1. This test could be considered as a two-group equivalent of the Bartlett's test.
2. If the test statistic is close to 1.0, the results are (of course) not significant.
3. The test assumes normality and independence of data.

Analysis of Variance

ANOVA is used for comparison of three or more groups of continuous data when the variances are homogeneous and the data are independent and normally distributed. A series of calculations are required for ANOVA, starting with the values within each group being added (ΣX) and then these sums being added (ΣΣX). Each figure within the groups is squared, and these squares are then summed (ΣX^2) and these sums added ($\Sigma\Sigma X^2$). Next the "correction factor" (CF) can be calculated from the following formula:

$$CF = \frac{\left(\sum_1^K \sum_1^N X\right)^2}{N_1 + N_2 + \ldots N_k},$$

where N is the number of values in each group and K is the number of groups. The total sum of squares (SS) is then determined as follows:

$$SS_{total} = \sum_1^K \sum_1^N X^2 - CF.$$

In turn, the sum of squares between groups (bg) is found from

$$SS_{bg} = \frac{\left(\sum X_1\right)^2}{N_1} + \frac{\left(\sum X_2\right)^2}{N_2} + \cdots \frac{\left(\sum X_k\right)^2}{N_k} - CF.$$

The sum of squares within group (wg) is then the difference between the last two figures, or

$$SS_{wg} = SS_{total} - SS_{bg}.$$

Now there are three types of degrees of freedom to determine. The first, total df, is the total number of data within all groups under analysis minus one ($N_1+N_2+\ldots N_k-1$). The second figure (the df between groups) is the number of groups minus one (K−1). The last figure (the df within groups or "error df") is the difference between the first two figures ($df_{total}-df_{bg}$).

The next set of calculations requires determination of the two mean squares (MD_{bg} and MS_{wg}). These are the respective sum of square values divided by the corresponding df figures (MS=SS/df). The final calculation is that of the F ratio. For this, the MS between groups is divided by the MS within groups ($F=MS_{bg}/MS_{wg}$).

A table of the results of these calculations (using data from Example 19 at the end of this section) would appear as follows:

	df	SS	MS	F
Bg	3	0.04075	0.01358	4.94
Wg	12	0.03305	0.00275	
Total	15	0.07380		

For interpretation, the F ratio value obtained in the ANOVA is compared to a table of F values. If F≤1.0, the results are not significant and comparison with the table values is not necessary. The degrees of freedom (df) for the greater mean square (MS_{bg}) are indicated along the top of the table. Then read

down the side of the table to the line corresponding to the df for the lesser mean square (MSwg). The figure shown at the desired significance level (traditionally 0.05) is compared to the calculated F value. If the calculated number is smaller, there is no significant differences among the groups being compared. If the calculated value is larger, there is some difference, but further (post hoc) testing will be required before we know which groups differ significantly.

Example 19

Suppose we want to compare four groups of dog kidney weights, expressed as percentage of body weights, following an inhalation study. Assuming homogeneity of variance (from Barlett's test), we could complete the following calculations:

	400 ppm	200 ppm	100 ppm	0 ppm
	0.43	0.49	0.34	0.34
	0.52	0.48	0.40	0.32
	0.43	0.40	0.42	0.33
	0.55	0.34	0.40	0.39
ΣX	1.93	1.71	1.56	1.38

$$\Sigma\Sigma X=1.93+1.71+1.56+1.38=6.58.$$

Next, the preceding figures are squared:

	400 ppm	200 ppm	100 ppm	0 ppm
	0.1849	0.2401	0.1156	0.1156
	0.2704	0.2304	0.1600	0.1024
	0.1849	0.1600	0.1764	0.1089
	0.3025	0.1156	0.1600	0.1521
$\Sigma X2$	0.9427	0.7461	0.6120	0.4790

$$\Sigma\Sigma X2=0.9427+0.7461+0.6120+0.4790=2.7798.$$

$$\mathrm{CF}=\frac{(6.58)^2}{4+4+4+4}=2.7060,$$

$$\mathrm{SS}_{\mathrm{total}}=2.7798-2.7060=0.0738,$$

$$\mathrm{SS}_{\mathrm{bg}}=\frac{(1.93)^2}{4}+\frac{(1.71)^2}{4}+\frac{(1.56)^2}{4}+\frac{(1.38)^2}{4}-2.7060$$
$$=0.04075,$$

$$\mathrm{SS}_{\mathrm{wg}}=0.07380-0.04075=0.03305.$$

$$\text{The total degrees of freedom (df)}=4+4+4+4-1=15,$$

$$\mathrm{df}_{\mathrm{bg}}=4-1=3\ \mathrm{df}_{\mathrm{wg}}=15-3=12,$$

$$\mathrm{MS}_{\mathrm{bg}}=\frac{0.4075}{3}=0.01358,$$

$$\mathrm{MS}_{\mathrm{wg}}=\frac{0.03305}{12}=0.00275,$$

$$\mathrm{F}=\frac{0.01358}{0.00275}=4.94.$$

Going to a table of F values, we find that for 3 dfbg (greater mean square) and 12 dfwg (lesser mean square), the 0.05 value of F is 3.49. As our calculated value is greater, there is a difference among groups at the 0.05 probability level. To determine where the difference is, further comparisons by a post hoc test will be necessary.

ASSUMPTIONS AND LIMITATIONS

1. What is presented here is the workhorse of toxicology—the one-way analysis of variance. Many other forms exist for more complicated experimental designs.
2. The test is robust for moderate departures from normality if the sample sizes are large enough. Unfortunately, this is rarely the case in toxicology.
3. ANOVA is robust for moderate departures from equality of variances (as determined by Bartlett's test) if the sample sizes are approximately equal.
4. It is not appropriate to use a t-test (or a 2-groups-at-a-time version of ANOVA) to identify where significant differences are within the design group. A multiple-comparison post hoc method must be used.

Post Hoc Tests

There is a wide variety of post hoc tests available to analyze data after finding significant result in an ANOVA. Each of these tests has advantages and disadvantages, proponents, and critics. Four of the tests are commonly used in toxicology and are presented here. These are Duncan's, Scheffe's, and Dunnett's t-test and Williams' t-test. Two other tests that are available in many statistical packages are Tukey's method and the Student-Newman-Keuls method (171, pp. 151–161).

If ANOVA reveals no significance, it is not appropriate to proceed to perform a post hoc test in hope of finding differences. To do so would only be another form of multiple comparisons, increasing the type I error rate beyond the desired level.

Duncan's Multiple Range Test

Duncan's (44) is used to compare groups of continuous and randomly distributed data (such as body weights, organ weights, etc.). The test normally involves three or more groups taken one pair at a time. It should only follow observation of a significant F value in the ANOVA and can serve to determine which group (or groups) differs significantly from which other group (or groups).

There are two alternative methods of calculation. The selection of the proper one is based on whether the number of data (N) are equal or unequal in the groups.

Groups with Equal Number of Data (N1=N2)

Two sets of calculations must be carried out. First, the determination of the difference between the means of pairs of groups; second, the preparation of a probability rate against which each difference in means is compared (as shown in the first of the two examples in this section).

The means (averages) are determined (or taken from the ANOVA calculation) and ranked in either decreasing or increasing order. If two means are the same, they take up two equal positions (thus, for four means we could have ranks of 1, 2, 2, and 4 rather than 1, 2, 3, and 4). The groups are then taken in pairs and the differences between the means $(\bar{X}_1 - \bar{X}_2)$, expressed as positive numbers, are calculated. Usually, each pair consists of a test group and the control group, though multiple test groups may be intracompared if so desired. The relative rank of the two groups being compared must be consid-

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ered. If a test group is ranked "2" and the control group is ranked "1," then we say that there are two places between them, whereas if the test group were ranked "3," then there would be three places between it and the control.

To establish the probability table, the standard error of the mean (SEM) must be calculated as presented earlier, or as

$$\sqrt{\frac{\text{error mean square}}{N}} = \sqrt{\frac{\text{mean square within group}}{N}},$$

where N is the number of animals or replications per dose level. The mean square within groups (MSwg) can be calculated from the information given in the ANOVA procedure (refer to the earlier section on ANOVA). The SEM is then multiplied by a series of table values (14,80) to set up a probability table. The table values used for the calculations are chosen according to the probability levels (note that the tables have sections for 0.05, 0.01, and 0.001 levels) and the number of means apart for the groups being compared and the number of "error" degrees of freedom (df). The "error" df is the number of df within the groups. This last figure is determined from the ANOVA calculation and can be taken from ANOVA output. For some values of df, the table values are not given and should thus be interpolated. Example 20 demonstrates this case.

Example 20

Using the data given in Example 19 (4 groups of dogs, with 4 dogs in each group), we can make the following calculations:

Ranks	1	2	3	4
Concentration	0 ppm	100 ppm	200 ppm	400 ppm
Mean kidney weight $(\bar{X})$	0.345	0.390	0.428	0.482

Groups compared	$\bar{X}_1-\bar{X}_2$	No of means apart	Probability
2 vs. 1 (100 vs 0 ppm)	0.045	2	p>0.05
3 vs. 1 (200 vs 0 ppm)	0.083	3	p>0.05
4 vs. 1 (400 vs 0 ppm)	0.137	4	0.01>p>0.001
4 vs. 2 (400 vs 100 ppm)	0.092	3	0.05>p>0.01

The mean square within groups from the ANOVA example was 0.00275. Therefore, the SEM= $\sqrt{0.00275/4}=0.02622$. The "error" d*f*(d*f*wg) was 12, so the following table values are used.

No. of means apart	Probability levels 0.05	0.01	0.001
2	3.082	4.320	6.106
3	3.225	4.504	6.340
4	3.313	4.662	6.494

When these are multiped by the SEM we get the following probability table:

No. of means apart	Probability levels 0.05	0.01	0.001
2	0.0808	0.1133	0.1601
3	0.0846	0.1161	0.1662
4	0.0869	0.1212	0.1703

Groups with Unequal Numbers of Data (N1≠N2)

This procedure is very similar to that discussed above. As before, the means are ranked and the differences between the means are determined $(\bar{X}_1-\bar{X}_2)$. Next, weighing values ("aij" values) are calculated for the pairs of groups being compared in accordance with

$$a_u = \sqrt{\frac{2N_iN_j}{(N_i+N_j)}} = \sqrt{\frac{2N_1N_2}{(N_1+N_2)}}$$

This weighting value for each pair of groups is multiplied by $(\bar{X}_1-\bar{X}_2)$ for each value to arrive at a "t" value. It is the "t" that will later be compared to a probability table.

The probability table is set up as above except that instead of multiplying the appropriate table values by SEM, SEM2 is used. This is equal to $\sqrt{\text{MS}_{\text{wg}}}$.

For the desired comparison of two groups at a time, the $(\bar{X}_1-\bar{X}_2)$. value (if N1=N2) is compared to

the appropriate probability table. Each comparison must be made according to the number of places between the means. If the table value is larger at the 0.05 level, the two groups are not considered to be statistically different. If the table value is smaller, the groups are different and the comparison is repeated at lower levels of significance. Thus, the degree of significance may be determined. We might have significant differences at 0.05 but not at 0.01, in which case the probability would be represented at $0.05>p>0.01$. Example 21 demonstrates this case.

Example 21

Suppose that the 400 ppm level from the above example had only 3 dogs, but that the mean for the group and the mean square within groups were the same. To continue Duncan's we would calculate the weighing factors as follows:

100 ppm vs. 0 ppm,

$$200 \text{ ppm vs. } 0 \text{ ppm } N_1 = 4; N_2 = 4 \; a_{ij} = \sqrt{\frac{2(4)(4)}{4+4}} = 2.00,$$

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$$400 \text{ ppm vs. } 0 \text{ ppm } N_2 = 3;\ N_4 = 4 a_{ij} = \sqrt{\frac{2(3)(4)}{3+4}} = 1.852,$$

400 ppm vs. 100 ppm.

Using the $\bar{X}_1 - \bar{X}_2$ from the above example we can set up the following tables:

Concentrations ppm	No. of means apart	$\bar{X}_1 - \bar{X}_2$	aij	$(\bar{X}_1 - \bar{X}_2)\cdot$aij
100 versus 0	2	0.045	2.000	2.000(.045)=.090
200 versus 0	3	0.083	2.000	2.000(.083)=.166
400 versus 0	4	0.137	1.852	1.852(.137)=.254
400 versus 100	3	0.092	1.852	1.852(.092)=.170

Next we calculate SEM2 as being $\sqrt{0.00275} = 0.05244$. This is multiplied by the appropriate table values chosen for 11 d*f*(d*f*wg for this example). This gives the following probability table.

	Probability levels		
No. of means apart	0.05	0.01	0.001
2	0.1632	0.2303	0.3291
3	0.1707	0.2401	0.3417
4	0.1753	0.2463	0.3501

Comparing the "t" values with the probability table values we get the following:

Comparison	Probability
100 ppm vs. 0 ppm	p>0.05
200 ppm vs. 0 ppm	p>0.05
400 ppm vs. 0 ppm	0.01>p>0.001
400 ppm vs. 100 ppm	0.05>p>0.01

ASSUMPTIONS AND LIMITATIONS

1. Duncan's assures a set alpha level or type I error rate for all tests when means are separated by no more than ordered step increases. Preserving this alpha level means that the test is less sensitive than some others, such as the Student-Newman-Keuls. The test is inherently conservative and not resistant or robust.

Scheffe's Multiple Comparisons

Scheffe's is another post hoc comparison method for groups of continuous and randomly distributed data. It also normally involved three or more groups (79, 131). It is widely considered a more powerful significance test than Duncan's.

Each post hoc comparison is tested by comparing an obtained test value (Fcontr) with the appropriate critical F value at the selected level of significance (the table F value multiplied by K–1 for an F with K–1 and N-K degrees of freedom2). Fcontr is computed as follows:

(a) Compute the mean for each sample (group);

(b) Denote the residual mean square by MSwg;

(c) Compute the test statistic as

$$F_{contr} = \frac{C_1\bar{X}_1 + C_2\bar{X}_2 + \ldots + C_k\bar{X}_k^2)}{(K-1)MS_{wg}(C_1^2/n_1 + \ldots + C_K^2/n_k},$$

where Ck is the comparison number such that the sum C1, C2…Ck=0 (see Example 22) and

2Where K=the number of groups and N=the total number of data.

Example 22

At the end of a short-term feeding study, the following body weight changes were recorded:

	Group 1	Group 2	Group 3	
		10.2	12.2	9.2
		8.2	10.6	10.5
		8.9	9.9	9.2
		8.0	13.0	8.7
		8.3	8.1	9.0
		8.0	10.8	
			11.5	
Totals	51.6	76.1	46.6	
Means	8.60	10.87	9.32	

$MS_{wg}=1.395$

To avoid logical inconsistencies with pairwise comparisons, we compare the group having the largest sample mean (group 2) with that having the smallest sample mean (group 1), then with the group having the next smallest sample mean, and so on. As soon as we find a nonsignificant comparison in this process (or no group with a smaller sample mean remains), we replace the group having the largest sample mean with that having the second largest sample mean and repeat the comparison process. Accordingly, our first comparison is between groups 2 and 1. We set $C_1=-1$, $C_2=1$, and $C_3=0$ and calculate our test statistic

$$F_{contr} = \frac{(10.87 - 8.60)^2}{(3-1)1.395(1/6+1/7)} = 5.97.$$

The critical region for F at $p \le 0.05$ for 2 and 11 degrees of freedom is 3.98. Therefore, these groups are significantly different at this level. We next compare groups

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2 and 3, using C1=0, C2=1, and C3=−1:

$$F_{contr} = \frac{(10.87 - 9.32)^2}{(3-1)1.395(1/7+1/5)} = 2.51.$$

This is less than the critical region value, so these groups are not significantly different.

ASSUMPTIONS AND LIMITATIONS

1. The Scheffe procedure is robust to moderate violations of the normality and homogeneity of variance assumptions.
2. It is not formulated on the basis of groups with equal numbers (as one of Duncan's procedures is), and if N1≠N2, there is no separate weighing procedure.
3. It tests all linear contrasts among the population means (the other three methods confine themselves to pairwise comparison, except they use a Bonferroni type correlation procedure).
4. The Scheffe procedure is powerful because of its robustness, yet it is very conservative. Type I error (the false-positive rate) is held constant at the selected test level for each comparison.

Dunnett's t-Test

Dunnett's t-test (45, 46) has as its starting point the assumption that what is desired is a comparison of each of several means with one other mean and only one other mean; in other words, that one wishes to compare each and every treatment group with the control group, but not compare treatment groups with each other. The problem here is that, in toxicology, one is frequently interested in comparing treatment groups with other treatment groups; however, if one does want only to compare treatment groups versus a control group, Dunnett's is a useful approach. In a study with K groups (one of them being the control), we will wish to make K-1 comparisons. In such a situation, we want to have a P level for the entire set of K-1 decisions (not for each individual decision). The Dunnett's distribution is predicated on this assumption. The parameters for using a Dunnett's table, such as found in his original article, are K (as above) and the number of degrees of freedom for mean square with groups (MSwg). The test value is calculated as

$$t = \frac{|T_j - T_i|}{\sqrt{2MS_{wg}/n}}$$

where n is the number of observations in each of the groups. The mean square within group (MSwg) is as we have defined it previously; Tj is the control group mean and Ti is the mean of, in order, each successive test group observation. Note that one uses the absolute value of the positive number resulting from subtracting Ti from Tj. This is to ensure a positive number for our final t.

Example 23 demonstrates this test, again with the data from Example 19.

Example 23

The means, N's, and sums for the groups previously presented in Example 19 are

	Control	100 ppm	200 ppm	400 ppm
Sum (ΣX)	1.38	1.56	1.71	1.93
N	4		44	4
Mean	0.345	0.39	0.4275	0.4825

The MSwg was 0.00275, and our test t for 4 groups and 12 df is 2.41. Substituting in the equation, we calculate our t for the control versus the 400 ppm to be

$$= \frac{|0.345 - 0.4825|}{\sqrt{2(0.00275)/4}}$$

$$= \frac{0.1375}{\sqrt{0.001375}}$$

$$= \frac{0.1375}{0.037081} = 3.708.$$

which exceeds our test value of 2.41, showing that these two groups are significantly different at $p \leq 0.05$. The values for the comparisons of the control versus the 200 and 100 ppm groups are then found to be, respectively, 2.225 and 1.214. Both of these are less than our test value, and therefore the groups are not significantly different.

ASSUMPTIONS AND LIMITATIONS

1. Dunnett's seeks to ensure that the type 1 error rate will be fixed at the desired level by incorporating correction factors into the design of the test value table.
2. Treated group sizes must be approximately equal.

Williams' t-Test

Williams' t-test (164, 165) is also popular, although its use is quite limited in toxicology. It is designed to detect the highest level (in a set of dose/exposure levels) at which there is no significant effect. It assumes that the response of interest (such as change in body weights) occurs at higher levels but not at lower levels and that the responses are monotonically ordered so that $X0 \leq Xi \ldots \leq Xk$. This frequently is not the case, however. The Williams' technique handles the occurrence of such discontinuities in a response series by replacing the offending value and the value immediately preceding it with weighted average values. The test also is adversely affected by any mortality at high dose levels. Such moralities "impose a severe penalty, reducing the power of detecting an effect not only at level K but also at all lower doses" (165, p. 529). Accordingly, it is not generally applicable in toxicology studies.

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Analysis of Covariance

ANCOVA is a method for comparing sets of data that consist of two variables (treatment and effect, with our effect variable being called the "variate") when a third variable (called the "covariate") exists that can be measured but not controlled and that has a definite affect on the variable of interest. In other words, it provides an indirect type of statistical control, allowing us to increase the precision of a study and to remove a potential source of bias. One common example of this is in the analysis of organ weights in toxicity studies. Our true interest here is the effect of our dose or exposure level on the specific organ weights, but most organ weights also increase (in the young, growing animals most commonly used in such studies) in proportion to increases in animal body weight. As we are not interested in the effect of this covariate (body weight), we measure it to allow for adjustment. We must be careful before using ANCOVA, however, to ensure that the underlying nature of the correspondence between the variate and covariate is such that we can rely on it as a tool for adjustments (3, 97).

Calculation is performed in two steps. The first is a type of linear regression between the variate Y and the covariate X. This regression, performed as described under the linear regression section, gives us the model

$$Y=a1+BX+e,$$

which in turn allows us to define adjusted means ($\bar{Y}$ and $\bar{X}$) such that $\bar{Y}_{1a}=\bar{Y}_1-(\bar{X}_1-X^*)$.

If we consider the case where K treatments are being compared such that K=1,2,...K, and we let Xik and Yik represent the predictor and predicted values for each individual i in group k, we can let Xk and Yk be the means. Then, we define the between-group (for treatment) sum of squares and cross products as

$$T_{xx}=\sum_{k-1}^{K}n_k(\bar{X}_K-\bar{X})^2,$$

$$T_{yy}=\sum_{k-1}^{K}n_k(\bar{Y}_K-\bar{Y})^2,$$

$$Txy=\sum_{k-1}^{K}n_k(\bar{X}_k-\bar{X})(\bar{Y}_k-\bar{Y}_k-\bar{Y}).$$

In a like manner, within-group sums of squares and cross products are calculated as

$$\sum xx=\sum_{k=1}^{k}\sum_{i}(X_{ik}-X_k)^2,$$

$$\sum yy=\sum_{k=1}^{k}\sum_{i}(Y_{ik}-Y_k)^2,$$

$$\sum xy=\sum_{k=1}^{k}\sum_{i}(X_{ik}-X_k)(Y_{ik}-Y_k),$$

where i indicates the sum from all the individuals within each group; f′=total number of subjects minus the number of groups

$$Sxx=Txx+\Sigma xx,$$
$$Syy=Tyy+\Sigma xx,$$
$$Sxy=Txy+\Sigma xy.$$

With these in hand, we can then calculate the residual mean squares of treatments (St2) and error (Se2):

$$St^2 = \frac{T_{yy} - \frac{S_{xy}^2}{S_{xx}} + \frac{\sum_{xy}^2}{\sum_{xx}}}{k-1},$$

$$Se^2 = \frac{\left(\sum_{yy} - \frac{\sum_{y}^{2}}{\sum_{xx}}\right)}{f-1.}$$

These can be used to calculate an F statistic to test the null hypothesis that all treatment effects are equal:

$$\mathrm{F} = \frac{\mathrm{St}^2}{\mathrm{Se}^2}.$$

The estimated regression coefficient of Y or X is

$$B = \frac{\sum_{xy}}{\sum_{xx}}.$$

The estimated standard error for the adjusted difference between two groups is given by

$$Sd = Se\frac{1}{n_i} + \frac{1}{n_j} + \frac{(X_i - X_j)^2}{\sum_{xx}},$$

where ni and nj are the sample sizes of the two groups. A test of the null hypothesis that the adjusted differences between the groups is zero is provided by

$$\mathrm{t} = \frac{\mathrm{Y_i - Y_j - B(X_i - X_j)}}{\mathrm{Sd}}.$$

The test value for the t is then looked up in the t-table with f-1 degrees of freedom. Computation is markedly simplified if all the groups are of equal size, as demonstrated in Example 24.

Example 24

An ionophere was evaluated as a potential blood-pressure-reducing agent. Early studies indicated that there was an adverse effect on blood cholesterol and hemoglobin levels, so a special study was performed

to evaluate this specific effect. The hemoglobin (Hgb) level covariate was measured at study start along with the percentage changes in serum triglycerides between study start and at the end of the 13-week study. Was there a difference in effects of the two ionopheres?

Ionophere A		Ionophere B	
Hgb (X)	Serum Triglyceride, %—Change (Y)	Hgb (X)	Serum Triglyceride, %—Change (Y)
7.0	5	5.1	10
6.0	10	6.0	15
7.1	−5	7.2	−15
8.6	−20	6.4	5
6.3	0	5.5	10
7.5	−15	6.0	−15
6.6	10	5.6	−5
7.4	−10	5.5	−10
5.3	20	6.7	−20
6.5	−15	8.6	−40
6.2	5	6.4	
7.8	0	6.0	−10
8.5	−40	9.3	−40
9.2	−25	8.5	−20
5.0	25	7.9	−35
		5.0	0
		6.5	−10

To apply ANCOVA using Hgb as a covariate, we first obtain some summary results from the data as follows:

	Ionophere A (Group 1)	Ionophere B (Group 2)	Combined
Σx	112.00	119.60	231.60
Σx^2	804.14	821.64	1625.78
Σy	−65.00	−185.00	−250.00
Σy^2	4575.00	6475.00	11050.00
Σxy	−708.50	−1506.50	−2215.00
$\bar{x}$	7.000	6.6444	6.8118
$\bar{y}$	−4.625	−10.2778	−7.3529
n	16	18	34

We compute for the ionophere group (i=1):

$$S_{xx}(1)=804.14-(112)^2/16=20.140,$$
$$S_{yy}(1)=4575.00-(-65)^2/16=4310.938,$$
$$S_{xy}(1)=-708.50-(112)(-65)/16=-253.500.$$

Similarly, for the ionophere B group (i=2), we obtain

$$S_{xx}(2)=26.964,$$
$$S_{yy}(2)=4573.611,$$
$$S_{xy}(2)=-277.278.$$

Finally, for the combined data (ignoring groups), we compute

$$S_{xx}=48.175,$$
$$S_{yy}=9211.765,$$
$$S_{xy}=-512.059.$$

The sums-of-squares can now be obtained as

$$TOT(SS) = 9211.8,$$

$$SSE = (20.140 + 26.964)(4310.938 + 4573.611) \frac{-[-253.500 - 277.28]^2}{(20.140 + 26.964)} = 2903.6,$$

$$SSG = \frac{(48.175)(9211.765) - (-512.059)^2}{48.175} - 2903.6 = 865.4,$$

$$SSX = (4310.938 + 473.611) - 2903.6 = 5980.9,$$

and the ANCOVA summary table can be completed as follows:

SOURCE	*df*	*SS*	*MS*	*F*
TREATMENT	1	865.4	865.4	9.2*
X (Hgb)	1	5980.9	5980.9	63.8
Error	31	2903.7	93.7	
Total	33	9211.8		

*Significant ($p<0.05$); critical F-value=4.16.

The F-statistics are formed as the ratios of effect mean-squares (MS) to the MSE (93.7). Each F-statistic is compared with the critical F value with 1 upper and 31 lower degrees of freedom. The critical F-value for $\alpha=0.05$ is 4.16.

The significant covariate effect (F=63.8) indicates that the triglyceride response has a significant linear relationship with HbAlc. The significant F-value for TREATMENT indicates that the mean triglyceride response adjusted for hemoglobin effect differs between treatment groups.

ASSUMPTIONS AND LIMITATIONS

1. The underlying assumptions for ANCOVA are fairly rigid and restrictive. The assumptions include the following:

a. The slopes of the regression lines of a Y and X are equal from group to group. This can be examined visually or formally (i.e., by a test). If this condition is not met, ANCOVA cannot be used.
b. The relationship between X and y is linear.
c. The covariate X is measured without error. Power of the test declines as error increases.
d. There are no unmeasured confounding variables.
e. The errors inherent in each variable are independent of each other. Lack of independence effectively (but to an immeasurable degree) reduces sample size.
f. The variances of the errors in groups are equivalent between groups.
g. The measured data which form the groups are normally distributed. ANCOVA is generally robust to departures from normality.
2. Of the seven assumptions above, the least robust are the first four.

Modeling

The mathematical modeling of biological systems, restricted even to the field of toxicology, is an extremely large and vigorously growing area. Broadly speaking, modeling is the principal conceptual tool by which toxicology seeks to develop as a mechanistic science. In an iterative process, models are developed or proposed, tested by experiment, reformulated, and so on in a continuous cycle. Such a cycle could also be described as two related types of modeling—explanatory (where the concept is formed) and correlative (where data are organized and relationships derived). An excellent introduction to the broader field of modeling of biological systems can be found in Gold (72).

In toxicology, modeling is of prime interest in seeking to relate a treatment variable with an effect variable and, from the resulting model, predict effects at exact points where no experiment has been done (but in the range where we have performed experiments, such as "determining" LD50s) to estimate how good our prediction is, and occasionally, simply to determine if a pattern of effects is related to a pattern of treatment.

For use in prediction, the techniques of linear regression, probit/logit analysis (a special case of linear regression), moving averages (an efficient approximation method), and nonlinear regression (for doses where data cannot be made to fit a linear pattern) are presented. For evaluating the predictive value of these models, both the correlation coefficient (for parametric data) and Kendall's rank correlation (for nonparametric data) are given. Finally, the concept of trend analysis is introduced and a method presented.

When we are trying to establish a pattern between several data points (whether this pattern is in the form of a line or a curve), what we are doing is interpolating. It is possible for any given set of points to produce an infinite set of lines or curves that pass near (for lines) or through (for curves) the data points. In most cases we cannot actually know the "real" pattern, so we apply a basic principle of science—Occam's razor. We use the simplest explanation (or, in this case, model) that fits the facts (or data). A line is, of course, the simplest pattern to deal with and describe, so fitting the best line (linear regression) is the most common form of model in toxicology.

Linear Regression

Foremost among the methods for interpolating within a known data relationship is regression—the fitting of a line or curve to a set of known data points on a graph and the interpolation ("estimation") of this line or curve in areas where we have no data points. The simplest of these regression models is that of linear regression (valid when increasing the value of one variable changes the value of the related variable in a linear fashion, either positively or negatively). This is the case we will explore here, using the method of least squares.

Given that we have two sets of variables, x (say, mg/kg of test material administered) and y (say, percentage of animals so dosed that die), what is required is solving for a and b in the equation $Y_i=a+bx_i$ (where the uppercase Y_i is the fitted value of y_i at x_i, and we wish to minimize $(y_i-Y_i)^2$). So we solve the equations

$$b=\frac{\sum x_1y_1-nx\bar{y}}{\sum x_1^2-n\bar{x}^2}$$

and

$$a=\bar{y}-b\bar{x},$$

where a is the y intercept, b is the slope of the time, and n is the number of data points. Use of this is demonstrated in Example 25.

Note that in actuality, dose-response relationships often are not linear and instead we must use either a

transform (to linearize the data) or a nonlinear regression method (65).
Note also that we can use the correlation test statistic (to be described in the correlation coefficient section) to determine if the regression is significant (and, therefore, valid) at a defined level of certainty. A more specific test for significance would be the linear regression analysis of variance (119). We start by developing the appropriate ANOVA table, as demonstrated in Example 25, then proceed to perform the linear regression portion of the ANOVA, as shown in Example 26.

Example 25

From a short-term toxicity study we have the following results:

Dose Administered (mg/kg)		% Animals dead	
xi,	x_i^2	yi	xiyi
1	1	10	10
3	9	20	60
4	16	18	72
5	25	20	100
Sums x1=13	$x_i^2 = 51$	yi=68	xiyi−242

$$\bar{x} = 3.25, \quad \bar{y} = 17,$$

$$b = \frac{242 - (4)(3.25)(17)}{51 - (4)(10.5625)} = \frac{21}{8.75} = 2.40,$$

$$a = 17 - (2.4)(3.25) = 9.20.$$

We therefore see that our fitted line is Y=9.2+2.4X.
These ANOVA table data are then used as shown in Example 26.

Linear regression analysis of variance

Source of variation (1)	Sum of squares (2)	Degrees of freedom (3)	Mean square (4) equal to $\frac{2}{3}$)
Regression	$b_1^2(\sum x_1^2 - n\bar{x}^2)$	1	By division
Residual	By difference	n−2	By division
Total	$\sum y_1^2 - n\bar{y}^2$	n−1	

We then calculate

$$F_{1,n-2} = \frac{\text{regression mean square}}{\text{residual mean square}}.$$

Example 26
We desire to test the significance of the regression line in Example 25:

$$\sum y_i^2 = 10^2 + 20^2 + 18^2 + 20^2,$$

Regression $SS=(2.4)^2[51-4(3.25)^2]=50.4$,
Total $SS=1224-4(17^2)=68.0$,
Residual $SS=68.0-50.4=17.6$,
$F_{1,2}=50.4/8.8=5.73$.

This value is not significant at the 0.05 level; therefore, the regression is not significant. A significant F value (as found in an F distribution table for the appropriate degrees of freedom) indicates that the regression line is an accurate prediction of observed values at that confidence level. Note that the portion of the total sum of squares explained by the regression is called the coefficient of correlation, which in the above example is equal to 0.862 (or 0.74). Calculation of the correlation coefficient is described later in this chapter.

Finally, we might wish to determine the confidence intervals for our regression line (i.e., given a regression line with calculated values for Yi given xi, within what limits may we be certain [with say a 95% probability] what the real value of Yi is)?

If we denote the residual mean square in the ANOVA by s^2, the 95% confidence limits for *a* (denoted by A, the notation for the true—as opposed to the estimated—value for this parameter) are calculated as

$$t_{n-2} = \frac{a - A}{\sqrt{\frac{s^2(\sum x^2)}{n\sum x_1^2 - n^2\bar{x}^2}}},$$

$$\frac{9.2 - \mathrm{A}}{\sqrt{\frac{8.8(51)}{4(51) - (16)(10.562)}}} = \frac{9.2 - \mathrm{A}}{\sqrt{\frac{448}{35.008}}}$$

$$= \frac{9.2 - \mathrm{A}}{3.58} = \pm 4.303,$$

$$9.2 - \mathrm{A} = \pm 15.405,$$

$$A = 9.2 \pm 15.405.$$

ASSUMPTIONS AND LIMITATIONS

1. All the regression methods are for interpolation, not extrapolation. That is, they are valid only in the range that we have data—the experimental region—not beyond.
2. The method assumes that the data are independent and normally distributed and it is sensitive to outliers. The x-axis (or horizontal) component plays an extremely important part in developing the least square fit. All points have equal weight in determining the height of a regression line, but extreme x-axis values unduly influence the slope of the line.
3. A good fit between a line and a set of data (i.e., a strong correlation between treatment and response variables) does not imply any casual relationship.
4. It is assumed that the treatment variable can be measured without error, that each data point is independent, that variances are equivalent, and that a linear relationship does not exist between the variables.
5. There are many excellent texts on regression, which is a powerful technique, including (53, 54), which are not overly rigorous mathematically.

Probit/Log Transforms and Regression

As we noted in the preceding section, dose-response problems (among the most common interpolation prob-

lems encountered in toxicology) rarely are straightforward enough to make a valid linear regression directly from the raw data. The most common valid interpolation methods are based on probability ("probit") and logarithmic ("log") value scales, with percentage responses (death, tumor incidence, etc.) being expressed on the probit scale whereas doses (Yi) are expressed on the log scale. There are two strategies for such an approach. The first is based on transforming the data to these scales, then doing a weighted linear regression on the transformed data (if one does not have access to a computer or a high-powered programmable calculator, the only practical strategy is not to assign weights). The second requires the use of algorithms (approximate calculation techniques) for the probit value and regression process and is extremely burdensome to perform manually.

Our approach to the first strategy requires that we construct a table with the pairs of values of xi and yj listed in order of increasing values of Yi (percentage response). Beside each of these columns a set of blank columns should be left so that the transformed values may be listed. We then simply add the columns described in the linear regression procedure. Log and probit values may be taken from any of a number of sets of tables and the rest of the table is then developed from these transformed x_i' and y_i' values (denoted as x_i' and y_i' A standard linear regression is then performed (see Example 27).

The second strategy we discussed has been broached by a number of authors (16, 32, 103, 12). All of these methods, however, are computationally cumbersome. It is possible to approximate the necessary iterative process using the algorithms developed by Abramowitz and Stegun (1), but even this merely reduces the complexity to a point where the procedure may be readily programmed on a small computer or programmable calculator.

Example 27

Percentage of animals killed xi	Probit of xi =xi	Dose of chemical (mg/kg) yi	Log of yi= y_i'	$(x_i')^2$	$x_i'y_i'$
2	2.9463	3	0.4771	8.6806	1.40568
10	3.7184	5	0.6990	13.8264	2.59916
42	4.7981	10	1.0000	23.0217	4.79810
90	6.2816	20	1.3010	39.4585	8.17223
98	7.2537	30	1.4771	52.6162	10.4190
	Σxi=24.9981			$\sum x_i'^2 = 137.6034$	$\sum x_i'y_i' = 27.68974$

Our interpolated log of the LD50 (calculated by using Y=−0.200591−0.240226 x, where x equals 5.000—the probit of 50%—in the regression equation) is 1.000539. When we convert this log value to its linear equivalent, we get an LD50 of 10.0 mg/kg.

Finally, our calculated correlation coefficient is r=0.997. A goodness-of-fit of the data using chi-square may also be calculated.

ASSUMPTIONS AND LIMITATIONS

1. The probit distribution is derived from a common error function, with the midpoint (50% point) moved to a score of 5.00.
2. The underlying frequency distribution becomes asymptotic as it approaches the extremes of the range. That is, in the range of 16%−84%, the corresponding probit values change gradually—the curve is relatively linear, but beyond this range they change ever more rapidly as they approach either 0% or 100%. In fact, there are no values for either of these numbers.
3. A normally distributed population is assumed, and the results are sensitive to outliers.

Moving Averages

An obvious drawback to the interpolation procedures we have examined to date is that they do take a significant amount of time (though they are simple enough to be done manually), especially if the only result we desire is an LD50, LC50, or LT50.

The method of moving averages (144, 161) gives a rapid and reasonable accurate estimate of this "median-effective-dose" (m) and the estimated SD of its logarithm.

Such methodology requires that the same number of animals be used per dosage level and that the spacing between successive dosage exposure levels be geometrically constant (i.e., levels of 1, 2, 4, and 8 mg/kg or 1, 3, 9, and 27 ppm). Given this and access to a table for the computation of moving averages, one can readily calculate the median effective dose with the formula (illustrated for dose):

log m=log D+d(K−1)/2+df, where m=median effective dose or exposure.

D=the lowest dose tested.

$\sum y_i' = 4.9542$

d=the log of the ratio of successivedoses/ exposures.

f=a table value taken from Gad (62) for the proper K (the total number of levels tested minus 1).

Example 28 demonstrates the use of this method.

Example 28

As part of an inhalation study, we exposed groups of 5 rats each to levels of 20,40, 80 and 160 ppm of a chemical vapor. These exposures killed 0, 1, 3, and 5 animals, respectively. From the N=5, K=3 tables on the r value 0, 1, 3, 5 line, we get an f of 0.7 and an σ_f^4 of 0.31623. We can then calculate the LC50 to be

LogLC50=1.30130+0.30103(2)/2+0.30103(0.7)
=1.30103+0.51175
=1.81278,
∴ LC50=65.0 ppm with 95% confidence intervals of
± 2.179 $d\sigma f$ or ±2.179(0.30103)(0.31623)
=±0.20743.

Therefore, the log confidence limits are 1.81278
± 0.20743=1.60535 to 2.02021; on the linear scale
=40.3 to 104.8 ppm

ASSUMPTIONS AND LIMITATIONS

1. A common misconception is that the moving average method cannot be used to determine the slope of the response curve. This is not true. Weil has published a straightforward method for determining slope in conjunction with a moving average determination of the LD50 (161).
2. The method also provides confidence intervals.

Nonlinear Regression

More often than not in toxicology we find that our data demonstrate a relationship between two variables (such as age and body weight) that are not linear. That is, a change in one variable (say age) does not produce a directly proportional change in the other (e.g., body weight), but some form of relationship between the variables is apparent. If understanding such a relationship and being able to predict unknown points is of value, we have a pair of options available to us. The first, which was discussed and reviewed earlier, is to use one or more transformations to linearize our data and then to make use of linear regression. This approach, though most commonly used, has a number of drawbacks. Not all data can be suitably transformed; sometimes the transformations necessary to linearize the data require a cumbersome series of calculations, and the resulting linear regression is not always sufficient to account for the differences among sample values—there are significant deviations around the linear regression line (i.e., a line may still not give us a good fit to the data or do an adequate job of representing the relationship between the data). In such cases we have available a second option—the fitting of data to some nonlinear function such as some form of the curve. This is, in general form, nonlinear regression and may involve fitting data to an infinite number of possible functions, but most often we are interested in fitting curves to a polynomial function of the general form

Y=a+bx+cx2+dx2+...,

where x is the independent variable. As the number of powers of x increases, the curve becomes increasingly complex and will be able to fit a given set of data increasingly well. Generally in toxicology, however, if we plot the log of a response (such as body weight) versus a linear scale of our dose or stimulus, we get one of four types of nonlinear curves. These are (137)

(1) Exponential growth, where
log Y=A(Bx), such as the growth curve for the log phase of a bacterial culture.
(2) Exponential decay, where
log Y=A(B−x), such as a radioactive decay curve.
(3) Asymptotic regression, where
log Y=A−B(px), such as a first-order reaction curve.
(4) Logistic growth curve, where
log Y=A/(1+Bpx), such as a population growth curve.

In all these cases, A and B are constant whereas p is a log transform. These curves are illustrated in Figure 7.7.

All four types of curves are fit by iterative processes (i.e., best guess numbers are initially chosen for each of the constants and, after a fit is attempted, the constants are modified to improve the fit). This process is repeated until an acceptable fit has been generated. Analysis of variance or covariance can be

used to objectively evaluate the acceptability of it. Needless to say, the use of a computer generally accelerates such a curve-fitting process.

ASSUMPTIONS AND LIMITATIONS

1. The principle of using least squares may still be applicable in fitting the best curve if the assumptions of normality, independence, and reasonably error-free measurement of response are valid.
2. Growth curves are best modeled using a nonlinear method.

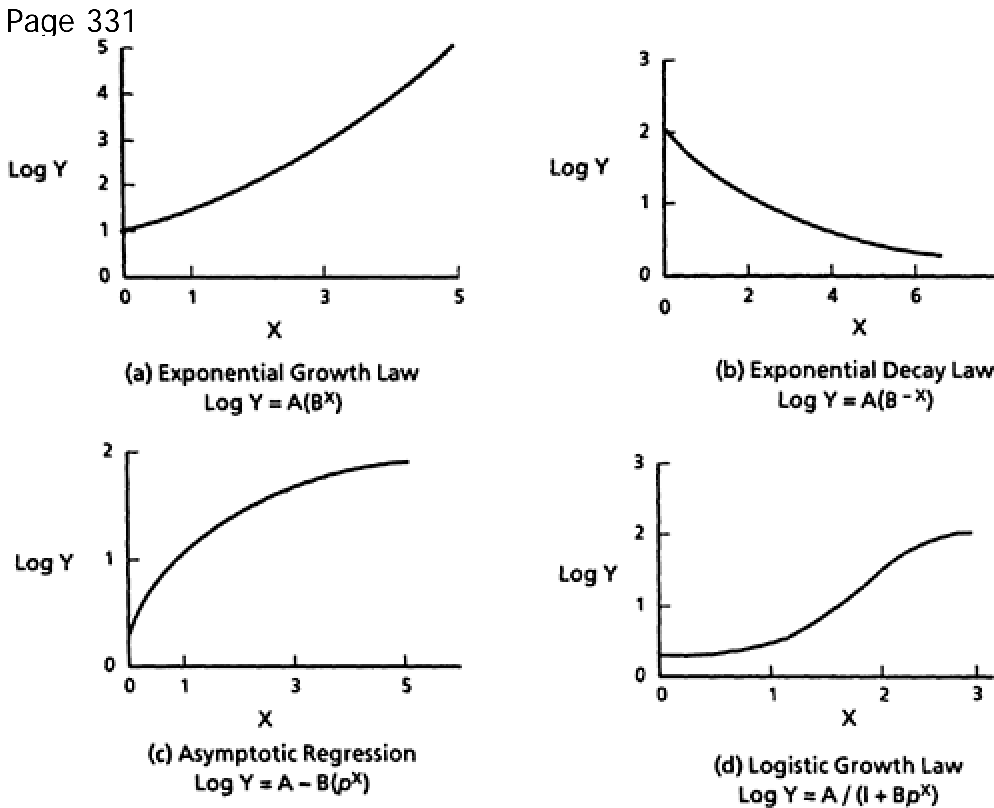

FIG. 7.7. Common curvilinear curves.

Correlation Coefficient

The correlation procedure is used to determine the degree of linear correlation (direct relationship) between two groups of continuous (and normally distributed) variables. It will indicate whether there is any statistical relationship between the variables in the two groups. For example, we may wish to determine if the liver weights of dogs on a feeding study are correlated with their body weights. Thus, we will record the body and liver weights at the time of sacrifice and then calculate the correlation coefficient between these pairs of values to determine if there is some relationship.

A formula for calculating the linear correlation coefficient (rxy) is as follows:

$$r_{xy} = \frac{N\sum XY - (\sum X)(\sum Y)}{\sqrt{N\sum X^2 - (\sum X)^2}\sqrt{N\sum Y^2 - (\sum Y)^2}},$$

where X is each value for one variable (such as the dog body weights in the above example), Y is the matching value for the second variable (the liver weights), and N is the number of pairs of X and Y. Once we have obtained rxy, it is possible to calculate tr, which can be used for more precise examination of the degree of significant linear relationship between the two groups. This value is calculated as follows:

$$t_r = \frac{r_{zy}\sqrt{N-2}}{\sqrt{1 - r_{zy}^2}}.$$

This calculation is also equivalent to r=sample covariance/(SxSy), as was seen earlier under ANCOVA. The value obtained for rxy can be compared to table values (137) for the number of pairs of data involved minus two. If the rxy is smaller (at the selected test probability level, such as 0.05), the correlation is not significantly different from zero (no correlation). If rxy is larger than the table value, there is a positive statistical relationship between the groups. Comparisons are then made at lower levels of probability to determine the degree of relationship (note that if rxy=either 1.0 or −1.0, there is complete correlation between the groups). If rxy is a negative number and the absolute is greater than the table value, there is an inverse relationship between the groups (i.e., a change in one group is associated with a change in the opposite direction in the second group of variables). Both computations are demonstrated in Example 29.

Because the comparison of rxy with the table values may be considered a somewhat weak test, it is perhaps more meaningful to compare the tr value with values in a t-distribution table for N-2 degrees of freedom (df), as is done for the Student's t-test. This will give a more exact determination of the degree of statistical correlation between the two groups.

Note that this method examines only possible linear relationships between sets of continuous, normally distributed data.

Example 29

If we computed the dog body weight versus dog liver weight for a study, we could have the following results:

	Body weight (kgj)		Liver weight (g)		
Dog#	X	X2	Y	Y2	XY
1	8.4	70.56	243	59049	2041.2
2	8.5	72.25	225	50625	1912.5
3	9.3	86.49	241	58081	2241.3
4	9.5	90.25	263	69169	2498.5
5	10.5	110.25	256	65536	2688.0
6	8.6	73.96	266	70756	2287.6
Sums	ΣX=54.8	ΣX2=503.76	ΣY=1494	ΣY2=373216	ΣXY=13669.1

$$r_{xy} = \frac{6(13669.1) - (54.8)(1494)}{\left(\sqrt{6(503.76) - (54.8)^2}\right)\left(\sqrt{6(373216) - (1494)^2}\right)}$$

$$= 0.381.$$

The table value for six pairs of data (read beside the N-2 value, or 6–2=4) is 0.811 at a 0.05 probability level. Thus, there is a lack of statistical correlation (at p=0.05) between the body weights and liver weights for this group of dogs.

The tr value for these data would be calculated as follows:

$$t_r = \frac{0.381\sqrt{6-2}}{\sqrt{1-(0.381)^2}} = 0.824.$$

The value for the t-distribution table for four df at the 0.05 level is 2.776; therefore, this again suggests a lack of significant correlation at p=0.05.

ASSUMPTIONS AND LIMITATIONS

1. A strong correlation does not imply that a treatment causes an effect.
2. The distances of data points from the regression line are the portions of the data not "explained" by the model. These are called residuals. Poor correlation coefficients imply high residuals, which may be due to many small contributions (variations of data from the regression line) or a few large ones. Extreme values (outliers) greatly reduce correlation.
3. X and Y are assumed to be independent.
4. Feinstein (55) has provided a fine discussion of the difference between correlation (or association of variables) and causation.

Kendall's Coefficient of Rank Correlation

Kendall's rank correlation, represented by τ(tau), should be used to evaluate the degree of association between two sets of data when the nature of the data is such that the relationship may not be linear. Most commonly, this is when the data are not continuous and/or normally distributed. An example of such a case is when we are trying to determine if there is a relationship between the length of hydra and their survival time in a test medium in hours, as is presented in Example 30. Both of our variables here are discontinuous, yet we suspect a relationship exists. Another common use is in comparing the subjective scoring done by two different observers.

Tau is calculated at τ=N/n(n−1), where n is the sample size and N is the count of ranks, calculated as N=4(nCi)−n(n−l), with the computing of nCi being demonstrated in the example.

If a second variable Y2 is exactly correlated with the first variable Y1, then the variates Y2 should be in the same order as the Y1 variates; however, if the correlation is less than exact, the order of the variates Y2 will not correspond entirely to that of Y. The quantity N measures how well the second variable corresponds to the order of the first. It has maximum value of n(n−1) and a minimum value of

$-n(n-1)$.

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A table of data is set up with each of the two variables being ranked separately. Tied ranks are assigned as demonstrated earlier under the Kruskal-Wallis test. From this point, disregard the original variates and deal only with the ranks. Place the ranks of one of the two variables in rank order (from lowest to highest), paired with the rank values assigned for the other variable. If one (but not the other) variable has tied ranks, order the pairs by the variables without ties (138).

The most common way to compute a sum of the counts is also demonstrated in Example 30. The resulting value of tau will range from −1 to+1, as does the familiar parametric correlation coefficient, *r*.

Example 30

During the validation of an in vitro method, it was noticed that larger hydra seem to survive longer in test media than do small individuals. To evaluate this, 15 hydra of random size were measured (mm) and then placed in test media. How many hours each individual survived was recorded over a 24-hour period. These data are presented below, along with ranks for each variable:

Length	Rank(R1)	Survival	Rank(R2)
3	6.5	19	9
4	10	17	7
6	15	11	1
1	1.5	25	15
3	6.5	18	8
3	6.5	22	12
1	1.5	24	14
4	10	16	6
4	10	15	5
2	3.5	21	11
5	13	13	3
5	13	14	4
3	6.5	20	10
2	3.5	23	13
5	13	12	2

We then arrange this based on the order of the rank of survival time (there are no ties here). We then calculate our counts of ranks. The conventional method is to obtain a sum of the counts, Ci, as follows: examine the first value in the column of ranks paired with the ordered column. In the following case this is rank 15. Count all ranks subsequent to it that ranks greater than 15. There are 14 ranks following the 2 and all of them are less than 15. Therefore, we count a score of C1=0. We repeat this process for each subsequent rank of R1, giving us a final score of 1 (by this point it is obvious that our original hypothesis—that larger hydrae live longer in test media than do small individual—was in error).

R2	R1	Following (R2) ranks greater than (R1)	Counts (Ci)
1	15	—	C1=0
2	13	—	C2=0
3	13	—	C3=0
4	13	—	C4=0
5	10	—	C5=0
6	6.5	10	C6=0
7	10	—	C7=0
8	6.5	—	C8=0
9	6.5	—	C9=0
10	6.5	—	C10=0
11	3.5	6.5	C11=0
12	6.5	—	C12=0
13	3.5	—	C13=0
14	1.5	—	C14=0
15	1.5	—	C15=0
			Ci=1

Our count of ranks, N, is then calculated as

$$N=4(1)-15(15-1)$$
$$=4-15(14)$$
$$=-206.$$

We can then calculate tau as

$$= \frac{-206}{15(15-1)}$$

$$= \frac{-206}{210}$$

$$= -0.9810.$$

In other words, there is a strong negative correlation between our variables.

ASSUMPTIONS AND LIMITATIONS

1. A very robust estimator that does not assume normality, linearity, or minimal error of measurement.

Trend Analysis

Trend analysis is a collection of techniques that have been "discovered" by toxicology since the mid-1970s (141). The actual methodology dates back to the mid-1950s (29).

Trend analysis methods are a variation on the theme of regression testing. In the broadcast sense, the methods are used to determine whether a sequence of observations taken over an ordered range of a variable (most commonly time) exhibit some form of pattern of change (either an increase-upward trend) associated with another

variable of interest (in toxicology, some form or measure of dosage or exposure).
Trend corresponds to sustained and systematic variations over a long period of time. It is associated with the structural causes of the phenomenon in question (e.g., population growth, technological progress, new ways of organization, or capital accumulation).
The identification of trend has always posed a serious statistical problem. The problem is not one of mathematical or analytical complexity but of conceptual complexity. This problem exists because the trend, as well as the remaining components of a time series, are latent (nonobservable) variables, and therefore, assumptions must be made on their behavioral pattern. The trend is generally thought of as a smooth and slow movement over a long term. The concept of "long" in this connection is relative, and what is identified as trend for a given series span might well be part of a long cycle once the series is considerably augmented. Often, a portion of a long cycle is treated as a trend because the length of the observed time series is shorter than one complete cycle.
The ways in which data are collected in toxicology studies frequently serve to complicate trend analysis, as the length of time for the phenomena underlying a trend to express themselves is frequently artificially censored. To avoid the complexity of the problem posed by a statistically vague definition, statisticians have resorted to two simple solutions. One consists of estimating trend and cyclical fluctuations together, calling this combined movement *trend-cycle;* the other consists of defining the trend in terms of the series length, denoting it as the longest nonperiodic movement.

Trend Models

Within the large class of models identified for trend, we can distinguish two main categories: deterministic trends and stochastic trends. Deterministic trend models are based on the assumption that the trend of a time series can be approximated closely by simple mathematical functions of time over the entire span of the series. The most common representation of a deterministic trend is by means of polynomials or of transcendental functions. The time series from which the trend is to be identified is assumed to be generated by a nonstationary process where the nonstationarity results from a deterministic trend. A classical model is the regression or error model (4), where the observed series is treated as the sum of a systematic part or trend and a random part or irregular part. This model can be written as

$$Z_t = Y_t + U_t',$$

where U_1 is a purely random process; that is, U_t ~i.i.d. $(0, \sigma_u^2)$ (independent and identically distributed with expected value 0 and variance σ_u^2).
Trend tests generally are described as "k-sample tests of the null hypothesis of identical distribution against an alternative of linear order"; i.e., if sample I has distribution function F1, i= 1, then the null hypothesis

$$H-:F1=F2-\ldots=Fk$$

is tested against the alternative

$$H1:F1>F2>\ldots=Fk$$

(or its reverse); there, at least one of the inequalities is strict. These tests can be thought of as special cases of tests of regression or correlation in which association is sought between the observations and its ordered sample index. They are also related to analysis of variance except that the tests are tailored to be powerful against the subset of alternatives H1, instead of the more general set $\{F1 \neq Fj, \text{some } i \neq j\}$.
Different tests arise from requiring power against specific elements or subsets of this rather extensive set of alternatives. The most popular trend test in toxicology is currently that presented by Tarone (141) in 1975 because it is that used by the National Cancer Institute in the analysis of carcinogenicity data. The Armitage and Doll method also is recommended by UIS and Canadian regulatory agencies. A simple but efficient alternative is the Cox and Stuart test (29) which is a modification of the sign test. For each point at which we have a measure (such as the incidence of animals observed with tumors), we form a pair of observations—one from each of the groups we wish to compare. In a traditional National Cancer Institute (NCI) bioassay this would mean pairing control with low dose and low dose with high dose (to explore a dose-related trend) or each time period observation in a dose group (except the first) with its predecessor (to evaluate time-related trend). When the second observation in a pair exceeds the earlier observation, we record a plus sign for that pair. When the first observation is greater than the second, we record a minus sign for that pair. A preponderance of plus signs suggests a downward trend whereas an excess of minus signs suggests an upward trend. A formal test at a preselected confidence

level can then be performed.

More formally put, after having defined what trend we want to test for, we first match pairs as (X_1-X_{1+c}), (X_2, X_{2+C}),...$(X_{n'-c}, X_{n'})$, where $c=n'/2$ when n' is even and $c=(n'+l)/2$ when n' is odd (where n' is the number of observations in a set). The hypothesis is then tested by comparing the resulting number of excess positive or negative signs against a sign test table such as are found in Beyer.

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Month of Study	Control Total X Animal with Tumors	Change (XA−B)	Low Doses Total Y Animals with Tumors	Change (YA−B)	Compared to Control (Y−X)	Total Z Animals with Tumors	Change (Za−b)	Compared to Control (Z-X)
12(A)	1	NA	0	NA	NA	5	NA	NA
13(B)	1	0	0	0	0	7	2	(+)2
14(C)	3	2	1	1	(−)1	11	4	(+)2
15(D)	3	0	1	0	0	11	0	0
16(E)	4	1	1	0	(−)1	13	2	(+)1
17(F)	5	1	3	2	(+)1	14	1	0
18(G)	5	0	3	0	0	15	1	(+)1
19(H)	5	0	5	2	(+)2	18	3	(+)3
20(1)	6	1	6	1	0	19	1	0
21(J)	8	2	7	1	(−)1	22	3	(+)1
22(K)	12	4	9	2	(−)2	26	4	0
23(L)	14	2	12	3	(+)1	28	2	0
24(M)	18	4	17	5	(+)1	31	3	(−)1
				Sum of signs Y−X	4+ 4− =0 (No trend)		Sum of signs Z-X	6+ 1− =5

Reference to a sign table is not necessary for the low-dose comparison (where there is no trend), but clearly shows the high dose to be significant at the p≤0.5 level.

We can, of course, combine a number of observations to allow ourselves to actively test for a set of trends, such as the existence of a trend of increasing difference between two groups of animals over a period of time. This is demonstrated in Example 31.

Example 31

In a chronic feeding study in rats, we tested the hypothesis that in the second year of the study there as a dose-responsive increase in tumor incidence associated with the test compound. We utilize below a Cox-Stuart test for trend to address this question. All groups start the second year with an equal number of animals.

ASSUMPTIONS AND LIMITATIONS

1. Trend tests seek to evaluate whether there is monotonic tendency in response to a change in treatment. That is, the dose response direction is absolute—as dose goes up, the incidence of tumors increases. Thus, the test loses power rapidly in response to the occurrences of “reversals”—for example, a low-dose group with a decreased tumor incidence. There are methods (47) that “smooth the bumps” of reversals in long data series. In toxicology, however, most data series are short (i.e., there are only a few dose levels).

Tarone’s trend test is most powerful at detecting dose-related trends when tumor onset hazard functions are proportional to each other. For more power against other dose-related group differences, weighted versions of the statistic are also available (19, 32).

In 1985, the United States *Federal Register* (53) recommended that the analysis of tumor incidence data is carried out with a Cochran-Armitage (8, 27) trend test.

The test statistic of the Cochran-Armitage test is defined as this term:

$$T_{CA} = \sqrt{\frac{N}{((N-r))r} \cdot \frac{\sum_{i=0}^{k}\left(R_1 - \frac{n_1}{N}r\right)d_1}{\sqrt{\sum_{i=0}^{k}\frac{n_i}{N}d_i^2 - \left(\sum_{i=0}^{k}\frac{n_i}{N}d_1\right)^2}}}$$

with dose scores *di.* Armitage’s test statistic is the square of this term (T^2_{CA}). As one-sided tests are carried out for an increase of tumor rates, the square is not considered. Instead, the above-mentioned test statistic, which is presented by Portier and Hoel (120), is used. This test statistic is asymptotically standard normal distributed. The Cochran-Armitage test is asymptotically efficient for all monotone alternatives (141) but this result only

holds asymptotically. Tumors are rare events, so the binominal proportions are small. In this situation approximations may become unreliable. Therefore, exact tests can be performed using two different approaches: conditional and unconditional are considered. In the first case, the total number of tumors *r* is regarded as fixed. As a result, the null distribution of the test statistic is independent of the common probability *p*. The exact conditional null distribution is a multivariate hypergeometric distribution.
The unconditional model treats the sum of all tumors as a random variable. Then the exact unconditional null distribution is a multivariate binomial distribution. The distribution depends on the unknown probability.

METHODS FOR THE REDUCTION OF DIMENSIONALITY

Techniques for the reduction of dimensionality are those that simplify the understanding of data, either visually or numerically, while causing only minimal reductions in the amount of information present. These techniques operate primarily by pooling or combining groups of variables into single variables, but may also entail the identification and elimination of low-information-content (or irrelevant) variables.
Descriptive statistics (calculations of means, SDs, etc.) are the simplest and most familiar form of reduction of dimensionality. Here we first need to address classification, which provides the general conceptual tools for identifying and quantifying similarities and differences between groups of things that have more than a single linear scale of measurement in common (e.g., which have both been determined to have or lack a number of enzyme activities). Then we will consider two collections of methodologies that combine graphic and computational methods, multidimensional/nonmetric scaling, and cluster analysis. Multidimensional scaling (MDS) is a set of techniques for quantitatively analyzing similarities, dissimilarities, and distances between data in a display-like manner. Nonmetric scaling is an analogous set of methods for displaying and relating data when measurements are nonquantitative (the data are described by attributes or ranks). Cluster analysis is a collection of graphic and numerical methodologies for classifying things based on the relationships between the values of the variables that they share.
The final pair of methods for reduction of dimensionality that will be tackled in this chapter are Fourier analysis and the life table analysis. Fourier analysis seeks to identify cyclic patterns in data and then either analyze the patterns or the residuals after the patterns are taken out. Life table analysis techniques are directed to identifying and quantitating the time course of risks (such as death or the occurrence of tumors).

Classification

Classification is both a basic concept and a collection of techniques that are necessary prerequisites for further analysis of data when the members of a set of data are (or can be) each described by several variables. At least some degree of classification (which is broadly defined as the dividing of the members of a group into smaller groups in accordance with a set of decision rules) is necessary prior to any data collection. Whether formally or informally, an investigator has to decide which things are similar enough to be counted as the same and develop rules for governing collection procedures. Such rules can be simple as “measure and record body weights only of live animals on study,” or as complex as that demonstrated by the expanded weighting classification presented in Example 32. Such a classification also demonstrates that the selection of which variables to measure will determine the final classification of data.

Example 32

I.	Is animal of desired species?	Yes/No
II.	Is animal member of study group?	Yes/No
III.	Is animal alive?	Yes/No
IV.	Which group does animal belong to?	
	A. Control	
	B. Low dose	
	C. Intermediate dose	
	D. High dose	
V.	What sex is animal?	Male/Female
VI.	Is the measured weight in acceptable range?	Yes/No

Classifications of data have two purposes (73, 81): data simplification (also called a descriptive function) and prediction. Simplification is necessary because there is a limit to both the volume and complexity of data that the human mind can comprehend and deal with conceptually. Classification allows us to attach a label (or name) to each group of data, to summarize the data (i.e., assign individual elements of data to groups and to characterize the population of the group), and to define the relationships between

groups (i.e., develop a taxonomy).

Prediction, meanwhile, is the use of summaries of data and knowledge of the relationships between groups to develop hypotheses as to what will happen when further data are collected (as when more animals or people are exposed to an agent under defined conditions) and as to the mechanisms which cause such relationships to develop. Indeed, classification is the prime device for the discovery of mechanisms in all of science. A classic example of this was Darwin's realization that there were reasons (the mechanisms of evolution) behind the differences and similarities in species that had caused Linaeus

to earlier develop his initial modern classification scheme (or taxonomy) for animals.
To develop a classification, one first sets bounds wide enough to encompass the entire range of data to be considered but not unnecessarily wide. This is typically done by selecting some global variables (variables every piece of data has in common) and limiting the range of each so that it just encompasses all of the cases on hand. Then one selects a set of local variables (characteristics which only some of the cases have, say, the occurrence of certain tumor types, enzyme activity levels, or dietary preferences) which thus serve to differentiate between groups. Data are then collected, and a system for measuring differences and similarities is developed. Such measurements are based on some form of measurement of distance between two cases (x and y) in terms of each single variable scale. If the variable is a continuous one, then the simplest measure of distance between two pieces of data is the Euclidean distance, (d[x, y]), defined as

$$= \frac{0.1375}{0.037081} = 3.708.$$

For categorical or discontinuous data, the simplest distance measure is the matching distance, defined as:

$$d(x, y) = \text{number of times } x_i \neq y_i.$$

After we have developed a table of such distance measurements for each of the local variables, some weighting factor is assigned to each variable. A weighting factor seeks to give greater importance to those variables that are believed to have more relevance or predictive value. The weighted variables are then used to assign each piece of data to a group. The actual act of developing numerically based classifications and assigning data members to them is the realm of cluster analysis and will be discussed later in this chapter. Classification of biological data based on qualitative factors has been well discussed (70, 73) and does an excellent job of introducing the entire field and mathematical concepts.
Relevant examples of the use of classification techniques range from the simple to the complex. Schaper et al. (130) developed and used a very simple classification of response methodology to identify those airborne chemicals which alter the normal respiratory response induced by CO_2. At the other end of the spectrum, Kowalski and Bender (98) developed a more mathematically based system to classify chemical data (a methodology they termed *pattern recognition*).

Statistical Graphics

The use of graphics in one form or another in statistics is the single most effective and robust statistical tool, and at the same time, one of the most poorly understood and improperly used. Graphs are used in statistics (and in toxicology) for one of four major purposes. Each of the four is a variation on the central theme of making complex data easier to understand and use. These four major functions are exploration, analysis, communication and display of data, and graphical aids. Exploration (which may be simply summarizing data or trying to expose relationships between variables) is determining the characteristics of data sets and deciding on one or more appropriate forms of further analysis, such as the scatter plot. Analysis is the use of graphs to formally evaluate some aspect of the data, such as whether there are outliers present or if an underlying assumption of a population distribution is fulfilled. As long ago as 1960 (5), some 18 graphical methods for analyzing multivariate data relationships were developed and proposed.
Communication and display of data are the most commonly used function of statistical graphics in toxicology, whether used for internal reports, presentations at meetings, or formal publications in the literature. In communicating data, graphs should not be used to duplicate data that are presented in tables, but rather to show important trends and/or relationships in the data. Though such communication is most commonly of a quantitative compilation of actual data, it can also be used to summarize and present the results of statistical analysis. The fourth and final function of graphics is one that is largely becoming outdated as microcomputers become more widely available. Graphical aids to calculation include nomograms (the classic example in toxicology of a nomogram is that presented by Litchfield and Wilcoxon for determining median effective doses) and extrapolating and interpolating data graphically based on plotted data.
There are many forms of statistical graphics (a partial list, classified by function, is presented in Table 7.6), and a number of these (such as scatter plots and histograms) can be used for each of a number of possible functions. Most of these plots are based on a Cartesian system (i.e., they use a set of rectangular coordinates), and our review of construction and use will focus on these forms of graphs.
'Construction of a rectangular graph of any form starts with the selection of the appropriate form of graph followed by the laying out of the coordinates (or axes). Even graphs that are going to encompass

multivariate data (i.e., more than two variables) generally have as their starting point two major coordinates. The vertical axis, or ordinate (also called the Y axis), is used to present an independent variable. Each of these axes is scaled in the units of measure that will most clearly present the trends of interest in the data. The range covered by the scale of each axis is selected to cover the entire region for which data is presented. The actual demarking of

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Table 7.6

Forms of statistical graphics (by function)

Data Summary	Two Variables	Three or More Variables
EXPLORATION		
Box and whisker plot	Autocorrelation plot	Biplot
Histogram	Cross-correlation plot	Cluster trees
Dot-array diagram	Scatter plot	Labeled scatter plot
Frequency polygon	Sequence plot	Glyphs and metroglyphs
Ogive		Face plots
Stem and leaf diagram		Fourier plots
		Similarity and preference maps
		Multidimensional scaling displays
		Weathervane plot
ANALYSIS		
Distribution Assessment	Model Evaluation and Assumption Verification	Decision Making
Probability plot	Average versus standard deviation	Control chart
Q-Q plot	Component-plus-residual plot	Cusum chart
P-P plot	Partial-residual plot	Half-normal plot
Hanging histogram	Residual plots	Ridge trace
Rootagram		Youden plot
Poissonness plot		
COMMUNICATION AND DISPLAY OF DATA		
Quantitative Graphics	Summary of Statistical Analyses	Graphical Aids
Line chart	Means plot	Confidence limits
Pictogram	Sliding reference distribution	Graph paper
Pie chart	Notched box plot	Power curves
Contour plot	Factor space/response	Nomographs
Stereogram	Interaction plot	Sample-size curves
Color Map	Contour plot	Trilinear coordinates
Histogram	Predicted response plot	
	Confidence region plot	

the measurement scale along an axis should allow for easy and accurate assessment of the coordinates of any data point, yet should not be cluttered.

Actual data points should be presented by symbols that present the appropriate indicators of location, and if they represent summaries of data from a normal data population, it would be appropriate to present a symbol for the mean and some indication of the variability (or error) associated with that population, commonly by using “error bars” which present the SD (or standard error) from the mean. If, however, the data are not normal or continuous, it would be more appropriate to indicate location by the median and present the range or semiquartile distance for variability estimates. The symbols that are used to present data points can also be used to present a significant amount of additional information. At the simplest level a set of clearly distinct symbols (circles, triangles, squares, etc.) are very commonly used to provide a third dimension of data (most commonly, treatment group), but by clever use of symbols, all sorts of additional information can be presented. Using a method such as Chernoff faces (79), in which faces are used as symbols of the data points (and various aspects of the faces present additional data, such as the presence or absence of eyes denoting presence or absence of a secondary pathological condition), it is

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FIG. 7.8. Acquisitions of postnatal development landmarks in rats.

possible to present a large number of different variables on a single graph.

The three other forms of graphs that are commonly used in toxicology are histograms, pie charts, and contour plots. Histograms are graphs of simple frequency distribution. Commonly, the abscissa is the variable of interest (such as lifespan or litter size) and generally is shown as classes or intervals or measurements (such as age ranges of 0 to 10 weeks, 10 to 20 weeks, etc.). The ordinate, meanwhile, is the incidence or frequency of observations. The result is a set of vertical bars, each of which represents the incidence of a particular set of observations. Measures of error or variability about each incidence are reflected by some form of error bar on top of or in the frequency bars, as shown in Figure 7.8. The size of class intervals may be unequal (in effect, one can combine or pool several small class intervals), but it is proper in such cases to vary the width of the bars to indicate differences in interval size.

Pie charts are the only common form of quantitative graphic technique that is not rectangular. Rather, the figure is presented as a circle out of which several "slices" are delimited. The only major use of the pie chart is in presenting a breakdown of the components of a group.

Typically the entire set of data under consideration (such as total body weight) constitutes the pie whereas each slice represents a percentage of the whole (such as the percentages represented by each of several organs). The total number of slices in a pie should be small for the presentation to be effective. Variability or error can be readily presented by having a subslice of each sector shaded and labeled accordingly.
Finally, there is the contour plot, which is used to depict the relationships in a three-variable, continuous data system. That is, a contour plot visually portrays each contour as a locus of the values of two variables associated with a constant value of the third variable. An example would be a relief map that gives both latitude and longitude of constant altitude using contour lines.
The most common misuse of graphs is to either conceal or exaggerate the extent of the difference by using an inappropriately scaled or ranged axis. Tufte (146) has termed a statistic for evaluating the appropriateness of scale size, the lie factor, defined as

$$\text{Lie factor} = \frac{\textit{Size of effect shown in graph}}{\textit{Size of effect in data}}.$$

An acceptable range for the lie factor is from 0.95 to 1.05. A value less than this range means the size of an effect is being understated, more than the effect is being exaggerated.
There are a number of excellent references available for those who would like to pursue statistical graphics more. Anscombe (7) presents an excellent short overview, whereas others (132, 145–147) provide a wealth of information.
Multidimensional and Nonmetric Scaling
Multidimensional scaling (MDS) is a collection of analysis methods for data sets that have three or more variables making up each data point. MDS displays the relationships of three or more dimensional extensions of the methods of statistical graphics.
MDS presents the structure of a set of objects from data that approximate the distances between pairs of the objects. The data, called similarities, dissimilarities, distances, or proximities, must be in such a form that the degree of similarities and differences between the pairs of the objects (each of which represents a real-life data point) can be measured and handled as a distance (remember the discussion of measures of distances under classifications). Similarity is a matter of degree, small differences between objects cause them to be "similar" (a high degree of similarity) whereas large differences cause them to be considered dissimilar (a small degree of similarity).
In addition to the traditional human conceptual or subjective judgments of similarity, data can be an "objective" similarity measure (the difference in weight between a pair of animals) or an index calculated from multivariate data (the proportion of agreement in the results of a number of carcinogenicity studies); however, the data must always represent the degree of similarity of pairs of objects.
Each object or data point is represented by a point in a multidimensional space. These plots or projected points are arranged in this space so that the distances between pairs of points have the strongest possible relation to the degree of similarity among the pairs of objects. That is, two similar objects are represented by two points that are close together, and two dissimilar objects are represented by a pair of points that are far apart. The space is usually a two- or three-dimensional Euclidean space, but may be non-Euclidean and may have more dimensions.
MDS is a general term that includes a number of different types of techniques; however, all seek to allow geometric analysis of multivariate data. The forms of MDS can be classified (170) according to the nature of the similarities in the data. It can be qualitative (nonmetric) or quantitative (metric MDS). The types can also be classified by the number of variables involved and by the nature of the model used—for example, classical MDS (there is only one data matrix, and no weighting factors are used on the data), replicated MDS (more than one matrix and no weighting), and weighted MDS (more than one matrix and at least some of the data being weighted).
MDS can be used in toxicology to analyze the similarities and differences between effects produced by different agents in an attempt to use an understanding of the mechanism underlying the actions of one agent to determine the mechanisms of the other agents. Actual algorithms and a good intermediate level presentation of MDS can be found in Davison (35).
Nonmetric scaling is a set of graphic techniques closely related to MDS, and is definitely useful for the reduction of dimensionality. Its major objective is to arrange a set of objects (each object, for our purposes, consisting of a number of related observations) graphically in a few dimensions while retaining the maximum possible fidelity to the original relationships between members (i.e., values that are most

different are portrayed as most distant). It is not a linear technique and it does not preserve linear relationships (i.e., A is not shown as twice as far from C as B, even though its "value difference" may be twice as much). The spacings (interpoint distances) are kept such that if the distance of the original scale between members A and B is greater than that between C and D, the distances on the model scale shall likewise be greater between A and B than between C

and D. Figure 7.5 uses a form of this technique in adding a third dimension by using letters to present degrees of effect on the skin.

This technique functions by taking observed measures of similarity or dissimilarity between every pair of M objects and then finding a representation of the objects as points in Euclidean space that the interpoint distances in some sense "match" the observed similarities or dissimilarities by means of weighting constants.

Cluster Analysis

Cluster analysis is a quantitative form of classification. It serves to help develop decision rules and then use these rules to assign a heterogeneous collection of objects to a series of sets. This is almost entirely an applied methodology (as opposed to theoretical). The final result of cluster analysis is one of several forms of graphic displays and a methodology (set of decision-classifying rules) for the assignment of new members into the classifications.

The classification procedures used are based on either density of population or distance between members. These methods can serve to generate a basis for the classification of large numbers of dissimilar variables, such as behavioral observations and compounds with distinct but related structures and mechanisms (63, 64), or to separate tumor patterns caused by treatment from those caused by old age (14).

There are five types of clustering techniques (52):

a. Hierarchical techniques: Classes are subclassified into groups, with the process being repeated at several levels to produce a tree that gives sufficient definition to groups.

b. Optimizing techniques: Clusters are formed by optimization of a clustering criterion. The resulting classes are mutually exclusive; the objects are partitioned clearly into sets.

c. Density- or mode-seeking techniques: Clusters are identified and formed by locating regions in a graphic representation that contains concentrations of data points.

d. Clumping techniques: A variation of density-seeking techniques in which assignment to a cluster is weighted on some variables so that clusters may overlap in graphic projections.

e. Others: Methods that do not clearly fall into classes a-d.

Romesburg (124) provides an excellent step-by-step guide to cluster analysis.

Fourier or Time Analysis

Fourier analysis (16) is most frequently a univariate method used for either simplifying data (which is the basis for its inclusion in this chapter) or for modeling. It can, however, also be a multivariate technique for data analysis. In a sense it is like trend analysis; it looks at the relationship of sets of data from a different perspective. In the case of Fourier analysis, the approach is by resolving the time dimension variable in the data set. At the most simple level it assumes that many events are periodic in nature, and if we can remove the variation in other variables because of this periodicity (by using Fourier transforms), we can better analyze the remaining variation from other variables. The complications to this are (a) there may be several overlying cyclic time-based periodicities, and (b) we may be interested in the time cycle events for their own sake.

Fourier analysis allows one to identify, quantitate, and (if we wish) remove the time-based cycles in data (with their amplitudes, phases, and frequencies) by use of the Fourier transform:

$$nJi=xiexp(-iwit),$$

where

n=length

J=The discrete Fourier transform for that case,

x=actual data,

i=increment in the series,

w=frequency,

t=time.

A graphic example of the use of Fourier analysis in toxicology is provided in Figure 7.9.

Life Tables

Chronic in vivo toxicity studies generally are the most complex and expensive studies conducted by a toxicologist. Answers to a number of questions are sought in such a study—notably if a material results in a significant increase in mortality or in the incidence of tumors in those animals exposed to it, but we are also interested in the time course of these adverse effects (or risks). The classic approach to assessing these age-specific hazard rates is by the use of life tables (also called survivorship tables).

It may readily be seen that during any selected period of time (ti) we have a number of risks competing to affect an animal. There are risks of (a) "natural death," (b) death induced by a direct or indirect

action of the test compound, and (c) death due to such occurrences of interest as tumors (77). We are indeed interested in determining if (and when) the last two of these risks become significantly different than the "natural" risks (defined as what is seen to happen in the control group). Life table methods enable us to make such determinations as the duration of survival (or time until tumors develop) and the probabil

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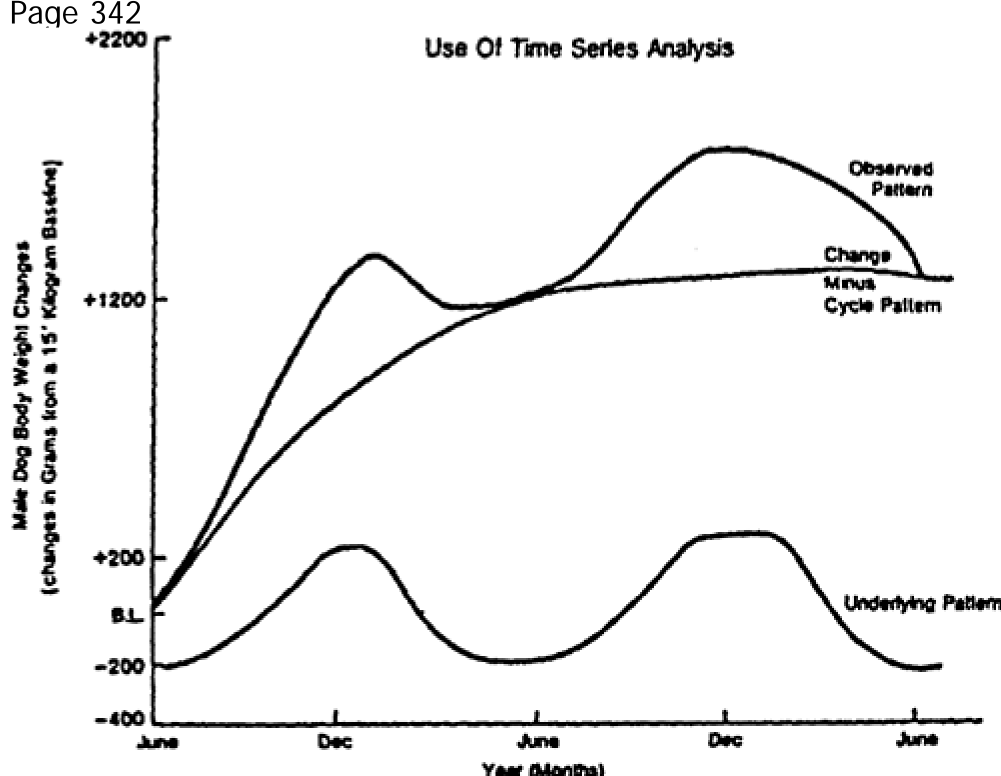

FIG. 7.9. Use of time series analysis.

ity of survival (or of developing a tumor) during any period of time.

We start by deciding the interval length (t_i) we wish to examine within the study. The information we gain becomes more exact as the interval is shortened, but as interval length is decreased, the number of intervals increases and calculations become more cumbersome and less indicative of time-related trends because random fluctuations become more apparent. For a 2-year or lifetime rodent study, an interval length of a month is commonly employed. Some life table methods, such as the Kaplan-Meyer, have each new event (such as a death) define the start of a new interval.

Having established the interval length, we can tabulate our data (33). We start by establishing the following columns in each table (a separate table being established for each group of animals—i.e., by sex and dose level):

(a) The interval of time selected (t_i).
(b) The number of animals in the group that entered that interval of the study alive (l_i).
(c) The number of animals withdrawn from study during the interval (such as those taken for an interim sacrifice or that may have been killed by a technician error) (ω_i).
(d) The number of animals that died during the interval (d_i).
(e) The number of animals at risk during the interval, $l_i = l_i - 1/2\omega I$, or the number on study at the start of the interval minus one half of the number withdrawn during the interval.
(f) The proportion of animals that died $= D_i = d_i / l_i$.
(g) The cumulative probability of an animal surviving until the end of that interval of study, $P_i = 1 - D_i$, or one minus the number of animals that died during that interval divided by the number of animals at risk.
(h) The number of animals dying until that interval (M_i).
(i) Animals found to have died during the interval (m_i).
(j) The probability of dying during the interval of the study $c_i = 1 - (M_i + m_i / l_i)$, or the total number of animals dead until that interval plus the animals discovered to have died during that interval divided by the number of animals at risk through the end of that interval.
(k) The cumulative proportion surviving, p_i, is equivalent to the cumulative product of the interval probabilities of survival (i.e., $P_i = p_1 \cdot p_2 \cdot p_3 \ldots P_x$).
(l) The cumulative probability of dying, C_i, equal to the cumulative product of the interval probabilities to that point (i.e., $C_i = c_1 \cdot c_2 \cdot c_3 \ldots c_x$).

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With such tables established for each group in a study (as shown in Example 33), we may now proceed to test the hypotheses that each of the treated groups has a significantly shorter duration of survival or that the treated groups died more quickly (note that plots of total animals dead and total animals surviving will give one an appreciation of the data, but can lead to no statistical conclusions).

Now, for these two groups, we wish to determine effective sample size and to compare survival probabilities in the interval months 14 to 15.

For the exposure group we compute sample size as

$$\ell_{E14-15}=\frac{0.8400(1-0.8400)}{(0.0367)^2}=99.7854$$

Likewise, we get a sample size of 98.1720 for the control group.

The standard error of difference for the two groups here is

$$SD=\sqrt{0.0367^2+0.0173^2}=0.040573$$

The probability of survival differences is PD= 0.9697–0.8400=0.1297. Our test statistic is then 0.1297/0.040573=3.196. From our z value table we see that the critical values are

$p\leq 0.05=1.960$

$p\leq 0.01=2.575$

$p\leq 0.001=3.270$

As our calculated value is larger than all but the last of these, we find our groups to be significantly different at the 0.01 level (0.01>p>0.001).

There are a multiplicity of methods for testing significance in life tables, with (as is often the case) the power of the tests increasing as does the difficulty of computation (30, 83, 126, 141).

We begin our method of statistical comparison of survival at any point in the study by determining the standard error of the K interval survival rate as (66)

$$S_K=P_k\sqrt{\sum_1^k\left(\frac{D_i}{1'_x-d_x}\right)}.$$

We may also determine the effective sample size (11) in accordance with

$$1_1=\frac{P(1-P)}{S^2}.$$

Example 33

Interval (months)	Alive at Beginning of interval	Animals withdrawn	Died during interval	Animals at risk	Proportion of animals dead	Probability of survival	Cumulative proportion surviving	Standard error of survival
				Test level 1				
Ii	*Ii*	*wi*	*di*	*Ii*	*Di*	*Pi*	*Pi*	*Si*
8–9	109	0	0	109	0	1.0000	1.0000	0.0000
9–10	109	0	2	109	0.0184	0.9816	0.9816	0.0129
10–11	107	0	0	107	0	1.0000	0.9816	0.0128
11–12	107	10	0	102	0	1.0000	0.9816	0.0128
12–13	97	0	1	97	0.0103	0.9897	0.9713	0.0162
13–14	96	0	1	96	0.0104	0.9896	0.9614	0.0190
14–15	95	0	12	95	0.1263	0.8737	0.8400	0.0367
15–16	83	0	2	83	0.0241	0.9759	0.8198	0.0385
16–17	81	0	3	81	0.0370	0.9630	0.7894	0.0409
17–18	78	20	1	68	0.0147	0.9853	0.7778	0.0419
18–19	57	0	2	57	0.0351	0.6949	0.7505	0.0446
Control Level								
11–12	99	0	1	99	0.0101	0.9899	0.9899	0.0100
12–13	98	0	0	98	0	1.0000	0.9899	0.0100
13–14	98	0	0	98	0	1.0000	0.9899	0.0100
14–15	98	0	2	98	0.0204	0.9796	0.9697	0.0172
15–16	96	0	1	96	0.0104	0.9896	0.9596	0.0198
16–17	95	0	0	95	0	1.0000	0.9596	0.0198

17–18	95	20	2	85	0.0235	0.8765	0.9370	0.0249
18–19	73	0	2	73	0.0274	0.9726	0.9113	0.0302

We may now compute the standard error of difference for any two groups (1 and 2) as

$$S_D = \sqrt{S_1^2 + S_2^2}.$$

The difference in survival probabilities for the two groups is then calculated as

$$PD=P1-P2.$$

We can then calculate a test statistic as

$$t' = \frac{P_D}{S_D}.$$

This is then compared to a z distribution table. If t'>z at the desired probability level, it is significant at that level. Example 33 illustrates the life table technique for mortality data. With increasing recognition of the effects of time (both as age and length of exposure to unmeasured background risks), life table analysis has become a mainstay in chronic toxicology. An example is the reassessment of the ED01 study (139), which radically changed interpretation of the results and understanding of underlying methods when adjustment for time on study was made.

Now, for these two groups, we wish to determine effective sample size and to compare survival probabilities in the interval months 14–15.

For the exposure group we compute sample size as

$$S_{xx} = T_{xx} + \sum_{xx}.$$

Likewise, we get a sample size of 98.1720 for the control group.

The standard error of difference for the two groups here is

$$SD = \sqrt{0.0367^2 + 0.0173^2} = 0.040573.$$

The probability of survival differences is PD=0.9697–0.8400=0.1297. Our test statistic is then 0.1297/0.040573=3.196. From our *z* value table we see that the critical values are

$$p \le 0.05 = 1.960,$$
$$p \le 0.01 = 2.575,$$
$$p \le 0.001 = 3.270.$$

As our calculated value is larger than all but the last of these, we find our groups to be significantly different at the 0.01 level (0.01>p>0.001).

The increased importance and interest in the analysis of survival data has not been restricted to toxicology, but rather has encompassed all of the life sciences. Those with further interest should consult Lee (101) or Elandt Johnson and Johnson (49), both general in their approach to the subject.

Multivariate Methods

In a chapter of this kind, an in-depth explanation of the available multivariate statistical techniques is an impossibility; however, as the complexity of problems in toxicology increases, we can expect to confront more frequently data that are not univariate but rather multivariate (or multidimensional). For example, a multidimensional study might be one in which the animals are being dosed with two materials that interact. Suppose we measure body weight, tumor incidence, and two clinical chemistry values for test material effects and interaction. Our dimensions, or variables, are now A=dose "x," B=dose "y," W=body weight, C=tumor incidence, D and E=levels of clinical chemistry parameters, and possibly also t (length of dosing).

These situations are particularly common in chronic studies (129). Though we can continue to use multiple sets of univariate techniques as we have in the past, there are significant losses of power, efficiency, and information when this is done, as well as an increased possibility of error (34).

Here we will also look briefly at the workings and uses of each of the most commonly employed multivariate techniques, together with several examples from the literature of their employment in toxicology and the other biological sciences. We shall group the methods according to their primary function: hypothesis testing (are these significant or not?), model fitting (what is the relationship between these variables, or what would happen if a population would be exposed to x?), and reduction of dimensionality (which variables are most meaningful?). It should be noted (and will soon be obvious), however, that most multivariate techniques actually combine several of these functions.

The most fundamental concept in multivariate analysis is that of a multivariate population distribution. By this point it is assumed that the reader is familiar with the univariate random variable and with such standard distributions as the normal distribution. Here we extend these to the multivariate normal distribution.

Multivariate data are virtually never processed and analyzed other than by computer. One must first set up an appropriate database file and then enter the data, coding some of them to meet the requirements of the software being used (e.g., if only numerical data are analyzed, sex may have to be coded as 1 for male and 2 for females).

Having recorded the data, it is then essential to review for suspect values and errors of various kinds. There

are many different types of suspect values, and it is helpful to distinguish among them:

(a) Outliers: These are defined to be observations that appear to be inconsistent with the rest of the data. They may be caused by gross recording or entering errors, but it is important to realize that an apparent outlier may occasionally be genuine and indicate a non-normal distribution or valuable data point.

(b) Inversions: A common type of error occurs when two consecutive digits are interchanged at the recording, coding, or entering stage. The error may be trivial if, for example, 56.74 appears as 56.47, but it may generate an outlier if 56.74 appears as 65.74.

(c) Repetitions: At the coding or entering stage, it is quite easy to repeat an entire number in two successive rows or columns of a table, thereby omitting one number completely.

(d) Values in the wrong column: It is easy to get numbers into the wrong columns.

(e) Other errors and suspect values: There are many other types of error, including possible misrecording of data of a minor nature.

The general term used to denote procedures for detecting and correcting errors is *data editing*. This includes checks for completeness, consistency, and credibility. Some editing can be done at the end of the data entry stage. In addition, many routine checks can be made by the computer itself, particularly those for gross outliers. An important class of such checks are range tests. For each variable an allowable range of possible values is specified and the computer checks that all observed values lie within the given range. Bivariate and multivariate checks are also possible. For example, one may specify an allowable range for some functions of two or more variables. A set of checks called "if-then" checks are also possible. For example, if both age and date of birth are recorded for each animal, then one can check that the answers are consistent. If the date of birth is given, then one can deduce the corresponding age. In fact, in this example the age observation is redundant. It is sometimes a good idea to include one or two redundant variables as a check on accuracy. Various other general procedures for detecting outliers are described by Barnett and Lewis (10).

When a questionable value or error is detected, the toxicologist must decide what to do about it. One may be able to go back to the original data source and check the observation. Inversions, repetitions, and values in the wrong column can often be corrected in this way. Outliers are more difficult to handle, particularly when they are impossible to check or have been misrecorded in the first place. It may be sensible to treat them as missing values and try to insert a value "guessed" in an appropriate way (e.g., by interpolation or by prediction from other variables). Alternatively, the value may have to be left as unrecorded and then either all observations for the given individual will have to be discarded or one will have to accept unequal numbers of observations for the different variables. With a univariate set of observations, the analysis usually begins with the calculation of two summary statistics, namely the mean and standard deviation. In the multivariate case, the analysis usually begins with the calculation of the mean and standard deviation for each variable, and, in addition, the correlation coefficient for each pair of variables is usually calculated. Their summary statistics are vital in having a preliminary look at the data.

The sample mean of the jth variable is given by

$$\bar{x}_j = \sum_{r=1}^{n} x_{rj}/n,$$

and the sample mean vector, x, is given by XT=[x1, x2, xn, ..., xn]. If the observations are a random sample from a population with mean $\bar{x}$, then the sample mean vector x is usually the point estimate of x, and this estimate can easily be shown to be unbiased.

The SD of the jth variable is given by

$$Sj = \sqrt{\left[\sum_{r=1}^{n} (x_{rj} - \bar{x}_j^2)/(n-1)\right]}.$$

The correlation coefficient of variables i and j is given by

$$r_{ij} = \sum_{r=1}^{n} (x_{ri} - \bar{x}_j)(x_{rj} - \bar{x}_j)/(n-1)S_i s_j.$$

These coefficients can be conveniently assembled in the sample correlation matrix, R, which is given by

$$R = \begin{bmatrix} 1 & r_{12} & \dots & r_{1n} \\ r_{21} & 1 & \dots & r_{2n} \\ \vdots & & & \\ r_n^1 & r_n^2 & \dots & 1 \end{bmatrix}.$$

Note that the diagonal terms are all unity.

The interpretation of mean and standard deviations is straightforward. It is worth looking to see if, for example, some variables have much higher scatter than others. It is also worth looking at the form of the distribution of each variable and considering whether any of the variables need to be transformed. For example, the logarithmic transformation is often used to reduce positive skewness and produce a distribution that is closer to normal.

One may also consider the removal of outliers at this stage.

There are three significant multivariate techniques that have hypothesis testing as their primary function: MANOVA, MANCOVA, and factor analysis.

MANOVA (multivariate analysis of variance) is the multidimensional extension of the ANOVA process we explored before. It can be shown to have grown out of Hotelling's T2 (88), which provides a means of testing the overall null hypothesis that two groups do not differ in their means on any of p measures. MANOVA accomplishes its comparison of two (or more) groups by reducing the set of p measures on each group to a simple number applying the linear combining rule Wi=WjXij (where Wj is a weighting factor) and then computing a univariate F-ratio on the combined variables. New sets of weights (wj) are selected in turn until that set which maximizes the F-ratio is found. The final resulting maximum F-ratio (based on the multiple discriminant functions) is then the basis of the significance test. As with ANOVA, MANOVA can be one way or higher order, and MANOVA has as a basic assumption a multivariate normal distribution.

Gray and Laskey (75) used MANOVA to analyze the reproductive effects of manganese in the mouse, allowing identification of significant effects at multiple sites. Witten et al. (167) utilized MANOVA to determine the significance of the effects of dose, time, and cell division in the action of abrin on the lymphocytes.

Multivariate analysis of covariance (MANCOVA) is the multivariate analog of analysis of covariance. As with MANOVA, it is based on the assumption that the data being analyzed are from a multivariate normal population. The MANCOVA test uses the two residual matrices using the statistic and is an extension of ANCOVA with two or more uncontrolled variables (or covariables). A detailed discussion can be found in Tatsuoka (142).

Factor analysis is not just a technique for hypothesis testing; it can also serve a reduction of dimensionality function. It seeks to separate the variance unique to particular sets of values from that common to all members in that variable system and is based on the assumption that the intercorrelations among the n original variables are the result of there being some smaller number of variables ("factors") that explain the bulk of variation seen in the variables. There are several approaches to achieving the end results, but they all seek a determination of what percentage of the variance of each variable is explained by each factor (a factor being one variable or a combination of variables). The model in factor analysis is y=Af xz, where

y=n dimensional vector of observable responses;

A=factor loading an n×q matrix of unknown parameters;

f=q dimensional vector of common factor;

z=n dimensional vector of unique factor.

Used for the reduction of dimensionality, factor analysis is said to be a linear technique because it does not change the linear relationships between the variables being examined.

Joung et al. (92) used factor analysis to develop a generalized water quality index that promises suitability across the United States, with appropriate weightings for 10 parameters. Factor analysis promises great utility as a tool for developing models in risk analysis, where a number of parameters act and interact.

Now we move on to two multivariate modeling techniques: multiple regression and discriminant analysis. Multiple regression and correlation seeks to predict one (or a few) variable from several others. It assumes that the available variables can be logically divided into two (or more) sets and serves to establish maximal linear (or some other scale) relationships among the sets;

The linear model for the regression is simply

$$Y=b_0+b_1X_1+b_2X_2+\ldots+b_pX_p,$$

where Y=the predicted value, b=values set to maximize correlations between X and Y, and X and Y=the actual observations (with X's being independent of predictor variables and Y's being dependent variables or outcome measures). One of the outputs from the process will be the coefficient of multiple correlation, which is simply the multivariate equivalent of the correlation coefficient *(r)*.

Schaeffer et al. (128) have neatly demonstrated the utilization of multiple regression in studying the contribution of two components of a mixture to its toxicologic action, using quantitative results from an Ames test as an end point. Paintz et al. (116) similarly used multiple regression to model the quantitative structure-activity relationships of a series of 14 l-benzoyl-3-methyl-pyrazole derivatives.

Discriminant analysis has for its main purpose finding linear combinations of variables that maximize the differences between the populations being studied, with the objective of establishing a model to sort objects into their appropriate populations with minimal error. At least four major questions are, in a

sense, being asked of the data:

1. Are there significant differences among the K groups?
2. If the groups do exhibit statistical differences, how do the central masses (or centroids, the multivariate equivalent of means) of the populations differ?
3. What are the relative distances among the K groups?

4. How are known (or at this point unknown) members allocated to *establish* groups? How do you predict the set of responses of characteristics of an as yet untried exposure case?

The discriminant functions used to produce the linear combinations are of the form

$$Di=di1Xi+di2Z2\ldots+dipZp,$$

where

D1=the score on the discriminant function I,

d's=weighing coefficients,

Z's=standardized values of the discriminating variables used in the analysis.

It should be noted that discriminant analysis can also be used for the hypothesis testing function by the expedient of evaluating how well it correctly classifies members into proper groups (say, control, treatment 1, treatment 2, etc.) Taketomo et al. (140) used discriminant analysis in a retrospective study of gentamycin nephrotoxicity to identify patient risk factors (i.e., variables which contributed to a prediction of a patient being at risk).

Finally, we introduce four techniques whose primary function is the reduction of dimensionality: canonical correlation analysis, principal components analysis, biplot analysis, and correspondence analysis.

Canonical correlation analysis provides the canonical R, an overall measure of the relationship between two sets of variables (one set consisting of several outcome measures, the other of several predictor variables). The canonical R is calculated on two numbers for each subject:

$$Wi=\Sigma wjXij \text{ and } Vi=\Sigma viYij,$$

where

X's=predictor variables,

Y's=outcome measures,

Wj and Vj=canonical coefficients.

MANOVA can be considered a special case of canonical correlation analysis. Canonical correlation can be used in hypothesis testing also for testing the association of pairs of sets of weights, each with a corresponding coefficient of canonical correlation, each uncorrelated with any of the preceding sets of weights, and each accounting for successively less of the variation shared by the two sets of variables. For example, Young and Matthews (169) used canonical correlation analysis to evaluate the relationship between plant growth and environmental factors at 12 different sites.

The main purpose of principal components analysis is to describe as economically as possible the total variance in a sample in a few dimensions: one wishes to reduce the dimensionality of the original data while minimizing the loss of information. It seeks to resolve the total variation of a set of variables into linearly independent composite variables that successively account for the maximum possible variability in the data. The fundamental equation is Y=AZ, where

A=matrix of scales eigenvectors,

Z=original data matrice,

Y=principal components.

The concentration here, as in factor analysis, is on relationships within a single set of variables. Note that the results of principal components analysis are affected by linear transformations.

Cremer and Seville (31) used principal components to compare the difference in blood parameters resulting from each of two separate pyrethroids. Henry and Hidy (84), meanwhile, used principal components to identify the most significant contributors to air quality problems.

The biplot display (58) of multivariate data is a relatively new technique but promises wide applicability to problems in toxicology. It is, in a sense, a form of exploratory data analysis, used for data summarization and description.

The biplot is a graphical display of a matrix Ynmx of N rows and M columns by means of row and column marker. The display carries one marker for each row and each column. The "bi" in biplot refers to the joint display of rows and columns. Such plots are used primarily for inspection of data and for data diagnostics when such data are in the form of matrices.

Shy-Modjeska et al. (135) illustrated this usage in the analysis of aminoglycoside renal data from beagle dogs, allowing the simultaneous display of relationships among different observed variables and presentation of the relationship of both individuals and treatment groups to these variables.

Correspondence analysis is a technique for displaying the rows and columns of a two-way contingency table as points in a corresponding low-dimensional vector space. As such, it is equivalent to simultaneous linear regression (for contingency table data, such as tumor incidences, which is a very common data form in toxicology). As such, it can be considered a special case of canonical correlation

analysis. The data are defined, described, and analyzed in a geometric framework. This is particularly attractive to such sets of observations in toxicology as multiple end point behavioral scores and scored multiple tissue lesions.

There are a number of good surveys of multivariate techniques available (9, 21, 133) that are not excessively mathematical. More rigorous mathematical treatments on an introductory level are also available (71). Most of the techniques we have described are available in the better computer statistical packages.

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Table 7.7 Classification of Data Commonly Encountered in Toxicology, by Type

Continuous normal:	Body weights
	Food consumption
	Organ weights: Absolute and relative
	Mouse Ear Swelling Test (MEST) measurements
	Pregnancy rates
	Survival rates
	Crown-Rump lengths
	Hematology (some)
	Clinical chemistry (some)
Continuous but not normal:	Hematology (some—WBC)
	Clinical chemistry (some)
	Urinalysis
Scalar data:	Neurobehavioral signs (some)
	PDI scores
	Histopathology (some)
Count data:	Resorption sites
	Implantation sites
	Stillborns
	Hematology (some—reticulocyte counts//Howel-Jolly//WBC differentials)
Categorical data	Clinical signs
	Neurobehavioral signs (some)
	Ocular scores
	GP sensitization scores
	Mouse ear swelling tests (MEST) sensitization
	Counts
	Fetal abnormalities
	Dose/mortality data
	Sex ratios
	Histopathology data (most)

DATA ANALYSIS APPLICATIONS IN TOXICOLOGY

Having reviewed basic principles and provided a set of methods for statistical handling of data, the remainder of this book will address the practical aspects and difficulties encountered in day-to-day toxicological work. As a starting point, we present in Table 7.7 an overview of data types actually encountered in toxicology, classified by type (as presented at the beginning of this book). It should be stressed, however, that this classification is of the most frequent measure of each sort of observation (such as body weight) and will not always be an accurate classification.

There are now common practices in the analysis of toxicology data, though they are not necessarily the best. These are discussed in the remainder of this chapter, which seeks to review statistical methods on a use-by-use basis and to provide a foundation for the selection of alternatives in specific situations.

Median Lethal and Effective Doses

For many years, the starting point for evaluating the toxicity of an agent was to determine its LD50 or LC50, which are the dose or concentration of a material at which half of a population of animals would be expected to die. These figures are analogous to the ED50 (effective dose for half a population) used in pharmacologic activities, and are derived by the same means. It is important to keep in mind the three dimensions of dose response.

As the dose increases,

Incidence of responders in an exposed population increases.

Severity of response in effected individuals increases.

Time to occurrence of response or of progressive stage of response decreases.

To calculate either the LD50 or LC50, we need, at each of several dosage (or exposure) levels, the number of animals dosed and the number that died. If we seek only to establish the median effective dose in a range-finding test, then 4 or 5 animals per dose level, using Thompson's method of moving averages, is the most efficient methodology and will give a sufficiently accurate solution. With two dose levels, if the ratio between the high and low dose is two or less, even total or no mortality at these two dose levels will yield an acceptably accurate medial lethal dose, although a partial mortality is desirable. If, however, we wish to estimate a number of toxicity levels (LD10, LD90) and are interested in more

precisely establishing the slope of the dose/lethality curve, the use of at least 10 animals per dosage level with the log/probit regression technique is the most common approach. Note that in the equation $Y_i=a+bx_1$, b is the slope of the regression line and that our method already allows us to calculate 95% confidence intervals about any point on this line. Note that the confidence interval at any one point will be different from the interval at other points and must be calculated separately. Additionally, the nature of the probit transform is such that toward the extremes—LD10 and LD90, for example—the confidence intervals will "balloon" (i.e., they become very wide). Because the slope of the fitted line in these assays has a very large uncertainty, in relation to the uncertainty of the LD50 itself (the midpoint of the distribution), much caution must be used with calculated Ldxs other than LD50s. The imprecision of the LD35, a value close to the LD50, is discussed by Weil (153), as is that of the slope of the log dose-probit line (152). Debanne and Haller (37) recently reviewed the statistical aspects of different methodologies for estimating a median effective dose.

There have been questions for years as to the value of LD50 and the efficiency of the current study design (which uses large numbers of animals) in determining it. As long ago as 1953, Weil et al. (154) presented forceful

arguments that an estimate having only minimally reduced precision could be made using significantly fewer animals. More recently, the last few years have seen an increased level of concern over the numbers and uses of animals in research and testing and have produced additional arguments against existing methodologies for determining the LD50 or even the need to make the determination at all (172). In response, a number of suggestions for alternative methodologies have been advanced (20, 38, 59).

Body and Organ Weights

Among the sets of data commonly collected in studies where animals are dosed with (or exposed to) a chemical are body weight and the weights of selected organs. In fact, body weight is frequently the most sensitive indication of an adverse effect. How to best analyze this and in what form to analyze the organ weight data (as absolute weights, weight changes, or percentages of body weight) have been the subject of a number of articles (90, 155, 156, 162).

Both absolute body weights and rates of body weight change (calculated as changes from a baseline measurement value that is traditionally the animal's weight immediately prior to the first dosing with or exposure to test material) are almost universally best analyzed by ANOVA followed by a post hoc test, if called for. Even if the groups were randomized properly at the beginning of a study (no group being significantly different in mean body weight from any other group, and all animals in all groups within two SDs of the overall mean body weight), there is an advantage to performing the computationally, slightly more cumbersome (compared to absolute body weights) analysis of changes in body weight. The advantage is an increase in sensitivity because the adjustment of starting points (the setting of initial weights as a "zero" value) acts to reduce the amount of initial variability. In this case, Bartlett's test is performed first to ensure homogeneity of variance, and the appropriate sequence of analysis follows.

With smaller sample sizes, the normality of the data becomes increasingly uncertain, and nonparametric methods, such as Kruskal-Wallis, may be more appropriate (171).

The analysis of relative (to body weight) organ weights is a valuable tool for identifying possible target organs (59). How to perform this analysis is still a matter of some disagreement, however.

Weil (155) presented evidence that organ weight data expressed as percentages of body weight should be analyzed separately for each sex. Furthermore, because the conclusions from organ weight data of males differed so often from those of females, data from animals of each sex should be used in this measurement. Others (17, 18, 157, 162) have discussed in detail other factors that influence organ weights and must be taken into account.

The two competing approaches to analyzing relative organ weights call for either

(1) calculating organ weights as a percentage of total body weight (at the time of necropsy) and analyzing the results by ANOVA, or

(2) analyzing results by ANCOVA, with body weights as the covariates, as discussed previously by the author (156).

A number of considerations should be kept in mind when these questions are addressed. First, one must keep a firm grasp on the difference between biological significance and statistical significance. In this particular case, we are especially interested in examining organ weights when an organ weight change is not proportional to changes in whole body weights. Second, we are now required to detect smaller and smaller changes while still retaining a similar sensitivity (i.e., the $p<0.05$ level).

There are several devices to attain the desired increase in power. One is to use larger and larger sample sizes (number of animals) and the other is to use the most powerful test we can; however, the use of even currently employed numbers of animals is being vigorously questioned and the power of statistical tests must, therefore, now assume an increased importance in our considerations.

The biological rationale behind analyzing both absolute body weight and the organ weight to body weight ratio (this latter as opposed to a covariance analysis of organ weights) is that in the majority of cases, except for the brain, the organs of interest in the body change weight (except in extreme cases of obesity or starvation) in proportion to total body weight. We are particularly interested in detecting cases where this is not so. Analysis of actual data from several hundred studies (unpublished data) has shown no significant difference in rates of weight change of target organs (other than the brain), compared to total body weight for healthy animals in those species commonly used for repeated dose studies (rats, mice, rabbits, dogs). Furthermore, it should be noted that analysis of covariance is of questionable validity in analyzing body weight and related organ weight changes, as a primary assumption is the independence of treatment—that the relationship of the two variables is the same for all treatments (123). Plainly, in toxicology this is not true.

In cases where the differences between the error mean squares are much greater, the ratio of F-ratios will diverge in precision from the result of the efficiency of covariance adjustment. These cases are where either sample sizes are much larger or where the differences between means themselves are much larger. This latter case is one that

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does not occur in the designs under discussion in any manner that would leave analysis of covariance as a valid approach because group means start out being very similar and cannot diverge markedly unless there is a treatment effect. As we have discussed earlier, a treatment effect invalidates a prime underpinning assumption of analysis of covariance.
Shirley and Newnham (134) have argued the case for ANCOVA but without providing answers to arguments presented above.
Clinical Chemistry
A number of clinical chemistry parameters are commonly determined on the blood and urine collected from animals in chronic, subchronic, and, occasionally, acute toxicity studies. In the past, and still in some places, the accepted practice has been to evaluate these data using univariate-parametric methods (primarily t-tests and/or ANOVA); however, this can be shown to be not the best approach on a number of grounds.
First, such biochemical parameters are rarely independent of each other. Neither is our interest often focused on just one of the parameters. Rather, there are batteries of the parameters associated with toxic actions at particular target organs. For example, increases in creatinine phosphokinase (CPK), γ-hydroxybutyrate dehydrogenase (γ-HBDH), and lactate dehydrogenase (LDH), occurring together, are strongly indicative of myocardial damage. In such cases we are not just interested in a significant increase in one of these, but in all three. Table 7.8 gives a brief overview of the association of various parameters with actions at particular target organs. A more detailed coverage of the interpretation of such clinical laboratory tests can be found in other references (65, 78, 103, 105).
Similarly, the serum electrolytes (sodium, potassium, calcium) interact with each other; a decrease in one is frequently tied, for instance, to an increase in one of the others. Furthermore, the nature of the data (in the case of some parameters), either because of the biological nature of the parameter or the way in which it is measured, is frequently either not normally distributed (particularly because of being markedly skewed) or not continuous in nature. This can be seen in some of the reference data for experimental animals in Mitruka and Rawnsley (109) or Weil (158) in, for example, creatinine, sodium, potassium, chloride, calcium, and blood.
Hematology
Much of what we said about clinical chemistry parameters is also true for the hematologic measurements made in toxicology studies. Which test to perform should be evaluated by use of a decision tree until one becomes confident as to the most appropriate methods. Keep in mind that sets of values and (in some cases) population distribution vary not only between species, but also between the commonly used strains of species, and that "control" or "standard" values will "drift" over the course of only a few years.
Again, the majority of these parameters are interrelated and highly dependent on the method used to determine them. RBC, platelet counts, and mean corpuscular volume (MCV) may be determined using a device such as a Coulter counter to take direct measurements, and the resulting data are usually stable for parametric methods. The hematocrit, however, may actually be a value calculated from the RBC and MCV values, and if so, is dependent on them. If the hematocrit is measured directly, instead of being calculated from the RBC and MCV, it may be compared by parametric methods.
Hemoglobin is directly measured and is an independent and continuous variable; however, and probably because at any one time a number of forms and conformations (oxyhemoglobin, deoxyhemoglobin, methemoglobin, etc.) of hemoglobin are actually present, the distribution seen is not typically a normal one, but rather may be a multimodal one. Here a nonparametric technique such as the Wilcoxon or multiple Rank-Sum is called for.
Consideration of the white blood cell (WBC) and differential counts leads to another problem. The total WBC is, typically, a normal population amenable to parametric analysis, but differential counts are normally determined by counting, manually, one or more sets of 100 cells each. The resulting relative percentages of neutrophils are then reported as either percentages or are multiplied by the total WBC count with the resulting "count" being reported as the "absolute" differential WBC. Such data, particularly in the case of eosinophils (where the distribution does not approach normality) should usually be analyzed by nonparametric methods. It is widely believed that "relative" (percentage) differential data should not be reported because they are likely to be misleading.
Lastly, it should always be kept in mind that it is rare for a change in any single hematologic parameter to be meaningful. Rather, because these parameters are so interrelated, patterns of changes in parameters should be expected if a real effect is present, and analysis and interpretation of results should focus on such patterns of changes. Classification analysis techniques often provide the basis for a
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useful approach to such problems.

Histopathologic Lesion Incidence

The last 30 years have seen increasing emphasis placed on histopathological examination of tissues collected

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Table 7.8 Association of changes in biochemical parameters with actions at particular target organs

PARAMETER	ORGAN SYSTEM								NOTES
	BLOOD	HEART	LUNG	KIDNEY	LIVER	BONE	INTESTINE	PANCREAS	
Albumin				↓	↓				Produced by the liver. Very significant reductions indicate extensive liver damage.
ALP (alkaline phosphatase)					↑	↑	↑		Elevations usually are associated with cholestasis. Bone alkaline phosphatase tends to be higher in young animals.
Bilirubin (total)	↑				↑				Usually elevated due to cholestasis—either due to obstruction or hepatopathy.
BUN (blood urea nitrogen)				↑	↓				Estimates blood filtering capacity of the kidneys. Doesn't become significantly elevated until kidney function is reduced 60%–75%.
Calcium				↑					Can be life threatening and result in acute death.
Cholinesterase				↑	↓				Found in plasma, brain and RBC.
CPK (creatinine phosphokinase)		↑							Most often elevated due to skeletal muscle damage but can also be produced by cardiac muscle damage. Can be more sensitive than histopathology.
Creatinine				↑					Also estimates blood filtering

PARAMETER	BLOOD	HEART	LUNG	ORGAN SYSTEM KIDNEY	LIVER	BONE	INTESTINE	PANCREAS	NOTES
									capacity of kidney as BUN does More specific than BUN.
Glucose								↑	Alterations other than those associated with stress are uncommon and reflect an effect on the pancreatic islets or anorexia.
GGT (gamma glutamyl transferase)					↑				Elevated in cholestasis. This is a microsomal enzyme and levels often increase in response to microsomal enzyme induction.
HBDH (hydroxybutyric dehydrogenase)		↑			↑				
LDH (lactic dehydrogenase		↑	↑	↑	↑				Increase usually due to skeletal muscle, cardiac muscle and liver damage. Not very specific.
Protein (total)				↓	↓				Absolute alterations are usually associated with decreased production (liver) or increased loss (kidney). Can see increase in case of muscle "wasting" (catabolism).

PARAMETER	BLOOD	HEART	LUNG	ORGAN SYSTEM KIDNEY	LIVER	BONE	INTESTINE	PANCREAS	NOTES
SGOT (serum glutamic-oxaloacetic transaminase); also called AST (aspartate amino transferase)		↑		↑	↑			↑	Present in skeletal muscle and heart and most commonly associated with damage to these
SGPT (serum glutamic-pyruvic transaminase); also called ALT (alanine amino transferase)					↑				Elevations usually associated with hepatic damage or disease.
SDH (sorbitol dehydrogenase)					↑ or ↓				Liver enzyme that can be quite sensitive but is fairly unstable. Samples should be processed as soon as possible.

Arrow indicates increase (↑) or decrease (↓).

from animals in subchronic and chronic toxicity studies. Whereas it is not true that only those lesions that occur at a statistically significant increased rate in treated/ exposed animals are of concern (for there are the cases where a lesion may be of such a rare type that the occurrence of only one or a few such in treated animals "raises a flag"), it is true that, in most cases, a statistical evaluation is the only way to determine if what we see in treated animals is significantly worse than what has been seen in control animals. Although cancer is not our only concern, this category of lesions is of greatest interest.

Typically, comparison of incidences of any one type of lesion between controls and treated animals are made using the multiple 2×2 chi-square test or Fisher's exact test with a modification of the numbers of animals as the denominators. Too often, experimenters exclude from consideration all those animals (in both groups) that died prior to the first animals being found with a lesion at that site. The special case of carcinogenicity bioassays will be discussed in detail later in this chapter.

An option that should be kept in mind is that frequently a pathologist cannot only identify a lesion as present, but also grade those present as to severity. This represents a significant increase in the information content of the data that should not be given up by performing an analysis based only on the perceived quantal nature (present/absent) of the data. Quantal data, analyzed by chi-square or Fisher's exact tests, are a subset (the 2×2 case) of categorical or contingency table data. In this case it also becomes ranked (or "ordinal") data—the categories are naturally ordered (e.g., no effect<mild lesion<moderate lesion<severe lesion). This gives a 2×R table if there are only one treatment and one control group, or an N×R ("multiway") table if there are three or more groups of animals.

The traditional method of analyzing multiple, crossclassified data has been to collapse the N×R contingency table over all but two of the variables and to follow this with the computation of some measure of association between these variables. For an N-dimensional table, this results in N (N−1)/2 separate analyses. The result is crude, "giving away" information and even (by inappropriate pooling of data) yielding a faulty understanding of the meaning of data. Though computationally more laborious, a multiway (N×R table) analysis should be used.

Reproduction

The reproductive implications of the toxic effects of chemicals are becoming increasingly important. Because of this, reproduction studies, together with other closely related types of studies (such as teratogenesis, dominant lethal, and mutagenesis studies, which are discussed later

in this chapter), are now a common companion to chronic toxicity studies.
One point that must be kept in mind with all reproduction-related studies is the nature of the appropriate sampling unit. What is the appropriate N in such a study? The number of individual pups, the number of litters, pregnant females? Fortunately, it is now fairly well accepted that the first case (using the number of offspring as the N) is inappropriate (162). The real effects in such studies actually occur in the female that was exposed to the chemical or that is mated to a male that was exposed. What happens to her and to the development of the litter she is carrying is biologically independent of what happens to every other female/litter in the stud. This cannot be said for each offspring in each litter (e.g., the death of one member of a litter can and will be related to what happens to every other member). The effect on all of the offspring might be similar for all of those from one female and different or lacking for those from another.
As defined by Oser and Oser (115), there are four primary variables of interest in a reproduction study. First, there is the fertility index (FI), which may be defined as the percentage of attempted matings (i.e., each female housed with a male) that resulted in pregnancy, with pregnancy being determined by a method such as the presence of implantation sites in the female. Second, there is the gestation index (GI), which is defined as the percentage of mated females, as evidenced by a vaginal plug being dropped or a positive vaginal smear, which deliver viable litters (i.e., litters with at least one live pup). Two related variables that may also be studied are the mean number of pups born per litter and the percentage of total pups per litter that are stillborn. Third, there is the viability index (VI), which is defined as the percentage of offspring born that survive at least 4 days after birth. Finally (in this four-variable system) there is the lactation index (LI), which is the percentage of animals per litter that survive 4 days and also survive to weaning. In rats and mice, this is classically taken to be 21 days after birth. An additional variable that may reasonably be included in such a study is the mean weight gain per pup per litter.
Given that our N is at least 10 pregnant animals, we may test each of these variables for significance using a method such as the Wilcoxon-Mann-Whitney U test, or the Kruskal-Wallis nonparametric ANOVA. If N is less than 10, we cannot expect the central limit theorem to be operative and should use the Wilcoxon sum of ranks (for two groups) or the Kruskal-Wallis nonparametric ANOVA (for three or more groups) to compare groups.

Developmental Toxicology

When the primary concern of a reproductive/developmental study is the occurrence of birth defects or deformations (terata, either structural or functional) in the offspring of exposed animals, the study is one of developmental toxicology (teratology). In the analysis of the data from such a study, we must consider several points.
First is sample size. Earlier a method to estimate sufficient sample size was presented previously. The difficulties with applying these methods here revolve around two points: (1) selecting a sufficient level of sensitivity for detecting an effect, and (2) factoring in how many animals will be removed from study (without contributing a datum) by either not becoming pregnant or not surviving to a sufficiently late stage of pregnancy. Experience generally dictates that one should attempt to have 20 pregnant animals per study group if a pilot study has provided some confidence that the pregnant test animals will survive the dose levels selected. Again, it is essential to recognize that the litter, not the fetus, is the basic independent unit for each variable.
A more fundamental consideration, alluded to in the section on reproduction, is that as we use more animals, the mean of means (each variable will be such in a mathematical sense) will approach normality in its distribution. This is one of the implications of the Central Limit Theorem: even when the individual data are not normally distributed, their means will approach normality in their distribution. At a sample size of 10 or greater, the approximation of normality is such that we may use a parametric test (such as a t-test or ANOVA) to evaluate results. At sample sizes less than 10, a nonparametric test (Wilcoxon rank-sum or Kruskal-Wallis nonparametric ANOVA) is more appropriate. Other methodologies have been suggested (100, 112) but do not offer any prospect of widespread usage. One nonparametric method that is widely used is the Mann-Whitney U test, which was described earlier. Williams and Buschbom (163) further discuss some of the available statistical options and their consequences, and Rai and Ryzin (22) have recommended a dose-responsive model.

Dominant Lethal Assay

The dominant lethal study is essentially a reproduction study that seeks to study the end point of lethality to the fetuses after implantation and before delivery. The proper identification of the sampling unit (the pregnant female) and the design of an experiment so that a sufficiently large sample is

available for analysis are the prime statistical considerations. The question of sampling unit has been adequately addressed in earlier sections. Sample size is of concern here because the hypothesis-testing techniques that are appropriate with small samples are of relatively low power, as the variability about the mean in such cases is relatively large. With sufficient sample size (e.g., from 30–50 pregnant females per dose level per week (11)),

variability about the mean and the nature of the distribution allow sensitive statistical techniques to be employed.
The variables that are typically recorded and included in analysis (for each level/week) are as follows: (a) the number of pregnant females, (b) live fetuses/pregnancy, (c) total implants/pregnancy, (d) early fetal deaths (early resorptions)/pregnancy, and (e) late fetal deaths/pregnancy.
A wide variety of techniques for analysis of these data have been (and are) used. Most common is the use of ANOVA after the data have been transformed by the arc sine transform (111).
Beta binomial (2, 151) and Poisson distributions (36) have also been attributed to these data, and transforms and appropriate tests have been proposed for use in each of these cases (in each case with the note that the transforms serve to "stabilize the variance" of the data). With sufficient sample size, as defined earlier in this section, the Mann-Whitney U test is recommended for use here. Smaller sample sizes necessitate the use of the Wilcoxon rank-sum test.
Diet and Chamber Analysis
Earlier we presented the basic principles and methods for sampling. Sampling is important in many aspects of toxicology, and here we address its application to diet preparation and the analysis of atmospheres from inhalation chambers.
In feeding studies we seek to deliver doses of a material to animals by mixing the material with their diet. Similarly, in an inhalation study we mix a material with the air the test animals breathe. In both cases we must then sample the medium (food or atmosphere) and analyze these samples to determine what levels or concentrations of material were actually present and to assure ourselves that the test material is homogeneously distributed. Having an accurate picture of these delivered concentrations and how they varied over the course of time is essential on a number of grounds:
1. The regulatory agencies and sound scientific practice require that analyzed diet and mean daily inhalation atmosphere levels be ±10% of the target level.
2. Excessive peak concentrations, because of the overloading of metabolic repair systems, could result in extreme acute effects that would lead to results in a chronic study that are not truly indicative of the chronic low-level effects of the compound, but rather of periods of metabolic and physiologic overload. Such results could be misinterpreted if true exposure or diet levels were not maintained at a relatively constant level.
Sampling strategies are not just a matter of numbers (for statistical aspects), but of geometry, so that the contents of a container or the entire atmosphere in a chamber is truly sampled, and of time, in accordance with the stability of the test compound. The samples must be both randomly collected and representative of the entire mass of what one is trying to characterize. In the special case of sampling and characterizing the physical properties of aerosols in an inhalation study, some special considerations and terminology apply. Because of the physiologic characteristics of the respiration of humans and of test animals, our concern is very largely limited to those particles or droplets that are of a respirable size. Unfortunately, "respirable size" is a complex characteristic based on aerodynamic diameter, density, and physiological characteristics. Unfortunately, while those particles with an aerodynamic diameter of less than 10 µm are generally agreed to be respirable in humans (i.e., they can be drawn down to the deep portions of the lungs), 3 µm in aerodynamic diameter is a more realistical value. Typically, it then becomes a matter of the calculation of measures of central tendency and dispersion statistics, with the identification of those values that are beyond acceptable limits (15).
Genotoxicity
Over the last 25 years a wide variety of tests (see ref. 96 for an overview of those available) for genotoxicity have been developed and brought into use. These tests give us a quicker and cheaper (though not as conclusive) way of predicting whether a material of interest is a mutagen, and possibly a carcinogen, than do longer-term, whole-animal studies.
How to analyze the results of the multitude of tests (Ames, DNA repair, micronucleus, chromosome abberation, cell transformation, and sister chromatid exchange, to name a few) is an extremely important question. Some workers in the field hold that it is not possible (or necessary) to perform statistical analysis and that the tests can simply be judged to be positive or not positive on the basis of whether or not they achieve a particular increase in the incidence of mutations in the test organism. Quantitations of potency are complicated by the fact that we are dealing with a nonlinear phenomenon, and although low dose of most genotoxins produce a linear response curve with increasing dose, the curve will flatten out (and even turn into a declining curve) as the higher doses provoke an acute response.
Several concepts different from those we have previously discussed need to be examined, for our

concern has now shifted from how a multicellular organism acts in response to one of a number of complex actions to how a mutational event is expressed, most frequently by a single cell. Given that we can handle much larger

numbers of experimental units in systems that use smaller test organisms, we can seek to detect both weak and strong mutagens.

Conducting the appropriate statistical analysis and using the results of such an analysis properly must start with an understanding of the biological system involved and, from this understanding, developing the correct model and hypothesis. We start such a process by considering each of five interacting factors (74, 150):

1. α, which is the probability of our committing a type I error (saying an agent is mutagenic when it is not, equivalent to our p in such earlier considered designs as the Fisher's exact test); false-positive.
2. β, which is the probability of our committing a type II error (saying an agent is not mutagenic when it is); false-negative.
3. Δ, our desired sensitivity in an assay system (such as being able to detect an increase of 10% in mutations in a population).
4. σ, the variability of the biological system and the effects of chance errors.
5. n, the single necessary sample size to achieve each of these (we can, by our actions, change only this portion of the equation) as n is proportional to

$$\frac{\sigma}{\alpha, \beta, \text{and } \Delta}$$

The implications of this are, therefore, that (a) the greater σ is, the larger n must be to achieve the desired levels of α, β, and Δ; (b) the smaller the desired levels of α, β, and/or Δ, if n is constant, the larger our σ is.

What is the background mutation level and the variability in our technique? As any good genetic or general toxicologist will acknowledge, matched concurrent control groups are essential. Fortunately, with these test systems, large n's are readily attainable, though there are other complications to this problem, which we shall consider later. An example of the confusion that would otherwise result is illustrated in the intralaboratory comparisons on some of these methods done to date, such as that reviewed by Weil (159).

New statistical tests based on these assumptions and on the underlying population distributions have been proposed, along with the necessary computational background, to allow one to alter one of the input variables (α, β, or Δ). A set that shows particular promise is that proposed by Katz (94, 95) in his two articles. He described two separate test statistics: Φ for when we can accurately estimate the number of individuals in both the experimental and control groups, and *θ* for when we do not actually estimate the number of surviving individuals in each group, we can assume that the test material is only mildly toxic in terms of killing the test organisms. Each of these two test statistics is also formulated on the basis of only a single exposure of the organisms to the test chemicals. Given this, then we may compute

$$\phi = \frac{a(M_E - 0.5) - Kb(M_C + 0.5)}{\sqrt{Kab(M_E + M_C)}},$$

where a and b are the number of groups of control (c) and experimental (e) organisms, respectively. N_C and N_E are the numbers of surviving microorganisms:

$$[K = N_E/N_C].$$

M_E and M_C are the numbers of mutations in experimental and control groups; μ_e and μ_c are the true (but unknown) mutation rates (as μ_c gets smaller, N's must increase).

We may compute the second case as

$$\theta = \frac{a(M_E - 0.5) + (M_C + 0.5)}{ab(M_E + M_C)}$$

with the same constituents.

In both cases, at a confidence level for I of 0.05, we accept that $\mu_c = \mu_e$ if the test statistic (either Φ or *θ*) is less than 1.64. If it is equal to or greater than 1.64, we may conclude that we have a mutagenic effect (at α=0.05).

In the second case (*θ*, where we do not have separate estimates of population sizes for the control and experimental groups), if K deviates widely from 1.0 (if the material is markedly toxic), we should use more containers of control organisms (tables for the proportions of each to use given different survival frequencies may be found in ref. 96). If different levels are desired, tables for *θ* and Φ may be found in Kastenbaum and Bowman (94).

An outgrowth of this is that the mutation rate per surviving cells (μc and μe) can be determined. It must be remembered that if the control mutation rate is so high that a reduction in mutation rates can be achieved by the test compound, these test statistics must be adjusted to allow for a two-sided hypothesis (48). The α levels may likewise be adjusted in each case, or tested for, if we want to assure ourselves that a mutagenic effect exists at a certain level of confidence (note that this is different from disproving the null hypothesis).

It should be noted that there are numerous specific recommendations for statistical methods designed for individual mutagenicity techniques, such as that of Bernstein et al. (13) for the Ames test.

Behavioral Toxicity

A brief review of the types of studies/experiments conducted in the area of behavioral toxicology and a classi

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fication of these into groups is in order. Although there are a small number of studies that do not fit into the following classification, the great majority may be fitted into one of the following four groups. Many of these points were first covered in earlier articles (61, 63).

The first type of study is observational. Observational score-type studies are based on observing and grading the response of an animal to its normal environment or to a stimulus that is imprecisely controlled. This type of result is generated by one of two major sorts of studies. Open-field studies involve placing an animal in the center of a flat, open area and counting each occurrence of several types of activities (grooming, moving outside a designated central area, rearing, etc.) or timing until the first occurrence of each type of activity. The data generated are scalar of either a continuous or discontinuous nature but frequently are not of a normal distribution. Tilson et al. (144) presented some examples of this sort.

Observational screen studies involve a combination of observing behavior and evoking a response to a simple stimulus, the resulting observation being graded as normal or as deviating from normal on a graded scale. Most of the data so generated are rank in nature, with some portions being quantal or interval. Irwin (89) and Gad (61) have presented schemes for the conduct of such studies, which became the basis of the commonly used functional observational battery. Table 7.9 gives an example of the nature (and of one form of statistical analysis) of such data generated after exposure to one material.

The second type of study is one that generates rates of response as data. The studies are based on the number of responses to a discrete controlled stimulus or are free of direct connection to a stimulus. The three most frequently measured parameters are licking of a liquid (milk, sugar water, ethanol, or a psychoactive agent in water), gross locomotor activity (measured by a photocell or elec tromagnetic device), or level pulling. Examples of such studies have been published by Annau (6) and Norton (114). The data generated are most often of a discontinuous or continuous scalar nature and are often complicated by underlying patterns of biological rhythm.

The third type of study generates a variety of data that are classified as error rate. These are studies based on animals learning a response to a stimulus or memorizing a simple task (such as running a maze or a Skinner box-type shock avoidance system). These tests or trials are structured so that animals can pass or fail on each of a number of successive trials. The resulting data are quantal, though frequently expressed as a percentage.

The final major type of study is that which results in data that are measures of the time to an endpoint. They are based on animals being exposed to or dosed with a toxicant and the time taken for an effect to be observed is measured. Usually the endpoint is failure to continue to be able to perform a task and can, therefore, be death, incapacitation, or the learning of a response to a discrete stimulus. Burt (22) and Johnson et al. (91) present data of this form. The data are always of a censored nature (i.e., the period of observation is always artificially limited, as in measuring time-to-incapacitation in combustion toxicology data, where animals are exposed to the thermal decomposition gases to test materials for a period of 30 minutes). If incapacitation is not observed during these 30 minutes, it is judged not to occur. The data generated by these studies are continuous, discontinuous, or rank in nature. They are discontinuous because the researcher may check, or may be restricted to checking for the occurrence of the endpoint only at certain discrete points in time. On the other hand, they are rank if the periods to check for occurrence of the endpoint are far enough apart, in which case one may actually only know that the endpoint occurred during a broad period of time, but not where in that period.

Table 7.9 Functional Observational Battery parameters showing significant differences between treated and control groups

	Rats (18-crown-6 animals given 40 mg/kg i.p.)				
Parameter	Control sum of ranks	Nc	18-crown-6 treated sum or ranks	NT	Observed difference in treated animals (as compared to controls)
Twitches	55.0	10	270.0	15	Involuntary muscle twitches
Visual placing	55.0	10	270.0	15	Less aware of visual stimuli
Grip strength	120.0	10	205.0	15	Considerable loss of strength, especially in hind limbs
Respiration	55.0	10	270.0	15	Increased rate of respiration
Tremors	55.0	10	270.0	15	Marked tremors

All parameters above are significant at $p<0.05$.

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There is a special class of test that should also be considered at this point—the behavioral teratology or reproduction study. These studies are based on dosing or exposing either parental animals during selected periods in the mating and gestation process or pregnant females at selected periods during gestation. The resulting offspring are then tested for developmental defects of a neurological and behavioral nature. Analysis is complicated by a number of facts:

(1) The parental animals are the actual targets for toxic effects, but observations are made on offspring.
(2) The toxic effects in the parental generation may alter the performance of the mother in rearing its offspring, which in turn can lead to a confusion of prenatal and postnatal effects.
(3) Different capabilities and behaviors develop at different times (discussed further later).

A researcher can, by varying the selection of the animal model (species, strain, sex), modify the nature of the data generated and the degree of dispersion of these data. In behavioral studies particularly, limiting the within-group variability of data is a significant problem and generally should be a highly desirable goal.

Most, if not all, behavioral toxicology studies depend on at least some instrumentation. Very frequently overlooked here (and, indeed, in most research) is that instrumentation, by its operating characteristics and limitations, goes a long way toward determining the nature of the data generated by it. An activity monitor measures motor activity in discrete segments. If it is a "jiggle cage"-type monitor, these segments are restricted so that only a distinctly limited number of counts can be achieved in a given period of time and then only if they are of the appropriate magnitude. Likewise, technique can also readily determine the nature of the data. In measuring response to pain, for example, one could record it as a quantal measure (present or absent), a rank score (on a scale of 1–5 for decreased to increased responsiveness, with 3 being "normal"), or as scalar data (by using an analgesia meter, which determines either how much pressure or heat is required to evoke a response).

Study design factors are probably the most widely recognized of the factors that influence the type of data resulting from a study. Number of animals used, frequency of measures, and length of period of observation are three obvious design factors that are readily under the control of the researcher and which directly help to determine the nature of the data.

Finally, it is appropriate to review each of the types of studies presently seen in behavioral toxicology, according to the classification presented at the beginning of this section, in terms of which statistical methods are used now and what procedures are recommended for use. The recommendations, of course, should be viewed with a critical eye. They are intended with current experimental design and technique in mind and can only claim to be the best when one is limited to addressing the most common problems from a library of readily and commonly available and understood tests.

Table 7.10 summarizes this review and recommendation process.

Carcinogenesis

The experimental evaluation of potentially carcinogenic substances is generally based on the exposure of nonhuman species to some relatively high dose and an attempt is made to predict the occurrence and level of

Table 7.10 Overview of statistical testing in behavioral toxicology: Those tests commonly used[a] as opposed to those recommended

Type of observation	Most commonly used procedures	Suggested procedures
Observational scores	Either Student's t-test or one-way ANOVA	Kruskal-Wallis nonparametric ANOVA or Wilcoxon Rank sum
Response rates	Either Student's t-test or one-way ANOVA	Kruskal-Wallis ANOVA or one way ANOVA
Error rates	ANOVA followed by a post-hoc test	Fisher's exact, or R×C chi-square, or Mann-Whitney U-test
Times to endpoint	Either Student's t-test or one-way ANOVA	ANOVA then a post-hoc test or Kruskal-Wallis ANOVA
Teratology and reproduction	ANOVA followed by a post-hoc test	Fisher's exact test, Kruskal-Wallis ANOVA, or Mann-Whitney U-test

[a]These are the most commonly used procedures. The reader need only look at the example articles cited in this chapter to verify this fact.

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Table 7.11 Sample size required to obtain a specified sensitivity at $p<0.05$

Background tumor incidence		Treatment Group Incidence									
	Pa	0.95	0.90	0.80	0.70	0.60	0.50	0.40	0.30	0.20	0.10
0.30	0.90	10	12	18	31	46	102	389			
	0.50	6	6	9	12	22	32	123			
0.20	0.90	8	10	12	18	30	42	88	320		
	0.50	5	5	6	9	12	19	28	101		
0.10	0.90	6	8	10	12	17	25	33	65	214	
	0.50	3	3	5	6	9	11	17	31	68	
0.05	0.90	5	6	8	10	13	18	25	35	76	464
	0.50	3	3	5	6	7	9	12	19	24	147
0.01	0.90	5	5	7	8	10	13	19	27	46	114
	0.50	3	3	5	5	6	8	10	13	25	56

aP=Power for each comparison of treatment group with background tumor incidence.

tumorogenesis in humans at much lower levels. This topic is addressed in Chapter 8 as well as in Gad (62).

The single most important statistical consideration in the design of carcinogenicity bioassays in the past was based on the point of view that what was being observed and evaluated was a simple quantal response (cancer occurred or it didn't) and that a sufficient number of animals needed to be used to have reasonable expectations of detecting such an effect. Though the single fact of whether or not the simple incidence of neoplastic tumors is increased due to an agent of concern is of interest, a much more complex model must now be considered. The time to tumor, patterns of tumor incidence, effects on survival rate, and age at first tumor all must now be included in a model.

Bioassay Design

As presented earlier in the section on experimental design, the first step that must be taken is to clearly state the objective of the study to be undertaken. Carcinogenicity bioassays have two possible objectives.

The first objective is to detect possible carcinogens. Compounds are evaluated to determine if they can or cannot induce a statistically detectable increase in tumor rates over background levels, and only by happenstance is information generated that is useful in risk assessment. Most older studies have such detection as their objective. Current belief is that at least two species must be used for detection, though the necessity of a second species (the mouse) is increasingly questioned.

The second objective for a bioassay is to provide a range of dose response information (with tumor incidence being the response) so that a risk assessment may be performed. Unlike detection, which requires only one treatment group with adequate survival times (to allow expression of tumors), dose response requires at least three treatment groups with adequate survival. We will shortly look at the selection of dose levels for this case; however, given that the species is known to be responsive, only one species of animal need be used for this objective.

To address either or both of these objectives, three major types of study designs have evolved. First is the classical skin painting study, usually performed in mice. A single, easily detected endpoint (the formation of skin tumors) is evaluated during the course of the study. Though dose response can be evaluated in such a study (dose usually being varied by using different concentrations of test material in volatile solvent), most often detection is the objective of such a study. Though others have used different frequencies of application of test material to vary dose, there are data to suggest that this only serves to introduce an additional variable (166). Traditionally, both test and control groups in such a test consist of 50 to 100 mice of one sex (males being preferred because of their very low spontaneous tumor rate). This design is also used in tumor initiation/promotion studies.

The second common type of design is the original National Cancer Institute (NCI) bioassay. The announced objective of these studies was detection of moderate to strong carcinogens, though the results have also been used in attempts at risk assessment. Both mice and rats were used in parallel studies. Each study used 50 males and 50 females at each of two dose levels (high and low) plus an equal-sized control group. The National Toxicology Program (NTP) has subsequently moved away from this design because of a recognition of its inherent limitations. More animals per group and more dose groups are now used.

Table 7.12 Average number of animals needed to detect a significant increase in the incidence of an event (tumors, anomalies, etc.) over the background incidence (control) at several expected incidence levels using the Fisher exact probability test (p=0.05)

Background incidence, %	Expected Increase in Incidence, %					
	0.01	0.1	1	3	5	10
0	46,000,000a	460,000	4,600	511	164	46
0.01	46,000,000	460,000	4,600	511	164	46
0.1	47,000,000	470,000	4,700	520	168	47
1	51,000,000	510,000	5,100	570	204	51
5	77,000,000	770,000	7,700	856	304	77
10	100,000,000	1,000,000	10,000	1,100	400	100
20	148,000,000	1,480,000	14,800	1,644	592	148
25	160,000,000	1,600,000	16,000	1,840	664	166

aNumber of animals needed in each group, controls as well as treated.

Finally, there is the standard industrial toxicology design, which uses at least two species (usually rats and mice) in groups of no fewer that 100 males and females each. Each study has three dose groups and at least one control. Frequently, additional numbers of animals are included to allow for interim terminations and histopathological evaluations. In both this and the NCI design, a long list of organs and tissues are collected, processed, and examined microscopically. This design seeks to address both the detection and dose response objectives with a moderate degree of success.

Selecting the number of animals to use for dose groups in a study requires consideration of both biological (expected survival rates, background tumor rates, etc.) and statistical factors. The prime statistical consideration is reflected in Table 7.11. It can be seen in this table that if, for example, we were studying a compound that caused liver tumors and were using mice (with a background or control incidence of 30%), we would have to use 389 animals per sex per group to be able to demonstrate that an incidence rate of 40% in treatment animals was significant compared to the controls at the p=0.05 level.

Perhaps the most difficult aspect of designing a good carcinogenicity study is the selection of the dose levels to be used. At the start it is necessary to consider the first underlying assumption in the design and use of animal cancer bioassays—the need to test at the highest possible dose for the longest practical period.

The rationale behind this assumption is that though humans may be exposed at very low levels, detecting the resulting small increase (over background) in the incidence of tumors would require the use of an impractically large number of test animals per group. This point is illustrated by Table 7.11, where, for instance, while only 46 animals (per group) are needed to show a 10% increase over a zero background (i.e., a rarely occurring tumor type), 770,000 animals (per group) would be needed to detect a tenth of a percent increase above a 5% background. As we increase dose, however, the incidence of tumors (the response) will also increase until it reaches the point where a modest increase (say, 10% over a reasonably small background level (say, 1%)) could be detected using an acceptably small-sized group of test animals (in Table 7.12 we see that 51 animals would be needed for this example case). There are, however, at least two real limitations to the highest dose level. First, the test rodent population must have a sufficient survival rate after receiving a lifetime (or two years) of regular doses to allow for meaningful statistical analysis. Second, we really want the metabolism and mechanism of action of the chemical at the highest level tested to be the same as at the low levels, where human exposure would occur. Unfortunately, toxicologists usually must select the high dose level based only on the information provided by a subchronic or range-finding study (usually 90 days in length), but selection of either too low or too high a dose will make the study invalid for detection of carcinogenicity and may seriously impair the use of the results for risk assessment.

There are several solutions to this problem. One of these solutions has been the rather simplistic approach of the NTP Bioassay Program, which is to conduct a 3-month range-finding study with sufficient dose levels to establish a level that significantly (10%) decreases the rate of body weight gain. This dose is defined as the maximum tolerated dose (MTD) and is selected as the highest dose. Two other levels, generally one half MTD and one quarter MTD, are selected for testing as the intermediate and low-dose levels. In many earlier NCI studies, only one other level was used.

The dose range-finding study is necessary in most cases, but the suppression of body weight gain is a

scientifically questionable bench mark when dealing with establishment of safety factors. Physiologic, pharmacologic, or metabolic markers generally serve as better indicators of systemic response than body weight. A series of well-defined acute and subchronic studies designed to determine the "chronicity factor" and to study onset of pathology can be more predictive for dose setting than body weight suppression.

Also, the NTP's MTD may well be at a level where the metabolic mechanisms for handling a compound at real-life exposure levels have been saturated or overwhelmed, bringing into play entirely artifactual metabolic and physiologic mechanisms (68). The regulatory response to questioning the appropriateness of the MTD as a high-dose level (82) has been to acknowledge that occasionally an excessively high dose is selected, but to counter by saying that using lower doses would seriously decrease the sensitivity of detection.

QUESTIONS

1. If the results of an analysis of variance establish that differences between groups is significant at the $p \leq 0.05$ level, what are the chances that the two groups are not different?

a) 5% or less. That is, one in twenty or less.

2. If a set of 17 measured values are ranked in ascending order, the median value is which value in order?

a) The 9th value.

3. Analysis of variance assumes that the data it is evaluating is from a normally distributed population. The test is robust for deviation from this assumption if what?

a) The sample size is large enough.

4. In what manner does a lag profit transformation of a dose response curve alter its shape?

a) It linearizes it.

5. Does the occurrence of outliers in a sample increase or decrease the sensitivity of any hypothesis testing (such as ANOVA)?

a) Decreases Sensitivity.

Groups of incidence data are usually compared by contingency table tests (such as Fishers Extract Test) of ANOVA?

a) Contingency table analysis.

REFERENCES

1. Abramowitz, M., and Stegun, I.A. (1964): *Handbook of Mathematical Functions*, pp. 925–964. National Bureau of Standards, Washington.

2. Aeschbacher, H.U., Vautaz, L., Sotek, J., and Stalder, R. (1977): Use of the beta binomial distribution in dominant-lethal testing for "weak mutagenic activity," Part 1. *Mutat. Res.*, 44:369–390.

3. Anderson, S., Auquier, A., Hauck, W.W., Oakes, D., Vandaele, W., and Weisburg, H.I. (1980): *Statistical Methods for Comparative Studies*. John Wiley & Sons, New York.

4. Anderson, T.W. (1971): *The Statistical Analysis of Time Series*. Wiley, New York.

5. Anderson, E. (1960): A semigraphical method for the analysis of complex problems. *Technomet.*, 2:387–391.

6. Annau, Z. (1972): The comparative effects of hypoxia and carbon monoxide hypoxia on behavior. In: *Behavioral Toxicology*, edited by B.Weiss and V.G.Laties, pp. 105–127. Plenum Press, New York.

7. Anscombe, F.J. (1973): Graphics in statistical analysis, *The American Statistician*, 27:17–21.

8. Armitage, P. (1955) Tests for linear trends in proportions and frequencies. *Biometrics.*, 11:375–386.

9. Atchely, W.R., and Bryant, E.H. (1975): *Multivariate Statistical Methods: Among Groups Covariation*. Dowden, Hutchinson and Ross, Stroudsburg.

10. Barnett, V., and Lewis, T. (1984): *Outliers in Statistical Data*, Edition 2. John Wiley, New York.

11. Bateman, A.T. (1977): The dominant lethal assay in the male mouse. In: *Handbook of Mutagenicity Test Procedures*, edited by B.J.Kilbey, M.Legator, W.Nichols, and C.Ramel, pp. 325–334. Elsevier, New York.

12. Beckman, R.J., and Cook, R.D. (1983): Outliers. *Technometrics*, 25:119–163.

13. Bernstein, L., Kaldor, J., McCann, J., and Pike, M.C. (1982): An empirical approach to the statistical analysis of mutagenesis data from the Salmonella test. *Mutation Res.*, 97:267–281.

14. Beyer, W.H. (1976): *Handbook of Tables for Probability and Statistics*. CRC Press, Boca Raton, FL.

15. Bliss, C.I. (1935): The calculation of the dosage-mortality curve. *Ann. Appl. Biol.*, 22:134–167.

16. Bloomfield, P. (1976): *Fourier Analysis of Time Series: An Introduction*. John Wiley, New York.

17. Boyd, E.M., and Knight, L.M. (1963): Postmortem shifts in the weight and water levels of body organs. *Tox. Appl. Pharm.*, 5:119–128.

18. Boyd, E.M. (1972): *Predictive Toxicometrics.* Williams & Wilkins, Baltimore.
19. Breslow, N. (1984): Comparison of survival curves. In: *Cancer Clinical Trials: Methods and Practice*, edited by M.F.Buse, M.J.Staguet, and R.F.Sylvester, pp. 381–406. Oxford University Press, Oxford.
20. Bruce, R.D. (1985): An up-and-down procedure for acute toxicity testing. *Fund. Appl. Toxicol.*, 5:151–157.
21. Bryant, E.H., and Atchely, W.R. (1975): *Multivariate Statistical Methods: Within-Groups Covariation.* Dowden, Hutchinson and Ross, Stroudsburg.
22. Burt, G.S. (1972): Use of behavioral techniques in the assessment of environmental contaminants. In: *Behavioral Toxicology*, edited by B.Weiss and V.G.Laties, pp. 241–263. Plenum Press, New York.
23. Chambers, J.M., Cleveland, W.S., Kleiner, B., and Tukey, P.A. (1983): *Graphical Methods for Data Analysis.* Wadsworth, Belmont.
24. Chernoff, H. (1973): The use of faces to represent points in K-dimensional space graphically. *J. Amer. Stat. Assoc.*, 68:361–368.
25. Cleveland, W.S., and McGill, R. (1984): Graphical perception: Theory, experimentation, and application to the development of graphical methods, *Journal of the American Statistical Association*, 79:531–554.
26. Cleveland, W.S. (1985): *The Elements of Graphing Data.* Wadsworth Advanced Books, Monterey, CA.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_361.html[2010/10/28 18:43:27]

27. Cochran, W.F. (1954): Some models for strengthening the common $x2$ tests. *Biometrics.*, 10:417–451.
28. Cochran, W.G., and Cox, G.M. (1975): *Experimental Designs.* John Wiley, New York.
29. Cox, D.R., and Stuart, A. (1955): Some quick tests for trend in location and dispersion. *Biomet.*, 42:80–95.
30. Cox, D.R. (1972): Regression models and life-tables. *J. Roy. Stat. Soc.*, 348:187–220.
31. Cremer, J.E., and Seville, M.P. (1982): Comparative effects of two pyrethroids dietamethrin and cismethrin, on plasma catecholamines and on blood glucose and lactate. *Toxicol. Appl. Pharmacol.*, 66:124–133.
32. Crowley, J., and Breslow, N. (1984): Statistical analysis of survival data. *Annual Review of Public Health*, 5:385–411.
33. Cutler, S.J., and Ederer, F. (1958): Maximum utilization of the life table method in analyzing survival. *J. Chron. Dis.*, 8:699–712.
34. Davidson, M.L. (1972): Univariate versus multivariate tests in repeated-measures experiments. *Psych. Bull.*, 77:446–452.
35. Davison, M.L. (1983): *Multidimensional Scaling.* John Wiley, New York.
36. Dean, B.J., and Johnston, A. (1977): Dominant lethal assays in the male mice: Evaluation of experimental design, statistical methods and the sensitivity of Charles River (CD1) mice. *Mutat. Res.*, 42:269–278.
37. Debanne, S.M., and Haller, H.S. (1985): Evaluation of statistical methodologies for estimation of median effective dose. *Tox. Appl. Pharm.*, 79:274–282.
38. DePass, L.R., Myers, R.C., Weaver, E.V., and Weil, C.S. (1984): An assessment of the importance of number of dosage levels, number of animals per dosage level, sex and method of LD50 and slope calculations in acute toxicity studies. In: *Alternate Methods in Toxicology, Vol. 2: Acute Toxicity Testing: Alternate Approaches*, edited by A.M.Goldberg. Mary Ann Liebert, Inc., New York.
39. Diamond, W.J. (1981): *Practical Experimental Designs.* Lifetime Learning Publications, Belmont, CA.
40. Diem, K., and Lentner, C. (1975): *Documenta Geigy Scientific Tables*, pp. 158–159. Geigy, New York.
41. Dixon, W.J. (1994): *BMD-Biomedical Computer Programs.* University of California Press, Berkeley.
42. Dixon, W.J., and Massey, F.J., Jr. (1969): *Introduction to Statistical Analysis*, Edition 3. McGraw-Hill, New York.
43. Draper, N.R., and Smith, H. (1998): *Applied Regression Analysis*, Edition 3. John Wiley, New York.
44. Duncan, D.B. (1955): Multiple range and multiple F tests. *Biomet.*, 11:1–42.
45. Dunnett, C.W. (1955): A multiple comparison procedure for comparing several treatments with a control. *J. Am. Stat. Assoc.*, 50:1096–1121.
46. Dunnett, C.W. (1964): New tables for multiple comparison with a control. *Biomet.*, 16:671–685.
47. Dykstra, R.L., and Robertson, T. (1983): On testing monotone tendencies. *J. Amer. Stat. Associ.*, 78:342–350.
48. Ehrenberg, L. (1977): Aspects of statistical inference in testing genetic toxicity. In: *Handbook of Mutagenicity Test Procedures*, edited by B.J.Kilbey, M.Legator, W.Nichols, and C.Ramel, pp. 419–459. Elsevier, New York.
49. Elandt-Johnson, R.C., and Johnson, N.L. (1980): *Survival Models and Data Analysis.* John Wiley, New York.
50. Engelman, L., and Hartigan, J.A. (1969): Percentage points of a test for clusters. *Journal of the American Statistical Association*, 64:1647–1648.
51. Everitt, B.S., and Hand, D.J. (1981): Finite mixture distributions. Chapman and Hall, New York.
52. Everitt, B. (1980): *Cluster Analysis.* Halsted Press, New York.
53. *Federal Register* (1985): No. 50, Vol. 50. Washington.
54. Federer, W.T. (1955): *Experimental Design.* Macmillan, New York.
55. Feinstein, A.R. (1979): Scientific standards versus statistical associations and biological logic in the analysis of causation. *Clin. Pharmacol. Therapeu.*, 25:481–492.
56. Finney, D.J., Latscha, R., Bennet, B.M., and Hsu, P. (1963): *Tables for Testing Significance in a 2×2 Contingency Table.* Cambridge University Press, Cambridge.
57. Finney, D.K. (1977): *Probit Analysis*, Edition 3. Cambridge University Press, Cambridge.
58. Gabriel, K.R. (1981): Biplot display of multivariate matrices for inspection of data and diagnosis. In: *Interpreting Multivariate Data*, edited by V.Barnett, pp. 147–173. John Wiley, New York.
59. Gad, S.C., Smith, A.C., Cramp, A.L., Gavigan, F.A., and Derelanko, M.J. (1984): Innovative designs

and practices for acute systemic toxicity studies. *Drug Chem. Toxicol.*, 7:423–434.
60. Gad, S.C., and Chengelis, C.P. (1992): *Animal Models in Toxicology.* Marcel Dekker, New York.
61. Gad, S.C. (1982): A neuromuscular screen for use in industrial toxicology. *J. Toxicol. Env. Health*, 9:691–704.
62. Gad, S.C. (1998): *Statistics and Experimental Design for Toxicologists.* CRC Press, Boca Raton, FL.
63. Gad, S.C. (1984): Statistical analysis of behavioral toxicology data and studies. *Arch. Toxicol. Suppl.*, 5:256–266.
64. Gad, S.C., Reilly, C., Siino, K.M., and Gavigan, F.A. (1985): Thirteen cationic ionophores: Neurobehavioral and membrane effects. *Drug and Chemical Toxicology*, 8:451–468.
65. Gallant, A.R. (1975): Nonlinear regression. *Am. Stat.*, 29:73–81.
66. Garrett, H.E. (1947): *Statistics in Psychology and Education*, pp. 215–218. Longmans, Green, New York.
67. Gaylor, D.W. (1978): Methods and concepts of biometrics applied to teratology. In: *Handbook of Teratology, Vol. 4,* edited by J.G. Wilson and F.C.Fraser, pp. 429–444. Plenum Press, New York.
68. Gehring, P.J., and Blau, G.E. (1977): Mechanisms of carcinogenicity: Dose response. *J. Environ. Path. Toxicol.*, 1:163–179.
69. Ghent, A.W. (1972): A method for exact testing of 2×2, 2×3, 3×3 and other contingency tables, employing binomiate coefficients. *American Midland Naturalist*, 88:15–27.
70. Glass, L. (1975): Classification of biological networks by their qualitative dynamics. *J. Theor. Bio.*, 54:85–107.
71. Gnanadesikan, R. (1977): *Methods for Statistical Data Analysis of Multivariate Observations.* John Wiley, New York.
72. Gold, H.J. (1977): *Mathematical Modeling of Biological System: An Introductory Guidebook.* John Wiley, New York.
73. Gordon, A.D. (1981): *Classification.* Chapman and Hall, New York.
74. Grafe, A., and Vollmar, J. (1977): Small numbers in mutagenicity tests. *Arch. Toxicol.*, 38:27–34.
75. Gray, L.E., and Laskey, J.W. (1980): Multivariate analysis of the effects of manganese on the reproductive physiology and behavior of the male house mouse. *J. Toxicol. Environ. Health*, 6:861–868.
76. Grubbs, F.E. (1969): Procedure for detecting outlying observations in samples. *Technometrics*, 11:1–21.
77. Hammond, E.C., Garfinkel, L., and Lew, E.A. (1978): Longevity, selective mortality, and competitive risks in relation to chemical carcinogenesis. *Environ. Res.*, 16:153–173.
78. Harris, E.K. (1978): Review of statistical methods of analysis of series of biochemical test results. *Ann. Biol. Clin.*, 36:194–197.
79. Harris, R.J. (1975): *A Primer of Multivariate Statistics*, pp. 96–101. Academic Press, New York.
80. Harter, A.L. (1960): Critical values for Duncan's new multiple range test. *Biomet.*, 16:671–685.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_362.html[2010/10/28 18:43:28]

81. Hartigan, J.A. (1983): Classification. *Encyclopedia of Statistical Sciences*, Vol. 2, edited by S.Katz and N.L.Johnson. John Wiley, New York.
82. Haseman, J.K. (1985): Issues in carcinogenicity testing: Dose selection. *Fund. App. Toxicol.*, 5:66–78.
83. Haseman, J.K. (1977): Response to use of statistics when examining life time studies in rodents to detect carcinogenicity. *J. Toxicol. Environ. Health*, 3:633–636.
84. Henry, R.D., and Hidy, G.M. (1979): Multivariate analysis of particulate sulfate and other air quality variables by principle components. *Atmos. Environ.*, 13:1581–1596.
85. Hicks, C.R. (1982): *Fundamental Concepts in the Design of Experiments.* Holt, Rinehart, and Winston, New York.
86. Hoaglin, D.C., Mosteller, F., and Tukey, J.W. (1983): *Understanding Robust and Explanatory Data Analysis.* John Wiley, New York.
87. Hollander, M., and Wolfe, D.A. (1999): *Nonparametric Statistical Methods*, Edition 2. John Wiley, New York.
88. Hotelling, H. (1931): The generalization of Student's ratio. *Ann. Math. Stat.*, 2:360–378.
89. Irwin, S. (1968): Comprehensive observational assessment. Systematic, quantitative procedure for assessing the behavioral and physiologic state of the mouse. *Psychopharmacologia.*, 13:222–257.
90. Jackson, B. (1962): Statistical analysis of body weight data. *Toxicol. Appl. Pharmacol.*, 4:432–443.
91. Johnson, B.L., Anger, W.K., Setzer, J.V., and Xinytaras, C. (1972): The application of a computer controlled time discrimination performance to problems. In: *Behavioral Toxicology*, edited by B.Weiss and V.G.Laties, pp. 129–153. Plenum Press, New York.
92. Joung, H.M., Miller, W.M., Mahannah, C.N., and Guitjens, J.C. (1979): A generalized water quality index based on multivariate factor analysis. *J. Environ. Qual.*, 8:95–100.
93. Kastenbaum, M.A., and Bowman, K.O. (1970): Tables for determining the statistical significance of mutation frequencies. *Mutat. Res.*, 9:527–549.
94. Katz, A.J. (1978): Design and analysis of experiments on mutagenicity. I: Minimal sample sizes. *Mutat. Res.*, 50:301–307.
95. Katz, A.J. (1979): Design and analysis of experiments on mutagenicity. II: Assays involving micro-organisms. *Mutat. Res.*, 64:61–77.
96. Kilbey, B.J., Legator, M., Nicholas, W., and Ramel, C. (1977): *Handbook of Mutagenicity Test Procedures*, pp. 425–433. Elsevier, New York.
97. Kotz, S., and Johnson, N.L. (1982): *Encyclopedia of Statistical Sciences, Vol. 1*, pp. 61–69. John Wiley & Sons, New York.
98. Kowalski, B.R., and Bender, C.F. (1972): Pattern recognition: A powerful approach to interpreting chemical data. *J. Amer. Chem. Soc.*, 94:5632–5639.
99. Kraemer, H.C., and Thiemann, G. (1987): *How Many Subjects? Statistical Power Analysis in Research.* Sage Publications, Newbury Park, California.
100. Kupper, L.L., and Haseman, J.K. (1978): The use of a correlated binomial model for the analysis of certain toxicological experiments. *Biomet.*, 34:69–76.
101. Lee, E.T. (1980): *Statistical Methods for Survival Data Analysis.* Lifetime Learning Publications, Belmont.
102. Litchfield, J.T., and Wilcoxon, F. (1949): A simplified method of evaluating dose effect experiments. *J. Pharmacol. Exp. Ther.*, 96:99–113.
103. Loeb, W.F., and Quimby, F.W. (1989): *The Clinical Chemistry of Laboratory Animals.* Pergamon Press, New York.
104. Marriott, F.H.C. (1991): *The Dictionary of Statistical Terms.* Longman Scientific & Technical, Essex, England.
105. Martin, H.F., Gudzinowicz, B.J., and Fanger, H. (1975): *Normal Values in Clinical Chemistry.* Marcel Dekker, New York.
106. Mendal, N.R., Finch, S.J., and Thode, H.C., Jr. (1993): Where is the likelihood ratio test powerful for detecting two component normal mixtures? *Biometrics*, 49:907–915.
107. Meyer, S.L. (1975): *Data Analysis for Scientists and Engineers*, pp. 17–18. John Wiley, New York.
108. Myers, J.L. (1972): *Fundamentals of Experimental Designs.* Allyn and Bacon, Boston.
109. Mitruka, B.M., and Rawnsley, H.M. (1977): *Clinical Biochemical and Hematological Reference Values in Normal Animals*. Masson, New York.
110. Montgomery, D.C., and Smith, E.A. (1983): *Introduction to Linear Regression Analysis.* John Wiley, New York.

111. Mosteller, F., and Youtz, C. (1961): Tables of the Freeman-Tukey transformations for the binomial and Poisson distributions. *Biometrika*, 48:433–440.
112. Nelson, C.J., and Holson, J.F. (1978): Statistical analysis of teratologic data: Problems and advancements.*J. Environ. Pathol. Toxicol.*, 2:187–199.
113. Nie, N.H., Hall, C.H., Jenkins, J.G., Steinbrenner, K., and Bent, D.H. (1995): *Statistical Package for the Social Sciences*. McGraw-Hill, New York.
114. Norton, S. (1973): Amphetamine as a model for hyperactivity in the rat. *Physiol. Behav.*, 11:181–186.
115. Oser, B.L., and Oser, M. (1956): Nutritional studies in rats on diets containing high levels of partial ester emulsifiers. II: Reproduction and lactation. *J. Nutr.*, 60:429.
116. Paintz, M., Bekemeier, H., Metzner, J., and Wenzel, U. (1982): Pharmacological activities of a homologous series of pyrazole derivatives including quantitative structure: Activity relationships (QSAR). *Agents Actions* (Suppl.), 10:47–58.
117. Peto, R., Pike, M.C., Armitage, P., Breslow, N.E., Cox., D.R., Howard, S.V., Kantel, N., McPherson, K., Peto, J., and Smith, P.G. (1977): Design and analysis of randomized clinical trials requiring prolonged observations of each patient, II, Analyses and examples, *British Journal of Cancer*, 35:1–39.
118. Peto, R., and Pike, M.C. (1973): Conservatism of approximation; (0—E) 2 E in the Log Rank Test for survival data on tumour incidence data, *Biometrics*, 29:579–584.
119. Pollard, J.H. (1977): *Numerical and Statistical Techniques*. Cambridge University Press, New York.
120. Portier, C., and Hoel, D. (1984): Type I error of trend tests in proportions and the design of cancer screens. *Comm. Stat. Theory Meth.*, A13:I-14.
121. Prentice, R.L. (1976): A generalization of the probit and logit methods for dose response curves. *Biomet.*, 32:761–768.
122. Rai, K., and Ryzin, J.V. (1985): A dose-response model for teratological experiments involving quantal responses. *Biomet.*, 41:1–9.
123. Ridgemen, W.J. (1975): *Experimentation in Biology*, pp. 214–215. Wiley, New York.
124. Romesburg, H.C. (1984): *Cluster Analysis for Researchers*. Lifetime Learning Publications, Belmont. 43:45–58.
125. Ryan, T.A., Joyner, B.L., and Ryan, B.F. (1996): *Minitab Reference Manual*, Duxbury Press, Boston.
126. Salsburg, D. (1980): The effects of life-time feeding studies on patterns of senile lesions in mice and rats. *Drug Chem. Tox.*, 3:1–33.
127. SAS Institute (1996): *SAS Users Guide 1996 Edition*. SAS Institute, Cary, NC.
128. Schaeffer, D.J., Glave, W.R., and Janardan, K.G. (1982): Multivariate statistical methods in toxicology, III: Specifying joint toxic interaction using multiple regression analysis. *J. Toxicol. Env. Health*, 9:705–718.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_363.html[2010/10/28 18:43:29]

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129. Schaffer, J.W., Forbes, J.A., and Defelice, E.A. (1967): Some suggested approaches to the analysis of chronic toxicity and chronic drug administration data. *Toxicol. Appl. Pharmacol.*, 10:514–522.
130. Schaper, M., Thompson, R.D., and Alarie, Y. (1985): A method to classify airborne chemicals which alter the normal ventilatory response induced by CO2. *Toxicol. Appl. Pharmacol.*, 79:332–341.
131. Scheffe, H. (1959): *The Analysis of Variance.* Wiley, New York.
132. Schmid, C.F. (1983): *Statistical Graphics.* John Wiley, New York.
133. Seal, H.L. (1964): *Multivariate Statistical Analysis for Biologists.* Methuen, London.
134. Shirley and Newman (1954).
135. Shy-Modjeska, J.S., Riviere, J.E., and Rawldings, J.O. (1984): Application of biplot methods to the multivariate analysis of toxicological and pharmacokinetic data. *Toxicol. Appl. Pharmacol.*, 72:91–101.
136. Siegel, S. (1956): *Nonparametric Statistics for the Behavioral Sciences.* McGraw-Hill, New York.
137. Snedecor, G.W., and Cochran, W.G. (1980): *Statistical Methods,* Edition 7. Iowa State University Press, Ames.
138. Sokal, R.R., and Rohlf, F.J. (1994): *Biometry,* Edition 3. W.H. Freeman, San Francisco.
139. SOT ED01 Task Force (1981): Reexamination of the ED01 study-adjusting for time on study. *Fundam. Appl. Toxicol.*, 1:8–123.
140. Taketomo, R.T., McGhan, W.F., Fushiki, M.R., Shimada, A., and Gumpert, N.F. (1982): Gentamycin nephrotoxicity application of multivariate analysis. *Clin. Pharm.*, 1:554–549.
141. Tarone, R.E. (1975): Tests for trend in life table analysis. *Biometrika,* 62:679–682.
142. Tatsuoka, M.M. (1971): *Multivariate Analysis.* John Wiley, New York.
143. Thompson, W.R., and Weil, C.S. (1952): On the construction of tables for moving average interpolation. *Biomet.*, 8:51–54.
144. Tilson, H.A., Cabe, P.A., and Burne, T.A. (1980): Behavioral procedures for the assessment of neurotoxicity. In: *Experimental and Clinical Neurotoxicology,* edited by P.S. Spencer and N. H.Schaumburg, pp. 758–766. Williams & Wilkins, Baltimore.
145. Tufte, E.R. (1990): *Envisioning Information.* Graphics Press, Cheshire, CT.
146. Tufte, E.R. (1983): *The Visual Display of Quantitative Information.* Graphics Press, Cheshire, CT.
147. Tufte, E.R. (1997): *Visual Explanations.* Graphics Press, Cheshire, CT.
148. Tukey, J.W. (1977): *Exploratory Data Analysis.* Addison-Wesley Publishing Co., Reading, PA.
149. Velleman, P.F., and Hoaglin, D.C. (1981): *Applications, Basics and Computing of Exploratory Data Analysis.* Duxbury Press, Boston.
150. Vollmar, J. (1977): Statistical problems in mutagenicity tests. *Arch. Toxicol.*, 38:13–25.
151. Vuataz, L., and Sotek, J. (1978): Use of the beta-binomial distribution in dominant-lethal testing for "weak mutagenic activity," Part 2. *Mutat. Res.*, 52:211–230.
152. Weil, C.S. (1975): Toxicology experimental design and conduct as measured by inter-laboratory collaboration studies. *J. Assoc. Off. Anal. Chem.*, 58:687–688.
153. Weil, C.S. (1972): Statistics versus safety factors and scientific judgment in the evaluation of safety for man. *Toxicol. Appl. Pharmacol.*, 21:459–472.
154. Weil, C.S., Carpenter, C.P., and Smith, H.I. (1953): Specifications for calculating the median effective dose. *Amer. Indust. Hyg. Assoc. Quart.*, 14:200–206.
155. Weil, C.S. (1962): Applications of methods of statistical analysis to efficient repeated-dose toxicological tests. I: General considerations and problems involved: Sex differences in rat liver and kidney weights. *Toxicol. Appl. Pharmacol.*, 4:561–571.
156. Weil, C.S., and Gad, S.C. (1980): Applications of methods of statistical analysis to efficient repeated-dose toxicologic tests. 2: Methods for analysis of body, liver and kidney weight data. *Toxicol. Appl. Pharmacol.*, 52:214–226.
157. Weil, C.S. (1973): Experimental design and interpretation of data from prolonged toxicity studies. In: *Proc. 5th Int. Congr. Pharmacol.*, Vol. 2, pp. 4–12. Beacon Press, San Francisco.
158. Weil, C.S. (1982): Statistical analysis and normality of selected hematologic and clinical chemistry measurements used in toxicologic studies. *Arch. Toxicol.*, (Suppl.) 5:237–253.
159. Weil, C.S. (1978): A critique of the collaborative cytogenetics study to measure and minimize interlaboratory variation. *Mutat. Res.*, 50:285–291.
160. Weil, C.S. (1952): Tables for convenient calculation of median-effective dose (LD50 or ED50) and instructions in their use. *Biomet.*, 8:249–263.
161. Weil, C.S. (1983): Economical LD50 and slope determinations. *Drug Chem. Toxicol.*, 6:595–603.
162. Weil, C.S. (1970): Selection of the valid number of sampling units and a consideration of their combination in toxicological studies involving reproduction, teratogenesis or carcinogenesis. *Food*

Cosmet. Toxicol., 8:177–182.
163. Williams, R., and Buschbom, R.L. (1982): Statistical Analysis of Litter Experiments in Teratology. *Battelle PNL-4425.*
164. Williams, D.A. (1971): A test for differences between treatment means when several dose levels are compared with a zero dose control. *Biomet.*, 27:103:117.
165. Williams, D.A. (1972): The comparison of several dose levels with a zero dose control. *Biomet.*, 28:519–531.
166. Wilson, J.S., and Holland, L.M. (1982): The effect of application frequency on epidermal carcinogenesis assays. *Toxicol.*, 24:45–53.
167. Witten, M., Bennet, C.E., and Glassman, A. (1981): Studies on the toxicity and binding kinetics of abrin in normal and Epstein Barr virus-transformed lymphocyte culture-I: Experimental results. *Exp. Cell. Biol.*, 49:306–318.
168. Woodward, W.A., Elliott, A.C., and Gray, H.L. (1985): *Directory of Statistical Microcomputer Software.* Marcel Dekker, New York.
169. Young, J.E., and Matthews, P. (1981): Pollution injury in Southeast Northumberland, England UK: The analysis of field data using economical correlation analysis. *Environ. Pollut. Sen. B. Chem. Phys.*, 2:353–366.
170. Young, F.W. (1985): Multidimensional scaling. In: *Encyclopedia of Statistical Sciences*, Vol. 5, edited by S.Katz and N.L.Johnson, pp. 649–659. John Wiley, New York.
171. Zar, J.H. (1974): *Biostatistical Analysis*, p. 50. Prentice-Hall, Englewood.
172. Zbinden, G., and Flury-Roversi, M. (1981): Significance of the LD50 test for the toxicological evaluation of chemical substances. *Arch. Toxicol.*, 47:77–99.
173. Conover, J.W., and Inman, R.L. (1981): Rank transformation as a bridge between parametric and nonparametric statistics, *The American Statistician*, 35:124–129.

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Chapter **8**

Quantitative Extrapolations in Toxicology

Joseph V.Rodricks, David W.Gaylor, and Duncan Turnbull

Principles and Methods of Toxicology,
Fourth Edition, edited by A.Wallace Hayes.
Taylor & Francis, Philadelphia © 2001.

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The purpose of this chapter is to describe the scientific basis for extrapolation of toxicity findings in laboratory animals to predict outcomes in human populations. Such extrapolation is often said to comprise two components, one qualitative in nature, the second quantitative. Qualitative extrapolations generally concern the nature of the toxic response (are the specific toxicity endpoints observed in test animals also expected in similarly exposed human beings?), while quantitative extrapolations concern issues such as the magnitude and duration of the dose at which human beings and test animals are expected to be at equal risk of toxicity. Although both types of extrapolation are important, scientific understanding of the basis for quantitative extrapolations across species is more limited than is the basis for qualitative extrapolations. At the same time, because results from animal toxicity studies have become such important determinants of regulatory and other public health protection activities, and because the latter typically require the establishment of quantitative limits on human exposure, questions regarding quantitative extrapola tions have come to be seen as ultimately of greater significance than those that are purely qualitative in character. Thus, as in many other areas in which science plays a significant role in the development of social policies, the questions that are of most importance tend to be those about which science has the least clear answers. It is therefore no surprise that there is so much public skepticism about the predictions of toxicologists and risk assessors.

The scientific basis for quantitative extrapolations across species is not, however, as feeble as it is sometimes made out to be by those who would minimize the importance of toxicology, and science in general, in regulatory and public health decisions. The principal purpose of this chapter is to describe that basis as it is understood today, and to point to the many active areas of research that are devoted to furthering that understanding. As has been the case in the evolution of other areas of science, the greatest understanding comes with the ability to provide quantitatively accurate, empirical descriptions

of physical and biological phenomena, and to build from these gen

erally applicable, predictive models of those phenomena. It is true, of course, that quantitative understanding never provides a complete description of these phenomena—the qualitative aspects will always be necessary to complete the picture.

Following brief discussions of the historical context of and need for interspecies extrapolation, and a description of the problem, the chapter continues with five sections on different aspects of the problem of extrapolation (these are outlined in the section on the need for extrapolation), and concludes with a discussion of the overall strategy for scientifically based, interspecies extrapolation.

HISTORICAL CONTEXT

The use of experimental animals to study biological phenomena arose in the mid 19th century, but their modern use in toxicology had its origin during the third decade of the 20th century, when several investigators began to study the effects of vitamins, minerals, and other food constituents. At about the same time, efforts were initiated to identify and breed species and strains of laboratory animals whose genetic and physiological characteristics, and nutritional requirements, could be sufficiently well defined that they could be reliably used in controlled experiments. By the mid-1930s a number of government and industrial laboratories in the United States, Europe, and Japan had begun the fairly routine use of laboratory animals to study occupational chemicals, and soon thereafter, reports of studies of food additives, pesticides, and pharmaceutical agents began to appear in the literature. These early efforts to use laboratory animals to investigate chemical toxicity were no doubt motivated by the belief that responses in animals were useful indicators of potential human responses, but there was little explicit discussion of how data from these studies should be used for that purpose.

Two FDA scientists, Arnold Lehman and O.Garth Fitzhugh, were perhaps the first toxicologists to deal explicitly with the issue. In a short but famous paper published in 1954 (39), the two scientists described the basis for the belief that results from animal studies could be used qualitatively to predict responses in humans, but that quantitative predictions were more problematic. To deal with this problem, Lehmann and Fitzhugh postulated that "average" humans would likely respond to a chemical exposure at a lower dose than would a group of experimental animals, and that within the human population, some individuals would respond at lower doses than would the "average" person. In modern parlance, these authors recognized that the *variability* in response at a given dose was likely to be much greater in a highly diverse human population than it is in a group of inbred, and otherwise homogeneous and healthy, experimental animals. They further recognized that if toxicology data from experimental animals were to be used to establish protective limits1 (what Lehman and Fitzhugh called "safe levels") for humans, it would be necessary to "adjust" the experimental results. From their discussion and review arose the concept of "safety factors": a factor of 10 was proposed to adjust (downward) the animal dose (specifically the no-observed-adverse-effect level, NOAEL) to estimate the NOAEL for the "average" human, and another factor of 10 to estimate the NOAEL for the "most sensitive" members of the human population. They offered the term *acceptable daily intake* (ADI) as their notion of a "safe level" of chronic chemical exposure for the general population, and the ADI was to be obtained by dividing experimental NOAELs from chronic animal toxicology studies by a factor of 100 (10×10). This system, though modified in several significant ways, remains in place today. It is used to establish various protective limits for chemical exposures in the general population. It is interesting that in the 1954 Lehman-Fitzhugh paper, an attempt is made to find an empirical basis for the two factors of 10, but the authors recognized that the database available for such an analysis was extremely limited (39).

Although the Lehman-Fitzhugh approach to quantitative extrapolation recognized the phenomena of inter-and intraspecies variability, it assumed implicitly that no toxic response was likely to occur in any individual unless exposures exceeded some threshold dose. The problem was to define that threshold for a large and diverse human population when the only significant data available arose from experimental studies. During the 1940s and 1950s an influential body of scientists working in the area of experimental carcinogenesis espoused the view that this particular class of toxic agents behaved biologically in ways that called into question the viability of the threshold concept (45). Government policies incorporated this view, which was until the 1970s captured by the phrase *no safe level*. By that time regulators saw that such a policy provided little useful guidance for decision making, and turned to the scientific literature to identify specific methodologies that could be applied to animal carcinogenicity data to estimate low-dose risks to humans for substances that might act by "no-threshold" mechanisms (45). The concept of safety also took a turn at this time, as scientists and decision makers recognized

1*Protective limits* is our term, used through the chapter as a description of any quantitative measure that is derived from toxicology or epidemiology data and that is intended to establish the upper limit on

exposure that is thought to be without significant risk of toxicity to humans. It is recognized that it is not possible to provide absolute assurance that such limits will protect every person in a population; moreover, exposures greater than these limits may pose no risk to many persons. The term is used here as a simple, one-phrase description of a concept that has in practice unfortunately attracted many different names (described later).

that safety could be defined only in relative terms. In an influential 1983 study issued by the National Research Council, the notion arose that decisions regarding levels of risk that were sufficiently small to ensure protection of the public health properly fell within the domain of *risk management:* Scientists have the task of assessing toxic risks and describing how their magnitudes change with exposure, but policymakers have the task of deciding how much risk reduction is needed to protect public health, and of how any needed risk reduction is to be achieved (45, 48).

As these developments regarding the uses of toxicology data in decision making evolved, so did the work of experimental toxicologists. Since the 1950s, animal studies have provided increasingly complete data on the effects of chemical exposures. Thus, increasing amounts of information have developed on the influences of dose, duration, and routes of exposure, on the roles of chemical kinetics and metabolism, and on the biological and molecular mechanisms underlying the production of toxicity. Experimentalists continue to find useful ways to examine the influences of exogenous compounds on a greater variety of targets, including complex systems of the body. Alongside the work of toxicologists must now be placed developments in the field of epidemiology, because these offer increased possibilities for testing hypotheses generated in the toxicology laboratory.

These various developments, which emerge more completely in the discussions to follow, place greater demands on the risk managers who must use the results of risk assessments in the formulation of health protection policies. At the same time, they provide better tools for risk assessment—of which interspecies extrapolation is a highly important component—and are proving to be of value in improving the scientific basis for risk-based decision making. This is, after all, what toxicology is about.

THE NEED FOR EXTRAPOLATION

Experimental and Epidemiological Methods and Their Limits

In the best of all possible worlds there would be no need for any form of extrapolation. Second best would be a world in which well-founded predictive models were available to extrapolate from one set of conditions to another. In the area of predictive toxicology, we now live in a third-best world, and perhaps our goal is to achieve the second best. The "best" world is probably not within our reach.

Why is extrapolation necessary? The answer to this question may seem so obvious that it is unnecessary to offer an explicit discussion. But because so many observers see science as a purely empirical subject in which all forms of extrapolation are merely speculative (a highly naive view of science), or as a subject in which extrapolation is justified only when well-supported predictive models have come to be available (a more credible and, indeed, a proper view of science), the question of "why engage in extrapolation?" is not as easy to answer as it might appear to be.

Given that there is a social need, expressed in many federal and state laws, to protect people from the toxic properties of chemicals in the environment, in foods, consumer products, medicines, and so on, it is necessary to rely upon one or both of the two basic methods available for acquiring toxicological data —the epidemiological and the experimental. Both have strengths and limits (Table 8.1), but neither method is capable of providing direct measurements of toxic risks that are applicable in all situations of potential interest. Animal studies allow us to understand toxicity characteristics of a chemical before human exposure is allowed to occur, whereas the epidemiological method generally does not. They also allow much more thorough examination of the effects of chemical exposure, under a much wider variety of conditions, than do the methods of epidemiology. They usually provide better information on dose-response characteristics, and also allow causal relations to be more readily established. They suffer, of course, one large disadvantage, in that they do not reveal responses directly in the species of interest. Thus, extrapolation from animal data is necessary in many if not most cases if anything at all is to be said about potential human risk and protection of human populations.

Both methods, experimental and epidemiological, suffer from additional limitations. Most importantly, the information they yield, even under the best conditions, is restricted to that portion of the dose-response curve that is within the detection power of the method used; in both cases the size of the population that can be studied, along with some other aspects of that population, is the principal determinant of detection power. Thus, empirical dose-response data will be limited to the relatively "high dose-high risk" portion of the dose-response curve. Most conditions about which toxicity dose-response data are sought concern exposures and doses that fall outside of (well below) the observable range, and therefore outside of the area of direct measurement.

It is also important to emphasize that although epidemiological data derive from studies in humans, there always remains the question of the representativeness of the studied population for the population whose risk is being assessed. The typical problem concerns the use of information obtained in occupational cohorts for assessing toxic risk in the general population.

Other extrapolation issues arise and cannot be avoided, because it is often necessary to reach decisions in the absence of complete data. The most common problems

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Table 8.1 Comparison of epidemiology and animal studies for identifying toxic properties

	Epidemiology studies	Animal studies
Opportunity to conduct study	Often not possible	Generally possible
Opportunity to obtain information prior to human exposure	No	Yes
Time requirements	Years to decades	Weeks to years
Species of interest	Yes[a]	No
Cause-effect determination	Difficult	Not as difficult
Opportunity to obtain quantitative dose-response data	Not frequently	Always

[a]Note that epidemiology studies may not provide data on both sexes or on all relevant subgroups of the human population.

arise when it is necessary to assess risk for a given route of exposure when data are available only for another route, and when the assessment concerns risks associated with chronic exposures when only relatively short-term data on toxicity are available.

Thus, if toxicity and epidemiology data are to be used at all to assess human risk and to establish protective limits, extrapolations will be necessary, sometimes several types.

Using Epidemiological and Experimental Data: Distinguishing "Prudence" From "Science"

When engaging in any form of extrapolation for which a reliable, well-established model or empirical basis is not available, judgments must be made that, strictly speaking, go beyond the realm of pure science (45). If such judgments are not introduced, then, as described earlier, no useful conclusions can be reached. Because of the social need to provide conclusions, however tentative and uncertain, judgments must be introduced, whether they concern qualitative or quantitative issues. The National Research Council (NRC), in its 1983 report on risk assessment in the federal government (45) and also in its 1994 report on the same subject (48) recommended that regulatory agencies use the best available science, but that the agencies also adopt guidelines that would specify what judgements they would make (these have come to be loosely called "defaults") to fill knowledge and data gaps. The NRC notion was that these defaults would be specified in advance, in the form of guidelines, and that they would be applied consistently, in specific risk assessments, to avoid case-by-case "manipulations" and to ensure explicitness in the assessment. The NRC also recommended flexibility in the use of defaults, so that chemical-specific data, if reliable, could be used in specific cases to replace one or more generic defaults.

The choice of defaults is a difficult topic, having both scientific and policy components. The U.S. Environmen tal Protection Agency (EPA) guidelines for carcinogen risk assessment can be consulted for information on the choice of defaults (73). The emphasis in this chapter is on the scientific basis for extrapolation and its limits, and the regulatory defaults are discussed only in passing. It is important, however, to attempt to distinguish what is in the area of well-established science from what is a "science-policy" choice. The truth is that all science is accompanied by uncertainty, and that there is no sharp distinction between what is known with such high certainty that little or no judgment is called for, and that which is insufficiently certain to stand on its own. (It would be interesting to poll the community of toxicologists on how much of any decision to extrapolate from experimental animals to humans they may think is based upon "science," and what part is based on "prudence"—"I'm not sure of its relevance to people but I'll use it anyway, because I want to be careful.")

DESCRIPTION OF THE PROBLEM

The several types of extrapolations necessary to assess risks of toxicity in human populations from data obtained in experimental animals are described in the next five sections of this chapter, along with what are called the empirical and biological bases for each. Both qualitative and quantitative aspects are described, with emphasis on the latter.

Cross-species extrapolations are those pertaining to the attempt to describe expected toxic responses and their relationship to dose in human populations based on responses and their relationship to dose observed experimentally in animals.

Within-species extrapolations are those pertaining to the attempt to describe the expected variability in response within human populations, based either on observations in limited segments of that

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population, or on responses predicted for limited segments of that population from observations in experimental animals.

Cross-dose extrapolations are those pertaining to the attempt to describe toxic responses and their relationship to dose for the range of the dose-response relationship that falls below the range that is subject to direct measurement, and within the range expected to be experienced by the population that is the subject of the risk assessment.

Cross-route extrapolations are those pertaining to the attempt to describe expected toxic responses and their relationships to dose in populations exposed by one route, based on responses and their relations to dose observed when exposure occurs by another route.

Cross-time extrapolations are those pertaining to the attempt to describe toxic responses and their relationships to dose for various exposure durations, based on responses and their relationships to dose observed over more limited exposure durations.

In each case the empirical and biological bases for such extrapolations are summarized. By *empirical* is meant an analysis based upon comparisons made in more limited circumstances of actual observations relevant to the extrapolation being considered. Thus, for example, compilations and comparisons of human and animal dose-response data for several carcinogens are available, and these comparisons allow at least limited generalizations to be made regarding cross-species extrapolations. By *biological* bases is meant the use of basic knowledge in biology to provide support for particular forms of extrapolation. Both the empirical and biological bases are limited, but often inferences drawn from them converge and thus may provide support for some aspects of extrapolation.

The emphasis in this chapter is on quantitative extrapolations and on the types of scientific data and theories that are regarded as the basis for such extrapolations. As noted in the concluding sections, practical applications of many of the issues discussed in the following are not yet generally available; the thrust of the discussion thus concerns the directions necessary to improve the scientific basis for human risk assessment.

CROSS-SPECIES EXTRAPOLATION

Defining the Problem

When extrapolating toxicological data from one species to another, it is necessary to consider the various factors that may differentially affect the response of dif ferent species to the exposure of interest. As a first approximation, of course, for both cross-species and within-species extrapolation, it is common to normalize the toxicant dose to the body weight, and express it as milligrams per kilogram body weight per day. This approach assumes that it is the concentration of the toxicant in the body or at the target site (or some measure directly proportional to it) that determines the magnitude of the toxic effect. Although this appears to address at least one of the differences between species (body size), there are many examples in the literature showing that direct extrapolation simply on the basis of body weight is not accurate. Other aspects of differences among species need to be considered.

Two broad, overlapping classes of factors may affect differential responses: those related to the "effective dose" of the toxicant (determined in part by its pharmacokinetic and metabolic behavior), and those related to the inherent sensitivity of different species. In the absence of differences in inherent sensitivity (often called pharmacodynamic2 differences), it is generally assumed that different species will show similar responses when exposed to the same effective dose of the toxicant. The effective dose may in turn be influenced by species differences in body size, life span, anatomy and physiology, and pharmacokinetics and metabolism. For a direct-acting toxicant that follows first-order kinetics, the effective dose may be simply proportional to the administered dose. For a chemical with a more complex pattern of kinetics and metabolism to generate a reactive metabolite, pharmacokinetic modeling may be needed to accurately predict the effective dose.

Two general approaches have been used to evaluate methods for interspecies extrapolation: empirical, and biologically based. Empirical approaches rely on collection of data from multiple species exposed to the same substances under comparable conditions, and evaluation of the data to identify consistent relationships among species that permit the prediction of the magnitude of the response in one species (typically humans) based on data from another species. This approach has the advantage of simplicity, but does not readily allow consideration of chemical-specific factors that may affect interspecies scaling. Biologically based approaches make use of scientific knowledge of factors that may influence interspecies scaling, such as anatomical and physiological parameters and especially pharmacokinetics, metabolism, and mechanism of action.

2 The terms *pharmacokinetic* and *pharmacodynamic* derive from the pharmacological sciences. They have been retained by many toxicologists to describe the behavior of substances having toxic effects.

Some have advocated the terms *toxicokinetics* and *toxicodynamics*, but, though those terms seem more descriptive, they have not become widespread in use.

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Empirical Approaches
Empirical approaches to cross-species scaling have been studied since the end of the nineteenth century, when Rubner (62) noted that oxygen utilization and caloric expenditure scaled among dogs of different sizes approximately on the basis of body surface area more closely than simply on body mass. Subsequently, this work was extended to other species and other parameters, including the development of criteria for selecting doses for cancer chemotherapeutic drugs (26, 53). These authors noted that, for a number of chemotherapeutic drugs, the repeat-dose LD10 in mice, rats, and hamsters (treated daily for 5 days), the maximum tolerated dose in dogs and monkeys (the highest daily dose that killed no animals), and the maximum tolerated dose used clinically in humans were more closely comparable when the doses were expressed on a milligrams per square meter body surface area basis than when expressed on the basis of body weight. Because of the difficulty in accurately measuring body surface area, as an approximation based on the relationship between the mass and surface area of a sphere, this surface-area scaling is generally approximated as (body weight)$^{2/3}$.
A more recent reevaluation of these and other repeat toxicity data by Travis and White (69), however, suggested that (body weight)$^{3/4}$ scaling gave a better correlation. Travis and White (69) reanalyzed the repeat toxicity data in mouse, rat, dog, rhesus monkey, and human with 14 chemotherapeutic drugs described by Freireich et al. (26), and similar data in mouse, hamster, rat, dog, monkey, and human for an additional 13 chemotherapeutic drugs. Based on all of these data sets, and the use of a multiple linear regression model, the exponent of body weight giving the best correlation (r^2=.96) among species was 0.73, with 95% confidence bounds of .69 to 0.77.
Recently, Rhomberg and Wolff (59) reported an analysis of cross-species scaling for acute oral LD50 values covering ten mammalian species and a wide range of chemical types. They used data from the NIOSH Registry of Toxic Effects of Chemical Substances (RTECS) database, which contains information on over 135,000 substances. Several thousand of these had oral LD50 data from at least two species to permit comparisons. Overall, based on the log-log scatter plots of LD50 values in pairs of species (e.g., rat vs. mouse; mouse vs. rabbit), and log-log plots of mean ratio of LD50 values versus body weight for all species-pair comparisons, body-weight scaling appeared to give a better correlation among species than did body surface area, (body weight)$^{2/3}$ or (body weight)$^{3/4}$. The authors suggested that the difference between the apparent optimal scaling procedure for acute lethality (mg/kg) and that for repeat-dose lethality [(mg/kg$^{2/3}$) or (mg/kg$^{3/4}$)] may be related to differences in the mechanism of lethality. With single acute doses, the lethal dose may depend upon the level of defense capacities or reserves, proportional to body mass; with repeated exposure, survival may, to a larger extent, be a function of repair or replacement rate, which may show scaling patterns more like those for basal metabolic rate or other rates. As discussed later, these rates tend to scale across species as a function of body surface area or (body weight)$^{3/4}$.
Empirical approaches have also been used extensively in attempts to identify the most appropriate approach for scaling of cancer data between species (2, 15, 28). In one of the most extensive of these studies, Allen et al. (2) evaluated 23 chemicals for which data permitted quantitative estimation of cancer potency in humans and animals. As their measure of "potency," Allen et al. (2) calculated "risk-related doses" (RRDs), defined as the average daily dose per kilogram body weight that would be expected to result in an extra cancer risk of 25% over a lifetime. Allen et al. examined several different ways of expressing the dose, different scaling procedures, different subsets of animal data (restricted by experimental design, route of exposure, species, sex, tumor types), different statistical measures (maximum likelihood estimates and lower confidence limits on RRD), and different ways of considering results from multiple studies of the same chemicals (median response, most sensitive species/sex combination). When the logarithms of the RRDs derived from epidemiological studies were plotted against the logarithms of the predicted RRDs from animal studies, the scaling procedure giving the strongest correlation (by a slight margin) between humans and animals was scaling on the basis of body weight. However, there were wide variations in the apparent relative potency for individual chemicals, and given the uncertainties in the experimental and epidemiological data, it was not possible to rule out surface-area scaling [(body weight)$^{2/3}$], or some intermediate procedure, such as (body weight)$^{3/4}$, as being appropriate. Wide variations in apparent relative potency were also reported in the other comparisons (14, 15, 28). These studies considered mostly potency comparisons between rats and mice. On average in these studies, rats appeared to be slightly more sensitive than mice when compared on a body-weight basis, as would be expected if the "correct" scaling was on the basis of (body weight)$^{2/3}$ or (body weight)$^{3/4}$. Because of the small sample sizes and wide variation for individual chemicals, however, these comparisons are of limited predictive value.

The U.S. Environmental Protection Agency (72) published an extensive discussion on the selection of a default scaling procedure for cancer risk assessment based on these and other considerations. Its conclusion was that, although there was considerable uncertainty in the

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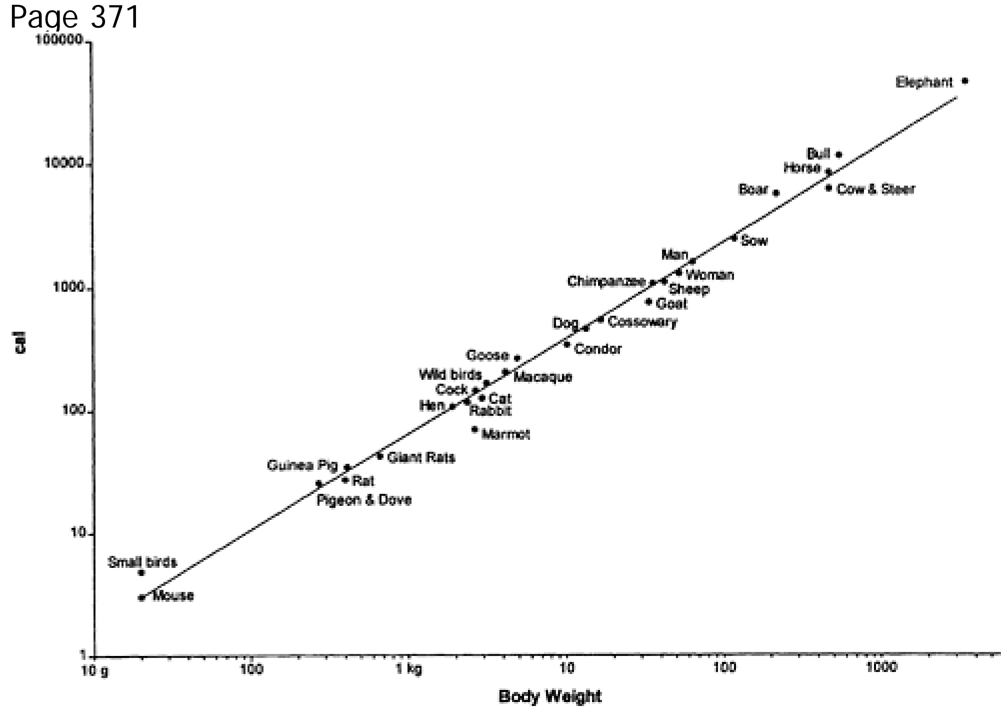

FIG. 8.1. Benedict's (7) mouse-to-elephant graph showing the relationship between species body weight and basal metabolic rate.

selection of any generic scaling procedure, the balance of evidence supported the use of (body weight)3/4 as a general procedure when no chemical-specific data provided support for an alternative procedure.

Biologically Based Approaches

Allometry

The work of Rubner (62) and others (e.g., refs. 1, 34, 35) on the relationship between body size and metabolism, mentioned previously, led to the study of allometry, the relationships between body weight and various biological and physiological parameters. A large number of biological and physiological parameters have been found to show a relation to body weight of the form:

$$Y=aWb$$

where *Y* is the biological parameter of interest (organ size, blood flow rate, basal metabolic rate, etc.), and *a* and *b* are constants relating *Y* to body weight *(W)*.

When the exponent *b* in the allometric equation is 1.0, cross-species scaling is based on relative body-weights. When it is 0.67, the biological or physiological measure is said to scale on the basis of relative surface areas.

In examining a wide number of measurements, measures of size (organ weights, blood volumes, etc.) tended to show an exponent *(b)* of approximately 1.0, while measures of rates (ventilation rate, basal metabolic rate, drug clearance rate, etc.) typically showed values closer to 0.67 (1). There was considerable variation in the best estimate of the exponent, *b,* for different parameters, and other authors have suggested that the value of *b* for metabolic and other rates falls closer to 0.75 (11, 54, 64). Figure 8.1, for example, shows a plot of basal metabolic rate versus body weight for warm blooded animals, ranging from mouse to elephant (7). The slope of this line corresponds to a *b* value of 0.76 (see ref. 19a).

Pharmacokinetics

Parameters affecting pharmacokinetic differences across species (organ sizes, physiological and metabolic

rates) are also subject to allometric scaling. As a result, provided a chemical follows the same pharmacokinetic pathways in different species, pharmacokinetic scaling will also tend to follow allometric scaling. Travis et al. (70) have suggested that for toxicants that are metabolically inactivated, scaling on the basis of (body weight)$^{3/4}$ provides the most accurate scaling across species. For reactive chemicals that are spontaneously inactivated, body weight scaling may provide a more accurate scaling metric.

All of the preceding discussion has centered on determining a generic procedure for extrapolating among species. As noted earlier, one of the most critical factors in cross-species extrapolation is the effective dose of the toxicant at the site of action. This is the purview of pharmacokinetic modeling. The use of pharmacokinetic modeling, particularly physiologically based pharmacokinetic (PBPK) modeling, in exposure estimation for risk assessment has been extensively discussed (46, 69a, 70) and is continually being updated and improved. For example, a series of increasingly comprehensive PBPK models have been applied to trichloroethylene over the past 10–15 years (see refs. 32, 66).

As discussed in Chapter 5, PBPK modeling uses physiological parameters, such as breathing rates, blood flow rates, and tissue volumes, to describe metabolic processes in physiologically realistic tissue groups (compartments), such as liver, lung, fat muscle, and other organs that are connected by venous and arterial blood flow. Each compartment is described mathematically by a set of differential equations. Such models can be used to relate exposure concentrations—for example, in air, water, or food—to concentrations of the parent compound or its metabolites in different tissues, including the target tissue. A major strength of PBPK models is that one can use the same model developed for a chemical in one species to predict chemical transport and metabolism in other species (including humans) by substituting species- specific physiological, biochemical, and metabolic parameters in the model.

Pharmacodynamics

Pharmacokinetics deals with the movement of chemicals in the body. Pharmacodynamics examines the interactions of chemicals and their metabolites with tissue constituents, their biological and physiological effects, and mechanisms of action. Even if one uses allometric scaling or pharmacokinetic modeling to identify an "equivalent" dose of a chemical at a target site, there may be differences in response in different species because of differences in pharmacodynamics or susceptibility. Thus, for example, a diverse group of chemicals, including unleaded gasoline, decalin, and d-limonene, cause nephrotoxicity and renal tumors in male rats by a species- and sex-specific mechanism involving binding to α2*u*-globulin (10, 68). This phenomenon does not occur in female rats, or in other species (including humans) that do not produce α2*u*-globulin. Nor does it occur in male Black Reiter rats, which also lack α2*u*-globulin (20).

A number of chemicals interact with glutathione and cause toxic effects when tissue glutathione levels are depleted. Knowledge of differences among species in the constitutive levels of glutathione in different tissue and its rate of synthesis can assist in predicting differences among species in sensitivity to such chemicals.

In some cases, data from in vitro studies can be used to assist in interspecies extrapolation. For example, chemicals that cause peroxisome proliferation in rodent liver have been studied in isolated hepatocytes to investigate differences among species in susceptibility (23, 50). Use of data from studies in isolated cells to predict effects in vivo, however, adds its own set of uncertainties, including those related to the representativeness of the cell or tissue samples used for the studies (49).

Application of Allometric Scaling

To apply allometric scaling in practice for converting a specific animal dose (in mg/kg body weight) to an equivalent human dose (also in mg/kg) the animal dose is multiplied by the ratio of animal to human body weight to the $(1-b)$ power. Thus, for scaling on the basis of (body weight)$^{3/4}$, the equivalent human dose is calculated as:

$$\text{Human dose (mg/kg)} = \text{animal dose (mg/kg)} \times \left(\frac{\text{animal body weight}}{\text{human body weight}}\right)^{1/4}$$

This scaling factor is derived as follows. For (body weight)^b scaling:

$$\frac{D_h}{(W_h)^b} = \frac{D_a}{(W_a)^b}$$

where D_h is the human dose (mg), D_a is the animal dose (mg), W_h is the human body weight (kg), and

Wa is the animal body weight. Rearranging this equation and dividing both sides by the human body weight gives:

$$\frac{D_{\mathrm{h}}}{W_{\mathrm{h}}}=\frac{D_{\mathrm{a}}}{W_{\mathrm{h}}}\times\left(\frac{W_{\mathrm{h}}}{W_{\mathrm{a}}}\right)^{b}$$

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Table 8.2 Cross-species scaling factors based on allometric scaling (based on equations described in text)

Allometric scaling	Mouse-human scaling factor	Rat-human scaling factor
W1.0	(0.035/70)0=1.0	(0.3 770)0=1.0
*W*0.75	(0.035/70)0.25=0.1 50	(0.3/70)0.25=0.256
*W*0.67	(0.035/70)0.33= 0.0794	(0.3/70)0.33=0.162

Note. Assumes human body weight of 70 kg, mouse body weight of 35 g, and rat body weight of 300 g. The scaling factor indicates the dose for a human (mg/kg/day) that is equivalent to 1 mg/kg/day for a mouse or rat. For example with *W*0.75 scaling, a dose of 1 mg/kg/day in a mouse is equivalent to 0.150 mg/kg/day for a 70-kg human.

Multiplying the top and bottom of the right side by the animal body weight *Wa* gives:

$$\frac{D_h}{W_h} = \frac{D_a}{W_a} \times \frac{(W_h)^b \times W_a}{(W_a)^b \times W_h}$$
$$= \frac{D_a}{W_a} \times \frac{(W_a)^{1-b}}{(W_h)^{1-b}}$$
$$= \frac{D_a}{W_a} \times \left(\frac{W_a}{W_h}\right)^{1-b}$$

Examples of the application of these relationships are illustrated in Table 8.2.

Current Trends

At the present time there is extensive work under way aimed at improving our ability to perform cross-species extrapolation, particularly in the areas of pharmacokinetic and pharmacodynamic modeling. As with other types of extrapolation, cross-species extrapolation is aided by an understanding of the mechanism of action of the toxicant under consideration. Because of the limitations of studying toxicants in humans, efforts are also underway to use isolated human cells to address some pharmacodynamic questions and, hence, improve the accuracy of extrapolation.

WITHIN-SPECIES EXTRAPOLATION—VARIABILITY IN HUMAN RESPONSE

Defining the Problem

The term *extrapolation* is, in this context, used somewhat differently than it is in the four other areas discussed in this chapter. The human population is without question highly *heterogeneous* with respect to all of the many differences that affect response to a given dose of a given chemical. If the distribution of toxic responses at a specified dose were known with accuracy, it would be possible to specify a dose at which a specified fraction of the population would be at a given risk of toxicity. Moreover, if the distribution of threshold doses for the population were known, it would be possible to specify the dose at which, for example, the vast majority of individuals would not be at risk; the specific fraction at the tail of the distribution that might remain at risk would be selected as a matter of policy (such a selection would be necessary on the assumption that any plausible distribution of population thresholds would not include an identifiable dose at which there were absolutely zero responders).

In practice such population distributions are not available, and the traditional "default" approach has been, first, to estimate a threshold dose for some hypothetical person described as "average" with respect to responsiveness, and, second, to divide this threshold estimate by a factor, usually 10, that is thought to lead to an estimate of the threshold dose somewhere near the tail of the underlying, but unknown, distribution at which the "most sensitive" members of the population are expected to be found. This estimate is usually considered protective for the "most sensitive" individuals and, necessarily, for all less sensitive individuals. Factors other than 10 have been used if there is some reason to believe that the data supporting a threshold represent individuals more or less sensitive than the hypothetical "average." This relatively crude procedure thus involves extrapolation from so-called "average" to "sensitive" individuals. Interestingly, there is no methodological tradition within the realm of carcinogen risk assessment that is specifically designed to account for population variability (48).

The traditional approach to deriving protective doses offers no insight into the degree of population protection provided. Whether it is underprotective or extraordinarily overprotective depends upon where on the true (but unknowable) distribution of threshold doses the protective dose happens to lie.

Movement away from the "default" approach toward a more scientifically rigorous one that provides some insight into the degree of protection from toxic risk provided at various doses depends upon the

development and incorporation of scientific information relevant to the question of population variability. The factors known to influence variability are described in the next subsection, and this is followed by a review of some empirical data on this question.

Biological Bases for Interindividual Variability

Three major sources of interindividual variability in response to a chemical exposure can be described. First,

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Table 8.3 Major sources of interindividual variability in responses to chemical exposures

Source	Cause of variability	Some major influences
Uptake	Differences in contact with and absorption of chemical from its environmental sources	Age Diet Smoking Health status of skin, respiratory tract, and gastrointestinal tract
Pharmacokinetics and metabolism	Differences in distribution, metabolism, and elimination, leading to different target site concentrations	Age Gender Health status Other exposures (dietary, drug, chemicals) Genetic polymorphisms Pregnancy
Response at target site(s) (pharmacodynamics)	Differences in biological response at a given target site concentration	Age Gender Health status Nutritional status Hormonal status Pregnancy Immune status Genetic polymorphisms

individuals vary in uptake of a chemical from the environment. Second, pharmacokinetic and metabolic behaviors of chemicals vary among individuals; and third, interindividual variability exists with respect to the response at the target site to a given dose (concentration×time) of a toxicologically active compound (pharmacodynamic differences). These three influences lead to variability in the size of the dose (concentration×time) of active compound (administered compound or, more often, a metabolite thereof) that reaches the target site, and the magnitude of the response to that dose, even when all individuals are exposed to identical concentrations of a chemical in their environment. In addition, variability exists with respect to the intake of a chemical because of differences in the nature and extent of human contacts with the environmental media in which the chemical is present. In Table 8.3 are listed some of the major contributors to interindividual variability in toxic response.

Although there is substantial empirical support for the fact of human variability, there is large uncertainty regarding its magnitude. In any given exposure situation, some factors may serve to increase the relative responsiveness of some individuals, while in other exposure situations these same individuals may be at less relative risk. Thus, for example, infants lacking metabolic capacity, which does not fully develop until about 1 year of age, may be less susceptible to substances requiring metabolic activation, yet be more sensitive to other substances because of their less than fully functional immune systems. The number of factors influencing responsiveness is so large and variable within the human population, and the cumulative direction of their effects (to increase or decrease sensitivity) so unpredictable in any given exposure situation, that no attempt to derive a generally applicable model of variability based on biological understanding of each of the factors known to influence it has proved successful. Instead, empirical evidence that captures the cumulative effects of all important influences has been generally regarded as of more value (48).

Empirical Approaches to Understanding Interindividual Variability

Little empirical data supported Lehmann and Fitzhugh's original 1954 proposal to extrapolate from "average" to "sensitive" individuals by the incorporation of the assumption of a 10-fold difference in sensitivity, and this assumption has become the standard "default" uncertainty factor, typically used by regulators when there is no known basis for another factor. Although it has never been explicitly described, the adoption of the 10-fold factor suggests that the total variability in response in the human population ranges over about 100-fold, assuming a symmetrical distribution of

responses about the average. Of course, the location of the "least" and "most" sensitive individuals on the actual distribution is unknown, so it is not possible to describe the actual range.

By the early 1980s, sufficient empirical information had accumulated to allow limited analysis of variability (21). Dourson and Stara's 1983 review of a number of data sets (21) concluded that a 10-fold factor was likely to reflect a wider range of interindividual variability than could be documented for the vast majority of chemicals. This analysis suggested that the 10-fold factor was adequately protective. Review of differences in human metabolism of chemicals (12) has found that a 10-fold factor covered the total range of variability for 80–95% of the population; this finding suggests that the range from "average" to "sensitive" is significantly less than 10-fold. LD50 ratios of adults to newborns for 238 chemicals have been evaluated (65) as a measure of intraspecies variability. Although it was found that the median ratio reflected only a 2.6-fold variability, and that 86% of the ratios were less than 10, the fact that most of the data derived from experimental animal studies casts some doubt on its applicability to humans.

In a review published in 1996, Dourson et al. (22) concluded, based on evaluations of the type just described, that:

In general, the default value of 10 for interhuman variability appears to be protective when starting from a median response, or by inference, from a NOAEL assumed to be from an average group of humans. However, when NOAEL's are available in a known sensitive human subpopulation, or if human toxicokinetics or toxicodynamics are known with some certainty, this default value should be adjusted or replaced accordingly. (p.111)

Some authors have proposed to examine variability separately for factors influencing delivery of target site dose (uptake, pharmacokinetics, metabolism) and those affecting response. One reviewer suggested that variability in the former factor was generally larger than it was for the latter, and he proposed that the 10-fold factor be subdivided into factors of 4 (pharmacokinetics) and 2.5 (pharmacodynamics) (58).

The limited empirical analysis supporting the factor of 10, or its subfactors, is perhaps reassuring. Little effort has been devoted to developing more complete descriptions of variability distributions. Heterogeneity in response might be derived by treating human data, where available, as animal data are often treated (56). Probit plots have, for example, been developed using data derived from studies in Iraq of neurobehavioral outcomes in humans exposed in utero to methylmercury. A probit plot is one useful way of describing the variability in thresholds among individuals in a population. If it is assumed that the distribution is lognormal in character, then a plot of probit against log dose yields a straight line, the slope of which reflects variability; steep slopes reveal less variability than do shallower ones. (Lognormal distributions arise when the factors contributing to variability act multiplicatively—addition of the logarithms of variables is identical to multiplying the variables themselves.) It has been suggested that some responses to methylmercury show a probit slope as low as 1, corresponding to a geometric standard deviation of 10 (56). It is inferred from this that 95% of the population would have thresholds spread over a range of 10,000-fold in dosage—from 100-fold lower to 100-fold higher than the threshold dose for individuals at the median. Such estimates provide the type of description of variability that could increase the level of risk information provided to decision makers, because they allow more explicit analysis of the areas of the distribution that might be selected as the focus of regulation. At the same time, numerous difficulties attend the use of such statistical methods, not the least of which is its failure to incorporate data on biological mechanisms. It nevertheless suggests the possibility of a more quantitative direction for this aspect of risk assessment.

QUANTITATIVE STRATEGIES FOR HIGH- TO LOW-DOSE EXTRAPOLATION

In the production and use of chemicals, it is necessary to consider the health risks and benefits. Both the degree of risks and benefits depend upon the amount of chemical present. For some chemicals, such as genotoxic carcinogens, trace amounts may pose a small risk. For other relatively nontoxic chemicals, large doses may be required before adverse health effects result. In either case, the goal is to eliminate or at least minimize the occurrence of adverse health effects. This generally entails establishing a dose-response curve that relates the incidence of disease, that is, the proportion of individuals that develop a disease, to the dose of a chemical. This provides a method for estimating doses associated with low probability of disease.

In the case of cancer, regulatory decisions regarding exposures to carcinogens are generally made to limit the estimated lifetime probability of cancer (risk) to less than 1 in 10,000 and often to less than 1 in 1,000,000 (60). This creates a difficult problem. For studies conducted in laboratory animals, it would require tens of thousands of animals to estimate the incidence of cancer with precision at doses producing cancer risks below 1 in 10,000. Resources simply are not available to conduct such studies.

Instead, experiments are conducted in animals at doses high enough to elicit potential toxic effects that can be observed in a moderate number, generally 50 or less, of animals per dose group. Such studies generally must produce incidence rates in excess of 10% in order to achieve statistical significance; this limit is 1,000 to

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100,000 times greater than the risks that are the subject of regulation. Often, these studies require doses that are tens, hundreds, or even thousands of times higher than human doses. In cancer studies, the highest dose generally is selected that is anticipated not to cause death, other than due to tumors, and does not cause average body weight losses greater than 10% compared to control animals. The use of high doses necessitates extrapolation to estimate the incidence of adverse health effects at human dose levels.
Occasionally, human data are available to access risk. Often these studies are based on occupational exposures that are higher than those experienced by the general public. Hence, extrapolation to lower doses is required, albeit much less than from animal studies.
Toxicity studies for drugs often are conducted from near human dose levels up to perhaps 100 times higher. In such studies, some extrapolation to lower dose levels may be required.
A question almost always arises from toxicological studies about extrapolating the results from high doses to lower doses experienced by humans. In the following sections the biological and empirical basis for low-dose extrapolation is summarized. Finally, the uncertainties and future directions for low-dose extrapolation are discussed.

Biologically Based Models for Low-Dose Extrapolation

Very few biological processes are understood well enough to make quantitative predictions of outcomes based on toxic insults. When we speak of biologically based dose-response models, it usually only means that some general principles are accepted that dictate to some extent the shape of the dose-response model. For example, it may be reasonable to assume that a threshold dose exists below which adverse health effects do not occur. Or, arguments may be presented that suggest no threshold dose exists. Arguments may be presented that support the saturation of toxic or detoxification pathways leading to asymptotic curves that flatten at high or low doses, respectively. Even if the general shape of a dose-response model can be established from knowledge of the biological mode of action or mechanisms, considerable variation may exist in available experimental data, resulting in imprecise estimates of risk particularly at doses below the experimental dose range. However, such estimates are generally considered more reliable than estimates based solely on empirical fits to data from an array of plausible dose-response models.

Threshold Doses

For noncancer endpoints, it generally is assumed that small doses of chemicals can be tolerated without any adverse health effects. Experimental data often are compatible with the existence of a threshold dose. However, experimental data can only demonstrate that an effect is likely to be within certain limits. For example, with no adverse effect in 100 animals, it can be stated with 95% confidence that the true incidence is likely to be less than 3%. Threshold doses generally cannot be estimated with precision even in animal studies with homogeneous animals. Estimation of threshold doses for a heterogeneous human population is even more problematic.
The general approach to safety assessment for noncancer effects is to establish an acceptable daily intake (reference dose) based on dividing an experimental no-observed-adverse-effect level (NOAEL) by a series of safety (uncertainty) factors up to 10 for each to allow for extrapolation from animals to humans, when necessary, and to allow for sensitive individuals in a population (6). Hopefully, this results in a reference dose (RfD) that is below the threshold dose for most individuals, resulting in negligible risks. Since the RfD based on animal data is generally a factor of 100 or more below doses that produce adverse effects in bioassays, it is presumed that risks are negligible at these lower doses. There generally is no dose-response modeling attempted to estimate the risk at or below the reference dose.
There is no explicit use of a dose-response curve in the safety assessment process described earlier. There is no estimate of the risk at the NOAEL and no extrapolation to lower risks at lower doses. Gaylor (27) demonstrated that estimated risks of embryo/fetal death and malformations at the NOAEL varied from 0 to 4.5% for typical bioassays. Leisenring and Ryan (40) show that risks at the NOAEL could be as high as 20% for quantal (incidence) data from typical bioassays. Recognizing the wide variation in risks at the NOAEL and the fact that smaller sample sizes result in larger NOAELs, Crump (16) suggested that rather than the NOAEL, the point of departure for RfDs should be a benchmark dose (BD) corresponding to a specified low level (1 to 10%) of risk. A lower confidence limit for the BD is used to allow for experimental variation, and an additional uncertainty factor is introduced to account for the point of departure being associated with a low level of risk.
The whole concept of threshold doses is challenged by the additivity-to-background argument. Obviously, all adverse health effects of concern occur in the human population. For those health effects

that may be the result of chemical exposure, either endogenous and/or exogenous, threshold doses have been surpassed for some individuals. Hence, the addition of any chemical dose, no matter how small, will have an additional effect if it augments an existing toxic chemical pathway (19). At low doses, the relationship between added health risk and dose is approximately linear (19). For this to be true,

the added chemical dose must not be a toxic effect that has a mechanism of action independent of existing mechanisms.

U-Shaped Dose-Response

For some essential vitamins and minerals, a U-shaped dose-response curve may occur. At deficient levels health risks increase, but health risks may also occur at excessive levels, such as for vitamin A and iron. Hence, there is an optimum dose or range that minimizes risk with risk increasing below and above the optimum level. If the dose-response can be modeled for both the deficient and excessive dose risks, assuming these two processes are independent, the overall risk can be estimated by adding the risk from the two components.

There is some evidence for hormetic effects. For example, low doses of a chemical may induce a detoxification process that actually lower risks, but becomes overwhelmed at higher doses. Such a process also may produce a U-shaped dose-response for risk.

Clearly, U-shaped dose-response curves require special attention. Risk assessment procedures that only consider high-dose risk may result in recommended doses that produce risks at low doses, particularly for essential or beneficial nutrients.

Noncancer Effects

The Michaelis-Menten equation is used widely in enzyme kinetics to relate the velocity (V) of an enzyme-mediated reaction to the substrate dose *(d)*, where *V* increases rapidly for small doses and then levels off, approaching a maximum rate (V max) at high doses:

$$V = \frac{V_{\max} d}{K + d}$$

where *K* is the dose at which *V* equals one-half of the maximum value (V_{max}). V_{max} and *K* are generally estimated by a double-reciprocal plot of 1/*V* versus 1/*d* that gives a linear relationship with an intercept of 1/V_{max} and slope of *K* times the intercept:

$$\frac{1}{V} = \frac{1}{V_{\max}} + \frac{K}{V_{\max}} \left(\frac{1}{d}\right)$$

In the event that an endogenous dose is present that is equivalent to d_e, then

$$V = \frac{V_{\max}(d + d_e)}{K + (d + d_e)}$$

Now nonlinear regression procedures are required to estimate the parameters.

The Hill equation is a generalization where

$$V = \frac{V_{\max}(d + d_e)^n}{K + (d + d_e)^n}$$

If $n>1$, the relationship between *V* and *d* is sigmoidal. If risk is proportional to the amount of V present, then

$$\text{Risk} = \frac{B(d + d_e)^n}{K + (d + d_e)^n}$$

where $B \leq 1$ when risk is expressed as a probability on the scale of 0 to 1. Even though there may be a biological basis for this dose-response curve, estimates of *B*, *K*, d_e, and *n* may be quite imprecise unless there are adequate numbers of animals over a wide range of doses. Also, the relationship between risk and *V* is crucial.

For developmental effects, Gaylor and Chen (29) proposed that birth defects may be related to decreased fetal weight. It was assumed that fetal growth was exponential and that the growth rate constant was effected by chemical exposure during gestation. Gaylor and Chen (29) suggested two models that fit a number of dose-response data about equally well for predicting the incidence *(P)* of a variety of structural malformations.

$$p=1-\exp[-b_0+b_1 d^k)]$$

and

$$P=1-\exp[-(b_0+b_1 d+b_2 d^2+\ldots+b_k d^k)]$$

where the *d* is the daily dose and the *b* terms and *k* are estimated from the data.

Leroux et al. (41) developed a mathematical model to describe aspects of the dynamic process of organogenesis, based on branching models of cell kinetics. The biological information incorporated in the model includes timing and rates of dynamic cell processes such as differentiation, migration, growth,

and replication. The dose-response models produced can explain patterns of malformation rates as a function of both dose and time of exposure.

Cancer

More attention has been devoted to dose-response modeling for cancer than to noncancer effects for risk assessment. For a genotoxic carcinogen, theoretically one molecule interacting with DNA at the right place and time could result in a mutation that initiates a carcinogenic process. Even though the probability that this event occurs is infinitesimally small, it argues against absolute threshold doses for genotoxic carcinogens. Further, the probability of an initiated cell is a stochastic process that is proportional to the number of molecules available to interact with DNA. This argues for low-dose linearity at the target site. As discussed earlier, additivity

of doses to background processes also argues for linear, nonthreshold dose-response for risk. The question is not, does low-dose linearity exist for genotoxic carcinogens; rather, the question is, over what dose range does linearity hold? High-dose studies may alter metabolic pathways and saturate detoxification processes. Nonlinear kinetics will result in nonlinear dose-response curves for risk, at least at high doses.

The most widely used model for estimating tumor risk is the multistage model of carcinogenesis. This model assumes that cancer is a progression of mutagenic or mutageniclike events that transform a normal cell into a malignant cell. These events may be the formation of DNA adducts, activation of oncogenes, deactivation of tumor suppressor genes, or mutations. The probability *(p)* of a tumor appearing within a specified period of time (t) is expressed in a simplified form as:

$$P=1-\exp[-(q_0+q_1d+q_2d^2+\ldots+q_kd^k)]t^k$$

where the *q* terms generally are restricted to be nonnegative and are estimated from tumor incidence data, and *d* is the active dose at the target tissue site. In practice, the term t^k is dropped and absorbed into the estimates of the *q* terms. In the absence of information to the contrary, it often is assumed that the target tissue dose is proportional to the administered dose. This model does not explicitly take into account pharmacokinetics, or the rates of cell proliferation, apoptosis, or DNA repair. Clearly, these factors may influence tumor incidence. However, the polynomial form of the model is flexible enough to provide an adequate fit to most data.

The value of *k* is the number of stages affected by the chemical. It is generally believed from the examination of data that *k* often ranges from 3 to 6. If only one stage is effected, a one-hit supralinear model is obtained:

$$P= 1-\exp[-(q_0+q_1d)]$$

This produces a concave dose-response that rises approximately linearly at low doses and then levels off.

At low doses the multistage model is approximately linear up to doses that double the background tumor incidence (19). The extra risk is approximately linear versus dose:

$$(P-P_0)/(1-P_0)\approx q_1d$$

where P_0 is the background tumor incidence. The upper confidence limit on the estimate of q1 is denoted as q_1^* When the dose-response has a sigmoid shape, $k\geq2$, at low doses the dose-response curves upward. Linear extrapolation tends to overestimate the risk in this range. The upper bound estimate of extra risk at low doses is given by

$$\text{Risk} \leq q_1^* \times d$$

which is the so-called linearized multistage estimate.

Crude tumor incidence rates (number of animals with tumors divided by the number of animals examined for a dose group) may provide poor fits for dose-response models. Animals may die at some doses before late occurring tumors appear, often resulting in lower crude incidence rates at higher doses than lower doses. To account for different noncancer mortality across dose groups, Peto et al. (51) proposed a dose-response trend test consisting of two parts: nonfatal tumors that are incidentally discovered when an animal dies from a cause other than the tumor type of concern or is sacrificed, and fatal tumors that cause the death of the animal. Bailer and Portier (4) and Bieler and Williams (9) provided a dose-response trend test that does not require knowledge of the cause of death but does utilize the age of death of the animal.

Moolgavkar and Venzon (44) and Moolgavkar and Knudson (43) introduced a two-stage model that included initiated cell cloning. This model considers the rate that normal cells are initiated, the birth and death rates of initiated cells, and the mutation rate of initiated cells to malignant cells. The exact solution that provides the probability of tumor by age t is given in a NRC report (47) as

$$P(t) = 1 - \left[\frac{2Ce^{-(\beta-\delta-\mu+C)t/2}}{(\beta-\delta-\mu+C)e^{-Ct}-(\beta-\delta-\mu-C}\right]^{v/\beta}$$

where $C=[(\beta+\delta+\mu)^2-4\beta\delta]^{1/2}$ and the parameters *v, β, δ,* and *μ* are defined as follows. It is assumed that the number of normal cells that are dividing per day remains relatively constant resulting in an average number, *v* of new initiated cells per day, β is the probability that an initiated cell undergoes division in a day, *δ* is the daily probability that an initiated cell is removed, and *μ* is the daily probability that an initiated cell divides into another initiated cell and a mutated cancer cell. In general, the values of these parameters as a function of dose are not known. However, changes in the tumor incidence can be calculated for changes in the parameters. For example, Gaylor and Zheng (30) showed that a

sustained increase of 20% in the net cell proliferation rate ($\beta-\delta$) can double the lifetime tumor incidence. Zheng et al. (74) extended the model to describe a sequence of mutational changes that constitute the G: C-A: T base substitution.

Empirical Models for Low-Dose Extrapolation

All empirical models must be biologically plausible if they are to fit bioassay data. The models described next do not arise from specific biologically based mechanisms, but generally accommodate saturation of detoxification and toxic processes. Unfortunately, several different

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models may fit bioassay data about equally well in the experimental dose range but unfortunately may result in widely different estimates of risk at low doses of interest (25). For this reason, linear extrapolation often is used to provide an upper bound on the risk for low doses of convex dose-responses that curve upward. Linear extrapolation does not mean that a linear model is fit to curvilinear dose-response data. A curvilinear model is still fit to the data and linear extrapolation is only used from the low end of the experimental dose range to an excess risk of zero at zero dose.

Statistical Models

The simplest model is linear where y is a response and d is dose:

$$y=b_0+b_1d$$

The intercept (b_0) representing the background response and b_1, the slope, are estimated from the data. A linear model often is used to approximate a dose-response curve over a limited range of doses. The response may be the incidence of risk of an adverse effect. A linear approximation may be adequate at low doses, but obviously must level off as the incidence (probability of risk) approaches 1.

The linear-quadratic model allows for some curvature, either up or down, in the dose-response curve:

$$y=b_0+b_1d+b_2d^2$$

This model often will fit data adequately up to the inflection point where the response curvature changes from convex to concave or concave to convex.

The probit model has a long history in biology (24). It belongs to a class of tolerance distributions. The probit model is based on the Gaussian (normal) bell-shaped distribution. The tolerance distribution describes the relative probability that an individual will respond—suffer an adverse health effect—at a dose d. If there is a large number of factors that act in an additive manner to determine the dose at which an individual responds, then by the Central Limit Theorem a normal distribution is obtained. Integrating (summing the relative probabilities) up to a dose d gives the proportion (probability) of individuals that develop an adverse effect at or below the dose d. The normal distribution is defined by the mean (μ) and standard deviation (σ). At the mean dose, 50% of the individuals are affected. Special probability (probit) graph paper is available such that plotting the proportion of effected individuals versus dose gives a straight line. The slope of the line is the reciprocal of the standard deviation.

Often, a probit model describes experimental data better when the logarithm of dose is used. This distribution could arise when a number of factors multiplicatively determine the dose at which an individual responds. The log-probit model has been used extensively for the analysis of dichotomous bioassay data (24). This model assumes that the distribution of log-dose thresholds is normal. For the log-probit model, the probability of a tumor induced by an exposure to a dose d of a chemical is given by

$$P(d)=\phi(b_0+b_1\log d)$$

where ϕ denotes the standard cumulative normal distribution.

Another model that has been used extensively in the analysis of dichotomous bioassay data is the log-logistic model (8):

$$P(d)=[1+\exp(b_0+b_1\ \log\ d)]^{-1}$$

where $P(d)$ is the probability (incidence) of an adverse effect by a specified time at a dose d, and b_0 and b_1 are estimated from the data.

Data from reproductive/developmental studies pose special problems where pregnant animals are dosed and the results are measured in the offspring/fetuses. Correlation of results among offspring/fetuses within a litter must be considered. Kodell et al. (37) assumed that the incidence of adverse effects for the offspring/fetuses of a litter behaves according to a binomial distribution and that the probability of adverse effects varies among litters according to a beta distribution. Further, it is assumed that the probability of an adverse effect may be a function of the size s (number of offspring/fetuses in a litter). The expected probability of an adverse effect for an offspring/fetus in a litter of size s at dose d is

$$P(d,s)=1-\exp\{-[b_0+b_1(s-\bar{s})]\}$$

for d less than or equal to a threshold dose of d_0 and s is the average litter size across all dose groups in a bioassay. For doses above the threshold dose of d_0,

$$P(d,s)=1-\exp(-\{b_0+b_1(s-\bar{s}) + [b_3+b_4(s-\bar{s})](d-d_0)^k\})$$

where the $b \geq 0$ and $k \geq 1$ are estimated from the data, and where $[b_0+b_1(s-\bar{s})]\geq 0$ and $[b_3+b_4(s-\bar{s})]\geq 0$ for all s

Kupper et al. (38) proposed a model for reproductive/developmental data of the form

$$P(d, s)=b0+b1s+[1-b0-b1s]/\ [1+\exp[b3+b4s-b5\log(d-d0)]$$

Ryan (63) discussed multivariate models that simultaneously consider two or more biological effects, such

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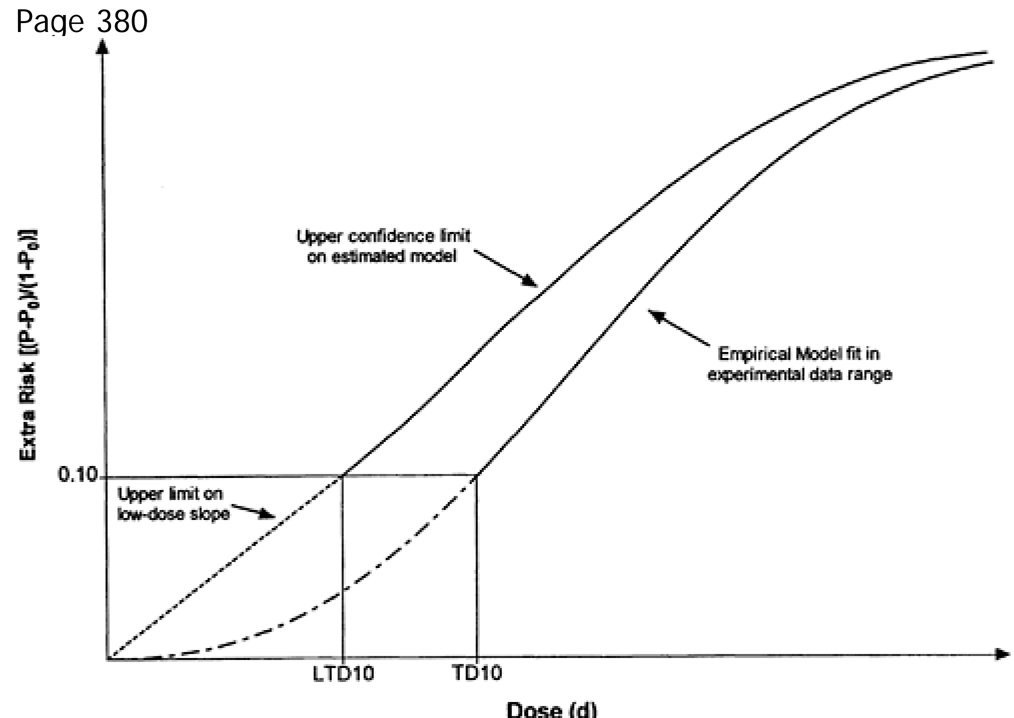

FIG. 8.2. Illustration of the low-dose extrapolation of the empirical model and likely overestimation of low-dose risk.

as the proportion of malformed fetuses in a litter and the proportion of dead/resorbed fetuses in a litter.

Microbial risk assessment is an area currently receiving more attention than in the past. Haas (33) proposed that the probability of infection could be described as a function of the number *N* of colony-forming-units by

$$P=1-[1+(N/\beta)]^{-\alpha}$$

where α and β are estimated from dose-response data.

Low-dose extrapolation using empirical models is suspect. The model may fit adequately in the experimental dose range, but there is little assurance of accuracy below the experimental dose range. However, linear extrapolation can serve as an upper bound estimate of risk for dose-responses that are convex (curve upward).

Recognizing that current methodology generally does not permit precise estimates of cancer risks below 10%, the proposed U.S. Environmental Protection Agency (73) carcinogen cancer risk assessment guidelines suggest estimating the dose associated with a 10% tumor risk (TD10) as a point of departure for low-dose extrapolation. In order to account for experimental variation, a lower confidence limit (LTD10) is calculated for this benchmark dose. If a nonlinear (thresholdlike) dose-response is likely below this benchmark dose, the LTD10 is divided by appropriate uncertainty (safety) factors to arrive at a reference dose that is not likely to cause an appreciable cancer risk. If a nonlinear dose-response below the benchmark dose cannot be justified, the regulatory default is linear extrapolation from the LTD10 to zero (Figure 8.2). This gives a low-dose cancer potency slope factor of 0.10/LTD10. This often is similar to the value of q_1^*, discussed earlier, which is estimated from the multistage model of carcinogenesis. If data are adequate, a benchmark dose below a 10% level of estimated cancer risk may be used as a point of departure for low-dose cancer risk assessment (73).

Uncertainties and Future Directions

There is a tremendous amount of research being conducted to examine the mechanisms of toxicity. Presumably this will provide better information to develop biologically based dose-response models that can facilitate low-dose extrapolation. In order to employ the

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Moolgavkar-Venzon-Knudson two-stage clonal model of carcinogenesis to estimate cancer risk, for example, it is necessary to identify directly or indirectly initiated cells and to obtain a measure of the rate at which initiated cells arise from normal cells, divide, and are removed, all as a function of dose of the carcinogen. Further, the mutation rate of an initiated cell to a malignant state is needed as a function of dose. Seldom will all of this information be available. Hence, assumptions regarding some of these rates generally will be needed in order to use this model. Currently, there are relatively large uncertainties, up to a factor of 100, for cancer risk estimates based on animal data (31). Many scientists have questioned the validity of long-term bioassays conducted at the maximum tolerated dose. Several authors (17, 55) have suggested that in the absence of extrapolatable data which can be extrapolated to low doses the same approach to safety assessment should be used for carcinogens as for noncancer effects. That is, establish a relatively safe dose and divide by a series of uncertainty factors to account for extrapolation from animals to humans, sensitive individuals, and other uncertainties to arrive at a relatively safe dose without any numerical estimates of risk. Several publications in recent years have addressed the size of uncertainty factors needed to provide adequate safety. Baird et al. (5) have shown that the products of current default values of 10 appear to provide adequate coverage for these uncertainties. Doubtless, research will continue to explore mechanisms of toxicity that will lead to better predictive models. Hopefully, the development of biomarkers of exposure will expand the use of human data for dose-response modeling.

CROSS-ROUTE EXTRAPOLATION

It is often necessary, particularly in the regulatory setting, to make some estimate of the likely toxic effect associated with exposure to a chemical by one route of exposure (e.g., inhalation) when toxicity data only from studies of exposure by a different route (e.g., oral) are available. Such extrapolation is subject to considerable uncertainty, and often is not attempted. In particular, chemicals quite frequently show route-specific toxic effects, especially at or near the site of administration (in the respiratory tract by inhalation, in the upper gastrointestinal tract by oral administration, or on the skin by dermal exposure). Such local effects may be indicative of a potential effect at a local site by another route of administration (e.g., a chemical that causes local dermal irritation when applied to the skin will very likely also cause local irritation when inhaled or ingested). However, quantitative extrapolation of such effects is generally not possible because of difficulties in identifying the effective dose of the toxicant at the target site and particularly because of differences in sensitivity of different tissues.

For systemic effects at distant sites, a crude cross-route adjustment can be performed (if absorption data are available) by simply normalizing the dose to a body-weight basis, and adjusting for relative absorption by the different routes of exposure. This procedure can be quite misleading, however. For example, if the chemical is subject to a significant first-pass effect in the liver (either activating a protoxicant, or inactivating a direct-acting toxicant), the ingestion and inhalation routes may show substantially different responses even with the same total absorbed dose. Clearly, if relevant metabolic and pharmacokinetic data are available, a preferred approach would be the development of a pharmacokinetic model that would take these factors into consideration (see Chapter 4).

CROSS-TIME EXTRAPOLATION: QUANTITATIVE STRATEGIES FOR ADJUSTING FOR DIFFERENCES IN EXPOSURE DURATIONS

Dosing animals with chemicals in toxicological studies may vary from a single administration to continuous exposure over the lifetime of the animal. Likewise, human exposure may vary from a single episode to continuous lifetime exposure. Often, a 2-year rodent lifetime is considered equivalent to a 70-year human lifetime. Hence, an exposure of an animal for 1 year is assumed to be equivalent to a 35-year exposure in humans. Generally, the durations of animal studies are chosen to mimic likely human exposure conditions. However, resources are not available to test chemicals at all of the possible human exposure conditions. Hence, statistical techniques have been devised to estimate the effects of short-term exposures from studies conducted with long-term exposures and vice versa.

Biological Models

Generally, estimates of cancer risk are based on the average daily lifetime exposure. That is, the total dose is divided by the number of days in a lifetime. This is a plausible approach for a genotoxic carcinogen where it is assumed that carcinogenesis is a stochastic process. In such a case, the probability of a biological event is proportional to the number of molecules of the chemical available to interact with biological matter. In order to predict biological effects for different durations of exposures, it is necessary that dose-response models contain a time or age element.

As discussed earlier, the multistage model of carcinogenesis does contain an element of time. Crump and Howe (18) provided estimates of risk as a function

of age and duration of exposure. Kodell et al. (36) showed that the use of the average daily lifetime dose may overestimate risk but never underestimates the cancer risk by more than a factor of *k* (generally 3 to 6) for a k-stage model. For example, based on the average daily lifetime dose, the estimated risk for exposure for one-tenth of a lifetime would be one-tenth of the risk for continuous lifetime exposure at that daily dose. According to Kodell et al. (36), this estimated risk should be multiplied by a factor of 3 to 6 to allow for exposure during a sensitive age.

For the Moolgavkar-Venson-Knudson two-stage clonal expansion model of carcinogenesis, Chen et al. (13) showed that the use of the average daily lifetime dose generally does not underestimate risk by more than a factor of ten. For exposures longer than one-tenth of a lifetime, the estimated risk would be the same as for a continuous lifetime exposure. For exposures for a fraction *(f)* less than one-tenth of a lifetime, the estimated risk is taken to be less than 10 times the upper limit on the estimated lifetime risk with continuous exposure. For the extreme case of only a single exposure to *N* milligrams of a carcinogen, the average daily lifetime (75-year) dose for a 70-kg person is $N/(70\times75\times365)=5\times10^{-7}\times N$ mg/kg. If q_1^* is the estimated upper bound of the cancer risk per milligram per kilogram body weight per lifetime daily exposure, the estimated risk from a single exposure is $5\times10^{-6}\times q_1^*$ where a factor of 10 is included to allow for exposure at a sensitive age.

Empirical Models

For noncancer effects, extrapolation of subchronic to chronic exposures is generally accompanied by an uncertainty factor of 10 (6). That is, it is assumed that an effect observed with a subchronic exposure is not likely to occur at less than one-tenth that dose for a chronic exposure. Swartout (67) compared NOAELs and LOAELs for subchronic and chronic exposures for about 100 cases. The median ratio of subchronic to chronic doses producing equivalent effects was 2 with a 95th percentile of 17. On the average, a chronic exposure to one-half of the dose for a subchronic exposure produced the same biological effect. For 5% of the cases, the chronic dose was less than 1/17th of the subchronic dose for the same biological effect. The convention default factor of 10 for subchronic to chronic extrapolation covered about 89% of the cases. Pieters et al. (52) conducted a similar study for 149 cases and obtained median ratio of subchronic to chronic doses for similar effects of 1.7 with a 95th percentile of 29.

Haber's Rule has been used extensively to make small extrapolations between durations of exposure. Haber's Rule states that equal biological effects are expected for equal exposures of concentration *(c)* times duration *(t)*. That is, equal values of $c\times t$ are expected to produce equal biological effects. For example, if the exposure duration is doubled, the concentration would need to be halved to obtain the same biological effect. A generalization of Haber's Rule is given by tenBerge et al. (71) where values of $c^n\times t$ are expected to produce equal biological effects. Estimation of the exponent *n* requires dose-response data collected for different durations of exposure. For several data sets, tenBerge et al. (71) observed that *n* varied from about 1 to 3 and tended to center around $n=2$. In the absence of duration-dose-response data, the recommended extrapolation to different durations of exposure is calculated on the basis of $c^2\times t$. For example, if the exposure time is increased by a factor of 4, the concentration needs to be halved to obtain an equivalent biological effect. In order to be conservative, it is recommended that $c^3\times t$ be used when extrapolating from long to shorter exposure times and $c\times t$ be used when extrapolating from short to longer exposure durations.

AN OVERALL STRATEGY FOR SCIENTIFICALLY BASED INTERSPECIES EXTRAPOLATION

Assessing risks to human populations from exposures to potentially toxic substances, based upon data from experimental studies, always requires cross-species and within-species extrapolations, almost always requires cross-dose extrapolations, and often requires cross-route and cross-time extrapolations. The need for specific types of extrapolation depends upon (a) the specific risk situation under assessment and (b) the nature of the data available for that assessment. Embarking upon a risk assessment thus requires, at the outset, a careful delineation of the problem to be evaluated. Once this is accomplished, efforts are made to collect all data that might be relevant to the risk question at hand. A matching of the data available with the problem to be assessed allows identification of the types of extrapolation that will have to be undertaken.

At the present stage of development of the toxicological sciences, most extrapolations are undertaken using the so-called "default" approaches discussed earlier. Increasingly, however, attempts are being made to search for the types of information needed to avoid resorting to such defaults, and to use approaches with more fully developed scientific bases.

Table 8.4 describes the types of inquiries that might be made to move toward a more purely science-based approach. It is assumed that the risk assessment problem to be addressed requires the use of

animal toxicology data (there is no significant epidemiology data available), and that all five forms of extrapolation will be required. Thus, for example, the assessment might involve chronic, gen

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Table 8.4 Overall strategy for science-based interspecies extrapolation: The search for information necessary to improve the scientific basis for human risk assessment based upon experimental data

Type of extrapolation	Type of inquiry
Cross-species	Can data be found for quantitative cross-species comparisons of target site doses and their relationships to administered doses?
	Can PBPK models be developed to accomplish above?
	Can mechanistic data be developed to estimate differences in target-site responsiveness across species?
Within species	Can quantitative estimates be developed, on a chemical-specific basis, of the ranges of human variability in uptake, pharmacokinetic and metabolic handing, and target-site responsiveness?
	Can methods be developed to integrate these sources of variability?
Cross-dose	Can the data necessary to apply biologically based models for low-dose extrapolation be identified or developed?
Cross-route	Can pharmacokinetic data be developed to permit accurate assessment of interroute differences?
Cross-time	Can empirical data be found to support extrapolations from one exposure duration to another? Are there biologically based mechanistic considerations to guide such extrapolations?

eral population exposure to a drinking-water or food contaminant for which the only available toxicity data involves gavage or even inhalation exposure over 90 days in one or more species of experimental animals. Before resorting to the usual defaults for each of these types of required extrapolation, and assuming that they are simply scientific uncertainties that cannot be overcome, it is now expected that toxicologists will inquire more fully, along the lines outlined in Table 8.4, into the possibility that alternative, data-based approaches can be developed. At the same time, it must be recognized that development of the data necesary for science-based extrapolations will necessarily introduce new uncertainties that have to be accommodated. Thus, while most would agree that reliable pharmacokinetic data can provide useful information on interspecies differences, it is likely that scientists will disagree on just how complete such data need to be before they can be used in risk assessment. Regulators typically display a high degree of skepticism about the incorporation of such data, and tend to remain close to the usual defaults, not because they dispute their relevance, but because they question their completeness. Thus, risk assessors must work to reach consensus not only on the types of data needed to improve risk assessments, but also on the difficult question of how complete they must be before they can be used for important public health decisions.

QUESTIONS

1. Why is it necessary to extrapolate?
2. How is a dose-response curve selected?
3. Does a threshold dose always exist? Ever exist?
4. Is the risk zero at the no-observed-adverse-effect level? Explain.
5. When does linear extrapolation to lower doses overestimate risk?
6. Describe the basis for a probit model.
7. Construct a numerical example that illustrates Haber's Rule.
8. When is a safety factor not an uncertainty factor?
9. What factors affect interspecies differences in response?
10. What factors account for variability in response within species?
11. What is the difference between variability and uncertainty?
12. What is the difference between a biologically based extrapolation and an empirically based extrapolation? Is one more reliable than the other?
13. How does science develop *general* explanations for phenomena such as interspecies differences in response?
14. Under what circumstances would cross-route extrapolation not be appropriate?

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REFERENCES

1. Adolph, E.F. (1949): Quantitative relations in the physiological constitutions of mammals. *Science,* 109:579.
2. Allen, B.C., Shipp, A.M., Crump, K.S., Kilian, B., Hogg, M.L., Tudor, J., and Keller, B. (1987): Investigation of cancer risk assessment methods: Summary. EPA/600/6–87/007a. NTIS PB88–127105. September.
3. Armitage, P., and Doll, R. (1961): Stochastic models for carcinogenesis. In: *Proc 4th Berkeley Symposium on Mathematical Statistics and Probability.* Vol. 4, pp. 19–38. University of California Press Berkeley, CA.
4. Bailer, A.J., and Portier, C.J. (1988): Effects of treatment-induced mortality and tumor-induced mortality on tests for carcinogenicity in small samples. *Biometrics,* 44:417–431.
5. Baird, S.J. S., Cohen, J.T., Graham, J.D., Shlyakhter, A.I., and Evans, J.S. (1996): Noncancer risk assessment: A probabilistic alternative to current practice. *Hum. Ecol. Risk Assess.,* 2:79–102.
6. Barnes, D.G., and Dourson, M. (1988): Reference dose (RfD): Description and use in health risk assessments. *Regul. Toxicol. Pharmacol.,* 8:471–486.
7. Benedict, F.G. (1938): *Vital Energetics: A Study in Comparative Basal Metabolism.* Carnegie Institute of Washington, Washington, DC.
8. Berkson, J. (1944): Application of the logistic function to bio-assay. *J. Am. Stat. Assoc.,* 39:357–365.
9. Bieler, G.S., and Williams, R.L. (1993): Ratio estimates, the delta method, and quantal response tests for increased carcinogenicity. *Biometrics,* 49:793–801.
10. Borghoff, S.J., Short, B.G., and Swenberg, J.A. (1990): Biochemical mechanisms and pathobiology of alpha 2μ-globulin nephropathy. *Annu. Rev. Pharmacol. Toxicol.,* 30:349–367.
11. Boxenbaum, H., and Ronfeld, R. (1983): Interspecies pharmacokinetic scaling and the Dedrick plots. *Am. J. Physiol.,* 245: R768-R775.
12. Calabrese, E.J. (1985): Uncertainty factors and interindividual variation. *Regul. Toxicol. Pharmacol.,* 5:190–196.
13. Chen, J.J., Kodell, R.L., and Gaylor, D.W. (1988): Using the biological two-stage model to assess risk from short-term exposures. *Risk Anal.,* 8:223–230.
14. Crouch, E. (1983): Uncertainties in interspecies extrapolations of carcinogenicity. *Environ. Health Perspect.,* 50:321–327.
15. Crouch, E., and Wilson, R. (1979): Interspecies comparison of carcinogenic potency. *J. Toxicol. Environ. Health,* 5:1095–1118.
16. Crump, K.S. (1984): A new method for determining allowable daily intakes. *Fundam. Appl. Toxicol.,* 4:854–871.
17. Crump, K.S. (1996): The linearized multistage model and the future of quantitative risk assessment. *Hum. Exp. Toxicol.,* 15:787–798.
18. Crump, K.S., and Howe, R.B. (1984): The multistage model with a time-dependent dose pattern: Applications to carcinogen risk assessment. *Risk Anal.,* 4:163–179.
19. Crump. K.S., Hoel, D.G., Langley, C.H., and Peto, R. (1976): Fundamental carcinogenic processes and their implications for low dose risk assessment. *Cancer Res.,* 36:2973–2979.
19a. Davidson, I.W. F., Parker, J.C., and Beliles, R.P. (1986): Biological basis for extrapolation across mammalian species, *Regul. Toxicol. Pharmacol. 6:*211–237.
20. Dietrich, D.R., and Swenberg, J.A. (1991): NCI-Black-Reiter (NBR) male rats fail to develop renal disease following exposure to agents that induce α-2u-globulin (α2u) nephropathy. *Fundam. Appl. Toxicol.,* 16:749–762.
21. Dourson, M.L., and Stara, J.F. (1983): Regulatory history and experimental support of uncertainty (safety) factors. *Regul. Toxicol. Pharmacol.,* 3:224–238.
22. Dourson, M.L., Felter, S.P., and Robinson, D. (1996): Evolution of science-based uncertainty factors. *Regul. Toxicol. Pharmacol.,* 24:108–120.
23. Elcombe, C.R., Bell, D.R., Elias, E., Hasmall, S.C., and Plant,N. J. (1996): Peroxisome proliferators: Species differences in response of primary hepatocyte cultures. *Ann. NY Acad. Sci.,* 804:628–635.
24. Finney, D.J. (1964): *Probit Analysis.* 2nd ed. Cambridge University Press, Cambridge.
25. Food and Drug Administration (1971): Advisory Committee on Protocols for Safety Evaluation. Panel on Carcinogenesis Report on Cancer Testing in the Safety Evaluation of Food Additives and Pesticides. *Toxicol. Appl. Pharmacol.,* 20:419–438.
26. Freireich, E.J., Gehan, E.A., Rall, D.P., Schmidt, L.H., and Skipper, H.E. (1966): Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man. *Cancer*

Chemother. Rep., 50:219–244.
27. Gaylor, D.W. (1992): Incidence of developmental defects at the no observed adverse effect level (NOAEL). *Regul Toxicol. Pharmacol.*, 15:151–160.
28. Gaylor, D.W., and Chen, J.J. (1986): Relative potency of chemical carcinogens in rodents. *Risk Anal.*, 6:283–290.
29. Gaylor, D.W., and Chen, J.J. (1993): Dose-response models for developmental malformations. *Teratology*, 47:291–297.
30. Gaylor, D.W., and Zheng, Q. (1996): Risk assessment of nongenotoxic carcinogens based upon cell proliferation/death rates in rodents. *Risk Anal.*, 16:221–225.
31. Gaylor, D.W., Chen, J.J., and Sheehan, D.M. (1993): Uncertainty in cancer risk estimates. *Risk Anal.*, 13:149–154.
32. Greenberg, M.S., Burton, G.A., Jr., and Fisher, J.W. (1999). Physiologically based pharmacokinetic modeling of inhaled trichloroethylene and its oxidative metabolites in B6C3F1 mice. *Toxicol. Appl. Pharmacol.*, 154:264–278.
33. Haas, C.N. (1983): Estimation of risk due to low doses of microorganisms: A comparison of alternative methodologies. *Am. J. Epidemiol.*, 118:573–582.
34. Kleiber, M. (1932): Body size and metabolism. *Hilgardia*, 6:315–353.
35. Kleiber, M. (1947): Body size and metabolic rate. *Physiol. Rev.*, 27:511–541.
36. Kodell, R.L., Gaylor, D.W., and Chen, J.J. (1987): Using average lifetime dose rate for intermittent exposures to carcinogens. *Risk Anal.*, 7:339–345.
37. Kodell, R.L., Howe, R.B., Chen, J.J., and Gaylor, D.W. (1991): Mathematical modeling of reproductive and developmental toxic effects for quantitative risk assessment. *Risk Anal.*, 11:583–590.
38. Kupper, L., Portier, C., Hogan, M., and Yamamoto, E. (1986): The impact of litter effects on dose-response modeling in teratology. *Biometrics*, 42:85–98.
39. Lehman, A.J., and Fitzhugh, O.G. (1954): 100-fold margin of safety. *Assoc. Food Drug Off. U.S. Q. Bull.*, 18:33–35.
40. Leisenring, W., and Ryan, L. (1992): Statistical properties of the NOAEL. *Regul. Toxicol. Pharmacol.*, 15:161–171.
41. Leroux, B.G., Leisenring, W., Moolgavkar, S.H., and Faustman, E.M. (1996): A biologically-based dose-response model for development toxicology. *Risk Anal.*, 16:449–458.
42. Lewis, S.C. (1993): Reducing uncertainty with adjustment factors. Improvements in non-cancer risk assessment. *Fundam. Appl. Toxicol.*, 20:2–4.
43. Moolgavkar, S.H., and Knudson, A.G. (1981): Mutation and cancer: A model for human carcinogenesis. *JNCI*, 66:1037–1052.
44. Moolgavkar, S.H., and Venzon, D.J. (1979): Two-event models for carcinogenesis: Incidence curves for childhood and adult tumors. *Math Biosci.*, 47:55–77.

45. National Research Council. (1983): *Risk Assessment in the Federal Government: Managing the Process.* National Academy Press, Washington, DC.
46. National Research Council.(1987):Drinking Water and Health *Water and Health Volume 8: Pharmacokinetics in Risk Assessment.* National Academy Press, Washington, DC.
47. National Research Council. (1993): *Issues in Risk Assessment.* National Academy Press, Washington, DC.
48. National Research Council. (1994): *Science and Judgement in Risk Assessment.* National Academy Press, Washington, DC.
49. Paine, A.J. (1996): Validity and reliability of in vitro systems in safety evaluation. *Environ. Toxicol. Pharmacol.,* 2:207–212.
50. Perrone, C.E., Shao, L., and Williams, G.M. (1998): Effect of rodent hepatocarcinogenic peroxisome proliferators on fatty acyl-CoA oxidase, DNA synthesis and apoptosis in cultured human and rat hepatocytes. *Toxicol. Appl. Pharmacol.,* 150:277–286.
51. Peto, R., Pike, M.C., Day, N.E., Gray, R.G., Lee, P.N., Parish, S., Peto,J., Richards, S., and Wahrendorf, J. (1980): Guidelines for simple, sensitive significance tests for carcinogenic effects in long-term animal experiments. *IARC Monogr. Suppl. 2* IARC, Lyon, France.
52. Pieters, M.N., Kramer, H.J., and Slob, W. (1998): Evaluation of the uncertainty factor for subchronic-to-chronic extrapolation: Statistical analysis of toxicity data. *Regul. Toxicol. Pharmacol.,* 27:108–111.
53. Pinkel, D. (1958): The use of body surface area as a criterion of drug dosage in cancer chemotherapy. *Cancer Res.,* 18:853–856.
54. Prothero, J.W. (1980): Scaling of blood parameters in mammals. *Comp. Biochem. Physiol. A.,* 67:649–657.
55. Purchase, I.F.H., and Auton, T.R. (1995): Thresholds in chemical carcinogenesis. *Regul. Toxicol. Pharmacol.,* 22:199–205.
56. Rees, D.C., and Hattis, D. (1994): Developing quantitative strategies for animal to human extrapolation. In: *Principles and Methods of Toxicology,* 3rd ed., edited by A.W.Hayes, pp. 275–315. Raven Press, New York.
57. Renwick, A.G. (1991): Safety factors and establishment of acceptable daily intake. *Food Addit. Contam.,* 8(2):135–150.
58. Renwick, A.G. (1993): Data-derived safety factors for the evaluation of food additives and environmental contaminants. *Food Addit. Contam.,* 10(3):275–305.
59. Rhomberg, L.R., and Wolff, S.K. (1998): Empirical scaling of single oral lethal doses across mammalian species based on a large database. *Risk Anal.,* 18:741–753.
60. Rodricks, J.V., Brett, S., and Wrenn, G. (1987): Significant risk decisions in federal regulatory agencies. *Regul. Toxicol. Pharmacol.,* 7:307–320.
61. Rodricks, J.V., Rudenko, L., Starr, T.B., and Turnbull, D. (1997): Risk assessment. In: *Comprehensive Toxicology. Vol. I. General Principles,* edited by J.Bond. Pergamon, Elsevier Science, New York.
62. Rubner, M. (1883): Ueber den einfluss der köpergrösse auf stoff und kraft wechsel. *Z. Biol,* 1919:535–562.
63. Ryan, L. (1992): Quantitative risk assessment for developmental toxicity. *Biometrics,* 48:163–174.
64. Schmidt-Nielson, K. (1984): *Scaling: Why Is Animal Size So Important?* Cambridge University Press, Cambridge.
65. Sheehan, D., and Gaylor, D.W. (1990): Analyses of the adequacy of safety factors. *Teratology,* 41:590–591.
66. Stenner, R.D., Merdink, J.L., Fisher, J.W., and Bull, R.J. (1998): Physiologically-based pharmacokinetic model for trichloroethylene considering enterohepatic recirculation of major metabolites. *Risk Anal.,* 18:261–269.
67. Swartout, J. (1996): Subchronic-to-chronic uncertainty factor for the reference dose. Abstr. F2.03. Society for Risk Analysis Annual Meeting, New Orleans, LA.
68. Swenberg, J.A., Short, B., Borghoff, S., Strasser, J., and Charbonneau, M. (1989): The comparative pathobiology of alpha 2μ-globulin nephropathy. *Toxicol. Appl. Pharmacol.,* 97:35–46.
69. Travis, C.C., and White, R.K. (1988): Interspecific scaling of toxicity data. *Risk Anal.,* 8:119–125.
69a Travis, C.C., White, R.K., and Arms, A.D. (1989): A physiologically based pharmacokinetic approach for assessing the cancer risk of tetrachloroethylene. In: *The Risk Assessment of Environmental Hazards,* edited by D.J.Paustenbach, Wiley Interscience, New York.
70. Travis, C.C., White, R.K., and Ward, R.C. (1990): Interspecies extrapolation of pharmacokinetics. *J.*

Theoret. Biol., 142:285–304.
71. tenBerge, W.F., Zwart, A., and Appelman, L.M. (1986): Concentration-time mortality response relationship of irritant and systemically acting vapours and gases. *J. Hazard. Mater.* 3:301–309.
72. U.S. Environmental Protection Agency. (1992): Draft report: A cross-species scaling factor for carcinogen risk assessment based on equivalence of mg/kg3/4/day. *Fed. Reg.*, 57:24152–24173.
73. U.S. Environmental Protection Agency. (1996): Proposed guidelines for carcinogen risk assessment. *Fed. Reg.*, 61:17960–18011.
74. Zheng, Q., Lutz, W.K., and Gaylor, D.W. (1997): A carcinogenesis model describing mutational events at the DNA adduct level. *Math. Biosci.*, 144:23–44.

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Chapter **9**

The Practice of Exposure Assessment

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Principles and Methods of Toxicology, Fourth Edition, edited by A.Wallace Hayes.
Taylor & Francis, Philadelphia © 2001.

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INTRODUCTION

Health risk assessment is the process wherein toxicology data from animal studies and human epidemiology are evaluated, a mathematical formula is applied to predict the response al low doses, and then information about the degree of exposure is used to predict quantitatively the likelihood that a particular adverse response will be seen in a specific human population (229, 257). More simply, risk assessment is a process by which scientists evaluate the potential for adverse health effects from exposure to naturally occurring or synthetic agents (335). Regulatory agencies have used the risk assessment process for nearly 50 years, most notably the U.S. Food and Drug Administration (U.S. FDA) (189). However, the difference between assessments performed in the 1950s and 1960s and those performed in the 1980s and 1990s is that dose-extrapolation models, quantitative

exposure assessments, and quantitative descriptions of uncertainty have been added to the process (63). Because of increased understanding of many relevant issues, the availability of desktop computers, and better quantitative methods for predicting the low-dose response (such as physiologically based pharmacokinetic ([PBPK] models), risk assessments conducted today provide more accurate risk estimates than in the past (257).

Since 1980, most environmental regulations and some occupational health standards have, at least in part, been based on health risk assessments (61, 390). They include standards for pesticide residues in crops, drinking water, ambient air, and food additives, as well as exposure limits for chemicals found in indoor air, consumer products, and other media. Risk managers increasingly rely on risk assessment to decide whether a broad array of risks are significant or trivial: an important task since, for example, more than 400 of the about 2000 chemicals routinely used in industry have been labeled carcinogens in various animal studies (234). In theory, the results of risk assessments in the United States should influence virtually all regulatory decisions involving so-called “toxic agents” (238, 278, 279).

The risk assessment process has four parts: hazard identification, dose-response assessment, exposure assessment, and risk characterization (234). Although progress has been made over the past 20 years in how to conduct and interpret toxicology and epidemiology studies (e.g., hazard identification), and scientists believe that they are doing a better job of dose-response extrapolation than in the past, most significant advances in the risk assessment process have occurred in the field of exposure assessment (405).

In recent years, an increasing number of environmental scientists have embraced the view that “toxicology data are important, but they do not mean much without quantitative information about human exposure.” For this reason, each year since about 1990, the toxicology community has shown increasing interest in understanding the exposure assessment field (246, 247). Fortunately, a significant amount of research has been conducted to identify better values for many exposure parameters, and major improvements have been made in applying these exposure factors to various scenarios. This chapter is intended to familiarize toxicologists, risk assessors, and others with this evolving field.

BASIC CONCEPTS

Description of Exposure Assessment

Exposure assessment is the step in the risk assessment process that quantifies the uptake of an agent resulting from contact with various environmental media (e.g., air, water, soil, food) (1, 257, 375). Exposure assessments can address past, current, or future anticipated exposures, although uncertainties can become significant when attempting to anticipate what might happen or estimate what happened long ago (98, 130, 262, 263, 294, 345).

Exposure assessment in various forms dates back at least to the early twentieth century, and perhaps earlier, particularly in the fields of epidemiology (106, 204), industrial hygiene (212, 256), and health physics (360). Epidemiology is the study of disease occurrence and the causes of disease. Exposure assessment combines elements of industrial hygiene, radiological health, and air pollution and relies upon aspects of statistics, biochemical toxicology, large-animal toxicology, atmospheric sciences, analytical chemistry, food sciences, physiology, environmental modeling, and others (377).

Fundamentally, an exposure assessment describes the nature and size of the various populations exposed to a chemical agent and the magnitude and duration of their exposures (371, 382). It determines the degree of contact a person has with a chemical and estimates the magnitude of the absorbed dose (69). Several factors need to be considered when estimating the absorbed dose, including exposure duration, exposure route, chemical bioavailability from the contaminated media (e.g., soil), and, sometimes, the unique characteristics of the exposed population (e.g., hairless mice absorb a greater percent of chemical than other mice). By definition, “duration” is the period of time over which the person is exposed.

Knowledge of the chemical concentration in an environmental medium is essential to determine the magnitude of the absorbed dose. This information is usually obtained by analytical measurements of samples of the contaminated medium (air, water, soil, sediment, food, or dust). Estimates can also be made using mathematical models, such as models relating air concentrations at various distances from a point of release (e.g., a smoke stack) to factors including release rate, weather conditions, distance, and stability of the agent (312, 430). Needless to say, a significant number of factors need to be considered to quantitatively evaluate a typical contaminated site (Figure 9.1).

In general, since about 1995, our ability to perform exposure assessments has matured to a degree that they will usually possess less uncertainty than other steps in the risk assessment. Admittedly, many factors should be considered when estimating exposure; for example, it is a complicated procedure to

understand the transport and distribution of a chemical that has been released into the environment. Nonetheless, available data indicate that scientists can now do an adequate job of quantifying chemical concentrations in various media, and the resulting uptake by exposed persons, if they account for the majority of factors that should be considered (268).

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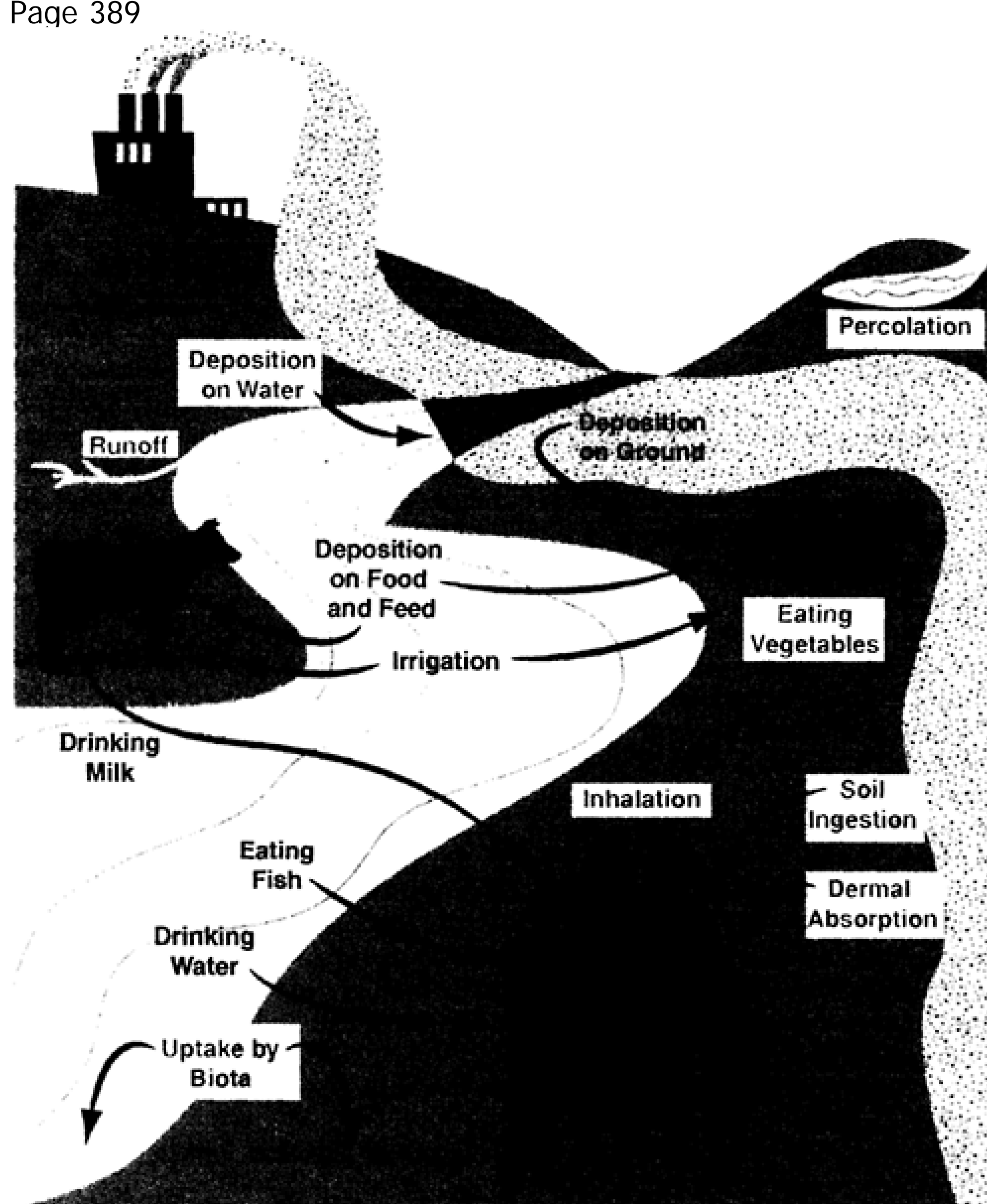

FIG. 9.1. Exposure pathways.

For those who wish to question this assertion, consider our level of confidence in animal bioassays which label a chemical a possible human carcinogen even though tumors were observed in a mouse liver only at the maximum tolerated dose (MTD). Similarly, consider our confidence in dose-extrapolation when three equally valid models yield risk estimates that are 1000-fold different at a typical environmental dose.

The primary routes of human exposure to chemicals in the ambient environment are dust and vapor inhalation, dermal contact with contaminated soils or dusts, and ingestion of contaminated food, water, dust, or soil. In the workplace, the predominant exposure route usually is inhalation, followed by dermal uptake and, to a lesser extent, dust ingestion due to hand-to-mouth contact (269). Uncertainty in environmental exposure assessment is usually less than in an occupational exposure assessment. In many workplaces, there can be large fluctuations in airborne concentrations, a significant difference in work practices of different persons, and there is real difficulty in measuring dermal uptake and incidental ingestion (166, 256, 259).

Scientists in the field of radiological health were the first to quantitatively estimate human uptake of environmental contaminants (301); health physics publications can be a source of valuable information when conducting assessments of chemical contaminants (20). This work, which was conducted after World War II, provided numerous methodologies for estimating human uptake of environmental contaminants. These have been refined

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over the past decade (161, 125, 218). The availability of information on the degree of exposure associated with various scenarios has increased dramatically over the past 15 years, as evidenced by the size of the recent *EPA Exposure Factors Handbook*, a four-volume document containing nearly 1000 pages of information on exposure assessment (382–384, 386).

The practice of exposure assessment, at least for regulatory purposes, has changed over time. For example, beginning in the late 1970s, regulatory policy in the United States encouraged or mandated the use of conservative approaches when conducting exposure assessments. This was codified in the U.S. EPA original document entitled *Risk Assessment Guidance for Superfund,* called RAGS (372). At that time, standardization of exposure assessments used to satisfy regulatory agencies was considered prudent because it guaranteed that risks would not be underestimated in order to ensure protection of public health. Beginning about 1985, concern evolved that repeated use of conservative exposure factor assumptions was producing unrealistically high estimates of exposure (81, 209, 242, 254, 259) and that the cost of achieving the recommended cleanup levels was becoming enormous. Thus, to improve the accuracy of many of the factors used in these assessments, a significant amount of new research was initiated.

Around 1990, risk assessors learned how to apply Monte Carlo techniques to evaluate both typical and highly exposed persons. Application of Monte Carlo techniques to exposure assessment has dramatically improved our understanding of the certainty of our estimates, and has decreased the problems associated with the repeated use of conservative assumptions, thereby altering the field permanently (44, 117, 353, 354). The U.S. EPA and other agencies have now embraced this approach, which is well described in the recent document called RAGS3A (a process for conducting probabilistic risk assessment) (392).

What Is Exposure?

Because there has been no agreed-upon definition of where or when exposure takes place, terminology used in published exposure assessment literature has been inconsistent. Although there is reasonable agreement that human exposure means contact with the chemical or the agent (4, 372, 375), there has not yet been widespread agreement as to whether this means contact with (1) the visible exterior of the person (e.g., an aluminum strip touches the skin but can't be absorbed), or (2) the so-called exchange boundaries where absorption takes place (skin, lungs, gastrointestinal tract) (377). The differing definitions have led to some ambiguity in the use of terms and units for quantifying exposure.

Some scientists find it helpful to think of the human body as having a hypothetical outer boundary that separates the inside of the body from the outside (377). The outer boundary of the body consists of the skin and openings into the body, such as the mouth, nostrils, or punctures and lesions in the skin. In most exposure assessments, chemical exposure is defined as contact of the chemical with some part of this boundary. An exposure assessment is the quantitative or qualitative evaluation of that contact. It describes the intensity, frequency, and duration of contact, the route of the chemical across the boundary (exposure route, e.g., dermal, oral, or respiratory), the resulting amount of chemical actually crossing the boundary (dose), and the amount of chemical absorbed (internal dose) (192, 375). These categories can be further refined as described below; however, a very workable quantitative definition of exposure is to think of it as "the product of (concentration), (time), and (duration), or rate of transport of toxicant (mg/cm2-min)" (166).

Depending on the purpose of the exposure assessment, the numerical output of these analyses may be an estimate of either exposure or dose. If an exposure assessment is being done as part of a risk assessment in support of an epidemiologic study, for example, sometimes only qualitative exposure levels are all that can be provided. In these situations, categories like low-, medium-, and high-level exposure may be used. In contrast, most assessments of environmental or occupational exposure conducted in recent years attempt to quantitatively predict the absorbed dose (mg/kg-d) and, occasionally, the circulating blood level or the concentration of the toxicant in the target organ (260, 292).

Concepts of Exposure, Intake, Uptake, and Dose

The process of a chemical entering the body can be described in two steps—contact (exposure), followed by actual entry (crossing the boundary). Absorption, either upon crossing the boundary or subsequently, leads to the availability of an amount of chemical to biologically significant sites within the body (target tissue dose). Although the description of contact with the outer boundary is simple conceptually (e.g., mg benzene/cm2 skin), estimating the degree to which a chemical crosses this boundary is somewhat more complex.

In the early 1990s, some scientists described the transport of chemicals into the body as involving two

separate steps: intake and uptake. Intake involved physically moving the chemical in question through an opening in the outer boundary (usually the mouth or nose), typically via inhalation, eating, or drinking. Normally, the chemical was contained in a medium such as air, food, water, or dust/soil. Here, the key question was the mass inhaled

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or ingested. Uptake, in contrast to intake, involved absorption of the chemical through the skin or across other barriers.

Today, most persons tend to lump intake and uptake together, simply calling the amount of chemical entering the body "uptake." In some cases, chemicals are absorbed completely, so systemic absorption (uptake) is the same as that eaten or in contact with the skin (intake). In other cases, the chemical is often contained in a carrier medium, and the medium itself typically is not absorbed at the same rate as the "contaminant of interest," so estimates of the amount of chemical crossing the boundary cannot be made directly. For example, benzene on the surface of a contaminated soil particle will move quickly through the skin, but benzene in the center of the soil particle may never completely reach the surface, and therefore it is not bioavailable and will not enter the bloodstream. Of course, for many inorganic chemicals like arsenic or lead in soil, oral and dermal bioavailability can be very low since the chemical is bound to the interstices of the soil particle and, therefore, uptake is very low. In short, if a chemical cannot be released from a media, it has no bioavailability and, consequently, since there is no uptake the chemical does not pose a risk.

Dermal absorption is an example of direct uptake across the outer boundary of the body. A chemical uptake rate is the amount of chemical absorbed per unit of time. In this process, mass transfer occurs by diffusion, so uptake will depend on the concentration gradient across the boundary, permeability of the barrier, and other factors (192, 214, 215). Chemical uptake rates can be expressed as a function of the exposure concentration, permeability coefficient, and surface area exposed, or as flux (269).

Bioavailability

The study of the bioavailability of chemicals in various media began around 1980 and continues to be an important topic (305).

Most studies are of oral bioavailability, although the dermal and inhalation bioavailability of chemicals have also been evaluated. This area of research has been a bit confusing due to a lack of standard terminology (159). A recent review paper by Ruby et al. (305) is probably the most authoritative one on this topic. The following definitions should be used in future assessments:

Bioavailability: Oral bioavailability is defined as the fraction of an administered dose that reaches the central (blood) compartment from the gastrointestinal tract. Bioavailability defined in this manner is commonly referred to as *absolute bioavailability*, and is equal to the oral absorption fraction.

Relative bioavailability: Relative bioavailability refers to comparative bioavailabilities of different forms of a substance or for different exposure media containing the substance (e.g., bioavailability of a metal from soil relative to its bioavailability from water), expressed in this document as a relative absorption factor (RAF).

Relative absorption factor: The RAF describes the ratio of the absorbed fraction of a substance from a particular exposure medium relative to the fraction absorbed from the dosing vehicle used in the toxicity study for that substance (the term *relative bioavailability adjustment*, RBA, is also used to describe this factor).

Bioaccessibility: The oral bioaccessibility of a substance is the fraction that is soluble in the gastrointestinal environment and is available for absorption. It is imortant to note that the bioaccessible fraction is not necessarily equal to the RAF (or RBA) but depends on the relation between results from a particular in vitro test system and an appropriate in vivo model.

There are both in vitro and in vivo tests for evaluating bioavailability, and many different approaches have been suggested over the past 20 years (159, 165, 321, 358).

As noted by Ruby et al. (305), a number of in vitro tests are available. Simple extraction tests have been used for several years to assess the degree of metals dissolution in a simulated gastrointestinal-tract environment. The predecessor of these systems was developed originally to assess the bioavailability of iron from food, for studies of nutrition. In these systems, various metal salts or soils containing metals are incubated in low-pH solution for a period intended to mimic residence time in the stomach. The pH is then increased to near neutral, and incubation continues for a period intended to mimic residence time in the small intestine. Enzymes and organic acids are added to simulate gastric and small-intestinal fluids. The fraction of lead, arsenic, or other metals that dissolves during the stomach and small-intestinal incubations represents the fraction that is bioaccessible (i.e., is soluble and available for absorption).

A number of in vivo tests have also been used with varying success. For example, gastrointestinal absorption of lead in humans varies with the age, diet, and nutritional status of the subject as well as with the chemical species and the particle size of lead that is administered. For example, age is a well-established determinant of lead absorption; adults typically absorb 5–7% of lead ingested from dietary

sources, while estimates of lead absorption from dietary sources in infants and children range from 40 to 53%. For the purpose of modeling exposure to lead in soil, the U.S. EPA currently assumes that the absolute bioavailability of lead in diet and water is 50% and that

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the absolute bioavailability of lead in soil is 30% for children. This corresponds to a soil RAF of 0.60 (60%) for the bioavailability of soil lead relative to lead in water (i.e., RAF=0.3/0.5) (305).
The results of bioavailability studies need to be considered in virtually all assessments involving human exposure. Often the effects in uptake will be minor, while in other cases one may find that insignificant quantities of a chemical are absorbed even though the applied dose or exposure is quite high (358, 396).

Applied Dose or Potential Dose

Applied dose has been defined as the amount of chemical available at the absorption barrier (skin, lung, gastrointestinal tract) (377). It is useful to know the applied dose if a relationship can be established between the applied dose and the internal dose, a relationship that can sometimes be established experimentally. This relationship can be estimated either through modeling or by direct measurement. For example, some researchers have analyzed phenol concentrations in the blood of volunteers over time after placing their hands in a bucket of nitrobenzene in an attempt to quantify the flux rate (273, 275). Usually it is difficult to measure the applied dose directly, as many of the absorption barriers are internal to the human, and not localized in such a way to make measurement easy. An approximation of applied dose can be made, however, using the concept of potential dose (377).
Potential dose is simply the amount of chemical that is ingested or inhaled, or the amount of chemical contained in material applied to the skin. It is a useful term or concept in those instances when there is a measurable amount of chemical or transport medium. The potential dose for ingestion and inhalation is analogous to the administered dose in a dose-response experiment.
For the dermal route, potential dose is the amount of chemical applied, or the amount of chemical in the medium applied (for example, as a small amount of soil deposited on the skin). Note that because all of the chemical in the soil particulate is not contacting the skin, this differs from exposure (the concentration in the particulate times the duration of contact) and applied dose (the amount in the layer actually touching the skin) (377).
As previously noted, the amount of chemical that reaches the exchange boundaries of the skin, lungs, or gastrointestinal tract may often be less than the potential dose if the material is only partly bioavailable. For example, only about 0.001% to 1.0% of dioxins or polycyclic aromatic hydrocarbons (PAHs) on fly ash in contact with the skin are likely to penetrate (321). When bioavailability data are known, adjustments to the potential dose should be made to convert it to the absorbed or internal dose (159, 321).

Internal Dose

The amount of chemical that has been absorbed and is available for interaction with biologically significant receptors (e.g., target organs) is called the *internal dose.* Estimating internal dose is the first objective of a good exposure assessment (288, 381).
Transport models are available to assist in this process (219). Once absorbed, the chemical can be metabolized, stored, excreted, or transported within the body. The amount transported to an individual organ, tissue, or fluid of interest is termed the *delivered dose* (68). The delivered dose may be only a small part of the total internal dose. For example, although 1 mg of PCB may be absorbed into the body, at any given time the amount in the liver (the target organ) may only be 0.001 mg. Work to refine the techniques used to estimate delivered dose has been among the most exciting areas of exposure assessment research over the past 15 years. Currently, the best approach to estimate delivered dose is to directly measure blood or to use physiologically based pharmacokinetic (PBPK) models (12, 194).
The *biologically effective dose* (BED), or the amount that actually reaches cells, sites, or membranes where adverse effects occur (235), may represent only a fraction of the delivered dose, but it is obviously the best one for predicting adverse effects. To understand BED is the ultimate goal of exposure assessment. Thus far, toxicologists have rarely been able to estimate BED or measure it for most chemicals (377), but models allow us to estimate it.
Currently, most risk assessments dealing with environmental chemicals (as opposed to pharmaceutical assessments) rely upon dose-response relationships based on the potential (administered) dose or the internal dose, because our understanding of how to estimate the delivered dose or the biologically effective dose is insufficient for most chemicals. In general, the best method currently available for estimating the dose to the target organ is to use PBPK models. These have been developed for about 60 high-volume industrial chemicals (193) (Table 9.1).
Often it is more convenient in risk assessment to refer to dose rates, or the amount of a chemical dose (applied or internal) per unit time (e.g., mg/d), or as dose rates on a per unit body weight basis (e.g., mg/kg-d). Most exposure data found in the various editions of the U.S. EPA *Exposure Factors Handbook*

and other guidance documents are presented as dose rates (e.g., grams of fish consumed each day) rather than as dose (8, 382–384).

Exposure and Dose Relationships

Depending on the purpose of the exposure assessment, different estimates of exposure and dose may require

Table 9.1 Examples of PBPK models for toxic substances

Benzene	Lead
Benzo[a]pyrene	Methanol
Butoxyethanol	Methoxyethanol (2-ME)
Carbon tetrachloride	Methyl ethyl ketone (MEK)
Chlorfenvinphos	Nickel
Chloralkanes	Nicotine
Chloroform	Parathion
Chloropentafluorobenzene	Physostigmine
cis-Dichlorodiammine platinum	PBB
Dichloroethane	PCBs
Dichloroethylene	Styrene
Dichloromethane	Toluene
Dieldrin	TCDF
Diisopropylfluorophosphate	TCDD (Dioxin)
Dimethyloxazolidine dione	Tetrachloroethylene
Dioxane	Trichloroethane
Ethylene oxide	Trichloroethylene
Ethyoxy ethanol (2-EE)	Trichlorotribluoroethane
Formaldehyde	Vinyl chloride
Hexane Hexavalent chromium	Vinylidene fluoride Xylene
Kepone	

Note. This table is an expansion of one presented in a paper by Leung and Paustenbach (193).

calculation. Often, estimates of uptake will be presented in units used in the toxicology study which may not be useful for exposure calculations.

When risk is a function of time of exposure, exposure or dose profiles can be very useful. In these profiles, the exposure concentration or dose is plotted as a function of time (19). Concentration and time are used to depict exposure, while amount and time characterize dose.

Such profiles are important for use in risk assessment where the severity of the effect depends on the pattern by which the exposure occurs, rather than on the total (integrated) exposure. For example, a developmental toxicant may only produce effects if exposure occurs during a particular stage of development. As shown in Figure 9.2, during the time above a certain dose rate (the shaded portion), there was an increased risk to the fetus of certain birth defects. Similarly, a single acute exposure to very high dose may induce adverse effects, even if the average is much lower than apparent no-effect levels. To understand the probability of an adverse effect, one must generally consider the pharmacokinetics of the specific chemical. For example, for a chemical that has a long biologic half-life, internal exposure continues long after the chemical is ingested because blood levels remain high until the substance is completely released from poorly perfused tissues like adipose, and then is metabolized and/or eliminated.

In general, there is a need to consider the time elements of exposure assessment. Specifically, it is useful to understand the relationship between the biological half-lives of toxicants and the subsequent critical time element of their exposure. Indeed, the appropriate consideration of these elements should drive the specified averaging times for both toxicant exposure limits and exposure assessment (258, 295).

If a chemical or agent causes its biological damage quickly and is gone from the body in a short time, then how we test its toxicity is critical. For example, consider

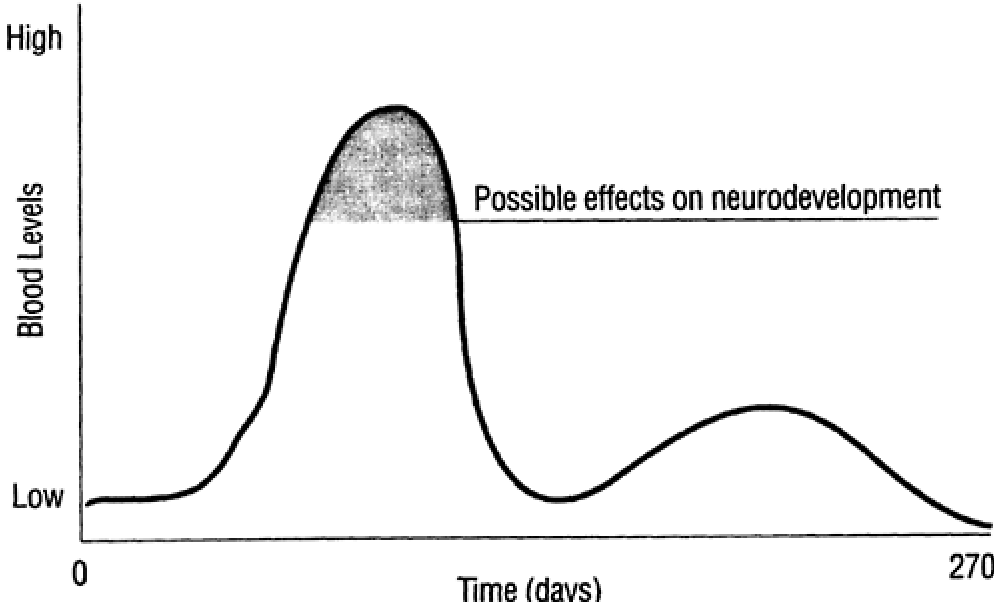

FIG. 9.2. Time course of exposure to a developmental toxicant. Note that the shaded portion represents the blood concentration of toxicant which is necessary to offer some probability that an inverse effect might occur.

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a material with a half-life of a few minutes in the body. If we were to test it by spreading or apportioning the daily dose of this material over 24 h via inhalation, it will produce a different toxicity than if the animal got the same amount in a couple of 1-h inhalation exposures. The same dose of this quick-acting material would do much more damage amassed in a bolus dose of a few minutes or even an hour or two than spread over 24 h.

Thus, dosing times in toxicological studies should be commensurate with the biological half-life for the relatively quick-acting chemicals. We often need to measure the exposure over an appropriately short period of time where the worst-case exposure may occur. The same logic also holds for the dermal (topically applied) and oral (normally ingested) exposures in that they should occur in time frames that are comparable to what we would expect in use. Bolus dosing by gavage or injection would, of course, be worst case.

On the other hand, if the biological half-life of the compound is longer than a few days, then relatively high spikes of exposure over a day or two are not particularly significant from a health impact perspective. What is important from a chronic toxicity perspective for these types of compounds is, of course, the weighted average over a significantly longer time period.

As such, it would only seem appropriate to use an annual average exposure when you are dealing with a compound with a very long (greater than 90 d) half-life in the body and no evidence of acute toxicity at high short-term dose rates (289, 291, 295).

Aggregate exposure is the sum total of exposure to a chemical via all routes of exposure (and all media). It is now commonplace to add as many as 6 to 10 different exposure sources per route (e.g., DDT in different fruits and vegetables) and 3 exposure routes (e.g., DDT via food, air, and dermal contact). The units of aggregate exposure are concentration times duration. Aggregate exposure has been considered in complex assessments of the past 10 yr, such as for incinerators, but it came to the fore with the passage of the Food Quality Protection Act (FQPA) in 1996. At least one major symposium and the resulting publication tackle this subject. Ample guidance from regulatory agencies and examples of how to perform these assessments will undoubtedly be published over the next 5 yr (161).

Integrated exposure is the total "area under the blood concentration vs. time curve" (AUC) of the exposure profile. An exposure profile (a picture of the exposure concentration over time) is particularly useful when trying to understand occupational exposure because it contains more information than a numerical estimate of the integrated exposure, including the duration and periodicity of exposure, the peak exposure, and the shape of the area under the time-concentration curve (Figure 9.2). The risk posed by most systemic toxicants with chronic effects are often best understood by evaluating the blood concentration versus time relationship.

The last way to characterize exposure is the time-weighted average (TWA). This is particularly relevant when conducting a carcinogen risk assessment. In cancer risk assessments, the time over which exposure is integrated is usually 70 yr (377). A TWA dose rate is the total dose divided by the time period of dosing, usually expressed in units of mass per unit time, or mass/time normalized to body weight (e.g., mg/kg-d). TWA dose rates such as the lifetime average daily dose (LADD) are used in dose-response equations to estimate lifetime risk.

Measures of Dose

For risk assessment purposes, dose estimates should be expressed in a manner that can be compared with available dose-response data from animal or human studies. For example, if data on human exposure are in milligrams of lead per deciliter of blood (mg/d1), it would be best to use the blood concentrations in an animal or human study to predict the risk. Frequently, dose-response relationships are based on potential dose (called administered dose in animal studies), although dose-response relationships are sometimes based on internal dose. These differences need to be accounted for. The measure of dose selected should be based on the mode of action of the adverse effect (10, 13, 19, 375, 381). For example, to assess a nasal irritant, the airborne concentration of the chemical is a relevant dose, and an even better dose metric would be mg of chemical contacting a square centimeter of nasal mucosa.

Doses may be expressed in several different ways. Solving Eq. (1), for example, gives the dose rate over the time period of interest. The dose per unit time is the dose rate, which has units of mass per time. The most common dose measure is average daily dose (ADD), which is used to predict or assess the noncarcinogenic effects of a chemical.

$$\mathrm{ADD}=(C\cdot \mathrm{IR}\cdot \mathrm{B})/(\mathrm{BW}\cdot \mathrm{AT}) \qquad (1)$$

where ADD is the potential average daily dose, BW the body weight, *B* the bioavailability, AT the time period over which the dose is averaged (days), C the mean exposure concentration, and IR the

ingestion rate.

The following presents a typical calculation.

Example Calculation 1: Determining the Average Daily Dose

A typical American eats a certain amount of lettuce over a lifetime (about 2000 kg). Assume that on any given week, the maximum quantity ingested is 0.5 kg, and the maximum on any one day is 0.04 kg/d. Assume that

the typical aldrin residue is 4 mg/kg on all lettuce ingested over the person's lifetime. What is the ADD of aldrin for the maximum week? Assume the oral bioavailability of aldrin in lettuce is 90%.

Given:

$$C=4\text{mg/kg(aldrin)}$$
$$\text{BW}=70\text{kg}$$
$$\text{AT}=7\text{d}$$
$$\text{IR}=0.5\text{kg}$$
$$\text{B}=0.9$$

Therefore:

$$\text{ADD}=(C\cdot \text{IR}\cdot \text{B})/(\text{BW}\cdot \text{AT})$$
$$\text{ADD}=(4\ \text{mg/kg})(0.5\ \text{kg})(0.9)/(70\ \text{kg})(7\ \text{d})$$
$$\text{ADD}=0.004\ \text{mg/kg-d}$$

When the primary health risk posed by a chemical is cancer or another chronic effect, then the biological response is usually described in terms of lifetime probabilities (e.g., the increased risk of developing cancer during a 70-yr lifetime is 2 in 100,000). In these circumstances, even though exposure may not occur over the entire lifetime, doses are usually presented as LADDs (377). The LADD takes the form of Eq. (2), with lifetime (LT) replacing the averaging time (AT):

$$\text{LADD}=(\text{C}\cdot \text{IR}\cdot \text{B})/(\text{BW}\cdot \text{LT}) \qquad (2)$$

Example Calculation 2: Determining the Lifetime Average Daily Dose

Assume that the reasonable maximum ingestion lifetime uptake of lettuce over a 70-yr lifetime (99% person) is 14,000 kg and that it contains 4mg/kg of aldrin. What is the LADD? Given:

$$C=4\ \text{mg/kg (aldrin in lettuce)}$$
$$\text{IR}=14,000\ \text{kg}$$
$$B=0.9$$
$$\text{BW}=70\text{kg}$$
$$\text{LT}=70\ \text{yr}=25,550\ \text{d}$$

where

$$\text{LADD}=(\text{C}\cdot \text{IR}\cdot \text{B})/(\text{BW}\cdot \text{LT})$$

Then

$$\text{LADD}=(4)(14,000)(0.9)/(70)(25,550)$$
$$\text{LADD}=0.028\ \text{mg/kg-d}$$

Although other measures of chronic dose may be more appropriate for predicting the hazard posed by specific chronic toxicants, such as an area under the blood concentration (AUC) curve or the peak target tissue concentration, the LADD is the most common dose metric used in carcinogen risk assessment (Figure 9.3).

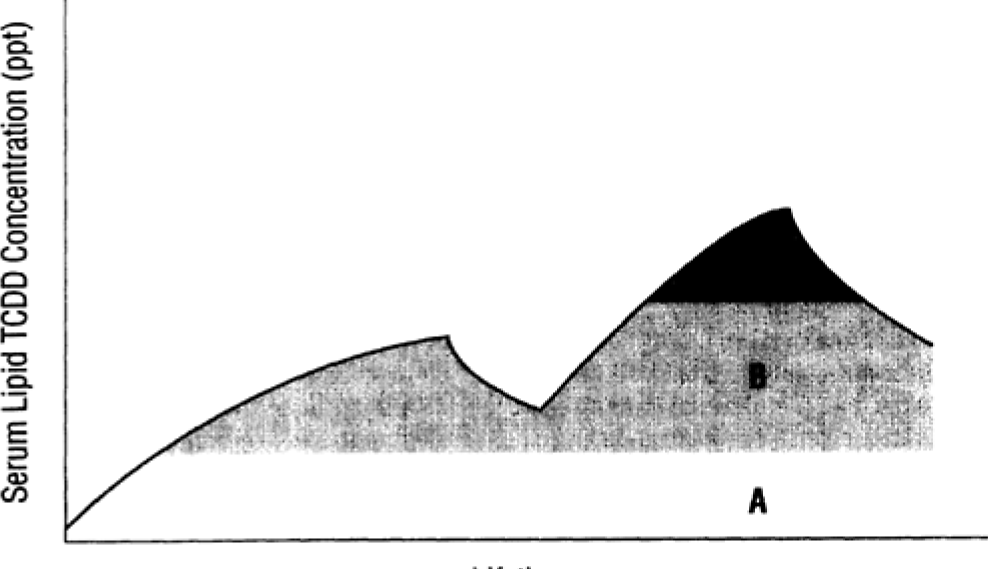

FIG. 9.3. Theoretical concentration versus time curve for TCDD illustrating one possible relationship between AUC and response. This figure illustrates the possible combination of AUC and thresholds for production of various responses: area A, no effect; area B, enzyme induction occurs; area C, significant

increased cell proliferation. From Reference 19.

CONCEPTUAL APPROACHES TO EXPOSURE ASSESSMENT

Quantifying Exposure

Although exposure assessments are conducted for a variety of reasons, the process of estimating exposure can be approached using one of the following three methods (377):

1. Direct measurement: The exposure can be measured at the point of contact (the outer boundary of the body) while it is taking place, measuring the exposure concentration and time of contact and integrating them (point-of-contact measurement). An example is the measurement of the amount of contaminated soil on an exposed hand of someone digging a hole to plant a tree. The relevant exposure information would be contaminant concentration in soil (μg/g), surface area of the hand in contact with the soil (100 m2), and time of exposure (2 h).
2. Exposure scenario: Sometimes one is concerned about an exposure that may or may not occur, so a hypothetical exposure scenario is developed. In these assessments, specific data cannot actually be collected, but relevant information can be found. For example, if an incinerator were built, it would not be known today how much of each chemical in the airborne emissions would reach the various compartments in the environment (food, soil, sediment, surface water), but one can describe what would be likely to occur (a scenario).
3. Biomonitoring: Sometimes historical exposure can be estimated based on the amount of chemical in the body or being eliminated in breath, urine, or feces. In recent years, doses have been reconstructed through internal indicators (biomarkers, body burden, excretion levels) for persistent organics and several metals. Among the best examples are lead in blood, phenol in urine, volatile hydrocarbons in the breath, and dioxins in blood fat.

These three approaches to exposure quantification (or dose) are independent because each is based on different assumptions and/or data. The fact that they are independent measures is useful in verifying or validating the results of the various approaches. Each of the three has strengths and weaknesses; using them in combination can considerably strengthen the credibility of an exposure assessment (236, 375, 395). For example, results of the exposure assessment would be validated if one could mathematically predict the absorbed dose per day of a chemical, estimate the resulting blood concentrations, and confirm these estimates by sampling the blood of the exposed population (268).

Estimates Based on Direct Measurement

Point-of-contact or direct exposure assessment evaluates the exposure as it occurs. Measuring chemical concentrations at the interface between the person and the environment as a function of time yields an exposure profile. The best known example of point-of-contact measurement is the output of a radiation dosimeter. This small badgelike device measures radiation exposure as it occurs and provides an integrated exposure estimate for the period of time over which the measurement has been taken (377). The Total Exposure Assessment Methodology (TEAM) studies (369) conducted by the U.S. EPA made use of direct measurements. In the TEAM studies, a small pump with a collector and an absorbent was attached to a person's clothing to measure his or her exposure to airborne solvents or other pollutants as it occurred, just as has been done in industrial hygiene studies of the past 60 yr (7). In both of these examples, the measurements are taken at the interface between the person and the environment while exposure is occurring.

The area of exposure assessment known as agricultural hygiene has developed very sophisticated techniques for estimating the uptake (absorption) of chemicals during the mixing and application of pesticides. These have been described in numerous articles (179, 180). A recent paper by Kissel and Fenske (175) is also useful.

Providing that the measurement devices are accurate, the direct measurement method likely gives the best exposure value for the period of time over which the measurement was taken. It is often expensive, however, to use these techniques to evaluate persons in the community, and measurement devices and techniques do not currently exist for all chemicals (at least at ambient concentrations).

Estimates Based on Exposure Scenarios

Using the exposure scenario approach, the assessor attempts to estimate or predict chemical concentrations in a medium or location and link this information with the time that individuals or populations are in contact with the chemical. An exposure scenario is the set of assumptions describing how this contact takes place. This is, by far, the most common approach to exposure assessment. Such an approach is necessary when trying to predict the impact of events that may occur in the future, such as building a new manufacturing facility or introducing a new pesticide or herbicide (239, 261, 262, 284).

The first step to building a scenario is to determine the concentration of the contaminated media. This is

typically accomplished indirectly by measuring,

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modeling, or using existing data on concentrations in the media of concern, rather than at the point of contact (for example, pesticide residues on food or metal emissions on residential soils). Often, we assume that the concentration in the bulk medium is the same as the concentration at the point of exposure. This can be a source of potential error and should be discussed in the uncertainty analysis. For example, over the past 20 yr, most assessments of the hazard posed by contaminated soil were based on soil samples collected in the top 6 in of soil, even though most persons were exposed routinely to the surface soil (usually the top 2½ in). Arguments can be made in either direction about the appropriateness of this assumption.

The next step in conducting an exposure scenario is to estimate the contact time, identify who is likely to be exposed, and then develop estimates of the exposure frequency and duration. Like chemical concentration characterization, this is usually done indirectly using demographic data, survey statistics, behavior observation, activity diaries, activity models, or, in the absence of more substantive information, assumptions about behavior (386, 387).

Chemical concentration and population characterizations are ultimately combined in an exposure scenario. One of the major problems in evaluating dose equations is that the limiting assumptions used to derive them (e.g., steady-state assumptions) do not always hold true. Two approaches to this problem are available: (1) to evaluate the exposure or dose equation under conditions when the limiting assumptions do hold true, or (2) to build a dynamic model that accounts for both accumulation and degradation. The microenvironment method, which is usually used to evaluate air exposures, is an example of the first approach. This method evaluates segments of time and location when the assumption of constant concentration is approximately true, and then sums the time segments to determine the total exposure for the respiratory route, effectively removing some of the uncertainty (282). In occupational hygiene, this is done by combining time-motion data with short-term air concentration data. While exposure concentration and time of contact may be estimated in some situations, the concentration and time of contact can be measured for each microenvironment. This avoids much of the error due to summing average values in cases where concentration and time of contact vary widely.

In the second approach, a computer model can efficiently predict dose if enough data are available (51, 204, 430). When conducting modeling, there are various tools used to describe parameter variation, such as Monte Carlo analysis, and these may be necessary in some assessments.

Estimating Exposure Using Biological Monitoring

Exposure can often be estimated after it has taken place. The key factor is whether the biological half-life of the chemical is sufficiently long to allow for accurate measurement. If a total dose is known or can be reconstructed, and information about intake and uptake rates is available, an average past exposure rate can be estimated (19, 276, 309, 333, 346). Dose reconstruction relies on measuring biological fluids (blood, urine), hair, nails, or feces after exposure, intake, and uptake have already occurred, and using these measurements to back-calculate dose (19). However, data on body burden levels or biomarkers cannot be used directly unless a relationship can be established between these levels (or biomarker indications) and internal dose.

Biological monitoring can be used to evaluate the amount of a chemical in the body by measuring one or more parameters (Table 9.2). In general, if these measurements can be made and the biologic half-life is acceptable, then past exposure estimates can be reasonably accurate. Not all of these can be measured for every chemical (377). Following is a list of possible measurements:

- The concentration of the chemical itself in biological tissues or sera (blood, urine, breath, hair, adipose tissue, etc.).
- The concentration of the chemical's metabolite(s).
- The biological effect that occurs as a result of human exposure to the chemical (e.g., alkylated hemoglobin or changes in enzyme induction).
- The amount of a chemical or its metabolites bound to target molecules.

The results of biomonitoring can be used to estimate chemical uptake during a specific interval, if background levels do not mask the marker and the relationship between uptake and the selected marker is known (89). The sampling time for biomarkers is often critical. Establishing a correlation between exposure and measurement of the marker, including pharmacokinetics, is necessary to properly back-calculate historical exposure (377).

The strengths of this method are that it demonstrates that exposure and absorption of the chemical has actually taken place, and theoretically it can give a good indication of past exposure. The drawbacks are that (1) it will not work for every chemical because of interferences or the reactive nature of the

chemical, or because the biological half-life of the agent is too short; (2) that the approach has been applied to only a few chemicals; (3) that data relating internal dose to exposure are needed; and (4) that it may be expensive.

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Table 9.2

Examples of types of measurements to characterize exposure-related media and parameters

Type of measurement (sample)	Usually attempts to characterize (whole)	Examples	Typical information needed to characterize exposure
Breath	Total internal dose for individuals or population (usually indicative of relatively recent exposures).	Measurement of volatile organic compounds (VOCs), alcohol. (Usually limited to volatile compounds.)	1. Relationship between individuals and population; exposure history (i.e., steady-state or not) pharmacokinetics (chemical half-life), possible storage reservoirs within the body. 2. Relationship between breath content and body burden.
Blood	Total internal dose for individuals or population (may be indicative of either relatively recent exposures to fat-soluble organics or longterm body burden for metals).	Lead studies, pesticides, heavy metals (usually best for soluble compounds, although blood lipid analysis may reveal lipophilic compounds).	1. Same as for breath. 2. Relationship between blood content and body burden.
Adipose	Total internal dose for individuals or population (usually indicative of longterm averages for fat-soluble organics).	NHATS, dioxin studies, PCBs (usually limited to lipophilic compounds).	1. Same as for breath. 2. Relationship between adipose content and body burden.
Nails, hair	Total internal dose for individuals or population (usually indicative of past exposure in weeks to months range; can sometimes be used to evaluate exposure patterns).	Heavy metal studies (usually limited to metals).	1. Same as for breath. 2. Relationship between nails, hair content and body burden.
Urine	Total internal dose for individuals or population (usually indicative of elimination rates); time from exposure to appearance in urine may vary, depending on chemical.	Studies of tetrachloroethylene and trichloroethylene.	1. Same as for breath. 2. Relationship between urine content and body burden.

Note. From Reference 385.

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For those chemicals where biological monitoring can be used to estimate past exposure, the information obtained can be invaluable for conducting retrospective exposure assessments that can be used in epidemiology studies. Some examples of chemicals for which past exposure can reliably be estimated include several metals, as well as numerous large organic chemicals (e.g., DDT, chlordane, dioxin, polybrominated biphenyl [PBB], PCB) (19).

INFORMATION UPON WHICH EXPOSURE ASSESSMENTS ARE BASED

Comprehensive exposure assessment of a complex scenario may require several hundred exposure factors to estimate the various chemical concentrations in one of several dozen different media. Among the most complex exposure assessments are those that address the risks posed by airborne emissions from combustors (125, 281, 388) (Figure 9.4). To estimate the concentration, numerous dispersion models, as well as fate and transport models, may be required. In addition, the assessor may need to search the literature to identify relevant studies from as many as 10 related fields of research. Sometimes, hundreds of published papers and government guidance documents need to be evaluated, used, and cited. In short, the exercise can be formidable, especially for assessments involving food chain contamination. Equally difficult and highly complex exposure assessments are those that attempt to estimate the uptake of fish by various members of the angling public (428).

Obtaining Data on Intake and Uptake

The numerous editions of the *Exposure Factors Handbook* (382–384, 386) present statistical data on many of the factors used to assess exposure, including intake rates, and these provide citations for primary references. Today, this series of publications represents the most comprehensive, single source of exposure assessment information. Some of the many intake factors in the various volumes include:

- Drinking water consumption rates.
- Breast milk ingestion rates for infants.
- Consumption rates for homegrown fruits, vegetables, beef, and dairy products.

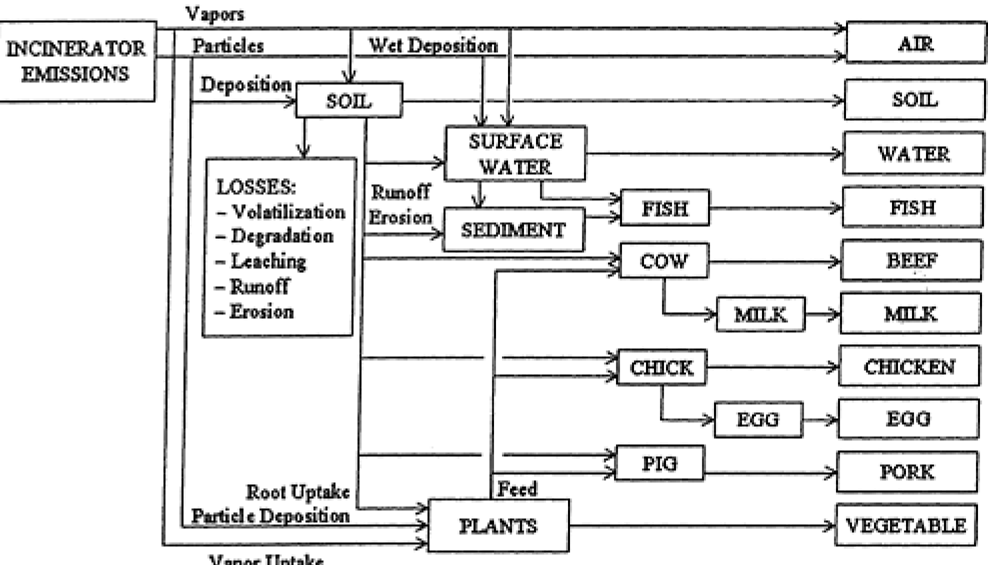

FIG. 9.4. EPA's conceptual approach to dealing with direct and indirect exposure pathways as illustrated by assessments of incinerator emissions.

- Consumption rates for recreationally caught fish and shellfish.
- Incidental soil ingestion rates.
- Pulmonary ventilation rates.
- Surface area of various parts of the human body.
- Body weight for various age groups.

Table 9.3 presents examples of some of the standard or default exposure factors used in risk assessment.

The *Exposure Factors Handbook* is updated routinely to include additional factors and to include new research data on previously discussed factors. It also provides default parameter values, which can be used when site-specific data are not available. Obviously, general default values should not be used in place of known, valid data that are more relevant to the assessment being conducted. The U.S. EPA handbook, though substantial, may not contain all available information on exposure factors or relevant studies, so a supplemental literature search should be conducted to ensure that pertinent literature has been identified. As discussed later, if a probabilistic or Monte Carlo assessment is to be conducted, the document titled *Risk Assessment Guidance for Superfund: Process for Conducting Probabilistic Risk Assessment* (392) and other publications should be consulted.

Concentration Measurements in Environmental Media

Sometimes the existing chemical concentration data are inadequate to conduct a proper site-specific assessment. In these cases, concentration data can be gathered by conducting a new field study, or by evaluating data from past field studies and using them to estimate concentrations. Media measurements taken close to the point of contact are preferable to measurements far removed geographically or temporally. As the distance from the point of contact increases, the certainty of the data at the point of contact usually decreases, and the obligation for the assessor to show relevance of the data to the assessment at hand becomes greater. For example, an outdoor air measurement, no matter how close it is taken to the point of contact, cannot by itself adequately characterize indoor exposure (267). Concentrations often vary considerably from place to place, seasonally and over time due to changing emission and use patterns (259, 312, 313). This needs to be considered not only when designing studies to collect new data, but also when evaluating the applicability of existing measurements as estimates of exposure concentrations in a new assessment. It is of particular concern when the measurement data will be used to extrapolate to long time periods, such as lifetimes. Transport and dis

Table 9.3 Some standard regulatory default assumptions used i in exposure assessment

Variable	Assumption
Drinking water	2 L/d (RME adult)
	1.4 L/d (adult ave.)
	1.0 L/d(child)
	0.1 L/d (incidental ingestion during swimming)
Soil (ingestion)	200 mg/d (child ave.)
	800 mg/d (child 90th percentile)
	100 mg/d (adult)
Food	2000 g/d (adult total)
Beef (home-grown)	44 g/d (ave.)
	75 g/d (RME) 100 g/d (all sources)
Dairy (home-grown)	160 g/d (ave.)
	300 g/d (RME)
	400 g/d (all sources)
Fruit (home-grown)	28 g/d (ave.)
	42 g/d (RME)
	140 g/d (all sources)
Vegetables (home-grown)	50 g/d (ave.)
	80 g/d (RME)
	200 g/d (all sources)
Sport fish	30 g/d (ave.)
	140 g/d (RME)
Inhalation	10 m3/d (ave. 8-h shift.)
	20 m3/d (adult ave.)
	30 m3/d (RME)

Body weight	13.2 kg (2-5 yr)
	20.8 kg (6 yr)
	70 kg (adult ave.)
Lifespan	70 yr
Exposed skin area	0.2 m2 (adult ave.)
	0.53 m2 (adult RME)
	1.94 m2 (male bathing)
	1.69 m2 (female bathing)
Showering	7 min (ave.)
	(5-min. shower uses 40 gal)
	12 min (90th percentile)
Residence time	9 yr (ave.)
	30 yr (RME)

Note. RME, reasonable maximum exposure; ave., average.

persion models are frequently used to help answer these questions (51).

The exposure assessor is likely to encounter several different types of measurements. One type often used to understand concentration trends is outdoor fixed-location monitoring. This measurement is used by the U.S. EPA and other groups to provide a record of pollutant concentration at one place over time. Nationwide air and water monitoring programs have been established so that baseline values in these environmental media can be documented. Although it is not practical to set up a national monitoring network to gather data for a particular exposure assessment, data from existing

networks can be evaluated for relevance to an exposure assessment. These data are often far removed from the point of contact. Adapting data from previous studies usually presents specific challenges. Indoor air contaminant concentrations can vary as much as or more than those in outdoor air (298, 410—413). Consequently, indoor exposure is best represented by measurements taken at the point of contact. However, because pollutants such as carbon monoxide can exhibit substantial outdoor penetration, indoor exposure estimates should consider potential outdoor, as well as indoor, sources of the contaminant(s) under evaluation (247, 377).

Contaminant concentrations in food and drinkingwater measurements can also be measured. General characterization of these media, such as market basket studies (where representative diets are characterized), shelf studies (where foodstuffs are taken from store shelves and analyzed), or drinking-water quality surveys, are usually far removed from the point of contact for an individual, but may be useful in evaluating exposure concentrations for a large population. Measurements of tap water or foodstuffs in a home and how they are used are closer to the point of contact. In evaluating the relevance of data from previous studies, variation in the distribution systems must be considered, as well as the space-time proximity (377).

Consumer or industrial product analysis is sometimes done to characterize the chemical concentrations in products. Product formulations can change substantially over time, similar products do not necessarily have similar formulations, and regional differences in product formulation can also occur. These should be considered when determining the relevance of existing data and when setting up sampling plans to gather new data (377).

Another type of concentration measurement is the microenvironmental measurement. Rather than using measurements to characterize the entire medium, this approach defines specific zones in which the concentration in the medium of interest is thought to be relatively homogeneous, and then characterizes the concentration in that zone (283, 375). Typical microenvironments include the home or parts of the home, office, automobile, or other indoor settings. Microenvironments can also be divided into time segments (e.g., kitchen during the day, kitchen during the night). This approach can produce measurements that are closely linked with the point of contact, both in location and time, especially when new data are generated for a particular exposure assessment. The more specific the microenvironment, however, the greater is the burden on the exposure assessor to establish that the measurements are representative of the population of interest.

The concentration measurement that provides the closest link to the actual point of contact is personal monitoring. In virtually all cases, if available, this information should be the basis of exposure assessments of individuals. An obvious exception is the work environment where lapel sampling is conducted while the person is wearing a respirator; in this case, personal sampling would not reflect genuine exposure.

Models and Their Role

Often the most critical element in an exposure assessment is estimating pollutant concentrations at exposure points. This is usually carried out by combining field data and modeling results. In the absence of field data, this process often relies on the results of mathematical models of aerial dispersion, such as ISCLT, or of water movement, such as MODFLOW (107, 364, 366–368). The U.S. EPA Science Advisory Board and others have recommended that modeling ideally should be linked with monitoring data in regulatory assessments, although this is not always possible.

A modeling strategy has several aspects, including setting objectives, model selection, obtaining and installing the code, calibrating and running the computer model, and validation and verification. Many of these aspects are analogous to the quality assurance and quality control measures applied to measurements.

Regardless of whether models are extensively used in an assessment or whether a formal modeling strategy is documented in the exposure assessment plan, when computer simulation models such as fate and transport models and exposure models are used in exposure assessments, the assessor must be aware of the performance characteristics of the model and state how the exposure assessment requirements are satisfied by the model (51).

The site must be characterized if models are to be used to simulate pollutant behavior at a specific site. Site characterization for any modeling study includes examining all data on the site, such as source characterization, dimensions and topography of the site, location of receptor populations, meteorology, soils, geohydrology, and ranges and distributions of chemical concentrations. For exposure models that simulate both chemical concentration and time of exposure (through behavior patterns), data on these two parameters must be evaluated (262, 264, 375).

Criteria are provided by the U.S. EPA (368) for selecting surface water models and groundwater models, respectively; the reader is referred to this document for details. Similar selection criteria exist for air dispersion models.

A primary consideration in selecting a model is whether to perform a screening study or a detailed evaluation. A screening study makes a preliminary evaluation of a site

or a general comparison between several sites. It may be generic to a type of site (i.e., an industrial segment or a climatic region) or may pertain to a specific site for which sufficient data are not available to properly characterize the site. Screening studies can help direct data collection at the site by, for example, providing an indication of the level of detection and quantification that would be required and the distances and directions from a point of release where chemical concentrations might be expected to be highest.

An example of a screening-level modeling effort would be to estimate the amount of lead deposited by an incinerator onto local crops using a basic air dispersion model, without considering local geographical or weather conditions. The next level of complexity would consider the presence of mountains, their proximity to the stack, the local weather patterns, and the number of atmospheric inversions per year. A higher level of analysis could incorporate yet other, more subtle factors.

The value of the screening-level analysis is that it is simple to perform and may indicate that no significant contamination exists. Screening-level models are frequently used to get a first approximation of the concentrations that may be present. Often these models use very conservative assumptions; that is, they tend to overpredict concentrations or exposures. If the results of the conservative screening procedure predict concentrations or exposures at less than a predetermined no-concern level, then more detailed analysis is probably not necessary. If the screening estimates are above that level, refinement of the assumptions or a more sophisticated model are necessary to generate a more realistic estimate (377).

Screening-level models also help the user conceptualize the physical system, identify important processes, and locate available data. The assumptions used in the preliminary analysis should represent conservative conditions, such that the predicted results overestimate potential conditions, limiting false negatives. If the limited field measurements or screening analyses indicate that a contamination problem may exist, then a detailed modeling study may be useful.

In contrast, the purpose of the detailed evaluation is to use the best data available to make the best estimate of spatial and temporal chemical distributions of a specific site. Detailed studies typically require higher quality data and more sophisticated models.

Accounting for Background Concentrations

Background exposure to so-called "toxic" or industrial chemicals, especially environmentally persistent ones, can occur due to natural or anthropogenic sources (355). In most exposure assessments, background soil concen trations are the focus of attention, but the same issue can be relevant when evaluating sediments, ambient air, groundwater, and vegetation (foodstuffs). At some sites, it is important that these so-called "background" concentrations be accounted for because removing the quantity of toxicant due to humans may, in fact, not appreciably change the concentrations or be sufficient to reduce the risk to acceptable levels. For example, naturally occurring concentrations of lead, arsenic, and cadmium, in some locations, may be higher than cleanup levels established by various regulatory agencies (94, 95). The exposure assessor should try to determine local background concentrations by gathering data from nearby locations clearly unaffected by the site under investigation, or by referring to published works that have assessed this issue. Statistical approaches are applicable to address this issue (245).

Description of Background Levels

When assessing soils, background levels can be viewed in at least four different ways (107):

• "Pristine" levels—Some would like to equate background levels with those associated with the "pristine" state, that is, soils or landscapes unaffected by human activity. This rather idealistic situation probably no longer exists; even in Antarctica, mercury and dioxin concentrations can be detected in some media. Toxic elements mainly associated with the solid phase of some natural material (such as soil dust, plant or volcanic ash, vegetable matter) are relatively mobile in a global sense. For example, Nriagu (243) has suggested that about 40 million tons of heavy metals have been dispersed atmospherically over the many centuries of human activity.

Increases in pollutant metal concentrations have been measured up to 60 km from smelters, and automotive lead (fine particles) has been measured in soils and rainfall up to about 50 km downwind from major cities. Soil contamination up to 50–100m from highways by automotive lead (coarser particles) is an example of short-range transport, and contrasts with transport of toxic metals on a continental or global scale (e.g., contamination of the Greenland ice sheets from the northern United States, mercury in the Florida Everglades due to aerial releases in South America, the snows of the New Zealand Alps by soil dust from inland Australia) (107).

• "Normal" levels—The question could be asked: Are soils contaminated at farms in the higher rainfall

areas of the Appalachians, which used to receive automotive exhaust particulates from

the New York metropolitan area 200 km away? These soils are not pristine, but the chemical concentrations are perfectly safe for growing food, raising farm animals, and residential living. Soils from such areas would have a range of what is often called "normal" background values. To most exposure assessors, this mosaic of normal soils, which is only affected by the minor pollution of everyday activities associated with modern rural and urban life, should be the basis for defining background values. Statistically, this range of normal background values would constitute a single lognormally distributed population. Obviously, one needs to exclude the outliers or "hot" spots due to a geochemical anomaly, or localized pollution arising from either industrial emissions, disposal of waste products, or intensive (excessive) use of farm chemicals (107).

• Historically polluted regions—Local community and regulatory policies often affect what are defined as background levels. A community with highly developed environmental consciousness may insist on very low, possibly unreasonable, reference values. Some densely populated areas with historically derived pollution, perhaps from former mining activities, may sustain apparently healthy populations who pragmatically must accept higher "background" level values. The cities of Philadelphia, Baltimore, and New York, and parts of Japan, for example, may fit in this category.

• Geochemical variation—Background levels of some potentially toxic elements may vary among geographical regions because of differences in soil type. The resulting concentrations are often called naturally occurring levels.

An important factor is the composition of rocks and sediments that weather from soils. Some extreme examples are high concentrations of nickel, cobalt, and chromium in igneous rocks such as basalts that cover extensive areas in western Victoria and Tasmania, Australia; and high concentrations of boron in soils on marine sediments in the Riverina, Mallee, and Wimmera districts of South Australia, and in Victoria, Eyre Peninsula and parts of western Australia.

Some regulatory agencies have provided written guidance describing how to select soil or sediment cleanup values that account for background chemical concentrations. In past years, these have varied significantly, but within the past 5 yr, there appears to be some convergence regarding the definition of background, how to measure it, and how it should affect exposure assessment calculations.

ESTIMATING UPTAKE VIA THE SKIN

When attempting to predict chemical risks in the environmental or occupational setting, the dermal exposure route can vary in its importance from negligible to significant. In most evaluations of hazardous waste sites and ambient air or water contaminants, this is not a major route of exposure. Although the uptake of chemicals via the skin has generally been overlooked in most workplace exposure assessments, it probably represents a substantial portion of the exposure for many occupations. Even though gloves are more frequently used than in years past and training has increased on the possible hazards of dermal exposure, there is still ample evidence to indicate that, in order to conduct a complete exposure assessment, this route deserves attention (192, 253, 265).

In addition to the risks associated with systemic toxicity due to uptake via the skin, it is sometimes necessary to evaluate the allergic contact dermatitis hazard (ACD). In recent years, techniques have been developed to quantitatively predict the likelihood of elicitation and induction of ACD (164, 240). Some regulatory agencies are concerned with ACD and have developed cleanup standards based on this health endpoint.

In the workplace, a worker's skin frequently comes into contact with solvents or chemicals mixed in water (aqueous materials). In most environmental settings where persons can be exposed to contaminated soil or contaminated water, dermal uptake must be assessed. Fortunately, a good deal of research has been conducted to understand the rate at which chemicals pass through the skin.

Percutaneous absorption of neat chemicals (i.e., the pure liquid) was often studied in humans until the late 1970s (100, 101, 113, 184, 225, 274, 275, 347). Because of the potential toxicity of many chemicals and improved laboratory techniques, in vivo human studies have been largely supplanted by experiments with laboratory animals, in vitro studies, or athymic rodents grafted with human skin (178). Historical research has shown that, in general, chemical penetration of the human skin is similar to that of a pig or monkey, and much slower than that of the rat and rabbit (26). Thus, for many chemicals, there is some level of confidence that the rate of dermal uptake of a chemical by humans can be inferred from animal data.

Starting in the 1980s, in vitro studies using human skin began to be conducted on a more routine basis. In these studies, a piece of excised skin is attached to a diffusion apparatus with a top chamber to hold the applied chemical and a temperature-controlled bottom chamber containing saline or other fluids (plus a sampling port to withdraw fractions for analysis) (123). Although human forearm skin is optimal,

it is difficult to obtain, so abdominal or breast skin is commonly used. Generally,

a properly conducted in vitro test can be a reasonably good predictor of the absorption rate in vivo (34). However, due to the fragile nature of the technique, these studies must be carefully interpreted (22). Often, depending on the conditions of the test, the results are not applicable to humans.

Aside from neat liquids and exposure to contaminated water, dermal exposures can also occur through contact with dust or dirt on surfaces, and by way of contact with soil or dust-bound contaminants (267). Few studies (96, 119, 156, 169, 177, 191, 286, 300, 317) have directly estimated soil loading on human skin, and only one of them attempted to measure dermal contact of contaminated equipment by workers (206). The available studies probably provide sufficient data to generate point estimates of soil adherence and perhaps can provide a reasonable probability density function (PDF) for most persons exposed to contaminated soils. The degree of representativeness of the data to the general population is difficult to assess (48).

Recently, a few studies measured the adherence of soil to multiple skin surfaces (hands, forearms, lower legs, faces, and feet) under ambient and recreational conditions (156, 177). Dermal loading on the hands was found to vary over five orders of magnitude and to be dependent on the type of activity. Differences between pre- and postactivity adherence demonstrated the episodic nature of dermal contact with soil. However, due to the activity-dependent nature of soil exposure, data from these studies must be interpreted for their relevance to the type of activity, frequency, duration, and otherwise site-specific nature of exposure. The various studies involving contaminated soil are informative for providing an estimate of exposure; however, they are probably a couple orders of magnitude greater than what might be expected in an occupational setting. Nonetheless, this work is a "starting point" for bracketing potential exposure to dusts in the workplace.

Recently, there has been a reasonable level of research investigating exposure to house dust. The basis for this concern has been increasing evidence that controlling exposure to house dust, especially in homes located near sites with considerable surface soil contamination, is more important for reducing the health hazard than remediating the soil (267). Numerous papers in recent years have shown that in-house exposure to toxics is much greater than that encountered due to ambient (so-called environmental) contamination (247, 405).

Along these lines, and of particular interest to those who study indoor exposure, is the recent work to develop standardized approaches for collecting wipe samples and estimating the amount of dust loading on the palm of the hand (197, 198). Although dermal absorption of toxicants in house dust will almost always pose a relatively low dermal uptake hazard, the uptake of toxicants due to hand-to-mouth contact can be substantial (267).

Quantitative Description of Dermal Absorption

For the purposes of risk assessment, percutaneous absorption is defined as transport of externally applied chemicals through cutaneous structures and the extracellular medium to the bloodstream (375, 391). In many settings, such as for agricultural workers, platers, mechanics, and others, dermal uptake is the primary route of exposure. The simplest way to describe the rate of skin absorption is to apply Fick's first law of diffusion at steady state (349, 418):

$$J = dQ/dt = KC/e \approx K_p C \qquad (3)$$

where J $(=dQ/dt)$ is the chemical flux or rate of chemical absorbed (mg/cm2-h), D is the diffusivity in the stratum corneum (cm2/h), K the stratum corneum/vehicle partition coefficient of the chemical (unitless), ∇C the concentration gradient (mg/cm3), e the thickness of the stratum corneum (cm), K_p the permeability coefficient (cm/h), and C the applied chemical concentration (mg/cm3). The concentration gradient is equal to the difference between the concentration above and below the stratum corneum. Because the concentration below is small compared to the concentration above, ∇C can be approximated as equal to the applied chemical concentration. From the preceding equation, it can be seen that the rate of absorption is directly proportional to the applied concentration. The diffusivity represents the rate of migration of the chemical through the stratum corneum. Since the stratum corneum has a nonnegligible thickness, there is a period of transient diffusion (lag time), during which the transfer rate rises to reach a steady state. In these studies, the steady state is maintained indefinitely, provided the system remains constant. Depending on the type of chemical, the lag time can range from minutes to days (192). From an exposure assessment standpoint, if the exposure duration is shorter than the lag time, it is unlikely that there will be any significant systemic absorption (142, 349). The partition coefficient (K_p) is one of the key parameters that influences the degree to which a chemical penetrates the skin (14, 122, 128, 142, 349). Fatty chemicals tend to accumulate in the stratum corneum. Conversely, the stratum coraeum is an effective barrier for hydrophilic substances, which tend to have low skin absorption rates. Because stratum corneum/vehicle partition coefficients are

difficult to measure, the three parameters (*D*, *k*, and *e*) are combined to give an overall permeability coefficient *(Kp)*. It is noteworthy that Eq. (3) only approximates most in vivo exposure situations

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in which true steady-state conditions are rarely attained. In spite of its limitations, this equation has yielded satisfactory estimations of the actual absorption rates of chemicals for many situations (Table 9.4).

Pharmacokinetic Models for Estimating the Uptake of Chemicals in Aqueous Solution

Pharmacokinetic models predict the uptake of a chemical through the skin based on fundamental thermodynamics. Several different models have been proposed. For example, a four-compartment pharmacokinetic model was developed in 1982 (128). This model, which uses first-order rate constants, describes chemical movement through the compartments representing the various skin structures. It has been used successfully to predict the chemical disposition in the skin and plasma as a function of their physicochemical properties, and when an input rate constant to the skin surface is added to the model, it can be used to assess vehicle effects. A similar model that treats the barrier membrane as a series of spaces filled with immiscible liquids has also been developed (14); its advantage is that it allows examination of non-steady-state conditions where Fick's law does not apply.

Under an infinite-dose situation where the amount of a chemical lost by penetration is too small to alter the applied concentration (e.g., where one is swimming), the rate of absorption is essentially linear once steady-state has been reached. In the finite-dose system, however, the chemical solution is applied as a thin film and the concentration decreases as penetration proceeds (e.g., a splash). All other model parameters being the same, penetration is reduced under finite-dose conditions. This is because the chemical concentration is continuously reduced over time, resulting in a decrease in the gradient across the stratum corneum. These modeling results indicate that the mechanism by which fluxes are affected must be considered when extrapolating to non-steady-state conditions.

Although classic pharmacokinetic modeling like that described by Guy et al. (42) can provide a good mathematical description of the disposition of chemicals, it does not depict exactly the biological processes in the intact animal. Fortunately, due to recent improvements in available computer hardware and software, pharmacokinetic methods based on physiological principles are now feasible alternatives for analysis of in vivo skin penetration studies. These so-called PBPK models realistically describe the disposition of the chemical in the intact animal in terms of rates of blood flow, permeability of membranes, and partitioning of chemicals into tissues (12, 14). Characterizing dermal absorption in terms of actual anatomical, physiological, and biochemical parameters facilitates extrapolations to the real species of interest, humans.

In 1991, a PBPK model was developed to describe percutaneous absorption of volatile organic contaminants in dilute aqueous solutions (316). The exposure scenario modeled was either hand or full-body immersion into a vessel of solute-contaminated water. Modeling results suggested that chemical uptake in aqueous solutions is most markedly influenced by epidermal blood flow rates, followed by epidermal thickness and lipid content of the stratum corneum. In general, thicker and fattier skin provides a better barrier to dermal penetration of chemicals. These are precisely the principles under which barrier creams offer their protection for increasing the effective thickness and lipophilicity of the skin. This model also predicted that the dose of some volatile organic chemicals in water absorbed through the skin during a 20-min bath may be equivalent to the amount inhaled (316).

Among the most complex and best validated of the various models for dermal uptake of liquids is that developed by McDougal (213). This team has successfully predicted dermal uptake rates for humans for more than a dozen chemicals based on animal data. One advantage of dermal PBPK models over traditional in vivo methods is their ability to accurately describe nonlinear biochemical and physical processes. For example, describing skin penetration based on blood concentrations or excretion rates as "percent absorbed" assumes that all processes have a simple linear relationship with the exposure concentration. This is often not the case. The kinetics become nonlinear when the absorption, distribution, metabolism, or elimination of a chemical is saturated at high exposure concentrations. This model and models developed since then address this phenomenon in a reasonable manner.

Factors Used to Estimate Dermal Uptake

Many factors need to be quantitatively accounted for in order to estimate the likely systemic uptake of a chemical that comes into contact with the skin, either as a liquid or when present in soil or dust (269, 376).

Dermal Bioavailability

The typical media of concern for assessing cutaneous contact to environmental chemicals, in contrast with occupational exposure, are house dust, soil, fly ash, and sediment. In the workplace, dermal uptake is due to direct contact with liquids and contact with surfaces contaminated with dirt or liquids. A number of parameters can influence the degree of cutaneous bioavailability of chemicals in complex

matrices. These may include aging (time following contamination), soil type (e.g., silt, clay, and sand), type and concentration

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Table 9.4 Human cutaneous permeability coefficient values for some industrial chemicals in aqueous medium

	MW	*Kow*	Observed	Calculated*a*
Organic chemicals				
2-Amino-4-nitrophenol	154.13	21.380.00066		0.019
4-Amino-2-nitrophenol	154.13	9.120.0028		0.0081
Aniline	93.12	7.940.041b		0.091
Benzene	78.11	134.900.11		0.39
p-Bromophenol	173.02	389.050.036		0.25
Butane-2, 3-diol	90.12	0.12<0.00005		0.0009
n-Butanol	74.12	7.590.0025		0.024
2-Butanone	72.10	1.940.0045		0.007
Carbon disulfide	76.14	100.000.54b		0.3
Chlorocresol	142.58	1258.930.055		1.31
S-Chlorophenal	128.56	147.910.033		0.19
p-Chlorophenal	128.56	257.040.036		0.34
Chloroxylenal	156.61	1621.810.059		1.35
m-Cresol	108.13	100.000.015		0.18
o-Cresol	108.13	100.000.016		0.18
p-Cresol	108.13	85.110.018		0.15
Decanol	158.28	37153.520.08		30.11
2, 4-Dichlorophenol	163.01	1995.260.06		1.5
1, 4-Dioxane	88.10	0.380.00043		0.0016
Ethanol	46.07	0.490.0008		0.0036
2-Ethoxyethanol	90.12	0.290.0003		0.0013
Ethylbenzene	106.16	1412.541.215*b*		2.65
Ethylether	74.12	6.760.016		0.022
p-Ethylphenol	122.17	549.540.035		0.79
Heptanol	116.20	257.040.038		0.41
Hexanol	102.17	107.150.028		0.21
Methanol	32.04	0.170.0016		0.0026
Methyl hydroxybenzoate	152.15	91.200.0091		0.082
β-Naphthol	144.16	691.830.028		0.7
3-Nitrophenol	139.11	100.000.0056		0.11
4-Nitrophenol	139.11	81.280.0056		0.09
Nitrosodiethanolamine	134.13	0.130.0000055		0.0005
Nonanol	144.26	2951.210.06		2.99
Octanol	130.22	933.250.061		1.19
Pentanol	88.15	36.310.006		0.091
Phenol	94.11	32.360.0082		0.074
Propanol	60.09	2.000.0017		0.0088
Resorcinol	110.11	6.030.00024		0.011
Styrene	104.14	891.250.635*b*		1.72
Thymol	150.21	1995.260.053		1.84
Toluene	92.13	489.781.01		1.15
2, 4, 6-Trichlorophenol	197.46	2344.230.059		1.02
3, 4-Xylenol	122.16	169.820.036		0.25
Inorganic chemicals				
Cobalt chloride	129.84		0.0004	
Lead acetate	325.29		0.0000042*b*	
Mercuric chloride	271.50		0.00093	
Nickel chloride	129.60		0.001	
Nickel sulfate	154.75		<0.000009	
Silver nitrate	169.87		<0.00035*b*	
Sodium chromate	161.97		0.0021*b*	

Note. From Reference 192.
a Permeability coefficients calculated using equation presented in Leung and Paustenbach (192).

b All the observed permeability coefficients were obtained by using in vitro techniques except those denoted with superscript *b*, which were determined in vivo.

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of cocontaminants (e.g., oil and other organics), and the concentration of the chemical contaminant in the media (321). The bioavailability of a chemical in soil will usually be affected by its physicochemical properties. High-molecular-weight chemicals tend to bind to soil/dust and be less water soluble, while smaller molecules will frequently be water soluble, less tightly bound, and relatively bioavailable (159, 215). The cutaneous bioavailability of perhaps 20 to 30 chemicals in soils has been determined in animals (159, 321, 326, 358, 359, 420). These studies show that different media and different chemicals can yield dramatically different cutaneous bioavailabilities. The results of these studies, for example, produce values of bioavailability for different chemicals that range from 0.001 to 3% for chemicals in soil.

Skin Surface Area

There is an abundance of information about the surface area of different portions of the body. One simple approach is to use the "rule of nines" for estimating the surface area of the human body (334): the head and neck are 9%, upper limbs are each 9%, lower limbs are each 18%, and the front or back of the trunk is 18% (254). The U.S. EPA has estimated an exposed surface area (arms, hands, legs, and feet) of 2900 cm2 for children 0 to 2 yr old; 3400 cm2 for children 2 to 6 yr old; and 2940 cm2 for adults (an adult is assumed to wear pants, an open-neck short-sleeve shirt, shoes, and no hat or gloves) (377). When assessing chemical exposure in the ambient environment, most of the necessary surface area information can be found in the U.S. EPA *Exposure Factors Handbook* (386). Table 9.5 presents the skin surface areas commonly used when conducting exposure assessments (334). A distribution plot of skin area versus body weight has been developed (42).

Soil Loading on the Skin

A key factor to consider when estimating dermal uptake via soil is the soil-to-skin adherence rate. Values of 0.5 to 0.6mg/cm2 and 0.2 to 2.8 mg/cm2 have been reported for adults and children, respectively (59, 96, 259, 286). Recent works by Finley et al. (119), Kissel et al. (177), and Holmes et al. (156) have built on prior studies to show that dermal loading can vary significantly among different activities and different people. Based on data collected in past studies, in 1992 the U.S. EPA suggested a default soil-to-skin adherence rate of 0.2mg/cm2 (median) and 1.0mg/cm2 (95th percentile) for an adult. The recent edition of the *Exposure Factors Handbook* gives considerable attention to this topic (382, 384). One approach to improving dermal uptake calculations is to use area-weighted adherence factors as recently suggested by the U.S. EPA.

Table 9.5 Representative surface areas of the human body (Adult male)

Body portion	Area (cm2)
Whole body	18,000
Head and neck	1,620
Head	1,260
Back of head	320
Neck	360
Back of neck	90
Torso	6,480
Back	2,520
Chest	2,520
Sides	1,440
Upper limbs	3,240
Upper arms (elbow-shoulder)	1,440
Lower arms (elbow-wrist)	1,080
Hands	720
Palms	360
Upper arms (back of)	360
Lower arms (back of)	270
Lower limbs	6,480
Thighs	3,240
Lower legs (knee-ankle)	2,160
Feet	1,080
Soles of feet	540
Thighs (back of)	810
Lower legs (back of)	540
Perineum	180

Note. Data adapted from Reference 334.

Interpreting Wipe Samples

In some workplaces, wipe sampling has been conducted historically to assess the degree of surface contamination. Hospitals were among the first occupational settings, as long ago as 1940, to rely on this method to determine microbial levels in operating rooms. In pharmaceutical manufacturing, wipe sampling has been used as an indicator of hygienic conditions since the 1960s. The health physics profession has utilized wipe samples extensively as an indicator of the need for better housekeeping and decontamination; this group performed most of the early work in quantifying the relationship of wipe sample concentrations to dermal and oral uptake.

Over the years, few papers have discussed how to collect and interpret wipe samples (60, 109, 110, 114, 176, 210). When the primary effect of a chemical is skin discoloration, allergic contact dermatitis (ACD), or

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chloracne, wipe sampling was nearly always the preferred approach for assessing the acceptability of the workplace (rather than relying on air samples). Beginning in the 1980s, a substantial number of wipe samples were collected in office buildings contaminated with dioxins and furans after electrical transformer fires to estimate the potential human exposure (222). The interpretation of these data was often mishandled and, as a result, a number of decisions by risk managers were less than optimal, resulting in significant unnecessary expenses. Today, better approaches are available.

Although wipe sampling data have generally been used as an indicator of cleanliness (60), these data can also be used to estimate systemic uptake of a contaminant if the degree of skin contact with the contaminated surfaces is known. While historical wipe sampling methods were rather imprecise, they were useful for obtaining a rough estimate of the possible exposure, which could be refined later by other means, such as biological monitoring.

If one knows that wipe sampling results are representative of what comes into contact with the hands (i.e., actually able to be absorbed), then the procedures for converting wipe sample data to estimates of systemic uptake are straightforward (35). For example, if one knows the number of times a surface (e.g., valve handle, instrument controller, or drum) is touched, the surface area of the hand touching these items (usually the palm), and the percutaneous chemical absorption rate, then the uptake can be estimated using wipe sample information. The best wipe sampling data were those collected in a reliable and consistent manner, with a focus on the mass per unit area. Hand wash sampling is often more representative than wipe samples (115).

Until recently, no standardized approaches existed for conducting wipe sampling. Differences in the use of wetting agent (acetone, methylene chloride, water, saline, isopropanol, and ethanol) and sampling media (paper, cotton, and synthetic fibers) produced drastically different results. In some procedures, especially those that used methylene chloride (in which the paint was concurrently stripped by the solvent), the chemical in the paint matrix was assumed to be bioavailable (a completely unreasonable assumption). Clearly, much of the previous work, which measured the amount of chemical released following aggressive scrubbing of the contaminated surfaces with detergent or solvent, did not reflect a realistic exposure scenario. Thus, there has been a need for standard techniques that attempt to mimic the conditions in which a hand comes into contact with a contaminated surface (210). Some of the techniques have been developed by hygienists involved in agricultural exposure assessment (179, 187, 188, 277).

In an attempt to fill this need, fairly sophisticated work to standardize these procedures has been conducted by researchers at Rutgers University. In fact, some of their wipe sampling procedures and devices have been patented (197). They have also developed a dry contact sampling device (198) that offers promise for understanding the hazard from surface dusts. The implications from recent wipe sampling research are that: (1) a minimum number of samples is needed to have statistical confidence; (2) the pressure applied to the cloth during sample collection should be standardized; (3) neat solvent should not be used as a collection media; (4) the size of the sample area needs to be sufficient to collect enough contaminant for quantification; and (5) the technique should be validated by using glove analyses.

Estimating the Dermal Uptake of Chemicals in Soil

One of the most frequently occurring exposure scenarios involving environmental exposures is that of contaminated soil (259). Unfortunately, dermal uptake of chemicals found on soil has rarely been evaluated experimentally (192). A model to estimate the amount of an organic chemical in soil that crosses the stratum corneum into the underlying tissue layer has been developed (215). To differentiate this absorptive process from bioavailability, which also includes transport into blood, McKone refers to the percentage of available chemical as an uptake fraction. The approach is based on the fugacity concept, which measures the tendency of a chemical to move from one phase to another. Because the skin has a fat content of about 10% and soil has an organic carbon content on the order of 1 to 4%, an organic chemical in soil placed on the skin will move from the soil to the underlying adipose layers of the skin. However, this transfer depends on the period of time between deposition on the skin and removal by evaporative processes. The mass-transfer coefficients of the soil-to-skin layer and the soil-to-air layer define the rate at which these competing processes occur.

Results of this model suggest that the chemical uptake fraction in soil varies with the exposure duration, soil deposition rate, and physical properties of the chemical, and is particularly sensitive to the values of Kow, as well as the mass or depth of soil deposited on the skin. When the amount of soil on the skin is low (<1 mg/cm2), a high uptake fraction, approaching unity in some cases, is predicted. With higher soil loading (20 mg/cm2), an uptake of only 0.5% is predicted. Because of the diverse variations of the

uptake fraction with soil loading, results obtained from experiments with a single soil loading should be applied with caution to human soil-exposure scenarios.

The dermal uptake of chemicals in soil is a complex process, but its behavior is predictable if the controlling factors are accounted for and quantified (192, 215). In

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situations involving a relatively thin layer of a chemical on the skin for purposes of screening assessments, a few generalizations can be made. First, for chemicals with a high *K*ow and a low air:water partition coefficient, it is reasonable to assume 100% uptake in 12 h. Second, for chemicals with an air:water partition coefficient greater than 0.01, the uptake fraction is unlikely to exceed 40% in 12 h. Third, for chemicals with an air:water partition coefficient greater than 0.1, one can expect less than 3% uptake in 12 h. In most occupational settings, contaminated soil will rarely be in contact with the skin for greater than 4 h before it is washed off. Consequently, this should be accounted for when attempting to predict systemic uptake.

Dermal Uptake of Contaminants in Soil

To estimate chemical uptake, one needs to know the percutaneous absorption rate, the exposed skin area, the chemical concentration, and the exposure duration. One scenario would be a thin film of chemical on the skin. For this finite-dose scenario, Eq. (5) is useful:

$$\text{Uptake (mg)} = (C)(A)(x)(f)(t) \qquad (5)$$

where *C* is the concentration of the chemical (mg/cm2), *A* the skin surface area (cm2), *x* the thickness of the film layer (cm), *f* the absorption rate (percent per hour), and *f* the duration of exposure (h).

Another scenario would be an excess amount of a chemical on the skin (i.e., infinite dose). In this case, the thickness of the chemical layer is not calculated and steady-state kinetics are assumed. For a chemical in an aqueous or gaseous media:

$$\text{Uptake (mg)} = (C)(A)(Kp)(t)(d) \qquad (6)$$

where *K*p is the permeability coefficient (cm/h) and *d* is the distribution factor.

For a neat liquid chemical,

$$\text{Uptake (mg)} = (A)(J)(t) \qquad (7)$$

where *J* is the flux of chemical (mg/cm2-h).

The U.S. EPA has suggested using the following equation for estimating percutaneous absorption of chemicals in soil (376):

$$\text{Uptake (mg)} = (C)(A)(r)(B) \qquad (8)$$

where *C* is the concentration of the chemical in soil (mg/g), *A* the skin surface area (cm2), *r* the soil-to-skin adherence rate (g/cm2), and *B* the cutaneous bioavailability (unitless).

Example Calculation 3: Skin Uptake of a Chemical in Soil

A person gardens with soil contaminated on average with 250 ng dioxin/soil (250 ppb). Assuming that the person's hands and lower arms are in contact with the soil, the soil loading is equal to 0.2mg/cm2, and the cutaneous bioavailability of dioxin in soil is 1% (321), what is the plausible uptake of dioxin by this person [using Eq. (8)]? Assume that the person washes his or her hands every 4 h and the exposed area of skin is 1800 cm2.

$$\text{Uptake (ng)} = (C)(A)(r)(B)$$

where:

$$C = 250 \text{ ng/g}$$
$$A = 1800 \text{ cm2}$$
$$r = 0.2 \text{ mg/cm2}$$
$$B = 0.01$$

By substitution:

$$\text{Uptake} = \left(\frac{250\,\text{ng TCDD}}{1\,\text{g soil}}\right)\left(\frac{0.2\,\text{mg soil}}{\text{cm}^2\,\text{skin}}\right)\left(\frac{1\,\text{g}}{10^3\,\text{mg}}\right) \times (1800\,\text{cm}^2\,\text{skin})(0.01) = 0.9\,\text{ng TCDD}$$

Note: A preferred method for performing this calculation, if data are available, is to use a flux rate (ng/cm2-h) for the chemical. Assume that rate is 500 ng/cm2-h:

$$\text{Uptake (ng)} = (C)(J)(A)(t)$$

where

$$J = 500 \text{ ng/cm2-h}$$
$$t = 4\text{h}$$

By substitution:

$$\text{Update} = \left(\frac{250\,\text{ng TCDD}}{1\,\text{g soil}}\right)\left(\frac{1\,\text{g}}{10^9\,\text{ng}}\right)(1800\,\text{cm}^2\,\text{skin})(4\text{h}) \times \left(\frac{500\,\text{ng}}{\text{cm}^2 - \text{h}}\right) = 0.9\,\text{ng TCDD}$$

Uptake of Chemicals in an Aqueous Matrix

Published estimates of dermal uptake of chemicals in water have generally focused on evaluating workplace or environmental exposure. A number of different scenarios have been evaluated (50, 117a, 168, 173, 314). If interested in the possible uptake of a chemical present in water, the amount of chlordane absorbed through the skin by a man swimming for 4 h in water containing 1 ppb chloroform has been estimated (168). This is useful to compare various approaches. For example, the amount of chloroform absorbed by a boy swimming for 3 h in

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water has been calculated (277). Some have compared the amounts absorbed through the skin during a 10-min shower versus a 20-min bath with water containing 1 ppb 1,1,1-trichlorethane (171).

About 10 yr ago, it was recognized that in the indoor environment, oral exposure to volatile chemicals present in drinking water may not necessarily represent the vast majority of the risk. Specifically, it was found that inhalation exposure due to the release of vapors from liquids to which people were in close contact could be relatively high. For example, comparisons have been made of the chloroform concentration in exhaled breath after a shower to that after an inhalation-only exposure (168). The concentration after showering was about twice that after the inhalation-only exposure, indicating that the absorbed dose from the skin is approximately equivalent to that from inhalation absorption.

Example Calculation 4: Skin Uptake of a Chemical From Water

A person has filled his swimming pool with shallow well water contaminated with 0.002mg/ml (2 ppb) toluene. What is the plausible dermal uptake of toluene while swimming in the contaminated water for half an hour? Assume that 18,000cm2 of skin is exposed and the Kp is 1.01 cm/h. From Eq. (6):

$$\text{Uptake} = (C)(A)(Kp)(t)(d)$$

where

C= 0.002 mg/ml
A=18,000 cm2
Kp=1.01 cm/h
t=0.5h
d=distribution factor (1 ml of water covers 1 cm3)

By substitution:

$$\text{Uptake} = (0.002\ \text{mg/ml})(l8{,}000\ \text{cm2})(l.01\ \text{cm/h})(0.5\text{h}) \times (1\ \text{ml water}/1\ \text{cm3}) = 18\text{mg}$$

Percutaneous Absorption of Liquid Solvents

While the percutaneous absorption of chemical solutes generally proceeds by simple diffusion, the skin uptake of neat chemical liquids is not necessarily exclusively governed by Fick's law. Consequently, the uptake of neat liquid through the skin needs to be estimated using direct in vivo skin contact techniques. Table 9.6 presents the percutaneous absorption rates of some neat industrial liquid solvents that have been determined in human volunteer studies.

Table 9.6 Absorption rates of some neat industrial liquid chemicals in human skin in vivo

Chemical	Absorption rate (mg/cm2-h)
Aniline	0.2–0.7
Benzene	0.24–0.4
2-Butoxyethanol	0.05–0.68
2-(2-Butoxyethoxy)ethanol	0.035
Carbon disulfide	9.7
Dimethylformamide	9.4
Ethylbenzene	22–23
2-Ethoxyethanol	0.796
2-(2-Ethyoxyethoxy)ethanol	0.125
Methanol	11.5
2-Methoxyethanol	2.82
2-(2-Methoxyethoxy)ethanol	0.206
Methyl butyl ketone	0.25–0.48
Nitrobenzene	2
Styrene	9–15
Toluene	14–23
Xylene (mixed)	4.5–9.6
m-Xylene	0.12–0.15

Note. From Reference 192.

Example Calculation 5: Skin Uptake of a Neat Liquid Chemical

Due to carelessness or a leak, the inside of a glove becomes contaminated with 2-methoxyethanol. How much can be absorbed if a worker wears the contaminated glove on one hand for half an hour? Assume the surface area of exposed skin is 360 cm2 and the flux rate is 2.82 mg/cm2-h. From Eq. (7),

$$\text{Uptake} = (A)(J)(t)$$

where

A=360 cm2
J=2.82mg/cm2-h

$t=0.5\text{h}$

By substitution,

$$\text{Uptake}=(360\ \text{cm}^2)(2.82\ \text{mg/cm}^2\text{-h})(0.5\text{h})=508\ \text{mg}$$

To understand the relative hazard from skin exposure versus inhalation exposure, the dose of 2-methoxyethanol absorbed by the same worker via inhalation for 8 h (10 m^3

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Table 9.7 Percutaneous absorption rates for chemical vapors in vivo

Chemical	Skin update in combined exposure (%)[a]	Permeability coefficient *K*p (cm/h) Rat	Human
Styrene	9.4	1.75	0.35–1.42
m-Xylene	3.9	0.72	0.24–0.26
Toluene	3.7	0.72	0.18
Perchloroethylene	3.5	0.67	0.17
Benzene	0.8	0.15	0.08
Halothane	0.2	0.05	
Hexane	0.1	0.03	
Isoflurane	0.1	0.03	
Methylene chloride		0.28	
Dibromomethane		1.32	
Bromochloromethane		0.79	
Phenol			15.74–17.59
Nitrobenzene			11.1
1,1,1-Trichloroethane			0.01

Note. Rat data from Reference 214.

[a]In combined exposure, rats are simultaneously absorbing chemical vapors by inhalation and by whole-body absorption through the skin.

of air inhaled), assuming a threshold limit value (TLV) of 16mg/cm3, can be estimated and compared to the dose due to inhalation exposure. Assume an 80% inhalation uptake efficiency.

$$\text{Inhalation uptake} = (16\text{mg/m}^3)(10\text{m}^3)(0.8) = 128 \text{ mg}$$

Thus, the uptake of 2-methoxyethanol following 30 min of skin exposure of a single hand can be as much as 4 times that from inhalation for 8 h at the TLV concentration, a presumably safe level of exposure. From this example, it is clear that the cutaneous route of entry can, in some situations, significantly contribute to the total absorbed dose, especially in the occupational setting.

Percutaneous Absorption of Chemicals in the Vapor Phase

Until the 1990s, it was generally assumed that the plausible dose resulting from vapors absorbed through the skin was too low to pose a hazard. Only a few studies have examined this issue (173, 184, 391). A few clinical reports have encouraged some limited in vivo research to evaluate the absorption of several chemicals in the gaseous phase through the human skin (Table 9.7). A chamber system to measure the whole-body percutaneous absorption of chemical vapors in rats has been described by McDougal et al. (214), and this approach has produced some interesting results (208). In this system, chemical flux across the skin is determined from the chemical concentration in blood during exposure by using a PBPK model. In most cases, vapor absorption through the skin amounts to less than 10% of the total dose received from a combined skin and inhalation exposure. While there is good agreement between the rat and human in the relative ranking of the permeability coefficients among the chemicals studied, for an individual chemical the rat skin appears to be two to four times more permeable than the human skin. These observations are consistent with previously reported data (22, 34, 274, 275, 321).

It is generally not necessary to account for the contribution from percutaneous uptake of vapors when the occupational exposure limit (OEL) is used as a guideline for acceptable exposure, because uptake of vapors through the skin is usually inherent in the data; that is, the studies of animals or humans from which data were collected were usually exposed via inhalation (whole body) so dermal uptake of the vapor occurred. However, although good work practices and the law require that situations where persons are placed in atmospheres that are life-threatening, sometimes in emergency situations,

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airline (supplied air) respirators or self-contained breathing apparatus (SCBA) are worn in environments containing chemical concentrations 10-fold to 1000-fold greater than the TLV. In these cases, it is important to account for vapor uptake through either exposed or covered skin.

Although nearly all data on vapor absorption involve bare skin, the role of clothing in preventing skin uptake has occasionally been evaluated. For example, a study of workers wearing denim clothing indicated no decreased uptake of phenol vapors (274), but found a 20% and 40% reduction in uptake of nitrobenzene (273) and aniline vapors, respectively (100). Although standard clothing may slightly decrease the amount of a chemical transferred from air through the skin, it can be a significant source of continuous exposure if the clothing has been contaminated.

Example Calculation 6: Skin Uptake of a Chemical Vapor

Assume that a person needs to repair a leaking pump, so he enters a room wearing an airline respirator. Assume the room contains 500 mg/m3 nitrobenzene (100 times the current TLV) and it takes 30 min to repair the pump. How much nitrobenzene might be absorbed through the skin?

The head, neck, and upper limbs are assumed to be exposed (surface area=4860 cm2), and the rest of the body (surface area=13,140 cm2) is covered with clothing. Assume the percutaneous *K*p of nitrobenzene is 11.1cm/h, and that the clothing has reduced the skin uptake rate of vapors by about 20% (273). From Example 5.

$$\text{Uptake} = (C)(A)(K_p)(t)$$

$$\text{Uptake through exposed skin} = (500\,\text{mg/m}^3)(4860\,\text{cm}^2) \times (11.1\,\text{cm/h})(0.5\,\text{h}) \times (1\,\text{m}^3/10^6\,\text{cm}^3) = 13.5\,\text{mg}$$

$$\text{Uptake through clothing} = (500\,\text{mg/m}^3)(13{,}140\,\text{cm}^2) \times (11.1\,\text{cm/h})(0.8)(0.5\,\text{h}) \times (1\,\text{m}^3/10^6\,\text{cm}^3) = 29\,\text{mg}$$

$$\text{Total uptake} = 13.5 + 29 = 42.5\,\text{mg}$$

From this example, it is clear that if one enters an environment containing a high concentration of an airborne contaminant, even if a supplied-air respirator is worn, the degree of skin uptake of the vapor may be worthy of evaluation to ensure that the worker is protected. These kinds of calculations sometimes have to be conducted in difficult work environments that are in a state of alert (e.g., submarines, chemical plants during emergency situations, etc.).

ESTIMATING INTAKE VIA INGESTION

If the appropriate information is available, estimating the intake of various chemicals due to ingestion is a relatively straightforward exercise. In general, one is concerned with the ingestion of the following media: drinking water, other liquids, food, soil, and house dust. Drinking-water contamination may occur because of soil contamination from leaking underground storage tanks, landfills, or hazardous waste sites, as well as discharges from contaminated streams or water transport systems. Nearly all foods in Western society contain a number of intentional and unintentional chemicals, including pesticide residues, naturally occurring chemicals, and food additives that serve as preservatives or enhancers of taste or visual appeal. Soils are ingested as a result of eating incompletely washed vegetables, hand-to-mouth contact, and through direct ingestion by children. Soils are also ingested when particles too large to reach the lower respiratory tract are inhaled (and then are swallowed). House dust contaminated with a number of chemicals can be ingested due to contact with foods and hand-to-mouth activities (267).

Estimating Intake of Chemicals in Drinking Water

Estimating the magnitude of the potential dose of toxics from drinking water requires knowledge of the amount of water ingested, the chemical concentrations in the water, and the chemical bioavailability in the gastrointestinal tract. The amount of water ingested per day varies with each person and is usually related to the amount of physical activity. A good deal of literature has addressed the amount of water ingested by persons engaged in different kinds of activities (364, 366, 386).

Currently, the U.S. EPA suggests that when little is known about the specifics of exposure, a value of 2 L/d for adults and 1 L/d for infants (body weight of less than 10 kg) should be used as the default value. These rates include drinking water consumed in the form of juices and other beverages.

Numerous studies cited in the U.S. EPA *Exposure Factors Handbook* (383, 384) have generated data on drinking-water intake rates. In general, these sources support the U.S. EPA use of 2 L/d for adults and 1 L/d for children as upper percentile tap-water intake rates. Many of the studies have reported fluid intake rates for both total fluids and tap water. Total fluid intake is defined as consumption of all types of fluids including tap water, milk, soft drinks, alcoholic beverages, and water intrinsic to purchased foods. Total tap water is defined as water consumed directly from the tap as a bev-

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Table 9.8 Summary of tap-water intake by age

Age group	Intake (ml/d)		Intake (ml/kg-d)	
	Mean	10th–90th Percentiles	Mean	10th–90th Percentiles
Infants (<1 yr)	302	0–649	43.5	0–100
Children (1-10 yr)	736	286–1,294	35.5	12.5–64.4
Teens (11-19 yr)	965	353–1,701	18.2	6.5–32.3
Adults (20-64 yr)	1,366	559–2,268	19.9	8.0–33.7
Adults (65+yr)	1,459	751–2, 287	21.8	10.9–34.7
All ages	1,193	423–2,092	22.6	8.2–39.8

Note. From Reference 108.

erage or used to prepare foods and beverages (i.e., coffee, tea, frozen juices, soups, etc.). Data for both consumption categories are presented in numerous publications. Table 9.8 presents typical information reported from these studies (377).

All currently available studies on drinking water intake are based on short-term survey data. Although short-term data may be suitable for obtaining mean intake values that are representative of both shortand long-term consumption patterns, upper percentile values may be different for short-term and long-term data because there is generally more variability in short-term surveys. It should also be noted that most of the currently available drinking water surveys are based on recall. This may be a source of uncertainty in the estimated intake rates because of the subjective nature of this type of survey technique (377).

To estimate the intake of toxics via direct ingestion of drinking water, the calculation is straightforward:

$$\text{Intake}=(V)(C)(B)$$

where *V* is the volume of water (L/d), *C* the concentration of chemical in water (μg/L), and *B* the bioavailability (unitless).

One of the more interesting observations of the past 15 yr is that ingestion of contaminated drinking water is sometimes not the primary route of exposure to the toxicant in drinking water. Uptake of volatile chemicals via inhalation can be nearly as great in some homes as ingestion, which is the result of the presence of these chemicals in air due to showering, off-gases from the dishwasher, and other opportunities for volatilization of the chemical (168, 171, 173, 208, 391).

The Importance of Soil Ingestion When Estimating Human Exposure

Between 1980 and 1995, predicted risks associated with the ingestion of contaminated soil were the primary drivers for remediating many (if not most) hazardous waste sites. As discussed by Paustenbach et al. (259), there was no better example than the site in Times Beach, MO. Hundreds of millions of dollars can be needed to clean up these kinds of sites to levels that would not pose a significant risk if children actually ate significant quantities of contaminated soil. Because of the expense of remediation, a good deal of research has been conducted over the past 15 yr to attempt to quantitatively understand this route of exposure.

Clearly, the ingestion of soil and house dust is a potential source of human exposure to toxicants (296–298). The potential for contaminant exposure via this source is greater for children because they are more likely to ingest greater quantities of soil than adults. Inadvertent soil ingestion among children may occur through the mouthing of objects or hands. Mouthing behavior is considered to be a normal phase of childhood development. Adults may also ingest soil or dust particles that adhere to food, cigarettes, or their hands. Deliberate soil ingestion is defined as pica and is considered to be relatively uncommon. Because normal, inadvertent soil ingestion is more prevalent and data for individuals with pica behavior are limited, the focus of most exposure assessments is on normal levels of soil ingestion that occur as a result of mouthing or unintentional hand-to-mouth activity (73, 174, 259, 377).

Mouthing activities by children, which are generally accepted as normal and commonplace [e.g., Barltrop (23) estimated that almost 80% of all children at age

1 yr exhibited mouthing tendencies], are potential exposure routes to trace amounts of soil and/or dust adhering to fingers, hands, and objects placed in the mouth. The available data indicate that soil exposure occurs through several indirect routes:

1. Soil contributes to house dust (e.g., by local dust deposition, and mud and dirt carried in by shoes and pets, etc.).
2. House dust (fine particles) adheres to objects and to children's hands.
3. Children ingest dust particles when sucking and mouthing objects and fingers.

Obviously, in some situations, exposure may be direct (a child playing outdoors may eat dirt directly). In other situations, oral exposure to chemicals in soil may occur via contamination of domestic water supplies or contamination of fruit and vegetable produce grown onsite. However, the content and concentration of dusts in the indoor environment, which may represent the most important source of indirect exposure to soil, need to be better understood (107, 267).

Many studies have been conducted to estimate the amount of soil ingested by children. Most of the early studies attempted to quantify the amount of soil ingested by measuring the amount of dirt present on children's hands and making generalizations based on behavior. More recently, soil intake studies have been conducted using a methodology that measures trace elements in feces and soil that are believed to be poorly absorbed in the gut. These measurements are used to estimate the amount of soil ingested over a specified period of time.

Studies off Soil Ingestion

In light of the importance of soil ingestion for estimating human exposure to contaminated soil, several literature surveys have been undertaken to identify the typical amount of soil consumed by children and adults (107, 174, 259, 377, 382). Research evaluating lead uptake by children from ingestion of contaminated soil, paint chips, dust, and plaster provides the best source of information. Walter et al. (417) estimated that a normal child typically ingests very small quantities of dust or dirt between the ages of 0 to 2 yr, the largest quantities between 2 to 7 yr, and nearly insignificant amounts thereafter. In the classic text by Cooper (71), it was noted that the desire of children to eat dirt or place inedible objects in their mouths becomes established in the second year of life and disappears more or less spontaneously by the age of 4 to 5 yr. A study by Charney et al. (65) also indicated that mouthing tends to begin at about 18 mo and continues through 72 mo, depending on several factors such as nutritional and economic status, as well as race. Work by Sayre et al. (310) indicated that ages 2 to 6 yr are the important years, but that "intensive mouthing diminishes after 2 to 3 years of age."

An important distinction that is often blurred is the difference between the ingestion of very small quantities of dirt due to mouthing tendencies and the disease known as pica. Children who intentionally eat large quantities of dirt, plaster, or paint chips (1 to 10 g/d), and consequently are at greater risk of developing health problems, can be said to suffer from the disease known as pica. This disease is known as geophagia if the craving is for dirt alone. Geophagia, rather than pica, is generally of greatest concern in areas with contaminated soil.

Duggan and Williams (99) have summarized the literature on the amount of lead ingested through dust and dirt. In their opinion, a quantity of 50 mg lead was the best estimate for daily ingestion of dust by children. Lepow et al. (191) estimated an ingestion rate equal to 100 to 250 mg/d (specifically, 10 mg ingested 10 to 25 times a day). Barltrop (23) and Barltrop et al. (25) also estimated that the potential uptake of soils and dusts by a toddler is about 100 mg/d. In a Dutch study, the amount of lead on hands ranged from 4 to 12ng. By assuming maximum lead concentrations of 500 ng/g (concentrations were typically lower) and complete ingestion of the contents adsorbed to a child's hand on 10 separate occasions, the amount of ingested dirt would equal 240 mg. Thus, in order to eat 10,000 mg soil/d, the rate once suggested by the Centers for Disease Control (174), children would have to place their hands into their mouths 410 times a day, a rate that seems improbable (232, 252).

A report by the National Research Council (232) addressing the hazards of lead suggested a rate of 40 mg/d. Day et al. (88) measured the amount of dirt transferred from children's hands (age range from 1 to 3 yr) to a sticky sweet, and estimated a daily intake of 2 to 20 sweets would lead to dirt intake of 10 to 1000 mg/d. Bryce-Smith (36) estimated 33 mg/d. In its document addressing lead in air, the U.S. EPA assumed that children ate 50 mg/d of household dust, 40 mg/d of street dust, and 10 mg/d of dust derived from their parents' clothing (i.e., a total of 100 mg/d).

Kimbrough et al. (174) used a series of assumptions about soil exposure when estimating the possible risks of contaminated soil at Times Beach, MO, based upon unpublished observations about children's behavior and hand-mouth activity. A few years later, Kimbrough noted that their estimate of up to 10,000 mg/d was clearly an exaggeration and her personal estimate would be nearer 50 mg/d (107).

La Goy (186) based his soil ingestion estimates upon a review of the literature, in particular using empirical data

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derived by Binder et al. (28) and Van Wijnen et al. (397). Similarly, Paustenbach et al. (264) based their estimates upon a review of the literature, including the mass-balance quantitative study conducted by Calabrese et al. (55).

De Silva (90, 91) adopted a different approach that may overcome some of the uncertainties inherent in the assumptions of the above indirect studies. She applied a "slope factor" increase of 0.6mg/dl in children's blood lead levels for each 1000 ppm increase in soil lead [this factor was developed by Barltrop et al. (25) following his work on blood lead levels in children from villages on old mining sites]. De Silva then deduced that an increase of 0.6mg/dl in blood indicates an extra oral intake of 3.75 mg lead/d, based upon a U.S. EPA (367) calculation that an increase of 1.0 mg lead/d in children's diets produces an increase of 0.16 in the blood lead level. With a soil lead value of 1000 ppm, 3.75 mg soil would contain 3.75 mg lead, suggesting that 3.75 mg/d (say 4mg) of soil was ingested by the children. However, the slope factor used here may not be the most appropriate, since mining soil wastes typically have larger sized particles, which tends to decrease lead bioavailability compared with soil contaminated by lead smelter activity, and therefore reduces the slope factor.

A major step forward beyond estimating soil ingestion using indirect measurements has been the attempt to study tracer elements found in soil with elements measured in the urine and feces of children. Several studies have been conducted thus far that have used this approach (28, 53, 55–57, 90, 91, 336–339). One early tracer study evaluated the amount of soil eaten by 24 hospitalized and nursery-school children. They analyzed the amount of aluminum, titanium, and acid-soluble residue in the feces of children aged 2 to 4 yr. The data were normally distributed. They found an average of 105 mg/d of soil in the feces of nursery children, and 49 mg/d in hospitalized children. Even with the limited number of children in the study, the difference between the two groups was significant ($p<.01$). If the value for the hospitalized children is assumed to be the background level because these substances are taken in from nonsoil sources (e.g., diet and toothpastes), the estimated average amount of soil ingested by the nursery school children would be 56 mg/d. This value is in the lower range of estimates in the literature and supports the use of 100 mg/d as a conservative uptake of soil by toddlers (ages 2 to 4 yr).

There have been two major studies completed by Calabrese et al. (53, 55–57; 52, 58, 336, 341). In the first, they quantitatively evaluated 6 different tracer elements in the stools of 65 school children aged 2 to 4 yr. They attempted to evaluate children from diverse socioeconomic backgrounds. This study, conducted in Massachusetts, was more definitive than prior investigations because they analyzed the children's diets, assayed for the presence of tracers in the diapers, assayed house dust and surrounding soil, and corrected for the pharmacokinetics of the tracer materials.

In the second study, soil ingestion estimates were obtained from a stratified, simple random sample of 64 children aged 1–4 years residing on a superfund site in Montana. The study was conducted during the month of September for 7 consecutive days (58). Soil ingestion was estimated by each soil tracer via traditional methods as well as by an improved approach using five trace elements (A1, Si, Ti, Y, and Zr), called the Best Tracer Method (BTM), which substantially corrects for error due to misalignment of tracer input and output as well as error occurring from ingestion of tracers from nonfood, nonsoil sources, while being insensitive to the particle size of the soil/dust ingested. According to the BTM, the median soil ingestion was less than 1 mg/day while the upper 95% was 160mg/day. No significant age (1 year vs. 2, vs. 3) or sex-related differences in soil ingestion were observed. These estimates are lower than estimates observed in the first study, which was conducted in New England during September and October.

Based on the series of papers by the researchers at the University of Massachusetts (53, 55–57; 52, 336), a few generalizations can be made. These studies were difficult to conduct and interpret. Second, only children from a single climate were studied, and it can be expected that rates vary with the amount of time spent indoors and outdoors. Third, only a handful of children have been studied (less than 500), so it is not possible to characterize the percentage of children who might tend to ingest large quantities of soil or house dust. Fourth, the relevant amount of soil or house dust ingested indoors versus outdoors is not known yet. In most cases, the contaminant concentrations in dust can be quite different when found in a carpet versus the yard (267). Fifth, although there is some degree of uncertainty in the results of the various studies, it appears that a best estimate of soil intake for most children resides in the area of 10 to 25 mg/d. It appears that perhaps 1 to 5% of the children may ingest much larger amounts during certain days or weeks (e.g., 2000 mg/d), but these tendencies do not occur on a chronic basis.

It has been proposed that one can estimate uptake over the period of one year and they have proposed

lifetime values (337). Recent work by Calabrese and Stanek (52) suggests that prior work yielded reasonable results for purposes of risk assessment. Most of the values discussed here are presented in Table 9.9. As mentioned previously, another area of research impacting exposure assessments of contaminated soil, which has been and continues to be actively pursued, is the bioavailability of the contaminant in the soil matrix (159).

Table 9.9 Values for childhood and adult soil ingestion rates that have been used in health risk assessments conducted between 1984 and 2000

Author	Age	Soil and dust (mg/d)
Barltrop (24)	2–6 yr	100
Lepow et al. (190)	2–6 yr	100–250
Day et al. (88)	2–6 yr	10–1000
Kimbrough et al. (174) (CDC)	0–9 mo	0
	9–18 mo	1000
	1.5–3.5 yr	10,000
	3.5–5 yr	1000
	5+ yr	100
Hawley (149)	0–2 yr	Negligible
	2–6 yr	90
	6–18 yr	21
	18–70 yr	57
La Goy (186)	1–6 yr	500 (max.)
	1–6 yr	100 (ave.)
Calabrese et al. (55)	1–4 yr	27–85 (mean)
		9–16 (median)
Paustenbach (262)	2–4 yr	25–50
	Adults	2–5
De Silva (91)	Children	~4
U.S. EPA (386)	Children	200
Calabrese & Stanek (52)	Children	30–60 (best estimate)

What is the Significance of Pica?

There appears to be some confusion in the literature over what constitutes "pica." Pica can be defined as "the habitual ingestion of substances not normally regarded as edible," but some authors have included mouthing and sucking activities in their definitions (201). Others appear to assume that all children with pica necessarily must be habitual soil eaters. In fact, pica behavior may be generalized to the ingestion of many different (nonfood) substances, or may be specific to one substance such as paper, soap, or earth. It is likely that repetitive pica behavior specifically for dirt, or habitual "geophagia," rarely occurs in the general population in most industrialized countries (84, 350).

Pica should, therefore, be considered a "normal" temporary phenomenon in some children. In the general population, the prevalence of both mouthing and pica, and the range of articles ingested, has been shown to decrease with age (23). In the 1-yr-old age group, 78% of children mouthed objects and 35% ingested them; this behavior decreased at the age of 4 yr, when 33% were mouthing and only 6% had pica.

It is also relevant to note that in certain circumstances, pica for soil may be culturally determined (such as eating clay, high in silicon and aluminum, for its medicinal properties in the relief of stomach discomfort and diarrhea by some Aborigines; or the custom of eating earth during pregnancy in certain cultures) (107). For example, some women in the southern portions of the United States report a craving for and eat certain clays during pregnancy.

Pica may be associated with physical disorders, including iron deficiency. However, it has been debated whether pica represents a cause or an effect of these deficiencies. Pica can also be associated with mental illness. It has also been reported that 25% of institutionalized mentally handicapped adults indulged in pica of one kind or another (including bizarre objects ranging from rags and string to rocks, insects, and feces) (84).

Calabrese and Stanek (52) have indicated that in their studies, they have observed great variability in soil ingestion by children. They have noted, for example, that some children are highly variable in their soil ingestion activities, displaying little propensity for soil ingestion on one day while ingesting copious amounts the next day. While there has not been any concerted focus on the soil pica child, the available data indicate that some children ingest over 50 g of soil on particular days. They note that while it is true that some children will ingest large amounts of soil, it is far from certain whether soil pica is behavior that only a small subgroup displays over a limited number of years (e.g., one to six) or whether most children, on occasion, display this behavior or some combination of both behavioral patterns. Clearly, additional work is needed to understand this topic.

Soil Ingestion by Adults

For most persons beyond the ages of 5 to 6 yr, the daily uptake of dirt due to intentional ingestion is generally thought to be quite low. With the exception of some lower income persons who eat clays due to tradition or mineral deficiency, adults will not usually intentionally ingest dirt or soil. However, there are two other important ways in which adults eat dirt—incidental hand-to-mouth contact and through dust on vegetables. It has been shown that most soil ingested from crops comes from leafy vegetables. Interestingly, investigations at nuclear weapons trials have shown that particles exceeding 45 μm are seldom retained on leaves. Further, superficial contamination by smaller particles is readily lost from leaves, usually by mechanical processes or rain, and certainly by washing (308). As a result, unless the soil contaminant is absorbed into the plant, superficial contamination of plants by dirt will rarely present a health hazard (207, 259).

The estimated deposition rate of dust from ambient air in rural environments is about 0.012 mg/cm2-d, assuming that rural dust contains about 300 mg/g of lead (the substance for which these data were obtained). The U.S. EPA has estimated that even at very high air concentrations (0.45 mg/m3 total dust), it is unlikely that surface deposition alone can account for more than 0.6 to 1.5mg lead/g dust (2 to 5 μg/g lettuce) on the surface of lettuce during a 21-d growing period (252). These data suggest that daily ingestion of dirt and dust by adults due to eating vegetables is unlikely to exceed about 0 to 5 mg/d even if all of the 137 g of leafy and root vegetables, sweet corn, and potatoes consumed by adult males each day were replaced by family garden products.

In its document on lead, the U.S. EPA used worst-case assumptions to estimate that persons could ingest up to 100 mg of lead each day due to unwashed vegetables. The actual uptake by adults from vegetables should actually be much less, and is probably negligible, because the U.S. EPA estimate assumed that all of the suspended dust is contaminated; persons do not wash the vegetables; garden vegetables are eaten throughout the year, rather than only during the growing season; and persons actually replace most vegetables with their own garden products.

With respect to the second route—incidental ingestion—only a very limited amount of work has been conducted which addresses dust ingestion via this route. It has been suggested that the primary route of uptake will be through accidental ingestion of dirt on the hands, which may be of special concern to smokers, who tend to have more frequent hand-to-mouth contact. It is true that before the importance of this route of entry was recognized, persons who worked in lead factories between 1890 and 1920 probably received a large portion of their body burden of lead due to poor hygiene and ingestion of dust; however, such conditions are now rare in the United States and most developed countries.

Some persons have evaluated the exposure experience of agricultural workers who apply or work with pesticide dusts. Due to the frequency and degree of pesticide exposure during its manufacture or application, these data do not appear to be appropriate surrogates for estimating soil uptake from the hands of persons who live on or near sites having contaminated soil. In addition, most of the published studies on pesticides involve liquids such as the organophosphates, rather than "soil-like" particles. Exposure studies of persons who apply granular pesticides might be more useful for defining upper bound estimates of dermal exposure than estimates based on dusty workplaces (181).

At least one study has been conducted to specifically address soil uptake by adults involved in remediating waste sites (52, 284, 339: also see ref. 431). The results suggest that the amount of soil eaten by these workers is much less than the default value of 100 mg/d suggested by the U.S. EPA in a number of guidance documents and risk assessments. Based on all the available data, it appears that a value of 5–25 mg/day for soil ingestion by most adults is a reasonable one.

Estimating the Intake of Chemicals Via Food

Without question, the information necessary to accurately estimate the ingestion of xenobiotics via foods is one the most complex of all exposure calculations. The hundreds of different possible foods and dozens of different chemicals that can be present as a pesticide residue, coupled with the background concentrations of various chemicals in soil, make this a formidable task.

The methodology for estimating uptake via ingestion must account for the quantity of food ingested each day, the concentration of contaminant in the ingested material, and the bioavailability of the contaminant in the media. Over the past 20 yr, a significant amount of work has been directed at understanding these exposure factors. Specifically, an entire volume of the U.S. EPA *Exposure Factors Handbook* (Volume II) is devoted to this topic (386).

The approach to estimating uptake via foods was first applied in the late 1940s by the Food and Drug Administration (361) and had not changed appreciably through 2000. However, because of the passage of the Food Quality Protection Act of 1996 (FQPA), the methodology for estimating uptake of chemicals from foods will be changing dramatically over the next 5–10 yr. Specifically, the FQPA requires that all pesticide residues from foods be added together, in a prescribed manner based on target organ, with the goal of understanding the total daily dose of all residual pesticides in the diet. Then, if necessary, the pesticide manufacturers are expected to calculate the necessary residue level that their chemical may have in a particular food so that the total dose does not exceed a fraction of the acceptable daily intake (ADI). Since there are hundreds of foods and dozens of residues, this presents a formidable challenge.

Ingestion of contaminated fruits and vegetables is a potential pathway of human exposure to chemicals. Fruits and vegetables may become contaminated by several different pathways. Ambient air pollutants may be deposited on or absorbed by plants, or dissolved in rainfall or irrigation waters that contact the plants. Plant roots may also absorb pollutants from contaminated soil and groundwater. The addition of

pesticides, soil additives, and fertilizers may also result in food contamination (386). Formulas are available to predict the concentration of chemicals from the soil, which have deposited from the air, and remain after treatment with a pesticide.

The primary information source on consumption rates of fruits and vegetables among the U.S. population is the U.S. Department of Agriculture (USDA) Nationwide Food Consumption Survey (NFCS) and the USDA Continuing Survey of Food Intakes by Individuals (CSFII). Data from the NFCS have been used in various studies to generate consumer-only and per-capita intake rates for individual fruits and vegetables, as well as total fruits and total vegetables. CSFII data from the 1989–1991 survey have been analyzed by the U.S. EPA to generate per-capita intake rates for various food items and food groups (362, 365, 386).

Consumer-only intake is defined as the quantity of fruits and vegetables consumed by individuals who ate these food items during the survey period. Per-capita intake rates are generated by averaging consumer-only intakes over the entire population of users and nonusers. In general, per-capita intake rates are appropriate for use in exposure assessment for which average dose estimates for the general population are of interest, because they represent both individuals who ate the foods during the survey period and individuals who may eat the food items at some time but did not consume them during the survey period. Total fruit intake refers to the sum of all fruits consumed in a day, including canned, dried, frozen, and fresh fruits. Likewise, total vegetable intake refers to the sum of all vegetables consumed in a day, including canned, dried, frozen, and fresh vegetables.

Intake rates may be presented on either an as-consumed or a dry-weight basis. As-consumed intake rates (g/d) are based on the weight of food in the form in which it is consumed. In contrast, dry-weight intake rates are based on the weight of food consumed after the moisture content has been removed. In calculating exposures based on ingestion, the unit of weight used to measure the contaminant concentration in the produce will vary. Intake data from the individual NFCS and CSFII components are based on "as eaten" (i.e., cooked or prepared) forms of the food items or groups. Thus, no corrections are required to account for changes in portion sizes from cooking losses (249, 363).

Estimating source-specific exposures to chemicals in fruits and vegetables may also require information on the amount of fruits and vegetables exposed to or protected from contamination as a result of cultivation practices, the physical nature of the food product itself (i.e., those having protective coverings that are removed before eating would be considered protected), or the amount grown beneath the soil (i.e., most root crops such as potatoes). The percentages of foods grown above and below ground will be useful when the contaminant concentrations in foods are estimated from concentrations in soil, water, and air. For example, vegetables grown below ground would more likely be contaminated by soil pollutants, but leafy above-ground vegetables would more likely be contaminated by deposition of air pollutants on plant surfaces. Some examples of various exposure factors and confidence ratings for liquids and food are presented in Table 9.10 (386).

Individual average daily intake rates calculated from NFCS and CSFII data are based on averages of reported individual intakes over 1 d or 3 consecutive days. Such short-term data are marginally suitable for estimating mean average daily intake rates representative of both short-term and long-term consumption. However, the distribution of average daily intake rates generated using short-term data (e.g., 3 day) does not necessarily reflect the long-term distribution of average daily intake rates. The distributions generated from short-term and long-term data will differ to the extent that each individual's intake varies from day to day; the distributions will be similar to the extent that individuals' intakes are constant from day to day (386).

Day-to-day intake variation among individuals will be greatest for food items or groups that are highly seasonal, and for items or groups that are eaten year-round but are not typically eaten every day. For these foods, the intake distribution generated from short-term data will not reflect long-term distribution. On the other hand, for broad categories of foods (e.g., vegetables), which are eaten on a daily basis throughout the year with minimal seasonality, the short-term distribution may be a reasonable approximation of the true long-term distribution, although it will show somewhat more variability.

Other relevant fruits and vegetables intake studies include the U.S. EPA Dietary Risk Evaluation System (DRES), Office of Pesticide Programs (OPP). The OPP uses the DRES (formerly the Tolerance Assessment System) to assess the dietary risk of pesticide use as part of the pesticide registration process (249, 363). The OPP sets tolerances for specific pesticides on raw agricultural commodities based on estimates of dietary risk. These estimates are calculated using pesticide residue data for the food item of concern and relevant consumption data. Intake rates are based primarily on the USDA 1977–1978 NFCS, although intake rates for some food items are based on estimations from production volumes or other data (i.e., some items were assigned an arbitrary value of 0.000001 g/kg-d) (386). The OPP has calculated per-capita intake rates of individual fruits and vegetables for 22 subgroups of the population (age, regional, and seasonal) by determining the composition of NFCS food items and disaggregating

complex food dishes into their component raw agricultural commodities (RACs) (386, 422). The advantage of using these data is that complex food dishes have been disaggregated to provide intake rates for a very large number of fruits and vegetables. These data are also based on the individual body weights of the respondents. Therefore, using these data to calculate

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Table 9.10 Summary of default exposure factor recommendations and confidence ratings for citizens of United States

Exposure factor	Recommendation	Confidence rating
Drinking-water intake	21 ml/kg-d or 1.4 L/d (average)	Medium
rate	34 ml/kg-d or 2.3 L/d (90th percentile) Percentiles and distribution also included	Medium
	Means and percentiles also included for pregnant and lactating women	
Total fruit intake rate	3.4 g/kg-d (per capita average)	Medium
	12.4 g/kg-d (per capita 95th percentile) Percentiles also included	Low
	Means presented for individual fruits	
Total vegetable intake	4.3 g/kg-d (per capita average)	Medium
rate	10 g/kg-d (per capita 95th percentile) Percentiles also included	Low
	Means presented for individual vegetables	
Total meat intake rate	2.1 g/kg-d (per capita average)	Medium
	5.1 g/kg-d (per capita 95th percentile) Percentiles also included	Low
	Percentiles also presented for individual meats	
Total dairy intake rate	8.0 kg-d (per capita average)	Medium
	29.7 g/kg-d (per capita 95th percentile) Percentiles also included	Low
	Means presented for individual dairy products	
Grain intake	4.1 g/kg-d (per capita average)	High
	10.8 g/kg-d (per capita 95th percentile) Percentiles also included	Low in long-term upper percentiles
Breast-milk intake rate	742 ml/d (average)	Medium
	1,033 ml/d (upper percentile)	Medium
Fish intake rate	General population	
	20.1 g/d (total fish) average	High
	14.1 g/d (marine) average	High
	6.0 g/d (freshwater/estuarine) average	High
	63 g/d (total fish) 95th percentile long-term Percentiles also included	Medium
	Serving size	
	129 g (average)	High
	326 g (95th percentile)	High
	Recreational marine anglers	
	27 g/d (finfish only)	Medium
	Recreational freshwater	
	8 g/d (average)	Medium
	25 g/d (95th percentile)	Medium
	Native American subsistence population	
	70 g/d (average)	Medium
	170 g/d (95th percentile)	Low

Note. From Reference 385.

chemical exposure may provide more representative estimates of potential dose per unit body weight. However, because the data are based on the NFCS short-term dietary recall, the same limitations discussed previously for other NFCS data sets also apply here. In addition, consumption patterns may have changed since the data were collected in 1977–1978. The OPP is in the process of translating consumption information from the USDA CSFII 1989–1991 survey to be used in DRES (386).

The USDA has also conducted a study entitled *Food and Nutrient Intakes of Individuals in One Day in the U.S.* (362, 386). The USDA calculated mean intake rates for total fruits and total vegetables using NFCS data from 1977–1978 and 1987–1988, and CSFII data from 1994–1995 (362, 386). Mean per-capita total intake rates are based on intake data for 1 d from the 1977–1978 and 1987–1988 USDA and NFCS, respectively. Data from both surveys are presented in the *Exposure Factors Handbook* to demonstrate that although the 1987–1988 survey had fewer respondents, the mean per-capita intake rates for all individuals agree with the earlier survey. Also, slightly different age classifications were used in the two surveys, providing a wider range of age categories from which exposure assessors may select appropriate intake rates. The age groups used in this data set are the same as those used in the 1987–1988 NFCS. Information for per-capita intake rates and consumer-only intake rates for various ages of individuals is also available. Intake rates for consumers-only were calculated by dividing the per-capita consumption rate by the fraction of the population using vegetables or fruits in a day (386).

The advantages of using these data are that they provide intake estimates for all fruits, all vegetables, or all fats combined. Again, these estimates are based on 1-d dietary data which may not reflect usual consumption patterns (386).

Intake of Fish and Shellfish

Contaminated finfish and shellfish are potential sources of human exposure to persistent chemicals and metals. Pollutants are carried in surface waters, but also may be stored and accumulated in sediments as a result of complex physical and chemical processes. Consequently, various aquatic species can be exposed to pollutants and may become sources of contaminated food (386).

Accurately estimating exposure to various chemicals in a population that consumes fish from a polluted water body requires an estimation of caught-fish intake rates by fishermen and their families. Commercially caught fish are marketed widely, making the prediction of an individual's consumption from a particular commercial source difficult. Because the catch of recreational and subsistence fishermen is generally not diluted in this way, these individuals and their families represent the population that is most vulnerable to exposure by intake of contaminated fish from a specific location (386).

Over the years, fish consumption survey data have been collected using a number of different approaches, which need to be considered when interpreting the survey results. Generally, surveys are either "creel" studies in which fishermen are interviewed while fishing, or broader population surveys (using mailed questionnaires or phone interviews). Both data types can be useful for exposure assessment purposes, but somewhat different applications and interpretations are needed. In fact, creel study results have often been misinterpreted because of inadequate knowledge of survey principles (280, 285, 386).

The typical survey seeks to draw inferences about a larger population from a smaller sample of that population. The larger population from which the survey sample is taken and to which the survey results are generalized describes the target population of the survey. In order to generalize from the sample to the target population, the probability of being sampled must be known for each member of the target population. This probability is reflected in weights assigned to each survey respondent, with weights being inversely proportional to sampling probability. When all members of the target population have the same probability of being sampled, all weights can be set to 1 and essentially ignored (102, 306).

In a mail or phone study of licensed anglers, the target population generally involves all licensed anglers in a particular area, and in these studies, the sampling probability is essentially equal for all target population members. In a creel study, the target population is anyone who fishes at the locations being studied; generally in a creel study, the probability of being sampled is not the same for all members of the target population. For instance, if the survey is conducted for 1 d at a site, then it will include all persons who fish there daily, but only about 1/7 of the people who fish there weekly, 1/30 of the people who fish there monthly, etc. In this example, the probability of being sampled (or inverse weight) is seen to be proportional to the frequency of fishing. However, if the survey involves interviewers who revisit the same site on multiple days, and persons who are only interviewed once for the survey, then the probability of being in the survey is not proportional to frequency; in fact, it increases less proportionally with greater frequency of fishing. If the same site is surveyed every day of the survey period with no

reinterviewing, all members of the target population would have the same probability of being sampled, regardless of fishing frequency, implying that the survey weights should all equal 1 (102, 302).
On the other hand, if the survey protocol calls for individuals to be interviewed each time an interviewer

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encounters them (i.e., without regard to whether they were previously interviewed), then the inverse weights will again be proportional to fishing frequency, no matter how many times interviewers revisit the same site. Note that when individuals can be interviewed multiple times, the results of each interview are included as separate records in the database, and the survey weights should be inversely proportional to the expected number of times that an individual's interviews are included in the database (102, 302, 386).

Fish and shellfish exposure assessments are among the most complicated of all assessments (227). A significant portion of the *Exposure Factors Handbook* addresses this topic (386). Recently, fairly complex Monte Carlo methods have been applied to resolve many of the difficulties estimating exposure of anglers and their families (428).

Aggregate Exposure and FQPA

Pesticides are regulated under both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Federal Food, Drug, and Cosmetics Act (FFDCA). In 1996, Congress passed the Food Quality Protection Act (FQPA) that amended both FIFRA and FFDCA. These laws mandated the U.S. EPA to register pesticides and set tolerances based on a safety determination, a reasonable certainty that use of a given pesticide or consumption of raw agricultural commodity of processed foods that contain the pesticide and its residues will cause no harm to human health or the environment. The U.S. EPA evaluates risks posed by the use and usage of each pesticide to make a determination of safety. Based upon this determination, the agency regulates pesticides to ensure that use of the chemical is not unsafe.

In the past, the U.S. EPA evaluated the safety of pesticides based on a single-chemical, single-exposurepathway scenario. However, FQPA requires that the agency consider aggregate exposure in its decisionmaking process. Section 408(a)(4)(b)(2)(ii) of FFDCA specifies with respect to a tolerance that there must be a determination "that there is a reasonable certainty that no harm will result from aggregate exposure to the pesticide chemical residue, including all anticipated dietary exposures and all other exposures for which there is reliable information." Section (b)(2)(C)(ii)(I) states that "there is a reasonable certainty that no harm will result to infants and children from aggregate exposure to the pesticide chemical residues" *Aggregate dose* is defined as the amount of a single substance available for interaction with metabolic processes or biologically significant receptors from multiple routes of exposure. *Aggregate risk* is defined as the likelihood of the occurrence of an adverse health effect resulting from all routes of exposure to a single substance. Conversely, *cumulative risk* is defined as the likelihood of the occurrence of an adverse health effect resulting from all routes of exposure to a group of substances sharing a common mechanism of toxicity.

As shown in Figure 9.5, the most basic concept underlying all aggregate exposure assessments is that exposure occurs to an individual. The integrity of the data concerning this exposed individual must be maintained throughout the aggregate exposure assessment. In other words, each of the individual "subassessments" must be linked back to the same person (394). Because exposures are based on that received by a single individual, aggregate exposure assessments must agree in time, place, and demographic characteristics. Each of these parameters have imbedded attributes that must be matched to create a reasonable assessment. Some of these imbedded attributes include:

• Time (duration, daily, seasonally).
• Place (location and type of home, urbanization, watersheds, region).
• Demographics (age, gender, reproductive status, ethnicity, personal preference).

To develop realistic aggregate exposure and risk assessments requires that the appropriate temporal, spatial, and demographic exposure factors be correctly assigned. Examples of some of these factors include sexand age-specific body weights, regional specific drinking-water concentrations of the pesticide being considered, seasonally based pesticide residues in food, and frequency of residential pest control representative of housing type. Once an aggregate exposure and risk assessment is completed for one individual, population and subpopulation distributions of exposures and risk may be constructed by probabilistic techniques (161).

An aggregate exposure and risk assessment is distinct from a cumulative risk assessment. Cumulative risk is defined as "the measure or estimate of distributions of exposures (doses) for a set of chemicals that act by a common mechanism of toxicity" (390). Cumulative risk assessment evaluates risks from multiple chemicals via all routes and pathways of exposure. The cumulative risk assessment considers the combined toxicological effect of a group of chemicals with a common mechanism of toxicity. The definition of a common mechanism of toxicity is "two or more pesticide chemicals that produce an adverse effect(s) to human health by the same, or essentially the same, sequence of major biochemical

events. The underlying basis of the toxicity is the same, or essentially the same, for each chemical" (390). Specific guidance concerning conducting a cumulative risk assessment is currently being developed (161).

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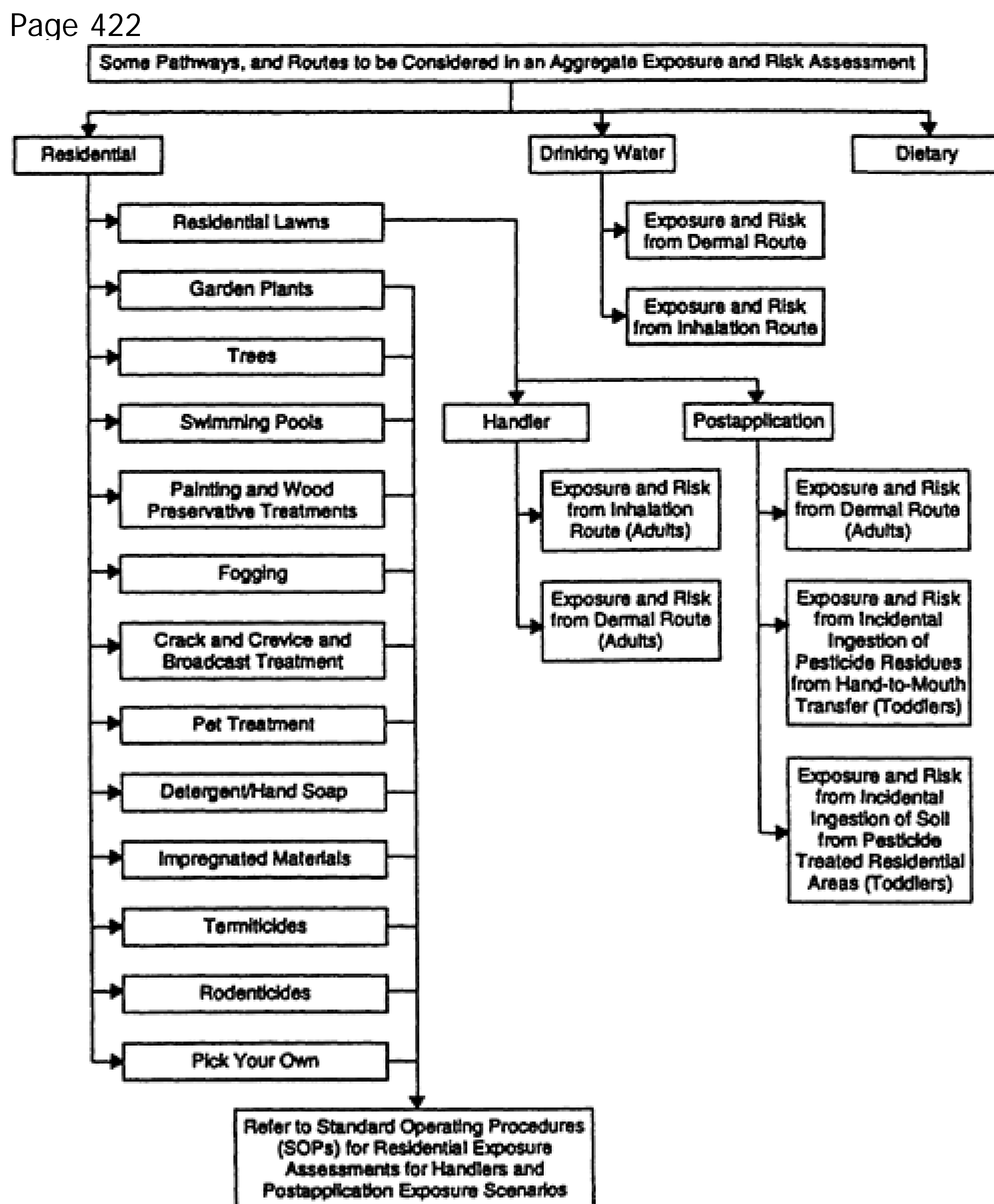

FIG. 9.5. Factors to consider in an aggregate exposure assessment of a pesticide. From Reference 394.

Breast Milk

Breast milk is a potential source of exposure to toxic substances for nursing infants. Lipid-soluble chemical compounds accumulate in body fat and may be transferred to breastfed infants in the lipid portion of breast milk. Because nursing infants obtain most (if not all) of their dietary intake from breast milk, they are especially vulnerable to exposures to these compounds. In fact, some models predict that the peak body burdens of certain chemicals (like dioxin) can reach their lifetime peak (μg/kg) on the last day of nursing at age 12–24 mo. Estimating the magnitude of the potential dose to infants from breast milk and the resulting body burdens of blood levels is quite complicated. It requires information on the quantity of breast milk consumed per day and the duration (months) over which breastfeeding occurs. Information on the fat content of breast milk is also needed for estimating dose from breast-milk residue concentrations that have been indexed to lipid con

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Table 9.11 Default values for daily intakes of breast milk

Age	Number of infants surveyed at each time period	Mean intake (ml/d)	Range of daily intake (ml/d)
Completely breast-fed			
1 mo		11600 ±159	426–989
3 mo		2833	645–1000
6 mo		1682	616–786
Partially breast-fed			
1 mo		4485 ±79	398–655
3 mo		11467 ±100	242–698
6 mo		6395 ±175	147–684
9 mo		3<554	451–732

Note. From Reference 249. Data expressed as mean ± standard deviation.

tent (331, 386). Until recently, these were considered the key parameters but it is now clear that one must account for the quickly increasing body weight of the infant, the differences in the blood perfusion of the fat compartments in the infant versus the mother, and other factors (172, 185).

Several studies have generated data on breast-milk intake (182, 230, 241, 332). Typically, breast-milk intake has been measured over a 24-h period by weighing the infant before and after each feeding without changing its clothing (test weighing). The sum of the difference between the measured weights over the 24-h period is assumed to be equivalent to the amount of breast milk consumed daily. Intakes measured using this procedure are often corrected for evaporative water losses (insensible water losses) between infant weighings (230). Neville et al. (241) evaluated the validity of the test-weight approach among bottle-fed infants by comparing the weight of milk taken from bottles with the difference between the before and after feeding weights of infants. Once corrected for insensible water loss, test-weight data were not significantly different from bottle weights. Conversions between weight and volume of breast milk consumed are made using the density of human milk (approximately 1.03 g/ml) (230). Recently, techniques for measuring breast-milk intake using stable isotopes have been developed; however, few data based on this new technique have been published (230).

Studies among nursing mothers in industrialized countries have shown that infant intake averages approximately 750 to 800 g/d (728 to 777 ml/d) during the first 4 to 5 mo of life, with a range of 450 to 1200 g/d (437 to 1165 ml/d) (230, 386). Similar intakes have also been reported for developing countries (230, 386). Infant birth weight and nursing frequency have been shown to influence the rate of intake (230, 386). Infants who are larger at birth and/or nurse more frequently have been shown to have higher intake rates. Also, breast-milk production among nursing mothers has been reported to be somewhat higher than the amount actually consumed by the infant (102, 386).

Like exposure assessments of fishes, techniques for estimating chemical uptake by children of breast milk continue to evolve. One of the more interesting papers on this topic was recently presented by Kerger et al. (172), which put into question most of the prior calculation approaches for estimating dose to nursing infants based, in part, on work conducted by Kreuzer et al. (185). A portion of the U.S. EPA *Exposure Factors Handbook* addresses this topic, and a few published papers have offered some novel approaches (331, 386, 387). Some examples of breast-milk intake rates are presented in Table 9.11. A distribution for breast-milk consumption has been suggested by Kerger et al. (1999).

ESTIMATING UPTAKE VIA INHALATION

Estimating intake via inhalation depends on only a few exposure factors, such as inhalation rate; airborne chemical concentration; bioavailability; and, if it is a particle, particle size. In general, uncertainty in estimates of intake via inhalation is among the smallest for all exposure calculations.

Inhalation rates are known to vary directly with the amount of physical activity of the persons being evaluated. The default value used by the U.S. EPA and others is 20 m3/d. When conducting occupational exposure assessments, it is common to assume that workers inhale about 10 m3 in an 8-h workday (377).

Airborne chemical concentrations are obtained through either direct measurement or modeling. The form of the chemicals in the air will be a gas (includes vapors) or particles (dusts or fumes). Generally, it is assumed that virtually all of the vapors or gases will be absorbed if inhaled (9, 184, 377). This may not be the case for volatile chemicals, if the concentration in the blood is approaching steady state. In those cases, a significant fraction of the inhaled vapors will be present in the exhaled breath and, therefore, not absorbed (275).

It is usually assumed that if particles enter the lower respiratory tract, they will eventually be absorbed unless the chemical is highly insoluble. Generally, it is assumed that particles less than 150 *μ*m in diameter are inhalable, but virtually all particles greater than 10 μm will be captured in the upper respiratory tract (nose and throat) and then ingested. It has often been assumed that particles less than 10 μm in diameter will be captured in the lower respiratory tract and the majority of these (by mass rather than particle number) will eventually be absorbed. Notably, for some chemicals, the adverse effect is related to the particle size, so this must be taken into account. For example, it is thought that beryllium particles should be collected in several different size fractions and that the severity of the adverse effect varies according to particle size.

Various Inhalation Rates

A significant amount of research has been done to correlate various inhalation rates with different tasks and body weights. Most studies on this subject have been summarized in the most recent U.S. EPA *Exposure Factors Handbook* (382). Data are available for dozens of different levels of physical activity and the distributions for several populations are presented.

A number of equations have been proposed for predicting the inhalation rate based on body weight (386). The *Exposure Factors Handbook* and other sources provide a number of tables that relate physical activity with inhalation rate (see Table 9.12).

Bioavailability of Airborne Chemicals

Because the mass of particles inhaled is usually quite small, and because most particles less than 10 μm in diameter are thought to be fairly easily absorbed, it is generally assumed that particles are 100% bioavailable after they

Table 9.12 Daily inhalation rates estimated from daily activities

	Inhalation rate (IR)		Daily inhalation rate (DIR)a (m3/d)
Subject	Resting (m3/h)	Light activity (m3/h)	
Adult man	0.45	1.2	22.8
Adult woman	0.36	1.14	21.1
Child (10 yr)	0.29	0.78	14.8
Infant (1 yr)	0.09	0.25	3.76
Newborn	0.03	0.09	0.78

Note. From Reference 334. Assumptions made were based on 8 h resting and 16 h light activity for adults and children (10 yr); 14 h resting and 10 h light activity for infants (1 yr); 23 h resting and 1 h light activity for newborns.

$$^{a}\mathrm{DIR} = \frac{1}{T}\sum_{i=1}^{k} \mathrm{IR}_i t_i$$

where IRi is corresponding inhalation rate at ith activity, ti, is hours spent during the ith activity, *k* is number of activity periods, and *T* is total time of the exposure period (i.e., a day).

are trapped in the lower lung. Likewise, it is generally assumed that most vapors and gases are completely absorbed (100% bioavailable) if they reach the lower respiratory tract. Both are conservative assumptions that should be reassessed on a case-by-case basis.

ROLE OF UNCERTAINTY ANALYSIS

Exposure assessment uses a wide array of information sources and techniques. Even when actual exposurerelated measurements exist, assumptions or inferences will still be required. Most likely, data will not be available for all aspects of the exposure assessment and these data may be of questionable or unknown quality. In these situations, the exposure assessor will have to rely on a combination of professional judgment, inferences based on analogy with similar chemicals and conditions, estimation techniques, and the like. The net result is that the exposure assessment will be based on a number

assumptions with varying degrees of uncertainty (377).

The decision analysis literature has focused on the importance of explicitly incorporating and quantifying scientific uncertainty in risk assessments (227, 302). Reasons for addressing uncertainties in exposure assessments include (377):

- Uncertainty information from different sources and of different quality must be combined.
- A decision must be made about whether and how to expend resources to acquire additional information (e.g., production, use, and emissions data; environmental fate information; monitoring data; population data) to reduce the uncertainty.

• So much empirical evidence exists that biases may occur, resulting in so-called best estimates that are not very accurate. Even when all that is needed is a best-estimate answer, the quality of the answer may be improved by incorporating a frank discussion of uncertainty into the analysis.
• Exposure assessment is an iterative process. The search for an adequate and robust methodology to handle the problem at hand may proceed more effectively, and to a more certain conclusion, if the associated uncertainty is explicitly included and it can be used as a guide in the process of refinement.
• A decision is rarely made on the basis of a single piece of analysis. Further, it is rare for there to be one discrete decision; a process of multiple decisions spread over time is the more common occurrence. Chemicals of concern may go through several levels of risk assessment before a final decision is made. During this process, decisions may be made based on exposure considerations. An exposure analysis that attempts to characterize the associated uncertainty allows the user or decision maker to conduct a better evaluation in the context of the other factors being considered.
• Exposure assessors have a responsibility to present not just numbers, but also a clear and explicit explanation of the implications and limitations of their analyses. Uncertainty characterization helps to achieve this.

Essentially, constructing scientifically sound exposure assessments and analyzing uncertainty go hand-in-hand. The reward for analyzing uncertainties is knowing that the results have integrity or that significant gaps exist in available information that can make decision making a tenuous process.

Variability Versus Uncertainty

While some authors treat variability as a specific component of uncertainty, the U.S. EPA (62) and others advise risk assessors (and, by analogy, the exposure assessor) to distinguish between variability and uncertainty (236). Specifically, uncertainty represents a lack of knowledge about factors affecting exposure or risk, whereas variability arises from true heterogeneity across people, places, or time. In other words, uncertainty can lead to inaccurate or biased estimates, whereas variability can affect the precision of the estimates and the degree to which they can be generalized.

Variability and uncertainty can complement or confound one another. The National Research Council (236) has drawn an instructive analogy based on estimating the distance between the earth and the moon. Prior to fairly recent technological developments, it was difficult to accurately measure this distance, resulting in measurement uncertainty. Because the moon's orbit is elliptical, the distance is a variable quantity. If only a few measurements were taken without knowledge of the elliptical pattern, then either of the following incorrect conclusions might be reached:
• The measurements were faulty, thereby ascribing to uncertainty what was actually caused by variability; or
• The moon's orbit was random, thereby not allowing uncertainty to shed light on seemingly unexplainable differences that are in fact variable and predictable.

A more fundamental error in this situation might be to incorrectly estimate the true distance and assume that a few observations were sufficient. This latter pitfall—treating a highly variable quantity as if it were invariant or only uncertain—is most relevant to the exposure or risk assessor (377).

Now consider a situation that relates to exposure, such as estimating the average daily dose by one exposure route—ingestion of contaminated drinking water. Suppose that it is possible to measure an individual's daily water consumption (and concentration of the contaminant) exactly, thereby eliminating uncertainty in the measured daily dose. The daily dose still has an inherent day-to-day variability, however, because of changes in the individual's daily water intake or concentration of the contaminant in the water (377).

Clearly, it is impractical to measure the individual's dose every day. For this reason, the exposure assessor may estimate the average daily dose (ADD) based on a finite number of measurements, in an attempt to "average out" the day-to-day variability. The individual has a true (but unknown) ADD, which has now been estimated based on a sample of measurements. Because the individual's true average is unknown, it is uncertain how close the estimate is to the true value. Thus, the variability across daily doses has been translated into uncertainty in the ADD. Although the individual's true ADD has no variability, the estimate of the ADD has some uncertainty (377).

The preceding discussion pertains to the ADD for one person. Now consider a distribution of ADDs across individuals in a defined population (e.g., the general U.S. population). In this case, variability refers to the range and distribution of ADDs across individuals in the population. By comparison, uncertainty refers to the exposure assessor's state of knowledge about that distribution, or about parameters describing the distribution

(e.g., mean, standard deviation, general shape, various percentiles) (377).

As noted by the National Research Council (1994), the realms of variability and uncertainty have fundamentally different ramifications for science and judgment. For example, uncertainty may force decision makers to judge how probable it is that exposures have been overestimated or underestimated for every member of the exposed population, whereas variability forces them to cope with the certainty that different individuals are subject to exposures both above and below any of the exposure levels chosen as a reference point (377).

Types of Variability

Variability in exposure is related to an individual's location, activity, and behavior or preferences at a particular point in time, as well as pollutant emission rates and physical/chemical processes that affect concentrations in various media (e.g., air, soil, food, and water). The variations in pollutant-specific emissions or processes, and in individual locations, activities, or behaviors are not necessarily independent of one another. For example, both personal activities and pollutant concentrations at a specific location might vary in response to weather conditions, or between weekdays and weekends (377).

At a more fundamental level, three types of variability can be distinguished:

- Variability across locations (spatial variability).
- Variability over time (temporal variability).
- Variability among individuals (inter-individual variability).

Spatial variability can occur both at regional (macroscale) and local (microscale) levels. For example, fish intake rates can vary depending on the region of the country. Higher consumption may occur among populations located near large bodies of water such as the Great Lakes or coastal areas. As another example, outdoor pollutant levels can be affected at the regional level by industrial activities and at the local level by activities of individuals. In general, higher exposures tend to be associated with closer proximity to the pollutant source, whether it be an industrial plant or related to a personal activity such as showering or gardening. In the context of exposure to airborne pollutants, the concept of a "microenvironment" has been introduced to denote a specific locality (e.g., a residential lot or a room in a specific building) where the airborne concentration can be treated as homogeneous (i.e., invariant) at a particular point in time.

Temporal variability refers to variations over time, whether long or short term. Seasonal fluctuations in weather, pesticide applications, use of woodburning appliances, and fraction of time spent outdoors are examples of longer term variability. Examples of shorter term variability are differences in industrial or personal activities on weekdays versus weekends or at different times of the day.

Interindividual variability can be either of two types: (1) human characteristics such as age or body weight, and (2) human behaviors such as location and activity patterns. Each of these variabilities, in turn, may be related to several underlying phenomena that vary. For example, the natural variability in human weight is due to a combination of genetic, nutritional, and other lifestyle or environmental factors. Variability arising from independent factors that combine multiplicatively generally will lead to an approximately lognormal distribution across the population, or across spatial/temporal dimensions (377; 153, 154).

Monte Carlo Analysis

Among the most promising and exciting techniques to emerge in the area of exposure assessment in recent years is the application of Monte Carlo or other probabilistic analyses to environmental health issues (98, 223, 379). Monte Carlo analysis has existed as an engineering analytical tool for many years, but the development of the personal computer and software [e.g., Crystal Ball (Decisioneering, Boulder, CO), @RISK (Palisades Corp., Newfield, NY)] has allowed its application to new areas. As discussed previously, one criticism of many exposure assessments has been a reliance on overly conservative assumptions about exposure, as well as the problem of how to properly account for the highly exposed (but usually small) populations that do exist (44,242). The Monte Carlo technique offers an approach to addressing this issue.

The probabilistic or Monte Carlo model accounts for the uncertainty in select parameters evaluating the range and probability of plausible exposure levels. Instead of specifying input parameters as single values, this model allows for consideration of the probability distributions. The Monte Carlo statistical simulation is a statistical model in which the input parameters to an equation are varied simultaneously. The values are chosen from the parameter distributions, with the frequency of a particular value being equal to the relative frequency of the parameter in the distribution. The simulation involves the following three steps:

1. The probability distribution of each equation parameter (input parameter) is characterized, and the distribution is specified for the Monte Carlo simulation. If the data cannot be fit to a distribution, the data are "bootstrapped" into the simulation,

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meaning that the input values are randomly selected from the actual data set without a specified distribution.
2. For each iteration of the simulation, one value is randomly selected from each parameter distribution, and the equation is run. Many iterations are performed, such that the random selections for each parameter approximate the distribution of the parameter. Five thousand iterations are typically performed for each dose equation.
3. Each iteration of the equation is evaluated and saved; hence a probability distribution of the equation output (possible doses) is generated.
This technique generates distributions that describe the uncertainty associated with the risk estimate (resultant doses). The predicted dose for every 50th percentile to the 95th percentile of the exposed population and the true mean are calculated. Using these models, the assessor is not forced to rely solely on a single exposure parameter or the repeated use of conservative assumptions to identify the plausible dose and risk estimates. Instead, the full range of possible values and their likelihood of occurrence is incorporated into the analysis to produce the range and probability of expected exposure levels (15, 43, 49, 98, 120, 332).
The methodology is illustrated in the following examples. The first example characterizes the time needed to go shopping. Time spent shopping each month (minutes) is estimated by the product of two parameters: the number of trips per month and the total time spent in the store (minutes). Total time spent in the store is the sum of time spent shopping and time spent waiting in line. Using Monte Carlo techniques, a distribution of likely values is associated with each of these parameters. These distributions depend upon the detail of information available to characterize each parameter. For example, the distribution compares all of the information, such as those days when the line at the check-out counter is short, as well as those when the line is long. It is noteworthy that each parameter has a different distribution: lognormal, gaussian, and square. Total time spent shopping is then calculated repeatedly by combining parameter values that are randomly selected from these distributions. The result is a distribution of likely time spent shopping each month. Using this technique, information concerning each parameter is carried along to the final estimate.
The second example, which directly applies to toxicologists, is to build a distribution that describes the various soil ingestion rates for children. As shown in Figure 9.6, the three pertinent distributions are the basis for constructing the overall exposure distribution.
Most of the variables used in an exposure assessment actually exist as ranges, rather than single point values and this is captured in Monte Carlo analysis. For instance, the common assumption that adult body weight is 70 kg will be replaced by the appropriate distribution (i.e., normal) of body weights (including maximum, minimum, mean, and standard deviation). Using this approach, virtually every exposure variable, whether physiological, behavioral, environmental, or chronological, can be replaced with a probability distribution (15, 43, 45–47, 49, 119, 120, 133, 162, 226, 282, 306, 332, 351, 353, 354, 357). Since no population (or individual) is exposed to a single concentration; breathes, eats, or drinks at a single rate; or is exposed for the same length of time, it is not appropriate to assess them as such. To be protective, high values are employed, resulting in the problems of compounding conservatisms mentioned previously (5, 44, 81, 124, 209).
The probabilistic analysis addresses the main deficiencies of the point estimate approach because it imparts more information to risk managers and the public, and uses all of the available data (27, 221). The range of values (i.e., the distribution) for all the variables used in an exposure assessment is determined (e.g., normal, lognormal, uniform, triangular, etc.) and combined into a "distribution of distributions." Because of the extrapolations involved and the assumptions made, the area of single greatest uncertainty in risk assessment is associated with the dose-response evaluations.
It should also be clear that, in addition to exposure variables, data forming the basis of the toxicological criteria (carcinogenic potency factors [CPFs] and reference doses [RfDs]) are also amenable to Monte Carlo-style analysis where a robust database exists (21, 33, 76, 78, 79, 111, 112, 152, 319, 322–324, 398). As with exposure variables, the advantage to this approach is that it allows all data to be used (and weighted appropriately, where necessary), thus avoiding reliance on a single experiment or endpoint.
Probabilistic analyses have in recent years been recognized in regulatory guidance (376), and the U.S. EPA Risk Assessment Forum has published a document of principles for conducting Monte Carlo analyses (76) (Table 9.13). Recently, the U.S. EPA (392) published a comprehensive guidance document on how to conduct Monte Carlo assessments.
Like traditional exposure analysis, one challenge to performing a Monte Carlo analysis properly is having

appropriate distributions for use in the analysis. Numerous studies on individual variables have been published in the risk assessment literature (27, 41, 42, 73, 74, 117, 129, 315, 330, 337, 338, 387), and the impact on the distributions employed on the outcome has also been discussed (40, 72, 143, 146, 148, 154). It should be pointed out that these techniques can be combined with other advanced risk assessment methods (i.e., PBPK modeling) to further reduce uncertainty in risk estimates (77, 325). Recently,

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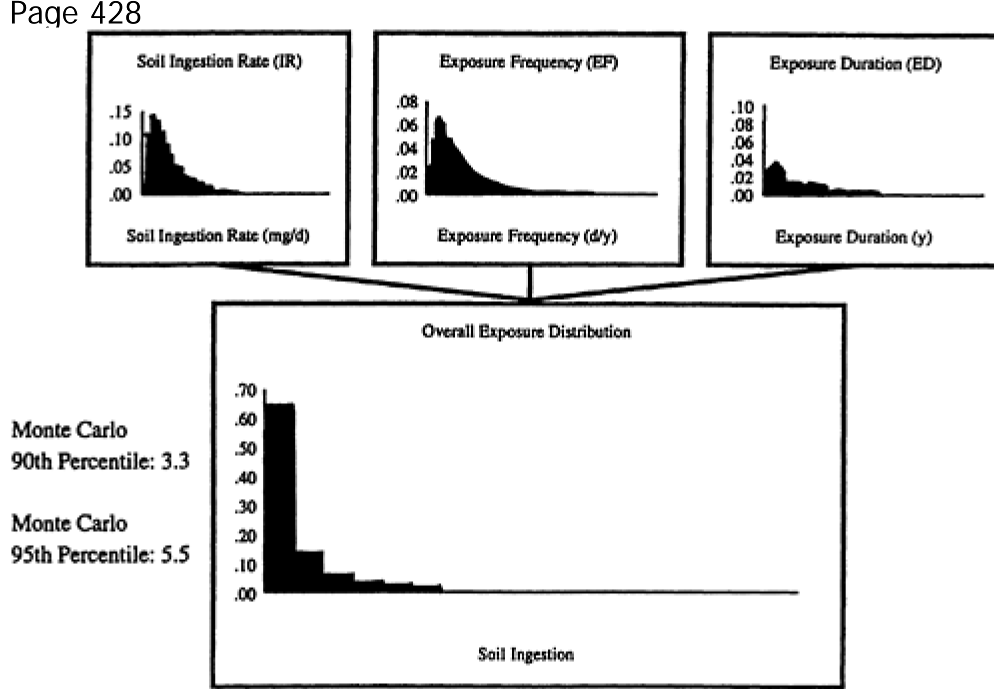

FIG. 9.6. Example of how probability density functions (distributions) for three different related exposure factors are combined to form a distribution for the amount of soil ingested by a population of children. The Monte Carlo technique allows the risk assessor to account for the variability in many exposure parameters within a population and then produce a distribution that characterizes the entire population.

two-dimensional Monte Carlo analyses have been developed that take into account both variability and uncertainty (82, 399). Information appropriate to probabilistic analyses can often be found in published papers in fields quite distant from the environmental sciences.

Case Study Using Monte Carlo Technique

An example might be useful (129). Assume that persons are likely to be exposed to contaminated drinking water at the maximum contaminant limit (MCL). Concern has been raised that these regulatory limits are not sufficiently protective, and that certain federal and state regulatory programs (i.e., Resource Conservation and Recovery Act) are justified in requiring groundwater remediation to levels below that of drinking water standards. To test this supposition, it is necessary to evaluate the possible incremental cancer risk of exposure via tap-water ingestion, dermal contact with water while showering, inhalation of indoor vapors, and ingestion of produce irrigated with groundwater, using a probabilistic approach. Probability density functions for each exposure variable (e.g., water ingestion, skin surface area, fraction of exposed skin, showering time, inhalation rate, air exchange and water use rates, exposure time, etc.) are then identified and used in the appropriate exposure equation to calculate dose and risk. A commercially available software package (i.e., @RISK) could be used to conduct the Monte Carlo analysis (357).

Some have suggested that the Latin hypercube (LHC) approach offers some advantages to traditional approaches for identifying the correct number of iterations. Often, one can reach convergence sooner with LHC than the Monte Carlo option in @RISK/Crystal Ball. In addition, LHC is more reproducible (to the hundredth decimal place). The Monte Carlo option needs more iterations to reach convergence.

The results of such an analysis are presented in Table 9.14 (129). The risk associated with exposure to water at the current maximum contaminant level (MCL) level for four different contaminants, as well as the 50th

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Table 9.13 U.S. EPA guiding principles for Monte Carlo analysis

1. Conduct preliminary sensitivity analyses to identify important contributors to the assessment endpoint and its variability and uncertainty.
2. Based on the results of the sensitivity analyses, include probabilistic assessments only for the important pathways and parameters.
3. Use the entire database of information when selecting input distributions.
4. When using surrogate data, identify sources of uncertainty, and whenever possible, validate the use of these data by collecting site/case specific data.
5. If empirical data are collected for use in the assessment, use collection methods that improve the representativeness and quality of these data (especially at the tails of the distribution.)
6. Identify when expert judgment, rather than hard data, is used in the assessment.
7. Separate uncertainty and variability during the analysis.
8. Use appropriate methods to address uncertainty and variability, e.g., two-dimensional Monte Carlo.
9. Discuss the numerical stability of estimates at the tails of the distribution.
10. Identify which sources of uncertainty are addressed by the assessment, and which are not.
11. Provide a detailed description of all models used.
12. Provide a detailed description of the input distributions, including a distinction between variability and uncertainty in these distributions, and a graphical representation of the probability density and cumulative distribution functions.
13. Provide a graphical representation of the probability density and cumulative distribution functions of each output distribution.
14. Consider the potential covariance between important parameters. If the covariance cannot be determined, evaluate the impact of a range of potential covariances on the output distributions.
15. Present point estimates and identify where they fall on the exposure distribution. If there are large differences between point estimates and Monte Carlo estimates, explain if the differences are due to changes in the data or models used.
16. Present results in a tiered approach.

Note. From Reference 161.

Table 9.14 Risks calculated for exposure to four halogenated solvents in water using probabilistic analysis at the MCL level and for the 50th and 95th percentile exposure

Chemical	50th Percentile risk	95th Percentile risk	MCL risk
Tetrachloroethylene	0	0.000005	0.000007
Chloroform	0.000009	0.00014	0.000017
Bromoform	0.000002	0.000016	0.000023
Vinyl chloride	0.000005	0.000029	0.000054

Note. Adapted from Reference 117.

and 95th percentile of exposure as determined by the probabilistic analysis, are shown. At the 50th percentile level ("the best estimate"), the risk ranges from 6×10^{-7} (tetrachloroethylene) to 9×10^{-6} (chloroform), while at the 95th percentile ("the upper-bound risk"), these risks range from 4×10^{-6} (tetrachloroethylene) to 1.5×10^{-4} (chloroform). These values can be compared to the point estimate risks calculated for the MCLs, which range from 7×10^{-6} (tetrachloroethylene) to 5.4×10^{-5} (vinyl chloride). For the 50th percentile (average) person, all calculated risks are within the range of "acceptable" risks adopted by regulatory authorities for Superfund sites (1×10^{-4} to 1×10^{-7}). For the 95th percentile person (upper bound), the risks are still mostly below the 1×10^{-4} benchmark risk level generally used to separate acceptable from unacceptable risks. For tetrachloroethylene,

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these results are 30 (50th percentile) to 3 (risk at the MCL) times below the reasonable maximum exposure (RME) risk of 2×10^{-5} developed by combining the 95th percentile values for each exposure variable using standard U.S. EPA risk assessment methodologies. This point estimate is greater than the 99th percentile of risk and is consistent with statements regarding the conservatism of the RME approach. These results suggest that chemical residues in drinking water at the MCL levels will be health protective and that remedial goals based on de minimis requirements (1×10^{-6}) might be unnecessarily low (129).

In terms of estimates for the reasonably maximally exposed (RME) individual, which often serve as the basis for regulatory decisions, several observations on the utility of probabilistic assessment can be made. First, exposure assessments that incorporate two to three direct exposure pathways usually show that the 95th percentile probabilistic estimates are three to five times below the traditional RME estimates. Second, for multipathway assessments that contain several indirect exposure pathways, the 95th percentile probabilistic estimates can be as much as an order of magnitude below the RME estimates. Third, when the number of distributions used in the exposure assessment is 10 or more, the difference between the 50th and 95th percentile estimates may be between 5 and 10. Finally, in such assessments, the difference between the RME estimates and the 95th percentile probabilistic estimates can be as high as 100 (117). In the probabilistic approach to estimating exposure and risk, the complete range of potential risks can be illustrated along with the likelihood estimates and estimates of uncertainty associated with such risks. While the availability and confidence of distributions for exposure variables differ, risk assessors ought to take advantage of this and similar approaches in their risk assessments to advance and improve the process. Additionally, since the highest degree of uncertainty in risk assessment tends to be the CPFs, attention ought to be directed to applying probabilistic analysis to the development of toxicity criteria in a similar manner (111, 116, 398).

Sensitivity Analysis

In addition to establishing exposure and risk distributions, probabilistic analysis can also identify variables with the greatest impact on the estimates and illuminate uncertainties associated with exposure variables through sensitivity analysis (30, 160, 287, 299, 319). This provides some insight into the confidence that resides in exposure and risk estimates, and has two important results. First, it identifies the inputs that would benefit most from additional research to reduce uncertainty and improve risk estimates. Second, assuming that a thorough assessment has been conducted, it is possible to phrase the results in more accessible terms, such as "the risk assessment of PCBs in smallmouth bass is based on a large amount of high-quality reliable data, and we have high confidence in the risk estimates derived. The analysis has determined that 90 percent of the increased cancer risk could be eliminated through a ban on carp and catfish, but there is no appreciable reduction in risk from extending such a ban to bass and trout." Such a description provides all stakeholders with considerably more information than a simple point estimate of risk based on a traditional exposure and risk assessment (257).

If the most "sensitive" exposure variables are based on limited or uncertain data, confidence in these estimates will be poor. Robust data sets, on the other hand, lead to increased confidence in the resulting estimates. In the preceding example involving smallmouth bass, sensitivity is defined as the ratio of the relative change in risk produced by a unit relative to change in the exposure variables used. A Gaussian approximation (the product of the normalized sensitivity and the standard deviation of the distribution) of intake was used to allow both sensitivity and uncertainty to be gauged. In this case, the true mean of each distribution was chosen as the baseline point value, and the differential value for each variable was calculated by increasing this value by 10%. For each variable, the differential value was substituted, the risks recalculated, and the baseline value replaced (129). Sensitivity was calculated using the following formula:

$$\text{Sensitivity} = \frac{|\text{Risk}_{\text{baseline}} - \text{Risk}_{10\%}|}{|X_{\text{baseline}} - X_{10\%}|} \cdot [\sigma]$$

where Xbaseline and X10% are baseline and differential values for the variable *X*, and σ is the standard deviation for the distribution of variable *X*. The sensitivity of each variable relative to one another is assessed by summing the unitless sensitivity values and determining the relative percent that each variable contributes to the total.

Table 9.15 indicates which are the most important variables in a probabilistic analysis of tetrachloroethylene in household tap water. In this case, the most sensitive exposure variables in household exposure to tap water are exposure time in shower and exposure duration. Relatively small changes in these variables will result in relatively large changes in the risk estimates. Since these

estimates are based on actual time-use studies and census information, this suggests a high level of confidence can be placed on this estimate, particularly if site-specific data are being used. If the critical variables (in terms of sensitivity) were not based on robust data, this would suggest that the risk assessment could be improved by additional research on these exposure variables. It is interesting that the form of the distribution chosen for

Table 9.15 Results of sensitivity analysis for tetrachloroethylene exposure in household water

Exposure variable	Sensitivity (unitless)	Percentage rank
Shower exposure time	0.000004	55.0%
Exposure duration	0.000001	20.0%
Plant-soil partition factor	0	8.4%
Water ingestion rate	0	4.6%
Surface area of exposed skin	0	4.4%
Body weight	0	3.8%
Dermal permeability constant	0	1.8%
Skin fraction contacting water	0	1.5%

Note. Adapted from Reference 117.

the variables is less important than the validity of the data (118). When the empirical distribution of the tapwater ingestion rate from Ershow and Cantor (108) was substituted with a lognormal distribution developed by Roseberry and Burmaster (303), the resultant change in the risk estimates was less than 1% (357).

In this case, the value of the sensitivity analysis is that it allows input variables to be ranked in order of importance and confidence in the output to be established to a higher degree than previously possible. As pointed out by the U.S. EPA, where possible, exposure assessors should report variability in exposures as numerical distributions and should characterize uncertainty as probability distributions. They need to identify clearly where they are using point estimates for 'bounding' potential exposure variables or estimates; these point estimates should not be misconstrued to represent, for example, the upper 95th percentile when information on the actual distribution is lacking (324). As noted by the U.S. EPA, such explicit presentation of the data reduces the temptation to use the exposure assessment process for veiling policy judgments (141).

ISSUES IN EXPOSURE ASSESSMENT

The field of exposure assessment will continue to benefit from ongoing research efforts. The following are some fruitful areas of ongoing research.

Bioavailability

Applied research that will improve the practice of exposure assessment include bioavailability, speciation, chemical fate, and the role of biological monitoring. Bioavailability has become an increasingly important issue in the exposure assessment process (159, 202, 266, 305, 311). Alexander (3) has shown that a variety of organic chemicals in soil lose the ability to interact with biological

Table 9.16 Effect of matrix and aging on the bioavailability of lead from soil

Treatment			
Lead acetate (ppm diet)	Soil lead (ppm)	Tibial lead (standard deviation)	Relative lead absorption
—	—	0.3 (0.3)	—
—	11.3	0	—
50	—	247 (10)	100
50	11.3	130 (30)	53
—	706	40 (6)	16
—	995	108 (26)	44
—	1080	37 (7.3)	15
—	1260	53.6 (7)	22
—	10420	173 (22)	70

Note. Lead acetate in the diet results in an increase in tibial lead, while lead acetate mixed with soil is only 50% as well absorbed. Aged lead from garden soil must reach high levels before significant absorption occurs. Adapted from Reference 64.

receptors over time, despite the fact that the chemical concentration in soil remains largely the same. The alteration in bioavailability extends across the various routes of exposure as well (307, 321, 326, 358, 359, 420, 421). Inorganic compounds, even those posing a potentially significant degrees of hazard (i.e., cyanide) react similarly (85–87, 318). These losses in hazard potential are presumably due to irreversible chemical interactions with soil constituents. Table 9.16 indicates that the bioavailability of lead added to soil is immediately halved and that it is further reduced over time (64). This would suggest that an assumption of 100% bioavailability of this compound (and many others) from soil is erroneous. It is also clear that the environmental media in which the compound occurs will influence its uptake into the body (307). The U.S. EPA recognized this fact when it developed two reference doses (RfDs) for manganese depending on whether it occurred in solid matrices (e.g., food, soil, etc.) or water

(390). One simple method to improve bioavailability estimates is to conduct extractions under more biologically relevant conditions.

Bench-scale extraction experiments in simulated gastric fluids or sweat can be used to inexpensively and accurately measure how readily environmental residues can be released from the media in which they occur (157, 304, 305). As with inhalation or ingestion of vapors or solutions, both the release and absorption rates of agents from an environmental matrix (i.e., soil) across biological membranes need to be incorporated into the risk assessment when such data are available, and generated when absent. This need is particularly great when assessing dermal exposure. The problem for materials in aqueous solutions is less problematic than from solid

matrices (192). For liquids, permeability constants expressed in terms of agent weight per unit area per time (mg/cm2-min) have been developed for a number of agents, and in vivo and in vitro techniques or mathematical models exist to develop similar flux rates if needed (22, 26, 29, 31, 123, 142, 217, 316, 349, 419, 420). From soil, however, the typical approach in many risk assessments has been to assume a constant percent absorbed from soil adhered to skin as a default. For volatiles, an absorption rate of 25% has sometimes been used. For semivolatiles and inorganics, absorption rates of 10% and 1% have been used, respectively.

Some experimental data for absorption are available for a few agents (e.g., PCBs, DDT, dioxin, benzo[a]pyrene, etc.), suggesting that the simple assumption of a constant percentage absorbed may overestimate or underestimate the dose depending on the agent, cocontaminants, soil type, exposure duration, and similar considerations (321, 326, 420, 421). The impact of using this default approach results in an instantaneous dermal dose being assumed, regardless of whether the soil remains in contact with the skin for 1 min or 1 day. This assumption, together with the questionable route-to-route adjustment of toxicity criteria from oral to dermal, results in a predicted dermal absorption of agents from soil, which arguably should present a minor risk in most cases, being a major driver in the risk assessment of soilbound contaminants.

Chemical Fate

Risk assessors ought to incorporate information on the fate of chemicals in the environment in their exposure estimates, whenever possible (86). Many organic compounds tend to degrade over time, and may disappear from exposed surfaces relatively quickly or otherwise change (6, 255). As already suggested, inorganic compounds may also undergo changes in the environment over time that affect their fates (64, 318). Influencing factors include degradation by sunlight, soil and water microbes, evaporation, and chemical interactions. The resultant changes can dramatically alter the outcome of exposure assessments (87, 266). For instance, much of the criticism of incinerators has focused on the inhalation risk of dioxin emitted from the stacks. As it turns out, the environmental half-life of dioxin (as a vapor) is only 90 min because of photolytic degradation. In contrast, the half-life for dioxin in soil or fly-ash is 50 to 500 yr. The focus of concern is often not the main risk issue when environmental fate is considered because levels and availability change over time (269). Incorporation of half-life data into risk assessments can have substantial benefits for improving understanding of the potential exposures and risks associated with a specific situation (32).

In a similar manner, the risk from persistent contaminants (i.e., DDT) in fish has usually been assessed using results from the analysis of raw fish fillets in combination with assumptions about the size and number of fish meals. The effects of cleaning and cooking on these residues are not typically considered, but have been shown to be reduced substantially in many cases (i.e., 50% or greater) when cooked or processed (224, 427). Since many of these risk assessments form the basis of fish advisories or bans with potentially significant economic repercussions, there is obviously an important reason to make these exposure estimates as accurate as possible. Additionally, since there are known health benefits to fish consumption, making recommendations against eating fish based on theoretical risk deserves careful evaluation (352).

Biomarkers and Molecular Epidemiology

The past decade has witnessed a dramatic increase in the level of research activity, derivation of theoretical constructs, and development of practical applications for the direct measurement of biological events or responses that result from human exposure to xenobiotics (89, 147). These measurements, conveniently grouped under the descriptor "biological markers" or "biomarkers," reflect molecular and/or cellular alterations that occur along the temporal and mechanistic pathways connecting ambient exposure to a toxicant and eventual disease. As such, an almost limitless array of biomarkers is theoretically available for assessment, and only a minute fraction of these has been recognized and investigated to date (147, 155, 220). Some events that can technically be classified as biomarkers of chemical exposure (e.g., hematological changes following high levels of exposure to lead or benzene, acetylcholinesterase inhibition by organophosphates) have been measured for decades. However, the recent surge of interest in this field has been driven by technical advances in analytical chemistry and molecular genetic techniques and by the recognition that "classical" toxicology and epidemiology may not be able to alone resolve critical questions regarding causation of environmentally induced disease (89).

Biomarkers are an important component of the emerging discipline of molecular epidemiology, which seeks to expand the capabilities and overcome the limitations of classical epidemiology by incorporating biological measurements collected in exposed humans (89,429). Early efforts at utilizing biomarkers to

make quantitative estimates of exposure and to predict human cancer risk were made by Ehrenberg and Osterman-Golkar (105). Using ethylene oxide as a model xenobiotic, these investigators explored the use of macromolecular reaction products (i.e., hemoglobin

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adducts) as internal dosimeters. By employing hemoglobin adduction data, they predicted the level of ambient ethylene oxide that would correspond to a tumorigenic dose of g-radiation, which they termed the "rad-equivalent dose." Seminal work in the area of biomarkers as applied to the molecular epidemiology of cancer was performed by Perera and Weinstein (271), who proposed the use of such techniques to identify environmental contributors to human cancer incidence. Important early applications of biomarkers to characterize environmental and occupational exposure have also been explored by several other groups in the United States and abroad.

As presented in the original NRC report, biomarkers of internal dose reflect the absorbed fraction of a xenobiotic, that is, the amount of material that has successfully crossed physiological barriers to enter the organism. Consequently, the magnitude of the biomarker accounts for bioavailability and is influenced by numerous parameters such as route of exposure, physiological characteristics of the receptor, and chemical characteristics of the xenobiotic. Generally, simple measurement of xenobiotic levels in biological media (blood, tissue, urine) can provide data on internal dose, and this is called biomonitoring (17, 18, 203). Biomarkers, on the other hand, reflect internal dose (in terms of proximity to downstream events in the sequence) and could include the measurement of a metabolite in selected biological media, particularly if such metabolite is active or critical to the toxic effects seen (89).

Very useful exposure biomarkers for reactive xenobiotics or their activated (i.e., electrophilic) metabolites are macromolecular reaction products. Substantial research effort has been devoted to the use of protein and DNA adducts as molecular dosimeters. Ehrenberg and co-workers first proposed using hemoglobin (Hb) adducts to monitor the internal dose of alkenes and epoxides such as ethylene oxide over two decades ago (105). This methodology has since evolved into a widely-used and highly sensitive technique for quantitating N-terminal Hb adducts of a variety of xenobiotic metabolites in human blood. Hb adducts have been employed as internal exposure biomarkers for aromatic amines, nitrosamines, polycyclic aromatic hydrocarbons (PAHs), and other compounds (89).

Protein and DNA adducts can be considered as biomarkers of either internal or biologically effective dose (BED), depending upon how close their relationship is to actual disease occurrence. The NRC report defined BED as dose at the site of action, dose at the receptor site, or dose to target macromolecules (233). This definition is troublesome since, strictly speaking, complete characterization of molecular site and mechanism of action for a given xenobiotic would be necessary in order to assign a particular measured end point as a marker of biologically effective dose. For example, protein adducts cannot be considered as effective dose biomarkers for carcinogens, since they do not satisfy these criteria. Ambiguity exists even for DNA adducts, since in no reported instance has xenobiotic-induced adduction of a specific base within a particular DNA sequence in a target cell type been unequivocally linked to a specific clinical outcome in people (89). Despite these uncertainties, adducts in total lymphocyte DNA are considered as appropriate BED biomarkers for carcinogens, based upon the postulated mechanism of chemical carcinogenesis and limited experimental data indicating correlations between DNA adducts in lymphocytes and target tissues (89).

Ideally, a biomarker should be biologically relevant, sensitive, and specific (i.e., valid). In addition, it should be readily accessible, inexpensive, and technically feasible. This combination of requirements is rarely achieved, and some trade-off is inevitable in order to obtain useful biomarker data in a timely manner. A few promising examples are presented in Table 9.17. The validation process for a biomarker involves determining the relationship between the biological parameter measured and both upstream and downstream events in the continuum, that is, the dose-response curve must be characterized (89). For example, an Hb adduct considered for use as an exposure biomarker for a xenobiotic should exhibit a predictable relationship to ambient exposure level. In addition, if used as a surrogate for DNA adduction, then a reproducible correlation between Hb and DNA adducts must be demonstrated. Biological relevance refers to the nature of the phenomenon being measured and its mechanistic involvement in the pathway from exposure to disease. For biomarkers of exposure, disease relevance is not as critical a requirement as is a predictable exposure-response relationship; the opposite is true for biomarkers of effect (89).

There are a few terms that are used when discussing biomarkers that help define their usefulness. *Sensitivity*, for example, reflects the ambient exposure level that can be detected by means of the biomarker. Highly sensitive markers are necessary to quantitate the low ambient levels typical of environmental exposures in industrialized Western nations. *Specificity* is the probability that the biomarker is indicative of actual exposure to the specific xenobiotic that it is designed to detect. For example, certain macromolecular adducts can be derived from exposure to a number of chemical species and are thus less specific than one unique to a single compound. Biomarkers must also be reasonably

accessible. Thus, with the exception of occasional tissue biopsies, samples for use in exposure biomarker studies generally consist of blood, urine, milk, or other readily obtainable biological media. Since these are rarely target tissues for toxicological or carcinogenic effects, how the concen

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Table 9.17 Biomarkers examined for selected occupational and environmental chemicals

Chemical	Biomarker		
	Exposure	Effect	Susceptibility
PAH	DNA adductsa	*hprt* mutation	GST-M1
	Hb adducts	*gpa* mutation	NAT-2
	SA adducts	*fes* oncogene activation	CYP1A1
	Urinary 1-HPa	*ras* p21 level	CYP1A2
	Sister chromatid exchange (SCE)	DNA single-strand breaks	
	SCE (high-frequency cells)	Chromosomal aberrations Micronuclei	
1,3-Butadiene	Hb adductsa	*hprt* mutation	
	Sister chromatid exchange (SCE)	Chromosomal aberrations	
	Urinary metabolites	Micronuclei *ras* oncogene activation	
Acrylamide	Hb adductsa		
	Urinary metabolites		

Note. From Reference 89.

aBiomarkers for which cumulative data indicate best correlation with ambient exposure.

tration relates to the incidence of disease must be inferred. Finally, cost and technical feasibility are important considerations in selection of appropriate biomarkers for applied studies (89).

Statistical and Analytical Issues

Despite the use of precise and reproducible analytical methods, we often do not have enough data of chemical concentrations to estimate exposure with great certainty. Due to resource availability, over the past 15–20 yr it has often been the case that a single round of analytical results or samples collected for other purposes (140) serves as input and the surrogate for long-term or lifetime exposure. As noted previously, chemical concentrations vary over both time and space, which makes the task of dose estimation all the more difficult (224). For instance, to use the (estimated) average dose to predict the typical lifetime dose may seriously overestimate or underestimate the actual dose. Additionally, the average dose may be less important in the biological scheme of things than peak exposures or exposures at specific times (i.e., developmental effects), and ought to be considered as such in the evaluation of exposure (11). Techniques do exist for estimating long-term exposure from short-term data (37, 38, 328), but the reliability of these estimates is uncertain. Similarly, various mathematical or bench-scale models exist that have been used to estimate exposure in the absence of measurements or long-term monitoring data (342). As has been noted on several occasions, "all models are wrong, but some are useful," and risk assessors should carefully evaluate mesoscale and microscale models, as well as model outputs, for relevance and accuracy. Often, field measurements can serve as useful and relatively inexpensive "reality checks" on model results.

Equally important in exposure assessment are the statistics used to analyze field data. Environmental data are most often lognormally distributed. Under such conditions, a geometric average is generally assumed to be a better measure of the central tendency of data than the arithmetic mean (80). Despite this, the arithmetic mean (and the 95% upper confidence limit of the arithmetic mean) is typically used to identify environmental concentrations for use in exposure assessment. Since the advances in analytical chemistry have improved our ability to measure trace amounts of chemicals in different media and identify potential sources in some situations, less reliance should be placed on the use of mathematical models to predict the distribution of chemical and physical agents in the environment, and actual field data should be collected.

Another important issue in exposure assessment is how the analytical limit of detection (LOD) is handled in calculations. An agent reported as a nondetect may be treated as a numerical zero, or occurring at the LOD or some fraction of the LOD, typically one-half, for purposes of calculating statistics. The manner in which

censured data are assessed may affect the outcome of the risk assessment process (132, 144, 150, 158, 251, 272, 290, 356). For instance, analysis of highly contaminated samples or samples containing interfering substances may result in high LODs. Under such conditions and in the absence of additional analysis, assuming that nondetects are present at one-half the LOD could result in the exposure assessment and subsequent risk assessment being driven by compounds that are not truly present in the environmental media. When such an approach is used on a site that may be only 2 to 10% contaminated (based on surface area), the predicted severity of the level of contamination will be much higher than what actually occurs (80).

The practical result of these decisions can be illustrated by considering the following 11 data points resulting from analysis of field samples: Nondetect (ND), ND, ND, ND, ND, 5, 6, 6, 8, 55, and 500 ppm. The results are lognormally distributed as expected. The detection limit is 0.05 ppm, and nondetects are assumed to be present at one-half the detection limit (0.025 ppm). Using these assumptions, the arithmetic mean of the data set is 52.7 ppm, while the geometric mean is 1.3 ppm. The practical consequence of choosing one descriptor over the other may be to mischaracterize the dose and ultimately the risk, and this could influence regulatory decisions involving remediation or regulation.

CLOSING THOUGHTS

The field of exposure assessment has evolved significantly over the past 20 yr. We have learned a great deal about where people are exposed to xenobiotics and the relative degree of exposure. Not that long ago, most of our concerns were about industrial chemicals in our water, ambient air, and the soil. Today, we know that indoor exposure to particles, vapors, and gases (influenced by smoking) often represents the predominant source of exposure for most persons. A greater portion of our work in the future will undoubtedly focus on better understanding of both occupational and indoor exposure, rather than environmental exposures.

It is my personal view that of the four portions of a risk assessment, exposure assessment has made the biggest improvement in quality over the 20-yr history of health risk assessment. Usually, exposure assessments will contain less uncertainty than other steps in a risk assessment, especially the dose-response portion. Admittedly, there are a large number of factors to consider when estimating exposure, and it is a complicated procedure to understand the transport and distribution of a chemical that has been released into the environment. Nonetheless, the available data indicate that scientists can do an adequate job of quantifying the concentration of the chemicals in the various media and the resulting uptake by exposed persons if they account for all the factors that should be considered.

There are at least 11 significant lessons we have learned about conducting exposure assessments in recent years. Had we not had to learn through experience, avoiding these lessons could potentially have saved the United States hundreds of millions of dollars and thousands of person-years of work. First, experience has shown that in our attempts to be prudent, we placed too much emphasis on the "so-called" maximally exposed individual (MEI) (117, 242). Often, the results of those analyses were misinterpreted by the public and/or misrepresented by some scientists or lawyers. Often, as a result, poor decisions were made by risk decision makers.

Second, as we have learned how to accurately characterize the risks of exposure for about 95% of the population, more emphasis has been placed on evaluating the various special groups (e.g., Eskimos, subsistence fishermen, dairy farmers). Although the risk for these populations, who can be exposed to particularly high doses (the 95–99.99% group) needs to be understood, the typical levels of exposure for the majority of the population should be the initial focus of the assessment. Perhaps the most significant change in exposure asessment of the past three years has been the national interest in characterizing the risks to children.

The third lesson is a variation of the second: Do not allow the repeated use of conservative assumptions to dictate the results of the assessment. In recent years, many investigators have addressed this issue and have demonstrated its importance. Monte Carlo techniques can generally be successful in addressing this problem.

Fourth, we have learned that risk managers and the public want to understand the statistical confidence in our estimates of risk. Sensitivity analyses can yield important information about the critical exposure variables (132, 144, 290, 356). Further, most risk assessments can benefit from analyses of both variability and uncertainty. Without these, risk managers are not fully informed.

Fifth, we have improved our techniques for statistically handling data; and particularly for samples that have no detectable amount of a contaminant. In the past, regulatory agencies have used the limit of detection (LOD) of the analysis or one-half the LOD in the exposure calculations relying on the premise that the contaminant might be present at that level. We learned that when such an approach is used

(without reflection) on a site that may only be 2–10% contaminated (based on surface area), the impact of a few samples on the results could lead us to improper conclusions about the actual level of risk to persons who live there or nearby.

Sixth, we have gained a significant degree of confidence in our ability to estimate historical exposures; so-called

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dose-reconstruction or retrospective risk assessments, a term that this author coined in 1983. Over the past ten years, for use in epidemiology studies, the likely exposure of workers and/or those in the community nearly forty to fifty years ago have been estimated using chemical usage and emission data, measured data, and models (145, 265, 294, 333, 345, 424).

Seventh, we now understand the need to quantitatively account for indirect pathways of exposure. For example, the uptake of a contaminant in water by humans due to ingestion is obvious (and direct), but the uptake of the same contaminant by garden vegetables due to watering and the uptake via the inhalation of volatile contaminants from the water while showering are indirect pathways that had not always been evaluated in assessments. Perhaps the most important indirect route of exposure, which had not been considered before 1986 when regulating airborne nonvolatile chemicals, was the ingestion of particulate emissions that have deposited onto soil and plants and were subsequently eaten by grazing animals (125, 388). Much additional research in this area will be conducted, and the results will probably change our views about the hazards posed by numerous chemicals.

Eighth, we have learned that children and their exposure patterns are unlike those of adults. As some have said, in more ways than one, children are not miniature adults! Their intake of certain foods, percentage of time spent outdoors, proximity to carpets, and inhalation rates per body weight are all different than adults.

The ninth lesson learned is to use biological monitoring to validate or confirm the predicted degree of human exposure. Over the past 5 to 10 yr, analytical chemists have increased their ability to detect very small quantities of dozens of chemicals in blood, urine, hair, feces, breath, and fat. For many chemicals, these data represent a direct indicator of recent exposure, and in some cases (like PCBs and dioxins), chronic exposure. Validation of our exposure assessments should be one of the major areas of study during the next decade (through both biomonitoring and molecular epidemiology).

Tenth, it has become clear that in most cases, the most significant risks due to exposure to chemicals occur in the workplace. Even though great strides have been made in industrial hygiene over the past 50 yr, the doses to which persons can legally be exposed are much greater (often by a factor of 100) than those to which most persons not in the community will ever be exposed.

Eleventh, and perhaps most important, we have learned that (for most persons) exposures to chemicals and bacteria in the home pose a greater risk than to those in the ambient air or through the ingestion of water. Many fine studies conducted in the 1970s through the current day continue to show that in-home exposures to most chemicals are often about 2–20 times greater than that present in the ambient environment (83, 131, 167, 183, 195, 196, 247, 407). Recently, more than 200 scientists in the fields of epidemiology, exposure assessment, and medicine signed a document called a "consensus statement," which states that future research should focus on personal monitoring; especially of the indoor environment (104).

We have come a long way in a short time. Several professional societies, including the International Society of Exposure Analysis (ISEA), Society for Risk Analysis (SRA), American Industrial Hygiene Association (AIHA), Air and Waste Management Association (AWMA), American Chemical Society (ACS), Society of Toxicology (SOT), International Society for Regulatory Toxicology and Pharmacology (ISRTP), and others, have placed an emphasis on improving the practice of exposure assessment. All indications are that the information we have gained has significantly improved the quality of recent risk assessments, and it can be expected that due to better exposure assessments, future decisions by risk managers will be much better informed.

QUESTIONS

1. What are the routes of exposure normally considered in an exposure assessment?
Answer: Dermal, inhalation, and ingestion
2. What is the definition of exposure assessment?
Answer: Exposure assessment is the step in the risk assessment that quantifies the uptake of an agent resulting from contact with various media (e.g., air, water, soil and food). These assessments can address past, current or future anticipated exposures.
3. When estimating uptake through the skin, what are the factors to be considered?
Answer. Percutaneous absorption rate, surface area of exposed skin, the chemical concentration, exposure duration, and interspecies scaling factor (if data were not collected using human skin).
4. What is a PB-PK model and why are they considered an important improvement over traditional toxicological methods?
Answer. A physiologically-based pharmacokinetic (PB-PK) model is a quantitative description of the absorption, distribution, metabolism and excretion (ADME) of a chemical in living organism (fish,

laboratory animal or human). These models are usually capable of scaling-up animal data to predict the behavior of the toxicologically important substance (parent or metabolite) in humans, thus representing a major improvement over traditional qualitative or semi-quantitative approaches.

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5. Uncertainty analyses are an important component of exposure assessments. In these analyses, uncertainty is contrasted with variability. What is the difference between these two terms and give an example?

Answer. Uncertainty represents a lack of knowledge about factors affecting exposure. For example, if one can precisely measure a particular value, such as the amount of chicken eaten by a specific person on a particular day, then there would be no uncertainty in the measurement. On the other hand, if one wanted to understand the ingestion of chicken during adulthood, this would vary from day to day and week to week; thus this would represent variability. To understand the degree of variability, which could be measured, then a large number of measurements would be necessary. The three most common forms of variability are the variability across locations, variability over time, and variability among individuals.

6. Over the past ten years, monte carlo or probabilistic techniques have become an important and useful component of exposure assessment. Describe the technique and discuss what is learned from their application.

Answer. Monte carlo techniques attempt to describe the uncertainty in select exposure parameters without having to make a particular measurement during every event over a lifetime. For example, these techniques allow one to estimate with confidence the daily ingestion of water by a typical adult male without having to collect every glass of water drunk by a person (or group of persons) over their lifetimes. The technique generates distributions that describe the uncertainty associated with the risk estimate. By using this approach, the assessor is not forced to rely solely on a single exposure parameter or the repeated use of conservative assumptions to identify the possible dose and risk estimates for a population of persons.

REFERENCES

1. Agency for Toxic Substances and Disease Registry. (1995): *Public health assessment guidance manual.* Lewis, Ann Arbor, MI.
2. Aggazzotti, G., Fantuzzi, G., Righi, E., Tartoni, P., Cassinadri, T., and Predieri, G. (1993): Chloroform in alveolar air of individuals attending indoor swimming pools. *Arch. Environ. Health*, 48:250–254.
3. Alexander, M. (1995): How toxic are chemicals in soil? *Environ. Sci. Technol.*, 29:2713–2717.
4. Allaby, M. (1983): *A dictionary of the environment*, 2nd ed., p. 195. New York University Press, New York.
5. Allen, B., Gentry, R., Shipp, A., and Van Landingham, C. (1998): Calculation of benchmark doses for reproductive and developmental toxicity observed after exposure to isopropanol. *Regul. Toxicol. Pharmacol.*, 28:38–44.
6. American Chemical Society. (1983): *Fate of chemicals in the environment*, edited by R.L.Swann and A.Eschenroeder. ACS Symp. Ser. 225. American Chemical Society, Washington, DC.
7. American Conference of Governmental Industrial Hygienists (ACGIH). (1998): *Industrial hygiene instruments handbook.* American Conference of Governmental Industrial Hygienists, Cincinnati, OH.
8. American Industrial Health Council. (1994): *Exposure factors sourcebook.* American Industrial Health Council, Washington, DC.
9. Andelman, J.B. (1985): Human exposures to volatile halogenated organic chemicals in indoor and outdoor air. *Environ. Health Perspect.*, 62:313–318.
10. Andersen, M.E., and Conolly, R.B. (1998): Mechanistic modeling of rodent liver tumor promotion at low levels of exposure: An example related to dose-response relationships for 2,3,7,8-tetraclorodibenzo-p-dioxin. *Hum. Exp. Toxicol.*, 17(12):683–690.
11. Andersen, M.E., MacNaughton, M.G., Clewell, H.J., and Paustenbach, D.J. (1987): Adjusting exposure limits for long and short exposure periods using a physiological pharmacokinetic model. *Am. Ind. Hyg. Assoc. J.*, 48(4):335–343.
12. Andersen, M.E., Clewell, H.J. III, Gargas, M.L., MacNaughton, M.G., Reitz, R.H., Nolan, R.J., and McKenna, M.J. (1991): Physiologically based pharmacokinetic modeling with dichloromethane, its metabolite, carbon monoxide, and blood carboxyhemoglobin in rats and humans. *Toxicol. Appl. Pharmacol.*, 108:14–27.
13. Andersen, M.E., Clewell, H.J., and Krishnan, K. (1995): Tissue dosimetry, pharmacokinetics modeling and interspecies scaling factors. *Risk Anal.*, 15:533–537.
14. Anderson, B.D., Higuchi, W.I., and Raykar, P.V. (1988): Heterogeneity effects on permeability: Partition coefficient relationships in human stratum corneum. *Pharmacol. Res.*, 5:566–573.
15. Anderson, P.D., and Yuhas, A.L. (1996): Improving risk management by characterizing reality: A benefit of probabilistic risk assessment. *Hum. Ecol. Risk Assess.*, 2:55–58.

16. Antoine, S.R., DeLeon, I.R., and O'Dell-Smith, R.M. (1986): Environmentally significant volatile organic pollutants in human blood. *Bull. Environ. Contam. Toxicol.*, 36:364–371.
17. Ashley, D.L., Bonin, M.A., Cardinali, L., McCraw, J.M., and Wooten, J.V. (1994): Blood concentrations of volatile organic compounds in a nonoccupationally exposed US population and in groups with suspected exposure. *Clin. Chem.*, 40:1401–1404.
18. Ashley, D.L., Bonin, M.A., Cardinali, F.L., McCraw, J.M., and Wooten, J.V. (1996): Measurement of volatile organic compounds in human blood. *Environ. Health Perspect.*, 104(suppl. 5):871–877.
19. Aylward, L.L., Hays, S.M., Karch, N.J., and Paustenbach, D.J. (1996): Relative susceptibility of animals and humans to the cancer hazard posed by 2,3,7,8-tetrachlorodibenzo-/?-dioxin using internal measures of dose. *Environ. Sci. Technol.*, 30(12):3534–3543.
20. Baes, C.F. III, Sharp, R.D., Sjoreen, A., and Shor, W.R. (1984): *A review and analysis of parameters for assessing transport of environmental released radionuclides through agriculture.* ORNL-5786. U.S. Department of Energy, Oak Ridge National Laboratory, Oak Ridge, TN.
21. Baird, S.J.S, Cohen, J.T., Graham, J.D., Shlyakhter, A.I., and Evans, J.S. (1996): Noncancer risk assessment: a probabilistic alternative to current practice. *Hum. Ecol. Risk Assess.*, 2:79–102.
22. Barber, E.D., Teetsel, N.M., Kolberg, K.F., and Guest, D. (1992): A comparative study of the rates of in vitro percutaneous absorption of eight chemicals using rat and human skin. *Fundam. Appl. Toxicol.*, 19:493–497.
23. Barltrop, D. (1966): The prevalence of pica. *Am. J. Dis. Child.*, 112(2): 116–123.

24. Barltrop, D. (1973): Sources and significance of environmental lead for children. *Proc. Int. Symp. Environmental Health Aspects of Lead.*, Commission of European Communities, Center for Information and Documentation, Luxembourg.
25. Barltrop, D., Stehlow, C.D., Thornton, I., and Webb, J.S. (1975): Absorption of lead from dust and soil. *Postgrad. Med. J.*, 5:801–804.
26. Bartek, M.J., LaBudde, J.A., and Maibach, H.I. (1972): Skin permeability *in vivo:* Comparison in rat, rabbit, pig and man. *J. Invest. Dermatol.*, 58:114–123.
27. Beck, B.D., and Cohen, J.T. (1997): Risk assessment for criteria pollutants versus other noncarcinogens: The difference between implicit and explicit conservatism. *Hum. Ecol. Risk Assess.*, 3:617–626.
28. Binder, S., Sokal, D., and Maughan, D. (1986): Estimating soil ingestion: The use of tracer elements in estimating the amount of soil ingested by young children. *Arch. Environ. Health*, 41:341–345.
29. Bogen, K.T. (1994): A note on compounded conservatisms. *Risk Anal.*, 14:379–382.
30. Bogen, K.T., and Spear, R.C. (1987): Integrating uncertainty and interindividual variability in environmental risk assessment. *Risk Anal.*, 7:427–435.
31. Bogen, K.T., Keating, G.A., Meissner, S., and Vogel, J.S. (1998): Initial uptake kinetics in human skin exposed to dilute aqueous trichloroethylene. J. *Expos. Anal. Environ. Epidemiol.*, 8:253271.
32. Borgert, S.J., Roberts, S.M., Harbison, R.D., and James, R.C. (1995): Influence of soil half-life on risk assessment of carcinogens. *Regul. Toxicol. Pharmacol.*, 22:143–151.
33. Boyce, C.P. (1998): Comparison of approaches for developing distributions for carcinogenic potency factors. *Hum. Ecol, Risk Assess.*, 4:527–578.
34. Bronaugh, R.L., Stewart, R.F., Congdon, E.R., and Giles, A.L., Jr. (1982): Methods for *in vitro* percutaneous absorption studies: I. Comparison with *in vitro* results. *Toxicol. Appl. Pharmacol.*, 62:474–480.
35. Brouwer, D.H., and Van Hemmen, J.J. (1992): Elements of a sampling strategy for dermal exposure assessment (abstr.). *Proc. Int. Occup. Hyg. Assoc.*, First International Scientific Conference, 710 December. Brussels, Belgium.
36. Bryce-Smith, D. (1974): Lead absorption in children. *Phys. Bull.*, 25:178–181.
37. Buck, R.J., Hammerstrom, K.A., and Ryan, P.B. (1995): Estimating long-term exposures from short-term measurements. *J. Expos. Anal. Environ. Epidemiol.*, 5:359–374.
38. Buck, R.J., Hammerstrom, K.A., and Ryan, P.B. (1997): Bias in population estimates of long-term exposure from short-term measurements of individual exposure. *Risk Anal.*, 17:455–465.
39. Buckley, T.J., Prah, J.D., Ashley, D., Wallace, L.A., and Zweidinger, R.A. (1997): Body burden measurements and models to assess inhalation exposure to methyl tertiary butyl ether (MTBE). *J. Air Waste Manage. Assoc.*, 47(7).
40. Bukowski, J., Korn, L., and Wartenberg, D. (1995): Correlated inputs in quantitative risk assessment: The effects of distributional shape. *Risk Anal.*, 15:215–219.
41. Burmaster, D.E. (1998): A lognormal distribution for time spent showering. *Risk Anal.* 18:33–36.
42. Burmaster, D.E. (1998): Lognormal distributions for skin area as a function of body weight. *Risk Anal.*, 18:27–32.
43. Burmaster, D.E., and Anderson, P.D. (1994): Principles of good practice for the use of Monte Carlo techniques in human health and ecological risk assessment. *Risk Anal.*, 14:477–491.
44. Burmaster, D.E., and Harris, R.H. (1993): The magnitude of compounding conservatisms in Superfund risk assessments. *Risk Anal.*, 13:131–134.
45. Burmaster, D.E., and Huff, D.A. (1997): Using lognormal distributions and lognormal probability plots in probabilistic risk assessments. *Hum. Ecol Risk Assess.*, 3:223–234.
46. Burmaster, D.E., and Maxwell, N.I. (1991): Time and loading-dependence in the McKone model for dermal uptake of organic chemicals from a soil matrix. *Risk Anal.*, 11:491–497.
47. Burmaster, D.E., and Thompson, K.M. (1995): Back calculating cleanup targets in probabilistic risk assessments when the acceptability of cancer risk is defined under different risk management policies. *Hum. Ecol. Risk Assess.*, 1:101–120.
48. Burmaster, D.E., and Thompson, K.M. (1997): Estimating exposure point concentrations for surface soils for use in deterministic and probabilistic risk assessments. *Hum. Ecol. Risk Assess.*, 3:363–384.
49. Burmaster, D.E., and von Stackelberg, K. (1991): Using Monte Carlo simulations in public health risk assessments: Estimating and presenting full distributions of risk. J. *Expos. Anal. Environ. Epidemiol.*, 1:491–521.
50. Byard, J. (1989): Hazard assessment of 1,1,1-trichloroethane in groundwater. In *The risk*

assessment of environmental and human health hazards: A textbook of case studies, edited by D.J. Paustenbach, pp. 331–334. John Wiley & Sons, New York.
51. Calabrese, E.J., and Kostecki, P.T. (1992): *Risk assessment and environmental fate methodologies.* Lewis, Ann Arbor, MI.
52. Calabrese, E.J., and Stanek, E.J. III. (1998): Soil ingestion in children and adult: A dominant influence in site-specific risk assessment. *Environ. Law Reporter*, 28:10,660–10, 671.
53. Calabrese, E.J., and Stanek, E.J. III. (1991): A guide to interpreting soil ingestion studies, II: Qualitative and quantitative evidence of soil ingestion. *Regul. Toxicol. Pharmacol.*, 13:278–292.
54. Calabrese, E.J., and Stanek, E.J. III. (1991): Evidence of soil pica behavior and quantification of soil ingestion. *Human Exp. Toxicology*, 10:245–249.
55. Calabrese, E.J., Barnes, R., Stanek, E.J.III, Pastides, H., Gilbert, C.E., Veneman, P., Wang, X.R., Lasztity, A., and Kostecki, P.T. (1989): How much soil do young children ingest: An epidemiologic study. *Regul. Toxicol. Pharmacol.*, 10:123–137.
56. Calabrese, E.J., Stanek, E.J., Gilbert, C.E., and Barnes, R.M. (1990): Preliminary adult soil ingestion estimates: Results of a pilot study. *Regul. Toxicol. Pharmacol.*, 12:88–95.
57. Calabrese, E.J., Stanek, E.J., and Barnes, R. (1996): Methodology to estimate the amount and particle size of soil ingested by children: implications for exposure assessment at waste sites. *Regul. Toxicol. Pharmacol.*, 24:264–268.
58. Calabrese, E.J., Stanek, E.J. III, Pekow, P., and Barnes, R.M. (1997): Soil ingestion estimates for children residing on a Superfund site. *Ecotoxicol. Environ. Safety*, 36:258–268.
59. California Department of Health Services. (1986): *Development of applied action levels for soil contact: A scenario for the exposure of humans to soil in a residential setting.* California Department of Health Services, Sacramento, CA.
60. Caplan, K. (1993): The significance of wipe samples. *Am. Ind. Hyg. Assoc. J.*, 53(2):70–75.
61. Carnegie Commission on Science, Technology, and Government. (1993): *Risk and the environment. Improving regulatory decision-making.* Carnegie Commission on Science, Technology, and Government, New York.
62. Carrington, C.D., and Bolger, P.M. (1998): Uncertainty and risk assessment. *Hum. Ecol. Risk Assess.*, 4:253–258.
63. Center for Risk Analysis. (1994): *Historical roots of health risk assessment.* Harvard University, School of Public Health, Cambridge, MA.
64. Chaney, R.L., Sterrett, S.B., and Mielke, H.W. (1984): The potential for heavy metal exposure from urban gardens and soils. *Proc. Symp. Heavy Metals in Urban Gardens*, edited by J.R.Preer,

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_438.html[2010/10/28 18:44:13]

pp. 37–44. Agricultural Experiment Station, University of District of Columbia, Washington, DC.
65. Charney, E., Sayre, J., and Coulter, M. (1980): Increased lead absorption in inner city children: Where does the lead come from? *Pediatrics*, 65:226–231.
66. Clayton, C.A., Perritt, R.R., Pellizzari, E.D., Thomas, K.W., Whitmore, R.W., Ö zkaynak, H., Spengler, J.D., and Wallace, rL.A. (1993): Particle Total Exposure Assessment Methodology (PTEAM) study: Distributions of aerosol and elemental concentrations in personal, indoor, and outdoor air samples in a Southern California community. *J. Expos. Anal. Environ. Epidemiol.*, 3:227–250.
67. Cleek, R.L., and Bunge, A.L. (1993): A new method for estimating dermal absorption from chemical exposure. 1. General approach. *Pharm. Res.*, 10(4):497–506.
68. Clewell, H.J. (1995): The application of physiologically based pharmacokinetics modeling in human health risk assessment of hazardous substances. *Toxicol. Lett.*, 79:207–217.
69. Committee on Advances in Assessing Human Exposure to Airborne Pollutants. (1991): *Human exposure assessment for airborne pollutants.* National Research Council Board of Environmental Studies and Toxicology, National Academy of Science. National Academy Press, Washington, DC.
70. Conner, J.M., Oldaker, G.B. III, and Murphy, J.J. (1990): Method for assessing the contribution of environmental tobacco smoke to respirable particles in indoor microenvironments. *Environ. Technol.*, 11:189–196.
71. Cooper, M. (1957): *Pica*, pp. 60–74. Charles C.Thomas, Springfield, IL.
72. Cooper, J.A., Ferson, S., and Ginzburg, L. (1996): Hybrid processing of stochastic and subjective uncertainty data. *Risk Anal.*, 16:785–792.
73. Copeland, T.L., Paustenbach, D.J., Harris, M.A., and Otani, J. (1993): Comparing the results of a Monte Carlo analysis with EPA's reasonable maximum exposed individual (RMEI): A case study of a former wood treatment site. *Regul. Toxicol. Pharmacol.*, 18:275–312.
74. Copeland, T.L., Holbrow, A.H., Otani, J.M., Connor, K.T., and Paustenbach, D.J. (1994): Use of probabilistic methods to understand the conservatism in California's approach to assessing health risks posed by air contaminants. *J. Air Waste Manage. Assoc.*, 44:1399–1413.
75. Costa, M., Zhitkovich, A., Harris, M., Paustenbach, D., and Gargas, M. (1997): DNA-protein crosslinks produced by various chemicals in cultured human lymphoma cells. *J. Toxicol. Environ. Health*, 30:101–116.
76. Cox, L.A., Jr. (1996): More accurate dose-response estimation using Monte Carlo uncertainty analysis: The data cube approach. *Hum. Ecol. Risk Assess.*, 2:150–174.
77. Cronin, W.J., Oswald, E.J., Shelley, M.L., Fisher, J.W., and Fleming, C.D. (1995): A trichloroethylene risk assessment using a Monte Carlo analysis of parameter uncertainty in conjunction with physiologically-based pharmacokinetic modeling. *Risk Anal.*, 15:555–566.
78. Crouch, E.A.C. (1996): Uncertainty distributions for cancer potency factors: Combining epidemiological studies with laboratory bioassays—The example of acrylonitrile. *Hum. Ecol. Risk Assess.*, 2:130–149.
79. Crouch, E.A.C. (1996): Uncertainty distributions for cancer potency factors: Laboratory animal carcinogenicity and interspecies extrapolation. *Hum. Ecol. Risk Assess.*, 2:103–129.
80. Crump, K.S. (1998): On summarizing group exposures in risk assessment: Is an arithmetic mean or a geometric mean more appropriate? *Risk Anal.*, 18:293–297.
81. Cullen, A.C. (1994): Measures of compounding conservatism in probabilistic risk assessment. *Risk Anal.*, 14(4):389–393.
82. Cullen, A.C., and Frey, H.C. (1999): *Probabilistic techniques in exposure assessment.* Plenum Press, New York.
83. Daisey, J.M., Hodgson, A.T., Fish, W.J., Mendell, M.J., and Ten Brinke, J. (1994): Volatile organic compounds in 12 California office buildings: Classes, concentrations, and sources. *Atmos. Environ.*, 28(22):3557–3562.
84. Danford, D.C. (1982): Pica and nutrition. *Annu. Rev. Nutr.*, 2:303–322.
85. Davis, A., Ruby, M.V., and Bergstrom, P.D. (1992): Bioavailability of arsenic and lead from the Butte, Montana, mining district. *Environ. Sci. Technol.*, 26:461–468.
86. Davis, A., Drexter, J.W., Ruby, M.V., and Nicholson, A. (1993): Micromineralogy of mine waste in relation to lead bioavailability, Butte, Montana. *Environ. Sci. Technol.*, 27:1415–1425.
87. Davis, A., Bloom, N.S., and Que Hee, S.S. (1997): The environmental geochemistry and bioaccessability of mercury in soils and sediments: A review. *Risk Anal.*, 17:557–569.
88. Day, J.P., Hart, M., and Robinson, M.S. (1975): Lead in urban street dust. *Nature (Lond.)*, 253:343–345.

89. DeCaprio, A.P. (1997): Biomarkers: Coming of age for environmental health and risk assessment. *Environ. Sci. Technol.*, 31(7):1837–1848.
90. de Silva, P.E. (1991): Assessment of Health Risk to Residents of Contaminated Sites. AMCOSH, Occupational Health Services Report to Gas and Fuel Corporation, Melbourne, Australia.
91. de Silva, P.E. (1994): How much soil do children ingest—A new approach. *Appl. Occup. Environ. Hyg.*, 9:40–43.
92. Delzell, E., Sathiakumar, N., Hovinga, M., Macaluso, M., Julian, J., Larson, R., Cole, P., and Muir, D.C. (1996): A follow-up study of synthetic rubber workers. *Toxicology*, 113(13):182–189.
93. Dockery, D.W., Schwartz, J., and Spengler, J.D. (1992): Air pollution and daily mortality: Associations with particulates and acid aerosols. *Environ. Res.*, 59:362–73.
94. Dragun, J. (1998): *The soil chemistry of hazardous materials*, 2nd ed. Amherst Scientific, Amherst, MA.
95. Dragun, J., and Chiasson, A. (1991): *Elements in North American soil.* Hazardous Materials Control Resources Institue, Greenbelt, MD.
96. Driver, J.H., Konz, J.J., and Whitmyre, G.K. (1989): Soil adherence to human skin. *Bull. Environ. Contam. Toxicol.*, 17(9):1831–1850.
97. Duan, N. (1991): Stochastic microenvironmental models for air pollution exposure. *J. Exp. Anal. Environ. Epidemiol.*, 1(2):235–257.
98. Duan, N., and Mage, D.T. (1997): Combination of direct and indirect approaches for exposure assessment. *J. Exp. Anal. Environ. Epidemiol.*, 7(4):439–470.
99. Duggan, M.J., and Williams, S. (1977): Lead-in-dust in city streets. *Sci. Total Environ.*, 7:91–97.
100. Dutkiewicz, T., and Piotrowski, J. (1961): Experimental investigations on the quantitative estimation of aniline absorption in man. *Pure Appl. Chem.*, 3:319–323.
101. Dutkiewicz, T., and Tyras, H. (1967): A study of the skin absorption of ethylbenzene in man. *Br. J. Ind. Med.*, 24:330–332.
102. Ebert, E., Harrington, N., Boyle, K., Knight, J., and Keenan, R. (1993): Estimating consumption of freshwater fish among Maine anglers. *North Am. J. Fisheries Manage.*, 13:737–745.
103. Ebert, E.S., Price, P.S., and Keenan, R.E. (1994): Selection of fish consumption estimates for use in the regulatory process. *J. Expos. Anal. Environ. Epidemiol.*, 4:373–394.
104. Editor. (2000): Pollution monitoring should get personal, scientists say. *Environment. Sci. Tech.*, February 1, pp. 64–65.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_439.html[2010/10/28 18:44:13]

105. Ehrenberg, L., and Osterman-Golkar, S. (1980): Alkylation of macromolecules for detecting mutagenic agents. *Teratogen. Carcinogen. Mutagen.* 1(1):105–127.
106. Eisenbud, M. (1978): *Environment, technology, and health: Human ecology in historial perspective.* New York University Press, New York.
107. El Saadi, O., and Langley, A. (1994): The health risk assessment and management of contaminated sites. *Proc. National Workshop on the Health Risk Assessment and Management of Contaminated Sites.* South Australian Health Commission, Adelaide.
108. Ershow, A.G., and Cantor, K.P. (1989): *Total tapwater intake in the United States: Population-based estimates of quantities and sources.* Life Sciences Research Office, Federation of American Societies for Experimental Biology, Bethesda, MD.
109. European Center for Ecotoxicology and Toxicology of Chemicals (ECETOC). (1993): Percutaneous absorption. Monograph 20. Brussels: European Center for Ecotoxicology and Toxicology of Chemicals.
110. European Center for Ecotoxicology and Toxicology of Chemicals (ECETOC). (1993): *Strategy for assigning a "skin notation."* Revised ECETOC Document 31. European Center for Ecotoxicology and Toxicology of Chemicals, Brussels.
111. Evans, J.S., Graham, J.D., Gray, G.M., and Sielken, R.L., Jr. (1994): A distributional approach to characterizing low-dose cancer risks. *Risk Anal.,* 14:25–33.
112. Evans, J.S., Gray, G.M, Sielken, R.L., Jr., Smith, A.E., Valdez-Flores, C., and Graham, J.D. (1994): Use of probabilistic expert judgment in uncertainty analysis of carcinogenic potency. *Regul. Toxicol. Pharmacol.,* 20:15–36.
113. Feldmann, R.J., and Maibach, H.I. (1974): Percutaneous penetration of some pesticides and herbicides in man. *Toxicol. Appl. Pharmacol.* 28:126–132.
114. Fenske, R.A. (1993): Dermal exposure assessment techniques. *Ann. Occup. Hyg.,* 37:687–706.
115. Fenske, R., and Lu, C. (1994): Determination of handwash removal efficiency: Incomplete removal of chloropyrifos from skin by standard handwash techniques. *Am. Ind. Hyg. Assoc. J.,* 55:425–432.
116. Finley, B., Kirman, C., Scott, P., Spivack, A., Bernhardt, T., Warmerdam, J., and Pittignano, A. (2000): A probabilistic risk assessment of a PCB-contaminated waterway: A case study. *J. Soil Contam.,* in press.
117. Finley, B.L., and Paustenbach, D.J. (1994): The benefits of probabilistic exposure assessment: Three case studies involving contaminated air, water, and soil. *Risk Anal.,* 14(l):53–73.
118. Finley, B.L., Scott, P., and Paustenbach, D.J. (1993): Evaluating the adequacy of maximum contaminant levels as health protective cleanup goals: An analysis based on Monte Carlo techniques. *Regul. Toxicol. Pharmacol.,* 18:438–455.
119. Finley, B.L., Scott, P.K., and Mayhall, D.A. (1994): Development of a standard soil-to-skin adherence probability density function for use in Monte Carlo analyses of dermal exposure. *Risk Anal.,* 14:555–569.
120. Finley, B.L., Proctor, D., Scott, P., Harrington, N., Paustenbach, D., and Price, P. (1994): Recommended distributions for exposure factors frequently used in health risk assessment. *Risk Anal.,* 14(4):533–553.
121. Fitzgerald, E.F., Hwang, S.-A., Brix, K.A., Bush, B., Cook, K., and Worswick, P. (1995): Fish PCB concentrations and consumption patterns among Mohawk women at Akwesasne. *J. Expos. Anal, Environ. Epidemiol.,* 5:1–20.
122. Flynn, G.L. (1990): Physicochemical determinants of skin absorption. In *Principles of route-to-route extrapolation for risk assessment,* edited by T.R.Gerrity and C.J.Henry, pp. 93–127. New York: Elsevier.
123. Frantz, S.W. (1990): Instrumentation and methodology for *in vitro* skin diffusion cells. In *Methods for skin absorption,* edited by B.W. Kemppainen and W.G.Reifenrath, pp. 35–59. CRC Press, Boca Raton, FL.
124. Frey, H.C., and Rhodes, D.S. (1998): Characterization and simulation of uncertainty frequency distributions: Effects of distribution choice, variability, uncertainty, and parameter dependence. *Hum. Ecol. Risk Assess.,* 4:423–469.
125. Fries, G.F., and Paustenbach, D.J. (1990) Evaluation of potential transmission of 2,3,7,8-tetrachlorodibenzo-p-dioxin-contammated incinerator emissions to humans via foods. *J. Toxicol. Environ. Health,* 29:1–43.
126. Fries, G.F., Paustenbach, D.J., Mathur, D.B., and Luksemburg, W.J. (1999): A congener specific evaluation of transfer of chlorinated dibenzo-p-dioxins and dibenzofurans to milk cows following ingestion of pentachlorophenol-treated wood. *Environ. Sci. Technol.,* 33(8): 1165–1170.
127. Gallacher, J.E., Elwood, P.C., Phillips, K.M., Davies, B.E., and Jones, D.T. (1984): Relation between

pica and blood lead in areas of differing lead exposure. *Arch. Dis. Child.*, 59:40–44.
128. Gargas, M.L., Burgess, R.J., Voisard, D.E., Cason, G.H., and Andersen, M.E. (1989): Partition coefficients of low molecular weight volatile chemicals in various liquids and tissues. *Toxicol. Appl. Pharmacol.*, 98:87–99.
129. Gargas, M.L., Finley, B.L., Paustenbach, D.J., and Long, T.F. (1999): Environmental health risk assessment: Theory and practice. In *General and applied toxicology*, vol. 3, eds. B. Ballantyne, T.Marrs, and T.Syversen, 2nd ed., pp. 1749–1809. Macmilllan, London.
130. Georgopoulos, P.G., and Lioy, P.J. (1994): Conceptual and theoretical aspects of human exposure and dose assessment. *J. Exp. Anal. Environ. Epidemiol.*, 4:253–285.
131. Gesell, T.F., and Prichard, H.M. (1980): The contribution of radon in tap water to indoor radon concentrations. In *Natural radiation environment III*, vol. 2, edited by T.F.Gesell and W. M.Lowder, pp. 1347–1363. Department of Energy, Washington, DC. CPMF-780422.
132. Gilbert, R.O. (1987): *Statistical methods for environmental pollution monitoring.* Van Nostrand Reinhold, New York.
133. Glickman, T.S. (1986): A methodology for estimating time-of-day variations in the size of a population exposed to risk. *Risk Anal.*, 6:317–323.
134. Goldstein, B. (1995): Risk management will not be improved by mandating numerical uncertainty analysis for risk assessment. *University of Cincinnati Law Review*, 63:1599–1610.
135. Gómez, M.R. (1997): Factors associated with exposure in occupational safety and health administration data. *Am. Ind. Hyg. Assoc. J.*, 58(3): 186–195.
136. Gómez, M.R. (1997): Recommendations for optimizing the usefulness of existing exposure databases for public health applications. *Am. Ind. Hyg. Assoc. J.*, 58(3): 181–182.
137. Gómez, M.R. (2000): Exposure assessment must stop being local. *Appl. Occup. Environ. Hyg.*, 15(1): 15–20.
138. Gómez, M.R., and Rawls, G.R. (1995): Conference on occupational exposure databases: A report and look at the future. *Appl. Occup. Environ. Hyg.*, 10(4):238–243.
139. Goodman, M., Paustenbach, D., Sipe, K., Malloy, C.D., Chapman, P., Figueroa, R., Zhao, K., and Exuzides, K.A. (2000): Epidemiologic study of pulmonary obstruction in workers occupationally exposed to ethyl and methyl cyanoacrylate. J. *Toxicol. Environ. Health A*, 59:135–163.
140. Graham, J.D., Green, L., and Roberts, M.J. (1988): *In search of safety: Chemicals and cancer risks*, pp. 80–114. Harvard University Press, Cambridge, MA.

141. Graham, J., Berry, M., Bryan, E.F., Callahan, M.A., Fan, A., Finley, B., Lynch, J., McKone, T., Ozkaynak, H., Sexton, K., and Walker, K. (1992): The role of exposure databases in risk assessment. *Arch. Environ. Health*, 47:408–420.
142. Guy, R.H., Hadgraft, J., and Maibach, H.I. (1982): A pharmacokinetic model for percutaneous absorption. *Int. J. Pharmacol.*, 11:119–129.
143. Haas, C.N. (1997): Importance of the distributional form in characterizing inputs to Monte Carlo risk assessments. *Risk Anal.*, 17:107–113.
144. Haas, C.N., and Scheff, P.A. (1990): Estimation of averages in truncated samples. *Environ. Sci. Technol.*, 24:912–919.
145. Hallock, M.F., Smith, T.J., Woskie, S.R., and Hammond, S.K. (1994): Estimation of historical exposures to machining fluids in the automotive industry. *Am. J. Ind. Med.*, 26:621–634.
146. Hamed, M.M., and Bedient, P.B. (1997): On the effect of probability distributions of input variables in public health risk assessment. *Risk Anal.*, 17:97–105.
147. Hattis, D.B. (1986): The promise of molecular epidemiology for quantitative risk assessment. *Risk Anal.*, 6(2):181–194.
148. Hattis, D., and Burmaster, D. (1994): Assessment of variability and uncertainty distributions for practical risk analyses. *Risk Anal*., 14:713–729.
149. Hawley, J.K. (1985): Assessment of health risk from exposure to contaminated soil. *Risk Anal.*, 5(4):289–302.
150. Helsel, D.R. (1990): Less than obvious: statistical treatment of data below the detection limit. *Environ. Sci. Technol.*, 24:1766–1774.
151. Hewitt, D.J., Millner, G.C., Nye, A.C., Webb, M., and Huss, R. G. (1995): Evaluation of residential exposure to arsenic in soil near a Superfund site. *Hum. Ecol. Risk Assess.*, 1:323–335.
152. Hill, R.A., and Hoover, S.M. (1997): Importance of the dose-response model form in probabilistic risk assessment: A case study of health effects from methylmercury in fish. *Hum. Ecol. Risk Assess.*, 3:465–481.
153. Hoffman, F.O., and Hammonds, J.S. (1992): *An introductory guide to uncertainty analysis in environmental and health risk assessment.* Martin Marietta. ES/ER/TM-35.
154. Hoffman, F.O., and Hammonds, J.S. (1994): Propagation of uncertainty in risk assessments: The need to distinguish between uncertainty due to lack of knowledge and uncertainty due to variability. *Risk Anal.*, 14:707–711.
155. Holdway, D.A. (1996): The role of biomarkers in risk assessment. *Hum. Ecol. Risk Assess.*, 2:263–267.
156. Holmes, K.K., Kissel, J.C., and Richter, K.Y. (1996): Investigation of the influence of oil on soil adherence to skin. *J. Soil Contam.*, 5(4):301–308.
157. Horowitz, S.B., and Finley, B.L. (1993): Using human sweat to extract chromium from chromite ore processing residue: Applications to setting health-based cleanup levels. *J.Toxicol. Environ. Health*, 40:585–599.
158. Horwitz, W. (1984): Effects of scientific advances on the decision-making process: Analytical chemistry. *Fundam. Appl. Toxicol.*, 4:S309-S317.
159. Hrudey, S.E., Chen, W., and Rousseaux, C. (1996): *Bioavailability.* CRC-Lewis Publishers, New York.
160. Iman, R.L., and Helton, J.C. (1991): The repeatability of uncertainty and sensitivity analyses for complex probabilistic risk assessments. *Risk Anal.*, 11:591–606.
161. International Life Science Institute (ILSI). (1998): *Aggregate exposure assessment.* Washington, DC.
162. Israeli, M., and Nelson, C.B. (1992): Distribution and expected time of residence for U.S. households. *Risk Anal.*, 12:65–72.
163. Jayjock, M.A. (1997): Uncertainty analysis in the evaluation of exposure. *Am. Ind. Hyg. Assoc. J.*, 58(5):380–382.
164. Jayjock, M.A. (1998): Risk assessment of contact allergens. *Am. J. Contact Dermatitis*, 9(3): 155161.
165. Jayjock, M.A., Hazelton, G.A., Lewis, P.G., and Wooder, M.F. (1996): Formulation effect on the dermal bioavailability of isothiazolone biocide. *Food Chem. Toxicol.*, 34(3):277–282.
166. Jayjock, M.A., Lynch, J.R., and Nelson, D.I. (2000): *Risk assessment: Principles for the industrial hygienist.* ACGIH Press, Cincinnati, OH.
167. Jenkins, P.L., Phillips, T.J., Mulberg, E.J., and Hui, S.P. (1992): Activity patterns of Californians:

Use of and proximity to indoor pollutant sources. *Atmos. Environ.*, 26A(12):2141–2148.
168. Jo, W.K., Weisel, C.P., and Lioy, P.J. (1988): Routes of chloroform exposure and body burden from showering with chlorinated tap water. *Risk Anal.*, 10:575–580.
169. Johnson, J.E., and Kissel, J.C. (1996): Prevalence of dermal pathway dominance in risk assessment of contaminated soils: A survey of Superfund risk assessments, 1989–1992. *Hum. Ecol. Risk Assess.*, 2:356–365.
170. Johnson, T.R. (1995): Recent advances in the estimation of population exposure to mobile source pollutants. *J. Expos. Anal. Environ. Epidemiol.*, 5(4):551–571.
171. Kerger, B., and Paustenbach, D. (2000): Exposure to 1,1,1 TCE vapors in a home due to contaminated groundwater. *Risk Anal.*, in press.
172. Kerger, B.D., Stabile, L, and Copeland, T.L. (1999): *Mass balance problems with the USEPA model for estimating dioxin dose via breast milk.* Society for Risk Analysis Annual Meeting, Atlanta, GA, 3–7 December.
173. Kezic, S., Mahieu, K., Monster, A.C., and de Wolff, F.A. (1997): Dermal absorption of vaporous and liquid 2-methoxyethanol and 2-ethoxyethanol in volunteers. *Occup. Environ. Med.*, 54:38–43.
174. Kimbrough, R.D., Falk, H., Stehr, P., and Fries, G.F. (1984): Health implications of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) contamination of residential soil. *J. Toxicol. Environ. Health*, 14:47–93.
175. Kissel, J., and Fenske, R. (2000): Improved estimation of dermal pesticide dose to agricultural workers upon reentry. *Appl. Occup. Environ. Hyg.* 15(3):284–290.
176. Kissel, J.C., Richter, K.Y., and Fenske, R.A. (1996): Factors affecting soil adherence to skin in hand-press trails. *Bull. Environ. Contam. Toxicol.* 56:722–728.
177. Kissel, J.C., Richter, K.Y., and Fenske, R.A. (1996): Field measurement of dermal soil loading attributable to various activities: Implications for exposure assessment. *Risk Anal.*, 16(1):115–125.
178. Klain, G.J., and Black, K.E. (1990): Specialized techniques: Congenitally athymic (nude) animal models. In *Methods for skin absorption*, edited by B.W.Kemppainen and W.G.Reifenrath, pp. 165–174. CRC Press, Boca Raton, FL.
179. Knaak, J.B., Iwata, Y., and Maddy, K.T. (1989): The worker hazard posed by reentry into pesticide-treated foliage: Development of safe reentry times, with emphasis on chlorhiophos and carbosulfan. In *The risk assessment of environmental hazards; a textbook of case studies*, edited by D.J.austenbach, pp. 797–842. John Wiley and Sons, New York.
180. Knaak, J.B., Dary, C.C., Patterson, G., and Blancato, J.N. (2001): The worker hazard posed by reentry into pesticide-treated foliage: Reassessment of reentry intervals using foliar residue transfer-percutaneous absorption PB-PK/PD models, with emphasis on isofenphos and parathion. In *Human and ecological risk assessment: Theory and practice*, edited by D.J.Paustenbach. J.Wiley and Sons, New York.
181. Knarr, R.D., Cooper, G.L., Brian, E.A., Kleinschmidt, M.G., and Graham, D.G. (1985): Worker exposure during aerial application of a liquid and a granular formulation of ordram selective herbicide to rice. *Arch. Environ. Contam. Toxicol.*, 14:523–527.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_441.html[2010/10/28 18:44:15]

182. Kohler, L., Meeuwisse, G., and Mortensson, W. (1984): Food intake and growth of infants between six and twenty-six weeks of age on breast milk, cow's milk formula, and soy formula. *Acta Paediatr. Scand.*, 73:40–48.
183. Krieger, R.I., Ross, J.H., and Thongsinthusak, T. (1992): Assessing human exposures to pesticides. *Rev. Environ. Contam. Toxicol.*, 128:1–15.
184. Krivanek, N.D., McLaughlin, M., and Fayweather, W.E. (1978): Monomethylformide levels in human urine after repetitive exposure to dimethylformamide. *J. Occup. Med.*, 20:179–187.
185. Kreuzer, P.E., Csanady, G.Y., Baur, C., Kessler, W., Papke, O., Greim, H., and Filser, J.Y. (1997): 2, 3, 7, 8 Tetrachlorodibenzo-p-dioxin (TCDD) and congeners in infants:A toxicokinetic model of human lifetime body burdens. *Arch. Toxicol.*, 71:383–400.
186. La Goy, P.K. (1987): Estimated soil ingestion rates for use in risk assessment. *Risk Anal.*, 7(3):355–359.
187. Lavy, T., Shepard, J., and Bouchard, D. (1980): Field worker exposure and helicopter spray pattern of 2,4,5-T. *Bull. Environ. Contam. Toxicol.*, 24(l):90–96.
188. Lavy, T., Walstad, J., Flynn, R., and Mattice, J. (1982): (2,4-Dichlorophenoxy)acetic acid exposure received by aerial application crews during forest spray operations. J. *Agric. Food Chem.*, 30:375–361.
189. Lehmann, A.J., and Fitzhugh, O.G. (1954): 100-fold margin of safety. *Q. Bull. Assoc. U.S. Food & Drug Administration*, 18:33.
190. Lepow, M.L., Bruckman, L., Robino, R.A., Markowitz, S., Gillette, M., and Kapish, J. (1974): Role of airborne lead in increased body burden of lead in Hartford children. *Environ. Health Perspect.*, 6:99–101.
191. Lepow, M.L., Bruckman, L., Gillette, M., Markowitz, S., Robino, R., and Kapish, J. (1975): Investigations into sources of lead in the environment of urban children. *Environ. Res.*, 10:415–426.
192. Leung, H.W., and Paustenbach, D.J. (1994): Techniques for estimating the percutaneous absorption of chemicals due to environmental and occupational exposure. *Appl, Environ. Occup. Hyg.*, 9(3):187–197.
193. Leung, H.W., and Paustenbach, D.J. (1995): Physiologically based pharmacokinetic and pharmacodynamic modeling in health risk assessment and characterization of hazardous substances. *Toxicol. Lett.*, 79:55–65.
194. Lilly, P.D., Andersen, M.E., Ross, T.M., and Pegram, R.A. (1998): A physiologically based pharmacokinetic description of the oral uptake, tissue dosimetry, and rates of metabolism of bromodichloromethane in the male rat. *Toxicol. Appl. Pharmacol.*, 150(2):205–217.
195. Lioy, P., Waldman, J.M., Greenberg, A., Harkov, R., and Pietarinen, C. (1988): The Total Human Environmental Exposure Study (THEES) to Benzo(a)pyrene: Comparison of the inhalation and food pathways. *Arch. Environ. Health*, 43(4):304–312.
196. Lioy, P., Waldman, J.M., Buckley, T., Butler, J., and Pietarinen, C. (1990): The personal, indoor, and outdoor concentrations of PM-10 measured in an industrial community during the winter. *Atmos. Environ.*, 24B(1):57–60.
197. Lioy, P.J., Wainman, T., and Weisel, C. (1993): A wipe sampler for the quantitative measurement of dust on smooth surfaces: Laboratory performance studies. *J. Exp. Anal. Environ. Epidemiol.*, 3:315–320.
198. Lioy, P.J., Yiin, L.M., Adgate, J., Weisel, C., and Rhoads, G.G. (1998): The effectiveness of a home cleaning intervention strategy in reducing potential dust and lead exposures. *J. Exp. Anal. Environ. Epidemiol.*, 8(1): 17–35.
199. Lipton, J., Shaw, W.D., Holmes, J., and Patterson, A. (1995): Short communication: Selecting input distributions for use in Monte Carlo analysis. *Regul. Toxicol. Pharmacol.*, 21:192–198.
200. Lotens, W.A., and Wammes, L.J.A. (1993): Vapour transfer in two-layer clothing due to diffusion and ventilation. *Ergonomics*, 36(10):1223–1240.
201. Lourie, R.S., and Cayman, E.M. (1963): Why children eat things that are not food. *Children*, 10:143–146.
202. Lucier, G.W., and Schecter, A. (1998): Human exposure assessment and the national toxicology program. *Environ. Health Perspect.*, 106:623–626.
203. Lynch, A.L. (1994): *Biological monitoring.* Wiley, New York.
204. Lynch, J.R. (1985): Measurement of worker exposure. In *Patty's industrial hygiene and toxicology*, vol. 3a, *The work environment*, eds. L.J.Cralley and L.V.Cralley, 2nd ed., pp. 569–615. Wiley-Interscience, New York.
205. Mage, D., Wilson, W., Hasselblad, V., and Grant, L. (1999): Assessment of human exposure to ambient particulate matter. *J. Air Waste Manage.*, 49:174–183.

206. Marlow, D., Sweeney, M.H., and Fingerhut, M. (1990): *Estimating the amount of TCDD absorbed by workers who manufactured 2,4,5-T.* Tenth Annual International Dioxin Meeting, Bayreuth, Germany.
207. Martin, W.E. (1964): Loss of Sr-90, Sr-89 and I-131 from fallout of contaminated plants. *Radiat. Bot.*, 4:275–281.
208. Mattie, D.R., Bates, G.D., Jr., Jepson, G.W., Fisher, J.W., and McDougal, J.N. (1994): Determination of skin: Air partition coefficients for volatile chemicals: Experimental method and applications. *Fundam. Appl. Toxicol.*, 22:51.
209. Maxim, D. (1989): Problems associated with the use of conservative assumptions in exposure and risk analysis. Chapter 14. In *The risk assessment of environmental and human health hazards: A textbook of case studies*, edited by D.J.Paustenbach, pp. 526–560. J.Wiley and Sons, New York.
210. McArthur, B. (1992): Dermal measurement and wipe sampling methods: A review. *Appl. Occup. Environ. Hyg.*, 7:599–606.
211. McBride, S.G., Ferro, A., Ott, W., Switzer, P., and Hildemann, L. (1999): Investigation of the proximity effect for pollutants in the indoor environment. *J. Exp. Anal. Environ. Epidemiol.*, 9(6):602–621.
212. McCord, C.P. (1943): *Industrial hygiene for engineers.*, Chicago: Martin Press.
213. McDougal, J.N. (1996): Physiologically-based pharmacokinetic modeling. In *Dermatoxicology*, edited by F.N.Marzulli and H.I.Maibach. Taylor and Francis, Washington, DC.
214. McDougal, J.N., Jepson, G.W., Clewell H.J. III, Gargas, M.L., and Andersen, M.E. (1990): Dermal absorption of organic chemical vapors in rats and humans. *Fundam. Appl. Toxicol.*, 14:299–308.
215. McKone, T.E. (1990): Dermal uptake of organic chemicals from a soil matrix. *Risk Anal.*, 10:407–419.
216. McKone, T.E. (1991): Human exposure to chemicals from multiple media and through multiple pathways: Research overview and comments. *Risk Anal.*, 11(1):5–10.
217. McKone, T.E. (1993): Linking a PB-PK model for chloroform with measured breath concentrations in showers: Implications for dermal exposure models. *J. Exp. Anal. Environ. Epidemiol.*, 3:339–365.
218. McKone, T.E., and Bogen, K.T. (1991): Predicting the uncertainties in risk assessment. *Environ. Sci. Technol.*, 25:16–74.
219. McKone, T.E., and Bogen, K.T. (1992): Uncertainties in health-risk assessment: An integrated case study based on tetrachloroethylene in California groundwater. *Regul. Toxicol. Pharmacol.*, 15:86–103.
220. McMillan, A., Whittemore, A.S., Silvers, A., and DiCiccio, Y. (1994): Use of biological markers in risk assessment. *Risk Anal.*, 14(5):807–813.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_442.html[2010/10/28 18:44:15]

221. Mertz, C.K., Slovic, P., and Purchase, L.F.H. (1998): Judgments of chemical risks: Comparisons among senior managers, toxicologists, and the public. *Risk Anal.*, 18:391–403.
222. Michaud, J.M., Huntley, S.L., Sherer, R.A., Gray, M.N., and Paustenbach, D.J. (1994): PCB and dioxin re-entry criteria for building surfaces and air. J. *Exp. Anal. Environ. Epidemiol.*, 4(2): 197–227.
223. Morgan, M.D., and Henrion, M. (1990): *Uncertainty: A guide to dealing with uncertainty in quantitative risk and policy analysis.* Cambridge University Press, Cambridge.
224. Morgan, J.N., Berry, M.R., and Graves, R.L. (1997): Effects of commonly used cooking practices on total mercury concentration in fish and their impact on exposure assessments. *J. Exp. Anal. Environ. Epidemiol.*, 7:119–133.
225. Mraz, J., and Nohova, M. (1992): Percutaneous absorption of N, N-dimethylformamide in humans. *Int. Arch. Occup. Environ. Health*, 64:79–83.
226. Murray, D.M., and Burmaster, D.E. (1992): Estimated distributions for total body surface area of men and women in the United States. J. *Exp. Anal. Environ. Epidemiol.*, 2:451–462.
227. Murray, D.M., and Burmaster, D.E. (1994): Estimated distributions for average daily consumption of total and self-caught fish for adults in Michigan angler households. *Risk Anal.*, 14:513–520.
228. National Academy of Public Administration (NAPA). (1995): Setting Priorities, Getting Results: A New Direction for EPA. Washington, DC.
229. National Academy of Sciences. (1983): *Risk assessment in the federal government: Managing the process.* National Academy Press, Washington, DC.
230. National Academy of Sciences. (1991): *Nutrition during lactation.* National Academy Press, Washington, DC.
231. National Committee on Radiation Programs. (1996): *A guide for uncertainty analysis in dose and risk assessments related to environmental contamination.* Commentary No. 14. National Committee on Radiation Programs, Scientific Committee, Washington, DC.
232. National Research Council. (1974): *Lead in the environment.* National Academy Press, Washington, DC.
233. National Research Council. (1987): Biological markers in environmental health research. Committee on Biological Markers of the National Research Council. *Environ. Health Perspect.*, 74:39.
234. National Research Council. (1989): *Improving risk communication.* National Academy Press, Washington, DC.
235. National Research Council. (1990): *Human exposure assessment for airborne pollutants: Advances and applications.* Committee on Advances in Assessing Human Exposure to Airborne Pollutants, Committee on Geosciences, Environment, and Resources, NRC. National Academy Press, Washington, DC.
236. National Research Council. (1994): *Science and judgment in risk assessment.* National Academy Press, Washington, DC.
237. National Research Council. (1994): *Building consensus through risk assessment.* National Academy Press, Washington, DC.
238. National Research Council. (1996): *Understanding risk: Informing decisions in a democratic society.* National Academy Press, Washington, DC.
239. Nessel, C.S., Butler, J.P., Post, G.B., Held, J.I., Gochfeld, M., and Gallo, M.A. (1991): Evaluation of the relative contribution of exposure routes in a health risk assessment of dioxin emissions from a municipal waste incinerator. *J. Expos. Anal. Environ. Epidemiol.*, 1:283–308.
240. Nethercott, J., Paustenbach, D.J., Adams, R., Horowitz, S., Finley, B.E., Fowler, J., Marks, J., Morton, C., and Taylor, J. (1994): A study of chromium induced allergic contact dermatitis with 54 volunteers: Implications for environmental risk assessment. *Occup. Environ. Med.*, 51(6):371–380.
241. Neville, M.C., Keller, R., Seacat, J., Lutes, V., Neifert, M., Casey, C., Allen, J., and Archer, P. (1988): Studies in human lactation: Milk volumes in lactating women during the onset of lactation and full lactation. *Am. J. Clin. Nutr.*, 48:1375–1386.
242. Nichols, A.L., and Zeckhauser, R.J. (1988): The perils of prudence: How conventional risk assessments distort regulations. *Regul. Toxicol. Pharmacol.*, 8:61–75.
243. Nriagu, J. (1979): *Heavy metals in the environment.* John Wiley and Sons, New York.
244. Ott, W., Thomas, J., Mage, D., and Wallace, L. (1988): Validation of the Simulation of Human Activity and Pollutant Exposure (SHAPE) model using paired days from the Denver, CO, carbon monoxide field study. *Atmos. Environ.*, 22:249–267.
245. Ott, W.R. (1995): *Environmental statistics and data analysis.* CRC Lewis, Boca Raton, FL.
246. Ott, W.R. (1995): Human exposure assessment: the birth of a new science. *J. Expos. Anal.*

Environ. Epidemiol., 5(4):449–472.
247. Ott, W.R., and Roberts, J.W. (1998): Everyday exposure to toxic pollutants. *Sci. Am.*, 278:86–91.
248. Özkaynak, H., Xue, J., Spengler, J., Wallace, L., Pellizzari, E., and Jenkins, P. (1996): Personal exposure to particles and metals: Results from the particle TEAM study in Riverside, CA. *J. Expos. Anal. Environ. Epidemiol.*, 26(1):57–78.
249. Pao, E.M., Fleming, K.H., Guenther, P.M., and Mickle, S.J. (1982): *Foods Commonly Eaten by Individuals: Amount per Day and per Eating Occasion.* Home Economics Report No. 44. U.S. Department of Agriculture, Beltsville, MD.
250. Park, C.N., and Snee, R.D. (1983): Quantitative risk assessment: State-of-the-art for carcinogenesis. *Fundam. Appl. Toxicol.*, 3:320–333.
251. Parkin, T.B., Melsinger, J.J., Chester, S.T., Starr, J.L., and Robinson, J.A. (1988): Evaluation of statistical estimation methods for lognormally distributed variables. *Soil Sci. J.*, 52:323–329.
252. Paustenbach, D.J. (1987): Assessing the potential environmental and human health risks of contaminated soil. *Comments Toxicol.*, 1:185–220.
253. Paustenbach, D.J. (1988): Assessment of the developmental risks resulting from occupational exposure to select glycol ethers within the semiconductor industry. *J. Toxicol Environ. Health*, 23:29–75.
254. Paustenbach, D.J. (1989): A survey of environmental risk assessment. In *The risk assessment of environmental and human health hazards: A textbook of case studies*, edited by D.J. Paustenbach, pp. 139. J. Wiley and Sons, New York.
255. Paustenbach, D.J. (1989): *The risk assessment of environmental hazards: A textbook of case studies.* John Wiley & Sons, New York.
256. Paustenbach, D.J. (1990): Health risk assessment and the practice of industrial hygiene. *Am. Ind. Hyg. Assoc. J.*, 51(7):339–351.
257. Paustenbach, D.J. (1995): The practice of health risk assessment in the United States (1975–1995): How the U.S. and other countries can benefit from that experience. *Hum. Ecol. Risk Assess.*, 1(1):29–79.
258. Paustenbach, D.J. (2000): Pharmacokinetics and unusual work schedules. In *Patty 's industrial hygiene and toxicology*, Chapter 40, pp. 1787–1901. John Wiley and Sons, New York.
259. Paustenbach, D.J., Shu, H.P., and Murray, F.J. (1986): A critical examination of assumptions used in risk assessment of dioxin contaminated soil. *Regul. Toxicol. Pharmacol.*, 6:284–307.
260. Paustenbach, D.J., Clewell, H.J. III, Gargas, M.L., and Andersen, M.E. (1988): A physiologically-based pharmacokinetic model for carbon tetrachloride. *Toxicol. Appl. Pharmacol.*, 96:191–211.
261. Paustenbach, D.J., Rinehart, W.E., and Sheehan, P.J. (1991): The health hazards posed by chromium-contaminated soils in residen

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_443.html[2010/10/28 18:44:16]

tial and industrial areas: Conclusions of an expert panel. *Regul. Toxicol. Pharmacol.*, 13:195–222.
262. Paustenbach, D.J., Meyer, D.M., Sheehan, P.J., and Lau, V. (1991): An assessment and quantitative uncertainty analysis of the health risks to workers exposed to chromium contaminated soils. *Toxicol. Ind. Health*, 7:159–196.
263. Paustenbach, D.J., Jernigan, J., Bass, R., Kalmes, R., and Scott, P. (1992): A proposed approach to regulating contaminated soil: Identify safe concentrations for seven of the most frequently encountered exposure scenarios. *Regul. Toxicol. Pharmacol.*, 16:21–56.
264. Paustenbach, D.J., Wenning, R.J., Lau, V., Harrington, N.W., Rennix, D.K., and Parsons, A.H. (1992): Recent developments on the hazards posed by 2,3,7,8-tetrachlorodibenzo-p-dioxin in soil: Implications for setting risk-based cleanup levels at residual and industrial sites. *J. Toxicol. Environ. Health*, 36:103–148.
265. Paustenbach, D.J., Price, P.E., Bradshaw, R.D., Ollison, W., Peterson, D., and Blank, C. (1992): Re-evaluation of benzene exposure for the pliofilm workers (1939–1976). *J. Toxicol. Environ. Health*, 36:177–232.
266. Paustenbach, D.J., Bruce, G.M., and Chrostowski, P. (1997): Current views on the oral bioavailability of inorganic mercury in soil: The impact on health risk assessments. *Risk Anal.*, 17:533–545.
267. Paustenbach, D.J., Finley, B.L., and Long, T.F. (1997): The critical role of house dust in understanding the hazards posed by contaminated soils. *Int. J. Toxicol.*, 16:339–362.
268. Paustenbach, D.J., Hays, S., Sururi, S., and Underwood, P. (1997): Comparing the estimated uptake of TCDD using exposure calculations with the actual uptake: A case study of residents of Times Beach, Missouri. *Proc. Int. Dioxin Conf.*, Indianapolis, IN.
269. Paustenbach, D.J., Leung, H.W., and Rothrock, J. (1999): Health risk assessment. In *Occupational skin disease*, edited by R.Adams, 3rd ed., pp. 291–323. W.B.Saunders, Philadelphia, PA.
270. Pellizzari, E.D., Perritt, R.L., and Clayton, C.A. (1999): National human exposure assessment survey (NHEXAS): Exploratory survey of exposure among population subgroups in EPA Region V. *J. Expos. Anal. Environ. Epidemiol.*, 9:4955.
271. Perera, F.P., and Weinstein, I.B. (1982): Molecular epidemiology and carcinogen-DNA adduct detection: New approaches to studies of human cancer causation. *J. Chron. Dis.*, 35(7):581–600.
272. Perkins, J.L., Cutter, G.N., and Cleveland, M.S. (1990): Estimating the mean, variance, and confidence limits from censored (limit of detection), lognormally-distributed exposure data. *Am. Ind. Hyg. Assoc. J.*, 51:416–419.
273. Piotrowski, J.K. (1967): Further investigations on the evaluation of exposure to nitrobenzene. *Br. J. Ind. Med.*, 24:60–65.
274. Piotrowski, J. (1971): Evaluation of exposure to phenol: Absorption of phenol vapor in the lungs and through the skin and excretion of phenol in urine. *Br. J. Ind. Med.*, 28:172–178.
275. Piotrowski, J. (1977): *Exposure tests for organic compounds in industrial toxicology.* National Institute for Occupational Safety and Health, Cincinnati, OH.
276. Plato, N., Krantz, S., Gustavsson, P., Smith, T.J., and Westerholm, P. (1995): A cohort study of Swedish man-made mineral fiber (MMMF) production workers. Part I: Fiber exposure assessment in the rock/slag wool production industry 1938–1990. *Scand. J. Work Environ. Health*, 21:345–352.
277. Popendorf, W.J., and Leffingwell, J.T. (1982): Regulating OP pesticide residues for farmworker protection. In *Residue review 82*, pp. 125–201. Springer-Verlag, New York.
278. Presidential/Congressional Commission on Risk Assessment and Risk Management (CRAM). (1997): *Framework for environmental health risk management, Final report*, Vol. 1, U.S. Government Printing Office, Washington, *DC.*
279. Presidential/Congressional Commission on Risk Assessment and Risk Management (CRAM). (1997): *Risk assessment and risk man-agement in regulatory decision-making, Final report*, Vol. 1, U.S. Government Printing Office, Washington, DC.
280. Price, P.S., Su, S.H., and Gray, M.N. (1994): The effect of sampling bias on estimates of angler consumption rates in creel surveys. *J. Expos. Anal. Environ. Epidemiol.*, 4:355–372.
281. Price, P.S., Su, S.H., Harrington, J.R., and Keenan, R.E. (1996): Uncertainty and variation in indirect exposure assessments: An analysis of exposure to tetrachlorodibenzo-p-dioxin from a beef consumption pathway. *Risk Anal.*, 16:263–277.
282. Price, P.S., Curry, C.L., Goodrum, P.E., Gray, M.N., McCrodden, J.I., Harrington, N.W., Carlson-Lynch, H., and Keenan, R.E. (1996): Monte Carlo modeling of time-dependent exposures using a microexposure event approach. *Risk Anal.*, 16:339–348.

283. Price, P.S., Scott, P.K., Wilson, N.D., and Paustenbach, D.J. (1998): An empirical approach for deriving information on total duration of exposure from information on historical exposure. *Risk Anal.*, 18:611–619.
284. Proctor, D.M., Zak, M.A., and Finley, B.L. 1997. Resolving uncertainties associated with the construction worker soil ingestion rate: A proposal for risk-based remediation goals. *Hum. Ecol. Risk Assess.*, 3:299–304.
285. Puffer, H.W., Azen, S.P., Duda, M.J., and Young, D.R. (1981): Consumption Rates of Potentially Hazardous Marine Fish Caught in the Metropolitan Los Angeles Area. EPA Grant R807 120010.
286. Que Hee, S.S., Peace, B., Clark, C.S., Boyle, J.R., Bornschein, R. L., and Hammond, P.B. (1985): Evolution of efficient methods to sample lead sources, such as house dust and hand dust, in the homes of children. *Environ. Res.*, 38:77–95.
287. Rai, S.N., and Krewski, D. (1998): Uncertainty and variability analysis in multiplicative risk models. *Risk Anal.*, 18:37–45.
288. Ramsey, J., and Andersen, M. (1984): A physiologically based description of the inhalation pharmacokinetics of styrene in rats and humans. *Toxicol. Appl. Pharmacol.*, 73:159–175.
289. Rappaport, S.M. (1985): Smoothing of exposure variability at the receptor—Implications for health standards. *Ann. Occup. Hyg.*, 29:201–214.
290. Rappaport, S.M., and Selvin, J. (1987): A method for evaluating the mean exposure from a lognormal distribution. *Am. Ind. Hyg. Assoc. J.*, 48:374–379.
291. Rappaport, S.M., and Spear, R.C. (1988): Physiological damping of exposure variability during brief periods. *Ann. Occup. Hyg.*, 32:21–33.
292. Reitz, R.H., Gargas, M.L., Andersen, M.E., Provan, W.M., and Green, T.L. (1996): Predicting cancer risk from vinyl chloride exposure with a physiologically based pharmacokinetic model. *Toxicol. Appl. Pharmacol.*, 137:253–267.
293. Rhomberg, L.R. (1997): A survey of methods for chemical risk assessment among federal regulatory agencies. *Hum. Ecol. Risk Assess.*, 3:1029–1196.
294. Ripple, S.R. (1992): Looking back: The use of retrospective health risk assessment. *Environ. Sci. Tech.*, 26:1270–1277.
295. Roach, S.A. (1966): A more rational basis for air-sampling programs. *Am. Ind. Hyg. Assoc. J.*, 27:1–12.
296. Roberts, J.W., and Dickey, P. (1995): Exposure of children to pollutants in house dust and indoor air. *Rev. Environ. Contam. Toxicol.*, 143:59–78.
297. Roberts, J.W., Budd, W.T., Ruby, M.G., Camann, D.E., Fortmann, R.C., Lewis, R.G., Wallace, L.A., and Spittler, T. M. (1992): Human exposure to pollutants in the floor dust of homes and offices. *J. Expos. Anal. Environ. Epidemiol. Suppl.*, 1:127–146.
298. Roberts, J.W., Budd, W.T., Chuang, J., and Lewis, R.G. (1993): Chemical Contaminants in House Dust: Occurrences and Sources.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_444.html[2010/10/28 18:44:17]

EPA/600/A-93/215. U.S. Environmental Protection Agency, Washington, DC.
299. Robinson, R.B., and Hurst, B.T. (1997): Statistical quantificationof the sources of variance in uncertainty analysis. *Risk Anal.*, 17:447–454.
300. Roels, H.A., Buchet, J.P., Lauwenys, R.R., Claeys-Thoreau, F., Lafontaine, A., and Verduyn, G. (1980): Exposure to lead by oral and pulmonary routes of children living in the vicinity of a primary lead smelter. *Environ. Res.*, 22:81–94.
301. Romney, E.M., Lindberg, N.G., Hawthorne, H.A., Bystrom, B. B., and Larson, K.H. (1963): Contamination of plant foliage with radioactive nuclides. *Annu. Rev. Plant Physiol.*, 14:271–279.
302. Roseberry, A.M., and Burmaster, D.E. (1991): A note: Estimating exposure concentrations of lipophilic organic chemicals to humans via finfish. *J. Expos. Anal. Environ. Epidemiol.*, 1:513–521.
303. Roseberry, A.M., and Burmaster, D.E. (1992): Lognormal distributions for water intake by children and adults. *Risk Anal.*, 12:99–104.
304. Ruby, M.V., Davis. A., Kempton, J.H., Drexter, J.W., and Bergstrom, P.D. (1992): Lead bioavailability under simulated gastric conditions. *Environ. Sci. Technol.*, 26:1242–1248.
305. Ruby, M.V., Schoof, R., Brattin, W., Goldade, M., Post, G., Harnois, M., Mosby, D.E., Casteel, S.W., Berti, W., Carpenter, M., Edwards, D., Cragin, D., and Chappell, W. (1999): Advances in evaluating the oral bioavailability of inorganics in soil for use in human health risk assessment. *Environ. Sci. Technol.*, 33(21):3697–3705.
306. Ruffle, B., Burmaster, D.E., Anderson, P.D., and Gordon, H.D. (1994): Lognormal distributions for fish consumption by the general U.S. population. *Risk Anal.*, 14(4):395–404.
307. Ruoff, W.L., Diamond, G.L., Velazquez, S.F., Stiteler, W.M., and Gefell, D.J. (1994): Bioavailability of cadmium in food and water: A case study on the derivation of relative bioavailability factors for inorganics and their relevance to the reference dose. *Regul. Toxicol. Pharmacol.*, 20:139–160.
308. Russell, R.S. (1966): Entry of radioactive materials into plants. In *Radioactivity and human diet*, edited by R.S.Russell, chap. 5. Pergamon Press, New York.
309. Sathiakumar, N., Delzell, E., Hovinga, M., Macaluso, M., Julian, J.A., Larson, R., Cole, P., and Muir, D.C. (1998): Mortality from cancer and other causes of death among synthetic rubber workers. *Occup. Environ. Med.*, 55(4):230–235.
310. Sayre, J.W., Charney, E., Vostal, J., and Pless. B. (1974): House and hand dust as a potential source of childhood lead exposure. *Am. J. Dis. Child.*, 127:167–170.
311. Schoof, R.A., and Nielsen, J.B. (1997): Evaluation of methods for assessing the oral bioavailability of inorganic mercury in soil. *Risk Anal.*, 17:545–555.
312. Scott, P.K., Sung, H., Finley, B.L., Schulze, R.H., and Turner, D. B. (1997): Identification of an accurate soil suspension/dispersion modeling method for use in estimating health-based soil cleanup levels of hexavalent chromium in chromit-ore processing residues. *J. Air Waste Manage.*, 47(7):753–765.
313. Scott, P.K., Harris, M.A., Rabbe, D.E., and Finley, B.L. (1997): Background air concentrations of Cr(VI) in Hudson County, New Jersey: Implications for setting health-based standards for Cr(VI) in soil. *J. Air Waste Manage*. 47:592–600.
314. Scow, K., Wechsler, A.E., Stevens, J., Wood, M., and Callahan, M.A. (1979): Identification and Evaluation of Waterborne Routes of Exposure From Other Than Food and Drinking Water. U.S. Environmental Protection Agency, Washington, DC. EPA/440/4–79/016.
315. Sedman, R., Funk, L.M., and Fountain, R. (1998): Distribution of residence duration in owner occupied housing. *J. Exp. Anal. Environ. Epidemiol.*, 8:51–57.
316. Shatkin, J.A., and Brown, H.S. (1991): Pharmacokinetics of the dermal route of exposure to volatile organic chemicals in water. A computer simulation model. *Environ. Res.*, 56:90–108.
317. Sheppard, S.C., and Evenden, W.G. (1994): Contaminant enrichment and properties of soil adhering to skin. *J. Environ. Qual.*, 23:604–613.
318. Shifrin, N.S., Beck, B.D., Gauthier, T.D., Chapnick, S.D., and Goodman, G. (1996): Chemistry, toxicology, and human health risks of cyanide compounds in soils at former manufactured gas plant sites. *Regul. Toxicol. Pharmacol.*, 23:106–116.
319. Shlyakhter, A.I. (1994): An improved framework for uncertainty analysis: Accounting for unsuspected errors. *Risk Anal.*, 14:441–447.
320. Shlyakhter, A., Goodman, G., and Wilson, R. (1992): Monte Carlo simulation of rodent carcinogenicity bioassays. *Risk Anal.*, 12:73–82.
321. Shu, H., Paustenbach, D., Murray, F.J., Marple, L., Brunck, B., Dei Rossi, D., and Teitelbaum, P. (1988): Bioavailability of soil-bound TCDD: Dermal bioavailability in the rat. *Fundam. Appl. Toxicol.*,

10:648–654.
322. Sielken, R.L., Jr. (1989): Useful tools for evaluating and presenting more science in quantitative cancer risk assessments. *Tox. Subst. J.,* 9:353–404.
323. Sielken, R.L., Jr., and Stevenson, D.E. (1997): Opportunities to improve quantitative risk assessment. *Hum. Ecol. Risk Assess.*, 3:479–490.
324. Sielken, R.L., Jr., and Valdez-Flores, C. (1996): Comprehensive realism's weight-of-evidence based distributional dose-response characterization. *Hum. Ecol. Risk Assess.*, 2:175–193.
325. Simon, T. (1997): Combining physiologically based pharmacokinetic modeling with Monte Carlo simulation to derive an acute inhalation guidance value for trichloroethylene. *Regul. Toxicol. Pharmacol.*, 26:257–270.
326. Skrowronski, G.A., Turkall, R.M., and Abdel-Rahman, M.S. (1988): Soil absorption alters bioavailability of benzene in dermally exposed male rats. *Am. Ind. Hyg. Assoc. J.*, 49:506–511.
327. Slob, W. (1994): Uncertainty analysis in multiplicative models. *Risk Anal.*, 14(4):571–576.
328. Slob, W. (1996): A comparison of two statistical approaches to estimate long-term exposure distributions from short-term measurements. *Risk Anal.*, 16:195–200.
329. Smith, A. (1994): Risk evaluation of breast milk. *J. Toxicol. Environ. Health,*
330. Smith, A.E., Ryan, P.B., and Evans, J.S. (1992): The effect of neglecting correlations when propagating uncertainty and estimating population distribution of risk. *Risk Anal.*, 12:467–474.
331. Smith, A.H. (1987): Infant exposure assessment for breast milk dioxins and furans derived from waste incineration emissions. *Risk Anal.*, 7(3):347–353.
332. Smith, R.L. (1994): Use of Monte Carlo simulation for human exposure assessment at a Superfund site. *Risk Anal.*, 14(4):433–439.
333. Smith, T.J., Hammond, S.K., and Wong, O. (1993): Health effects of gasoline exposure: I: Exposure assessment for U.S. distribution workers. *Environ. Health Perspect.*, 101(6):13–21.
334. Snyder, W.S. (1975): *Report of the task group on reference man.* International Commission on Radiological Protection Pub. 23. Pergamon Press, New York.
335. Society of Toxicology. (2000): Risk assessment: What's it all about? *Communique,* special issue, pp. 4. Society of Toxicology, Reston, VA.
336. Stanek, E.J. III, and Calabrese, E.J. (1991): A guide to interpreting soil ingestion studies, I: Development of a model to estimate the soil ingestion detection level of soil ingestion studies. *Regul. Toxicol. Pharmacol.*, 13:263–277.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_445.html[2010/10/28 18:44:17]

337. Stanek, E.J. III, and Calabrese, E.J. (1995): Daily estimates of soil ingestion in children. *Environ. Health Perspect.*, 103:276–285.
338. Stanek, E.J. III, and Calabrese, E.J. (1995): Improved soil ingestion estimates for use in site evaluations using the best tracer method. *Hum. Ecol. Risk Assess.*, 1:133–157.
339. Stanek, E.J. III, and Calabrese, E.J. (1995): Soil ingestion estimates for use in site evaluation based on the best tracer method. *Hum. Ecol. Risk Assess.*, 1:133–156.
340. Stanek, E.J. III, and Calabrese, E.J. (1998): Prevalence of soil mouthing/ingestion among healthy children aged 1 to 6. *Soil Contam.*, 2:27–42.
341. Stanek, E.J. III, Calabrese, E.J., and Xu, L. (1997): Soil ingestion in adults: Results of a second pilot study. *Ecotoxicol. Environ. Safety*, 36:249–257.
342. Stanek, E.J. III, Calabrese, E.J., and Xu, L. (1998): A caution for Monte Carlo risk assessment of long term exposures based on short term exposure data. *Hum. Ecol. Risk Assess.*, 4:409–422.
343. Stern, A.H., Korn, L.R., and Ruppel, B.E. (1996): Estimation of fish consumption and methylmercury intake in the New Jersey population. *J. Expos. Anal. Environ. Epidemiol.*, 6:503–525.
344. Stewart, P., and Stenzel, M. (2000): Exposure assessment in the occupational setting. *Appl. Occup. Environ. Hygiene*, 15:435–444.
345. Stewart, P.A., and Herrick, R.F. (1991): Issues in performing retrospective exposure assessment. *Appl. Occup. Environ. Hyg.*, ???
346. Stewart, P.A., Lees, P.S.J., and Francis, M. (1996): Quantification of historical exposures in occupational cohort studies. *Scand. J. Work Environ. Health*, 22:405–414.
347. Stewart, R.D., and Dodd, H.C. (1964): Absorption of carbon tetrachloride, trichloroethylene, tetrachloroethylene, methylene chloride, and 1,1,1-trichloroethane through the human skin. *Am. Ind. Hyg. Assoc., J.* 25:439–446.
348. Stone, R.A., Marsh, G.M., Youk, A.O., Smith, T.J., and Quinn, M.M. (1996): Statistical estimation of exposure to fibres in jobs for which no direct measurements are available. *Occup. Hyg.*, 3:91–101.
349. Surber, C., Wilhelm, K.P., Maibach, H.I., Hall, L.L., and Guy, R.H. (1990): Partitioning of chemicals into human stratum corneum: Implications for risk assessment following dermal exposure. *Fundam. Appl. Toxicol.*, 15:99–107.
350. Taylor, E.R. (1983): How much soil do children eat? In *The health risk assessment and management of contaminated sites*, edited by O.El Saadi and A.Langley, pp. 7277. South Australian Health Commission, Adelaide.
351. Taylor, A.C., Evans, J.S., and McKone, T.E. (1993: The value of animal test information in environmental control decisions. *Risk Anal.*, 13:403–412.
352. Thomas, K.W., Sheldon, L.S., Pellizzari, E.D., Handy, R.W., Roberds, J.M., and Berry, M.R. (1997): Testing duplicate diet sample collection methods for measuring personal dietary exposures to chemical contaminants. *J. JExpos, Anal. Environ. Epidemiol.*, 7:17–36.
353. Thompson, K.M., and Burmaster, D.E. (1991): Parametric distributions for soil ingestion by children. *Risk Anal.*, 11:339–342.
354. Thompson, K.M., Burmaster, D.E., and Crouch, E.A.C. (1992): Monte Carlo techniques for quantitative uncertainty analysis in public health risk assessments. *Risk Anal.*, 12(1):53–63.
355. Travis, C.C., and Hester, S.T. (1990): Background exposure to chemicals: What is the risk? *Risk Anal.*, 10:463–466.
356. Travis, C.C., and Land, M.L. (1990): Estimating the mean of data sets with nondetectable values. *Environ. Sci. Technol.*, 24:961–962.
357. Trowbridge, P.R., and Burmaster, D.E. (1997): A parametric distribution for the fraction of outdoor soil in indoor dust.*J. Soil Contam.*, 6:161–168.
358. Umbreit, T.H., Hesse, E.J., and Gallo, M.A. (1986): Acute toxicity of TCDD contaminated soil from an industrial site. *Science*, 232:497–499.
359. Umbreit, T.H., Hesse, E.J., and Gallo, M.A. (1986): Comparative toxicity of TCDD contaminated soil from Times Beach, Missouri and Newark, New Jersey. *Chemosphere*, 15:121–2124.
360. Upton, A.C. (1988): Evolving perspectives on the concept of dose in radiobiology and radiation protection. *Health Phys.*, 55(4):605–614.
361. U.S. Department of Agriculture. (1972): *Food consumption: Households in the United States, seasons and year 1965–1966.* U.S. Department of Agriculture, Washington, DC.
362. U.S. Department of Agriculture. (1980): Food and Nutrient Intakes of Individuals in One Day in the United States, Spring 1977. Nationwide Food Consumption Survey 1977–1978. U.S. Department of Agriculture, Washington, DC. Preliminary Report 2.

363. U.S. Department of Agriculture. (1992): Food and Nutrient Intakes by Individuals in the United States, 1 Day, 1987–88. Nationwide Food Consumption Survey 1987–1988. U.S. Department of Agriculture, Washington, DC. Human Nutrition Information Service, NFCS Report 87–1-1.
364. U.S. Environmental Protection Agency. (1983–1989): *Methods for assessing exposure to chemical substances, Vol. 113*. U.S. Environmental Protection Agency, Washington, DC. Office of Toxic Substances, Exposure Evaluation Division, EPA/560/5–85/002, NTIS PB86–107067.
365. U.S. Environmental Protection Agency. (1984) *An estimation of the daily food intake based on data from the 1977–1978 USDA nationwide food consumption survey*. U.S. Environmental Protection Agency, Washington, DC. Office of Radiation Programs, EPA/520/1–84/015.
366. U.S. Environmental Protection Agency. (1985): *Development of statistical distributions or ranges of standard factors used in exposure assessments*. U.S. Environmental Protection Agency, Washington, DC. Office of Health and Environmental Assessment, EPA/600/8–85/010.
367. U.S. Environmental Protection Agency. (1986): *Guidelines on air quality models* (rev.). U.S. Environmental Protection Agency, Research Triangle Park, NC. Office of Air Quality Planning and Standards, EPA/450/2–78/027R.
368. U.S. Environmental Protection Agency. (1987): *Selection criteria for mathematical models used in exposure assessments: Surface water models*. U.S. Environmental Protection Agency, Washington, DC. Office of Health and Environmental Assessment, Office of Research and Development, EPA/600/8–87/042, NTIS PB88–1399287 AS.
369. U.S. Environmental Protection Agency. (1987): *The Total Exposure Assessment Methodology (TEAM) study. Volume 1: Summary and analysis*. U.S. Environmental Protection Agency, Office of Acid Deposition, Environmental Monitoring and Quality Assurance, Research and Development, EPA/600/6–87/002a.
370. U.S. Enviornmental Protection Agency. (1987): *Methods for assessing exposure to chemical substances, Vol. 7, Methods for assessing consumer exposure to chemical substances*. U.S. Environmenmental Protection Agency, Washington, DC. Office of Toxic Substances, EPA/560/5–857007.
371. U.S. Environmental Protection Agency. (1988): Proposed guide lines for exposure-related measurements. *Fed. Reg.*, 53(232):48830–48853.
372. U.S. Environmental Protection Agency. (1989) *Risk assessment guidance for Superfund. Volume I. Human health evaluation manual (Part A)*. Interim final. U.S. Environmental Protection Agency, Washington, DC. Office of Emergency and Remedial Response, EPA/540/1–89/002.

373. U.S. Environmental Protection Agency. (1991): *Risk assessment guidance for Superfund (RAGS). Volume I: Human health evaluation manual (HHEM) (Part B). Development of risk-based pre-liminary remediation goals.* U.S. Environmental Protection Agency, Washington, DC. Office of Emergency and Remedial Response, EPA/540/R-92/003, NTIS PB92–963333.
374. U.S. Environmental Protection Agency. (1991): *Time spent in activities, locations, and microenvironments: A California-national comparison.* Office of Research and Development, Las Vegas, NV. Environmental Monitoring Systems Laboratory, EPA/600/4–91/006.
375. U.S. Environmental Protection Agency. (1992): *Supplemental guidance to RAGS: Calculating the concentration term.* U.S. Environmental Protection Agency, Washington, DC. Office of Solid Waste and Emergency Response, OSWER Directive 9285.7–081.
376. U.S. Environmental Protection Agency. (1992): *Dermal exposure assessment: Principles and applications.* U.S. Environmental Protection Agency, Washington, DC. Office of Health and Environmental Assessment, Office of Research and Development, EPA/600/8–91/011.
377. U.S. Environmental Protection Agency. (1992): Guidelines for exposure assessment; Notice. *Fed. Reg.*, 57(104):22888–22938.
378. U.S. Environmental Protection Agency. (1992): Safeguarding the future: Credible science, credible decisions. Report of the Expert Panel on the Role of Science at EPA. Washington, DC, EPA/600/9–91/050.
379. U.S. Environmental Protection Agency. (1995): *Guidance for risk characterization.* U.S. Environmental Protection Agency, Washington, DC. Science Policy Council.
380. U.S. Environmental Protection Agency. (1996): *Summary report for the workshop on Monte Carlo analysis.* U.S. Environmental Protection Agency, Washington, DC. Office of Research and Development, EPA/630/R-96/010.
381. U.S. Environmental Protection Agency (EPA). (1996): Draft guidelines for carcinogen risk assessment. *Fed. Reg.*, 61(79):17960–18011.
382. U.S. Environmental Protection Agency. (1996): *Exposure factors handbook. Volume I of III: General factors—SAB Review Draft.* U.S. Environmental Protection Agency, Washington, DC. Office of Research and Development, EPA/600/P-95/002Ba.
383. U.S. Environmental Protection Agency. 1996d. *Exposure factors handbook. Volume II of III: Food ingestion factors—SAB Review Draft.* Washington, DC: U.S. Environmental Protection Agency, Office of Research and Development, EPA/600/P-95/002Bb.
384. U.S. Environmental Protection Agency. 1996e. *Exposure factors handbook. Volume III of III: Activity factors—SAB Review Draft.* U.S. Environmental Protection Agency, Washington, DC. Office of Research and Development, EPA/600/P-95/002P.
385. U.S. Environmental Protection Agency. (1997): *Lognormal distribution in environmental applications.* U.S. Environmental Protection Agency, Washington, DC. Office of Solid Waste and Emergency Response, EPA/600/R-97/006.
386. U.S. Environmental Protection Agency. (1997): *Exposure factors handbook* (update to the May 1989 edition). U.S. Environmental Protection Agency, Washington, DC. EPA/600/P-95/002Fa.
387. U.S. Environmental Protection Agency. (1997): *Guiding principles for Monte Carlo analysis.* U.S. Environmental Protection Agency, Washington, DC. Office of Research and Development, Risk Assessment Forum, EPA/630/R-97 7001.
388. U.S. Environmental Protection Agency. (1997): *Methodology for assessing health risks associated with multiple exposure pathways to combustor emissions.* U.S. Environmental Protection Agency, Washington, DC. National Center for Environmental Assessment, NCEA-C-0238.
389. U.S. Environmental Protection Agency. (1997): *The parameter guidance document.* U.S. Environmental Protection Agency, Cincinnati, OH.
390. U.S. Environmental Protection Agency. (1998): *Integrated risk information system (IRIS).* U.S. Environmental Protection Agency, Washington, DC.
391. U.S. Environmental Protection Agency. (1999): *Risk assessment guidelines for dermal assessment.* U.S. Environmental Protection Agency, Washington, DC.
392. U.S. Environmental Protection Agency. (1999): *Risk assessment guidance (RAGS3A) for conducting probabilistic risk assessment.* U.S. Environmental Protection Agency, Washington, DC.
393. U.S. Environmental Protection Agency. (1999): *Sociodemographic data used for identifying potentially highly exposed populations.* U.S. Environmental Protection Agency, Washington, DC. EPA/600/R-99/060.
394. U.S. Environmental Protection Agency. (1999): *Guidance for performing aggregate exposure and*

risk assessments under the Food Quality Protection Act. (Draft). U.S. Environmental Protection Agency, Washington, DC. Office Periodicals Program.
395. U.S. Office of Science and Technology Policy. (1993): *Researching health* risks. Office of Technology Assessment, Washington, DC.
396. Van Den Berg, M., Olie, K., & Hutzinger, O. (1984): Uptake and selective retention in rats of orally administered chlorinated dioxins and PCDF from fly ash. *Chemosphere*, pp. 531–544.
397. Van Wijnen, J.H., Clausing, P., and Brunekreef, B. (1990: Estimated soil ingestion by children. *Environ. Res.*, 51:147–162.
398. Velazquez, S.F., McGinnis, P.M., Vater, S.T., Stiteler, W.S., Knauf, L.A.. and Schoeny, R.S. (1994): Combination of cancer data in quantitative risk assessments: Case study using bromodichloromethane. *Risk Anal.*, 14:285–292.
399. Vose, D. (1996): *Quantitative risk analysis: A guide to Monte Carlo simulation modelling.* Wiley, New York.
400. Wallace, L.A.(1986): The Total Exposure Assessment Methodology (TEAM) study: Direct measurement of personal exposures through air and water for 600 residents of several U.S. cities. In *Pollutants in a multimedia environment*, edited by Y.Cohen, pp. 289–315. Plenum Press, New York.
401. Wallace, L, A. (1987) *The TEAM study: Summary and analysis: Volume I.*U.S. Environmental Protection Agency, Washington, DC, EPA 600/6–87/002a, NTIS PB 88–100060.
402. Wallace, L.A. (1989): The Total Exposure Assessment Methodology (TEAM) study: An analysis of exposures, sources, and risks associated with four chemicals. *J. Am. Coll. Toxicol.*, 8:883–895.
403. Wallace. L.A. (1993): A decade of studies of human exposure: What have we learned? *Risk Anal.*, 13(2):135–143.
404. Wallace, L.A. (1996): Environmental exposure to benzene: An update. *Environ. Health Perspect.*, 104(suppl. 6):1129–1136.
405. Wallace, L.A. (1998): The Weslowski Lecture. Personal correspondence.
406. Wallace, L. (2000): Correlations of personal exposure to particles with outdoor air measurements: A review of recent studies. *Appl. Occup. Environ.*, in press.
407. Wallace, L.A. (2000): Real-time monitoring of particles, PAH, and CO in an occupied townhouse. *Appl. Occup. Environ. Hyg.*, 15:19.
408. Wallace, L.A., and Pellizzari, E.D. (1995): Recent advances in measuring exhaled breath and estimating exposure and body burden for volatile organic compounds (VOCs). *Environ, Health Perspect.*, 103:95–98.
409. Wallace, L.A., and Slonecker, T. (1997): Ambient air concentrations of fine manganese (PM2.5) in U.S. national parks and in California and Canadian cities: The possible impact of adding MMT to unleaded gasoline. *J. Air Waste Manage. Assoc.*, 47:(6)642–652.

410. Wallace, L.A., Pellizzari, E., Hartwell, T., Sparacino, C., Sheldon, L., and Zelon, H. (1985): Personal exposures, indoor-outdoor relationships and breath levels of toxic air pollutants measured for 355 persons in New Jersey. *Atmos. Environ.*, 19:1651–1661.
411. Wallace, L.A., Pellizzari, E.D., Hartwell, T.D., Whitmore, R., Sparacino, C., and Zelon, H. (1986): Total Exposure Assessment Methodology (TEAM) study: Personal exposures, indoor-outdoor relationships, and breath levels of volatile organic compounds in New Jersey. *Environ. Int.*, 12:369–387.
412. Wallace, L.A., Pellizzari, E.D., Hartwell, T.D., Sparacino, C., Whitmore, R., Sheldon, L., Zelon, H., and Perritt, R. (1987): The “TEAM” study: Personal exposures to toxic substances in air, drinking water, and breath of 400 residents of New Jersey, North Carolina, and North Dakota. *Environ. Res.*, 43:290–307.
413. Wallace, L.A., Pellizzari, E., Leaderer, B., Hartwell, T., Perritt, R., Zelon, H., and Sheldon, L. (1987): Emissions of volatile organic compounds from building materials and consumer products. *Atmos. Environ.*, 21:385–393.
414. Wallace, L.A., Pellizzari, E., and Wendel, C. (1991): Total volatile organic concentrations in 2700 personal, indoor, and outdoor air samples collected in the US EPA TEAM studies. *Indoor Air* 4:465–477.
415. Wallace, L.A., Duan, N., and Ziegenfus, R. (1994): Can long-term exposure distributions be predicted from short-term measurements? *Risk Anal.*, 14:75–85.
416. Wallace, L.A., Buckley, T., Pellizzari, E.D., and Gordon, S. (1996): Breath measurements as VOC biomarkers: EPA’s experience in field and chamber studies. *Environ. Health Perspect.*, 104(suppl. 5):861–869.
417. Walter, S.D., Yankel, A.J., and von Lindern, I.H. (1980): Age-specific risk factors for lead absorption in children. *Arch. Environ. Health*, 35:53–58.
418. Wepierre, J., and Marty, J.P. (1979): Percutaneous absorption of drugs. *Trends Pharmacol. Sci.*, 1:23–26.
419. Wester, R.C., and Noonan, P.K. (1980): Relevance of animal models for percutaneous absorption. *Int. J. Pharmacol.*, 7:99–110.
420. Wester, R.C., Bucks, D.A. W., and Maibach, H.I. (1993): Percutaneous absorption of contaminants from soil. In *Health risk assessment: Dermal and inhalation exposure and absorption of toxicants*, edited by R.G.M.Wang, J.B.Knaak, and H.I. Maibach. CRC Press, Boca Raton, FL.
421. Wester, R.C., Maibach, H.L, Sedik, L., Melendres, J., Wade, M., and DiZio, S. (1993): Percutaneous absorption of pentachlorphenol from soil. *Fundam. Appl. Toxicol.*, 20:68–71.
422. White, S.B., Peterson, B., Clayton, C.A., and Duncan, D.P. (1983): The Construction of a Raw Agricultural Commodity Consumption Database. Interim Report 1. Prepared by Research Triangle Institute for U.S. Environmental Protection Agency. Office of Pesticide Programs.
423. Whitmore, R.W., Immerman, F.W., Camann, D.E., Bond, A.E., Lewis, R.G., and Schuam, J.L. 1994. Non-occupational exposures to pesticides for residents of two U.S. cities. *Arch. Environ. Contam. Toxicol.* 26:47–59.
424. Widner, T. (2000): Dose-reconstruction for radionuclides and chemicals released from the federal nuclear facility in Oak Ridge, Tennessee. In *Human and ecological risk assessment: Theory and practice*, edited by D.J.Paustenbach. John Wiley and Sons, New York.
425. Wilschut, A., and ten Berge, W.F. (1995): Two mathematical skin permeation models for vapours. Abstracts of presentations at the Fourth International Prediction of Percutaneous Penetration Conference, La Grande Motte, April 1995. *Prediction of Percutaneous Penetration*, vol. 4a. 3M Medica.
426. Wilschut, A., ten Berge, W.F., Robinson, P.J., and McKone, T.E. (1995): Estimating skin permeation. The validation of five mathematical skin permeation models. *Chemosphere*, 30:1275–1296.
427. Wilson, N.D., Shear, N.D., Paustenbach, D.J., and Price, P.S. (1998): The effect of cooking practices on the concentration of DDT and PCB compounds in the edible tissue of fish. *J. Expos. Anal. Environ. Epidemiol.*, 8:423–440.
428. Wilson, N.D., Price, P., and Paustenbach, D.J. (2000): An assessment of the risk of DDT and PCB in fish from the Palos Verdes shelf. In *Human and ecological risk assessment: Theory and practice*, edited by D.J.Paustenbach. John Wiley & Sons, New York.
429. Wolfe, D.A. (1996): Insights on the utility of biomarkers for environmental impact assessment and monitoring. *Hum. Ecol. Risk Assess.*, 2:245–250.
430. Zannetti, P. (1992): Particle modeling and its application for simulating air pollution phenomena. In *Environmental modeling*, edited by P.Melli, chap. 11. Macmillan, UK.
431. Zartarian, V.G., Ferguson, A.C., and Leckie, J.O. (1998): Quantified mouthing activity data from a four-child pilot field study. *J. Expos. Anal. Environ. Epidemiol.*, 8(4):543–553.

432. Zweig, G., Leffingwell, J.T., and Popendorf, W.J. (1985): The relationship between dermal pesticide exposure by fruit harvesters and dislodgeable foliar residues. *J. Environ. Sci. Health B*, 20:27–60.

Chapter **10**

Epidemiology for Toxicologists

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Principles and Methods of Toxicology,
Fourth Edition, edited by A.Wallace Hayes.
Taylor & Francis, Philadelphia © 2001.

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The search for scientific "truth" regarding the causes of human disease is a laborious multistep process, a winnowing of a large number of postulated hypotheses down to the few that can be supported with data derived from testing and observation. It depends upon the replication of results, coherence of evidence from many different fields and, ultimately, an understanding of the underlying biological mechanisms of action. In evaluating the potential human health effects of chemical exposures, there are three major sources of scientific information used by the courts, various government agencies, and the larger scientific community: experimental laboratory research, controlled clinical investigations, and observational epidemiology studies. These three are not mutually exclusive in method or thought; each makes a unique contribution toward understanding the etiologies of human disease; but each has certain inherent limitations. Ultimately, the determination of causation depends upon the demonstration of a meaningful elevated risk for the disease among those with the "exposure" and a biological explanation for the excess. The former can only be obtained via epidemiology studies; the latter usually comes from an interplay of information derived from experimental laboratory research and controlled clinical investigations.

Toxicology is one of the key experimental disciplines. In toxicology, investigators are able to carefully con trol the exposures of genetically homogeneous groups of animals, some purposefully bred so that they exhibit a marked predilection for specific diseases. The latter can provide toxicologists and pharmacologists with important clues about the biological mechanisms which predispose, aggravate, or cause disease. Toxicologists also have the opportunity of evaluating each and every subject to the same exquisite detail. Therefore, at least in theory, the experimental method can provide comprehensive results unperturbed by extraneous variables; but it is increasingly being recognized that this approach does not automatically eliminate, or reduce to a level of insignificance, all forms of technical bias (97). Nor does it protect against personal bias, a problem that can plague all types of research irrespective of the affiliations of the investigators (70, 74, 118). Toxicology also has become stylized to the extent that the format of research results is both predictable and quantitative. This has made it very convenient for information derived from toxicology experiments to be used in quantitative risk assessments and related regulations. Paradoxically, this convenience facilitates a major violation of scientific principles: an

extrapolation beyond the data to make inferences about health effects not only for levels of exposure that were not administered but also for species who were not studied.

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Clinical investigators also administer measured doses according to a predetermined schedule, but do so to the species of major interest, humans. Although this type of work is "controlled," it is at best quasi-experimental because humans are not passive participants in health research. At the very least, they must consent to be studied. Some do not and key characteristics of those who do and do not may be markedly different. In other words, most study groups used in clinical trials are not random samples of the general population. This means that care must be exercised in extrapolating the findings too broadly.

On the plus side, clinical research can collect data both on objective signs of pathology and also on more subjective symptoms, data that animal research cannot provide. On the negative side, an equivalent and comprehensive data set for each study subject may not be obtainable. For example, histopathology of all the organs is only available on those few of the deceased whose next-of-kin allow an autopsy to be performed. Any study of humans also means that the research subjects are not homogeneous with regard to either genetic makeup or alternative exposures such as diet, personal habits, or medications. To address this problem, randomization of subjects to different treatment groups is utilized during study design, the assumption being that key alternative variables are randomly distributed across groups and confounding thereby controlled. Sometimes it doesn't work.

There are two major strengths of observational epidemiology research. One, it studies humans. Two, it deals with the effects of real exposures—actual levels, durations and patterns of exposure to individual agents and to mixtures. If epidemiology studies are well done, they furnish results that reasonably can be extended to larger populations. Unfortunately, epidemiologists are often forced to handle exposure as a qualitative variable (either as a yes/no or some variation of high, medium and low). This can limit the utility of the research results for those who require quantitative information. In addition, because the research is observational in the sense that the investigators simply observe natural experiments and do not exercise control over the key variables, epidemiologists routinely must grapple with a number of technical biases that are largely transparent to those in the other two fields. If these biases—in particular, selection, misclassification, and confounding—are not adequately addressed during study design or data analysis, the study results may be unduly imprecise and important associations missed. Alternatively, the results may be relatively precise but precisely inaccurate, thereby leading to interpretations that are incorrect. Some of these problems can be exacerbated if the epidemiologist utilizes secondary sources of data (data originally gathered for purposes other than the specific research project, possibly even for reasons unrelated to research), especially if the methods for the original data collection process were poorly documented.

There is one other point that differentiates epidemiology from the other two fields. Although acceptance into the American College of Epidemiology provides an imprimatur of professional competence, in practice epidemiology requires no graduate degree, certification, or licensure. Some enter the field with strong statistical skills; others have extensive training in human biology. The formal graduate degree programs require a proficiency in both; but anyone with any level of education can gather a data set, analyze it, report the results, and call their efforts epidemiology research. There is no law against it and no professional body condemns the practice. Epidemiologists can be very egalitarian. The rule of thumb for the consumer of epidemiology results, therefore, especially if a report appears in other than the peer reviewed literature, is *caveat emptor*.

Epidemiology has been defined as the study of the distribution and determinants of disease in humans (75). Although commonly used, this definition is incomplete. Although epidemiologists certainly search for the factors associated with human disease, they also attempt to identify both interventions that likely will benefit those who are at risk for getting the condition (perhaps because of unique patterns of exposure to combinations of putative agents or a genetic predisposition for reacting adversely to such exposures) and treatments that will help control or cure any significant pathology once it occurs. They also, implicitly or explicitly, try to determine which agents do not cause a specific disease, which interventions will not be successful, and which treatments are not effective.

As with toxicology and clinical research, good epidemiology is an amalgam of subject-specific knowledge and methods. And just as there are clinical specialties and areas of expertise in toxicology that have evolved with the growing complexities of each of those two fields, epidemiology is divided into a number of overlapping subgroups: occupational, environmental, reproductive, cardiovascular, cancer, infectious disease, molecular, genetic, nutritional, medical device, clinical, etc. Some of these are subdivided still further, for example, AIDS is a subcategory of viral which, in turn, is a subset of infectious disease. Although certain knowledge and techniques may be unique to a subgroup, many concepts are common across the discipline.

The primary objective of this chapter is to introduce those concepts that span the field so that toxicologists might become better consumers of the epidemiology literature. Table 10.1 provides an outline of the major topics: data, measures of disease frequency, measures of risk and association, methods, and issues. All of these are interrelated so the order of presentation is somewhat arbitrary; but the first three set the stage for the last one,

Table 10.1 Major topics

Data
- Prevalence
- Incidence

Measures of disease frequency
- Prevalence rate
- Incidence rate

Measures of risk and association
- Absolute risk
- Relative risk
- Standardized mortality ratio (SMR)
- Proportional mortality ratio (PMR)
- Rate difference
- Attributable risk

Methods
- Cohort
- Case control
- Cross-sectional
- Case studies or case series

Issues
- Selection
- Misclassification
- Confounding
- Chance
- Causation

in particular the key issues impacting validity—selection, misclassification, and confounding. Each of these in turn is presented to show how it can bias the measures of association. The intent is to make the readers more sensitive to possible flaws so that they might determine for themselves how well the investigators recognized a potential problem and addressed it during study design, data collection or data analysis, and during data interpretation.

Most of the major points are illustrated with recent examples from the epidemiology literature or, to a lesser extent, with toxicology or clinical references. In part, this was done to emphasize the point that all science—experimental, quasi-experimental, and observational—is based upon assumptions that may not be correct. Some of these assumptions are relatively innocuous. Conversely, some are so important that, if violated, the science is severely flawed and any policies based upon that science likely also are flawed. Those are the two poles of a continuum; but validity is not a dichotomous variable like pregnancy. The challenge for the consumer of scientific reports is to determine which provide useful information, which do not, and which lie somewhere in between—intriguing enough to warrant additional research, but not strong enough to merit intervention. This winnowing equates to first, identifying potential problems that may have compromised the validity of the research; second, determining the probability to which the problems occurred; and third, estimating the impact, if any, they had upon the results.

This chapter is by design a limited overview. For those who wish to have a more detailed presentation of the field, the list of references includes a number of recently published textbooks (19, 48, 62, 98, 101). Note, throughout this chapter key "terms of art" are emphasized with italics. A small number of these are defined in the book's glossary; but most can be found in Last's *Dictionary of Epidemiology* (72), an invaluable resource to any technical library. Additionally, at the end of the chapter there is an appendix: the *Guidelines for Good Epidemiology Practices for Occupational and Environmental Epidemiologic Research* (GEPs), an analog of *Good Laboratory Practices* (GLPs).

DATA AND MEASURES OF DISEASE FREQUENCY

Data, information, and knowledge are related but not equivalent terms. Data are the raw materials gathered by the investigator during the course of an investigation, information is analyzed data, and knowledge is meaningful information that can be used to predict or solve problems. By way of analogy, data are bricks, information the wall, and knowledge the building. Just as good bricks can be put together poorly to build an unstable wall, so can data be valid, but aggregated in a fashion that produces useless information. Pseudoknowledge may be a function of either bad data or useless information, just as a structurally unsound building may result from poor quality bricks or an unstable

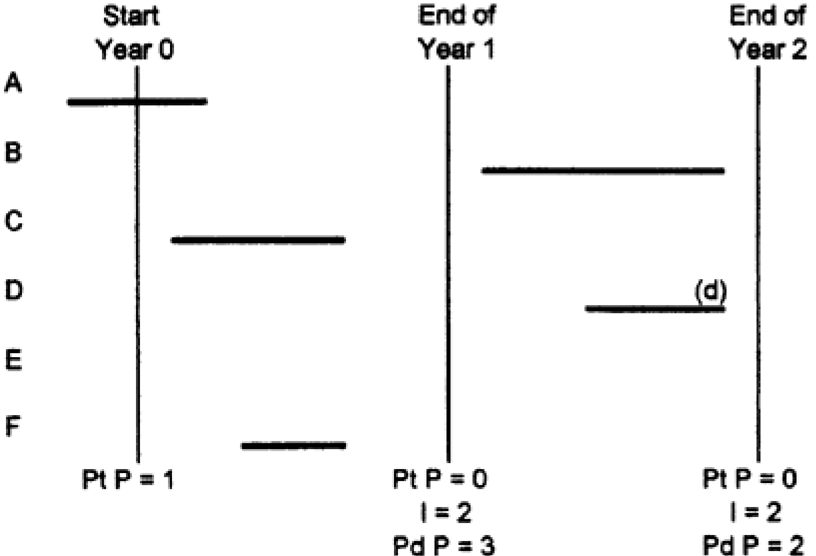
wall.

Prevalence and Incidence

For the epidemiologist, there are two general types of data: *prevalence* and *incidence*. Prevalence is what is observed at a single point; a snapshot of what is prevalent, what exists, at a specific point in chronological or biological time. For example, the number of toxicologists currently employed by federal regulatory agencies is prevalence data.

Incidence is the number of incidents, of new events, that develop over time. Because it represents a delta, a change, incidence data have to be gathered at two or more points in time. In a sense, if prevalence is a snap-shot, incidence is a movie. The number of toxicologists hired by federal agencies in any given year is an example of incidence data. They didn't work for the agencies at the start of the year (observation point one); but they did work for the government later in the year (observation points two or more).

Parenthetically, when epidemiologists speak of prevalence data they are usually referring to *point prevalence* but they may *mean period prevalence*. Period prevalence is a combination of what exists at the beginning as well as what occurs during a specified period. The number of toxicologists who were employed by federal agencies at any time during a given year is period prevalence data. It includes those who were working at the beginning of the year (point prevalence data) and those who were hired during the year (incidence data). Period prevalence may or may not be the same as the number of those who were employed at the end of the year (more point prevalence data) because some toxicologists may have left government employment during the period of observation.
Whether data are period prevalence or incidence can sometimes be difficult to discern, because both refer to events occurring during a span of time. The key is whether the data represent a combination of existing and new events (period prevalence) or just new events (incidence). Unless otherwise noted, the term prevalence is used in this chapter as a synonym for point prevalence.
The difference between prevalence and incidence data is important for at least three reasons. One, incidence data can be used to evaluate cause and effect; prevalence data usually cannot, at least not without additional assumptions. Two, because prevalence data can be gathered at a single point in time, it is much easier to obtain and therefore many reports in the medical literature are based upon prevalence data. Three, the medical literature often incorrectly uses the two terms interchangeably. As a consequence, reports that use valid prevalence data to develop nonsense information about cause and effect appear in even the most prestigious journals.
Although the two are different, they are related. Prevalence *(P)* is a function of both the incidence *(I)* and the duration *(D)* of the disease *($P=I\times D$)*. What that means is a chemical may not cause a disease, may not increase the incidence of the disease, but it may still be associated with a higher prevalence of the condition. Whether that is good news or bad depends upon the circumstances. For example, the incidence of diabetes may be quite stable in a population; but if that population is given access to a chemical called insulin, the prevalence of the condition likely will increase dramatically. It will increase because the insulin extends the duration of the disease by allowing more of the afflicted to live longer. Conversely, the prevalence of minor birth defects (prevalence because the events are measured at a single point in biological time, birth) may be lower among live children born to women exposed to some agent, not because the agent prevents the development of minor defects in utero, but because the agent and causes major malformations that lead to early spontaneous abortions.



Pt P is point prevalance; I is incidence; and Pd P is period prevalence.
FIG. 10.1. Prevalence versus incidence (time-limited condition).

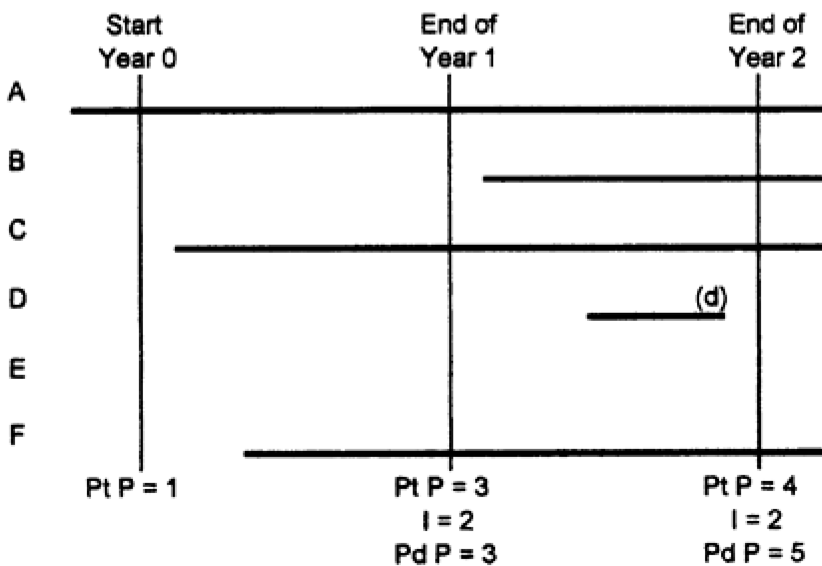


FIG. 10.2. Prevalence versus incidence (condition chronic).

The incidence of minor defects is quite stable in this example, but their duration has been shortened and therefore fewer malformations observed among live births.

Figures 10.1 and 10.2 illustrate these points. In both, a group of six patients (A through F) is observed for 2 years. In Figure 10.1, the condition is time limited. It either spontaneously resolves, is cured through some treatment, or the patient dies. The point prevalence at the initial baseline observation (year 0) is one. Two additional cases subsequently occur and all three resolve before year's end. At the end of year 1, the point prevalence is zero, the incidence is two, and the period prevalence is three. During the following year, two more cases develop and one patient dies (patient D) of an unrelated cause before the end of the year. Therefore, at year's end, the point prevalence is zero, the incidence two, and the period prevalence also two.

In Figure 10.2, the condition is chronic, perhaps because, like diabetes, it has been extended through treatment. Note the incidence is exactly the same as in the previous example. It is two in each year. However, the extended duration has impacted both measures of prevalence. The point prevalence at the time of the three observations is respectively one, three, and four. The period prevalence for the first year is three. For the second, it is five even though patient D died before year's end. Each of these three measures of disease provides valuable information, but using either type of prevalence data for interpretations about cause and effect depends upon assumptions about *incidence time* (i.e., when the health event actually occurred) and disease duration that are often untestable or incorrect.

Rates

Technically, the terms incidence and prevalence refer to numerator data; however, in both the epidemiology and medical literature, these terms often are used interchangeably with, respectively, *incidence rate* and *prevalence rate*. An incidence rate is the number of new events of a disease in a defined population that occur during some specified period of time. A prevalence rate is the number of cases of disease observed in a defined population at a point in time. In both, the numerator is a subset of the denominator. Obviously, errors of count in either the numerator or the denominator can impact the accuracy of a rate. Nonetheless, in some technical reports the former may not be a subset of the latter and the description as to how either was compiled may be less than clear.

Rather than presenting a rate as the actual numerator (the exact number of new events observed) in comparison to the actual denominator (the precise count of the group under study) at or during the period of observation, for convenience a rate is usually given as the number of cases per 100 or per 1000 or per 10,000. For example, if there were 486 persons in the study group and 5 new events occurred during a 12-month period of observation, the incidence rate might be presented as 1.0 per 100 per year (5 divided by 486 times 100) or, alternatively, as 10.3 per 1000 per year.

As opposed to toxicology, in epidemiology the study groups can be either *fixed* or *open* (sometimes called *dynamic*). In a fixed study group, those included are defined at the start and followed over time. If no losses occur during the period of study, the group may be called a *closed population*. In an open study group, individuals may be added or lost during the time of study. Just the events that occur and just that time that passes during the period each individual was under observation are counted. This so-called *person-time experience* assumes that observing 10 people for 1 year is the same as observing 1 person for 10 years. In some situations, the assumption is appropriate. In others, it may not be. Determining which is which depends upon the underlying biological model.

MEASURES OF RISK AND ASSOCIATION

Absolute Risk

It is an immutable fact of life that we are all going to get ill at sometime and ultimately we are all going to die. On a personal level, the questions for each of us are by what disease and when? Epidemiologists are also interested in those questions, but they are particularly interested in whether the disease occurs more frequently or more severely in association with some type of exposure. In other words when it comes to identifying the causes of disease, what is at issue is whether the *absolute risk* for a specific disease among the exposed is greater than the absolute risk of that same disease in the unexposed.

Relative Risk

An incidence rate provides a measure of absolute risk. The ratio of the incidence rates in two different groups is a *rate ratio*, *risk ratio*, or *relative risk*, a key measure of association between exposure and disease. If the relative risk *(RR)* is appreciably greater than 1 among those with a particular exposure, it is evidence that the agent may be causing the disease. May be. Conversely, if the relative risk is below 1, the agent may be protecting against the disease. May be. And if the relative risk approximates 1, there may be no meaningful association between the two variables. Once again, may be. May be is an important caveat in all three situations because how well the *apparent relative risk* (the number derived as the result of a particular investigation) corresponds to the *true relative risk* (the actual underlying biological truth) depends not only on the statistical stability of the estimate of relative risk, but also on how well the potential technical biases of selection, misclassification, and confounding were controlled in study design, during data collection, and by data analyses.

Standardized Mortality Ratio (SMR)

In cohort mortality studies, the measure of association may be provided as a *standardized mortality ratio* or SMR. Because this is simply the ratio of the number of deaths observed in the study group to the number that would have been expected if the study group had the same death rate as a reference (i.e., standard) population, it is sometimes presented as *observed to expected* deaths or

O/E. By convention, this measure of association is given as a percentage, but the interpretations parallel those of the relative risk. A SMR of 150 is analogous to a RR of 1.5; a SMR of 75 to a RR of 0.75; and a SMR of 100 to a RR of 1.0. Because the observed number of deaths occurs in discrete increments and the number of expected deaths is for all intents and purposes a continuous variable (i.e., the expected deaths might be a biologically impossible number such as 1.27365…), by convention many epidemiologists will not calculate a SMR if the number of observed deaths is less than 2 (19). They may simply provide the two numbers, the observed and the expected, or just give a confidence interval. Sometimes, they'll do neither and merely indicate that the numbers were too small to be meaningful
Note, the "controls," the comparison group, in a SMR analysis are essentially those in a hypothetical group statistically constructed from the reference population to have approximately the same age, race, and gender characteristics as the exposed group. For many occupational studies, the mortality experience of United States white males is used as a reference even if a small number of those in the occupational cohort are of a different race or ethnic group, the assumption being that the calculations of expected deaths will be adequate. Using a SMR approach also means the investigator is assuming that no one in the reference population was exposed to the agent of interest. If the exposure is relatively rare among those in the reference population, the assumption is probably reasonable because the mortality experience of those few who were exposed would have had very little impact on the population statistics. On the other hand, if the exposure is relatively common—for example, something like chlorinated drinking water—the assumption may be unreasonable and another type of study would have to be done to obtain valid information.

An interpretation of a "crude" measure of association assumes that both the exposed group and the reference population had similar habits regarding smoking, dietary preferences, medical care, etc. This assumption may be incorrect. For example, a number of years ago a study was done in California of men who lived in communities adjacent to petrochemical facilities along the Sacramento river (8). The comparison population was composed of those who lived in the same county, but remote from the industrial sector. An apparent elevated risk for lung cancer was discovered and the finding was initially presumed, at least by the news media, to be due to emissions from the petrochemical plants. However, during this first stage of the investigation, no attempt had been made to control for the effects of cigarette smoking. For efficiency, that activity had been deferred to subsequent stages of the research. It was later found that those who lived near the plants were largely blue collar workers and those who lived elsewhere tended to be white collar professionals who commuted to work in San Francisco. Why was this important? Blue collar workers on average smoke more than white collar workers (at least that has been past experience) and therefore blue collar workers have higher rates of lung cancer. When the subsequent research gathered the necessary data and adjusted for these differences, the elevated risk disappeared (20). As a result, the proposed intervention strategies changed from targeting industrial emissions to implementing smoking cessation programs.

The SMR can also be an acronym for a *standardized morbidity ratio*. Instead of death being the outcome of interest, it is illness; but the calculations and the resultant interpretations are basically the same. So too are the underlying assumptions. If the assumptions were violated to the degree that the study results were affected, the reader should look for confirmation elsewhere.

Proportional Mortality Ratio (PMR)

Neither the RR or the SMR should be confused with a *PMR*, a *proportional mortality (or morbidity) ratio*. The PMR is a measure of the relative importance of an individual category of disease among those with disease. As such, both numbers in the ratio are "numerator data." Although it is a convenient measure to obtain, it must be used with caution in etiologic research because it compares proportions and not rates. It makes the assumption that a higher proportion of a particular disease is the same as an increased frequency of that disease. Because a PMR calculation works like a teeter-totter, that assumption may be invalid. Although a higher proportion of disease A may be due to an increased incidence of disease A, it also simply may be a function of a lower frequency (and therefore a lower proportion) of some other condition, disease B. For example, a higher PMR for cancer among an occupational group with a certain exposure may mean that more of those with the exposure were developing (and dying) from cancer than those in the standard population; but it is also consistent with the interpretation that those with the exposure were not dying more often from cancer, they were just dying less often from noncancer events. In other words, in a PMR analysis an apparently "adverse" finding may be spurious, for example, solely a function of the *healthy worker effect* (79).

Rate Difference and Attributable Risk

With two incidence rates it is possible to calculate not only a rate ratio, but also a *rate difference*. If the

association between the exposure and the disease is truly causal, the rate difference provides a measure of the excess

burden of disease an exposed population might expect to experience as a result of the exposure. Stated another way, it represents the amount of the disease that would never have occurred if the exposure had been prevented. In such situations, it may be called an *attributable risk, attributable risk percent, attributable fraction,* or a number of other related terms as derived for just the exposed group or for the general population as a whole. Unfortunately, some will calculate a risk difference and use the term "attributable" even when causation has not been established.

Note the two measures, the rate ratio and the rate difference, provide very different information. The higher a RR is above one, the greater the likelihood that there is a true cause-and-effect relationship; but a high RR for a very rare disease among a few individuals with a unique exposure may be of *de minimis* concern from a public health perspective, whereas a lower RR for a relatively common condition might equate to an enormous number of cases. By way of example, it is generally accepted that excess exposures to vinyl chloride monomer cause angiosarcoma of the liver. The RR for this association is quite high; but the total number of excess cases, worldwide, approximates 100. By way of contrast, the RR for heart disease among cigarette smokers is only about 1.5; but the rate difference equates to a large number of cases—many, many orders of magnitude more than 100. This is because both the disease and the exposure are relatively common. From a public health perspective, it is much more important to control the excess risk of disease related to smoking than it is the risk associated with vinyl chloride monomer. Yet for the purpose of establishing a cause for the disease, it took many fewer epidemiology studies to establish an etiologic association between vinyl chloride monomer and liver angiosarcomas than it did for cigarettes and cardiovascular problems.

Rate ratios and rate differences are derived from research in which two groups are defined based upon exposure status and the disease patterns of each are followed forward in time. On occasion, it is easier to get groups based upon whether they do or do not have a specific disease and then collect data on previous exposures. For example, it may be more convenient to identify all those who developed lung cancer during some period, possibly via the use of data from a tumor registry, and identify a comparable group of healthy individuals from the general population, perhaps by means of random digit dialing within the same area codes as the cases. Gathering data on previous exposures from those in each group (or from their next of kin) would allow the calculation of an *odds ratio* or *OR,* that is, the odds of having been exposed to a particular agent given one had the disease versus the odds of having been exposed to that same agent among the healthy controls. If a study is done properly, the OR will approximate the RR. For example, if the RR for getting lung cancer among cigarette smokers is 10, the OR of having been a cigarette smoker among those with lung cancer also will be about 10. For simplicity, the rest of the text will focus predominately on two measures of association, the relative risk and the odds ratio.

METHODS

Cohort

Over the years, epidemiologists have developed a variety of methods to evaluate cause and effect. The most intuitively obvious, and the most analogous to the approach used in toxicology, is the *cohort* study. A cohort is simply a group with some common characteristic, for example, gender, ethnic background, health behavior, or exposure to a particular chemical or medicine. In a cohort study, the health experiences of at least two cohorts are compared, one with an exposure to the agent of interest and one without. Ideally, multiple cohorts, each with a different level of exposure, are identified so that the effects of low, medium, and higher levels of exposure can be assessed. Irrespective of the number of groups, conceptually exposure status is determined first and health data—on subsequent mortality, morbidity, blood cholesterol levels, etc.—are then gathered forward in time. If the exposure status is determined in the present and the health data then gathered into the future, the term *prospective cohort study* is used.

Prospective cohort studies, for all of their advantages, may not be the method of choice in preliminary investigations of the causes of disease, especially if the disease has a long latency. As opposed to toxicologists who dose animals of species with relatively short life spans (a standard chronic feeding study of mice takes 2 years), epidemiologists examine a long-lived species, humans; therefore if they only did prospective cohort studies of chronic disease, they likely would complete very few projects during their professional careers. To overcome this problem, epidemiologists often times will use historical records—personnel files, medical archives, industrial hygiene reports, etc.—to define their exposed and unexposed study groups at some arbitrary date in the past. They will then gather health data on each individual in the study groups from that point up to the present. These are sometimes called *retrospective cohort studies* or, to differentiate them from the case control method that also

gathers data on former events, they may be labeled *nonconcurrent prospective studies* or *historical prospective studies* or even *retrospective prospective studies*. Irrespective of whether the starting point for a cohort study is at the present or in the past, the results

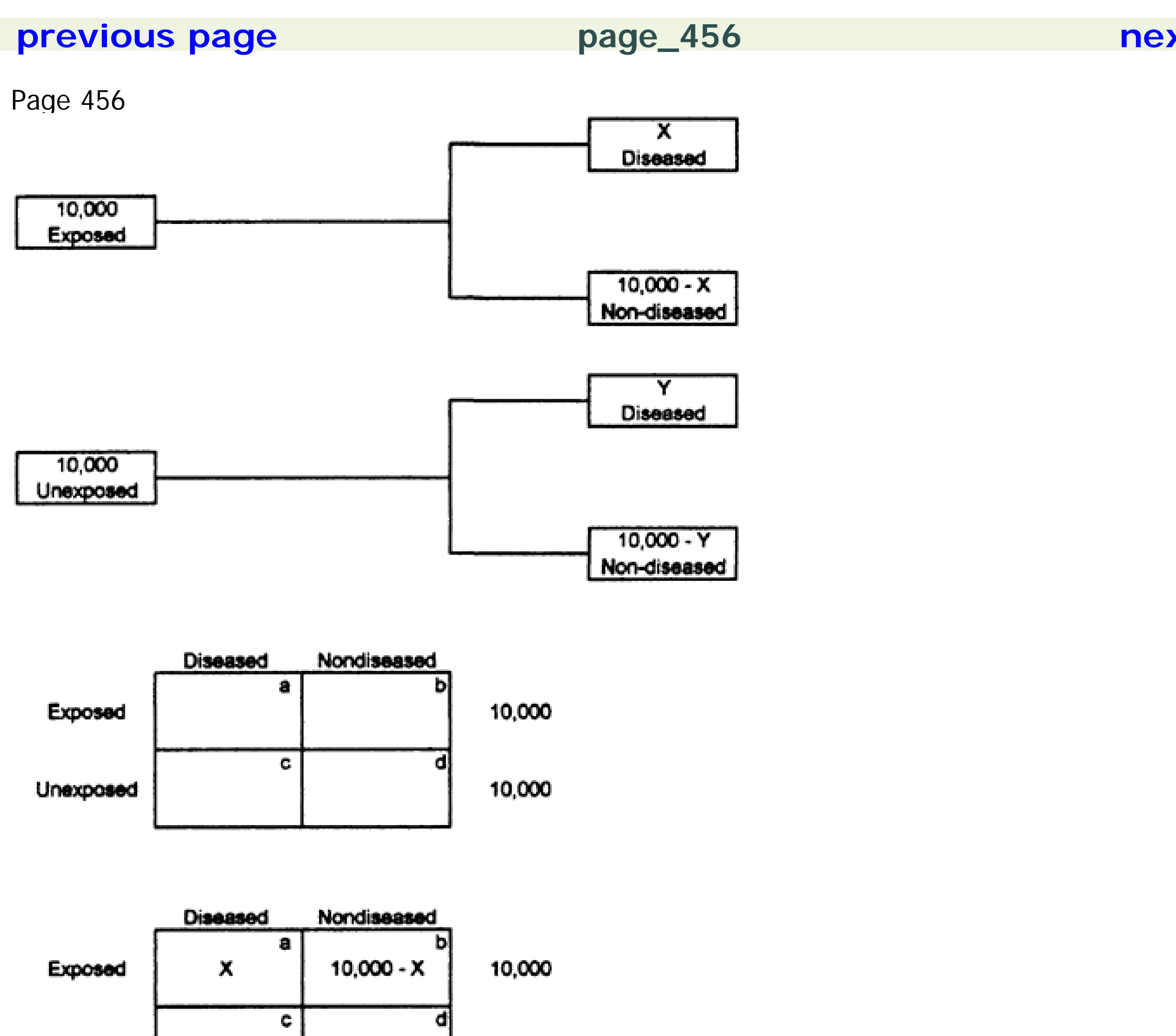


FIG. 10.3. Illustration of the cohort method.

are based on incidence data presented as relative risks (and, if appropriate, risk differences). Figure 10.3 illustrates how this is done. Two groups of healthy individuals are identified at a point in time. One group is selected because they have (or had) a known or presumptive exposure to a specific agent; the second, because they don't have (and ideally never had) the exposure. The health experience of those in each group is then compiled in an equivalent fashion over some defined period of time. This health experience is converted into incidence rates and the rates compared by means of a relative risk.

It is possible to calculate relative risks for all health events combined (e.g., total causes of mortality) or for any number of distinct outcomes (e.g., just deaths due to angiosarcoma of the liver). When the cohort approach is used in exploratory data analysis, it can be considered "an exposure in search of a disease," a hypothesis generating exercise. If it targets just one or a limited number of specific associations of a priori concern, it is akin to hypothesis testing. Many epidemiology studies are a combination of both and it may be difficult for the reader to discern which associations were of concern at the beginning of the research and which were simply serendipitous findings (3). At times, it is possible to make this determination only by reviewing the original study protocol—if there was one.

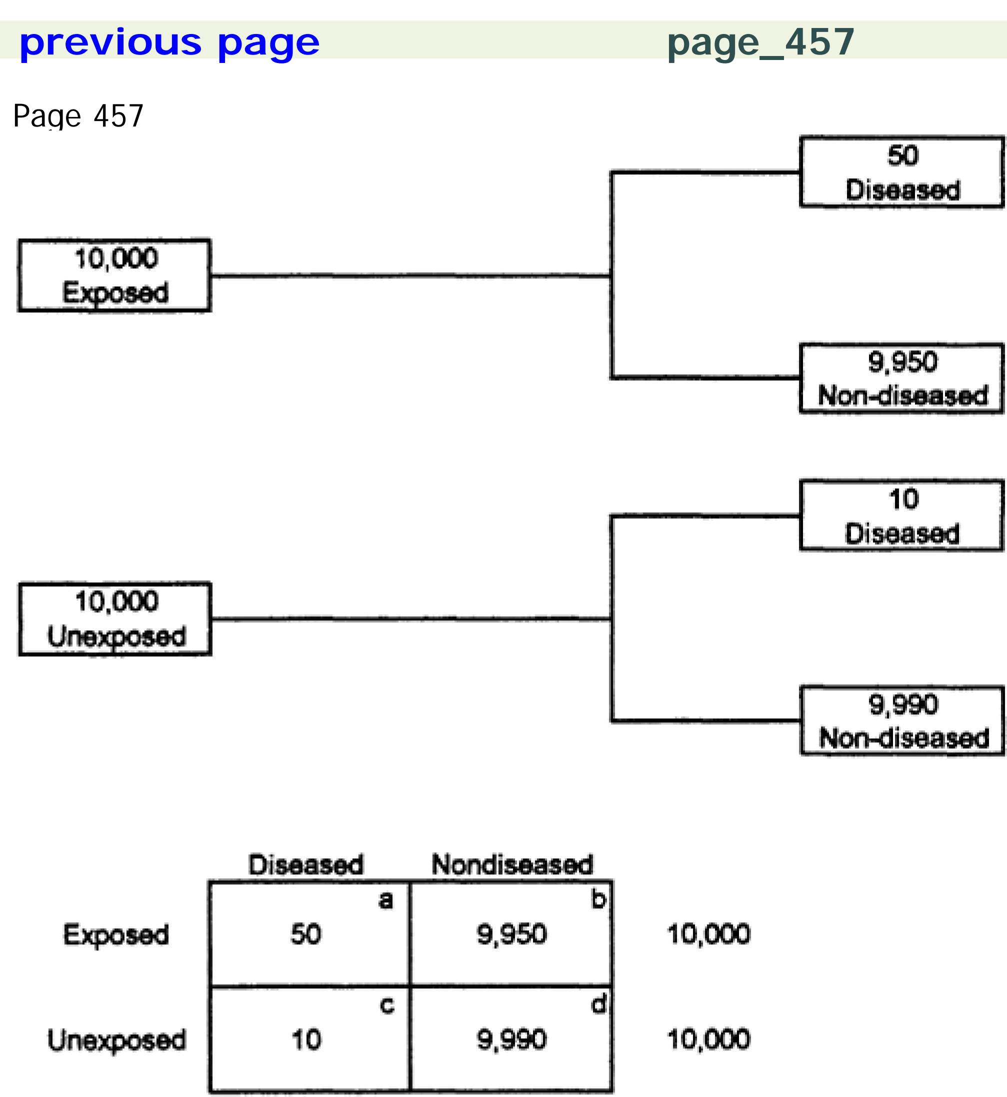
In the example shown in Figures 10.3, there were 10,000 individuals in each group at the start of the study. Therefore, the marginals for the 2 by 2 table are both 10,000. During the period of study, *X* individuals in the exposed group were observed to have developed the disease (cell a) whereas the

remainder, 10,000 minus *X*, did not (cell b). Therefore, the incidence rate for the exposed is *X* divided by 10,000 (10,000 being the totals of those in cells a and b). Among the unexposed, *Y* developed the same disease (cell c) and 10,000 minus *Y* did not (cell d). The incidence rate among the unexposed is therefore *Y* divided by 10,000. Dividing *X* over 10,000 by *Y* over 10,000 gives the relative risk. Because both groups had the same denominator, this particular RR simplifies to *X/Y*. In real life that seldom happens.

Hypothetically, the investigators might have found that 50 individuals among the exposed developed the disease and only 10 among the unexposed (Figure 10.4). After plugging these numbers into the table, the resultant calculations would produce a RR of 5. The exposed had five times the risk of developing the disease as did the unexposed—assuming there was no selection, misclassification, or confounding bias, and the finding was not a chance occurrence.

Note, in a cohort study those in both groups must be free of the condition at the start of the investigation. This implies that no one in either the exposed or the unexposed group is eligible until they are first examined and determined to be disease free. In other words, the first step of any prospective incidence study is, conceptually, a *cross-sectional* or *prevalence study*. The data from this cross-sectional study, even though they are collected on two or more "cohorts," cannot be used to make interpretations concerning etiology. They are prevalence data.

In actual practice, it may be impossible to determine baseline health status. For example, in a nonconcurrent cohort morbidity study, an investigator cannot go back in time to examine the study participants in any of the groups. Moreover, even when the study has a prospective orientation, it may not be feasible to examine those in the control group if a SMR-type approach is used, because that would mean everyone in the standard group (e.g., the U.S. white male population) would have to be examined, a logistical impossibility. Nevertheless, if the natural history of the health condition is well understood, adjustments can be made to overcome this problem. With diseases of long latency, the investigators might simply ignore the health data from the first couple of years. For mortality research, a person might be pre



	Diseased	Nondiseased	
Exposed	a 50	b 9,950	10,000
Unexposed	c 10	d 9,990	10,000

Relative Risk = (50/10,000) divided by (10/10,000)
RR = 50/10
RR = 5

FIG. 10.4. Illustration of the cohort method, RR=5.

sumed living at the start of the study if he or she was then employed, paying taxes, or receiving retirement benefits.

A cohort study can be a very labor-intensive process. Exposure histories have to be compiled and validated. Study subjects (or their next of kin) may have to be traced and contacted and data obtained on personal habits, hobbies, and a host of other variables. Medical records then must be collected and coded. Many things can complicate the process.

The first major obstacle is simply finding the study subjects. In our society, it is not unusual for someone to change their residence multiple times during his or her lifetime. Women may leave the workforce, get married, and, in the process, assume a new last name. Conversely, someone may have a name so common that it is very difficult to determine which “John Miller” is the right study subject and which is not.

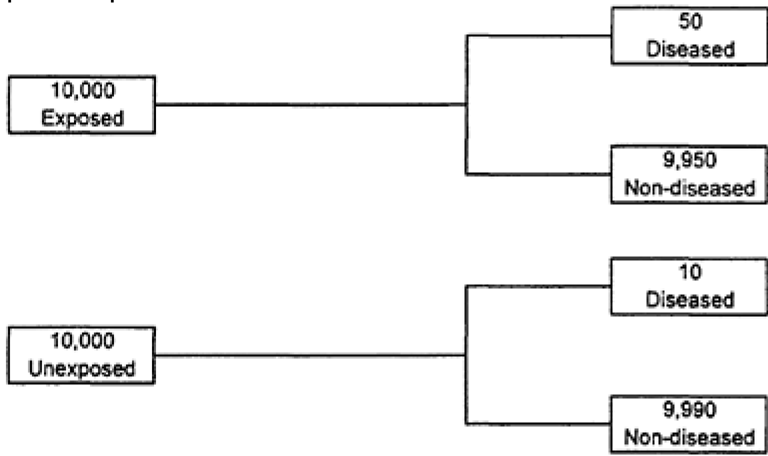
A second major obstacle is finding comparable health data on each individual. The amount of medical information can vary from person to person simply because of differences in health care-seeking behavior. The study subjects may have many different physicians, each providing a different level of care, possessing diverse diagnostic skills, and having office records with unique formats. Many states and municipalities have disparate rules governing access to government records such as death certificates. In addition, litigation and regulations may obstruct the process of data collection (5, 7, 29).

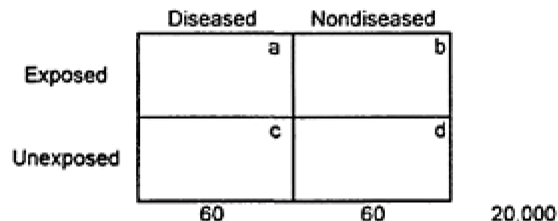
Case Control

Even if all of these obstacles can be satisfactorily addressed, it means that a great deal of effort may be needed to gather a lot of data that produce relatively little useful information. In the hypothetical example, 20,000 individuals were tracked to identify the 60 who actually got the disease. To overcome the inefficiencies of cohort studies, epidemiologists developed the *case control* method. With case control studies, the past exposures of those with some disease are compared to the past exposures of those who don't have the disease. For

example, smoking histories might be compared between men who do and do not have lung cancer. Due to the fact that data are gathered from the past, a case control study may be called a *retrospective study* or a *trohoc* (cohort spelled backwards) to differentiate it from a retrospective cohort study. Because the study participants for a case control investigation are first determined in the disease axis of the 2×2 table and data are then gathered on exposure status to fill in each of the four cells, it makes no sense to calculate incidence rates or relative risks. Instead a different measure of association is used based on the odds of past exposure. The odds of past exposure are calculated respectively for the diseased group and for the nondiseased control group. These odds are then compared to develop an OR. Because the magnitude of the OR in well-done case control research closely approximates that of the underlying RR, it allows the interpretations of a case control study to parallel those of the cohort method: An OR appreciably above one suggests a causal association between the disease and the exposure; an OR appreciably below one suggests protection; and a ratio near one, plus or minus, suggests no association between the exposure and the disease.

If we go back to the hypothetical example, there were 60 new cases of disease in this closed population (Figure 10.5). Knowing the age, gender, race, and perhaps other key characteristics of the diseased, 60 "matched" nondiseased individuals could be randomly selected from the remainder of the 20,000. These 120 would constitute the bottom marginals of the 2×2 table, 60 in each column. Data could then be collected on past exposure.





	Diseased	Nondiseased	
Exposed	a 50	b 30	
Unexposed	c 10	d 30	
	60	60	20,000

Odds Ratio = odds of exposure among the diseased versus the odds of exposure among the nondisease

Odds Ratio = 50/10 divided by 30/30

OR = 5

the cross-product ratio is *ad* divided by *bc*

OR = 50 x 30 divided by 30 x 10

OR = 5

FIG. 10.5. Illustration of the case control method, OR=5.

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In this particular example, 50 of the diseased group would end up in cell a and 10 in cell c. Among the nondiseased, approximately 30 would end up in cell b and another 30 in cell d. Parenthetically, the nondiseased in each exposure category were approximately the same, 9950 and 9990. Therefore, random sampling of 60 from the aggregate 19,940 should select equal numbers from each group: 30 and 30. With data in all four cells, the odds of exposure among the patients would be 50 to 10 (5 to 1) and the odds of exposure among the controls would be 30 to 30 (1 to 1), giving an odds ratio or OR of 5. Conveniently, the calculations for case control studies often simplify to a *cross-product ratio,* ad divided by bc. In the example, 50 times 30 divided by 30 times 10 simplifies to 5.
For this case control study, an evaluation of just 120 individuals provided the same information as a study of 20,000. In the context of the time, effort, and cost, the need to gather data on such a limited number of study subjects can be a tremendous advantage. Paradoxically, the small size of the study can also be a problem because seemingly minor amounts of bias can have a dramatic impact on the OR. Unfortunately, avoiding inadvertent bias can be more difficult in case control studies because the health outcome has already occurred. If the investigators or the study subjects are not properly "blinded," this knowledge can impact both the participants in the research and the data they provide. Social forces still may influence the results even if "blinding" is incorporated. For example, those with disease may be more inclined to participate in the research because they have a greater need to understand why they became ill. Furthermore, the cases who selectively participate may expend greater effort toward trying to remember their past exposures and thereby may provide more comprehensive or more valid data than the controls. This is called *recall* or *rumination bias*. It is a type of *information bias* that very often leads to overestimates of risk.
There is one other problem. A case control study may utilize a combination of prevalence and incidence data instead of just incidence data and thereby limit its utility for etiologic interpretations. For all of these reasons, results from case control studies are considered "lesser evidence" than those derived from cohort research.
Even with its limitations, a case control approach can be very attractive. Because the two groups are initially defined based on disease status, data on any number of exposures can be collected. As a consequence, a large number of different associations can be evaluated simultaneously and rapidly reduced to just a few that deserve further study. For that reason, an exploratory case control study can be considered "a disease in search of an exposure," the mirror image of the hypothesis-generating exercise done in a cohort mode.
A case control study also can focus in depth on just one disease-exposure association, testing a hypothesis derived from case reports or other types of research with much greater sophistication than might be feasible in a cohort study. In certain situations, it can be advantageous to use the cohort and the case control approaches in series to generate a relatively small and well-defined number of hypotheses. Such a *nested case control study* can combine the strengths of both methods. For example, the cohort approach could be used to identify a cluster of disease within a broadly defined group, perhaps all those ever employed at a multiple chemical manufacturing facility, and a case control study could then be implemented within the larger cohort not only to narrow the focus to those few agents that appear to be most important for that particular disease but also to do so with proper adjustments for confounding. This integrated approach, therefore, can achieve both efficiency and rigor.
Cohort and case control studies are sometimes called *analytic* research—in contrast to other types of epidemiology investigations that are simply *descriptive* of time, place, and person. In theory, the term "analytic" should be restricted to those studies that are designed to test a priori hypotheses; but in practice, it is often used more broadly to refer to any cohort or case control research, irrespective of whether it generates or tests hypotheses. That is unfortunate because it blurs the distinction between these two important concepts and the role each plays in the search for the causes of human disease.
As illustrated by the cartoon strip Sally Forth (Figure 10.6), determining disease etiology is basically a four-step process. Step 1 is formulating a theory. Step 2 is testing that theory. To be tested, it must be constructed in a form that is refutable. Although the theory may be stated as "exposure X is associated with an increased risk to disease Y," conceptually it has to be tested in the null (i.e., exposure X *is not* associated with an increased risk to disease Y) and the null disproved (4). If the null is rejected, that is, if an epidemiology study detects an apparent increased risk for disease among those with a particular exposure and the reasons for this excess are not otherwise obvious, step 3 is research to understand the underlying biological mechanisms of the association. Finally, step 4 is to be confident that the key results derived in the second and third steps were not statistical flukes or the consequence of one or more technical biases, each has to be replicated. As a rule of thumb, the more important the

association, the more important is the need to replicate the findings.

Case Studies or Case Series

Hypotheses for analytic epidemiology may originate from toxicology studies or from epidemiology investiga

SALLY FORTH BY GREG HOWARD

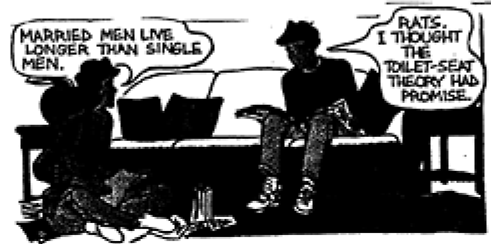

FIG. 10.6. Determining the etiology of human disease. Copyright 1996. Reprinted with permission of King Features Syndicate.

tions; but many evolve from clinical observations and are published in the form of *case studies* or *case series*. Although based a great deal on intuition, a case study is a time-honored way for a physician to develop new theories about the causes of human disease. It has been said, with some justification, that every human carcinogen was first identified by an astute clinician who published his findings in the form of a case study or case series. Nonetheless, that does not mean case studies can be used to unerringly identify new etiologic associations. Although the theories derived from case studies are not always wrong, history teaches that they are seldom right (9, 31, 95). Determining which is which depends upon data developed by others using experimental, quasi-experimental, and observational research. If we go back to the 2×2 table, we can see why.

To test a hypothesis about a new cause for human disease (to identify an elevated risk in analytic epidemiology research), data are needed in all four cells of the 2×2 table—data that are properly defined on both variables. Case studies tend to focus just on those in one of the four cells, cell a, the exposed with disease. Very little, if any, data are gathered by the clinician on those in the other three cells. Furthermore, those from whom data are gathered are a *convenience sample*. They are not a representative sample of any well-defined group, especially not a representative sample of the healthy—irrespective of their exposure history. They are not because physicians tend to direct their efforts toward diagnosing and treating those with medical problems.

In Figure 10.7, examples a through c, the three 2×2 tables represent the three possible types of association. In the first, the 50 in cell a translates to a RR of 5; in the second, the 30 to a RR of 1; and in the third, the 20 to a RR of 0.5. The three relative risks have very different meanings. Although it is conceivable that any clinician practicing in a community might become suspicious if a cluster of three or so patients came to him with the same rare disease and all had a similar exposure history, based upon the information available he would not be able to determine whether the cluster was a subset of those in cell a from example 10.7a, or from 10.7b, or from 10.7c. Most clusters, however provocative, are meaningless (96). Furthermore, additional case reports do not satisfy the need for replication and confirmation. Once a testable hypothesis has been formulated, additional case reports proposing the same hypothesis contribute nothing.

By way of example, in the silicone breast implant controversy, it was originally hypothesized that women who received this medical device were at increased risk for breast cancer. The theory was based on clinical observations and concern was increased because of an animal toxicology study that demonstrated an Oppenheimer

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Example a. Relative Risk is 5

	Diseased	Nondiseased	
Exposed	50	9,950	10,000
Unexposed	10	9,990	10,000
	60	19,940	20,000

Example b. Relative Risk is 1

	Diseased	Nondiseased	
Exposed	30	9,950	10,000
Unexposed	30	9,990	10,000
	60	19,940	20,000

Example c. Relative Risk is 0.5

	Diseased	Nondiseased	
Exposed	20	9,950	10,000
Unexposed	40	9,990	10,000
	60	19,940	20,000

A physician sees three patients with the condition and all three were exposed to the same chemical leading him to conclude that the disease in all three was caused by the chemical exposure. Is he correct?

FIG. 10.7. Case studies and case series.

effect, the tumorigenic properties of foreign bodies as observed in rodents (12, 45, 83). As a result of subsequent research, both experimental and observational, something between examples 10.7b and 10.7c is now thought to most closely approximate the association between silicone breast implants and human breast cancer. It is being theorized that these medical devices or the materials from which they were constructed offer some type of protective effect against breast cancer (15, 120). The current data-based theory is in exact opposition to the hypothesis originally derived from the case reports. Interestingly, no action has been taken on this information. Why? Probably because even though the epidemiology study results have been reasonably consistent and demonstrate coherence with the findings of the experimental animal research, and the public health implications of such an association could be profound considering both the frequency and the life-threatening characteristics of the cancer, the underlying biological mechanisms of protection have not been identified.

ISSUES

Peer review is an imperfect process. Even the most prestigious journals publish findings that are wrong. As a consequence, everything must be read with a degree of healthy skepticism (90). This can be difficult enough within a single field; but it is truly a daunting task when a scientist tries to evaluate the merit of work from a different discipline. If a toxicologist understands the basics of data, measures of

disease frequency, measures of association and methods, the epidemiology literature can be screened fairly rapidly using the "mantra" of *selection, misclassification, confounding, chance*, and *causation*. Consultation with an epidemiologist or biostatistician might still prove necessary; but only for the smaller number of studies.

The order of this mantra is important. If there are obvious technical biases related to selection, misclassification or confounding, it may make very little sense to spend time trying to evaluate the merit of the investigators statistical machinations, much less to assume the findings of statistical significance have biological meaning. It is no accident that the scientific literature has a highly stylized format: some variation of abstract, introduction, methods and materials, results, discussion, and conclusion. This format allows the reader to rapidly focus on the key components of the work. If the authors provide a one-sided presentation of the topic in the introduction, supply insufficient detail regarding their methods and materials, or do not critique their own work in the discussion—pointing out the potential biases of selection, misclassification, and confounding and how they were addressed—the reader should exercise extreme caution before accepting either the results or the conclusions, even as provisional truth.

Selection

In epidemiology, the term *bias* is used to denote a deviation from the truth; but not necessarily to imply that the deviation occurred intentionally (3, 125). *Selection bias* refers to errors that are related to systematic differences between those who are and are not selected into a study. Therefore, even if the data gathered are valid for the examined, it may be inappropriate to use any information derived from these data for purposes of extrapolation to a larger population. For example, the results of a study of hormone replacement therapy among women cannot logically be extended to men. In epidemiology research, various types of selection bias can be introduced by the study subjects, by the investigators, or even by traditional medical practice and other social forces.

Self-selection occurs in both clinical research and some epidemiology studies. It is well recognized that those who

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participate in controlled clinical investigations, those who actually sign informed consents, may not be representative of the general population. Therefore, even with subsequent randomization of treatment, care must be taken before extending the study results too broadly. A similar problem occurs in observational studies in which some type of active participation, some type of action on the part of the study subjects, is required. For example, informed consent is needed for any epidemiology study in which biological samples are collected. Usually the more invasive the procedure, the more disinclined are the potential subjects to participate, and the greater the potential for bias. However in other situations, this bias may be less obvious or, paradoxically, so obvious that it is largely overlooked. For example, how many times have you received a questionnaire in the mail and, rather than filling it out, tossed it away? By doing so, you introduced a *potential participation bias* into that investigator's work.

In certain types of observational research, self-selection is not a problem. Projects that can be conducted without the active cooperation of the subjects often are able to achieve close to 100% follow-up. For example, occupational cohort mortality studies that utilize personnel records and industrial hygiene reports to identify the exposed, and death certificates to document the cause of death, can be conducted with little or no self-selection (84).

The same arguably holds for some studies that utilize medical records, but only if the medical records relate to the total health experience of a well-defined population. Such is the case in certain countries with socialized medicine in which all the hospital and clinic records are available for the entire citizenry. In the United States, such opportunities are rare and even those few are disappearing rapidly. For example, Mayo Clinic is a world renowned referral center, providing both state-of-the-art medical treatment and highly sophisticated research on the underlying mechanisms of disease. In addition, it serves most of the primary medical needs for those who live in the relatively isolated community of Rochester, Minnesota, and shares medical records by agreement with the few other primary care facilities that operate in that area (49, 50). Having access to the total health experience on those in the community has allowed the Mayo epidemiologists to focus some of their research just on the residents and thereby to conduct high quality population-based epidemiology research that had minimal self-selection or referral bias. Recently, ostensibly for reasons of privacy and confidentiality, the state legislature passed a law requiring study-specific informed consent from all study subjects before any of their data may be utilized for research purposes, even if the patients had previously expressed a willingness to have their medical records used for any such activities

WANTED

Kansas farmers suffering from nonHodgkins lymphoma especially those exposed to herbicdes such as 2,4-D. Needed to interview for research article. Please send name, address, and phone number to Box P7629 Classified Dept. The Star, 1729 Grand, KC., MO 64108

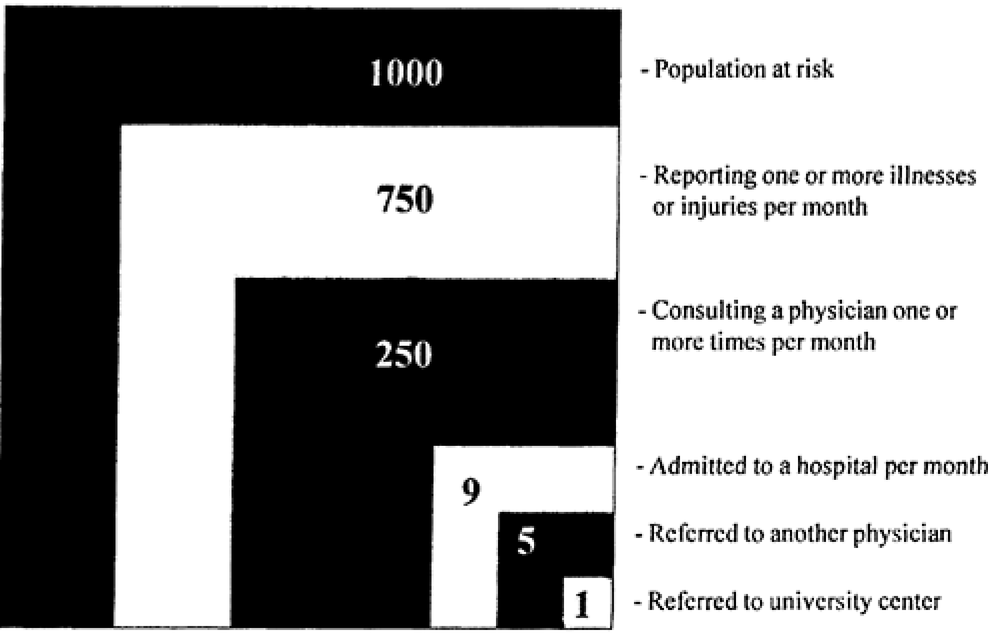
FIG. 10.8. Recruitment of study subjects.

(69). This action by the Minnesota legislature, although undoubtedly politically expedient, will not only complicate the logistics of future research at Mayo Clinic, it unfortunately may also compromise the validity of the work.

Either intentionally or not, investigators can introduce selection bias when they decide who to study, especially if they make a greater effort to get participation among the exposed than the unexposed, or the diseased rather than the healthy. Figure 10.8 is an advertisement that appeared in a Kansas paper in the late 1980s. It apparently was placed by investigators who wished to identify more subjects for a research project and thereby improve its statistical power. What they presumptively did not recognize was that by recruiting simultaneously on both health outcome (non-Hodgkin's lymphoma) and exposure (2,4-dichlorophenoxyacetic acid), they would introduce a significant selection bias into their work, one potentially so severe as to invalidate any of their findings.

More recently, a study was published in the *Journal of the American Medical Association* of children with esophageal dysfunction who had been born to mothers with silicone breast implants (73). Once again, the key study subjects had been selected on the dual characteristics of health outcome and exposure. The investigators characterized their work as a case control study and indicated they had findings that were supportive of a cause and effect association. The fact that their report was little more than a case series was missed during the peer review process and corrected later in the form of an obscure errata, and apparently only then because of ad hoc peer review, that is, a series of highly critical letters to the editor (25, 36, 39, 87).

Figure 10.9 illustrates the dynamic which leads to *Berkson's bias*, a particular type of selection bias that occurs as a result of the patterns of referral, either self-referral or physician-referral (51). Although there is some merit in asserting that the 250 individuals who



Source: Gehlbach SH. Interpreting the Medical Literature. New York: McGraw-Hill, Inc., 1993. (51).
FIG. 10.9. Berkson's bias: potential selection bias by referral.

initially consulted a physician represent those with the more definitive illness among the 1000 in the population at risk and thus were legitimate subjects for etiologic research, it is less likely that the same thing can be said about the 5 referred to a specialist or the 1 who finally ended up at a university center. Patients seen by specialists or at tertiary referral centers include a disproportionate number whose disease is complicated, obscure, or atypical. In our chemophobic society, these patients also may be referred because of a suspicion that the condition is related to what Peter Huber has called the latest *terror du jour* (64). A spuriously elevated relative risk will predictably be found in any research in which the study subjects are selected on the joint characteristics of the condition of interest and the putative agent of concern.

Even if no formal study is conducted, the specialist may develop a marked suspicion concerning the presumptive cause for the condition—and then act upon that presumption. Once it becomes known in the community that a physician or a referral center is interested in patients with a particular condition, especially when it occurs in conjunction with exposure to a specific agent, additional referrals or self-referrals further compromise the value of the sample for etiologic research (115). Ironically, the more caring the physician in the sense of being more willing to provide therapy to those who have been unsuccessfully treated or refused treatment by others, the more that physician becomes a magnet for these patients.

The 1995 publication by Robinson and colleagues entitled "Analysis of explanted silicone implants: A report of 300 patients" illustrates a number of different types of selection bias (93). Among the 300 women who Dr. Robinson explanted over the course of 3 years, 214 (71.3%) reportedly had "disruption" (defined as frank rupture of an implant or severe silicone bleed). Interestingly, these authors noted that there was "virtually no difference in the disruption rates between those patients relating symptoms to their implants and those who did not (71.8% versus 70.9%)," suggesting health complaints were not a consequence of implant status. Nonetheless, they extrapolated from this sample to predict that most implants will lose their integrity somewhere between 8 and 14 years and recommended that all gel-filled implants be removed "preferably before 8 years from implantation."

Robinson et al. based their "rates," their interpretations, and formulated a policy of explantation on

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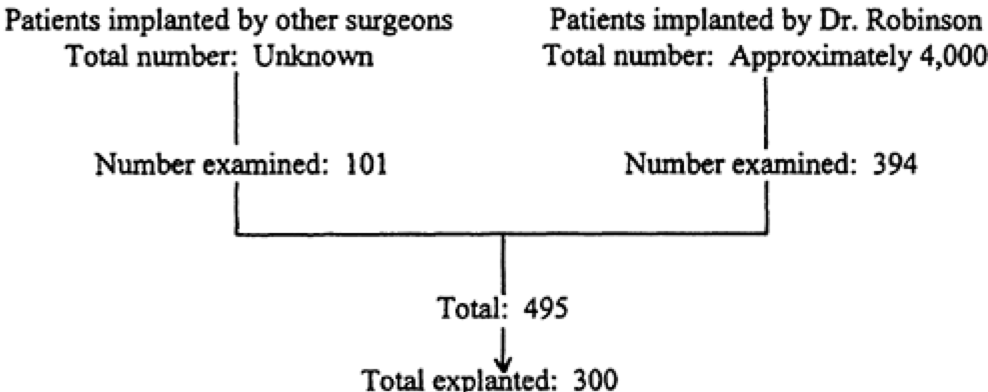
"Materials and Methods

From February 1, 1991 to January 1, 1994, 495 patients consulted O. Gordon Robinson with concerns about their silicone-gel implants. Of these 394 were his patients and 101 had been operated on elsewhere. All 495 had the same consultation and were offered implant removal. Of the 495 patients, 300 elected implant removal, and these constitute our study group."



Excerpted from Robinson et al. Analysis of explanted silicone implants: a report of 300 patients. *Ann Plast Surg* 1995;34:1-7

FIG. 10.10. Convenience sample.

data from a denominator of 300; but that was not the group they actually studied (Figure 10.10). According to the paper, Dr. Robinson saw 495 women who would have been eligible for this investigation, 101 who had been implanted by other surgeons, and 394 of his own patients. The 300 were drawn from the 495, but note, even if he had studied all 495, he still would not have been able to develop rates that were free of potential selection bias. Even with 100% participation of his sample, he would not have been able to develop rates that meaningfully could be extrapolated back to a larger group. That is because the 495 were a convenience sample, an ill-defined and likely highly biased sample of the larger population from whence they came. The larger population included all of Dr. Robinson's implant patients and, by implication, all the breast implant patients of the other 15 to 20 plastic surgeons who practiced concurrently in the same community (2). Court records indicate that Dr. Robinson implanted approximately 4000 women and it is quite possible that at least some of the surgeons in his community implanted comparable numbers (92).

So what can we make of the Robinson information? The data collected for this report were prevalence data. Although gathered over a 3-year period, for the individual study subjects they were obtained at a single point in biological time, time of surgical explantation. Because they had prevalence data, the researchers could not differentiate between events that occurred at the time of surgical implantation, during the period the implant was within the body, or at explantation. Therefore, their interpretation that implant failure was a function of the aging of the device, presumptively related to biological degradation of the silicone elastomer shell, required assumptions (e.g., the incidence time of rupture was just before explantation) that were not adequately addressed in this research.

Based on the work of others, at least some of those assumptions appear to be incorrect. Rapaport et al. have found an appreciable number of implant ruptures occur secondary to micropunctures caused by needles or other medical devices used during the implant procedure (88). Others have done work that expands on this observation (13). Brandon and colleagues, using lot-matched controls, reported that the material properties of the silicone shell were not affected by implantation for time periods up to 21 years, and concluded "that the silicone elastomer undergoes little or no change during implantation" (14). Also, Slavin and Goldwyn noted that approximately 25% of the implant ruptures they observed occurred during the explant procedure (110). At least two other mechanisms contribute to implant ruptures in vivo: closed capsulotomies (manual compression of the breast to rupture the tissue capsule surrounding the medical device) and so-called "fold flaws" (disruption of the elastomer by excessive

flexing at the site of folds in the shell). Both involve mechanical trauma. Obviously, dif

ferent approaches might better be used to prevent, control, or otherwise address implant ruptures caused by different mechanisms.

Setting aside the questions of the validity of the data and the causes of implant rupture, if the 300 who were explanted were a representative sample of the ever-implanted, then it is quite possible that a high proportion of implanted women have "disrupted" implants. Further, if "disruption" equates to implant rupture, either overt or occult, it suggests there may be a high rupture rate for these medical devices, at least for those brands and models favored by Dr. Robinson and his colleagues (22). On the other hand, if the 214 with disrupted implants are the majority of those in the numerator of a true rate, especially if disruption does not equate to rupture, then it is likely that the actual rupture rate is quite low, quite possibly a single-digit phenomenon. Of course if neither scenario is correct, then the information is invalid and has no utility at all. Furthermore—and in spite of the question about rupture rates—if these authors are correct in their observation that there is a lack of association between implant integrity and health outcome, a conclusion reached independently by others, is it good public health policy to expose all implanted women to the predictable risks of explant surgery (17, 131)? Probably not.

There are a number of lessons to be learned from this report. One, not understanding the difference between prevalence and incidence data can lead to flawed interpretations (26–28, 53–55). Two, selection bias can occur even when 100% of those selected for the study participate because the selection process itself may be flawed. Three, anytime there is less than 100% participation among those originally selected, even in a descriptive study of just the exposed group, the results are susceptible to an additional selection bias. Particularly troublesome are those situations in which the participation rates differ between the groups in analytic research—that is, among the exposed and the unexposed for a cohort study or the diseased and the healthy in a case control study—because this suggests the reasons for participation may not have been equivalent and therefore there may have been a spurious correlation between health outcome and exposure among one group or the other. The consequence of selection bias is an incorrect measure of association, possibly an underestimate of risk but often an overestimate. Complicating the situation still further, the dynamics of selection bias can change over time as a result of a well-publicized environmental controversy, a lawsuit, a provocative news program, or any number of other things. Thus, different types of selection bias can wax and wane. Four, flawed studies can lead to flawed policies, policies that ironically may put those whom they are designed to protect at greater risk.

In evaluating the literature, the reader needs to ask two questions related to selection bias. One, was the sample that the investigators were attempting to study truly representative of some larger group? Two, were the researchers successful in getting participation from all or a large majority of those they sought to study? An individual epidemiology report probably will have little or no value if the answer to either question is "no." The operative term in the last sentence is "probably." It is important to note that not every potential selection bias is real and therefore not every study in which there is less than 100% participation need be dismissed as meaningless. The question is how one determines whether a study with less than optimal participation provides relatively unbiased results. Usually one cannot make that determination from the single study. The question can only be addressed in the context of the larger body of literature. If the results of the potentially flawed study are comparable with those of other work in which selection bias is a lesser concern, the consistency suggests a cross-validation of findings. On the other hand, if the results of multiple studies are markedly different, it raises concern that the findings of one or more of the reports are biased.

Misclassification

Measurement or *misclassification bias,* also called *information bias,* is systematic error arising from the inaccurate measurement or inappropriate classification of subjects on the study variables—either exposure (to the putative agent or confounder) or health outcome. At some level, all measurement or classification is inaccurate. The errors may be large or small and, in turn depending upon the use to which the data are put, these errors may be important or meaningless. For example, in measuring blood pressure some physicians routinely round up to the next increment of 5 (e.g., 140 mm Hg systolic and 90 diastolic, or 145 and 95, etc.), others round down, and still others record to the closest unit of 2. The experienced clinician tends to make these measurements consistently on the same two of the five Korotkoff sounds; but which two may vary from physician to physician (47). These variations from the true blood pressure probably have very little importance in the clinical setting if the patient is consistently measured and treated by the same physician; but they could be very important if treatment is provided by multiple physicians. They also could be important if the clinical data were used to judge the relative efficacy of a variety of treatments as administered by different physicians.

Misclassification can be introduced into an epidemiology study by the study subjects, the measurement tool, the observer, or even, after the fact, by the consumer

of the research findings. For example, Edwards and associates conducted interviews to gather data on alcohol consumption (37). They observed that men reported significantly lower age-adjusted mean levels of alcohol use when a third party was present during the interview (probably the spouse in most instances). Conversely, study subjects may over-report specific conditions. Cautioning against placing too much reliance on self reported data, Star et al. noted that the self-reported diagnosis of rheumatoid arthritis could be confirmed in only about 20% of elderly women, a finding replicated more broadly across age strata by Sanchez-Guerrero and colleagues in a larger study of nurses (102, 116).

Overreporting or underreporting by study subjects may be a function of a number of factors unrelated to the biology of the disease (59). For example, medical students tend to develop the symptoms of the latest disease they are studying even to the extent that some male students reportedly have complained of sympathetic labor pains during their obstetrics training! To address such *reporting bias,* Turner and associates emphasized the importance of "double blinding" in clinical trials of pain medications, positing that even inadvertent clues of voice inflection or facial expression by an "unblinded" investigator could influence how a patient might report his or her symptoms (124).

There are various techniques that an epidemiologist might use to avoid or reduce the potential for either purposeful or unintentional misreporting. Concealing the intent of the research from the study subjects is one; but such "blinding" of subjects is increasingly difficult to use in a climate of mandated informed consent and almost instantaneous dissemination of news about the latest health controversy. Another approach is to add a dummy health variable whose association with the exposure is biologically implausible. For example, a query about dental caries could be incorporated into a study evaluating the effects of exercise on angina. If there is a strong correlation between the frequency with which the study subjects reported the dummy variable and the health outcome of concern, one should suspect a misreporting problem. In such a situation, it may be necessary to validate the reports—perhaps, if feasible, by examining a subset of the respondents or via review of medical records that predate the controversy or by use of a biological marker such as salivary cotinine for cigarette smoking (71, 94, 128).

At a minimum, the processes by which the data were collected should be well defined. Even then, there could be problems. It is well recognized by the seasoned researcher that mechanical or electronic instruments of assessment periodically must be calibrated to assure a consistency of measurement over time. To achieve validity, they need to be calibrated to an external standard. Yet the application of other data collection tools like questionnaires may be less than rigorous. With survey instruments, the order in which the questions were posed can be important. Even if the questionnaire is not open-ended, the words themselves may have alternative connotations for different ethnic or racial groups. To the extent possible, epidemiology research should use tools whose strengths and limitations are well recognized, or should incorporate a validation pilot into the research project.

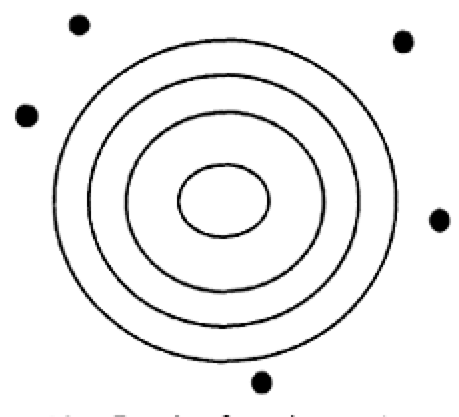
Diagnostic bias, a type of observer bias, occurs when a physician's diagnosis is influenced by his or her knowledge of certain exposures or surrogates of such exposures. In a study of eosinophilia-myalgia syndrome, Wagner et al. found up to a sixfold increase in diagnosing the condition when physicians were told the patients had ingested L-tryptophan, even though use of this dietary supplement was not part of the definition for the condition (129). In unpublished work, Cook submitted a series of chest x-rays to a board-certified radiologist and resubmitted the same x-rays about 1 month later. As is customary, a short description of each patient accompanied his respective radiograph. During one submission the patients were identified as office workers, during the other as pipe-fitters. When presented as pipe-fitters, they were more frequently diagnosed as having asbestosis.

Even laboratory tests that logically should be free of this bias may not be. In attempting to satisfy himself of the utility of a new laboratory test that purported to distinguish between those who did and did not have a particular environmental exposure, Young found a stronger correlation between test positivity and a history of exposure than the correlation with actual exposure, even when some of the histories had been fabricated. He concluded that "since the test costs at least $350, it is probably wise for surgeons to advise their patients to find a better way to spend their money" (130).

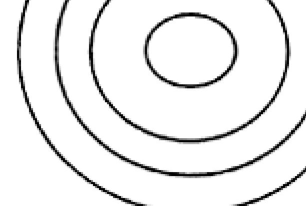
Conflict of interest is another form of observer bias. It occurs when special interests of the investigators unintentionally or intentionally compromise observer objectivity. Journal requirements for disclosure of the authors affiliations and financial interests are an attempt to control this bias, or at least make the readers aware of its potential; but this implies that affiliation and money are the only threats to objectivity (41, 56, 77, 97). There are others that are much more insidious: power, prestige, position, promotion, social philosophy, and a need to publish being just a few. The best protection against observer bias, whatever the cause, is for the investigators to be aware that it might exist and design it

out at the project's inception. This equates to a well-thought out protocol, review and approval of the protocol by an appropriate third party, slavish adherence to the approved procedures, and possibly even independent oversight of the conduct of the research. In other words, it requires good epidemiology practices.

RELIABILITY
(Precision, Repeatability)
versus
ACCURACY



Not Precise but Accurate



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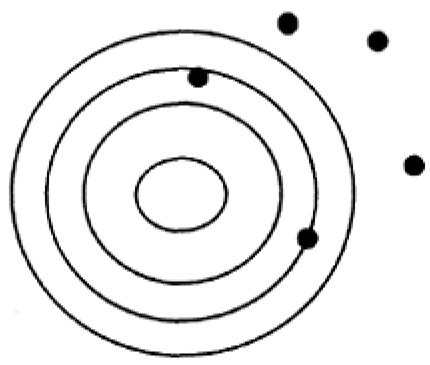

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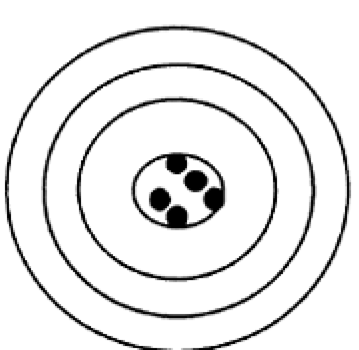

Both Precise and Accurate

FIG. 10.11. Precision and accuracy.

Misclassification can even occur after a study has been published! In the Robinson et al. article, silicone breast implants were classified as "disrupted" if, at time of explantation, the device shell was broken or it was simply judged subjectively that there was an excess of what appeared to be silicone gel surrounding the intact implant (93). Others, referencing the published paper, erroneously have implied that all the "disrupted" implants were ruptured (17, 53). Familiarity with the literature is the best protection against this form of misclassification bias. For those new to a field or an issue, it may be necessary to go back to the original sources before accepting the conclusions of a literature review or a meta-analysis.

Sensitivity and Specificity

Consistency or precision of measurement, although important, does not assure the absence of measurement bias, whatever its underlying cause. As illustrated in Fig ure 10.11, it is possible to be precise and precisely inaccurate. What is more important is accuracy, that is the validity of the data. The key measures of validity are *sensitivity* and *specificity*. Sensitivity is a measure of how well the test

identifies a true condition (disease or exposure). Specificity is a measure of how well it documents a true noncondition (the absence of disease or exposure).

It is actually more complicated than that. A number of tests that are used in medicine, like blood cholesterol or antinuclear antibody status, do not clearly separate the normal individuals from those who are abnormal (62, 121). The distribution of values in each group overlap (Figure 10.12). In such situations, the operational diagnostic break point between the two can be somewhat arbitrary. It can be set to identify all the true abnormals (point A), all the true positives, but only at the expense of accepting a certain number of false positives, of

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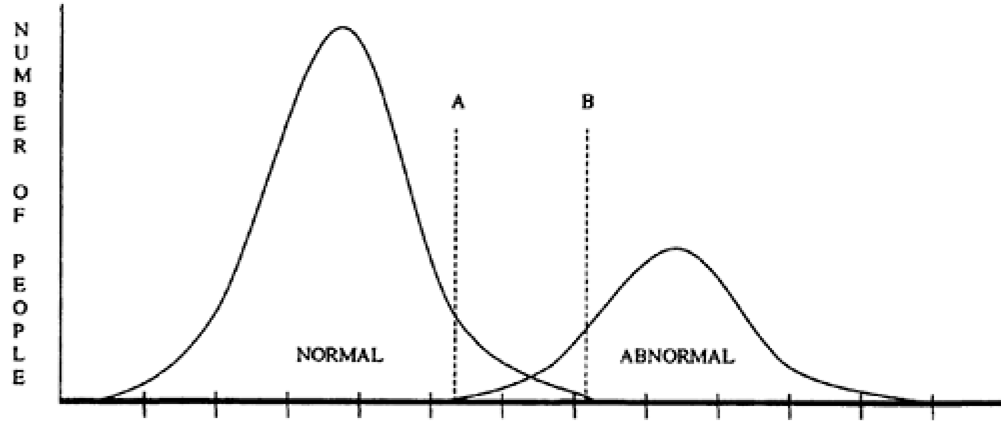
SENSITIVITY versus SPECIFICITY



TEST RESULTS

FIG. 10.12. Sensitivity versus specificity.

incorrectly labeling some normal individuals as abnormal. Or the break point could be moved to correctly identify all the true normals (point B), but only at the expense of getting more false negatives, of misclassifying some of the abnormal patients. In other words, where the break point is set can impact the sensitivity and specificity of a test; if sensitivity is improved it usually means the specificity has been compromised, and visa versa. For example, one could arbitrarily call all chemicals human carcinogens. Such an "error on the side of caution" would certainly correctly label all the true carcinogens, would guarantee a sensitivity of 100%; but the specificity of such a strategy would be abysmal because the number of false positives would be huge.

Note, to determine the sensitivity and specificity of a test, its results must be compared to those of a *gold standard*, an accepted test or procedure that reliably determines the presence or absence of the condition. Why then is it necessary to have the new test? Why not just use the gold standard? The new test may be needed because the gold standard is not as useful a tool in the clinical setting. It might be more expensive, inconvenient, invasive, or dangerous.

Paradoxically, data gathered by means of a gold standard actually might have little utility for etiologic research in and by itself, but still may be important for the development of tools that can be used in such investigations. Explantation, for example, is the gold standard for determination of implant rupture (17). However, for both technical and ethical reasons, it can only be used to collect prevalence data. There are other noninvasive techniques like mammography, ultrasound, and magnetic resonance imaging (MRI) that can collect incidence data and at lesser risk to the patient; but the relative validity of each can only be established via the gold standard of explantation.

Given there is a suitable gold standard, Figure 10.13 shows how these measures are calculated. TP is true positive, FN is false negative, FP is false positive, and TN is true negative. Sensitivity is obtained by dividing TP by (TP+FN), specificity by dividing TN by (FP+TN). For this particular example, the sensitivity and specificity are both 90%, quite good for most clinical tests (101). Sensitivity reflects how well, given the condition is actually present, the test detects the condition. Conversely, specificity is a measure of how well, given the condition is really absent, the test does not erroneously document its presence.

Predictive Value Positive and Predictive Value Negative

In real life, whether the condition is actually present or not is unknown before the test is performed. That's the reason for doing the test! For most investigations, what is of greatest interest is the predictive capabilities of a test: how well, given the test result is positive or negative, it respectively predicts the presence or absence of the condition. These measures, *predictive value positive* (PV+) and *predictive value negative* (PV−), can be obtained by making calculations in the vertical axis of the 2×2 table (Figure 10.14). In this example, the PV+ is 50% and the PV− is 98.8%. What this indicates is that among those who are diagnosed as having an illness on the basis of an "abnormal" test result, only 50% of them are truly diseased; and among those whose test result was in the "normal" range, 98.8% are actually healthy.

The two sets of measures are related but not equivalent. Although the sensitivity and specificity are relatively

	Test positive	Test negative	
Disease present	TP	FN	TP + FN
Disease absent	FP	TN	FP + TN
			TOTAL

Sensitivity = TP/(TP+FN)

Specificity = TN/(FP+TN)

	Test positive	Test negative	
Disease present	90	10	100
Disease absent	90	810	900
			1,000

Sensitivity = 90/100 or 90%

Specificity = 810/900 or 90%

FIG. 10.13. Sensitivity and specificity.

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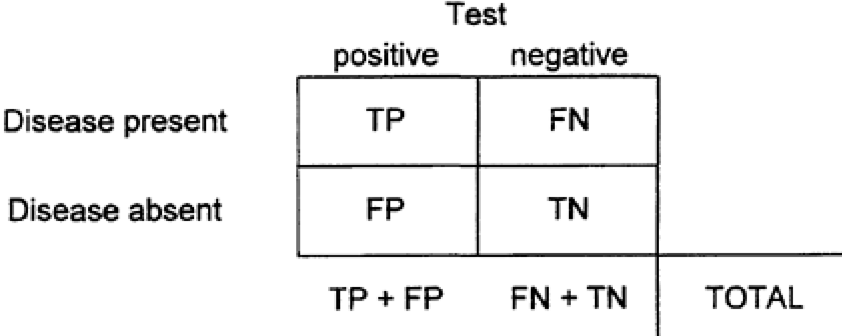

Predictive Value Positive = TP/(TP+FP)

Predictive Value Negative = TN/(FN+TN)

	Test positive	Test negative	
Disease present	90	10	
Disease absent	90	810	
	180	820	1,000

Predictive Value Positive = 90/180 or 50%

Predictive Value Negative = 810/820 or 98.8%

FIG. 10.14. Predictive value positive and predictive value negative.

stable attributes of a test, the predictive values vary widely as a function of the background frequency of the condition being studied. Figure 10.15 illustrates this point. For a given test, the less frequent the condition, the lower the PV+ and the higher the PV−. When the disease frequency drops to 1 in a 1000, the PV+ is less than 1%. In other words, if used as a screening tool, the test would label patients as "abnormal" incorrectly more than 99 times out of a hundred. This interplay between the underlying validity of a test and the relative frequency of the condition being studied impacts not only epidemiology, it also has important implications for medical surveillance (and the government regulations that mandate, fund, or otherwise support such procedures) as well as toxicology, in particular as it impacts risk assessment.

For example, a blue ribbon panel of experts recently recommended that routine mammography screening be restricted to women over 50 or those in high risk groups. In both, the frequency of breast cancer is orders of magnitude higher than it is in the general population of younger aged women. This recommendation ignited a fire storm of controversy and the panel, in part apparently due to pressure from Congress, subsequently modified its recommendation to include younger women (40). This probably will prove to be a mistake. Predictably what will happen is that the medical system will be flooded with false positives (38).

There are a number of downsides to a false-positive breast cancer screening test among younger women. One, a false-positive test can severely frighten patients. Even among those subsequently told the test was incorrect, many will retain a lingering anxiety at the very least. Two, an abnormal mammogram is often checked by means of a biopsy. This surgical procedure is associated with a cer

Given:

Sensitivity=90%
Specificity=90%

Disease Frequency	PV+	PV−
1 in 10	50%	98.8%
1 in 100	8.3%	99.9%
1 in 1,000	0.9%	99.99%

FIG. 10.15. Predictive values as a function of diseasef requency.

tain small but predictable risk of infection, bleeding, loss of sensation, and adverse reactions to anesthesia. For women without breast cancer, it is an unnecessary risk. Three, false positives put a strain on our medical care delivery system and misdirect limited resources. Four, even procedures that produce false results cost money, a financial burden that must be borne by the individual patient in the form of direct payments and by society at large in the form of increased insurance premiums and higher taxes. Five, arguably of greatest importance, some young women after one, two, or more false positive reports may lose confidence in the procedure. A certain number of these will drop out of the mammography program and never re-enroll. This means that they will not get the screening test later when they would benefit from it most.

The results of toxicology studies are not immune to this problem, in part because their results are routinely extrapolated to humans. If a high dose of an agent is found to cause tumors among rodents, it is current policy to assume it will cause some form of cancer in humans at lower levels of exposure. Unfortunately, although the sensitivity of toxicology research is quite high (but not perfect), its PV+ for extrapolations between different species of rodents is low, in the order of 50% in a study of various chemicals purposefully selected because of their presumed carcinogenicity (67). Arguably its PV+ is even lower for humans, especially for chemicals being tested simply to satisfy a mandated protocol (see questions).

Nondifferential and Differential Misclassification

Descriptive epidemiology research such as that done by Robinson and colleagues focuses on a single group (93). Nonetheless, misclassification obviously can produce erroneous information. In analytic epidemiology, the problem is compounded because data are gathered on and compared between two or more groups. This can lead to errors which are either *nondifferential* or *differential*.

With the *nondifferential mismeasurement* or *misclassification*, error is equivalent across all study groups. For example, an investigator may wish to compare the effects of growth between two groups with different dietary habits. As the health outcome, height might be assessed and recorded to the nearest inch. Even if the measurements were made carefully, two individuals with identical recorded values could easily vary in height by a half inch or more. In spite of the mislabeling, if the measurements were conducted consistently, the rank order of the various study subjects by height, short to tall, would be reasonably accurate. Furthermore, this would be the situation irrespective of the exposure group to which a particular individual might belong. As a consequence, meaningful comparisons could be made between the two groups. What the nondifferential misclassification might do is add a degree of statistical variability to the data and thereby bias the measure of association, the RR or OR, towards the null—to a greater or lesser extent depending upon the magnitude of the bias.

With *differential misclassification bias*, the relative invalidity of the data varies by study group. This may give rise to unpredictable shifts of the RR or OR either toward or away from the null and may generate marked under- or overestimates of RR depending on the size and direction of the differential error. It can even generate large measures of apparent excess risk where none really exists. In addition, this error is magnified as a function of the underlying frequency of the condition. Figure 10.16 illustrates these points. In this example, the respective sensitivities and specificities were quite high; but a slightly better job of identifying problems was done among the employees exposed: there was a better sensitivity, but also a concomitant decrement in the measure of specificity. Although the *true relative risk* was 1, that is, there was no excess risk in either group, the *apparent relative risks* among the exposed employees in plant A were quite high.

Does this happen in real life? Yes. Dr. Irving Selikoff justifiably has been recognized as one of the icons of modern occupational medicine. In 1968, he and his associates published a paper in the *Journal of the American Medical Association* entitled "Asbestos exposure, smoking and neoplasia" in which they reported an excess risk of lung cancer among insulation workers (104). Quoting from the article, "A copy of the death certificate was obtained for each of the 94 deaths. In addition we examined hospital records, postmortem findings (41 cases), as well as the surgical and pathological reports when surgery was performed (39 cases). We also re-examined histologic

Givens:

True relative risk=1, i.e., background incidence rate equivalent in the exposed and the unexposed

Differential misclassification fixed

Sensitivity and specificity among the exposed: 95% and 90%, respectively

Sensitivity and specificity among the unexposed: 90% and 99%, respectively

Background Incidence Rate	Apparent Relative Risk
10 per 100 per year	1.9

1 per 100 per year	5.5
1 per 1,000 per year	9.2

FIG. 10.16. Risk estimates in presence of differential misclassification.

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specimens. It was found that the death certificate was inaccurate in 14 instances." For comparison numbers, that is, expected deaths by cause, they used "United States 1964 life tables for white males." In their well-meaning attempts to be thorough, they introduced a significant bias into their study because, by using multiple sources to diagnose the exposed and only one source (life tables based on death certificates) to determine cause of death among the unexposed, they compromised their study results with differential misclassification. As a consequence, they overestimated the true relative risk.

Differential misclassification continues to contaminate research and clinical practice. Propelled by a series of case reports in the late 1980s and early 1990s, a cascade of systemic diseases were alleged to have been caused by silicone breast implants (105, 108, 114). Many of these theories have been evaluated in case control and cohort studies and found wanting (33, 119). An unresolved question is whether women with implants are at higher risk to something called atypical connective tissue disease (ACTD) or, by some, siliconosis and, more recently, systemic silicone related disease (SSRD) (107, 111). One of the problems with this alleged condition is that no one can define it well enough so that it can be rigorously studied to determine whether it occurs uniquely or more frequently among women with breast implants. Investigators who have tried have reported that the condition is essentially the same as fibromyalgia or chronic fatigue syndrome and the risks appear to be equivalent in women who both do and do not have breast implants (21). However, those who allege a unique disease does exist and waits to be discovered criticize this work as "studying the wrong disease" (112, 132).

To address this presumptive shortcoming, one physician proposed epidemiologists utilize a set of criteria that he developed to diagnose SSRD in his medical practice (113). It is based on a series of inclusionary, exclusionary, and relatively nonspecific clinical criteria. By definition, one of the two inclusionary criteria are needed to make a diagnosis, either "current or past silicone gel-filled breast implants" or a "local disease" such as capsular contracture or implant rupture. Parenthetically, neither of the latter could occur unless a woman had an implant. They are, therefore, surrogates for the exposure of interest, silicone breast implants.

Restricting a diagnosis of SSRD to those with the joint characteristics of exposure and health outcome means that two women with exactly the same signs and symptoms, one with silicone breast implants and the other without, could never be classified as having the same condition. By means of the inclusionary criteria, Dr. Solomon assured, however inadvertently, that any epidemiology study that used his definition would be biased by essentially a 100% differential misclassification (113). In theory, any etiologic research based on his definition would produce a spurious elevated relative risk that approached infinity.

Similar biases occur in toxicology research, both nondifferential and differential. The traditional acceptance of tumors, benign and malignant, as a surrogate for cancer is one form of misclassification. In well-conducted studies, it probably is nondifferential; but any time the methods for disease determination differ between the exposed animals and the controls, it could be differential. For example, if more histopathological slides are made or read for the exposed animals than the controls, it is more likely that small occult tumors will be found among the exposed. This is differential misclassification, one that would introduce an overestimate of risk.

The reader of scientific reports can garner clues regarding the potential for misclassification bias, both nondifferential and differential, from the Methods and Materials section of these articles. The variables, both exposure and disease, should be well defined and equivalent throughout. If they are not, the reasons for the differences should be discussed. The techniques for data collection should be reasonable and applied consistently across all groups. It is important to be particularly vigilant if a study report did not discuss the potential for misclassification bias and how it was addressed. This suggests the authors were either naïve regarding the problem or chose to ignore it. In either situation, the potential for bias could be high.

Confounding

A potential *confounder* is a determinant for the disease in question, an alternative "cause" whose effects may confound or confuse the results of an epidemiology study. It can either be an agent itself or a surrogate for that agent. For instance, age is a surrogate for a constellation of biological, environmental, and social factors that individually and in aggregate are associated with increased risks to certain diseases. The same can be said for race and ethnic background. One of the two major characteristics of a confounder is that it is a "cause" for the disease under study. Different agents have different effects. None are universal confounders.

A confounder is not the same as an *effect modifier,* although an agent, depending on the study, can be one, both, or neither. With effect modification, there is a nonuniformity of effect across various levels of

the effect modifier (98). For example, the consequences of exposure to pathogenic organisms varies by immunization status.

In addition to being an alternate cause, the other major attribute of a confounder is that it must be unequally distributed across study groups. That is, *confounding* only occurs when a determinant of the outcome of interest

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(a confounder) is unequally distributed among the exposed and the unexposed in a cohort study or among the diseased and the nondiseased in a case control study. As with the biases related to selection and misclassification, the degree of differential distribution determines the direction and magnitude of the error. In addition, the relative "potency" of the confounder can also, to a greater or lesser extent, influence the apparent relative risk or odds ratio.

For example, cigarette smoking is one of the major determinants of lung cancer. Any epidemiology study investigating the carcinogenic potential of a particular agent vis-à-vis lung cancer has to take this into consideration, has to "control for smoking." If not, the greater the proportion of the exposed who are or were smokers, the greater will be the overestimate of actual risk. On the other hand, if more controls smoked, the true risk to the putative agent will be underestimated.

Smoking is also associated with mortality due to cardiovascular disease; but not to the degree to which it causes lung cancer. In other words, equivalent amounts of unequal distribution between the two study groups may not have the same impact on the measures of risk for different conditions because the potency, the biological activity of a confounder, varies from disease to disease. With lung cancer, smoking equates to a relative risk of perhaps 10, whereas for cardiovascular mortality the relative risk lies closer to 1.5, and for still other diseases it has a RR which approximates 1 (no effect).

Very few, if any, diseases have only one etiology. Even a rare malignancy like angiosarcoma of the liver has a number of alternative causes aside from vinyl chloride monomer (42). Agents with high potency are relatively easy to discern. It is those with lesser biological activity that are more difficult to identify. An indeterminate number of the latter undoubtedly have not yet been discovered. Theoretically, because all the causes for the various diseases are unknown, some level of confounding may occur in any epidemiology study (and any toxicology study for that matter). In addition, it is highly likely that there are synergistic and antagonistic actions between various agents, both exogenous and endogenous, further complicating the picture. Evidence is also growing that suggests that effects seen at high dose may actually be reversed at low dose, a phenomenon that makes the interpretation of the dose response curve more challenging (18).

In experimental studies, the number of variables are purposefully kept to a minimum and ostensibly all of them are under the control of the investigators. Those who conduct observational studies of humans do not have the same advantages. The number of variables are only limited by life itself. Each participant in an epidemiology study has his or her own unique genetic makeup and own unique pattern of extraneous exposures (diet, medications, personal habits, etc.). Although either or both may be only weak confounders for a particular health outcome under investigation, they may be one reason why epidemiology research, especially any single study, has difficulty in reliably identifying putative agents with lesser biological potency, with true relative risks less than 3 or so (123). That is because even in the absence of selection and misclassification biases, the signal may be swamped by the noise of uncontrolled confounding. In epidemiology, the signal-to-noise ratio is improved via more research, especially more targeted research. As the exposure-disease associations become more focused, the relative risks should increase in size. If they don't, be suspicious of claims of causation. Also be suspicious of etiologic interpretations based on one study unless there is supporting evidence.

Control of Confounding

Confounding can be addressed through study design, data analysis, or a combination of both. For example, if smoking is a confounder for a particular disease (i.e., those who smoke get the disease more frequently than those who don't smoke, but those who don't smoke still get the disease), confounding by smoking can be dealt with via a technique called *subject category restriction;* that is, by restricting the study subjects (both those exposed to the putative agent and the controls) to just those who never smoke. This design strategy simplifies the analysis and interpretation of the data; but it also restricts how broadly the results can be extrapolated. If only nonsmokers are studied, the results derived from the sample usually only apply to the larger population of nonsmokers. Comparable information about smokers must come from another study restricted to exposed and unexposed individuals, all of whom smoked.

Alternatively, if a certain number of subjects are being evaluated and it is known that a proportion of those in the exposed and unexposed groups were smokers, controlling for confounding could be attempted at the analysis stage of the research, possibly by means of a *stratified data analysis* whereby different strata of smokers are analyzed and the results combined across strata. With the advent of high speed computers, ever more sophisticated statistical techniques have been developed to control confounding; but most of these incorporate assumptions that may or may not be valid depending upon the circumstances and, because they involve complex calculations within a "black box," it is often

impossible for the reader (and perhaps even the investigators) to assess the relative impact of the various assumptions on the results.

Matching on potential confounders—age, race, gender, smoking, etc.—is an intuitively attractive way of address

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ing confounding that combines elements of both study design and data analysis. However, it is not a panacea (98). Not only may it be difficult to do properly, it also places certain constraints on the types of information that can be developed. And it may lead to *overmatching,* that is, to matching on surrogates of exposure or health outcome (54).

No matter what method is used to prevent or control confounding, decisions about which specific potential confounders might be important need to be made at the stage of protocol development, if for no other reason than to assure that adequate data are collected. Obviously, it would be impossible to control for smoking during the analysis stage of the research if no data had been collected concerning cigarette smoking.

Confounding is not restricted to epidemiology research. It also occurs in toxicology. For example, Hart and associates have explored the impact of food intake in laboratory animals (61). They noted that animals fed *ad libitum* have poorer health and longevity than those whose diet has been restricted. The total caloric load appears to play a role, but trace contaminants may also be important. As reported recently by Paolini and colleagues, most standardized diet formulations used by cancer research laboratories worldwide, "contain the well-known mutagenic carcinogenic element manganese at the same level and, in some cases, at an even higher level (up to ninefold) compared to that used to study the carcinogenicity of manganese itself' (85). Obviously, the more animals eat, the higher their caloric load and the higher their dose of this carcinogen. However, the amount ingested could be an unintended consequence of the experiment, for example, ever larger amounts of the test chemical mixed with the food may make the food less and less palatable. For those experiments in which ingestion varied by dose level of the experimental agent, it is quite possible that the results reflect a measure of confounding, and perhaps effect modification. Paolini et al. also summarized a number of problems with using historical controls. For example, "B6C3F1 mice have a higher natural incidence of tumors than humans, and this incidence has also changed over time, increasing in excess of 50% over a period of just 10 years."

Although it is impossible to control for all possible confounders in any single study, the reader of epidemiology reports should determine whether attempts were made to control those factors which likely would have had the greatest impact on the results. As with other types of potential bias, a paper can offer a number of clues as to how well this issue was or was not properly managed. If confounding was ignored or obviously inadequately addressed, be skeptical of the information. Look for confirmation in other work which did try to minimize confounding.

Chance

Within the mantra of selection, misclassification, confounding, chance, and causation, the rubric *"chance"* covers all things mathematical and statistical and some that are methodological. For example, did the investigators add, subtract, multiply, and divide properly? Were the number of subjects consistently the same in the abstract, results, discussion, and tables? With more complex statistical procedures, especially those conducted in the mode of exploratory data analysis, it is possible for even the most seasoned epidemiologist to inadvertently lose part of a data set or to ignore a key assumption and thereby produce erroneous results. If numbers are inconsistent within a report, do the authors explain why? If they do, does the explanation seem appropriate or does it smack of gerrymandering or numerology? If either of the latter, look for confirmation of the results elsewhere. Or look for a correction published as an errata in a subsequent issue of the journal.

The term "statistical significance" is used by both epidemiologists and toxicologists. It means that within some acceptable measure of statistical "wobble" two findings were not equivalent, a measure of association such as the relative risk was different than 1, a trend was found, two variables were highly correlated, etc. It is not the same as biological significance because it does not speak to the underlying validity of the data. As a consequence, if it is apparent that the data set in a research study is likely biased by selection, misclassification, or confounding, it may make very little sense to analyze the data or to accept any information resulting from a data analysis.

Nor does statistical significance equate to cause and effect even in situations where the underlying data may be valid. For example, Vojandi, Campbell, and Brautbar published a study in 1992 that compared the results of a large number of tests of immune function among women who had breast implants for more than 10 years with those of a sex- and age-matched control group composed of women who did not have these medical devices (127). In this study, they identified a number of differences ($p<.001$) between the exposed and the unexposed and concluded that "these immunological abnormalities in individuals who underwent silicone breast augmentation indicate a mechanism of tissue injury to these patients causing autoimmune diseases or syndromes…"

Their data may have been valid; but their inference regarding breast implants was not. In the study, all of the implanted women had "symptomatology in relation to the musculoskeletal and nervous system" and all the unimplanted women did not. In the context of the 2×2 table, they only collected data for two of the four cells, a and d: the exposed/diseased and the

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unexposed/healthy. As a consequence, they could not disentangle the two variables and determine whether the implants "caused" the disease. They could not calculate a relative risk or odds ratio and therefore could not determine whether women with breast implants were more likely to get disease. At best, what their study could do was to appraise the efficacy of their test battery for differentiating between those who did and did not have disease, irrespective of exposure (24). But even for that, the exercise was of little utility because the "disease" was so poorly defined and the battery of tests so broad.

p Values and Confidence Intervals

Increasingly, epidemiologists are moving away from the use of *p values* and toward *confidence intervals (CI)* (43). *p* values, although useful, can obscure important characteristics of the underlying data set. By itself, a *p* value less than .05 or .01 suggests a finding that deviates from the null (e.g., a RR that differs from 1), but not whether the result is higher or lower. Nor does it necessarily provide insight regarding statistical power. With confidence intervals, one set of numbers representing the range of values which are consistent with the data observed, for example, the 95% confidence interval, provides not only an indication of where the point estimate of risk lies relative to the null, but also gives the reader a sense of the underlying variability of the data and, therefore, the *statistical power* of the study to detect a problem given one exists. If 1 lies within a 95% CI, it indicates the finding is not statistically significantly different from 1. If the lower value of a 95% CI is greater than 1, the estimate of risk is statistically significantly elevated. If the upper value is less than 1, it is statistically significantly decreased. Furthermore, the width of a confidence interval is an indication of the power of that study, at least for that particular outcome. If narrow, the power of the study was large. Conversely, if wide, the power was low.

It is important to note that a study result may have a wide confidence interval and still be valid. Statistical power and study validity are not equivalent concepts. One addresses precision, the other accuracy. In fact, a result from a small study relatively unbiased by selection, misclassification, and confounding may be more valid than the result from a larger study that has a narrower confidence interval. Although the former may have limited utility in and by itself to support or refute causation as a consequence of its low power, when combined with the results of other studies of comparable quality, it may prove to be very valuable. This is the rationale underlying *meta-analysis*.

Meta-Analysis

Meta-analysis refers to the use of statistical tools to combine the results of different studies. Originally, it was confined to randomized controlled clinical trials, to combining results of multiple small studies of the equivalent design, that is, those with identical dosing regimens and comparable, well-defined outcomes. It is increasingly being used to aggregate the findings of multiple epidemiology studies, even when their results were derived by means of disparate methods (e.g., cohort and case control studies), the sample sizes varied by orders of magnitude, the categories of exposure differed, and the disease outcomes were similar but not equivalent (11). Although some decry the use of meta-analysis for this purpose, others view it as an important adjunct to the traditional, more subjective literature review. Done properly, meta-analysis promises not only an aggregate quantitative measure of risk that has a narrower confidence interval than each individual study, but it also facilitates the identification of any studies that may be outliers, perhaps because of various types of technical bias or differences in study design.

Meta-analysis is not the same as *data pooling*. Whereas meta-analysis depends upon the research results as obtained from epidemiology reports, pooling refers to the aggregation of the actual raw data from many different studies and the subsequent analysis of this larger, single data set. Conceptually, pooling has some advantages over meta-analysis; but in practice it also has a number of disadvantages, a major one being access to the data. Unlike meta-analysis where the results have been distributed publicly via the scientific journals, data are not as readily available. In part, this is because of concerns related to protecting the privacy of individual study subjects and the confidentiality of their data (6).

The validity of a meta-analysis is dependent upon the validity of the studies included in the exercise. To address this problem, some have suggested that a priori rules must be established with respect to which studies to include or exclude. Unfortunately, these rules may reflect the personal biases of the person doing the meta-analysis. For that reason, a type of sensitivity analysis is arguably a better approach (86). In this type of analysis, the results of all available studies are first evaluated together and then various combinations are used to better understand how the different methods, number of study subjects, classifications of exposure, or definitions of health outcome may have influenced the calculations. It can even be used to compare and contrast the results of different studies that may have

different types of bias and to explore whether potential bias is a likely explanation for why one or just a few of the studies seem to be outliers. If a comprehensive sensitivity analysis is conducted and the results published, readers also have the opportunity to make their own interpretations, something that can be difficult to do with the traditional literature review or even with pooling.

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There is one particular type of bias to which meta-analyses are particularly prone: *publication bias*. Publication bias is a type of selection bias. It refers to the tendency of authors to submit and editors preferentially to accept studies with provocative findings (3, 34, 68). This has also been called *positive results bias* and can be exacerbated by a *hot stuff bias* (100). The publication of "I had a patient like that too" case reports is an example of the latter. Such a flurry of case reports following the initial announcement of an interesting finding in either a medical journal or the popular press can give undue credibility to hypothesized associations, even if they are not real. There are a number of different approaches that can be used to assess the possibility of publication bias, but the best way to avoid it is to aggressively search for pertinent research reports, including those in the form of dissertations, abstracts, and publications in obscure journals (90).

Exploratory Data Analysis and Multiple Comparisons Bias

To the general public, all findings of statistical significance have basically the same merit. They either accept them as exact and correct or, when faced with apparent contradictions, become frustrated with science. The late Senator Edmund Muskie, following an exhaustive series of federal hearings in which various experts testified about a complex environmental issue, epitomized that frustration when he reportedly said that he wanted to meet a one-armed scientist, someone who did not always say, "On the one hand this, but on the other hand that."

Scientific discovery is not a destination. It is a journey with many side trips along the way. It starts with a hypothesis, a theory whose genesis may be any number of things ranging from the subjective (clinical observations that seem unusual for intuitive reasons) to the super quantitative (statistically significant findings derived during *exploratory data analysis* of a large medical data set, e.g., the health claim files of a private insurance company or of Medicare/Medicaid). Before these findings can be accepted as even provisional truth, they have to be confirmed by additional research, preferably well-focused hypothesis-testing research.

In both hypothesis-generating and hypothesis-testing exercises, the same statistical tools and the same levels of statistical significance may be used. Yet the findings of the former do not carry the same interpretive weight as those from the latter (109). That's because the former, in addition to uncontrolled confounding, are subject to a *multiple comparisons bias* (122).

The statistical tests used in health research factor in both a type I and a type II error. A type I is the error of rejecting a null hypothesis, of concluding that a difference exists when, in truth, it does not. By convention, the alpha level (the probability of a type I error) is usually set at .05 (that equates to a 95% CI). This means that a certain predictable number of statistically significant findings are incorrect, about 1 in 20. The greater the number of comparisons, the greater is the number of spurious associations that may be found, that is, the larger is the multiple comparisons bias. Various techniques have been developed to address this bias, the simplest perhaps being the *Bonferroni correction* in which the putative alpha is divided by the total number of comparisons and the "corrected alpha" used to determine the presence or absence of statistical significance (78). For example, if the study alpha level was preset at .05 and 10 comparisons were made, a Bonferroni-corrected 95% CI would, in essence, be a 99.5% CI.

In many studies in which a large number of comparisons are made, the authors will do a Bonferroni correction or some analogous procedure and report the confidence intervals with and without the adjustment. In others, they will not; but they will indicate the total number of comparisons and thus allow the reader to develop his or her own opinions about the merit of the findings. In still others, it may be difficult for the reader to recognize the potential for a multiple comparisons bias, especially if investigators practice surreptitious data dredging—engage in exploratory statistical analyses of large and diverse data sets, but selectively report only those results which support their own pet theories (82, 103, 122). Because few comparisons are presented, the reader is given the erroneous impression that only those few were considered and therefore they must have been of some a priori concern. This approach can be particularly attractive to quasi-scientific advocacy groups who recognize the publicity value of a statistically significant cluster.

Post Hoc Reasoning

The latter is but one of a number of variations on the theme of purposefully biased science (66, 81). In another, the investigator simply scans a data set and determines which hypotheses he wishes to test. Or he may gerrymander the data set and thereby construct an artificial cluster. In either case, by having foreknowledge of what the cluster is and where it is located in the data set, he can reduce the total number of actual statistical procedures and therefore, even with "overly conservative" corrections for multiple comparisons, claim to have refuted the null hypothesis. The nefarious may even point to a

hypothesis in a protocol that predated the formal statistical analysis. Although the work seems to fit the scientific method, giving the results an aura of biological credibility, the findings are a product of *post hoc reasoning*. They are worthless. Using this approach, statistically significant clusters even can be generated from a table of random numbers.

Investigators who are guilty of post hoc reasoning are sometimes derisively called *Texas sharpshooters* (58).

In most target shooting, one shoots at a bull's eye. The Texas sharpshooter first shoots at the side of the barn (perhaps from very close to the building) and then draws the bull's eye around the holes. By doing so, he claims his marksmanship is both precise and accurate.

If clusters of disease are the catalyst for an epidemiology study, they can introduce another form of self-fulfilling reasoning into the research. It occurs when an investigator stumbles upon a cluster of disease, perhaps in an occupational group, and then uses the cluster both to develop a hypothesis about one or more of the chemicals to which the group was exposed and also to test this hypothesis, that is, the cluster is incorporated into any subsequent analytic research. If the disease is rare, it is quite possible that an elevated relative risk will be found in the formal epidemiology study even if no new cases are discovered in the expanded cohort. Although the additional research in this situation may be designed, initiated, and conducted after the theory was developed, it will not be an independent test of hypothesis (3, 23, 44).

In summary, even the most precise results may be wrong, a consequence of simple mathematical errors, technical bias, or less innocent intent. Although exploratory data analysis is a valuable tool, more is not always better. This maxim applies equally well to epidemiology, toxicology, and clinical medicine (Figure 10.17). To be interpreted properly, the results of tests must be put in the context of the size of the data set, the number of tests that were performed, the body of information that is already available, and even, if possible, the mind-set of the investigators at the inception of the research. The latter may be obvious from the introduction of the paper or from the protocol; but sometimes it can only be surmised.

Causation

Even when selection, misclassification, and confounding are minimal, the identification of the causes of human disease is not simply an exercise of calculating which exposure-disease associations are statistically significant. It is a thoughtful process based upon the preponderance of evidence and a logical ordering of that information. Sir Bradford Hill, a British statistician/ epidemiologist, presented his criteria for determining causation in the mid-1960s and subsequently refined them for his textbook (63). These criteria are still in wide use. In interpreting data, he noted that an investigator must deal with two basic problems: *significance* (the statistical reliability of a finding) and *inference* (the deductions one might make from such a finding). With the former, he cautioned against overinterpreting statistical significance and also noted that, if absent, "chance is a not unlikely reason" for an apparent difference, for an apparent association, and for an apparent elevated relative risk. As for inference, he offered nine criteria for differentiating between "causation or merely association" when faced "with a clear and significant association

You seem to be in fine health but let's run a few tests. I'm sure we can find something wrong with you.

FIG. 10.17. Multiple comparisons bias in clinical medicine.

Table 10.2 Hill criteria for causation

Strength of the association
Consistency
Specificity
Temporal relationship
Biological gradient
Biological plausibility
Coherence of the evidence
Experiment
Reasoning by analogy

Adapted from Reference 63.

between some form of sickness and some feature of the environment" (Table 10.2).

His first criterion was *strength of the association;* in other words, the size of the relative risk or odds ratio. Obviously not every statistically significant relative risk is meaningful; but the larger the number, the less likely any observed association is simply the result of random error or the consequence of selection, misclassification, and confounding. The question is, "How large is large enough?" For isolated findings, seasoned epidemiologists are reluctant to accept relative risks of less than 3 or 4 (123).

Sir Bradford's second criterion was *consistency*, the finding of similar relative risks for the same condition and exposure in different epidemiology studies conducted by different investigators on different groups of participants. In part, this is important because it is unlikely that the equivalent errors would be replicated in all the studies. Therefore, a finding that is consistent across many studies is more likely true. It logically follows that a summary measure of risk as derived from consistent findings will more likely reflect the underlying biological truth than the results of any single study. As mentioned earlier, meta-analysis provides such a summary measure. It is a way of teasing out a signal from the cacophony of noise that is inherent to epidemiology. If statistically significant, the findings of multiple small studies may be biologically important; but also meaningful can be the absence of elevated risks in study after study after study or as de Grasse Tyson has emphasized, "Null results matter, too" (31). Although it is theoretically impossible to prove the negative, when multiple studies fail to identify an association between disease and a particular exposure, pragmatic scientists conclude proof of causation is lacking and move on.

As his third criterion, Sir Bradford offered *specificity*, elevated risks to a single or small number of well defined health problems. When many disparate conditions are attributed to an agent, at some point it becomes question able whether any of them are a likely consequence of exposure. The need for specificity also applies to the disease itself. No meaningful body of etiologic research can be conducted to determine if a condition occurs more frequently among the exposed if the "disease" cannot be defined because, perforce, each individual study would be evaluating a different outcome. The same holds for exposure. Although the initial stages of investigation may incorporate broader categories of disease such as "pulmonary disease" and mixtures of chemicals, knowledge comes with focus.

Sir Bradford's fourth criterion dealt with the *temporal relationship* of the exposure and the disease or, as he put it, "which is the cart and which is the horse?" In cross-sectional or prevalence research, it is often impossible to make this determination. Conditions with long latency or those whose signs and symptoms wax and wane over time can further complicate the picture (124). Nonetheless, if the condition occurs before the exposure, it cannot have been caused by the exposure.

His fifth criterion was *biological gradient,* that is, if small doses cause harm, do larger doses cause greater harm? Parenthetically, this is not a variation of the assumption inherent to quantitative risk assessment, that is, that if large doses are associated with health problems, lesser doses cause lesser problems (18). Something akin to linear extrapolation back through zero exposure must be assumed for the latter. Such an assumption is not required for the former.

Biological plausibility was presented as a sixth criterion. This he implicitly categorized one of the lesser tier of criteria because "what is biologically plausible depends on the biological knowledge of the day." Some consider this necessary to prove causation, that is, that the underlying mechanisms of action must be understood before cause and effect can be accepted. For many, it is too stringent a requirement. They are satisfied if a meaningful association is found for a risk factor even if the exact causal agent and the process by which it works is unknown. In a sense, biological plausibility also is a lesser criterion because it is subordinate to other criteria. For example, a biologically plausible explanation for a disease excess is meaningless if there is no disease excess.

The seventh criteria addressed the *coherence of the evidence,* the amalgamation of what is known

concerning the natural history and biology of the disease, the presumptive actions of the etiologic agent, the results of experimental research on animals, and the contributions of other types of information. The evidence can come from within a single study or across studies from many different disciplines. Cigarette smoking, for example, is associated with both an increase in lung cancer and an excess risk for a constellation of other diseases, in part because smoke is a mixture of noxious agents. Although lung cancer may be the outcome of interest in a particular

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study, say one evaluating the impact of low levels of smoking, an increase in both lung cancer and the other pertinent diseases would add coherence to any evidence of harm. As for multidisciplinary evidence, the decrease of mammary tumors among methylnitroso urea-exposed animals implanted with silicone gel-filled devices adds credibility to the epidemiology findings of lower breast cancer risk among women with silicone breast implants (120).

The next attribute was *experiment;* but not necessarily in the context of a laboratory experiment. He also considered the removal of the presumptive etiologic agent a type of experiment. If a problem resolves following such removal, it may provide support for cause and effect; but even this is not absolute proof. Diseases wax and wane. If the putative exposure is removed at the apex of disease severity, resolution may take place coincidentally and the condition, in the absence of exposure, may return at a later date. If that were the case, it would suggest the original "experiment" was incomplete and therefore lent fallacious support to conclusions about cause and effect. To eliminate this possibility, there must be adequate follow-up of the patients following removal of the putative agent. Even then, there are a number of other things that can confound such experiments. Humans react to subliminal clues, exhibiting both placebo and nocebo effects, and these can present as either subjective symptoms or more objective signs of disease (117). Resolution of a condition may be related to concomitant treatment. Alternatively, its original presentation and subsequent resolution can be due to malingering (106).

Sir Bradford's ninth and final criterion was *reasoning by analogy,* that is, if agent *X* can cause disease *Y,* then perhaps a material similar to X can cause a disease comparable to Y. Some have argued that because new environmental immunologically mediated diseases such as eosinophilia myalgia secondary to L-tryptophan exposure are still being identified, it is possible (they imply probable) that silicone is also associated with a new disease. However, eosinophilia myalgia has a relatively short latency. It also has a characteristic clinical presentation. Both of these attributes are missing with silicone. If there is an epidemic of a unique autoimmune disease caused by silicone, it has not yet been discovered. If this is because it is a disease of long latency and therefore the epidemic has not yet occurred, one has to ask the question, "What then is the basis for the legal controversy?"

Legal Causation

At one time, courts tended to disregard epidemiology as simply a statistical exercise that provided information of little probative value; however, within the last 10 to 15 years, it has become key to the legal theory of causation as used in a particular type of litigation, that dealing with tort or product liability (10). Epidemiology research not only helps establish whether an agent is causally associated with a particular disease, but also whether the association supports a finding of "more likely than not." This equates to an attributable risk percent of greater than 50% and, with knowledge of the relative risk, can be calculated with the following formula: $AR\%=(RR-1)/RR$. For example, a relative risk of 3 would equate to an attributable risk percent of 67%.

As mentioned earlier, the various calculations regarding attributable risk have no meaning until causation for human disease is established, until an acceptable number of the Hill Criteria have been satisfied. Therefore, in theory, there are four characteristics of an exposure-disease association that are needed before a claim of causation logically can be accepted in legal deliberations. One, the putative agent must be a known cause of the disease. Two, the causal relationship must be more likely than not. Three, the plaintiff must have been exposed to the agent in adequate quantity and for sufficient duration. Four, the plaintiff must have developed the appropriate disease after the exposure. The first two deal with *general causation*. The last two pertain to *specific causation*. In tort liability cases, the plaintiff has the burden to prove all four, at least in theory. Trials are emotional events and jury deliberations can sometimes be more influenced by the subjective rather than the objective.

Prior to the 1993 Daubert decision, juries were the triers of fact and judges basically functioned as the umpires of the proceedings (30, 46, 89). They made rulings regarding process but few about content. The Daubert case changed that. After a series of appeals that went all the way to the Supreme Court, judges were given the additional responsibility of "gate-keepers." Juries retained the role of triers of fact; but judges were charged with determining which body of "facts" were relevant and reliable versus which were simply "junk science"—which testimony would "assist" the jury in their deliberations and "whether the 'probative value' of the testimony substantially outweighed the risks of prejudice, confusion or wasted time" (65). In practice, this means federal judges now must decide which "expert witnesses" can and cannot testify and what opinions they will be permitted to convey to the jury. Many state courts are also moving toward a process based upon the Daubert principles.

Some courts have done an impressive job in rendering judgments that included sophisticated legal

arguments well infused with scientific principles (80). Others have accomplished the same result with the help of outside experts employed directly by the court, an option acknowledged in the Daubert decision (60). Still others at the state level have yet to apply the Daubert principles, in part because some judges feel uncomfortable with their

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new role and in part because the new rules technically pertain to just the Federal judiciary (35). Lawyers and judges, even at the federal level, are still exploring the limits of the gatekeeper function and how certain statistical and epidemiologic thought might be translated into legal concepts. For example, statistical significance means a finding has a lower confidence limit above 1, that is, there is some assurance that the estimate of risk is different than 1. The legal notion of "more likely than not" requires a relative risk above 2; but it is unclear whether the key finding, to be admissible, has to be statistically significantly different than 1 or statistically significantly different than 2. When there is just one or a limited number of epidemiology studies, the latter makes more sense. However, the former is not inconsistent with epidemiology opinion when there are a large number of studies which have similar results.

Clinical Causation

Neither epidemiology causation (what Sir Bradford Hill called "medical causation") nor legal causation should be confused with *clinical causation*. The primary goal of clinical medicine is diagnosis and treatment. In a sense, the major reason for a diagnosis is to predict which treatment will most successfully reverse, eliminate, or control a patient's troublesome symptoms or signs of pathology. If the diagnosis is correct, the resulting treatment works and the patient is well served. If not, the patient likely gets no better, possible may get worse, or even may develop additional adverse outcomes as a result of the inappropriate therapy.

Experienced clinicians are adept at the technique of *differential diagnosis*. Through the use of various signs, symptoms, and test results, and factoring in the risks inherent to alternative treatments, they identify the most probable diagnoses, weigh the merits of each, and use the resultant information to help select a treatment that likely will be most successful. If that particular treatment does not work, they move on to the next most likely diagnosis and a different treatment—and if that doesn't work to still another, continually balancing benefit and risk.

When clinicians speak of searching for the "cause" of a patient's problems, they usually are referring to identifying the most likely diagnosis, quite possibly one whose underlying mechanisms of action are unknown. Arguably, knowledge regarding the underlying cause of a particular disease is only important in the clinical setting if it materially impacts treatment decisions; for example, if a specific type of bacterial pneumonia is more efficaciously treated by a particular antibiotic. Also, the underlying causes are not initially discovered by the process of differential diagnosis. Such knowledge is derived from experimental animal research, controlled clinical investigations, and observational epidemiology studies. Contrary to what some physicians have asserted, differential diagnosis, no matter how sophisticated, does not obviate the need for etiologic research (1, 52, 57, 91). As the many programs of the National Institutes of Health demonstrate, research regarding cause and effect and that related to diagnosis and treatment are complementary but not equivalent.

Parenthetically, proper diagnoses are made by means of pattern recognition; by what Margolis has called "habits of the mind" (76). Within the context of clinical causation, this has a number of implications. One, the more extensive a physician's training and experience, the larger the number of mental templates he acquires against which he can compare the next patient's combination of signs, symptoms, and test results. Thus, even if the underlying etiology for a condition is unknown (i.e., the condition is "idiopathic"), a physician may develop successful strategies for treating the syndrome. Two, this knowledge, no matter how prodigious, is always finite. Physicians recognize this. They specialize so that they might concentrate their energy on developing in-depth knowledge within one sector of medical practice; and even within that specialty, they refer patients to their peers, a tacit acknowledgment that another physician may be better suited to diagnose and treat a particular individual. Three, because the number of templates increases as a direct result of experience, the more seasoned the clinician, the greater his ability to diagnose and paradoxically, the greater the potential for a multiple comparisons bias. The latter is reflected in case reports.

CONCLUSION

As de Grasse Tyson noted in his recent essay, entitled "Certain Uncertainties," "the frontier of science is a messy place" (32). As a consequence, to the uninitiated, science appears to provide contradictory and therefore unreliable findings, irrespective of whether the research is experimental, quasi-experimental, or observational; but perhaps more so for the latter (Figure 10.18). Part of the reason for the apparent inconsistencies is related to technical bias: selection, misclassification, and confounding; but part is due to overinterpretation of the findings of any single study, either by the study investigators or by the consumers of research reports.

One of the primary goals of any scientist should be the elimination of bias from his or her research. The

first step is to acknowledge that various types of bias exist. The second is to understand how they occur. The third is to develop methods and procedures to avoid, minimize, or control bias. Over the years, well-trained epidemiologists have found ways to address potential error and improve the validity of their research. The same can be said for toxicologists and clinical investigators. The scientific method has been core to all of these endeavors.

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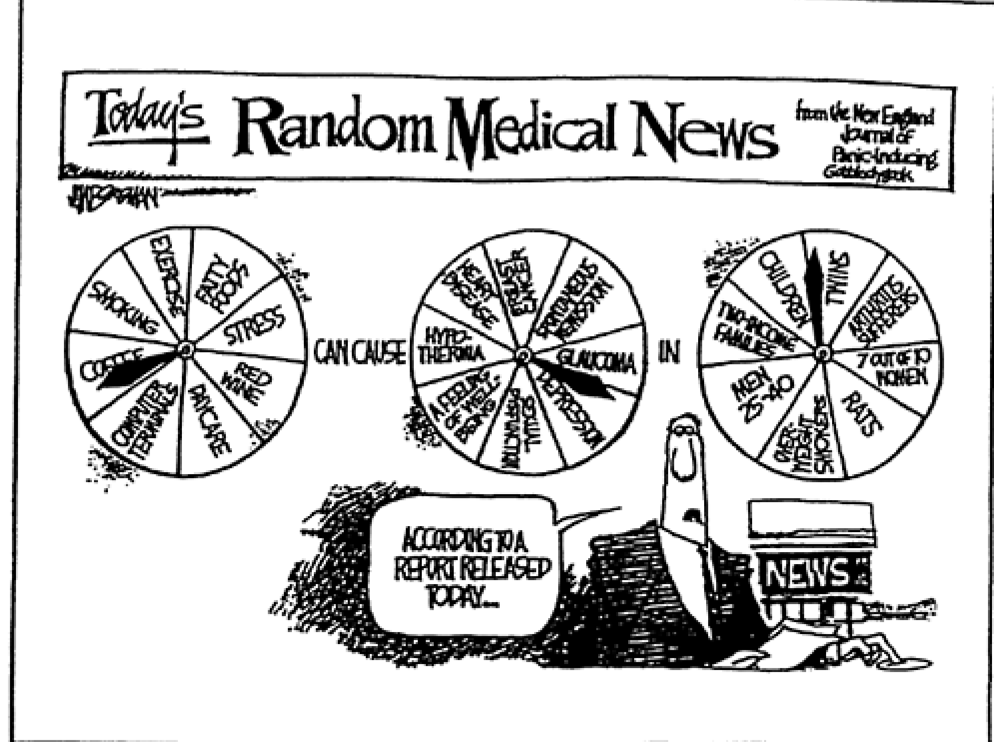

BY JIM BORGMAN

FIG. 10.18. Today's random medical news. Copyright 1997, *The Cincinnati Enquirer*. Reprinted with special permission of King Features Syndicate.

The scientific method is one of the major discoveries in human history (16, 126). It has allowed mankind to gain a more objective view of the universe; to better understand the workings of the atom; and to successfully identify the causes of disease and with that understanding to treat, control, and even eliminate some of the major scourges that once were endemic worldwide. Unfortunately, the scientific method can be laborious, inconvenient, time consuming, and expensive. The temptation to take shortcuts can be great; but history teaches that shortcuts often just lead to further confusion (99). Identifying the truth can be difficult enough even in the best of circumstances; but it is impossible with biased data, inappropriate methods, or muddled logic.

One of the responsibilities of the technical journals is to screen research papers and determine which have sufficient rigor in data, methods, and interpretation to warrant publication. However, scientific investigation is a human endeavor and peer review an imperfect process, therefore flawed studies still get published, even in the best of journals. For that reason, the ultimate judgment regarding the value of any single report or group of reports may have to be made by the consumer: the epidemiologist, toxicologist, physician, lawyer, judge, newspaper reporter, or other member of the general public less well versed in the scientific method. This chapter provides a conceptual framework whereby such winnowing of fact from fancy might be accomplished. Within a basic understanding of epidemiology data, measures of association, and methods, it is based on the mantra of selection, misclassification, confounding, chance, and causation.

QUESTIONS

Exercise One

Among 50 employees of a chemical company producing ethylmethyl chicken wire, the company phys

ician identified 2 last year with lung cancer. In the general population, the incidence rate of lung cancer is 5 per 1000 per year. What is the relative risk for lung cancer among the chemical company employees? Does this finding support the legal concept of "general causation"? What about "medical causation" as defined by Sir Bradford Hill?

Answer

Based upon the information provided, the presumptive incidence rate for lung cancer among the employees was 2 per 50 per year, which is the same as 4 per 100 or 40 per 1000 per year. Because the incidence rate in the general population is 5 per 1000 per year, the apparent relative risk is 40 divided by 5 or 8.0.

To support the legal concept of "general causation," two conditions must be satisfied. One, the putative agent must be a known cause of the condition. Two, the association must be "more likely than not." In other words, the relative risk must be above 2 and statistically significant. In this situation, we don't know whether ethylmethyl chicken wire is generally accepted as a known cause for lung cancer, in part because we have no knowledge of the results of any other experimental or observational research on this chemical. Furthermore, we don't know whether the finding in this particular study is statistically significantly greater than 2 or even whether it is significantly above 1. Calculating the 95% confidence interval could be done; but, with due consideration for the small number of cases that were observed, it is quite possible that this was a chance occurrence.

General causation aside, even if this had been the first report of lung cancer associated with the chemical, the data provided are insufficient to conclude that ethylmethyl chicken wire should be considered a potential risk factor for this type of malignancy. For example, we don't know how the two diagnoses of lung cancer were made. Were they based on x-ray or confirmed by biopsy? The former is much less likely to be correct than the latter. We also don't know whether the two lung cancers developed before or after first exposure. Although the determination of the exact incident time of a malignancy is usually impossible to determine, if the company had been producing the chemical for just a short time or the two employees had just recently been hired, the known latency of lung cancer would suggest that the condition predated any possible putative exposure. Moreover, who made the original diagnosis and when? If the diagnoses had been made earlier by the employees' personal health providers and only "identified" later by the company physician during the course of a routine clinic visit, the cancers could have been *prevalence* and not *incidence* cases.

In addition to more information about those in the numerator, what about the denominator? Did this company only have 50 employees? If there were considerably more and only 50 had been seen at the company's medical clinic, it is quite possible that a *selection bias* could have artificially inflated the incidence rate for lung cancer. If such was the case, the relative risk was seriously elevated. Furthermore, to calculate the incidence rate for the plant population, we had to assume the two who developed the condition, given they were diagnosed accurately, actually were exposed. If they worked in a part of the company remote from the production facilities, for example, in accounting or sales, an incorrect assumption that they were exposed could have introduced a *misclassification bias* into the calculations, one that resulted in another spurious elevation in relative risk.

Finally, the comparison incidence rate, the absolute risk among the unexposed, 5 per 1000 per year, was that of the general population. That means there were other causes for lung cancer, like smoking. Furthermore, the term "general population" suggests the rate was based upon the experience of both men and women. The two genders have distinctly different incidence rates of lung cancer. If the distribution of either the attribute of gender or smoking was not the similar in both the exposed and controls, then *confounding* could have biased the results.

Exercise Two

There are two hospitals in the same city, a smaller one with 30 births a month and a larger one with 300 births a month. At the end of the year, which hospital is likely to have experienced more months with more than 60% male births?

Answer

The sample size is smaller for the smaller hospital. As a consequence, the variability of the data is greater. Thus there is a greater chance that the smaller hospital will have more months with more than 60% male births.

Exercise Three

In a study of chemicals associated with site-specific neoplasia in rodents, Huff and colleagues reported that "25 chemicals were carcinogenic to the liver in both rats and mice, 9 chemicals caused liver cancer only in rats; 53 caused liver cancer only in mice; and in 226/313 studies, no chemically related liver

tumors were observed in either rats or mice" (67). They also stated that "the overall interspecies concordance in liver carcinogenicity is 80% (251/313)." That means if the mouse bioassay

were used as a screening test of the carcinogenic potential of chemicals for rats, it would have a sensitivity of 73.5% and a specificity of 81.0%. In this example, what was the predictive value positive and the predictive value negative of the mouse bioassay?

Answer

The easiest way to solve this problem is to set up a 2×2 table similar to that found in Figure 10.14. If the outcomes in rats is the "truth" that we wish to predict, there were 25 true positives (TP), 9 false negatives (FN), 53 false positives (FP), and 226 true negatives (TN).

Predictive value positive was TP/(TP+FP) or 25/78. In other words, less than a third (32.1%) of the chemicals that were positive in the mouse bioassay were actually carcinogenic to rats.

Predictive value negative was TN/(TN+FN) or 226/235. The mouse bioassay correctly predicted which chemicals would not cause liver tumors in rats 96.2% of the time.

Exercise Four

In Exercise Three, 34 of the 313 chemicals tested caused liver tumors in rats, about 1 in 10. If among additional chemicals to be tested randomly, only one in a hundred would actually cause liver tumors in humans, how predictive would be the results of the mouse bioassay? Calculate the predictive value positive, the predictive value negative, and the concordance. Assume the same level of sensitivity and specificity for the mouse bioassay as found with rats. For convenience, assume also that 3400 chemicals were tested.

Answer

Among the 3400 chemicals tested, 34 are actually human carcinogens. With a sensitivity of 73.5%, that would mean 25 would be true positives (TP) and 9 would be false negatives (FN). With a specificity of 81%, among the remaining 3366 chemicals, approximately 2726 would be true negatives (TN) and 640 would be false positives. Using these numbers, a 2×2 table can be set up, and the predictive values and the concordance calculated.

Predictive value positive would be TP/(TP+FP) or 25/665 or 3.8%. Of the 665 positive tests in mice, less than 4% of them would correctly predict what would happen in humans.

Predictive value negative would be TN/(TN+FN) or 2726/2735 or 99.7%. In other words, of the 2735 negative mouse bioassay studies, 99.7% would correctly predict that the chemicals would not produce cancer in humans.

Concordance would be (TP+TN)/(TP+FN+TN+ FP) or 2751/3400 or 80.9%, basically the same as that found by Huff for his interspecies study.

REFERENCES

1. *Allison v. McGhan Medical Corp.*, #1:93-CV-2051-RLV (N.D.Ga. November 3, 1998).
2. *American Society of Plastic and Reconstructive Surgery and the Plastic Surgery Education 1996 Combined Roster*. ASPRS/PSEF, Arlington Heights, IL.
3. Anderson, B. (1990): *Methodological Errors in Medical Research—An Incomplete Catalog.* Blackwell Scientific Publications, Oxford.
4. Angell, M. (1997): *Science on Trial: The Clash of Medical Evidence and the Law in the Breast Implant Case.* W.W.Norton & Company, New York.
5. Anon. (1997): Informed consent litigation could severely hamper epidemiologic research. In: *The Epidemiology Monitor*, edited by R.H.Bernier and V.M.Mason, pp. 18(8):1–3.
6. Anon. (1999): OMB explains how it intends to implement new requirements for release of research data collected under federal grant dollars. In *The Epidemiology Monitor*, edited by R.H. Bernier and V.M.Mason, pp. 20(3):7–10.
7. Anon. (1997): Pharmacoepidemiologists moving to protect access to medical record information. In: *The Epidemiology Monitor*, edited by R.H.Bernier and V.M.Mason, pp. 18(6): 1–3.
8. Austin, D.F. (1979): *Preliminary Report, Cancer Incidence Rates, Industrial and Non-Industrial Areas of Contra Costa County.* California Department of Health Services (unpublished). Emeryville, CA.
9. Bender, A.P., Williams, A.N., Johnson, R.A., and Jagger, H.G. (1990): Appropriate publish health responses to clusters: The art of being responsibly responsive. *Am. J. Epidemiol.*, 132:S48–S52.
10. Black, B. (1990): Matching evidence about clustered health events with tort law requirements. *Am. J. Epidemiol.*, 132:S79–S86.
11. Blair, A., Burg, J., Foran, J., Gibb, H., Greenland, S., Morris, Raabe, G., Savitz, D., Teta, J., Wartenberg, D., Wong, O., and Zimmerman, R. (1995): Guidelines for application of meta-analysis in environmental epidemiology. *Regul. Toxicol. Pharmacol.*, 22:189–197.
12. Brand, K.G., Johnson, K.H., and Buoen, L.C. (1976): Foreign body tumorigenesis. *CRC Crit. Rev. Toxicol.*, 4:353–94.

13. Brandon, H.J., Young, V.L., Jerina, K.L., Wolf, C., and Schorr, M.W. (1997): Diagnosis of breast implant failure mechanisms. *Presented at the 13th European Conference on Biomaterials,* Goteborg, Sweden. (4–7 September, 1997).
14. Brandon, H.J., Young, V.L., Wolf, C., and Jerina, K.L. (1997): Long-term material stability of explanted breast implants. *Plast. Surg. Forum,* XX:215–216.
15. Brinton, L.A., Malone, K.E., Coates, R.J., Schoenberg, J.B., Swanson, C.A., Daling, J.R., and Stanford, J.L. (1996): Breast enlargement and reduction: Results from a breast cancer case-control study. *Plast. Reconstr. Surg.,* 97:269–75.
16. Bronowski, J. (1956): *Science and Human Values.* Harper & Row, New York.
17. Brown, S.L., Silverman, B.G., and Berg, W.A. (1997): Rupture of silicone-gel breast implants: Causes, sequelae and diagnosis. *Lancet,* 350:1531–1537.
18. Calabrese, E.J., and Baldwin, L.A. (1997): A quantitatively-based methodology for the evaluation of chemical hormesis. *Hum. Ecol. Risk Assess.,* 3:545–554.

page_483

19. Checkoway, H., Pearce, N., and Crawford-Brown, D. (1989): *Research Methods in Occupational Epidemiology.* Oxford University Press, New York.
20. Cheevers, J. (1981): CC industry may not be cancer culprit. *Contra Costa Times.* Walnut Creek, California.
21. Chow, H.Y., Cash, J.M, Calabrese, H.H., and Wilke, W.S. (1996): Patients with chronic fatique syndrome (CFS) and silicone-associated disease (SAI) are similarly disabled. *Arthritis Rheum.*, 38 (Suppl. 9):S52.
22. Collis, N., and Sharpe, D.T. (1998): Rupture of silicone-gel breast implants. *Lancet*, 351:520.
23. Cook, R.R. (1981): Dioxin, chloracne and soft tissue sarcoma. *Lancet*, 1:618–619.
24. Cook, R.R. (1993): But is it significant? *Ann. Plast. Surg.*, 31:94–5.
25. Cook, R.R. (1994): Sclerodermalike esophageal disease in children breast-fed by mothers with silicone breast implants. *J. A. M. A.*, 272:767–768.
26. Cook, R.R., Curtis, J.M., Perkins, L.L., and Hoshaw, S.J. (1998): Rupture of silicone-gel breast implants. *Lancet*, 351:520–521.
27. Cook, R.R., Hoshaw, S.J., and Perkins, L.L. (1998): Failure of silicone gel breast implants: Analysis of literature data for 1652 explanted protheses. *Plast. Reconstr. Surg.*, 101:1162.
28. Cook, R.R., Hoshaw, S.J., and Perkins, L.L. (1999): Failure of silicone gel breast implants. *Plast. Reconstr. Surg.*, 103:1091–1092.
29. Cook, R.R., Tirey, S.L., Spadacene, N.W., and Woodbury, M. (1994): Access to data for epidemiological studies. In: *Environmental Epidemiology: Effects of Environmental Chemicals on Human Health*, pp. 231–244, edited by W.M.Draper. American Chemical Society, Washington, DC.
30. *Daubert v. Merrell Dow Pharmaceuticals, Inc.*, 509 U.S. 579 (1993).
31. de Grasse Tyson, N. (1998): Belly up to the error bar. *Natural History*, 11:70–74.
32. de Grasse Tyson, N. (1998): Certain uncertainties. *Natural History*, 10:86–88.
33. Diamond, B.A., Hulka, B.S., Kerkvliet, N.I., and Tugwell, P. (1998): Silicone breast implants in relation to connective tissue diseases and immunologic dysfunction: A report by a national science panel to the Honorable Sam C.Pointer, Jr., coordinating judge for the Federal Breast Implant Multi-District Litigation in *In re Silicone Breast Implants Products Liability Litigation (MDL 926)*, #CV 92–10000-S (N.D.AL November 17, 1998).
34. Dickersin, K. (1990): The existence of publication bias and risk factors for its occurrence. *J. A. M. A.*, 263:1385–1389.
35. *Dow Chemical Company v. Mahlum*, 970 P.2d 98 (Nev. Supreme Court 1998).
36. Editors of the JAMA. (1994): Correction: Incorrect study design in abstract. *J. A. M. A.*, 272:770.
37. Edwards, S.L., Slattery, M.L., and Ma, K. (1998): Measurement errors stemming from nonrespondents present at in-person interviews. *Ann. Epidemiol.*, 8:272–877.
38. Elmore, J.G., Barton, M.B., Moceri, V.M., Polk, S., Arena, P.J., and Fletcher, S.W. (1998): Ten-year risk of false positive screening mammograms and clinical breast examinations. *N. Engl. J. Med.*, 338:1089–1096.
39. Epstein, W.A. (1994): Sclerodermalike esophageal disease in children breast-fed by mothers with silicone breast implants. *J. A. M. A.*, 272:768–769.
40. Ernster, V.L. (1997): Mammography screening for women 40 through 49: A guidelines saga and a clarion call for informed decision making. *Am. J. Public Health*, 87:1103–1106.
41. Fairweather, W.E., Higginson, J., and Beauchamp, T.L., eds. (1991): *Ethics in Epidemiology.* Pergamon Press, New York.
42. Falk, H., Herbert, J., Crowley, S., Ishak, K.G., Thomas, L.B., Popper, H., and Caldwell, G. (1981): Epidemiology of hepatic angiosarcoma in the United States: 1964–1974. *Environ. Health Perspect.*, 40:107–113.
43. Feinstein, A.R. (1998): P-values and confidence intervals: Two sides of the same unsatisfactory coin. *J. Clin. Epidemiol.*, 51:355–360.
44. Fingerhut, M.A., Halperin, W.E., Marlow, D.A., Piactelli, D.A., Honchar, P.A., Sweeney, M.A., Griefe, A.L., Dill, P.A., Steenland, K., and Suruda, A.J. (1991): Cancer mortality in workers exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *N. Engl. J. Med.*, 324:212–218.
45. Food and Drug Administration. (1991): *Background Information on the Possible Health Risks of Silicone Breast Implants* (Revised February 8, 1991). FDA, Rockville, MD.
46. Foster, K.R., and Huber, P.W. (1997): *Judging Science: Scientific Knowledge and the Federal Courts.* MIT Press, Cambridge, MA.
47. Fraser, G.E. (1986): *Preventive Cardiology.* Oxford University Press, New York.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_483.html[2010/10/28 18:44:40]

48. Friedman, G.D. (1994): *Primer in Epidemiology,* 4th ed. McGraw-Hill, New York.
49. Gabriel, S.E., O'Flalon, W.M., Kurland, L.T., Beard, C.M., Woods, J.E., and Melton, L.J., III. (1994): Risk of connective-tissue diseases and other disorders after breast implantation. *N. Engl. J. Med.,* 330:1697–1702.
50. Gabriel, S.E., Woods, J.E., O'Fallon, W.M., Beard, C.M. , Kurland, L.T., and Melton, L.J., III. (1997): Complications leading to surgery after breast implantation. *N. Engl. J. Med.,* 336:677–682.
51. Gehlbach, S.H. (1993): *Interpreting the Medical Literature.* McGraw-Hill, New York.
52. Gershwin, E. (1997): Testimony, in *Spitzfaden v. Dow Corning Corporation* #CV 92–2589 (La. Civ. Dist. Ct. April 22, 1997).
53. Goldberg, E.P., Widenhouse, C., Marotta, J., and Martin, P. (1997): Failure of silicone gel breast implants: Analysis of literature data for 1652 explanted protheses. *Plast. Reconstr. Surg.,* 100:281–284.
54. Goldberg, E.P., Widenhouse, C., Marotta, J., and Martin, P. (1998): Failure of silicone gel breast implants: Analysis of literature data for 1652 explanted protheses. *Plast. Reconstr. Surg.,* 101:1163–1164.
55. Goldberg, E.P., Widenhouse, C., Marotta, J., and Martin, P. (1999): Failure of silicone gel breast implants. *Plast. Reconstr. Surg.,* 103:1092.
56. Goldwyn, R.M. (1997): Financial disclosure is not full disclosure. *Plast. Reconstr. Surg.,* 99:2034–2035.
57. Gorman C. (1999): The web of deceit. *Time,* 153(5):76.
58. Grufferman, S. (1982): Hodgkin's disease. In: *Cancer Epidemiology and Prevention,* p. 744, edited by D.Schottenfeld and J. F.Fraumeni. W.B.Saunders Company, Philadelphia.
59. Hahn, R.A. (1997): The nocebo phenomenon: Concept, evidence and implications for public health. *Prevent. Med.,* 26:607–611.
60. *Hall v. Baxter Healthcare Corporation*, 947 F. Supp. 1387 (D.OR 1996).
61. Hart, R.W., Neumann, D.A., and Robertson, M. (1995): *Dietary Restriction: Implications for the Design and Interpretation of Toxicity and Carcinogenicity Studies.* ILSI Press, Washington, DC.
62. Hennekens, C.H., and Buring, J.E. (1987): *Epidemiology in Medicine,* edited by S.L.Mayrent. Little, Brown and Company, Boston.
63. Hill, A.B. (1971): *Principles of Medical Statistics*, 9th ed. Oxford University Press, New York.
64. Huber, P. (1997): The health scare industry. *Forbes,* October 6, 1997:15.
65. Huber, P. (1998): Joiner, Scheffer and Kumbo: Refining the standards of evidence. *Civil Justice Memo,* 35:1–5.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_483.html[2010/10/28 18:44:40]

66. Huff, D. (1954): *How to Lie with Statistics.* W.W.Norton & Company, New York.
67. Huff, J., Cirvello, J., Haseman, J., and Bucher, J. (1991): Chemicals associated with site-specific neoplasia in 1394 long-term carcinogenesis experiments in laboratory rodents. *Environ. Health Perspect.*, 93:247–270.
68. Ioannidis, J.P.A. (1998): Effect of the statistical significance of results on the time to completion and publication of randomized efficacy trials. *J. A. M. A.*, 279:281–286.
69. Jacobsen, S.J., Xia, Z., Campion, M.E., Darby, C.H., Plevak, M. F., and Melton, L.J. (1997): Authorization for research use of medical records: Who declines. In: *Proceedings of The American College of Epidemiology Annual Scientific Sessions*, (abstract A-3). September 21–23, 1997, Cambridge, MA.
70. Kohn, A. (1986): *False Prophets: Fraud and Error in Science and Medicine.* Basil Blackwell, Oxford.
71. Kvien, T.K., Glennas, A., Knudsrod, O.G., and Smedstad, L.M. (1996): The validity of self-reported diagnosis of rheumatoid arthritis: Results from a population survey followed by clinical examinations. *J. Rheumatol.*, 23:1866–1871.
72. Last, J.M. (1995): *A Dictionary of Epidemiology*, 3rd ed. Oxford University Press, New York.
73. Levine, J.J., and Ilowite, N.T. (1994): Sclerodermalike esophageal disease in children breast-fed by women with silicone breast implants. *J. A. M. A.*, 271:213–216.
74. Lock, S. (1988): Misconduct in medical research: Does it exist in Britain? *B. M. J.*, 297:1531–1535.
75. MacMahon, B., and Pugh, T.F. (1970): *Epidemiology: Principles and Methods.* Little, Brown and Company, Boston.
76. Margolis, H. (1993): *Paradigms and Barriers: How Habits of the Mind Govern Scientific Beliefs.* University of Chicago Press, Chicago.
77. Marshall, E. (1997): Journals joust over conflict-of-interest. *Science*, 276:524.
78. Matthews, D.E., and Farewell, V. (1985): *Using and Understanding Medical Statistics.* Karger, Basel.
79. McMichael, A.J. (1976): Standardized mortality ratios and the "healthy worker effect"—scratching beneath the surface. *J. Occup. Med.*, 18:165–168.
80. *Merrell Dow Pharmaceuticals, Inc. v. Havner*, 953 S.W.2d 706 (Tex. 1997).
81. Michael, M., III, Boyce, W.T., and Wilcox, A.J. (1984): *Biomedical Bestiary: An Epidemiologic Guide to Flaws and Fallacies in the Medical Literature*. Little, Brown and Company, Boston.
82. Mills, J.L. (1993): Data torturing. *N. Engl. J. Med.*, 329:1196–1199.
83. Moore, G.E., and Palmer, W.N. (1977): Money causes cancer: Ban it! *J. A. M. A.*, 238:397.
84. Olsen, G.W., Lacy, S.E., Bodner, K.M., Chau, M., Arceneaux, T. G., Cartmill, J.B., Ramlow, J.M., and Boswell, J.M. (1997): Mortality from pancreatic and lymphopoietic cancer among workers in ethylene and propylene chlorohydrin production. *Occup. Environ. Med.*, 54:592–598.
85. Paolini, M., Biagi, G.L., and Cantelli-Forti, G. (1997): A hidden paradox in carcinogenesis bioassays. *J. Natl. Cancer Inst.*, 89:736.
86. Perkins, L.L., Clark, B.D., Klein, P.J., and Cook, R.R. (1995): A meta-analysis of breast implants and connective tissue diseases. *Ann. Plast. Surg.*, 35:561–570.
87. Placik, O.J. (1994): Sclerodermalike esophageal disease in children breast- fed by mothers with silicone breast implants. *J. A. M. A.*, 272:768–769.
88. Rapaport, D.P., Stadelmann, W.K., and Greenwald, D.P. (1997): Incidence and natural history of saline-filled implant deflations: Comparison of blunt-tipped versus curring and tapered needles. *Plast. Reconstr. Surg.*, 100:1028–1032.
89. Reed, M.E. (1997): *Daubert* and the breast implant litigation: how is the judiciary addressing the science. *Plast. Reconst. Surg.*, 100:1322–1326.
90. Riegleman, R.K., and Hirsch, R.P. (1996): *Studying a Study and Testing a Test: How to Read the Health Science Literature*, 3rd ed. Little, Brown and Company, Boston.
91. Roberts, H.J. (1988): Reactions attributed to aspartame-containing products: 551 cases. *J. Appl. Nutr.*, 40:85–94.
92. Robinson, O.G. (1994): Deposition testimony in *In re Silicone Breast Implants Product Liability Litigation (MDL 926)*, #CV 92-P-10000-S (N.D.AL March 12, 1994).
93. Robinson, O.G., Bradley, E.L., and Wilson, D.S. (1995): Analysis of explanted silicone implants: A report of 300 patients. *Ann. Plast. Surg.*, 34:1–7.
94. Roht, L.H., Vernon, S.W., Weir, F.W., Pier, S.M., Sullivan, P., and Reed, L.J. (1985): Community exposure to hazardous waste disposal sites: Assessing reporting bias. *Am. J. Epidemiol.*, 122:418–433.
95. Rothman, K.J. (1987): Clustering of disease. *Am. J. Public Health*, 77:13–15.
96. Rothman, K.J. (1990): A sobering start for the cluster busters' conference. *Am. J. Epidemiol.*,

132:S6–S13.
97. Rothman, K.J. (1993): Conflict of interest: The new McCarthyism in science. *J. A. M. A.*, 269:2782–2784.
98. Rothman, K.J., and Greenland, S. (1998): *Modern Epidemiology*, 2nd ed. Lippincott-Raven Publishers, Philadelphia.
99. Rousseau, D.L. (1992): Case studies in pathological science. *Am. Sci.*, 80:54–62.
100. Sackett, D.L. (1979): Bias in analytic research. *J. Chronic Dis.*, 32:51–63.
101. Sackett, D.L., Haynes, R.B., Guyatt, G.H., and Tugwell, P. (1991): *Clinical Epidemiology:* A Basic Science for Clinical Medicine, 2nd ed. Little, Brown and Company, Boston.
102. Sanchez-Guerrero J., Colditz, G.A., and Karlson, E.W. (1995): Silicone breast implants and the risk of connective tissue diseases and symptoms. *N. Engl. J. Med.*, 332:1666–1670.
103. Schneiderman, M.A. (1994): More on torturing data. *N. Engl. J. Med.*, 330:861–862.
104. Selikoff, I.J., Hammond, E.C., and Churg, J. (1968): Asbestos exposure, smoking and neoplasia. *J. A. M. A.*, 204:106–112.
105. Shoaib, B.O., Patten, B.M., and Calkins, D.S. (1994): Adjuvant breast disease: An evaluation of 100 symptomatic women with breast implants for silicone fluid injections. *Keio J. Med.*, 43:79–87.
106. Shorter, E. (1992): *From Paralysis to Fatigue: A History of Psychosomatic Illness in the Modern Era.* The Free Press, New York.
107. Silverman, S., Borenstein, D., Solomon, G., Espinoza, L., and Colin, M. (1996): Preliminary operational criteria for systemic silicone related disease (SSRD). *Arthritis Rheum.*, 39 (Suppl. 9):S51.
108. Silverstein, M.J., Handel, N., Gamagami, P., Waisman, J.R., Gierson, E.D., Rosser, R.J., Steyskal, R., and Colburn, W. (1988): Breast cancer in women after augmentation mammaplasty. *Arch. Surg.*, 123:681–685.
109. Skrabanek, P. (1994): The emptiness of the black box. *Epidemiology*, 5:553–555.
110. Slavin, S.A., and Goldwyn, R.M. (1995): Silicone gel implant explantation: Reasons, results and admonitions. *Plast. Reconstr. Surg.*, 95:63–69.
111. Solomon, G. (1993): Clinical and serologic features of 176 women with silicone implants: Evidence for a novel disease siliconosis. *Arthritis Rheum.*, 36 (Suppl. 9):S117.

112. Solomon, G., Espinoza, L., and Silverman, S. (1994): Breast implants and connective-tissue disease. *N. Engl. J. Med.*, 331:1231.
113. Solomon, G.E. (1996): Operational criteria for systemic silicone related disease (SSRD). Declaration submitted in *In re Breast Implant Litigation*, #92–182-JO-LEAD (E.D.NY August 2, 1996).
114. Spiera, H. (1988): Scleroderma after silcone augmentation mammoplasty. *J. A. M. A.*, 260:236–238.
115. Spiera, H., and Kerr, L.D. (1993): Scleroderma following silicone implantation: A cumulative experience of 11 cases. *J. Rheumatol.*, 20:958–961.
116. Star, V.L., Scott, J.C., Sherwin, R., Lane, N., Nevitt, M.C., and Hochberg, M.C. (1996): Validity of self-reported rheumatoid arthritis in elderly women. *J. Rheumatol.*, 23:1862–1865.
117. Staudenmayer, H. (1999): *Environmental Illness: Myth and Reality.* Lewis Publishers, London.
118. Steimle, S. (1998): Will Germany's Good Scientific Practice Guidelines prevent fraud? *J. Nat. Cancer Inst.*, 90:1694–1695.
119. Sturrock, R.D., Batchelor, J.R., Harpwood, V., Long, D.R., Milward, T.M., Silman, A.J., and Sloane, J.P. (1998): *Silicone Gel Breast Implants: The Report of the Independent Review Group.* Medical Device Agency of the British Department of Health, London.
120. Su, C.W., Dreyfuss, D.A., Krizek, T.J., and Leoni, K.J. (1995): Silicone breast implants and the inhibition of cancer. *Plast. Reconstr. Surg.*, 96:513–520.
121. Tan, E.M., Feltkamp, T.E.W., Smolen, J.S., Butcher, B., Dawkins, R., Fritzler, M.J., Gordon, T., Hardin, J.A., Kalden, J.R., Lahita, R.G., Maini, R.N., McDougal, J.S., Rothfield, N.F., Smeenk, R.J., Takasaki, Y., Wiik, A., Wilson, M.R., and Koziol, J.A. (1997): Range of antinuclear antibodies in "healthy" individuals. *Arthritis Rheum.*, 40:1601–1611.
122. Tannock, I.F. (1996): False-positive results in clinical trials: Multiple significance tests and the problem of unreported comparisons. *J. Nat. Cancer Inst.*, 88:206–207.
123. Taubes, G. (1995): Epidemiology faces its limits. *Science*, 269:164–169.
124. Turner, J.A., Deyo, R.A., Loeser, J.D., Von Korff, M., and Fordyce, W.E. (1994): The importance of placebo effects in pain treatment and research. *J. A. M. A.*, 271:1609–1614.
125. Ungar, W. (1998): Bias—it's everywhere! *Pharmacoepidemiol. Drug Safety*, 7:425–427.
126. Van Doren, C. (1991): The invention of the scientific method. In: *A History of Knowledge*, pp. 184–212. Ballantine Books, New York.
127. Vojandi, A., Campbell, A., and Brautbar, N. (1992): Immune functional impairment in patients with clinical abnormalities and silicone breast implants. *Toxicol. Ind. Health*, 8:415–29.
128. Wagenknecht, L.E., Burke, G.L., Perkins, L.L., Haley, N.J., and Friedman, G.D. (1982): Misclassification of smoking status in the CARDIA study: A comparison of self-report with serum cotinine levels. *Am. J. Public Health*, 82:33–36.
129. Wagner, K.R., Elmore, J.G., and Horwitz, R.I. (1996): Diagnostic bias in clinical decision making: An example of L-tryptophan and the diagnosis of eosinophilia-myalgia syndrome. *J. Rheum.*, 23:2079–2085.
130. Young, V.L. (1996): Testing the test: An analysis of the reliability of the silicone sensitivity test (SILS) in detecting immune-mediated responses to silcone breast implants. *Plast. Reconstr. Surg.*, 97:681–683.
131. Young, V.L., Elliott, L.F., Peters, W.J., and Lassus, C. (1997): Panel discussion: Management of displaced breast implants. *Aesthetic Surg. J.*, 17:247–253.
132. Zuckerman, D. (1999): Uncertainty about breast implants' safety won't stop thousands from trying them. *San Jose Mercury News*, C2, pp. 1–2, February 21, 1999.

APPENDIX

Guidelines for good epidemiology practices for occupational and environmental epidemiologic research

The *Guidelines for Good Epidemiology for Occupational and Environmental Epidemiologic Research* are included in this text by courtesy of the Chemical Manufacturers Association (CMA). They were developed by the CMA Epidemiology Task Group as part of the Epidemiology Resource and Information Center (ERIC) Pilot Project and, prior to publication in 1991, were modified following review and comment by an ad hoc panel of epidemiologists from academia, various government agencies, and the private sector. Although they do not have the force of law, they have been recognized by a number of groups and are analogous to the toxicology *Good Laboratory Practice Standards* or GLPs. The *Guidelines for Good Epidemiology Practices* (GEPs) were developed in part to provide an alternative to the GLPs, one that would appropriately address the issues confronted by epidemiologists conducting nonexperimental research.

The *Guidelines for Good Epidemiology Practices* address the conduct of studies generally undertaken to

answer questions about human health in relation to the work place or the environment. The GEPs propose minimum practices and procedures that should be considered in order to help ensure the quality and integrity of data used in epidemiologic research and to provide adequate documentation of the research methods. Epidemiologic studies often evolve through a number of stages that precede the development of a protocol, for example, proposals, feasibility studies, and measurement instrument validation. Although the GEPs are intended to address all activities that begin with protocol development, it was the opinion of the Task Group that adherence to the spirit of the guidelines would prove beneficial for those activities preceding protocol development as well as more informal investigations such as health hazard assessments/evaluations or small cluster investigations.

A copy of the original guidelines as published by ERIC can be obtained from the CMA. The complete document includes an introduction, eight sections, and three appendices. Only the eight sections are presented here, and in a slightly abridged form. A more detailed discussion of these GEPs can be found in the proceedings of a conference published in December 1991 issue of the *Journal of Occupational Medicine.*

I. Organization and Personnel

A. *Organizational Structure*

The organization or individual conducting the research shall be fully responsible for the operation and perform

ance of the research. The organization shall be a legal entity with a governing body that sets policy and that is fully responsible for the administrative aspects of the organization and its related research activities. The relationship, roles, and responsibilities of the organizations and/or individuals sponsoring or conducting the study should be carefully defined in writing.

B. *Personnel*

Personnel engaged in epidemiologic research and related activities shall have the education, training, and/or experience necessary to competently perform the assigned functions. The organization shall maintain a current summary of training and experience of these personnel. A job description for each individual engaged in or supervising activities shall be maintained and updated periodically.

II. Facilities, Resource Commitment, and Contractors

A. *Facilities*

Adequate physical facilities shall be provided to all those engaged in epidemiologic research and related activities. Sufficient resources, for example, office space, relevant equipment, and office/professional supplies, shall be available to ensure timely completion of all studies. Suitable storage facilities shall be available to maintain research materials in a safe and secure environment.

B. *Resource commitment*

Sufficient commitment shall be made at the beginning of each study to ensure its timely and proper completion.

C. *Contractors*

For the purposes of ensuring and documenting the contractor's conformance with the *Guidelines for Good Epidemiology Practices,* it is recommended that the study sponsor have the right during the course of the study, and for a reasonable period following completion of the study, to inspect the contractor's facilities, including equipment, technical records, and records relating to the work conducted under the sponsor's contract.

III. Protocol

Each study shall have a written protocol. This protocol must be approved before the study begins. The protocol should include the following:

A. A descriptive title.

B. The names, titles, degrees, addresses, and affiliations of the study director, principal investigator, and all coinvestigators.

C. The name and address of the sponsor.

D. An abstract of the protocol.

E. The proposed study tasks and milestones, including study approval date (date protocol signed by all signatories), study start date (first date that the protocol is implemented), periodic progress review dates, and estimated completion date.

F. A statement of the research objectives, specific aims, and rationale. The statement should identify the immediate purpose of the investigation. For example, it might indicate whether the study will be exploratory data analysis, hypothesis testing, or a combination of both.

G. A critical review of the relevant literature to evaluate applicable findings. This should include pertinent animal and human experiments, clinical studies, vital statistics, and previous epidemiologic studies. The literature review should be in sufficient depth to identify potential confounders and effect modifiers and to determine areas where new knowledge is needed.

H. A description of the research methods, including:

1. The overall research design and the reasons for choosing the proposed study design.
2. The data sources for exposure, health status, and risk factors.
3. Clear definitions of health outcomes, exposure, and other measured risk factors as well as selection criteria, as appropriate, for exposed and nonexposed persons, morbidity and mortality cases, and referent groups.
4. The project's study size and, if appropriate, statistical power.
5. The methods to be used in assembling the study data, including a description of, or reference to, methods used to control, measure, or reduce various forms of error—for example, bias due to selection, misclassification, interviewer, or confounding—and their impact on the study. Pretesting procedures for research instruments and any manuals and formal training to be provided to interviewers, abstractors, coders, or data entry personnel also should be described or referenced.
6. The procedures for handling the data in the analysis.
7. The methods for data analysis.
8. The major limitations of the study design, data sources, and analytic methods.

9. The criteria for interpreting the results.
I. A description of plans for protecting human subjects.

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J. The quality assurance and quality control procedures for all phases of the study. As appropriate, a certification and/or qualifications of any supporting laboratory or research groups.
K. A description of plans for disseminating and communicating study results.
L. The resources required to conduct the study.
M. The bibliographic references.
N. Addenda, as appropriate; for example informed consent forms, questionnaires, and representative samples of other documents to be used in the study.
O. A data protocol review and approval sign-off sheet for the study director, principal investigator, coinvestigators, and all reviewers.
P. The dated amendments to the protocol.

IV. Review and Approval

A. *Scientific Review*

The study protocol shall receive appropriate scientific review by qualified persons who are not part of the investigative team to ensure that the study is designed to address the objectives of the research and that the protocol is written according to the *Guidelines for Good Epidemiology Practices*. The nature and the circumstances of this review shall be documented.

B. *Ethical Review*

The ethical aspects of each study protocol shall be reviewed by an institutional review board or other comparable review procedure. This review should consider:
1. Obligations to research subjects.
2. Obligations to society.
3. Obligations to funders and employers.
4. Obligations to colleagues.

C. *Administrative Review*

The administrative aspects of the study protocol shall receive appropriate review and written approval by sponsors, contractors, and associated third parties to ensure that sufficient resources are available to complete the study in a timely and proper fashion.

V. Study Conduct

While the study director shall be responsible for the overall research program, the principal investigator shall be responsible for the individual research project, including the day-to-day conduct of the study, interpretation of the study data, and preparation of a final report. These responsibilities extend to all aspects of the study including periodic reporting of study progress as well as quality assurance. In some situations, the study director and the principal investigator may be the same person. To ensure the proper conduct of the study, personnel shall adhere to sound research principles and practices established according to the protocol. A protocol must be approved before the study begins. The study shall be conducted in accordance with the protocol; all deviations from the protocol shall be properly documented and authorized by the principal investigator. If a decision is made not to complete a research project, the reasons for that decision shall be put in writing, dated, and signed by the responsible party, that is, the individual who makes the decision to terminate the study.

A. *Protection of Human Subjects*

Procedures for protecting human subjects shall be followed. Confidential information about study subjects shall be protected using established procedures. If stipulated by the study protocol and/or required by an institutional review board, each study subject shall be informed about the purpose of the study and any risks associated with participating in the study. Written consent, if required, shall be obtained from each study subject before he/she participates in the study. Written consent shall include at a minimum:
1. The purpose of the research or study.
2. The names, addresses, and phone numbers of personnel available to answer questions about the research and the rights of study subjects.
3. The expected duration of a subject's participation
4. The eligibility requirements for study participation.
5. The possible benefits of the study results to the study subject or others.
6. A statement on the voluntary nature of participation in the study and the right of the study subject to discontinue participation at any time.
7. A statement of confidentiality of records identifying the study subject, including reasonable exceptions to absolute confidentiality, for example, sharing of information with the study subject's personal physician or as required by court order.

8. A description of any foreseeable risks or discomforts to the study subject.
9. A statement of the availability of the results.

B. *Data Collection and Verification*

All data collected for the study should be recorded directly, accurately, promptly, and legibly. The individuals responsible for the integrity of the data, computerized and hard copy, shall be identified. All procedures used to verify and promote the quality and integrity of the data shall be outlined in writing. A historical file of these procedures shall be maintained, including all revisions and the dates of such revisions. Any changes in data entries shall be documented.

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C. *Analysis*

All data management and statistical analysis programs and packages used in the analyses should be documented. All dated versions used in research shall be kept with accompanying documentation.

D. *Study Report*

Completed studies shall be summarized in a final report that accurately and completely presents the study objectives, methods, results, and the principal investigator's interpretation of the findings. Although the content and length of any technical publication based on the research may be subject to requirements of the particular journal, if a more comprehensive report is written, it should include:

1. A descriptive title.
2. An abstract.
3. The purpose (objectives) of the research as stated in the protocol.
4. The names, titles, degrees, addresses, and affiliations of the study director, principal investigator, and all the coinvestigators.
5. The name and address of the sponsor.
6. The dates on which the study was initiated and completed.
7. An introduction with background, purpose, and specific aims of the study.
8. A description of the research methods, including:
a. The selection of study subjects and controls.
b. The data collection methods.
c. The transformations, calculations, or operations on the data.
d. The statistical methods used in data analyses.
9. A description of circumstances that may have affected the quality or integrity of the data.
10. A summary of the data analyses, including sufficient tables, graphs, and illustrations to present the pertinent data and to reflect the analyses performed.
11. A statement of the conclusions drawn from the analyses of the data.
12. A discussion of the implications of the study results.
13. A list of references.
14. A statement describing the location where all source data and the final report are stored.
15. A dated study report review sign-off sheet for the study director, principal investigator, coinvestigators, and reviewers and/or auditors.

VI. Communication

Each organization shall predetermine procedures under which communications of the intent, conduct, results, and interpretations of an epidemiologic study will occur, including what function individuals associated with the research will fulfill. These individuals should include the principal investigator, study director, and/or the sponsor. This procedure may be documented in the form of a standard operating procedure, in the study protocol, or through contractual agreement. Government agencies shall be informed of study results in a manner that complies with applicable regulatory requirements. To the extent possible, scientific peers shall be informed of study results by publication in the scientific literature or via presentations at scientific conferences, workshops, or symposia. As feasible, all study subjects shall be informed of the study results and any interpretations of the study findings and conclusions. Information about the study results should be presented in language appropriate to the audience.

VII. Archiving

Physically secure archives must be designated for the orderly storage and expedient retrieval of all study-related material. An index shall be prepared to identify the archived contents and their location, and to identify by name and location any material that by their general nature are not retained in a specific study archive. Access to the archives shall be controlled and limited to authorized personnel only. Special procedures may be necessary to ensure that confidential information about study subjects is protected. Individual study archives should contain, or refer to, the following:

A. The original signed and dated study protocol and all approved modifications.
B. The original signed and dated final report of the study.
C. All source data and, where feasible, biological specimens. A printed sample of the master computer data files with reference to the location of the machine readable master.
D. Documentation adequate to identify and locate all computer programs and statistical procedures used, including version numbers where appropriate.
E. Copies of computer printouts, including relevant execution code, that form the basis of any tables, graphs, discussions, or interpretations in the final report. Any manually developed calculations shall be

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documented on a work sheet and similarly retained.

F. Correspondence pertaining to the study, standard operating procedures, informed consent releases, copies of all relevant representative material, copies of signed institutional review board and other external reviewer reports, and copies of all quality assurance reports and audits. As appropriate, this would

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include questionnaires, the name, make and model numbers of relevant measurement instruments and calibration information and procedures.

G. Original documents for the certain research materials that may be unique to the study such as laboratory notebooks and coder modification records.

VIII. Quality Assurance

Written procedures shall be established to ensure the quality of the data used in a study. These procedures shall address data collection and completeness, coding and computer input, storage and retrieval, and data validation and analysis. Any deviations from the GEPs shall be explained and documented in the final report. An individual who is not part of the investigative team should be assigned as a study quality assurance auditor. This individual shall, no less than annually, review study compliance with the written quality-assurance procedures. The study quality-assurance auditor shall prepare a written summary of the audit. The principal investigator should respond in writing to the audit report, including any remedial actions taken. Quality-assurance activities shall address the preceding sections of these guidelines as well as monitor conformance with established standard operating procedures.

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Chapter **11**

Food-Borne Toxicants

Chada S.Reddy and A.Wallace Hayes

Principles and Methods of Toxicology,
Fourth Edition, edited by A.Wallace Hayes.
Taylor & Francis, Philadelphia © 2001.

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Chemicals capable of causing adverse effects in humans are present in food as naturally occurring components, contaminants, intentional additives, or as components formed in the course of food processing. The simultaneous presence of dietary components capable of enhancing or protecting against the adverse effects of food toxicants has, without a doubt, contributed to the lack of correlation between experimental animal and epidemiological data. An understanding of such agents and direction and magnitude of their effects on specific toxicants in the complex dietary milieu is presently lacking. This together with a lack of good methods for risk extrapolation of mixtures of toxicants/anti-toxicants from animals to humans hampers our effort toward realistic human risk estimations related to food toxicants.

NATURAL TOXICANTS

Natural toxicants are derived from food and feed components of plants and animals. Acute intoxications from chemicals naturally occurring in the human diet are limited to individuals and to selected classes of compounds, for example, favism in individuals deficient in RBC glucose-6-phosphate dehydrogenase (123). Food derived from animal sources can also contain toxic components/contaminants that causes acute intoxications. However, long-term or delayed effects resulting from plant and animal toxins are more widespread and are likely to be more important.

Toxicants in Foods of Plant Origin

Foods of plant origin account for most (>70%) of the world's supply of protein. Although plants with obvious toxic effects have been excluded from human diet by trial and error, deleterious (toxic as well as antinutritive) effects from the following groups of compounds are deemed significant for human health.

Alkaloids

Nitrogenous heterocyclic organic compounds have protective roles against herbivorous consumption and attack by insects, parasites, and competitors. Major alkaloid groups of concern from the standpoint of human consumption include pyrrolizidines, xanthines, and solanines. Others including piperidines from *Conium* and tobacco; quinolizidines from *Lupinus;* and indolizidines from *Astragalus*, *Swainsona*, and red clover are mainly consumed by grazing animals and potentially can be transferred to humans through milk. For a review of toxic effects of alkaloids in humans and animals see Cheeke (38).

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Pyrrolizidine alkaloids (PAs) are a group of more than 250 geographically ubiquitous plant metabolites posing a major threat to animal health by their presence in plants including *Senecio, Crotalaria,* and *Heliotropium*, among others. Human exposure and possible health effects result from the wide use of coltsfoot *(Tussilago)*, comfrey *(Symphytum)* and petasites *(Petasites)*, as herbal remedies, foods (salads, etc.), and tea; contamination of food grains with seeds from PA-containing plants; honey derived from pansy ragwort *(Senecio sp.)* and Patterson's Curse *(Echium sp);* and/or through milk from animals grazing on these alkaloid-containing plants (44).

Among the several structural groups of PAs, the acyclic diesters and macrocyclic diesters such as retronecine, senecionine, and petasitenine are more toxic (77). Highly reactive pyrrole derivatives of PAs and/or their hydrolysis products formed by the action of mixed function oxidases are considered responsible for the toxic effects of PA (130). Huxtable (83) reviewed human intoxications with PA. Typically, liver involvement with venoocclusive disease is characterized by occlusion of small branches of the hepatic vein thus leading to ascites, edema, reduced urinary output, and high mortality which occur mostly in children. Survivors often manifest cirrhosis. Histologically, endothelial proliferation and medial hypertrophy lead to occlusion of small hepatic veins which then advances to centrilobular congestion resulting in sinusoidal widening and blood pooling. Necrosis and fibrosis ultimately result. Certain dehydro PAs, monocrotaline in particular, are known to induce similar occlusive changes in pulmonary arterioles, developing into pulmonary hypertension and right ventricular hypertrophy and, ultimately, to cor pulmonale (right heart congestive failure). Impairment of serotonin and norepinephrine clearance by endothelial cells appears to contribute to pulmonary hypertension (83).

Many PAs and their pyrrole metabolites are bifunctional alkylating agents crosslinking to macromolecules, including DNA (77, 83), thus accounting for their mutagenicity and carcinogenicity in experimental animals (77). At least six species of plants *(Senecio longilobus, Petasites japonicus, Tussilago farfara, Symphytum officinale, Farfugium japonicum,* and *Senecio cannabifolis)* and eight PAs have been shown to induce one or more of the following types of cancer: hepatic carcinoma, hepangioendothelial sarcoma in the liver, liver cell adenoma, cholangiosarcoma, astrocytoma, squamous cell carcinoma of the skin, pulmonary adenoma, adenocarcinoma of the small intestines, adenomyoma of the ileum, and rhabdomyosarcoma (77). One of the PAs, heliotrine, was shown to be teratogenic in rats. Oberved malformations included lower jaw hypoplasia, musculoskeletal defects involving ribs, and general growth retardation (77).

Solanum alkaloids, predominantly, solamine, chaconine, and tomatine are found in potato, eggplant, and tomato (species of *Solanum genus*) among others. Reviews on the biosynthesis, occurrence, and toxicology of solanum alkaloids include those by Sharma and Salunkhe (183) and Keeler et al. (94). Potatoes, especially the sprouted, greened, blighted, injured, or spoiled, have raised the most concern relating to alkaloid intoxication with over 200 cases of human poisonings documented (141). Signs of human intoxication, some of which may be related to the irritant and cholinesterase-inhibiting activity of the alkaloids, appear at >20 mg alkaloid/100 g of tuber and include headache, vomiting, diarrhea, neurological signs, debilitation, and death. Association of consumption of blighted potatoes to the incidence of anencephaly-spina bifida (ASB) in humans has been questioned by subsequent studies which failed to produce defects in animals consistent with ASB in humans (177). Although normal levels of glycoalkaloids in potato conform to the U.S. Department of Agriculture (USDA) guideline of 20 mg/100g of tuber, exposure to light, immature tubers, wounding of potatoes, and stresses such as fungal attack can increase its content severalfold (25). Baking, boiling, or microwaving do not destroy solanine or chaconine in potatoes. Some simple methods to prevent glycoalkaloid formation during storage appear to be protection of tubers from sunlight, γ-irradiation, soaking in water under controlled conditions, dipping damaged potatoes in emulsified water, treating potatoes with sprout inhibitors during storage, waxing, and heating, dipping in oils (corn, olive, or mineral), spraying tubers with lecithin (such as PAM), or simply spray rinsing tubers with an aqueous solution of an edible surfactant (Tween 85) (183).

Xanthine alkaloids. Three major related alkaloids, caffeine, theobromine, and theophylline, are found as major components of coffee *(Coffea arabica)*, cocoa *(Theobroma cocas)*, and tea *(Thea sinenis)*, respectively. Caffeine, in addition, is added to many beverages, foods, and medications (56). Caffeine-related adverse effects begin when 0.5–1.0 g of caffeine (10 cups of coffee) is ingested by an adult, leading to fatalities at 5 g in children and 5 to 10 g in adults (45, 56). Caffeine and other methylxanthines inhibit phosphodiesterase, leading to intracellular accumulation of cyclic AMP, blockage of adenosine receptors, and increased release of Ca2+ from the terminal cisternae of the sarcoplasmic reticulum (45). Major effects of xanthines involve the central nervous system (CNS) stimulation

(hyperesthesia to convulsions), emesis, cardiovascular effects (cardiac stimulation to arrhythmias), diuresis, and smooth muscle effects leading to decreased vascular resistance and bronchodilation (45). In addition, caffeine enhances gastric secretion of acid and pepsin. Caffeine and theobromine are mutagenic in bacterial systems and can potentiate DNA damage

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caused by other genotoxins but are neither directly carcinogenic in animals nor are they associated with human cancer (10, 45). Caffeine is teratogenic in experimental animals, causing mostly limb and facial defects (177). Although high caffeine consumption during pregnancy may increase the risk of low birth-weight babies, no correlation exists between caffeine consumption and birth defects in humans (190). In 1980, the U.S. Food and Drug Administration (FDA) however, issued a warning to pregnant women to limit coffee consumption (45).

Allergens

Food allergies are a group of disorders characterized by an exaggerated immunological response to a component of food. All foods are capable of eliciting an allergic reaction. Most allergens in foods are heat-stable proteins, glycoproteins, or peptides with a MW between 5000 and 70,000. Prevalence of food allergies appears to be age-dependent, with up to 8 percent of children under 3 years and 1.5 percent of adults (155, 174) affected. Milk (casein, β-lactoglobulin) and eggs (ovomucoid, ovalbumin) are the most commonly incriminated agents (13), followed by peanuts (Aha I & II, aglutinin), wheat (globulins, glutenine), soy proteins (in formulae), fish, crustaceans, tomatoes, strawberries, chocolate, and certain beverages (15, 26, 213). Although multiple tissues of the body are often affected, the skin (eczema and urticaria) and the respiratory tract (rhinitis, pneumonitis, asthma, etc.) account for 90 percent of food allergies (156). Abdominal distress, vomiting and diarrhea, hypotens in and shock secondary to hypovolemia, and nervous system involvement as indicated by headaches, convulsions, and behavioral problems (156) are also suggestive of allergic reaction to food. Rapid-onset allergies are dependent on reaction of the antigens with circulating antibodies of the IgE class (reagin) and the eventual release of vasoactive substances such as serotonin and histamine, sometimes leading to life-threatening anaphylaxis. Non-IgE-type food allergies are dependent on specifically sensitized lymphocytes that are attracted to the site of antigen exposure by lymphokines released by already existing T-lymphocytes and require several hours or days to fully manifest. Recent evidence suggests a role for food allergies in autoimmune disorders (celiac disease), juvenile or insulin-dependent diabetes mellitus, migraine, and arthritis in children (26, 97). Idiosyncratic reactions such as lactose intolerance, a result of genetic deficiency of lactase leading to luminal accumulation of lactic acid and osmotic diarrhea, must be differentiated from immunologically mediated allergic reactions.

Cyanogens

Cyanogenic glycosides which release highly toxic hydrocyanic acid on hydrolysis are derived not only from plants (more than 2000 species) but, also, from fungi, bacteria, and even members of the animal kingdom (140). Although cassava, sweet potatoes, yam, maize, millets, bamboo, sugarcane, peas, beans, almond kernel, lemon, lime, apple, pear, cherry, apricot, prune, and plum constitute sources for humans, poisonings are mainly associated with the consumption of improperly processed cassava in Africa, Asia, and Latin America (140, 158). Among more than 20 glycosides identified, only four (i.e., amygdalin, dhurrin, linamarin, and lotaustralin) appear to be of toxicological importance. Cyanogenic lipids, although of unknown toxicological significance, are also present in plants and yield carbonyl compounds and HCN upon hydrolysis (50).

The hydrolysis of the glycoside is triggered by physical disruption (mastication, trampling, etc.) or stress (drought, cooking, frost, etc.) and is catalyzed by β-glucosidase and hydroxynitrile lyase, which are present within the plant or in bacteria found in the gastrointestinal tract of humans and animals (158). The scheme of breakdown leading to the formation of glucose and hydrosynitrile from the glycoside, followed by breakdown of hydroxynitrile into carbonyl compounds and HCN, is presented in Figure 11.1. Rhodanese catalyzes the conversion of HCN to thiocyanate in the presence of thiosulfate (158).

Animals have often been acutely poisoned by young sorghum and arrow grass. Young bamboo shoots and tea made from peach leaves are examples of dietary sources of HCN poisoning in children. The minimal lethal dose of HCN in humans and animals is 0.5–3.5 mg/kg and 2–10 mg/kg, respectively. The acute effects of HCN result from its affinity toward metalloporphyrin-containing enzymes, more specifically, cytochrome oxidase. Cyanide concentration of only 33 μM can completely block electron transfer through the mitochondrial electron transport chain and thus prevent O2 utilization (158). Death results from generalized cytotoxic anoxia. Signs of acute cyanide poisoning in humans are hyperventilation, headache, nausea and vomiting, generalized weakness, coma, and death due to respiratory depression and failure. Treatment of acute cyanide intoxication involves, in addition to artificial respiration, the conversion of hemoglobin in the blood to methemoglobin with nitrites (sodium or amyl). Methemoglobin competes with cytochrome oxidase for HCN and forms cyanmethemoglobin. Co-administration of sodium thiosulfate will convert free cyanide present in the blood to thiocyanate, which is eliminated. As free cyanide in the blood decreases, additional cyanide dissociates from the

cyanmethemoglobin and is subsequently eliminated (36).
Tropical ataxic neuropathy (TAN), characterized by myelopathy, bilateral optical atrophy, deafness, and polyneuropathy; konzo, an irreversible upper moto-

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FIG. 11.1. Enzymatic hydrolysis of cyanogenic glycosides. Initially, the glycoside is hydrolyzed by a β-glucosidase releasing glucose and α-hydroxynitrile. The hydroxynitrile dissociates either enzymatically or nonenzymatically to yield HCN and the corresponding aldehyde or ketone.

neuron paralytic disease (20) of women and children; goiter; epigastric burning pain; dizziness; abdominal distension and vomiting (2)—all have been linked to longer-term consumption of cassava diets in Africa and other tropical countries (150). These diets were also poor in protein and sulfur-containing amino acids which can detoxify HCN to thiocyanoalanine and subsequently to the inert 2-amino-4-thiazolidine carboxylic acid (158). Although it is generally assumed that chronic effects of cyanogen exposure are due to thiocyanates, recent evidence suggests that the glycoside, linamarin, itself may be responsible for konzo in cassava-consuming populations (20).

Enzyme Inhibitors

Although plant and animal foods contain inhibitors of proteases, amylases, and lipases, only the inhibitors of proteases pose some hazard to human health, if any.

Kunitz inhibitor, the major protease inhibitor of soybeans, is a heat labile protease capable of inhibiting trypsin and, to a lesser degree, other proteases (93). The cationic form, which accounts for a majority of human trypsin activity, is only weakly inhibited, whereas the anionic form is fully inhibited (114). In addition, there appear to be a heat stable, lower molecular weight Bowman-Birk inhibitor, an insect protease inhibitor, and a papain inhibitor associated with raw soybeans. Egg white, milk, a number of varieties of beans, peas, cereal grains, alfalfa, and potatoes also have been shown to contain one or more protease inhibitors (86).

The major effects of protease inhibitors in animal diets include pancreatic hypertrophy, adenomas, and nodular hyperplasia associated with growth depression (115). Pancreatic hypertrophy is postulated to result from constant pancreatic hypersecretion necessitated by release of a humoral agent (possibly cholecystokinin pancreozymin) in the upper small intestine in response to the lack of free trypsin and chymotrypsin following their binding with the inhibitor (66). Although any single food source such as soybean is likely to be consumed by humans in quantities of toxicological significance, consumption of multiple sources of protease inhibitors may increase the risk of pancreatic hypertrophy and cancer. Ironically, soybean trypsin inhibitors are gaining attention for their inhibition of initiation and promotion, as well as dissemination of transformed cells during the carcinogenic process (46).

Estrogens

There are more than 220 species of plants containing estrogenic isoflavonoids (e.g., genistein, glycetein, daidzein) and/or their glycosides (genistin, glyectin, daidzin). Coumestans (e.g., coumestrol, 4-O-methylcoumestrol) and lignans represent other important groups of plant estrogens (3, 191). Phytoestrogens, although capable of causing infertility in animals heavily grazing on estrogen(coumestan)-containing forages (subterranean clover, alfalfa), have not been proven to cause human problems. Genistein (soybeans) appears to be the plant estrogen of most (if any) significance in human health (6). Zearalenone and zearalenol, two major resorcylic acid

lactone estrogens, are produced in corn in response to infection by toxigenic strains of the fungus *Fusarium roseum* and are discussed later. Human infants can be exposed to 4 mg/kg body weight or more of isoflavones from soy-based formula (6). Phytoestrogens bind to the same intracellular receptors as those that bind estradiol but at 20–200 times lower affinity, resulting in 500–10,000 times lower potency in vivo, compared to estradiol. Recently, Safe (173) estimated that human adults are exposed to 102 μg estrogenic equivalents (reflecting both potency and exposure) daily compared to 3.35 mg/day for estrogen replacement and 16.7 mg/day for oral contraceptives. Due to inefficient binding, phytoestrogens can actually impede the action of endogenous mammalian steroidal estrogen and at higher doses also induce antigonadotropic effects at the hypothalarnic, pituitary, and gonadal levels in both sexes (6). In addition to the above effects, genistein inhibits protein tyrosine kinases associated with a number of growth factors and other enzymes with roles in cell proliferation and differentiation (6).

The only reported animal effects of phytoestrogens include infertility in sheep fed subterranean clover and cattle consuming alfalfa (6), and feminization of males following exposure during development (40). In humans, however, although reversible changes in menstrual cycle and FSH and LH surges in premenopausal women appear to result from soy consumption, no developmental or infertility problems were noted in populations consuming large quantities of phytoestrogen (6). Phytoestrogens, on the contrary, may protect humans against coronary heart disease, cancer of the breast, prostate, and colon, and postmenopausal osteoporosis (4), possibly due to their antiestrogenic effects. Phytoestrogens are not mutagenic in the Ames assay (22) and appear to be noncarcinogenic when given orally (206). Whether exposure to relatively high levels of isoflavones in soy-based formulae in human infants leads to longer-term effects needs to be investigated.

Glucosinolates

Glucosinolates (GS) are a group of more than 100 flavor-imparting thioglucoside compounds found at up to 60 mg/g in crucifers such as broccoli, cabbage, brussels sprouts, cauliflower, calabrese, turnip, radish, horseradish, mustard, rapeseed, and related plants. Common names of some important GS include sinigrin, progoitrin, epi-progoitrin, glucobrassicin, and neoglucobrassicin. Not only the parent GS but, also, their products of plant and human digestive tract bacterial myrosinase (thioglucosidase) hydrolyze —isothiocyanates, nitriles, oxazo-lidinethione (OZT), and thiocyanate ions—contribute to their biological activity (208).

Although evidence is lacking in humans, in animals the thiocyanate ion inhibits the uptake of iodine by the thyroid, leading to iodine-reversible hyperplasia and hypertrophy of the thyroid (cabbage and legume goiter) and growth suppression. OZT also inhibits thyroxine synthesis and induces goiter (brassica seed goiter) in rats by inhibiting the incorporation of iodine into precursors of thyroxine (129). This condition is not reversible by iodine supplementation. In addition to goiter, epiprogoitrin and progoitrin also induce liver and kidney enlargement and death at 2.6 percent in the diet via their nitrile metabolites (203). Bile duct hyperplasia, hepatocyte necrosis, and megalocytosis of renal tubular epithelium were also seen in these animals (200).

Isothiocyanates are embryocidal and cause fetal weight reduction (25). Isothiocyanates and certain GS (e.g., sinigrin) are mutagenic in the Ames assay, whereas thiocyanates are not (25). Higher intake of cruciferous vegetables in humans and animals, however, may exert an anticarcinogenic effect attributable to the formation of isothiocyanates (at least seven), indoles, indole-3-car-binol, 3-indoleacetonitrile, and 3,3′-diindolylmethane (208). Anticarcinogenic effects of glucosinolates may result from their induction of phase II enzymes (quinone reductase) in the gastrointestinal (GI) tract and liver (197) and stimulation of apoptosis (188).

Lectins (Phytohemagglutinins)

Lectins are high MW (100,000 to 150,000), heat labile proteins, lipoproteins, or glycoproteins (up to >10% of total seed protein) detected in over 800 edible plant species, including 600 that belong to the leguminosae (beans, peas, etc.). In addition, lectins are also present in animals including sponges, crustaceans, mollusks, fish (blood), amphibian (eggs), and even mammals (tissue) (50). Their interactions with membrane glycoproteins (e.g., red blood cells causing hemolysis) made them suitable in the study of blood typing, tumor cell recognition, cell adhesion, signal transduction, mitogenesis, immune function, and cell death (139).

Binding of lectins from edible sources to cells in the crypts and villi of the intestines followed by nonspecific inhibition of active and passive absorption of many nutrients (amino acids, fats, vitamins, minerals, thyroxin, etc.) across the intestinal mucosa and necrosis of intestinal epithelial cells (96) appears to account for growth reduction and possibly goiter after long-term oral exposure to high levels

(86). Mortality following acute systemic lectin exposure is associated with damage to the liver (84) and other organs. The most toxic lectin, ricin from castor bean, can cause severe intestinal epithelial cell necrosis and death from multi-organ damage (204). Recent evidence has suggested that lipid peroxidation mediated by reactive oxygen species may be involved in ricin-induced thyroid damage (175). Epithelial-bound ricin is taken up by cells via clathrin-dependent as well as clathrin-independent endocytosis and then subjected

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to lysosomal degradation. A portion of ricin enters golgi and ER, inhibits protein synthesis, and causes cell death (176, 204). Less toxic lectins may act by the same mechanism to stimulate protein synthesis, mitogen activation, and immune stimulation.

Lipids

Adverse effects can result from naturally occurring plant lipids when factors such as the departure from established food use patterns, the use of new lipids in human diets, or inborn errors of metabolism are introduced. Erucic acid (cis-13-docosanoic acid) is a predominant component of rape (*Brassica napus* and *B. campestris*) and mustard (*B. hirta* and *B. juncea*) seeds. Canada, Argentina, Mexico, China, India, Pakistan, Japan, and several European countries are the major producers and users of these oils. Growth suppression, myocardial fatty infiltration, mononuclear cell infiltration, and fibrosis were observed in weanling rats fed erucic acid at levels supplying greater than 20 percent of the dietary calories. In addition, ducklings showed hydropericardium and cirrhosis, and guinea pigs developed splenomegaly and hemolytic anemia (131). Organ-specific inhibition of glutamate oxidation and adenosine triphosphate (ATP) synthesis in cardiac mitochondria (79) could be mechanistically involved in the pathogenesis of these lesions. In humans, however, although the long-term use of Lorenzo's oil (oleic acid and erucic acid) in the treatment of adrenoleukodystrophy or adrenomyeloneuropathy leads to thrombocytopenia and lymphopenia (202), adverse effects from dietary consumption of erucic acid have not been reported.

Refsum is disease is a genetic peroxisomal fatty acid oxidase and catalase deficiency resulting in an inability of the affected individuals to convert phytanic acid (3,7,11,15-tetramethylhexadecanoic acid, a product of chlorophyll metabolism in the rumen) from dairy products and ruminant fats to α-hydroxyphytanic acid in preparation for further oxidation. This results in accumulation of lipids containing phytanic acid in many tissues and a disorder characterized by poor physical and mental growth, blindness, deafness, and other neurological signs (38). Elimination of dairy and ruminant fats from the diet of these individuals results in partial remission.

Cyclopropene fatty acids such as sterculic acid (C19) and malvalic acid (C18) are natural components of oils from plants of the order *Malvales,* most important of which are cotton and kapok seeds. Cyclopropene fatty acids have been incriminated in the pink discoloration of egg whites and reduced egg production in cottonseed-fed laying hens, growth suppression and impaired female reproduction in rats, and increased saturated fatty acids (possibly causing atherosclerosis) in the tissues of pigs and other animals (131). Cyclopropene fatty acids are carcinogens and markedly increase the carcinogenicity of aflatoxin in trout (10).

Increased consumption of dietary polyunsaturated fatty acids to lower blood cholesterol, although beneficial in decreasing the incidence of coronary disease, has raised concern about adverse effects such as induction of Vitamin E deficiency (131). Carrol (32) reported a strong correlation between dietary fat and age-adjusted mortality rates for breast and intestinal cancer. Pancreatic cancer was found to be enhanced by a diet containing 20 percent corn oil but not by one containing 18 percent hydrogenated coconut oil and 2 percent corn oil (167). Unsaturated fatty acids are easily oxidized during cooking to a variety of mutagens, enols and other aldehydes, and alkoxy and hydroperoxy radicals (10). Lipid oxidation products alter signal transduction pathways (196) and thus enhance cell proliferation and promote carcinogenesis. Lipid-induced inhibition of immune responses and enhanced formation of some of the known tumor promotors such as prostaglandins and bile acids also have been reported (32). Interestingly, Hayasu et al. (74) showed that oleic and linoleic acids may, in fact, be antimutagenic. The overall effect of dietary fats may depend on the ratio of beneficial fatty acids to causative fatty acids for each effect.

Oxalates and Phytates

Certain plants including spinach, rhubarb, beet leaves, tea, and cocoa contain high (0.2–2.0% on a fresh weight basis) levels of oxalic acid. Although dietary oxalates present little problem in humans, cattle and sheep have been poisoned following ingestion of the toxic plants *Halogeton* and *Sarcobatus* (grease wood). Toxic signs result from binding of the oxalic acid to serum calcium leading to hypocalcemia, coagulation defects, and tetany. Degeneration and necrosis of kidneys and vasculature from Ca^{++} oxalate deposition may result in severe cases. Oxalates also interfere with absorption of calcium, iron, magnesium, and copper and inhibit succinate dehydrogenase and carbohydrate metabolism (151). Approximately 2.5 kg of tomato or 0.5 kg of spinach leaves need to be consumed to approach a lethal dose (5 g or more) of oxalates.

Phytic acid, the hexaphosphoric ester of myo-inositol, is present at high levels (up to 1.5 g%) in the bran and germ of wheat followed by other cereals, nuts, seeds, spices, and legumes (87). Phytates bind

di- and tri-valent metals in the order: $Cu^{++} > Zn^{++} > Co^{++} > Mn^{++} > Fe^{+++} > Ca^{++}$, causing mineral deficiencies (especially of Ca^{++} and Fe^{+++}) in developing countries that are heavily reliant on cereals as the exclusive source of protein. Inclusion of phytase, an enzyme that releases phosphate from plant phytic acid, in animal feeds ensures phosphate utilization and reduces environmental phosphate pollution from animal production. Supple

mentation with minerals and Vitamin D can antagonize most effects of oxalates and phytates (87).

Plant Phenolics

Plant phenolics comprise a group of several thousand substituted phenolic compounds occurring in trace amounts as esters or glycoconjugates. Acute human and animal poisonings are mostly caused by phenolics uncommon in human food and include coumarius, aflatoxins, and gossypol. Phenolics common in human foods belong to three general classes, that is, non-flavonoids (gallic, syringic, caffeic, and other acids); flavonoids (flavones such as tangeritin, flavonols such as kaempferol and quercetin, isoflavones such as coumestrol, aurones, chalcones, and anthocyanin pigments); and polyphenols (tannins and lignin). Polyphenols are widely distributed and present in relatively large amounts in cereals, millets, legumes, and fruits. Deleterious effects of long-term exposure of both hydrolyzable (polyphenolic acid) and condensed (polyflavonoid) tannins include reductions in the digestibility of foods and feeds, protein utilization, and body weight gain, damage to and sloughing of the mucosal lining of the gastrointestinal tract, and cancer of the mouth and esophagus (77, 164). In contrast to mild acute effects in humans, livestock losses can exceed $10 million annually, attributable to the toxic effects of hydrolyzable oak tannins consumed when other forages are unavailable (186).

Epidemiological correlation exists between high consumption of condensed tannins (sorghum and dark beer prepared from sorghum, tea, red wines, and areca nuts) and high rates of oral and esophageal cancer (50). Parenteral exposure to tannins reportedly has led to high incidence of liver and other tumors in rodents (77). On the other hand, a negative association between tea drinking and stomach cancer (192) and coffee consumption and kidney cancer (85) also exists.

Polyphenols, however, are not directly damaging to DNA (35) and experimental evidence of anticarcinogenic effects of penta-O-gallyl-beta-D-glucose and epigallocatechin gallate, two green tea tannins (64), exists. Flavonoids and non-flavonoids exert no less than 40 different physiological and pharmacological actions accounting for their therapeutic and health food use. These include anticoagulant, antihistaminic, antihypercholesterolemic, anti-inflammatory, antioxidant, antiproliferative, antipruritic, antipyretic, antirheumatic, antiseptic, antithrombogenic, antitumor, apoptotic, estrogenic, and vasoactive effects. Many if not all of these actions are based on their UV-absorbing, chelating, oxidative phosphorylation uncoupling and antioxidant properties. In addition, their sparing effect on vitamins C and E; induction of P450-mediated enzymes; and alteration of enzymes (phospholipases, ATPases, cyclo xygenases, lipoxygenases, protein kinases), oncogenes, and other signaling components critical for cell survival and proliferation (35, 61) contribute to an array of opposing effects in many systems.

Although human consumption of flavonoids alone can be greater than 1 g/day (87), the toxicological implications of exposure to flavonoids and other simple phenolics arise from lifetime exposure to them. Recently, high intake of flavonoid supplements was suggested to have caused acute renal failure due to hemolysis (116). More than 30 flavonoids, including the most abundant quercetin, kaempferol, myricetin, hesperetin, naringenin, wogonin, and norwogonin, as well as their glycosides have been shown to be mutagenic in bacterial and/or mammalian systems (77, 122). Quercetin induced hyperplasia and benign renal tubular epithelial tumors and appeared to enhance pancreatic pre-tumorous lesions induced by nitrosomethylurea in rats fed a 5% dietary level for 2 years (21, 54). The proponderance of evidence (77), however, points to the antagonistic effects of flavonoids against mutagenic and carcinogenic effects of a number of genotoxicants and promotors in many organs (18, 35, 77, 117). These conflicting results are likely due to differences in test systems and the dose levels used because phenolics exert antioxidant effects at low doses and prooxidant effects at higher doses (219).

On balance, however, both polyphenols and simple phenolics may play a protective role against the carcinogenic and oxidative influences (e.g., those causing coronary heart disease) in humans. Anticarcinogenic effects of phenolics are likely due to the inhibition of metabolic enzymes leading to reduced levels of reactive intermediates, induction of detoxifying enzymes such as glutathione-S-transferase, reduced formation of oxidation products, and/or inhibition of enzymes such as protein kinases and oncogenes that stimulate cell proliferation, among other mechanisms (117, 215). Cardiovascular protection appears to result from reduced low-density lipoprotein oxidation (antiatherogenic effect), reduced platelet aggregation (antithrombotic), vasodilation, relaxation of cardiovascular smooth muscle, and antihypercholesterolemic effects of flavonoids (61, 80).

Gossypol (1,1,6,6,7,7-hexahydroxy-5,5-diisopropyl-3, 3-dimethyl [2,2-binaphthalene]-8,8-dicarbovxaldehyde), a yellow phenolic pigment in cottonseed, can bind to proteins and minerals and reduce the biological availability of iron and lysine (86). Similar to other phenolics, free gossypol (>60 ppm) inhibits oxidative phosphorylation and causes myriad other effects leading to acute toxicity in

animals on a high cottonseed diet. In general, higher acute doses cause cardiac failure associated with liver and lung (pulmonary edema) damage whereas chronic exposure leads to general malnutrition and reproductive effects (35). Signs of gossypol toxicity include loss of

appetite and body weight; rough hair coat; edematous fluid in body cavities, lungs, and pericardium giving rise to gasping; hemorrhagic degenerative changes in liver, and necrosis of cardiac myocytes (223). Changes in plasma $K+$ (increase in calves and decrease in humans) may be responsible for gossypol toxicity. Olive discoloration of yolk and decreased egg hatchability occur in poultry (35). Male antifertility effects of gossypol in mammals are only partially reversible and include reduced sperm production as well as motility during the late stages of spermatogenesis, likely caused by mitochondial damage (160) or inhibition of protein kinases (199). Gossypol is not mutagenic in the Ames test (35) but appears to induce genetic damage (dominant lethal mutations) in rats and may be both an initiator and promotor of carcinogenesis (10).

Gossypol and polyphenol (tannin) toxicity can be prevented by the addition of iron, supplemental protein, vitamin K, and alkalinizing agents such as sodium hydroxide. In addition, non-ionic detergents such as Tween 80, methyl donors such as choline and methionine, and dehulling and peeling of grains and fruits have also been shown to counteract the toxic effects of tannins (49, 186). A glandless (gossypol-free) variety of cottonseed, although it is expected to eliminate gossypol toxicity in animals, appears to be more susceptible to insect attack and has yet to gain popularity.

Proteins and Peptides

In the average American diet protein supplies 15 percent of total calories. Long-term consumption of higher amounts of protein, especially animal-derived, may contribute to diabetes, renal glomerular sclerosis, Crohn's disease, and osteoporosis (by increased Ca^{++} loss from bones in response to acidosis) (23, 185, 217).

Protein toxicants such as allergens, hemagglutinins (lectins) and enzyme inhibitors have been discussed. Certain microbial protein toxins are discussed in subsequent sections. Toxic peptides from mushrooms are discussed below.

Mushroom peptides. Among several thousand species of mushrooms only about 100 may be toxic and 12 are known to contain lethal toxins (57, 108, 214).

Emesis and profuse diarrhea followed by rapidly developing hepatic and renal insufficiency (similar to acute hepatitis) and death are characteristic of poisoning by mushrooms related to *Amanita verna* (destroying angel) in the United States and *A. phalloides* (green death cap) in Europe (209). Approximately one-half of a mature cap of *A. phalloides* can be lethal in an adult (132). Among the three classes of thermostable peptide toxins (amatoxins, phallotoxins, and virotoxins) in the deadly Amanitas, the cyclic octapeptides, amatoxins (Figure 11.2), appear to be responsible for the observed clinical effects which begin to appear after a 12-hour latency period. Evidence of hepatotoxicity includes an increase in serum transaminases, decrease in blood glucose and clotting factors, and, occasionally, jaundice. Hepatogenous encephalopathy and renal failure may be present terminally.

Fatalities are common even following intensive symptomatic care, which includes fluid replacement, activated charcoal hemoperfusion, and forced diuresis, etc. In countries other than France and the United States, use of silibinin (from *Silybum marianum*), which prevents hepatocyte uptake of amatoxins, has produced beneficial effects in direct relationship with the speed of onset of therapy (132, 209). A return toward normal glucose, factor V, and fibrinogen is prognostic of recovery (132). Amatoxins inhibit RNA polymerase II by binding to the enzyme directly, and subsequently inhibit mRNA synthesis by blocking the formation of phosphodiester bonds at the elongation step, due to stabilization of the ternary complex of template, enzyme, and the nascent ribonucleotide chain (209).

The other two groups of polypeptide toxins, the phallotoxins and virotoxins, are capable of causing toxic effects only at relatively higher doses. The effects of phallotoxins include swelling of the liver due to engorgement of hepatic sinusoids with blood, and depletion of blood in the peripheral circulation, leading to shock. Reduction of cellular G-actin concentration by a combined effect of stimulated G-actin polymerization into F-actin, and inhibition of F-actin depolymerization leading to a loss of membrane elasticity and thus cell surface vesiculation may explain subsequent hepatocyte damage (209).

The toxic effects of several mushroom species that are rarely lethal are summarized in Table 11.1. Treatment in most cases is supportive. Other identified human conditions associated with mushroom production, commerce, and consumption are: hypersensitivity to edible mushrooms in certain populations; hypersensitive allergic alveolitis and other pulmonary allergic changes in mushroom workers from spores of certain edible mushrooms (mushroom worker's lung); hemolytic reactions following consumption of mushrooms belonging to the genera *Gyromitra*, *Boletus*, and *Paxillus;* and dermatitis (allergic) from contact with one or more species of the genera *Boletus*, *Lactarius*, *Calvaria*, and *Agaricus*.

Saponins

The saponins are bitter-tasting, steroidal (C27) or mono, di-, tri-, and sesqui-terpenoid (C30) glycosides

from plants, fish, and sponges, and are capable of reducing surface tension, hemolyzing red blood cells, and causing toxic effects in cold-blooded animals. Their occurrence, biological effects, and relevance to food,

	R_1	R_2	R_3	R_4
α - Amanitin	•OH	•OH	•NH_2	•OH
β - Amanitin	•OH	•OH	•OH	•OH
γ - Amanitin	•CH	•H	•NH_2	•OH
ε - Amanitin	•OH	•H	•OH	•OH
Amanin	•OH	•OH	•OH	•H
Amanullin	•H	•H	•NH_2	•OH
Amaninamide	•OH	•OH	•NH_2	•H

FIG. 11.2. The structures of amatoxins.

agriculture, and medicine are reviewed by Walker and Yamazaki (211, 212).

So far, d-limonene from citrus oils, ginseng saponins, and medicagenic acid and hederosides in alfalfa and *Hedera helix,* respectively, and oleanolic and ursolic acid in a variety of food, medicinal, and other plants, as well as their aglycones (sapogenins), have been studied to some extent. Their analgesic, anti-atherosclerotic, anticarcinogenic, anticholinergic, antihypercholesteremic, antihyperglycemic, anti-inflammatory, antitubercular, cardioprotective, diuretic, and hepatoprotective effects are likely to encourage increased dietary, supplemental, and medicinal utilization of saponin-containing plants such as ginseng (119, 124, 161, 218). Mechanisms of protection involve Ca^{++}-antagonistic and vasodilatory/venoconstrictive; immune-modulatory, bile acid-binding, anti-proliferative, and membrane permeabilizing; and antioxidant and anti-cytochrome P450 effects (119, 161). Feeding high levels of saponin from a variety of sources, however, resulted in lower growth rate; increased serum LDH and GOT associated with hepatocellular necrosis; and increased BUN, hematuria, and proteinuria associated with renal tubular necrosis (99, 143) in animals. Several steroidal and nonsteroidal saponins from pasture weeds such as *Hypericum perforatum* and *Narthecium ossifragum,* vines such as *Tribulus terrestris,* and tropical grasses such as *Brachiaria* and *Panicum* sp. cause primary or hepatogenic photosensitization in animals (34). Alpha-hederin, a saponin that induces metallothionein in maternal tissues, appears to induce visceral and skeletal defects in offspring born to exposed rat dams by possibly reducing zinc availability to the fetus (52). Similar to phenolics, the beneficial effects of saponins can be derived from daily doses present in a balanced diet.

Toxic Amino Acids

In general, foodstuffs do not contain individual amino acids of nutritional importance in amounts that cause adverse reactions. Therapeutic use of greater than ten times the required dose of amino acids, on the other hand, when given on an empty stomach, can lead to adverse effects, including gastric distress (essential amino acids), nausea, febrile reaction and/or headaches (methionine, isoleucine, and threonine), and disorientation (methionine and tryptophan) in psychiatric patients treated with monoamine oxidase inhibitors (73).

Monosodium glutamate (MSG) had long been used as a flavor enhancer in commercially processed

foods. MSG, as well as other acidic amino acids, but not basic or neutral amino acids, produced lesions in rats and mice

Table 11.1 Mushroom-induced syndromes

	Mushroom species	Toxic compound(s)	Effects	Mechanism	Prevention/ Treatment
Rapid onset:					
Syndrome	*Chlorophyllum molybditis* *Entoloma lividum* *Omphalotus olearius* *Paxillus involutus* *Trichodoma pardinum*	Many unknown	Emesis, diarrhea	Unknown	Cooking/Fluid replacement
Parasympathetic	*Inocyte* sp. *Clitocybe* sp. *Omphalotus illudens* *Amanita* sp.	Muscarine and related	Increased salivation, lacrimation, and urination; diarrhea; dyspnea; sweating; bradycardia; tremors, etc.	Parasympathetic stimulation	Avoid/Atropine
CNS Syndrome	*Psilocybe* sp. *Panaeolus* sp. *Copelandia* sp. *Gymnopilus* sp.	Psilocybin Psilocin	Hallucinations involving all sensations; hyperthermia, convulsions, coma, and death.	Serotonin agonist	Avoid/Diazepam and cooling
	Amanita pantheria *A. muscaria*	Ibotenic acid, muscinol, stizolobic and stizolobinic acid	Alternating depression and neuromuscular stimulation	Stimulation of bicuculinreactive postsynaptic receptors	Avoid/Diazepam and respiration
Alcohol sensitization	*Coprinus* sp. *Clitocybe claviceps* *Boletus luridus* *Verpa bohemica*	Coprine and others	Nausea, vomiting, headache, hypotension, tingling, palpitations, tachycardia; testicular damage, etc.	Inhibit acetaldehyde dehydrogenase	Avoid mushroom and Alcohol/ supportive
Delayed onset:					
Headache	*Gyromitra; esculenta* (false morel) *Gyromitra* sp.	Gyromitrin, Monomethyl hydrazine, etc.	Fatigue, head and body ache, vomiting, liver damage, death, carcinogenic	Interfere with pyridoxine?	Cook or dry, don't inhale vapors
Nephropathy	*Cortinarius* sp.	Orellanine Cortinarin	Polydypsia, oliguria, nausea, head and body aches, chills, etc., Renal tubular and liver necrosis, death	Membrane damage from oxygen-derived free radicals (similar to paraquat)	Hemodialysis
Carcinogenic	*Agaricus bisporus* (edible)	Agaritine, hydrazines	Lung tumors	Genotoxic	Cooking
Hepatotoxic	*Amanita phalloides* (Europe) *A. Virosa*	Amatoxins, phallotoxins, and virotoxins	Emesis and diarrhea, increase in serum enzymes, decrease in glucose and clotting factors, hepatic and	1. Inhibit RNA polymerase 2. Enhance G-actin	1. Correct glucose and clotting effects 2.

(United States) *Galerina* sp. *Lepiota* sp.	renal damage, jaundice, coma, and death	polymerization into F-actin 3. Inhibit F-actin depolymerization	Decontaminate 3. Penicillin and silobinin 4. Supportive 5. Transplant liver

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in the arcuate nucleus of the hypothalamus, retina, and lateral geniculate nucleus, and in other brain areas devoid of the blood-brain barrier (148). Numbness of the neck and back, weakness, and palpitations, typical signs of the so-called Chinese restaurant syndrome, were later found not to be associated with dietary MSG (198).

Hypoglycin A (β-methylene cyclopropyl alanine) and its γ-glutamyl conjugate, hypoglycin B, are components of the fruit of the plant, *Blighia sapida* (ackee in Jamaica and isin in Nigeria). Consumption of this fruit in the unripened stage has been associated with hypoglycemia, resulting from inhibition of gluconeogenesis involving inhibition of fatty acyl-CoA dehydrogenases and thus β-oxidation of fatty acids by cyclopropylacetyl CoA (a metabolite of hypoglycin A). Signs of intoxication include vomiting, convulsions, hypothermia, coma, and even death. Pretreatment with clofibrate (stimulator of peroxismal fatty acid oxidases) prevented many but not all signs, lesions, and biochemical effects (205).

Koa haoli *(Leucaena leucocephala),* a legume found in Hawaii, and other legume species belonging to the Mimosidae family, have potentially high nutritive value for animals and humans (144). However, use of these legumes is precluded in ruminants by the goitrogenic effect of the metabolite (3,4-dihydroxypyridine) of an unusual amino acid, mimosine [3-N-(3-hydroxypyridone-4)-2aminopropionic acid], present in this plant. Mimosine also causes reversible destruction of the hair follicle matrix (loss of hair), reduced bone strength and mineral composition in poultry, and growth depression in both ruminants and nonruminants. The ability of mimosine to chelate Zn and Mg, reduce plasma thyroid and other hormone levels (159), inhibit a large number of enzymes leading to DNA synthesis inhibition and cell-cycle arrest (88, 113) explains many of the effects.

Djenkolic acid, which is an amino acid that is structurally similar to cystine, is present in the djenkol bean *(Pithocolobium lobatum),* found in Sumatra and Java. It can neither substitute for cystine nor can it be totally metabolized, but it can crystallize in the kidney, causing hematuria and crystalluria (113).

Favism, a hemolytic disease (accompanied by jaundice and hemoglobiuuria) in persons genetically deficient in glucose-6-phosphate dehydrogenase (G6PD) and, thus, in NADPH and reduced glutathione content, results from the consumption of the amino acid, 3,4-dihydroxyphen-ylalanine, and the pyrimidine aglycones (divicine and isouramil) of the glycosides vicine and convicine in broad beans *(Vicia faba),* mainly in the Mediterranean region and in the Middle East (37). Ohga et al. (147) observed beneficial effects of human heptaglobin administration in managing this crisis.

The etiology of the neurological disease characterized by posterior sensory ataxia in cattle consuming cycads may be an amino acid, β-N-methylamino-L-alanine. Cer tain seleno-amino acids such as methylselenocysteine, selenocystathionine, selenocysteine, and selenomethionine in plants that grow on high Se soils (113), when incorporated into structural animal proteins, may, during longer-term exposure in livestock, produce defective hair and hooves that are eventually lost. In human beings, a syndrome characterized by abdominal distress, nausea, vomiting, diarrhea, and loss of scalp and body hair had been reported following consumption of coco de momo *(Lecythis ollaria)* nuts containing high levels of selenocystathionine (16).

The amino acids L-2,4-diaminobutyric acid (DABA), 3-N-oxalyl-L-2,3-diaminopropionic acid (ODAP) 3-cyanoalanine, 4-glutamylcyanoalanine, and related homologues, present in seeds of several species of *Lathyrus* and *Vicia sativa* in the Indian subcontinent, have been implicated in the pathogenesis of neurolathyrism, a syndrome characterized by muscular rigidity, weakness, paralysis of leg muscles, and death following long-term, high-level consumption of *L. sativus* seeds (204). The mechanism of action appears to involve irreversible binding of ODAP to the glutamate receptor, and enhanced release/reduced re-uptake of glutamine at relevant nerve terminals, leading to vascular degeneration and necrosis of neurons (152). In certain individuals, amino acids such as β-aminopropionitrile and the dipeptide (N-γ-glutamyl) aminopropionitrile as well as certain urides, hydrazides, and hydrazines from the green parts of *Lathyrus* and other plants, lead to osteolathyrism characterized by bone deformities and reduction in the tensile strength of the aorta (72) resulting from the irreversible inhibition of lysyl oxidase and interference with crosslinking of collagen (216).

Creeping indigo *(Indigofera endecapylla),* a tropical forage, contains a nitric oxide synthase inhibitor, indospicine, which causes liver damage in sheep, rats, and mice by inhibiting the incorporation of arginine, the amino acid it resembles, into protein (113, 154). 3-Nitropropionic acid, a neurotoxin capable of inhibiting mitochondrial succinate dehydrogenase and, thus, cellular respiration, is also present (7).

Vaso- and Psychoactive Substances

High levels of amines such as tyramine and its methyl derivatives octopamine, dopamine, epinephrine, norepinephrine, histamine, serotonin, and others are present in cheese, yeast products, fermented

foods, beer, wine, pickled herring, snails, chicken liver, coffee, broad beans, chocolate, and certain cream products, and in plants such as pineapple, banana, plantain, and avocado (121). Moderate amounts of cheese and yeast products often contain sufficient tyramine (10 mg) to cause severe hypertensive crisis in individuals treated with non-selective monoamine oxidase (MAO) inhibitors for mood disorders (19). Inhibition of MAO leads to a combined

vasopressor effect of unmetabolized biogenic as well as dietary amines. In addition, tyramine enhances release of catecholamines that are present in supranormal amounts in the adrenal medulla (19). Palpitations, migraine headaches, and, in some instances, intracranial bleeding and death may ensue. Use of selective (MAO-A or B) inhibitors for therapy appears not to sensitize individuals to dietary tyramine (101).

Psychoactive substances include CNS stimulants such as xanthines (caffeine, theophylline, and theobromine present in coffee, tea, and cocoa); depressants such as alcohol and high doses of atropine (from jimsonweed and henbane); and hallucinogens such as myristicin from nutmeg, psilocybin and psilocin from mushrooms, and nondietary sources of cocaine and lysergic acid derivatives (130). Chronic overindulgence in xanthine beverages may lead to restlessness, disturbed sleep, myocardial stimulation reflected as premature systoles and tachycardia (palpitations), and tremors. Herbs containing psychoactive agents include California poppy, catnip, cinnamon, hops, hydrangea, juniper, kola nut, nutmeg, periwinkle, thornapple, and wild lettuce (25). The essential oils of coffee and the tannins in tea may cause diarrhea and constipation, respectively (45). Caffeine is neither mutagenic by itself nor enhances the mutagenic effects of other compounds in mammalian cells and may actually be anticarcinogenic (39).

Vitamins and Antivitamins

Vitamins A (retinol), D, and pyridoxine have a lower safety margin (ten times the RDA) and should be used on a longer-term basis only under medical supervision (126). Other vitamins with a safety ratio of 50–100 relative to the RDA are generally safe. Therapeutic uses of vitamin A for night blindness, steatorrhea, hyperkeratosis, acne vulgaris, certain immune disorders, and cancer, along with daily consumption of carotenoids and vitamin A in plant and animal tissues (especially the liver), account for the total vitamin A exposure in humans (149). Oral doses of 18,000–60,000 IU/day and 100,000 IU/day can cause hypervitaminosis A in infants and adults, respectively, with premature epiphyseal closure and retardation of long bone growth in children; and headaches, blurred vision, fatigue, hair loss, drying and flaking of skin, pruritus, nose bleeds, anemia, and liver and spleen enlargement in adults (149). Therapy of acute promyelocytic leukemia with tretinoin (all-trans-retinoic acid) as an adjunct to antineoplastic therapy induces retinoic acid syndrome (fatal leukocytosis, body weight gain, respiratory distress, cardiac and renal failure) in 25 percent of cases (60). Excess vitamin A (retinoids) is teratogenic, causing craniofacial, thymic, heart, and CNS malformations subsequent to interaction with cellular retinoic acid/retinol binding proteins (47). The oral doses of vitamin A not exceeding 6000 IU/day are considered safe during pregnancy in human beings.

Vitamin D, following hydroxylation at C25 and C1 in the liver and kidney, respectively, functions to facilitate the action of parathyroid hormone to release Ca^{++} from bone and to promote intestinal absorption of Ca^{++} and inhibit its renal loss. Ergosterol (a plant steroid) converted to ergocalciferol (Vit-D2) by ultraviolet light, endogenous dehydrocholesterol (in skin) converted to cholecalciferol (Vit D3) by sunlight, and vitamin D-fortified milk are predominant sources of vitamin D for humans (149). While the recommended dose is 400 IU/day, excessive exposure to vitamin D from 1000–3000 IU/day in infants and 10,000–500,000 IU/ day in adults has resulted in toxicosis. Poisonings in dogs and cats have resulted from accidental consumption of insecticidal vitamin D packages. Toxic signs of vitamin D in humans, which are similar to those seen in laboratory animals, are a result of hypercalcemia leading to extraskeletal calcifications, especially blood vessel walls and kidneys, leading to hypertension, renal failure, and cardiac insufficiency (149). Recent evidence suggests that supplementation with vitamin D3 may lead to an increase in LDL and may negate putative cardioprotection by hormone replacement therapy in post-menopausal women (76), and may exacerbate intimal hyperplasia in balloon-damaged rat arteries (106).

Long-term supplemental use of vitamin E can result in coagulopathy (by reduced iron utilization and increased vitamin K requirement) as well as tumor promotion (both stage I and II); such use of pyridoxine can result in photoallergic reaction and sensory neuropathy (126, 137, 149). Table 11.2 lists examples of anti-vitamin factors in foods, the effects of which, in general, only manifest in individuals with already low levels of the vitamin in question.

Miscellaneous Plant Toxicants

A common human intoxication called milk sickness was one of the most dreaded diseases from colonial times to the early 19th century in an area extending from North Carolina to Virginia and to the midwestern United States (112). The disease manifested as weakness, nausea and vomiting, constipation, tremors, prostration, delirium, and even death, resulted from consumption of dairy products made from milk derived from cows (even healthy ones) grazing on white snakeroot

(Eupatorium rugosum) or rayless goldenrod *(Haplopappus heterophillus)*. The causative agent appears to be trematol, an unsaturated alcohol, in combination with a resin acid (112). Other plant toxins excreted through milk that pose toxic hazards for children and nursing animals include pyrrolizidine, piperidine, and quinolizidine alkaloids; sesquiterpene lactones of bitterweed and rubberweed; and glucosinolates (153). Animals grazing high Se forages

Table 11.2 Examples of anti-vitamin factors in foods

Vitamin	Antagonist(s)	Mechanism	Effect(s)	Source(s)
A	Lipoxidase Citral	oxidize β-carotene inhibit retinoic acid (P=450) dehydrogenase	Lower blood Vit A level Endothelial damage cardiovascular disease?	Soybeans Oranges
B Thiamine	Thiaminase, tannins, and ortho-catechols	inactivate thiamine	Neurological syndrome	Bracken fern other plants and seafood Ackee plum Cereal crops
Riboflavin Niacin Biotin Pyridoxine	Hypoglycin A Leucine? Avidin, Linatine, Agaritine	Irreversible binding Hydrazine metabolites condense with the vitamin	Vomiting, sickness Pellagra	Egg white Flaxseed, Shiitake mushroom Pea seedlings Soybeans
Pantothenic acid	Unknown			
B12	Unknown	Unknown		
C	Ascorbic acid oxidase	Oxidizes Vit C	Normally none	Fruits and vegetables
D	β-carotene, plant steroids	Reduce absorption	Rickets and osteomalacia	Green leafy vegetables
	(some) Unknown	Unknown		Soybeans
E	α-tocoferol oxidase	Oxidizes Vit E	Muscular dystrophy	Kidney beans and others
	Polyunsaturated fatty acids	Increases Vit E demand	Liver necrosis	Vegetable oils Beans
K	Diconmarol	Inhibit epoxide reductase	Hemorrhages	Sweet clover

may excrete high levels of Se in milk and contribute to chronic Se toxicosis in the offspring (153). Current processing methods have kept these conditions in check for the most part. In cattle, consumption of 5–10 pounds of snakeroot causes weakness and trembling of various groups of muscles, labored respiration, and death.

Fool's parsley *(Aethusa cynapium)* and other members of the *Umbelliferae* family contain highly toxic acetylene derivatives (e.g., aethusin) that are responsible for many human poisonings (114). Carotatoxin and other acetylene compounds in carrots are neurotoxic (25) but are not likely to cause problems in humans.

Purple mint *(Perilla frustescens)*, widely distributed in the United States and Japan, is used as a flavoring agent, for medicinal purposes, and as animal feed. The presence, in mint, of a ketone-substituted furan capable of causing acute pulmonary emphysema and other lung lesions in cattle and other animals (181) raises questions about the safety of these uses in humans and animals.

Cycads, the palm-like plants adapted for adverse climatic conditions of the tropical and subtropical areas of the world, are still used (seeds and stem) as a source of starch in Guam, Kenya, Amami Oshima, Miyako Island, and southern Japan by small groups of people (128). Adverse effects result from incomplete extraction of toxicants, including cycasin and β-N-methyla-mino-L-alanine (BMAA), during preparation of the flour. Several neurological conditions, including a paralytic disease (amyotrophic lateral sclerosis, ALS) and Parkinsonism-dementia (PD) were observed among the native Chamorro in Guam who consume cycad, gait disturbances, motor weakness, and paralysis in cattle grazing on cycads, and Parkinsonian features and degenerative changes in CNS motor neurons in monkeys—all appear to be related to either or both of these cycad toxicants (128, 190). Attenuation of the cycad-induced neurotoxic syndrome by AP7 and MK801, two selective antagonists of N-methyl-D-aspartate

receptor and its associated ion channel, suggests a role of the excitatory neurotransmitters in the causation of ALS-PD, other motor-system diseases (Huntington's chorea, Parkinson's disease, and olivopontocerebellar atrophy), and Alzheimer's disease (190). Other effects of cycasin, its aglycone, or cycad flour include hepatic necrosis, subserosal hemorrhages, accumulation of yellow fluid in serosal cavities, benign and malignant tumors in the liver, kidney, lungs, and gastrointestinal tract (mainly colon), neuroteratological effects in offspring, death in experimental animals, and mutagenic effects in a variety of in vitro and in vivo systems. Interestingly, cycasin is neither toxic nor carcinogenic when given parenterally to conventional rats or when given either orally or parenterally to germ-free rats, suggesting that the intestinal flora mediate cycasin toxicity. Bacterial β-glucosidase hydrolyzes cycasin to the active carcinogen, methylazoxymethanol (MAM), which produces hepatomas in rats. MAM spontaneously breaks down to methyldiazonium hydroxide which methylates hepatic DNA, RNA, and proteins (128). Certain cycad glycosides inhibit aromatase and may be useful in the treatment of estrogen-dependent cancer (102).

Toxicants in Foods of Animal Origin

The discussion on plant toxicants has included certain agents from this category (e.g., vasoactive agents in cheese, plant-derived toxicants in milk). Others such as bacterial toxins in meats are discussed in a subsequent section. Residues, in meats and milk, from pesticides, antibiotics, and growth promotants (including hormones such as estrogenic substances) are addressed briefly in the section on food additives and in the chapter dealing with pesticides. Natural toxicant hazards in foods of animal origin are mostly limited to those derived from marine sources.

Marine Toxins in Food

Of the many marine organisms containing toxins (up to 1200 species), only a few are involved in food poisoning. Problems of seafood poisoning have spread to inland areas from coastal fishing sites as a result of modern transportation and shipping. Toxicants may be produced by the fish itself, and/or by the marine plankton or algae consumed by the fish, with or without the aid of certain marine bacteria. Leftley and Hannah (111) and Russell and Dart (172) have presented a detailed discussion of the toxicological information relating to fish-borne toxins.

Shellfish poisoning is one of several (amnesic, digestive, neurotoxic-paralytic) disease entities resulting from the consumption of shellfish (clams, crustaceans, lobsters, mussels, oysters, scallops, etc.) that have ingested toxic marine algae, especially certain dinoflagellates. The shellfish are toxic during seasons of heavy algal bloom (such as red tide) containing 200 organisms/ml or more. Toxicity increases in proportion to the concentration of algae and disappears within two weeks after the toxic plankton has disappeared from the waters (171). Saxitoxin, neosaxitoxin, and gonyautoxins are the most potent of the more than 20 toxins present in the group of paralytic shellfish poison, produced by dinoflagollates belonging to *Alexandrium, Gymnodinium, Guanyalax,* and *Pyrodinium* sp.

Saxitoxin blocks the action potential in nerves and muscles by preferential blockade of inward flow of sodium ions with no effect on the flow of potassium or chloride ions (90). Consumption of 1 mg of the toxin (in 1 to 5 mussels or clams weighing 150 g each) can be mildly toxic whereas 4 mg can be fatal if not treated vigorously. Toxic symptoms begin as numbness of the lips, tongue, and fingertips within minutes after eating. Numbness then extends to the legs, arms, and neck and is followed by general muscular incoordination, which progresses to respiratory paralysis and death. Decreased heart rate and contractile force, headache, dizziness, increased sweating, and thirst may also be noted. Boiling in bicarbonate-treated water and discarding the broth is suggested as a means of preventing shellfish poisoning (71).

Diarrhetic shellfish poisoning results from consumption of shellfish contaminated by one of several species of *Dinophysis* that containing okadaic acid (OA) and/or dinophysistoxins (DPT). Both OA and DPT are powerful inhibitors of protein phosphatases and potent tumor promoters (101). Whether protein phosphatase inhibition by OA leads to the observed increase in the permeability of intestinal epithelial cells OA and the diarrhetic effect is unknown.

Neurotoxic shellfish poisoning is characterized by nausea, vomiting, diarrhea, chills, headache, muscle weakness and pain, eye and nasal irritation, and, in severe cases, paresthesia, difficulty in breathing, double vision, dysphonea, and dysphagia. It has been reported along the Gulf of Mexico, the eastern coast of Florida, and New Zealand following consumption of shellfish with a heavy load of *Gymnodinium breve* and/or similar organisms (111). The lipophilic polyether toxin, the brevitoxin, promotes $Na+$ influx and thus depolarization by its action on site-5 of the voltage-dependent $Na+$ channels (111).

Amnetic shellfish poisoning, characterized by short-term and sometimes permanent memory loss associated with gastrointestinal signs and hallucinatory state, has been reported mostly from coastal

areas in North America. Damage to the hippocampus, coma, and death result in severe cases. A water-soluble acidic non-protein amino acid, domoic acid (and its isomers),

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produced by the diatom *Pseudonitzschia* sp. and acting as a competitive glutamate antagonist at various sites, has been ascribed the etiological agent (111).

Between 300 and 400 tropical reef and semipelagic species of edible marine animals, including barracudas, groupers, sea basses, snappers, surgeon fishes, parrot fishes, jacks, wrasses, eels, and certain gastropods, accumulate in their liver and other viscera toxins capable of causing ciguatera poisoning, with an estimated 20,000 cases/year worldwide (118). The intoxication, common in the South Pacific and the Caribbean, appears to follow the spacial and temporal pattern of the distribution of a photosynthetic dinoflagellate *Gamblerdiscus toxicus,* which is consumed by the smaller herbivorous fish and, in turn, by the ciguatoxic fish (171).

Ciguatoxins, the colorless and heat stable lipophilic polyethers (MW of 1100), appear to play a major role in intoxication with some contribution from the water-soluble maitotoxin (111). Ciguatoxins increase membrane permeability to sodium ions causing depolarization of nerves. In addition, ciguatoxin inhibits subsequent inactivation of open Na^+ channels and possesses anticholinesterase activity in experimental animals (111, 171). Maitotoxin, on the other hand, inactivates voltage-dependent and receptor-mediated Ca^{++} channels leading to high intracellular Ca^{++} and cell death (111). In humans, tingling of the lips, tongue, and throat, followed by numbness, nausea, vomiting, abdominal pain, diarrhea, pruritus, bradycardia, dizziness, and muscle and joint pain occurs. Severe cases exhibit paresis of the legs and, infrequently, death due to cardiovascular and/or respiratory failure (111, 171). Prevention of ciguatera poisoning is difficult, although extensive evisceration of fish may help.

Pufferfish (fugu fish) poisoning, known to occur as far back as 2000–3000 B.C. in China and Japan, results from consumption of tetrodotoxin present in the liver and ovaries of puffer fish, ocean sunfishes, porcupine fishes, blue-ringed octopus, and certain amphibians of the family *Salamandridae* (91, 171). Toxin accumulation is greatest just prior to spawning in the spring. Tetrodotoxin (TTx), with a cyclic hemilactal structure, is highly lethal (LD50, 10 mg/kg) to all vertebrates and is active after boiling for one hour but is inactivated under alkaline conditions (63). Tetrodotoxin prevents the increase in the early Na^+ permeability in both motor and sensory neuronal membranes similar to that of saxitoxin (171). In humans, numbness of the lips, tongue, fingers, and arms, muscular paralysis and ataxia, hypotension, and respiratory paralysis leading to death progress rapidly beginning 30–60 minutes after consumption of 1 to 2 mg of tetrodotoxin (1–10 g of roe or liver). Although current treatment is only symptomatic, experimental evidence (33, 166) indicates that anti-TTx antibodies or 4-aminopyridine may be effective in antagonizing the cardiorespiratory effects of tetrodotoxin. Training of personnel in proper evisceration techniques and licensing of fugu restaurants is a must.

Scombroid poisoning is the most widespread fish-borne intoxication. It results from the consumption of inadequately preserved abalone, amberjack, bluefish, tuna, mackerel, mahi-mahi, and sardines in which histamine and saurine are produced by bacterial scombrotoxic action (118). Scombroid fish apparently has a sharp or peppery taste. Signs of intoxication include nausea, vomiting, diarrhea, epigastric distress, flushing of the face, throbbing headache, and burning of the throat followed by numbness and urticaria. Severe cases may lead to cyanosis and respiratory distress but, rarely, death. These signs appear within 2 hours of the meal and disappear in 16 hours (171). The disease readily responds to antihistamine treatment.

FOOD CONTAMINANTS

Although some naturally occurring toxicants and food additives impart resistance against pests to plants and help preserve and/or enhance the nutritional quality of the diet, respectively, the biological and synthetic industrial chemical contaminants sometimes increase the risk of food-borne illness and deserve a much broader margin of safety in their control than food additives. The FDA, in consultation with other federal agencies, establishes legal action levels, that is, the maximal level of a contaminant allowed in foods and feeds based on economic considerations and technological feasibility (75).

Bacterial Toxins

Foods contaminated with microbial agents are a major source of human disease estimated to afflict up to 80 million people and to cost $22 billion annually in the United States (8). With a few exceptions, these can be prevented by adequate cooking and proper cooling, storage, and reheating of cooked foods in clean containers (127). Bacterial food-borae disease may result from the consumption, in food, either of bacteria (e.g., *Salmonella* sp. and *Clostridium perfringens*) that can cause disease by multiplying in the intestinal mucosa where they may elaborate toxins (enterotoxins), or of a sufficient amount of preformed microbial toxins (staphylococcal enterotoxins and botulinum toxin). In addition to the above well-known etiologies, genetic changes in bacteria that increase virulence, changes in eating habits, altered food production and distribution systems, the increased number of immunocompromised

consumers, and improved detection systems have led to identification of other pathogens such as *Escherichia coli*, *Listeria* sp., and *Yersinia* sp. as causing food-borne illness.

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C. perfringens frequently causes food-borne infections which subsequently lead to sporulation of the organism in the large intestine. The enterotoxin, released during sporulation of the bacteria, is capable of causing fluid accumulation in the intestines. The α-toxin, possessing lethal, necrotizing, and hemolytic activities, is also produced by certain types. Among the five distinct types of *C. perfringens* (type A through E), type A is almost always involved in food-borne gastroenteritis and associated signs in human beings in the United States. Only meat and fish products are capable of providing all the amino acids and growth factors required for the growth of *C. perfringens.* Roast beef, beef stew, gravy, and meat pies for type A, and pork, other meats, and fish for type C, are frequently involved (27). Typically, foods involved are cooked at 100°C for less than an hour and are subsequently kept warm or slowly cooled. Spores that survive the heat shock multiply faster in the food than those not subjected to heat treatment, and elaborate the enterotoxin in the gut. The enterotoxin appears to form ion-permeable channels in the cell membrane, leading to movement of extracellular calcium and water into the cells, leading to cell death (194). Entry of the toxin into the blood stream leads to release of potassium from hepatocytes, hyperkalemic cardiac failure, and death (194). Due to the ubiquitous distribution of the organism in soil, and in the gastrointestinal tract of humans and animals, prevention of contamination is difficult. Multiplication and toxin production can be inhibited by heating food to the proper temperature (165–212°F), prompt and effective cooling, and avoiding prolonged reheating before consumption.

Staphylococcus aureus is probably the leading cause of food-borne disease worldwide. The organisms are gram-positive, non-motile, and non-spore-forming cocci that occur ubiquitously in the environment. Although humans are the leading source of food contamination by way of nasal discharge and infected cuts and wounds, the organism can be present in milk derived from mastitic cows and meat derived from arthritic poultry (136). Baked ham, poultry, fish and shellfish, meat and potato salads, cream-filled bakery goods, and high-protein leftover foods are frequently involved in such intoxication (27). Multiplication of *S. aureus* in raw food products is inhibited by other spoilage organisms present. As a result, mostly cooked products subsequently contaminated by infected handlers and stored at warm temperature for several hours before consumption cause intoxication. The causative agent is one of more than six immunologically distinct heat stable enterotoxic proteins (MW 26,000–34,000) whose secretion is genetically regulated during growth (A, D, and E by chromosomes or by plasmids B and C). In addition, *S. aureus* also produces many other substances such as coagulase, DNase, hemolysins, lipases, fibrinolysin, and hyaluronidase, that are toxic to one or more animal species. Although all strains of *S. aureus* are potentially pathogenic, the enterotoxin production is closely related to the presence of coagulase and DNase. Signs and symptoms begin one to six hours after consumption of contaminated food, and include nausea, salivation, vomiting, retching, occasional diarrhea, abdominal cramps, sweating, dehydration, and weakness followed by recovery in one to three days. Severe cases may show fever, chills, a drop in blood pressure, and prostration (136). Preventive measures effective against *S. aureus* food intoxication include education of food handlers regarding hygienic practices to reduce post-cooking contamination of high-protein foods and eliminating prolonged storage of cooked foods at room temperature before consumption.

Botulism is a neurotoxic syndrome caused by consumption of improperly cooked and stored foods containing one of seven (A through G) heat labile neurotoxins produced by *Clostridium botulinum.* It is an ubiquitous, anaerobic, gram-positive, and motile rod capable of forming heat-resistant spores. High moisture, a pH above 4.6, and prolonged anaerobic storage are required for sufficient toxin production (136). Common foods involved are home canned fruits and vegetables such as beans, corn, leafy vegetables, and, especially, peppers, all of which contain toxins A and B. Non-poultry meats contain toxin B, and cheese and other dairy products contain toxin A. Type E is isolated mostly from fish products (136). Types C and D, which cause botulism in animals and birds, do not affect humans. Botulinum toxin is stable in the acid pH of the stomach where it is protected from the gastric juice and pepsin by a nontoxic component of the toxin molecule. Once in the duodenum, it is activated by trypsin, with no change in molecular size, and subsequently absorbed into the lymphatics. The toxin irreversibly binds to the myoneural junction and, acting as a Zn endopeptidase, degrades peptides involved in the release of acetylcholine (ACh), thus inhibiting its release at the peripheral cholinergic nerve endings (136). Signs and symptoms of botulism usually appear 12–24 hours (range: 2 hours to 6 days) following consumption of the toxin-containing food. Initial signs of nausea, vomiting, and diarrhea are followed later by predominantly neurological symptoms including headache, dizziness, blurred and/or double vision, loss of light reflex, weakness of facial muscles, and pharyngeal paralysis (difficulty in speech and swallowing). Fever is absent. Sensory reflexes and mental alertness are intact. Paralysis of the respiratory muscles leads to failure of respiration and death, usually in 3–10 days (187). Food-borne

botulism can be prevented by proper canning technique, boiling vegetables for at least 3 minutes before serving, and discarding all swollen and damaged canned products. Treatment of botulism involves the use of

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monovalent (E), bivalent (A and B), or polyvalent (A, B, and E) antitoxin, recall of all involved commercial products, proper reporting, and epidemiological investigation. Boiling for 3 minutes or heating at 80°C for 30 minutes destroys the preformed toxin, and the use of salt, the antimicrobial compound nisin, polyphosphates, smoke, spices, lactic acid, and nitrite can inhibit the growth of *C. botulinum* and, thus, toxin formation (136). If the nitrite content of cured meats and fish as well as fermented sausages is reduced from current levels as a means of decreasing the carcinogenic dietary nitrosamines, it is conceivable that the incidence of botulism from the consumption of such foods could increase unless suitable replacements are found.

Food-borne disease outbreaks involving *Bacillus cereus* have occurred in Northern and Eastern Europe. A diarrheal illness involving a wide variety of meats and vegetables, various desserts, fish, pasta, milk, and ice cream (similar to that of *C. perfringens*), and a vomiting illness involving flour-based foods such as cereals and fried rice served in Chinese restaurants (similar to that of *S. aureus*) are both apparently caused by this organism (136). At least seven toxins including a heat stable (121°C for 90 minutes) emetic toxin and a heat labile (56°C for 5 minutes) enterotoxin contribute to the syndrome (136). The enterotoxin appears to disrupt cell membranes, leading to increased permeability, whereas the mechanism of emetic toxin is unknown.

Salmonella sp. consists of over 2200 serotypes possessing somatic O, flagellar H, and capsular Vi antigens, of which 50 serotypes commonly occur. *S. typhi, S. paratyphi,* and *S. sendai* are adapted to human hosts which serve as the sole carrier for those organisms. Feces of infected humans, domestic and wild animals, and birds serve as sources of contamination in a variety of meat and milk products, causing severe gastrointestinal signs along with fever, septicemia, shock, and the sequelae of embolism including pneumonia, meningitis, and abortion. Mortality is rare and occurs in very young, very old, and immunocompromised patients. Enteritis can result from bacterial multiplication within the mucosa or from enterotoxins secreted by some serotypes. Salmonella-free birds can be raised in salmonella-free environments using salmonella-free pelleted feed or by vaccination. Thorough cooking of meats; pasteurization of milk and dairy and egg products; prevention of cross-contamination between cooked and raw products; and, finally, testing, isolation, and treatment of carrier animals and food handling personnel are all extremely important in controlling the incidence of this most common food-borne disease (58).

Campylobacter jejuni and other species in this genus (*C. sputorum* etc.) are associated with up to 4 million cases of diarrheal illness similar in many respects to salmonellosis (9). Reactive arthritis, inflammation of the urethra and conjunctiva, and Guillain-Barré syndrome have been described as sequalae in occasional cases (9).

Escherichia coli, a close relative of the genus *Shigella,* has recently raised concern as a fatal, food-borne disease agent. There are more than 160 serotypes (based on O, H, or capsular K antigen), of which 43 have been associated with bloody diarrhea and hemolytic uremic syndrome (HUS) in humans (165). Pneumonia, meningitis, thrombotic and thrombocytopenric purpura, bladder and kidney infections, and septicemia may also result from *E. coli* infections. Based on virulence factors (which bestow the organism with the ability to attack, invade, and produce toxin in the host cells) located in the plasmids, five virotypes have been identified as pathogenic. These are: enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (Eagg EC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and enteroinvasive *E. coli* (EIEC). Serogroup O 157: H7, belonging to the EHEC group and producing a shiga-like toxin, may be the most common serotype causing bloody diarrhea and HUS mainly traceable to consumption of contaminated beef products (165). Less frequently, unpasteurized milk and juices; ham, turkey, and cheese sandwiches; dry fermented sausage; salad; and non-chlorinated water have been involved. Once in the intestines, *E. coli* produce shiga-like toxins SL1 and SL2 which similar to vicin, act by inactivating ribosomes to cause intestinal cell death (165).

Listeriosis, in addition to being transmitted by other routes, is an emerging food-borne disease resulting from the consumption of *Listeria monocytogenes-contami*nated soft cheeses, milk and other milk products, poultry, meat (especially delicatessen meats and frankfurters), and coleslaw and other products (salads, etc.) derived from contaminated vegetables. Food products are contaminated by contact with soil, feces, discharges, and urine from infected animals and humans. The clinical food-borne disease, occurring mostly in pregnant women, neonates, older, and immunocompromised adults, is characterized by gastrointestinal or flu-like symptoms within 12 hours of exposure followed by bacteremia leading to abortions, stillbirths, or premature births in pregnant women; meningitis, respiratory distress, and skin nodules in neonates; and meningitis-related signs in adults (41). The disease can be treated with antibiotics and other supportive measures. Prevention involves improvement

of sanitation of the environment and equipment, and education to identify and avoid contaminated food products.

Another recent controversy involves food-borne transmission of *spongiform encephalopathies* (SE) of animals (bovine SE [BSE] and scrapie of sheep and goats) to humans that may manifest as Creutzfeldt-Jakob (CJ) disease, characterized by fatal neuronal vacuolization and

cell death (70). Although experimental evidence suggests that these diseases can sometimes be orally transmitted, no definitive proof exists that human disease results from consumption of edible animal products or that toxins play a role in its pathogenesis (70). Nevertheless, the fact that BSE may be transmitted to other species should increase the vigilance of the veterinarians responsible to ensure that products from animals with progressive degenerative neurological disease do not enter the human food chain.

Yersiniosis resulting from consumption of improperly cooked chitterlings (porcine large intestines), cryptosporidiosis from contaminated water and unpasteurized apple cider, *Cyclospora* infection from contaminated water and fresh berries, and brucellosis from unpasteurized milk and meats from infected cattle, sheep, goats, and their products are other examples of potential food-borne outbreaks.

Prevention and Control of Microbial Food Hazards The National Animal Health Monitoring System (USDA) has stepped up efforts to monitor food animal and poultry health on the farm and thus develop strategies to deal with potential increases in existing as well as emerging food-borne disease threats. The USDA's Food Safety Inspection Service (FSIS) began implementing a hazard analysis and critical control point system (HACCP) for pathogen reduction in 1996 for all slaughter and processing operations. The HACCP directs each processing unit to: conduct a hazard analysis; identify critical control points at which a safety hazard can be prevented; establish limits at each point; develop monitoring procedures and corrective action when limits are exceeded; and implement record keeping that will allow subsequent verification for compliance by FSIS (78). Data for the *E. coli* and salmonella burden of carcasses are used as evidence of fecal and enteric pathogen reduction. Together, these two programs are aimed at minimizing overall food-borne disease from animal foods in the U.S. population. The approval, by FDA, of low-dose irradiation of red meats to control pathogens, coupled with previously approved irradiation of poultry for pathogen reduction, pork for control of trichinae, fruits, vegetables, and grains for insect control, and spices, seasonings, and dry enzymes used in food processing for microbial reduction (14), should not only contribute to the prevention of food-borne disease caused by microbial pathogens but also help in increasing the shelf life of such products without undesirable organoleptic, toxicological, or nutritional changes. Irradiation is yet to be approved for pathogen control of seafood products and is unsuitable for dairy products because of development of off-flavors and discoloration. In the final analysis, however, the key to minimizing the microbial food-borne illness is at the food preparer/consumer level in the form of proper canning, cooking at the correct temperature, hygienic service, and/or prompt and appropriate storage and reheating.

Mycotoxins

From the standpoint of human and animal health, toxigenic molds belonging to the genera *Aspergillus, Fusarium*, and *Penicillium* have received the most attention, owing to their frequent occurrence in food and feed commodities. Unfavorable conditions such as drought and damage to seeds by insects or mechanical harvesting can enhance fungal toxin (mycotoxin) production during both growth and storage thus making mycotoxicoses a problem of both developing and developed countries. Although more than a hundred mycotoxins have been identified, the public health significance of most remains unknown.

Aflatoxins

The aflatoxins are a group of highly substituted coumarins containing a fused dihydrofuran moiety (Figure 11.3) and produced by the molds *Aspergillus flavus* and *A. parasiticus*. Four major aflatoxins designated B1, B2, G1, and G2 (based on blue or green fluorescence under ultraviolet light) are produced in varying quantities in a variety of products that has not been adequately dried at harvest and has been stored at relatively high temperatures (30). Commodities most often shown to contain aflatoxins are peanuts, various other nuts, cottonseed, corn, and figs. Human exposure can occur from consumption of aflatoxins from these sources and the products derived from them, as well as from tissues and the milk (AFM1, a hydroxylated metabolite) of food animals consuming contaminated feeds. Aflatoxin B1 (AFB1), the most potent and the most commonly occurring aflatoxin, is acutely toxic (LD50, 0.3–0/9.0 mg/kg) to all species of animals, birds, and fishes (42). Acute effects of AFB1 in animals include death without signs, or signs of anorexia, depression, ataxia, dyspnea, anemia, and hemorrhages from body orifices. In subchronic cases icterus, hypoprothrombinemia, hematomas, and gastroenteritis are common. Chronic aflatoxicosis, characterized by bile duct proliferation, periportal fibrosis, icterus, and cirrhosis of the liver, and associated with loss of weight and reduced resistance to disease, is more prevalent in domestic animals and is also likely to occur in humans (151). Prolonged exposure to low levels of AFB1 in animals also leads to hepatoma, cholangiocarcinoma, or hepatocellular carcinoma and other tumors (30).

The National Research Council (146) reviewed epidemiological studies to conclude that the risk of

primary hepatocellular carcinoma from AFB1 exposure may be

AFLATOXINS

	R_1	R_2	R_3
Aflatoxin B_1	H	C	O
Aflatoxin G_1	H	O	O
Aflatoxin M_1	OH	C	O
Aflatoxicol	H	C	OH

TRICHOTHECENES

T-2 toxin $R_1 = R_2 = CH_3COO-$, $R_3 = (CH_3)_2\ CHCH_2COO-$;
HT-2 toxin $R_1 = OH$, $R_2 = CH_3COO-$, $R_3 = (CH_3)_2\ CHCH_2COO-$
Neosolaniol $R_1 = CH_3COO-$, $R_3 = OH$, $R_2 = CH_3COO-$;
Diacetoxyscirpenol $R_1 = R_2 = CH_3COO-$, $R_3 = H$;
Monoacetoxyscirpenol $R_1 = OH$, $R_2 = CH_3COO-$, $R_3 = H$.

ZEARALENONE

FIG. 11.3. The structure of the mycotoxins, aflatoxins, trichothecenes, ochratoxin A, and zearalenone.

one in 10,000 in the United States. However, in populations infected with hepatitis B, the risk may be 10 to 100 times higher. AFB1 is mutagenic following metabolic activation in many systems including HeLa cells, *Bacillus subtilis, Neurospora crossa,* and *Salmonella typhimurium* (30). A portion of AFB1 is metabolized by microsomal mixed-function oxidase system in the liver into a variety of reactive products (AFB1 2,3-epoxide, or e.g.) that from adducts with DNA. Hsieh (82) suggested that subsequent formation of repair-resistant adduct, apurination, or error-prone DNA repair may lead to single strand breaks, base-pair substitution, transversion, or frame shift mutations. Recent evidence has suggested that a higher proportion of liver cancer in high aflatoxin-exposure areas had mutations of the *ρ*53 gene involving G>T transversions of codon 249 (109) and that such effect-biomarkers as well as exposure biomarkers such as AFB1-DNA or AFB1-albumin adducts can be used to assess the effectiveness of preventive agents and strategies in public and animal health (95). In addition, AFB1 inhibits DNA synthesis, DNA-dependent RNA polymerase activity, and messenger RNA synthesis and protein synthesis (81) which may be related to several lesions and signs of aflatoxicosis including fatty liver (failure to mobilize fats from the liver), coagulopathy (inhibition of prothrombin synthesis), and reduced immune function.

Other less widespread human clinical syndromes in which aflatoxins have been implicated include childhood cirrhosis in India; possibly Reye's syndrome in many parts of the world, and, rarely, acute hepatitis (aflatoxicosis) in India, Taiwan, and certain countries in Africa (182).

Widespread concern regarding the toxic effects of aflatoxins in humans and animals and the possible transfer of residues from animal tissues and milk to humans has led to regulatory actions governing the interstate as well as global transport and consumption

of aflatoxin-contaminated food and feed commodities. Action levels for total aflatoxins in corn and other feed commodities used to feed mature nonlactating animals range from 100–300 ppb. For milk, the action level is 0.5 ppb. For other commodities destined for human consumption and interstate and potentially global commerce, the action limit is 20 ppb (FDA Compliance Policy Guides 7106.10, 7120.26, 7126.23).

Ergot Alkaloids

Ergotism, which is now rare, was first associated with the consumption of scabrous (ergotized) grain in the mid-16th century (St. Anthony's fire). Subsequent studies led to the identification of *Claviceps purpura* as the fungal agent invading rye, oats, wheat, and Kentucky bluegrass, and *C. paspali* invading Dallis grass. Lysergic acid derivatives, the peptides and the anime alkaloids of ergot, were identified as the causative agents of the gangrenous and CNS forms of the disease (104). The gangrenous form, resulting from a predominance of alkaloids with α (ergotoxine) and vasopressor (ergotanine) action (104), typically manifested as prickly and intense heat and cold sensations in the limbs, and swollen, inflamed, necrotic, and gangrenous extremities which eventually sloughed off. Convulsive ergotism, characterized by CNS signs, numbness, cramps, severe convulsions, and death. Abortions have been reported in animals.

Fumonisins

Fusarium moniliforme Sheldon is a common fungal contaminant of cereals, especially corn, around the world. Contamination of corn by *F. moniliforme* as well as its major metabolites, fumonisins B1 and B2, can induce one of several human and animal diseases including leukoencephalomalacia (LEM) in horses, pulmonary edema in swine, renal and hepatotoxicosis in horses, swine, and rats, and hepatocarcinogenic effect in rats (55). Recent evidence has suggested that FB1 increases chromosomal aberrations in primary rat hypatocytes (98) and developmental effects in the offspring of pregnant mice (163). Consumption of high levels of fumonisins in home-grown corn has been associated with an higher incidence of human esophageal cancer in certain regions of South Africa and China (125). Although the mechanisms of the toxic and carcinogenic effects are not understood, inhibition of spingolipid biosynthesis (210), enhancement of lipid peroxidation (1), elevated secretion of tumor necrosis factor-alpha (53), depletion of glutathione levels (89), elevated nitric oxide synthesis (169), induction of protein kinase C translocation via its action on phorbolester binding site (222), and inhibition of protein serine/threonine phosphotases (65) are among the changes that can explain some of the effects of FB1.

Ochratoxins

The ochratoxins, a group of seven isocoumarin derivatives linked with phenylalanine by an amide bond, are produced by *Aspergillus ochraceus* and *Penicillium verrucosum* (among others) in barley, corn, wheat, oats, rye, green coffee beans, peanuts, wine, cocoa, and dried fruits (180). In experimental animals ochratoxin A (OTA) produces predominantly renal proximal tubular lesions and liver degeneration. The acute oral LD50 of OTA ranges between 0.2 mg/kg for the dog and 59 mg/kg in mice. The association between consumption of a high level OTA in the diet and nephropathy in humans and swine in the Balkan countries and swine in Denmark and the United States has been clearly established (103, 120). Signs include lassitude, fatigue, anorexia, abdominal (epigastric or diffuse) pain, and severe anemia followed by signs of renal damage. Reduced concentrating ability, reduced renal plasma flow, and decreased glomerular filtration occur sequentially, accompanied by gross and microscopic renal changes including necrosis, fibrosis with some tubular regeneration, glomerular hyalinization, and interstitial sclerosis. Death results from uremia. Ochratoxins are teratogens and genotoxic carcinogens (180) inducing hepatomas and renal adenomas in mice (92).

Relevant cellular effects specific to OTA include alteration in enzymes involved in glucose metabolism and anion-transport leading to intracellular alkalinization and associated morphological change (105, 133). Creppy et al. (43) and Schramek et al. (178) proposed roles for free radicals and prostaglandins, and extracellular signal-regulated kinases, respectively, in ochratoxin-induced renal damage.

Psoralens

Psoralens are furocoumarin compounds that have been used in repigmenting achromatic skin lesions in an acquired disease called vitiligo, in some suntan lotions, and in drugs used to treat psoriasis (31). Abuse of such compounds can result in dermatitis following exposure to the sun along with nausea, vomiting, vertigo, and mental excitation. A phototoxic dermatitis in celery pickers has been linked to the presence of psoralens (8-methoxypsoralen, 5-methoxypsoralen, and trimethylpsoralen) in stalks infected with *Sclerotinia sclerotiorum* (pink rot), *S. rolfsii, Rhizoctonia solani,* or *Erwinia aroideae,* or in celery stalks soaked in 5 percent NaCl (182). Fig, parsley, parsnip, lime, and clove also contain psoralens. 8-Methoxypsoralen appears to undergo epoxidation of the furan ring similar to aflatoxins and may react

with DNA in a similar fashion. Treatment with 8-methoxypsoralen and ultraviolet light led to squamous cell carcinomas of the ear in mice (31).

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Unlike other photosensitizing agents, psoralens seem to act by photoreacting with DNA, and to a lesser extent, with RNA. The mechanism of psoralen photosensitivity appears to involve intercalation and crosslinking of psoralen in the DNA which occurs in three steps:
(a) reversible intercalation of psoralen between two pyrimidines on opposing sides of the helix;
(b) formation of a monoadduct with the 5, 6 double bond of the pyrimidine following absorption of 1 quantum of ultraviolet light; and
(c) crosslink formation by absorption of a second quantum of ultraviolet light and linking of the monoadduct to the 5, 6 double bond of thymidine (179). In general, there is an excellent correlation between photoadduct formation and photosensitization of psoralens.

Trichothecenes

Trichothecenes are a group of 12,13-epoxy trichothecenes produced by *Fusarium poae, F. tricinctum, F. graminearum, F. nivale, F. solani, Myrothecium roridum,* and *Stachybatrys atra,* among others, in cereal grains, including wheat. The group of macrocyclic trichothecenes including satratoxins, verrucarins, and roridins is produced mainly by *Stachybotrys* sp. in hay. Although more toxic, this group does not pose significant human health threat due to lack of prevalence. Group A trichothecenes (T-2 toxin, diacetoxyscirpenol) contain a side chain and are relatively less polar compared to group B (nivalenol, deoxynivalenol, fusarenon). A two-volume treatise about trichothecene toxins and their role in human and animal health is available (24).

Most trichothecenes of health significance are produced by *Fusarium* sp. Characteristic signs of alimentary toxic aleukia (ATA), caused by T-2 toxin and related trichothecenes, including radiometric damage such as irritation and necrosis of skin and mucous membranes, hemorrhage, destruction of the thymus and bone marrow, hematological changes, and nervous disturbances; necrotic angina and shock are common to all toxic syndromes (24). Feed refusal, vomiting, and immune suppression are common problems in farm animals, especially swine, in the midwestern United States and are associated predominantly with the presence of the trichothecene, deoxynevalenol (vomitoxin) in wheat and corn (151). Paradoxically, nivalenol and deoxynivalenol exposure of a prolonged duration induced autoimmune-like effects similar to human IgA nephropathy (170). Trichothecenes (T-2 toxin) can cause fetal death, abortions, and teratogenic effects (24). Although several trichothecenes are genotoxic in bacterial, yeast, and cell culture systems (98, 201), they exhibit no initiator or promoter effect in whole animal systems (107).

Metabolism of trichothecenes occurs rapidly through deacetylation and hydroxylation and subsequent glucuronidation in the liver and kidneys (24, 170), thus posing little problem of residues in meats from contaminated animals. Trichothecenes inhibit protein synthesis which either by itself or together with their ability to induce apoptosis (138) can explain many of their toxic effects. In addition, deoxynivalenol and possibly other trichothecenes affect serotonergic pathways in the brain and also induce expression of a number of cytokines (170). The significance of these effects in trichothecene intoxication in humans and animals is unknown.

Zearalenone

Zearalenone and zearalenol are nonsteroidal estrogenic contaminants (produced by *Fusarium roseum*) in grains such as corn, wheat, sorghum, barley, and oats. Zearalenone induces effects consistent with those produced by excessive steroidal estrogens, that is, anabolic and uterotropic activities and regulation of serum gonadotropins. Although swine appear to be the most sensitive species and exhibit signs of hyperestrogneic syndrome—that is, changes in serum leutinizing hormone, swollen and edematous vulva, hypertrophic myometrium, vaginal cornification and prolapse (in extreme cases), and infertility (151)—human exposure to zearalenone and its metabolites by way of cereal products can also be significant.

The mode of action of zearalenone involves interaction with estrogen receptors, translocation of the receptorzearalenone complex to the nucleus, combination with chromatin receptors, selective RNA transcription leading to biochemical effects including increased water and lowered lipid content in muscle, and increased permeability of the uterus to glucose, RNA, and protein precursors (67). Available evidence indicates that rapid conversion of zearalenone and zearalanol to conjugated metabolites to be excreted in urine and feces makes consumption of meat and milk from animals receiving Ralgro an insignificant risk to humans.

Zearalenone is genotoxic in bacterial systems (68), forms DNA adducts in female mouse tissues, and induces hepatocellular adenomas in female mice (157). Unknown presently is the carcinogenic risk to humans and whether potentiative interaction exists between the adverse effects of zearalenone and those of dietary or endogenous estrogens as well as the xenoestrogens in the environment.

Other Mycotoxins

A number of other mycotoxins (Table 11.3) have been identified either as contaminants in foods destined for human consumption, or as metabolites of fungi isolated from human foods (29). Although some of these have been associated with outbreaks of domestic animal diseases, no link between human consumption and disease

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Table 11.3 Miscellaneous Mycotoxins

Mycotoxin	Major producing organisms	Source of fungi	Principal toxic effects
Alternariol and alternariol methyl ether	*Alternaria* sp.	sorghum, peanuts, wheat	highly teratogenic to mice; cytotoxic to HeLa cells; lethal to mice
Altenuene, altenuisol	*Alternaria* sp.	peanuts	cytotoxic to HeLa cells
Altertoxin 1	*Alternaria* sp.	sorghum, peanuts, wheat	cytotoxic to HeLa cells; lethal to mice
Ascladiol	*Aspergillus clavatus*	wheat flour	lethal to mice
Austamide and congeners	*Aspergillus ustus*	stored foodstuffs	toxic to ducklings
Austadiol	*Aspergillus ustus*	stored foodstuffs	toxic to ducklings
Austin	*Aspergillus ustus*	peas	lethal to chicks
Austocystins	*Aspergillus ustus*	stored foodstuffs	toxic to ducklings; cytotoxic to monkey kidney epithelial cells
Chaetoglobosins	*Penicillium aurantiovirens* *Chaetomium globosum*	pecans	toxic to chicks; cytotoxic to HeLa cells
Citreoviridin	*Penicillium citreoviride*	rice	neurotoxic, producing convulsions in mice
Citrinin	*Penicillium viridicatum*, *Penicillium citrinum*	corn, barley	nephrotoxic, swine
Cyclopiazonic acid	*Penicillum cyclopium*	ground nuts, meat products	nephrotoxic, enterotoxic
Cytochalasins	*Aspergillus clavatus* *Phoma* sp. *Phomopsis* sp. *Hormiscium* sp. *Helminthosporium dematioideum* *Metarrhizium anisopliae*	rice, potatoes millet, pecans, tomatoes	Cytotoxic to HeLa cells, teratogenic to mice and chickens
Diplodiatoxin	*Diplodia maydis*	corn	nephrotoxic and enterotoxic to cattle and sheep
Emodin	*Aspergillus wentii*	chestnuts	lethal to chicks
Fumigaclavines	*Aspergillus fumigatus*	silage	enterotoxic to chicks
Kojic acid	*A spergillus fla vus*	squash, spices	lethal to mice
Malformins	*Aspergillus niger*	onions, rice	lethal to rats
Maltoryzine	*Aspergillus oryzae*	malted barley	hepatotoxic and causes paralysis
Moniliformin	*Fusarium moniliforme*	corn	cardiotoxic in rodents
Oosporein (chaetomidin)	*Chaetomium trilaterale*	peanuts	lethal to chicks
Paspalamines	*Claviceps paspali*	Dallisgrass	neurotoxic to cattle and horses; causes paspalum staggers
Patulin	*Penicillium urticae*	apple juice	lethal to mice; mutagenic; teratogenic to chicks; pulmonary effects in dogs; carcinogenic to rats

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Mycotoxin	Major producing organisms	Source of fungi	Principal toxic effects
Penicillic acid	*Penicillium* sp.	corn, dried beans	lethal to mice; mutagenic; carcinogenic to rats
PR toxin	*Penicillium roqueforti*	mixed grains	hepatotoxic and nephrotoxic to rats; abortion in cattle
Roseotoxin B	*Trichothecium roseum*	corn	toxic to mice and ducklings
Rubratoxins	*Penicillium rubrum*	corn	causes hemorrhage in animals; hepatoxic to cattle
Secalonic acids	*Aspergillus aculeatus Penicillium oxalicum*	rice, corn	lethal, cardiotoxic, lungirritant, and teratogenic to mice
Slaframine	*Rhizoctonia leguminicola*	red clover	salivation and lacrymation in horses and cattle
Sporidesmins	*Pithomyces chartarum*	pasture grasses	hepatotoxic, causes photosensitization in ruminants
Sterigmatocystin	*Aspergillus flavus*	mammals	mutagen, carcinogen, and hepatotoxic to mammals
Tenuazonic acid	*Alternaria* sp.	grains, nuts	lethal to mice hepatoxic to mice;
Terphenyllins	*Aspergillus candidus*	wheat flour	cytotoxic to HeLa cells
Tremorgenic Mycotoxins Fumitremorgens A and B	*Aspergillus fumigatus*	rice	neurotoxic (prolonged tremors and convulsions)
Paxilline	*Penicillium paxilli*	pecans	neurotoxic (prolonged tremors and convulsions)
Penitrems A, B, and C	*Penicillium cyclopium*	peanuts, meat products, cheese	neurotoxic (prolonged tremors and convulsions) to cattle, sheep, dogs, and horses
Tryptoquivalines	*Aspergillus clavatus*	rice	neurotoxic (prolonged tremors and convulsions)
Verruculogen (TR-1)	*Penicillium verru culosum*	peanuts	neurotoxic (prolonged tremors and convulsions)
Unidentified Toxin(s)	*Aspergillus terrus Balansia epichloe Epichloe typhina Fusarium tricinctum* and others	fescue grass	gangrene (Fescue foot); summer slump syndrome; fat necrosis and agalactia in cattle
Xanthoascin	*Aspergillus candidus*	wheat flour	hepatotoxic and cardiotoxic to mice

Condensed and modified from Refrence 29.

has been established. Others have been shown to induce toxic and lethal effects in laboratory animals with no association between consumption of these toxins by animals or humans and a disease syndrome. Several of these, for example, cytochalasins and secalonic acid D (162), have been used to expand our understanding of normal as well as abnormal cellular responses to xenobiotics.

Although it is difficult to assess the total significance of the consumption of mycotoxins in human foods, it is easy to conceive that such a task requires extensive research into hundreds of known and a potentially large number of as yet unknown mycotoxins. In spite of the vast number of toxic metabolites, reduction in mycotoxin levels in foods and feeds and the prevention of mycotoxicoses in humans and animals can be achieved for the most part by avoiding stress in crops and damage to seeds by pests and by mechanical harvesting. Rapid postharvest drying and avoiding conditions that promote mold growth during storage are equally important.

Pesticides

Pesticides are essential in agriculture. The National Monitoring Program for Food and Feed, comprised of three federal surveillance programs (i.e., the Total Diet Study of market foods by the FDA, nationwide monitoring of unprocessed food and feed by the FDA, and analysis of meat and poultry by the USDA), monitors residues of chlorinated hydrocarbon insecticides, organophosphates and carbamates, and, very infrequently, herbicides and inorganic pesticides such as Arsenic and bromide in various agricultural products. Despite the ban on DDT in 1972 and aldrin and dieldrin in 1975, a partial ban on heptachlor and chlordane in 1978, and a complete ban on heptachlor in 1983 and others later, residues of these and other chlorinated pesticides and their metabolites continue to appear, especially in dairy products, meat, fish, and poultry. In most cases, however, the daily intake of a pesticide did not exceed the ADI established by a Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Pesticide Residues. Intoxication with a pesticide usually results from accidental or suicidal ingestion, careless storage, or improper use. The toxic effects of individual pesticides are discussed in Chapter 13.

Toxic Metals

A high proportion of the general population's total daily exposure to metals occurs from their natural presence in foods. Beverages, water, air, and contact with metal-containing consumer products contribute to the rest. Children consume more calories per unit body weight and have a higher absorption rate than adults, placing them at a higher risk for metal toxicity than adults.

Industrial and agricultural uses of metal products pose the hazard of food-contamination associated with their use, storage, accidental spillage, and improper disposal. The recent decline in the use of heavy metal-based pesticides, including herbicides, makes acute poisonings from dietary toxic metals less likely. A decline in the use of containers with metal coatings that dissolve during food manufacture, cooking, and storage has also contributed to the decline in acute toxicities associated with metals. Food-borne intoxications from metals are mostly limited to long-term consumption of water and food products from environments that contain naturally high levels of metals (e.g., selenium and fluoride) or that are contaminated by mining, smelting, and industrial discharge (e.g., methylmercury and Minamata disease). The reader is referred to Chapter 14 for information on the toxic effects of metals.

FOOD ADDITIVES

The increasing demand for food by an ever-increasing world population, and for ready-to-eat foods because of changes in lifestyles in developed societies, has necessitated the use of chemical additives to help preserve, nutritionally fortify, and process foods. Concerns about adulteration (masking of low-quality food by chemical additives) and of toxic effects from chronic dietary chemical exposure have led to the passage of the Food and Drug Act of 1906; the Food, Drug, and Cosmetic Act of 1938; the Miller Pesticide Amendment of 1954; the Food Additive Amendment of 1958; the Color Additive Amendment of 1960; the Animal Drug Amendment of 1968; the Federal Food, Drug, and Cosmetic Act (FFDCA) of 1976; and the Federal Food Quality Protection Act (FFQPA) of 1996. The term, *food additive,* is defined in these acts as

"…any substance the intended use of which results or may reasonably be expected to result, directly or indirectly, in its becoming a component or otherwise affecting the characteristic of any food (including any substance intended for use in producing, manufacturing, packing, processing, preparing, treating, packaging, transporting, or holding food; and including any source of radiation intended for any such use), if such substance is not generally recognized, among experts qualified by scientific training and experience to evaluate its safety, as having been adequately shown through scientific procedures (or, in the case of substances used in food prior to January 1, 1958, through either scientific procedures or experience based on common use in food) to be safe under the conditions of its intended use…"

Food additives fall into two broad categories, direct and indirect. Direct additives are those intentionally added to foods to preserve or improve the quality of

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the product or to aid in the processing. Some examples of 32 functional classes of direct additives are antioxidants, inhibitors of bacterial and mold growth, vitamins and minerals, color, and antifoaming agents. Among the approximately 3000 direct additives, more than 500 are "generally recognized as safe" (GRAS), and about 150 are sanctioned as safe prior to September 6, 1958, with their number likely to increase. They are exempt from regulation as food additives unless the scientific review of these substances currently underway warrants reclassification in the future. Sucrose, corn syrup, dextrose, and salt (all on GRAS list) account for 93 percent, by weight, of all the food additives used. Indirect additives are chemicals that gain their way into foods unintentionally or unavoidably during some phase of production, processing, storage, or packaging. Components of packaging containers and materials that migrate into foods fall under this category. In addition, the presence of pesticide residues in crops and processed foods and residues from animal drugs in milk, meat, and eggs is allowed based on their potential health risk as balanced against the benefits of their use.

Safety Assessment of Food Additives

Foods in their natural forms are assumed safe unless they are "ordinarily injurious." The presence of avoidable contaminant(s) posing a risk of injury to health renders the food unsafe and subject to recall, while the presence of unavoidable contaminant(s) posing such risk is considered unsafe if it exceeds tolerance levels set by FDA (or EPA) or action levels (informal and not subject to law) set by FDA. Intentional additives other than those GRAS or those that are prior sanctioned (e.g., colors) are subject to regulations described above and can only be used at levels posing a risk at or below the level considered acceptable.

Safety evaluation of direct food additives involves establishment of a no observable adverse effect level (NOAEL) in experimental animals followed by establishment of acceptable daily intake (ADI) in the total diet for human beings using a suitable safety factor (usually 100). For unavoidable contaminants, tolerance levels are set to limit the quantity of the agent in each commodity based on risk-benefit-cost analysis.

The FDA-recommended safety testing approach is based on the concept of "level of concern" (28). Level of concern is based on the level of exposure, structural correlation with known toxic compounds (if no toxicity data are available), and existing toxicological data. Subjective categorization of additives into concern levels I, II, or III (level III being of highest concern) is done for compounds contributing <0.05 pmm, 0.05–1.0 ppm, and >1.0 ppm, respectively, to the total diet. Also, low, medium, or high toxicity or a structural similarity to com pounds with low, medium, or high toxicity places a compound in concern levels I, II, or III, respectively. Furthermore, formation of active metabolites would place the compound in level III.

Table 11.4 lists recommended toxicological tests for each level of concern. This testing scheme allows compounds producing effects only at high levels and those with lower levels of human exposure to be tested less extensively. Protocols for testing color additives and indirect food additives are similar to direct food additive testing. Safety testing of animal drugs and feed additives, however, is more complex not only because an additional animal species (target animal) is involved but also because of the necessity of understanding the impact of target animal metabolism on the diversity and toxic potential of the metabolites to humans, the need for the development of residue detection methods and elimination strategies in the target animal species, and to set maximum allowable residues (tolerance) of the parent compound and metabolites in tissues of the target animal.

Food and color additive use is regulated by a special anti-cancer clause, the Delaney Clause, in the Food Additive Amendment. The Delaney Clause prohibits the use of these additives "if they are found to induce cancer when ingested by man or animal, or if found, after tests which are appropriate to the evaluation of safety of such substances, to induce cancer in man or animal" (59). Although the addition of the Delaney Clause reflected the then-prevailing concern for cancer, subsequent scrutiny and recent developments in the understanding of the mechanisms of carcinogenesis required changes as reflected in the FQPA of 1996. Currently, the regulation of pesticide residues has become the responsibility of EPA and, thus, is not an issue for the FDA as a food additive. EPA can approve pesticide applications if it concludes that there is "a reasonable certainty that no harm will result from its aggregate exposure." This would translate to no greater than a risk of 1 in 1 million lifetime risk for carcinogens and levels with a 100-fold safety margin to NOAEL for threshold-limited effects. For already approved carcinogenic pesticides, EPA is allowed to retain tolerance posing greater than negligible risk (1 in 1 million) if the pesticide either protects consumers from a greater health risk or is necessary to avoid disruption of an adequate, wholesome, and economical food supply (134). In addition, the FQPA directs EPA to take higher intakes for certain pesticides in children into consideration in setting tolerances and to apply an

additional safety factor of 10 for threshold effects in calculating ADI.

Animal Drugs

All new animal drugs and feeds containing them must receive premarket clearance from the Bureau of Veterin

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Table 11.4 Test schedule recommended by FDA for additives at various concern levels

Concern Level I	Concern Level II	Concern Level III
1. Short-term (at least 28 days) feeding study in a rodent species	1. Subchronic feeding study in a rodent species	1. Chronic (at least 1 yr) feeding study in a rodent species
2. Short-term test for carcinogenic potential	2. Subchronic feeding study in a non-rodent species	2. Chronic (at least 1 yr) feeding study in a nonrodent species
	3. Multi- (at least two) generation reproduction study with teratology, in a rodent species	3. Multi- (at least two) generation reproduction study with teratology, in a rodent species
	4. Short-term test for carcinogenic potential	4. Short-term test for carcinogenic potential
		5. Carcinogenicity studies in two rodent species (test 1 can be a part of this)

ary Medicine of the FDA. Antibiotics and steroidal as well as nonsteroidal growth promotants are used in feeds for the prevention of disease or for growth promotion in raising 60 to 100 percent of food-producing animals. Two major concerns with the use of antibiotics in animal feeds are: the development of resistance in enteric bacteria in animals that could be transferred via plasmids to pathogenic bacteria in the gut of animals and, thus, to humans through meat or milk, making antibiotics currently used in human medicine ineffective; and, the presence of carcinogenic residues in meat or residues that form carcinogenic nitrosamines following reaction with nitrite in the meat. Increasing pressure on the FDA has already resulted in the exclusion of many antibiotics (kanamycin, gentamicin, semisynthetic penicillins, chloramphenicol etc.) as feed additives with exclusion of all likely in the future.

Similarly, concerns about the use of hormonal and other growth promotants in feeds are related to possible chronic toxic effects, mainly carcinogenicity. The synthetic estrogen, diethylstilbestrol (DES), was banned as a feed additive due to its carcinogenic effects and the lack of a method sensitive enough to detect residues of health significance (causing >1 life time cancer in 1 million population). Steroids approved for feed additive use in one or more animal species include estradiol, progesterone, testosterone, melengestrol acetate, and zearalanol. Other additives commonly used in animal production include monensin, iodides (EDDI), phenothiazine, and thiabendazole. Carcinogenic animal drugs can be approved for use by the FDA if an adequate withdrawal period is recommended between the last dose of the additive and slaughter to allow residues to fall below those judged capable of inducing greater than negligible (1 lifetime cancer in 1 million) incidence of cancer.

Toxic reactions associated with the use of selected additives that have been restricted, banned, or are currently being critically reviewed are presented below.

Direct Food Additives

Aspartame (Nutrasweet)

The use of aspartame (L-aspartyl-L-phenylalanine methyl ester) as a sweetener in a large variety of food products increases the likelihood that acceptable daily intake is exceeded. Previously suspected association between aspartame consumption and the anecdotal reports of headache, dizziness, and seizures in adults and hyperkinesia in children appears to be unsupported. Although nitrosation products of aspartame are moderately mutagenic (184), the question of the role of aspartame in the recent increase of lymphoma of the brain remains unresolved. The metabolism of aspartame to phenyl alanine led to the suggestion that patients with phenylketonuria should avoid aspartame-containing products.

Butylated Hydroxy Anisole (BHA)

Synthetic phenolic antioxidant BHA has been GRAS and used for decades as an antioxidant to retard the autoxidation of lipids and prevent rancidity in foods. Together with ascorbic acid, α-tocopherol, gallate esters, and BHT, it fulfills almost 100 percent of the antioxidant needs of foods. BHA may be a rodent carcinogen involving squamous cells in the forestomach but these cells are not the human equivalent of glandular or other cells. The carcinogenicity of BHA appears to involve O-demethylation of BHA to tertiarybutyl hydroquinone (TBHQ), oxidation of TBHQ to tertiary butyl semiquinone and tertiary butylquinone (TBQ), conjugation of TBQ with glutathione (GSH), and formation of DNA-reactive oxygen species including the hydroxyl radical (207). However, genotoxicity of BHA has not been demonstrated. Since humans lack a forestomach, evidence for direct genotoxic effect is lacking, and since human exposure is well below inducing nongenotoxic effects it is highly unlikely that BHA carcinogenicity in rodents is relevant to the safety of BHA in human foods.

Cyclamates

Sodium and calcium cyclamate were introduced as non-nutritive sweeteners in 1950 and were included in the 1959 GRAS list. Significant consumption of cyclamates in low-calorie foods and drinks followed. Subsequent demonstration of cancer-promoting or co-carcinogenic activity in animals led to the removal of cyclamates from the GRAS list and, thus, from food additive use in the United States, despite a lack of evidence in human beings (5). Cyclamates, however, are still used in many other countries.

Monosodium Glutamate (MSG)

MSG has been on the GRAS list since 1958, used as both a seasoning agent and a flavor enhancer with an ADI of 120 mg/kg. The demonstration of lesions in the retina and the lateral geniculate nucleus in MSG-exposed neonatal rats and mice led to a voluntary discontinuation of its use in infant foods in the United States. This appears justified since neonatal effects of MSG may last through adulthood (193). In adults, the MSG symptom complex (headache, muscle tightness, numbness/tingling, general weakness, and flushing, among others) occurs in a third of the population with a threshold dose of 2.5 g of MSG (220).

Nitrates, Nitrites, and Nitrosamines

Of the total daily dietary intake of nitrates of 10–150 mg/day (48), leafy vegetables contribute 99 percent. Nitrate use to cure meats (to give characteristic flavor and pink color, to prevent rancidity, and to prevent growth of the spores of *Clostridium botulinum*) contributes <0.1 mg/day (48). Nitrates can be reduced endogenously by microbial systems to nitrites which then oxidize the hemoglobin to methemoglobin (heme iron from ferrous to ferric state). Methemoglobin, being unable to combine with oxygen, following accumulation in sufficient quantities, can lead to anoxia. The use of water with high (>30 mg/1) nitrate content (from soils, fertilizers, etc.) in making baby formula and foods, spinach with high nitrate content, and occasionally meats with high levels of added nitrates and nitrites have resulted in life-threatening methemoglobinemia in humans, especially children. The consumption of plants high in nitrates by animals has caused significant economic loss for owners. In adult humans, however, the daily intake of nitrate and nitrite amount to less than 69 percent and 0.7 percent of the ADI of 3.64 and 0.135 mg/kg/day (48).

Nitrite reacts with secondary amines to form a variety of N-nitrosamines which are present in foods, pharmaceuticals, cosmetics, agricultural chemicals, tobacco, and tea. In vivo, nitrosamines are converted to unstable hydroxyalkyl compounds which subsequently form reactive alkyl carbonium ions capable of alkylating DNA (207). Nitrosamines are mutagens and rodent carcinogens, producing cancer in a variety of organs, including the liver, respiratory tract, kidney, urinary bladder, esophagus, stomach, lower gastrointestinal tract, and pancreas (207). Nitrite itself may promote carcinogenesis. Because they are not added to foods, however, nitrosamines are not subject to the restrictions of the Delaney Amendment. Inhibition of nitrosamine formation in foods by ascorbate, cysteine, gallic acid, tannins, sodium sulfite, and sodium erythorbate prompted the FDA to suggest that one of these compounds be concurrently added to meats during curing to reduce nitrite added from 200 to 120 ppm. Such a practice, however, is ill-advised until a suitable additive is found to deal with the threat of *C. botulinum* growth at reduced nitrate levels and while consumers accept the ensuing changes in the appearance and organoleptics properties of meat cured with this combination. Perhaps a change to lower nitrate-containing vegetables to reduce nitrate intake is a less dangerous option.

Saccharin

After having been in use since the beginning of the 20th century and surviving a ban and an attempted ban of its use due to suspected weak bladder cancer-promoting activity (142) at high doses, saccharin continues to be used as a sweetener in soft drinks and in table top uses. The average intake is 7.1

mg/day in the United States and 15.0 mg/day in Europe per capita, reaching as high as 25 mg/day in certain subpopulations (86). In 2000,

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Saccharin was delisted as a human carcinogen by the National Toxicology Program.

Safrole

Safrole and other alkenylbenzene compounds (β-asarone, methyleugenol, estragole, and isosafrole) are active components of many spice flavors. Sassafras, which contains high levels of safrole, has been used as a flavoring agent in sarsaparilla root beer. Safrole also is consumed in the form of sassafras oil and sassafras tea, the latter still occurring to a limited extent in the United States. A total dose of only 0.5–1.5 mg of safrole orally or intraperitoneally to infant male mice caused high liver tumor incidence. Dihydrosafrole, a synthetic safrole, caused esophageal tumors in rats, and some of the other natural alkenylbenzenes also are carcinogenic (135). These findings resulted in the FDA ban on the use of safrole, sassafras, and sassafras oil from commercial use in foods, including root beer, in 1960. Safrole continues to be used in the European Community, however. A metabolite, 1-hydroxy sulfate ester, is apparently the ultimate carcinogen, forming adducts with guanine and adenine (135).

Other chemical additives initially approved as safe by a regulatory body but now prohibited from use due to a potential risk or to lack of demonstration of safety include calamus and its derivatives in 1968 (containing aklenylbenzene flavoring agents); coumarin flavoring compounds in 1953; chlorofluorocarbon propellants in self-pressurized containers in 1978 (due to their role in the dissolution of the earth's ozone layer, which results in increased skin cancer risk from ultraviolet radiation); diethyl pyrocarbonate (DEPC), an antimicrobial agent in beers and juices (cold pasteurization) and a ferment inhibitor, in 1972, due to the presence of the carcinogen urethan in DEPC-treated products; and dulcin, a sweetener, in 1950, due to liver and bladder cancer in rats. On the other hand, the ultimate determination of the safety of recently approved additives such as the fat substitute, Olestra (long-chain fatty acid esters with sugar), must await longer-term consumer use.

Food Colors

Color is a quality of foods that makes them visually acceptable and aids in their recognition. Foods containing added colors include candy and confections, bakery goods, soft drinks, cereals, and dairy products such as butter, ice cream, and sherbet, margarine, snack foods, jams and jellies, and dessert powders. Following the passage of the Color Additive Amendment of 1960, 20 natural colors (compromising preparations such as dried algae meal, beet powder, grape skin extract, fruit juice, paprika, caramel, carrot oil, cochineal extract, ferrous gluconate, and iron oxide) were exempted from certification, whereas all the synthetic colors, including the ones approved prior to the amendment, were required to be retested if questions arose regarding their safety. A provisional certification was given to those in use that required further testing. Currently, there are seven certified synthetic colors (FD & C colors Blue No. 1, Red No. 3, Red No. 40, and Yellow No. 5 are permanently listed whereas FDB Blue No. 2, Green No. 3, and Yellow No. 6 are provisionally listed) with unlimited uses (according to good manufacturing practices) and one permanently listed color (Citrus Red No. 2) used only for coloring skins of oranges at 2 ppm. Several colors including Green No. 1, Green No. 2, Orange No. B, Red No. 2, Red No. 4, and Violet No. 1 were delisted due to concerns about their carcinogenicity and other chronic toxic effects. A controversy linking food colors to allergies and hyperkinesis in children failed to draw supportive evidence.

Packaging Materials

Packaging is an essential part of food processing that aids in the preservation of the wholesomeness of foods by preventing

(a) contamination or destruction by dirt, microorganisms, insects, and rodents,
(b) loss or gain of moisture, odors, flavors, or aroma, and
(c) deterioration from air, light, heat, and contaminating gases (69).

Other functions served by packaging include assembling a variety of items, convenient handling, labeling, and, finally, sales promotion. A variety of materials, ranging from metal foils to complex plastic substances, are in use. Examples of package modifications employing chemical additives are oleoresinous coating with or without suspended ZnO, which is used in the preservation of acid foods that do (e.g., seafood) or do not (e.g., cherries) produce sulfides; stabilizers to prevent degradation of plastic when exposed to heat and light; and hot-melt adhesives used to glue multilayered packages (tea, hydrated soups, potato chips, etc.). A complete list of additives approved for use in packaging is given by Gilchrist (69).

To approve new packaging material, FDA requires extraction studies involving one or more of aqueous (8% alcohol), alcoholic (50% alcohol), or lipid solvents (corn oil or triglycerides), followed by toxicity testing depending upon the extent of extraction, >1 ppm requiring extensive testing including chronic toxicity. The National Science Foundation (NSF) estimated that as many as 3000 chemicals may enter

foods indirectly from the process of packaging itself (75). A safety review by the FDA has resulted in banning the adhesive Flectol

H, polyurethane resins and curing agents food packaging adhesives containing 4,4-methylenebis (2-chloroanaline), and the synthetic chemicals, mercaptoimidazoline and 2-mercaptoimidazoline, used in the production of rubber articles (75). The use of polyvinylchloride for packaging liquors has been banned in the United States. Among the packaging-derived contaminants likely to be encountered in U.S. diets, benzene and vinyl chloride are known carcinogens; acrylonitrile, 1,3-butadiene, epidilorohydrin, formaldehyde, propylene oxide, and styrene oxide, are probable carcinogens; and 2,4-diaminololuene, dibutyl and diethylhexylphthalates, dimethylformamide, 1,4-dioxane, ethylacrylate, phenyl glycidil ether, styrene, and toluene diisocyanate are possible carcinogens (86, 146).

Toxic Factors Produced During Processing

Food processing is aimed at improving the quality of foodstuffs, ensuring safety, and enhancing the ease of preparation. This requires various chemical and physical treatments of food which may result in
(a) partial or complete destruction or removal of nutrients,
(b) inferior digestibility or utilization of nutrients, and
(c) generation of new, potentially harmful chemicals.

The first two effects can be overcome by nutritional supplementation. The latter represents a need for appropriate toxicological investigation. In addition, similar products can be formed during storage due to the continuous effects of heat, humidity, light, oxygen, and catalysts present in foods.

Formation of crosslinked amino acid side chains such as lysinoalanine, ornithinoalanine, and lanthionine, as well as racemization of amino acids to D-analog appear to take place during alkali treatment, for example, of soybean protein for preparing imitation meat (62). These products, especially lysinoalanine, have been shown to cause nephrocytomegaly (enlarged nuclei and cytoplasm) of the pars recta cells.

Nonenzymatic browning reactions (Maillard reactions) occurring during heating of foods (drying, frying, roasting, baking, and broiling) involve chemical interactions between amino acids and reducing sugars (aldoses and ketoses), forming mutagenic reductones, furans, amino-carbonyls, pyrazines, and other premelanoid secondary amine derivatives (Amadori and Heyns' products) which are hypothesized to inhibit growth, impair reproduction, damage the liver, cause allergies, play a role in aging, and induce lens lesions (86).

In general, high-protein foods appear to possess more mutagenic activity compared with foods rich in carbohydrates and/or fats. Pyrolysis of proteins and amino acids at high temperatures (300°C or more) yields a series of heterocyclic compounds which can be metabolized to mutagenic products were positive in one or more rodent species for carcinogenicity (86, 195). Using estimates of various heterocyclic amines ingested and cancer potencies in animal studies, Layton et al. (110) estimated that only 0.25 percent of human colorectal cancer may be due to these compounds. Certain cooking practices such as frying of high nitrite foods such as cured bacon results in the formation of nitrosamines whose carcinogenic effects have already been discussed.

A variety of polycyclic aromatic hydrocarbons (PAH) are formed in foods by pyrolysis during cooking or by their prior contact with petroleum and/or coal tar products. Although the carcinogenic effects of PAH are known, the contribution of dietary PAH to cancer in humans is likely to be insignificant.

Fats (polyunsaturated) undergo three basic changes during storage and/or heat treatments, that is, autoxidation, thermal oxidation, and thermal polymerization. Autoxidation occurs at below 100°C in the presence of enzymes (lipoxygenases) or upon exposure to light and results in the generation of hydroperoxides via a free-radical or singlet oxygen mechanism leading to rancidity (86). Hydroperoxides can be degraded into alkanes, aldehydes, and ketones, among others. Termination of peroxidative reactions generally involves scavenging of the radicals or their polymerization into nonreactive products. Lipid hydroperoxides, at subtoxic levels, can stimulate signal transduction mediated by Ca^{++} and protein phosphorylation by acting as second messengers in pathways involved in cell proliferation, chemotaxis, apoptosis, and other cellular mechanisms (196). High levels of rancid fats (5% or more of the diet) can cause decreased food consumption, diarrhea, weight loss, leukopenia, and hair loss. Hydroperoxides and/or their products (hydroxynonenal, melon-dialdehyde, etc.) can disrupt gap-junctional communication and form DNA adducts, and are mutagenic and carcinogenic, increasing the incidence of tumors and atherosclerosis (86, 221). Components of heated oils that fail to form adducts with urea, especially the cyclic monomeric fatty acids followed by polymers of fatty acids, appear to be toxic. Toxic effects include, in addition to those described above, hepatomegaly and carcinogenicity.

Yeast-fermented foods and beverages such as yogurt, cider, malt beverages, bread, soy sauce, wine, and sake, in addition to psychoactive and vasoactive amines discussed earlier, contain mutagenic and carcinogenic ethyl carbamate (urethane) derived, in the presence of heat and light, from arginine, asparagine, cyanogenic glycosides, or ethanol in the fermented commodity. Levels of <10 ppb or less

for soft drinks and <30–400 ppb for various alcoholic beverages have been recommended as acceptable by the FAO/WHO and the Canadian government, respectively (87).

The major fermentation product consumed by humans is ethanol which, in addition to death from toxic effects, contributes to human deaths from occupational as well as automobile accidents. The toxic effects of ethanol, although they can manifest themselves in many organ systems, display major CNS involvement (dependence and depression), and impact the developing fetus (fetal alcohol syndrome of mental deficiency and microcephaly) and the liver (hepatomegaly followed by cirrhosis). Mechanisms of ethanol toxicosis may involve the direct effects of alcohol, effects of its metabolite acetaldehyde, ethanol-induced malnutrition, ethanol-induced endotoxin release by intestinal bacteria that stimulate release of reactive chemicals by Kupfer's cells, ethanol-induced potentiation of other hepatotoxic agents, or a combination. An International Agency for Research on Cancer (IARC) expert panel considered ethanol a human carcinogen causing tumors of the oral cavity, pharynx, esophagus, and liver (189).

Toxic effects of processed food as a whole, however, cannot be estimated by simply adding up the toxic, mutagenic, and/or carcinogenic potential of the products present in it. This is due to the fact that chemical derivatives which both enhance as well as antagonize the myriad toxic effects of other dietary components are formed during processing (146). At present, these chemicals and their interactions with each other, for the most part, are unknown. As a result, the overall adverse effects of cooked foods can only be determined reliably based on the assessment of risk from the complex milieus of the product in question.

CARCINOGENS AND MUTAGENS IN FOODS

Cancer, a disease of most public concern for the past half a century, is, experimentally, a multistage process involving initiation (induction of DNA damage, thus resulting in a transformed cell), promotion (a nongenotoxic effect leading to a rapid multiplication of the transformed cell and, thus, establishment of a cancerous lesion), and progression. Multistage models involving a sequence of multiple genetic events with the incidence increasing in proportion to the exponent of time, however, seem to fit most human cancers (146). Naturally occurring food toxicants provide examples of both initiators and promoters, with up to 70 percent of cancer deaths being attributed to dietary factors (51). Examples of likely dietary carcinogens as reviewed by the National Research Council (NRC) (146) are provided in Table 11.5. This list includes carcinogens derived from natural products both by commercial processing (e.g., alcohol) as well as biotransformation in the body (e.g., allylisothiocyanate and nitrosamines); and initiators (e.g., aflatoxins, furocoumarins, pyrrolizidine alkaloids) as well as promoters (e.g., phorbol esters, fat, caffeine). In addition, residues of synthetic chemicals can be present in foods subsequent to their accidental contact or intentional use to increase production. An added dimension to diet is the formation of animal and possibly human carcinogens during cooking, including nitrosamines, aromatic hydrocarbons, amino acid pyrrolysates, carbolines, imidazoquinolines, quinoxalines, and fat oxidation products inducing cancer of the liver, stomach, intestines, zymbal and clitoral glands, skin, and oral cavity and others (146). Coffee, in addition to caffeine, is known to yield several animal carcinogens including caffeic acid, catechol, furfural, hydrogen peroxide, and hydroquinone during roasting and/or brewing (11). Carcinogenic natural pesticides are present in all classes of plant foods including fruits, vegetables, and spices (11).

Balancing this bewildering array of toxins and carcinogens, in almost every food item, is another group of chemicals capable of antagonizing these effects. The dietary antimutagens and anticarcinogens, whose mechanisms of action are not always understood, belong to a wide variety of chemical structures (Table 11.6). As with mutagens, multiple species of antimutagens/ anticarcinogens appear to be present in each dietary component (at least five are known in soybeans and three or more in broccoli). Interactions between carcinogens and anticarcinogens are complicated as indicated by the study of indole-3-carbinol, a component of cruciferous vegetables, known to inhibit mammary and forestomach neoplasia in rodents. When given as a pretreatment, indole carbinol reduced the carcinogenicity of aflatoxin B1, while exposure to indole carbinol after the carcinogen exposure resulted in an increase in aflatoxin carcinogenicity (17).

Natural versus Synthetic Chemicals

The widely held belief that naturally (free of synthetic chemicals) grown foods are inherently safer than those grown with the aid of synthetic chemicals is flawed. Certain natural chemicals in the human diet (such as indole carbinol in cruciferous vegetables) interact with the same receptor (Ah) as dioxin (TCDD), one of the most feared synthetic toxicants, interacts (12). An EPA reference dose, a dose estimated to produce one cancer in one million individuals (6 fg/kg/day), of TCDD is comparable to 5 mg of indole carbinol per 100 g of broccoli or cabbage, a level of exposure not unrealistic. The EPA ban on alar—using a worst-case scenario risk estimation in response to public outcry resulting from less-than-objective reporting by the media and a passive attitude by knowledgeable academicians and

researchers (168)—suggests that regulatory agencies also may subscribe to this misconception.

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The NRC (146), however, considers natural carcinogens at least as potent and, considering the extent of exposure, more potent than synthetic carcinogens. The fact that disproportionately fewer natural chemicals have been tested suggests a greater need to test natural chemicals, a daunting task considering that all toxicants in all classes of foods are not known and that exposures to those that are known are wide-ranging and not well documented.

Table 11.5 Naturally occurring carcinogens and potential carcinogens in the diet

Carcinogen/Mutagen	Major Foods Containing the Chemical
Alcohol	grains and fruits (derived from...)
Allylisothiocyanate	cabbage, collard greens, brussels sprouts, mustard (brown)
Caffeic acid, Caffeine, and Theobromine	coffee, cocoa, fruits, and vegetables
Cyclopropene fatty acids	cotton seed oil, kapok, and okra
Fat (unsaturated and cholesterol-containing)	vegetable and animal fats
Flavonoids (Quercetin, etc.)	vegetables, tea, coffee
Furocoumarins (psoralen)	celery, figs, parsley, parsnips
Gossypol	cottonseed oil
Hormones (estrogen, testosterone, progestins, and related)	meats as residues, supplements
Hydrazines (agaritine, gyromitrin)	mushrooms
d-Limonine	citrus juices
Methylazoxymethanol, cycasin	cycads
Mycotoxins (aflatoxins, fumonisins, ochratoxin A, sterigmatocystin)	corn, cottonseed, peanuts, wheat, and other grains
Nitrosamines	beets, celery, spinach, meat preserved in nitrite
Phorbol esters	croton oil, other Euphorbaceae (herbal teas)
Polyphenols (tannic acid)	beverages (tea, cider, cocoa, red wine), fruits
Ptaquiliside	bracken fern
Pyrrolizidine alkaloids	herbs, herbal teas, honey
Safrole, estragole, methyleugenol, piperine, etc.	nutmeg, other spices, black pepper
Cooked food carcinogens/mutagens	all cooked foods
Polyaromatic hydrocarbons (mono and dibenzo derivatives)	
Maillard reaction products	
Amino acid pyrrolysates (Trp-P-1-, Trp-P-2; Glu-P-1, Glu-P-2) Carbolines	
Imidazoquinolines and quinoxalines (IQ, MeIQ, MeIQx)	
Fat oxidation products	
Coffee-derived mutagens/carcinogens	

Table 11.6 Important antimutagens and anticarcinogens naturally occurring in foods

Class/subclass	Examples	Foods containing them
Alkaloids	indole-3-carbinol, caffeine	broccoli, cabbage, cauliflower, coffee
Amino acids	cysteine and tryptophan, curcumin	many plants and animals
Arylheptanoids		tumeric
Benzenoids	gingerol, paradol	ginger root and related plants
Cyclitols	myoinostol, phytic acid	wheat, other cereals, nuts, and meats?
Estrogens	sitosterol	soybeans, alfalfa, etc.
Fatty acid derivatives	conjugated linoleic and arachidonic acid	vegetable oils
Fiber	acid-soluble, neutral, etc.	fruits and vegetables, cereal bran
Minerals	Se, Ca++	crops grown on Se-containing soils, milk, meat
Phenolics		
Phenolic acids	gallic and protocatechuic acids,	many fruits and vegetables
Phenyl propanoids	caffeic, cinnamic, chlorogenic and ferrulic acids, enginol, myristicin	broccoli, other vegetables
Flavones	apigenin, myricetin, quercetin, robinetin, rutin	fruits, herbs, and vegetables soybeans and others
Isoflavones	biochanin A, genistein, daidzein, etc.	
Polyphenols		
Lignans	sesamin	sesame seed
Tannins	ellagic and tannic acids, epigallo-catechin-gallate	Chinese green tea, other teas, cereals, legumes, and fruits
Protease Inhibitors	antipain, elastatinal	
Porphyrins	chlorophyll, chlorophyllin, cytochrome C, hemin, hemoglobin, myoglobin	green leafy vegetables, meats
Sulfur-containing compounds	benzyl isothiocyanate, cysteanine, diallyl sulfide and disulfide, glutathione, isothiocyanate, phenethyl, sinigrin, sulforaphane	broccoli, cabbage, cauliflower, and others
Terpenoids		
Monoterpenes	carveol, limonene, menthols	citrus fruits, grapes, mint, other plants, wine
Diterpenes	cafestol, kahweol	coffee, variety of plants, sponges, corals, etc.
Triterpenes	glycerrhetinic acid, its glycoside, limonin, oleanolic acid, and ursolic acid	citrus fruits and a variety of medicinal plants
Sesquiterpenes	nerolidol	medicinal plants and herbs
Unidentified	unknown	in beef, cabbage, germinating wheat, mushrooms, etc.
Vitamins		
Carotenoids	canthaxanthin, α and β-carotene, fucoxanthin	fresh green, leafy vegetables
Others	vitamins A, C, E, and riboflavin	fruits and vegetables (fresh), meats, fish

Adding to this complexity is the fact that simultaneous exposure to two initiators, an initiator and a promotor, or to two promotors can lead to additive, multiplicative, and supramultiplicative carcinogenic responses in experimental settings (100). However, at exposure levels as low as those occurring in natural foods, the differences are lost so that the overall risk of a mixture becomes only additive (145). Furthermore, making the safety assessment of human dietary ingredients an almost impossible task is the presence of protective (anticarcinogens, etc.) agents in the same mixture, with interactive effects among themselves and their combined antagonistic effect against the effects of mixture of carcinogens and other toxicants present.

Testing various crude solvent extracts of each dietary ingredient or even selected total diets (composed of average daily per capita amounts of each of the common dietary ingredients in the United States, for example) may reduce the amount of testing. Interactions between components of various extracts and between extractable and non-extractable components will still need to be estimated or tested further. The best but not a perfect approach to a more realistic risk appraisal, however, may involve testing pelleted dehydrated edible whole product such as meat, fruit, or vegetable individually or in combination in animals at doses reflecting human intake. Such an approach not only reduces the cost of testing compared to an individual chemical testing strategy but, also, provides data more relevant to natural exposure to a complex diet.

At the same time, transgenic plants—with improved nutritional quality, capabilities to withstand processing, resistance to spoilage, lower levels of toxic compounds (using anti-sense or other recombinant DNA technology to inactivate gene[s] regulating biosynthesis and/or metabolism), and reduced susceptibility to fungal infestation—should be developed and the lower risk associated with consumption of such plants must be confirmed prior to extensive consumer use. Reduction in fungal susceptibility can be achieved by lowering the micro- and/or macronutrients in the plant needed for fungal growth, or other appropriate techniques (146). Success in risk reduction is also likely if preservation, processing, and storage methods to reduce the levels and/or the effects of natural toxicants in foods are developed and put in practice. Methods to process cassava root to extract/neutralize cyanide; evisceration of fugu fish to eliminate tetrodotoxicosis; waxing, heating, dipping in corn oil, spraying with lecithin, and/or immersion in dilute detergents of potatoes to reduce their glycoalkaloid content; and prudent addition of antioxidants to reduce formation of harmful agents such as lipid oxidation products and nitrosamines—all are examples of such processes. Last, but not the least, of the strategies in dealing successfully with natural dietary chemicals involves public education in avoiding/reducing exposure to natural dietary hazards.

CONCLUSION

Although there has been much progress in our understanding of identification and management of food-borne hazards, large gaps in knowledge exist in the areas of mechanisms of pathogenesis of known human intoxications associated with foods; interactions between multiple toxicants present simultaneously, between toxicants and nutritional components, and between toxicants and antitoxicants (including antimutagens and anticarcinogens) in foods; methods of realistic human health risk extrapolation from animal data; and the development of safer plant varieties and processing and cooking methodologies that minimize toxic hazards to consumers.

Considering the facts that natural dietary toxicants are, at least, as toxic as the synthetic ones and that human exposure to them is greater in quantity and consistency than to synthetic toxicants, U.S and worldwide research resources should be shifted to achieve a realistic balance, in the study of health hazards, more toward the natural dietary components. Present testing of purified individual food toxicants in animals is inadequate and must be succeeded by feeding realistic levels of such compounds in the complex milieus of the product in which the toxicant is naturally present (smoked meats, for example) or even, perhaps, in the total human diet. Realistically speaking, although this task is impossible because of the vastly variable composition of individual food ingredients as well as of the total human diet, we can edge closer to this goal by designing a diet containing various dietary ingredients (vegetables, fruits, grain, dairy products, and meats) at a level equal to percentages of their average per capita human consumption.

Education of the consumers to minimize dietary risks using practicable methods similar to those cited above and to shatter the myths that "natural is healthy" and "man made or synthetic is toxic" needs to be vigorously pursued. An educated populace is less likely to be unduly alarmed and is more likely to accept prudent regulatory actions resulting from realistic scenarios of risk estimation. Finally, application of newer molecular methodologies (such as PCR) to confirm intoxication from bacterial and other biotoxins and intensified activities by national animal health monitoring systems combined with more

rigorous application of HACCP will lead to a significant reduction in the currently widespread incidence of microbial diseases from food sources.

ACKNOWLEDGMENT
The authors acknowledge Kathy Craighead and Sarah Young for their excellent word processing skills and timely completion of this manuscript.

QUESTIONS: FOOD FOR THOUGHT

1. Design a Hazard Analysis Critical Control Point System for a food-borne microbial agent of your choice. Can this system, in principle, be used to control other toxic (both natural and synthetic) hazards? If not, is it possible to modify the HACCP system or to develop a similar system to suit such needs?
2. Using the information provided in this chapter (start with #2 of the recommended reading list) and other chapters of this book as well as other available resources (including your imagination), propose a strategy to test natural dietary toxicants in a way that allows more realistic extrapolation to humans than is currently used.
3. Do synthetic chemicals pose a greater hazard than food-borne chemicals in your informed opinion (use #3 of the recommended reading list and others)? If so, are we doing all we can to keep them out of our food supply? Is there more that can be done to achieve this goal? If not, how can we use our resources in the right context of food safety?

REFERENCES

1. Abado-Becognee, K., Mobio, T.A., Ennamany, R., Fleurat-Lessard, F., Shier, W.T., Badria, F., and Creppy, E.E. (1998): Cytotoxicity of fumonisin B1: Implication of lipid peroxidation and inhibition of protein and DNA syntheses. *Arch. Toxicol.*, 72(4):233–236.
2. Abuye, C., Kelbassa, U., and Wolde-Gebriel, S. (1998): Health effects of cassava consumption in South Ethiopia. *East African Med. J.*, 75:166–170.
3. Adams, H.R. (1989): Phytoestrogens. In: *Toxicants of Plant Origin*, Vol. IV, Phenolics, edited by P.R., Cheeke, pp. 23–51. CRC Press, Boca Raton, Florida.
4. Adlercreutz, H. (1995): Phytoestrogens: Epidemiology and a possible role in cancer protection. *Environ. Hlth. Perspect.*, 103 (supp 7):103–112.
5. Ahmed, F.E., and Thomas, D.B. (1992): Assessment of the carcinogenicity of the non-nutritive sweetener cyclamate. *Crit. Rev. Toxicol.*, 22:81–118.
6. Aldridge, D., and Tahourdin, C. (1998): Natural oestrogenic compounds. In *Natural Toxicants in Foods*, edited by D.H.Watson, pp. 54–83. CRC Press, Boca Raton, Florida.
7. Alston, T.A., Mela, L., and Bright, H.G. (1977): 3-Nitropropionate, the toxic substance of Indigofera, is a suicide activator of succinate dehydrogenase. *Proc. Natl. Acad. Sci. (USA)*, 74:3767–3771.
8. Altekruse, S.F., Swerdlow, D.L., and Wells, S.J. (1998): Factors in the emergence of food borne diseases. In: *Microbial Food Borne Pathogens*, edited by L.Tollefson, *Vet. Clin. N. Amer. (Food Anim. Pract.)* 14:1–15.
9. Altekruse, S.F., Swerdlow, D.L., and Stern, N.J. (1998): *Campylobacter jejuni.* In: *Microbial Food-Borne Pathogens*, edited by L.Tollefson, *Vet. Clin. N. Amer. (Food Anim. Pract.)*, 14:31–40.
10. Ames, B.N. (1983): Dietary carcinogens and anticarcinogens: Oxygen radicals and degenerative diseases. *Science*, 221: 1256–1264.
11. Ames, B.N., and Gold, L.S. (1990): Too many rodent carcinogens: Mitogenesis increases mutagenesis. *Perspect. Science*, 249: 970–971.
12. Ames, B.N., Profet, M., and Gold, L.S. (1990): Nature's chemicals and synthetic chemicals: Comparative toxicology. *Proc. Natl, Acad. Sci. (USA)*, 87:7782–7786.
13. Anderson, J.A. (1997): Milk, eggs, and peanuts: Food allergies in children. *Amer. Fam. Phys.*, 56:1365–1374.
14. Andrews, L.S., Ahmedna, M., Grodner, R.M., Liuzzo, J.A., Murano, P.S., Murano, E.A., Rao, R.M., Shane, S., and Wilson, P.M. (1998): Food preservation using ionizing radiation. *Rev. Environ. Contam. Toxicol.*, 154:1–53.
15. Angus, F. (1998): Nut allergens. In: *Natural Toxicants in Food*, edited by D.H.Watson, pp. 84–104. CRC Press, Boca Raton, Florida.
16. Aronow, L., and Kerdel-Vegas, F. (1965): Selino-cystathionine, a pharmacologically active factor in the seeds of Lecythis ollaria: Cytotoxic and depilatory effects. *Nature*, (London) 205: 1185–1186.
17. Bailey, G., Goeger, D., Hendricks, J., Nixon, J., and Pawlowski, N. (1985): Indole-3-carbinol promotion and inhibition of aflatoxin B1 carcinogenesis in rainbow trout. *Proc. Am. Assoc. Cancer Res.*, 26:115: (abst).
18. Balasubramanian, S., and Govindaswamy, S. (1996): Inhibitory effect of dietary flavonol, quercetin, on 7,12-cimethylbenzanthra-cine-induced hamster baccal pouch carcinogenesis. *Carcinogenesis*, 17:877–

879.
19. Baldessarini, R.J. (1985): Drugs and the treatment of psychiatric disorders. In: *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 7th ed., edited by A.G.Gilman, L.S.Goodman, T.W.Rall, and F.Murad, pp. 387–445. Macmillan Publishing Co. Inc., New York.
20. Banea-Muyambu, J.P., Tylleskar, T., Gitebo, N., Mtadi, N., Gebre-Medhim, M., and Rosling, A. (1997): Geographical and seasonal association between linamarin and cyanide exposure from cassava and the upper motor neuron disease Konzo in former Zaire. *Trop. Medi. Int. Health*, 2:1143–1151.
21. Barotto, N.N., Lopez, C.B., Eynard, A.R., Fernandez-Zapico, M.D., and Valentich, M.A. (1998): Quercetin enhances pre-tumorous lesions in the NMU model of rat pancreatic carcinogenesis. *Cancer Lett.*, 129:1–6.
22. Bartholomew, R.M., and Ryan, D.A. (1980): Lack of mutagenicity of some phytoestrogens in the Salmonella/mammalian microsome assay. *Mut. Res.*, 78:317–321.
23. Barzel, U.S., and Massey, L.K. (1998): Excess dietary protein can adversely affect bones. *J. Nutr.*, 128:1051–1053.
24. Beasley, V.R. (1989): *Trichothecene Mycotoxicosis: Pathophysiologic Effects*, Vols. 1–2, CRC Press, Boca Raton, Florida.
25. Beier, R.C. (1990): Natural pesticides and bioactive components in foods. *Rev. Environ. Contam. Toxicol.*, 113:47–137.
26. Bruggink, T. (1997): Food allergy and food intolerance. In: *Food Safety and Toxicity*, edited by J.DeVries, pp. 183–194. CRC Press, Boca Raton, Florida.
27. Bryan, F.L. (1979): Infections and intoxications caused by other bacteria. In: *Foodborne Infections and Intoxications*, 2nd ed.,

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_524.html[2010/10/28 18:45:04]

edited by H.Riemann, and F.L.Bryan, pp. 212–298. Academic Press, New York.
28. Bureau of Foods (1982): *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food.* U.S. Food and Drug Administration, Washington, D.C.
29. Busby, W.F., Jr., and Wogan, G.N. (1979): Foodborne mycotoxins and alimentary mycotoxicoses. In: *Foodborne Infections and Intoxications*, 2nd ed., edited by H.Riemann, and F.L.Bryan, pp. 519–610. Academic Press, New York.
30. Busby, W.F., Jr., and Wogan, G.N. (1981): Aflatoxins. In: *Mycotoxins and Nitroso Compounds: Environmental Risks,* Vol. 2, edited by R.C.Shank, pp. 3–28. CRC Press, Boca Raton, Florida.
31. Busby, W.F., Jr., and Wogan, G.N. (1981): Psoralens. *Mycotoxins and Nitroso Compounds: Environmental Risks,* Vol. 2, edited by R.C.Shank, pp. 105–119. CRC Press, Boca Raton, Florida.
32. Carrol, K.K. (1982): Dietary fat and its relationship to human cancer. In: *Carcinogens and Mutagens in the Environment*, Vol. 1, edited by H.F.Stich, pp. 31–38. CRC Press, Boca Raton, Florida.
33. Chang, F.C.T., Spriggs, D.L., Benton, B.J., Ketter, S.J., and Capucio, B.R. (1997): 4-aminopyridine reverses saxitoxin and tetrodotoxin-induced cardiorespiratory depression in chronically instrumented guinea pigs. *Fundam. Appl. Toxicol.*, 38:75–88.
34. Cheeke, P.R. (1996): Biological effects of feed and forage saponins and their impacts on animal production. *Adv. Exper. Med. Biol.*, 405:377–385.
35. Cheeke, P.R. (1989): Toxicants of plant origin. In: *Phenolics,* Vol.1–4, CRC Press, Boca Raton, Florida.
36. Chen, K.K., and Rose, C.L. (1952): Nitrite and thiosulfate therapy in cyanide poisoning. *J. A. M. A.*, 149:113–119.
37. Chevion, M., Mager, J., and Claser, G. (1983): Favism producing agents. In: *Handbook of Naturally Occurring Food Toxicants,* edited by M.Rechcigl, Jr., pp. 63–79. CRC Press, Boca Raton, Florida.
38. Chow, C.W., Poulos, A., Fellenberg, A.J., Christodoulon, J., and Danks, D.M. (1992): Autopsy findings in two siblings with infantile Refsum's disease. *Acta Neuropath.*, 83:190–195.
39. Chung, F.L., Wang, M., Rivenson, A., Iatropoulous, M.J., Reinhardt, J.C., Pittman, B., Ho, C.T., and Amin, S.G. (1998): Inhibition of lung carcinogenesis by black tea in Fischer rats treated with a tobacco-specific carcinogen: Caffeine as an important constituent. *Cancer Res.*, 58(18):4096–4101.
40. Clarkson, T.B. (1995): Estrogenic soybean isoflavones and chronic disease. *Trends Endocrin. Metab.*, 6:11–16.
41. Cooper, J., and Walker, R.D. (1998): Listeriosis. *Vet. Clin. N. Amer. (Food Anim. Pract.)*, 14(1):113–125.
42 Coulombe, R.A., Jr. (1991): Aflatoxins. In: *Mycotoxins and Phytoalexins,* edited by R.P.Sharma, and D.K.Salunkhe, pp. 103–143. CRC Press, Boca Raton, Florida.
43. Creppy, E.E., Baudrimont, I., and Betbeder, A.M. (1995): Prevention of nephrotoxicity of ochratoxin A, a food contaminant. *Toxicol. Lett.*, 82–83:869–877.
44. Crews, C. (1998): Pyrrolizidine alkaloids. In: *Natural Toxicants in Food*, edited by D.H.Watson, pp. 11–28. CRC Press, Boca Raton, Florida.
45. Daly, J.W. (1993): Mechanism of action of caffeine. In: *Coffee, Caffeine, and Health,* edited by S.Garattini, pp. 97–150. Raven Press. New York.
46. DeClerk, Y.A., and Inven, S. (1994): Protease inhibitors: Role and potential therapeutic use in human cancer. *Eur. J. Cancer*, 30A:2170–2180.
47. Dencker, L., Gustafson, A.L., Annerwall, E., Busch, C., and Eriksson, U. (1991): Retinoid-binding proteins in craniofacial development. *J. Craniofac. Gen. Dev. Biol.*, 11(4):303–314.
48. Derks, H.J.G.M., Groen, C., Olling, M., and Zeilmaker, M.J. (1997): Extrapolation of toxicity data in risk assessment. In: *Food Safety and Toxicity,* edited by J.DeVries, pp. 241–254. CRC Press, Boca Raton, Florida.
49. Deshpande, S.S., Sathe, S.K., and Salunkhe, D.K. (1984): Chemistry and safety of plant polyphenols. In: *Nutritional and Toxicological Aspects of Food Safety*, edited by M.Friedman, pp. 457–495. Plenum Press, New York.
50. Deshpande, S.S., and Sathe, S.K. (1991): Toxicants in plants. In: *Mycotoxins and Phytoalexins,* edited by R.P.Sharma, and D. K.Salunkhe, pp. 671–730. CRC Press, Boca Raton, Florida.
51. Doll, R., and Peto, R. (1981): The causes of cancer: Quantitative estimates of avoidable risks of cancer in the United States today. *J. Nat. Cancer Inst.*, 66:1193–1308.
52. Duffy, J.Y., Baines, D., Overmann, G.J., Keen, C.L., and Daston, G.P. (1997): Repeated administration of alpha-hederin results in alterations in maternal zinc status and adverse developmental outcome in the rat. *Teratol.*, 56(5):327–334.

53. Dugyala, R.R., Sharma, R.P., Tsunoda, M., and Riley, R.T. (1998): Tumor necrosis factor-alpha as a contributor in fumonisin B1 toxicity. *J. Pharm. Exper. Ther.*, 285(1):317–324.
54. Dunnick, J.K., and Hailey, J.R. (1992): Toxicity and carcinogeniality studies of quercetin, a natural component of foods. *Fnd. Appl. Toxicol.*, 19:423–431.
55. Dutton, M.F. (1996): Fumonisins, mycotoxins of increasing importance: Their nature and their effects. *Pharmacol. Ther.*, 70:137–161.
56. Ellenhorn, M.J., and Barceloux, D.G. (1988): *Medical Toxicology: Diagnosis and Treatment of Human Poisoning*, pp. 508–514, 606–613, Elsevier, New York.
57. Ellenhorn, M.J., Schonwald, S., Ordog, G., and Wesserberger, J. (1997): Natural toxins: Plants, mycotoxins, mushrooms. In: *Ellenhorn's Medical Toxicology*, pp. 1880–1896. Williams & Wilkins, Philadelphia.
58. Ekperigen, H.E., and Nagaraja, K.V. (1998): Salmonella. In: *Microbial Food-Borne Pathogens*, edited by L.Tollefson, *Vet. Clin. N. Amer. (Food Anim. Pract.)*, 14:17–29.
59. *Federal Food, Drug, and Cosmetic Act, as Amended.* (1976): U.S. Government Printing Office, Washington, D.C.
60. Fenaux, P., and DeBotton, S. (1998): Retinoic acid syndrome. Recognition, prevention, and management. *Drug Safety*, 18(4):273–279.
61. Formica, J.V., and Regelson, W. (1995): Review of the biology of quercetin and related bioflavonoids. *Food & Chem. Toxicol.*, 33(12):1061–1080.
62. Friedman, M., Gumbmann, M.R., and Masters, P.M. (1984): Protein-alkali reactions: Chemistry, toxicology, and nutritional consequences. In: *Nutritional and Toxicological Aspects of Food Safety*, edited by M.Friedman, pp. 367–412. Plenum Press, New York.
63. Fuhrman, F.A. (1983): Toxic constituents of animal foodstuffs: Eggs of fishes and amphibians. In: *Handbook of Naturally Occurring Food Toxicants*, edited by M.Rechcigl, Jr., pp. 301–311. CRC Press, Boca Raton, Florida.
64. Fujiki, H., Yoshizawa, S., Horiuchi, T., Sugamura, M., Yatsunami, J., Nishiwaki, S., Okabe, S., Nishiwaki-Matsushima, R., Okuda, T., and Sugimura, T. (1992): Anticarcinogenic effects of penta-O-gallyl-beta-D-glucose and epigallocatedmin gallate. *Prev. Med.*, 21:503–509.
65. Fukuda, H., Shima, H., Vesonder, R.F., Tokuda, H., Nishino, H., Katoh, S., Tamura, S., Sugimura, T., and Nagao, M. (1996): Inhibition of protein serine/threonine phosphatases by fumonisin

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_525.html[2010/10/28 18:45:05]

B1, a mycotoxin. *Biochem. Biophys. Res. Commun.*, 220(1):160–165.
66. Gallaher, D., and Schneeman, B.O. (1984): Nutritional and meolic response to plant inhibitors of digestive enzymes. *Adv. Exp. Med. Biol.*, 177:299–320.
67. Gentry, P.A. (1986): Comparative biochemical changes associated with mycotoxicosis other than aflatoxicosis and trichothecene toxicosis. In: *Diagnosis of Mycotoxicoses*, edited by J.R.Richard, and J.R.Thurston, pp. 125–139. Martinus Nijhoff Publishers, Dordrecht, Netherlands.
68. Ghedira-Chekir, L., Maaroufi, K., Zakhama, A., Ellouz, F., Dhouib, S., Creppy, E.E., and Bacha, H. (1998): Induction of a SOS repair system in lysogenic bacteria by zearalenone and its prevention by vitamin E. *Chemico-biol. Interact.*, 113(1):15–25.
69. Gilchrist, A. (1981): *Foodborne Disease and Food Safety*. American Medical Association, Monroe, Wisconsin.
70. Godon, K.A.H., and Houstead, J. (1998): Transmissible spongiform encephalopathies in food animals, *Vet. Clin. N. Amer. (Food Anim. Prac.)*, 14(1):49–70.
71. Halstead, B.W. (1978): *Poisonous and Venomous Marine Animals of the World.*. Darwin Press, Inc., Princeton, New Jersey.
72. Haque, A., Hossain, M., Lambein, F., and Bell, E.A. (1997): Evidence of osteolathyrism among patients suffering from neurolathyrism in Bangladesh. *Nat. Tox.*, 5(1):43–46.
73. Harper, A.E. (1973): Amino acids of nutritional importances. In: *Toxicants Occurring Naturally in Foods*, 2nd ed., edited by the Committee on Food Protection, NRC, pp. 130–152. National Academy of Sciences Press, Washington, D.C.
74. Hayatsu, S., Arimoto, K., Togawa, K., and Mokita, M. (1981): Inhibitory effects of the ether extract of human feces on activities of mutagens: Inhibition of oleic and linoleic acids. *Mutat. Res.*, 81:287–293.
75. Hayes, J.R., and Campbell, T.C. (1986): Food additives and contaminants. In: *Casarett and Doull's Toxicology, the Basic Science of Poisons*, 3rd ed., edited by C.D.Klaassen, M.O.Amdur, and J.Doull, pp. 771–800. Macmillan Publishing Co., New York.
76. Heikkinen, A.M., Tuppurainen, M.T., Niskanen, L., Komulainen, M., Penttila, I., and Saarikoski, S. (1997): Long-term vitamin D3 supplementation may have adverse effects on serum lipids during postmenopausal hormone replacement therapy. *Eur. J. Endocrin.*, 137(5):495–502.
77. Hirono, I. (1987): *Naturally occurring carcinogens of plant origin: Toxicology, Pathology, and Biochemistry*, pp. 1–227. Kodansha/Elsevier, New York.
78. Hogue, A.T., White, P.L., and Heninover, J.A. (1998): Pathogen reduction and hazard analysis and critical control point (NACCP) systems for meat and poultry. *Vet. Clin. N. Amer. (Food Anim. Pract.)*, 14(1):151–164.
79. Houtsmuller, U.M.T., Struijk, C.B., and Van Der Beek, A. (1970): Decrease in rate of ATP synthesis of isolated rat heart mitochondria induced by dietary erucic acid. *Biochime. Biophys. Acta*, 218:564–566.
80. Howard, B.V., and Kritchevsky, D. (1996): Phytochemicals and cardiovascular disease: A statement for health care professionals from the American Heart Association. *Circulation*, 95(11):2591–2593.
81. Hsieh, D.P.H. (1979): Basic meolic effects of mycotoxins. In: *Interactions of Mycotoxins in Animal Production*, pp. 43–55. National Academy of Science, Washington, D.C.
82. Hsieh, D.P.H. (1986): Genotoxicity of mycotoxins. In: *New Concepts and Developments in Toxicology*, edited by P.L.Chambers, P.Gebring, and F.Sakai, pp. 251–259. Elsevier Science Publishers, New York.
83. Huxtable, R.J. (1989): Human health implications of pyrrolizidine alkaloids and herbs containing them. In: *Toxicants of Plant Origin*, Vol. I, Alkaloids, edited by P.R.Cheeke, pp. 41–86. CRC Press, Boca Raton, Florida.
84. Ikeguonu, F.I., and Bassir, O. (1977): Effects of phytohemagglutinins from immature legume seeds on the function and enzyme activities of the liver and on the organs of the rat. *Toxicol. Appl. Pharmacol.*, 40:217–226.
85. Jacobsen, B.K., and Bjelke, E. (1982): Coffee consumption and cancer: A prospective study. In: Proceedings of the 13th International Cancer Congress, Seattle, Washington (abstr).
86. Janssen, M.M.T. (1997): Antinutritives; Food contaminants; Food additives; Nutrients. In: *Food Safety and Toxicity*, edited by J.De Vries, pp. 39–52; 53–62; 63–74; 75–98. CRC Press, Boca Raton, Florida.
87. Janssen, M.M.T., Put, H.M.T., and Nout, M.J.R. (1997): Natural toxins. In: *Food Safety and Toxicity*, edited by J.De Vries, pp. 7–38. CRC Press, Boca Raton, Florida.
88. Kalejta, R.F., and Hamlin, J.L. (1997): The dual effect of mimosine on DNA replication. *Exper. Cell Res.*, 231(1):173–183.

89. Kang, Y.J., and Alexander, J.M. (1996): Alterations of the glutathione redox cycle status in fumonisin B1-treated pig kidney cells. *J. Biochem. Toxicol.*, 11(3):121–126.
90. Kao, C.Y. (1967): Comparison of the biological actions of tetrodotoxin and saxitoxin. In: *Animal Toxins*, edited by F.E. Russell, and P.R.Saunders, pp. 109–114. Pergamon Press, Oxford.
91. Kao, C.Y. (1966): Tetrodotoxin, saxitoxin, and their significance in the study of excitation phenomena. *Pharmacol. Rev.*, 18:997–1049.
92. Kanisawa, M., and Suzuki, S. (1978): Induction of renal and hepatic tumors in mice by ochratoxin A, a mycotoxin. *Gann*, 69:599–600.
93. Kassell, B. (1970): Inhibitors of proteolytic enzymes. *Methods Enzymol.*, 19:839–906.
94. Keeler, R.F., Baker, D.C., and Gaffield, W. (1991): Solanum alkaloids. In: *Mycotoxins and Phytoalexins*, edited by R.P. Sharma, and D.K.Salunkhe, pp. 607–636. CRC Press, Boca Raton, Florida.
95. Kensler, T.W., Groopman, J.D., and Roebuck, B.D. (1998): Use of aflatoxin adducts as intermediate endpoints to assess the efficacy of chemopreventive interventions in animals and man. *Mut. Res.*, 402(1–2): 165–172.
96. King, T.P., Pusztai, A., and Clarke, E.M.W. (1980): Kidney bean lectin-induced lesions in rat small intestine, I. Light microscopic studies. *J. Comp. Pathol.*, 90:585–593.
97. Kitts, D., Yuan, Y., Joneja, J., Scott, F., Szilagyi, A., Amiot J., and Zarkadas, M. (1997): Adverse reactions to food constituents: Allergy, intolerance, and autoimmunity. *Can. J. Physiol. Pharm.*, 75(4):241–254.
98. Knasmuller, S., Bresgen, N., Kassie, F., Mersch-Sundermann, V., Gelderblom, W., Zohrer, E., and Eckl, P.M. (1997): Genotoxic effects of three fusarium mycotoxins, fumonisin B1, moniliformin and vomitoxin in bacteria and in primary cultures of rat hepatocytes. *Mut. Res.*, 391(1–2):39–48.
99. Kobayashi, M., Suzuki, K., Nagasawa, S., and Mimaki, Y. (1993): Purification of toxic saponins from narthecium asiaticum maxim. *J. Vet. Med. Sci.*, 55(3):401–407.
100. Kodell, R.L., Krewski, D., and Zielinski, J.M. (1991): Additive and multiplicative risks in the two-stage clonal expansion model of carcinogenesis. *Risk Anal.*, 11:483–490.
101. Korn, A., Wagner, B., Moritz E., and Dingemanse, J. (1996): Tyramine pressor sensitivity in healthy subjects during combined treatment with moclobemide and selegiline. *Eur. J. Clin. Pharm.*, 49(4):273–278.
102. Kowalska, M.T., Itzhak, Y., and Puett, D. (1995): Presence of aromatase inhibitors in cycads. *J. Ethnopharm.*, 47(3): 113–116.

103. Krogh, P., Hald, B., Plestina, R., and Ceovic, S. (1977): Balkan nephropathy and food-borne ochratoxin A: Preliminary results of a survey of foodstuffs. *Acta Pathol. Microbiol. Scand. Sect. B,* 85:238–240.
104. Kunkel, D.B., and Jallo, D.S. (1990): Ergot. In: *Clinical Management of Poisoning and Drug Overdose,* 2nd ed., edited by L. M.Haddad, and J.F.Winchester, pp. 1401–1406. W.B.Saunders Co., Philadelphia.
105. Kuramochi, G., Gekle, M., and Silbernagle, S. (1997): Derangement of pH homeostasis in the renal papilla: Ochratoxin A increases pH in vasa recta blood. *Nephron.,* 76(4):472–476.
106. Lamawansa, M.D., Wysocki, S.J., House, A.K., and Norman, P. E. (1996): Vitamin D3 exacerbates intimal hyperplasia in balloon-injured arteries. *Br. J. Surg.,* 83(8):1101–1103.
107. Lambert, L.A., Hines, F.A., and Eppleyl, R.M. (1995): Lack of initiation and promotion potential of deoxynivalenol for skin. *Food & Chem. Toxicol.,* 33(3):217–222.
108. Lampe, K.F. (1983): Mushroom poisoning. In: *Handbook of Nat-urally Occurring Food Toxicants,* edited by M.Rechcigl, Jr., pp. 193–212. CRC Press, Boca Raton, Florida.
109. Lasky, T., and Magder, L. (1997): Hepatocellular carcinoma p53 G >T transversions at codon 249: The fingerprint of aflatoxin exposure? *Environ. Health Perspect.,* 105(4):392–397.
110. Layton, D.W., Bogen, K.T., Knize, M.G., Hatch, F.T., Johnson, V.M., and Felton, J.S. (1995): Cancer risk of heterocyclic amines in cooked foods: An analysis and implications for research. *Carcinogenesis,* 16:39–52.
111. Leftley, J.W., and Hannah, F. (1998): Phycotoxins in seafood. In: *Natural Toxicants in Food*, edited by D.H.Watson, pp. 182–224. CRC Press, Boca Raton, Florida.
112. Lewis, W.H., and Elvin-Lewis, M.P.F. (1977): *Medical Botany: Plants Affecting Human Health*, p. 57. John Wiley & Sons, New York.
113. Liener, I.E. (1980): Miscellaneous toxic factors. In: *Toxic Constituents of Plant Foodstuffs*, 2nd ed., edited by I.E.Liener, pp. 429–467. Academic Press, New York.
114. Liener, I.E., and Kakade, M.L. (1980): Protease inhibitors. In: *Toxic Constituents of Plant Foodstuffs*, 2nd ed., edited by I.E. Liener, pp. 7–71. Academic Press, New York.
115. Liener, I.E. (1995): Possible adverse effects of soybean anti-carcinogens. *J. Nut.* 125(3 Suppl):744–750.
116. Lin, J.K., Chen, Y.C., Huang, Y.T., and Lin-Shiau, S.Y. (1997): Suppression of protein kinase C and nuclear oncogene expression as possible molecular mechanisms of cancer chemoprevention by apigenin and curcumin. *J. Cell. Biochem.,* Suppl 28–29:39–48.
117. Lin, J.L., and Ho, Y.S. (1994): Flavonoid-induced acute nephropathy. *Am. J. Kidney Dis.,* 3(3):433–440.
118. Lipp, E.K., and Rose, J.B. (1997): The role of seafood in food borne diseases in the United States of America. *Revue Scientifique et Technique*, 16:620–640.
119. Liu, J., Liu, Y., Bullock, P., and Klaassen, C.D. (1995): Suppression of liver cytochrome P450 by alpha-hederin: Relevance to hepatoprotection. *Toxicol. Appl Pharm.,* 134(1):124–131.
120. Lloyd, W.E., Daniels, G.N., and Stahr, H.M. (1985): Cases of nephrotoxic mycotoxicoses in cattle and swine in the United States. In: *Trichothecenes and Other Mycotoxins*, edited by J.Lacey, pp. 545–548. John Wiley & Sons, New York.
121. Lovenberg, W. (1973): Some vaso- and psychoactive substances in food. In: *Toxicants Occurring Naturally in Foods,* 2nd ed., edited by the Committee on Food Protection, NRC, pp. 170–188. National Academy of Sciences Press, Washington, D.C.
122. MacGregor, J.T. (1984): Genetic and carcinogenic effects of plant flavonoids: An overview. In: *Nutritional and Toxicological Aspects of Food Safety*, edited by M.Friedman, pp. 497–526. Plenum Press, New York.
123. Mager, J., Chevion, M., and Claser, G. (1980): Favism. In: Toxic Constituents of Plant Foodstuffs, 2nd ed., edited by I.E.Liener, pp. 266–294. Academic Press, New York.
124. Malinow, M.R., Bardana, E.J. Jr., Pirofsky, B., Craig, S., and McCluagblin, P. (1982): Systemic lupus erythematosus-like syndrome in monkeys fed alfalfa sprouts: Role of a non-protein amino acid. *Science,* 216:415–417.
125. Marasas, W.F. (1995): Fumonisins and their implications for human and animal health. *Nat. Toxins,* 3:193–198.
126. Marks, J. (1989): The safety of the vitamins: An overview. *Int. J. Vit. Nut. Res. Suppl*, 30:12–20.
127. Marth, E.H. (1981): Food-borne hazards of microbial origin. In: *Food Safety*, edited by H.R.Roberts, pp. 15–65. John Wiley & Sons, New York.

128. Matsumoto, H. (1983): Cycasin. In: *Handbook of Naturally Occurring Food Toxicants*, edited by M.Rechcigl, Jr., pp. 43–61. CRC Press, Boca Raton, Florida.
129. Matsumoto, T., Itoh, H., and Akiba, Y. (1968): Goitrogenic effects of 5-vinyl-2-oxazolidinethione, a goitrogen in rapeseed, in growing chicks. *Poultry Sci.*, 47:1323–1330.
130. Mattocks, A.R. (1986): *Chemistry and Toxicology of Pyrrolizidine Alkaloids.* Academic Press, New York.
131. Mattson, F.H. (1973): Potential toxicity of food lipids. In: *Toxicants Occurring Naturally in Foods*, 2nd ed., edited by the Committee on Food Protection, NRC, pp. 189–209. National Academy of Sciences Press, Washington, D.C.
132. McPartland, J.M., Vigaly, R.J., and Cubeta, M.A. (1997): Mushroom poisoning. *Am. Fam. Phys.*, 55:1797–1811.
133. Meisner, H., and Cimbala, M. (1985): Effect of ochratoxin A on gene expression in rat kidneys. In: *New Concepts and Developments in Toxicology*, edited by P.L.Chambers, P.Gehring, and F.Sakai, pp. 261–271. Elsevier Science Publishers, New York.
134. Merril, R.A. (1997): Food safety regulation: Reforming the Delaney clause. *Ann. Rev. Pub. Health*, 18:313–340.
135. Miller, J.A., Miller, E.C., and Phillips, D.H. (1982): The metabolic activation and carcinogenicity of alkenylbenzenes that occur naturally in many spices. In: *Carcinogens and Mutagens in the Environment*, Vol. 1, edited by H.F.Stich, pp. 93–96. CRC Press, Boca Raton, Florida.
136. Miller, I., Gray, D., and Kay, H. (1998): Bacterial toxins found in foods. In: *Natural Toxicants in Food*, edited by D.H.Watson, pp. 105–146. CRC Press, Boca Raton, Florida.
137. Mitchel, R.E., and McCann, R. (1993): Vitamin E is a complete tumor promoter in mouse skin. *Carcinogenesis*, 14(4):659–662.
138. Miura, K., Nakajima, Y., Yamanaka, N., Terao, K., Shibato, T., and Ishino, S. (1998): Induction of apoptosis with fusarenon-X in mouse thymocytes. *Toxicol.*, 127(1–3):195–206.
139. Mody, R., Joshi, S., and Chaney, W. (1995): Use of lectins as diagnostic and therapeutic tools for cancer. *J. Pharmacol. Toxicol. Meth.*, 33:1–10.
140. Montgomery, R.D. (1980): Cyanogens. In: *Toxic Constituents of Plant Foodstuffs*, 2nd ed., edited by I.E.Liener, pp. 143–160. Academic Press, New York.
141. Morris, S.C., and Lee, T.H. (1984): The toxicity and teratogenicity of Solanaceae glycoalkaloids, particularly those of the potato *(Solanum tuberosum):* A review. *Food Technol. Aust.*, 36:118–124.
142. Nakanishi, K., Hagiwara, A., Shibata, M., Imaida, K., Tetematsu, M., and Ito, N. (1980): Dose response of saccharin induction of urinary bladder hyperplasia in Fisher 344 rats pretreated with N-butyl N-(4-hydroxybutyl) nitrosamine. *J. Natl. Cancer Inst.*, 65:1005–1010.
143. Nakhla, H.B., Mohamed O.S., Abu, I.M., Fatuh, A.L., and Adam, S.E. (1991): The effect of *Trigonella foenum graecum*

(fenugreek) crude saponins on Hisex-type chicks. *Vet. Hum. Toxicol.*, 33(6):561–564.
144. National Academy of Sciences. (1977): *Leucaena, Promising Forage, and Tree Crop for the Tropics.* NAS Press, Washington, D.C.
145. National Research Council (NRC). (1988): Complex mixtures: Methods for in vivo toxicity testing. National Academy Press, Washington, D.C.
146. NRC. (1996): *Carcinogens and anticarcinogens in the human diet*, pp. 1–417. National Academy Press, Washington, D.C.
147. Ohga, S., Higashi, E., Nomura, A., Matsuzaki, A., Hirono, A., Miwa, S., Fujii, H., and Ueda, K. (1995): Haptoglobin therapy for acute favism: A Japanese boy with glucose-6-phosphate dehydrogenase Guadalajara. *Br. J. Haematol.*, 89(2):421–423.
148. Olny, J.W. (1982): The toxic effects of glutamate and related compounds in the retina and the brain. *Retina*, 2:341–359.
149. Omaye, S.T. (1984): Safety of megavitamin therapy. In: *Nutritional and Toxicological Aspects of Food Safety*, edited by M.Friedman, pp. 169–203. Plenum Press, New York.
150. Osuntokun, B.O. (1973): Ataxic neuropathy associated with high cassava diets in West Africa. In: *Chronic Cassava Toxicity*, edited by B.Nestel, and R.MacIntyre, pp. 127–138. International Development Research Center, Ottawa.
151. Osweiler, G.D., Carson, T.L., Buck, W.B., and Van Gelder, G.A. (1985): *Clinical and Diagnostic Veterinary Toxicology*, Kendall-Hunt Publishing Co., Dubuque, Iowa.
152. Padmanaban, G. (1980): Lathyrogens. In: *Toxic Constituents of Plant Foodstuffs*, 2nd ed., edited by I.E.Liener, pp. 239–263. Academic Press, New York.
153. Panter, K.E., and James, L.F. (1990): Natural plant toxicants in milk: A review. *J. Anim. Sci.*, 68:892–904.
154. Pass, M.A., Arab, H., Pollitt, S., and Hegarty, M.P. (1996): Effects of the naturally occurring arginine analogues indospicine and canavanine on nitric oxide mediated functions in aortic endothelium and peritoneal macrophages. *Nat. Toxins*, 4(3):135–140.
155. Pearl, E.R. (1997): Food allergy. *Lippincott's Primary Care Practice* 1:154–167.
156. Perlman, F. (1980): Allergens. In: *Toxic Constituents of Plant Foodstuffs*, 2nd ed., edited by I.E.Liener, pp. 295–327. Academic Press, New York.
157. Pfohl-Leszkowicz, A., Chekir-Ghedira, L., and Bacha H. (1995): Genotoxicity of zearalenone, and estrogenic mycotoxin: DNA adduct formation in female mouse tissues. *Carcinogenesis*, 16(10):2315–2320.
158. Poulton, J.E. (1983): Cyanogenic compounds in higher plants and their toxic effects. In: *Handbook of Natural Toxins, Vol 1, Plant and Fungal Toxins*, edited by R.F.Keeler, and A.T.Tu, pp. 117–160. Marcel Dekker, Inc., New York.
159. Puchala, R., Pierzynowski, S.G., Sahlu, T., and Hart, S.P. (1996): Effects of mimosine administered to a perfused area of skin in Angora goats. *Br. J. Nutr.*, 75(1):69–79.
160. Randel, R.D., Chase, C.C., and Wyse, S.J. (1992): Effect of gossypol and cottonseed products on reproduction in mammals. *J. Anim. Sci.*, 70:1628–1638.
161. Rao, A.V., and Sung, M.K. (1995): Saponins as anticarcinogens. *J. Nutr.*, 125(3 suppl):717S–724S.
162. Reddy, C.S., Hanumaiah, B., Hayes, T.G., and Ehrlich, K. (1986): Developmental stage specificity and dose response of secalonic acid D-induced cleft palate and the absence of cytotoxicity in the developing mouse palate. *Toxicol. Appl. Pharmaco.*, 84:346–354.
163. Reddy, R.V., Johnson, G., Rottinghaus, G.E., Casteel, S.W., and Reddy, C.S. (1996): Developmental effects of fumonisin B in mice. *Mycopathologia*, 134:161–166.
164. Reed, J.D. (1995). Nutritional toxicology of tannins and related polyphenols in forage legumes. *J. Anim. Sci.*, 43:1516–1528.
165. Riemann, H.P., and Oliver, D.O. (1998): Escherichia coli O157: H7. *Vet. Clin. N. Amer. (Food Anim. Pract.)*, 14(1):41–48.
166. Rivera, V.R., Pol, M.A., and Bignami, G.S. (1995): Prophylaxis and treatment with a monoclonal antibody of tetrodotoxin poisoning in mice. *Toxicon*, 33:1231–1237.
167. Roebuck, B.D., Yeager, J.D. Jr., Longnecker, D.S., and Wilpone, S.A. (1981): Promotion by unsaturated fat of azaserine-induced pancreatic carcinogenesis in the rat. *Cancer Res.*, 41:3961–3966.
168. Rosen, J.D. (1990): Much ado about alar. *Iss. Sci. Technol.*, VIII:85–90.
169. Rotter, B.A., and Oh, Y.N. (1996): Mycotoxin fumonisin B1 stimulates nitric oxide production in a murine macrophage cell line. *Nat. Toxins*, 4(6):291–294.
170. Rotter, B.A., Prelusky, D.B., and Pestka, J.J. (1996): Toxicology of deosynivalenol (vomitoxin). *J.*

Toxicol. Environ. Health, 48(1):1–34.
171. Russell, F.E. (1986): Toxic effects of animal toxins. In: *Casarett and Doull's Toxicology, the Basic Science of Poisons*, 3rd ed., edited by C.D.Klaassen, M.O.Amdur, and J.Doull, pp. 706–756. Macmillan Publishing Co., New York.
172. Russell, F.E., and Dart, R.C. (1991): Toxic effects of animal toxins. In: *Casarett and Doull's Toxicology, the Basic Science of Poisons*, 4th ed., edited by M.O.Amdur, J.Doull, and C. D.Klaassen, pp. 753–803. Pergamon Press, New York.
173. Safe, S.H. (1995): Environmental and dietary estrogens and human health: Is there a problem? *Environ. Health Perspect.*, 103:346–351.
174. Sampson, H.A. (1997): Food allergy. *JAMA*, 278:1888–1894.
175. Sandani, G.R., Soman, C.S., Deodhar, K.K., and Nadharni, G. D. (1997): Reactive oxygen species involvement in ricin-induced thyroid toxicity in the rat. *Hum. Exper. Toxicol.*, 16:254–256.
176. Sandvig, K., and Van Deurs, B. (1997): Endocytosis, intracellular transport and cytotoxic action of shiga toxin and ricin. *Physiol. Rev.*, 76:949–966.
177. Schardein, J.L. (1985): *Chemically Induced Birth Defects*, pp. 709–716. Marcel Dekker Inc., New York.
178. Schramek, H., Wilflingseder D., Pollack, V., Freudinger, R., Mildenberger, S., and Gekle, M. (1997): Ochratoxin A-induced stimulation of extracellular signal-regulated kinases 1/2 is associated with Madin-Darby canine kidney-C7 cell dedifferentiation. *J. Pharm. Exper. Ther.*, 283(3): 1460–1468.
179. Scott, B.R., Pathak, M.A., and Mohn, G.R. (1976): Molecular and genetic basis of furocoumarin reactions. *Mutat. Res.*, 39:29–74.
180. Scudamore, K.A. (1998): Mycotoxins. In: *Natural Toxins in Foods*, edited by D.H.Watson, pp. 147–181. CRC Press, Boca Raton, Florida.
181. Selman, I.E., Wiseman, A., Breeze, R.G., and Pirie, H.M. (1976): Fog fever in cattle: Various theories on its etiology. *Vet. Rec.*, 99:181–184.
182. Shank, R.C. (1981): Environmental toxicoses in humans. In: *Mycotoxins and Nitroso Compounds: Environmental Risks*, Vol. 1, edited by R.C.Shank, pp. 107–140. CRC Press, Boca Raton, Florida.
183. Sharma, R.P., and Salunkhe, D.K. (1989): Solanum glycoalkaloids. In: *Toxicants of Plant Origin*. Vol. I, Alkaloids, edited by P.R.Cheeke, pp. 179–236. CRC Press, Boca Raton, Florida.
184. Shephard, S.E. (1993): Mutagenic activity of peptides and artificial sweetener aspartame after nitrosation. *Food Chem. Toxicol.*, 31:323–329.
185. Shoda, R., Matsueda, K., Yamamoto, S., and Umeda, N. (1996): Epidemiologic analysis of Crohn's disease in Japan: Increased diet

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ary intake of n-6-polyunsaturated fatty acids and animal protein relates to increased incidence of Crohn's disease. *Am. J. Clin. Nutr.*, 63:741–745.
186. Singleton, V.L., and Kratzer, F.H. (1973): Plant phenolics. In: *Toxicants Occurring Naturally in Foods*, 2nd ed., edited by the Committee on Food Protection, NRC, pp. 309–345. National Academy of Sciences Press, Washington, D.C.
187. Smith, L.D. (1977): *Botulism: The Organism, Its Toxins, the Disease*. Charles C.Thomas Publishers, Springfield, Illinois.
188. Smith, T.K., Lund, E.K., and Johnson, I.K. (1998): Inhibition of dimethyl-hydrazine induced aberrant crypt foci and induction of apoptosis in rat colon following oral administration of the glucosinolate, sinigrain. *Carcinogens*, 19:267–273.
189. Snyder, R., and Andrews, L.S. (1996): The effects of solvents and vapors. In: *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 5th ed., edited by C.D.Klaassen, pp. 737–771. McGraw-Hill Co., New York.
190. Spencer, P.D., Nunn, P.B., Hugon, J., Ludolph, A.C., Ross, S. M., Roy, D.N., and Robertson, R.C. (1987): Guam amyotrophic lateral sclerosis-parkinsonism-dementia linked to a plant-excitant neurotoxin. *Science*, 237:517–522.
191. Stob, M. (1983): Estrogens. In: *Handbook of Naturally Occurring Food Toxicants*, edited by M.Rechcigl, Jr., pp. 81–100. CRC Press, Boca Raton, Florida.
192. Stocks, P. (1970): Cancer mortality in relation to national consumption of cigarettes, solid fuel, tea, and coffee. *Br. J. Cancer*, 24:215–225.
193. Stricker-Krongrad, A., Burlet, C., and Beck, B. (1998): Behavioral deficits in monosodium glutamate rats: Specific changes in structure and behavior. *Life Sci.*, 62:2127–2132.
194. Sugimoto, N., Horiguchi, Y., and Matsuda, M. (1996): Mechanism of action of *Clostridium perfringens* enterotoxin. In: *Natural Toxins II*, edited by B.R.Singh, and A.Tu, pp. 257–269. Plenum Press, New York.
195. Sugimura, T. (1986): Past, present, and future of mutagens in cooked foods. *Environ. Health Perspect.*, 67:5–10.
196. Suzuki, Y.J., Forman, H.J., and Sevanian, A. (1997): Oxidants as stimulators of signal transduction. *Free Radical Biol. Med.*, 22:269–285.
197. Talaley, P., and Zhang, Y. (1996): Chemoprotection against cancer by isothiocyanates and glucosinolates. *Biochem. Soc. Transact.*, 24:806–810.
198. Tarasoff, L., and Kelly, M.F. (1993): Monosodium L-glutamate: A double-blind study and review. *Food Chem. Toxicol.*, 31(2):1019–1035.
199. Teng, C.S. (1995): Gossypol-induced apoptotic DNA fragmentation correlates with inhibited protein kinase C activity in speratocytes. *Contraception*, 52:389–395.
200. Tookey, H.L., Van Etten, C.H., and Daxenbichler, M.E. (1980): Glucosinolates. In: *Toxic Constituents of Plant Foodstuffs*, 2nd ed., edited by I.E.Liener, pp. 103–142. Academic Press, New York.
201. Tsuda, S., Kosaka, Y., Murakami, M., Matsuo, H., Matsusaka, N., Taniguchi, K., and Sasaki, Y.F. (1998): Detection of nivalenol genotoxicity in cultured cells and multiple mouse organs by the alkaline single-cell gel electrophoresis assay. *Mut. Res.*, 415(3):191–200.
202. Unkrig, C.J., Schroeder, R., Scharf, R.E., and Aubourg, P. (1994): Lorenzo's oil and lymphocytopemia (letter) *New Engl. J. Med.*, 330:577.
203. Van Etten, C.H., and Tookey, H.L. (1983): Glucosinolates. In: *Handbook of Naturally Occurring Food Toxicants*, edited by M. Rechcigl, Jr., pp. 15–30. CRC Press, Boca Raton, Florida.
204. Van Genderen, H. (1997): Adverse effects of naturally occurring non-nutritive substances. In: *Food Safety and Toxicity*, edited by J.DeVries, pp. 147–162. CRC Press, Boca Raton, Florida.
205. Van Hoff, F., Hue, L., Vamecq, J., and Sherratt, H.S. (1985): Protection of rats by clofibrate against the hypoglycaemic and toxic efects of hypoglycin and pent-4-enoate. An ultrastructural and biochemical study. *Biochem. J.*, 229(2):387–397.
206. Verdeal, K., Brown, R.R., Richardson, T., and Ryan, D.S. (1980): Affinity of phytoestrogens for the estradiol-binding proteins and effect of coumestrol on growth of 7,12-dimethylbenz (A) anthracene-induced rat mammary tumors. *J. Natl. Cancer Inst.*, 64:285–290.
207. Verhagen, H. (1997): Adverse effects of food additives. In: *Food Safety and Toxicity*, edited by J.DeVries, pp. 121–132. CRC Press, Boca Raton, Florida.
208. Verkerk, R., Dekker, M., and Jongen, W.M.F. (1998): Glucosinolates. In: *Natural Toxicants in Foods*, edited by D.H. Watson, pp. 29–53. CRC Press, Boca Raton, Florida.
209. Vetter, J. (1998): Toxins of *Amanita phalloides*. *Toxicon*, 36:13–24.

210. Voss, K.A., Chamberlain, W.J., Bacon, C.W., Riley, R.T., and Norred, W.P. (1995): Subchronic toxicity of fumonisin B1 to male and female rats. *Food Addit. Contam.*, 12(3):473–478.
211. Walker, G.R., and Yamazaki, K. (1996): Saponins in food and agriculture. *Adv. Exp. Med. Biol.*, 404: pp. 1–422.
212. Walker, G.R., and Yamazaki, K. (1996): Saponins in traditional and modern medicine. *Adv. Exp. Med. Biol.*, 405: pp. 1–576.
213. Whitley, B.D., Holmes, A.R., Shepherd, M.G., and Ferguson, M. M. (1991): Peanut sensitivity as a cause of burning mouth. *Oral. Surg., Oral Med., Oral Path.*, 72:671–674.
214. Wieland, T., and Faulstich, H. (1983): Peptide toxins from *Amanita*. In: *Handbook of Natural Toxins*, Vol. 1., Plant and Fungal Toxins, edited by R.F.Keeler, and A.T.Tu, pp. 117–160. Marcel Dekker, Inc., New York.
215. Williamson, G., Faulkner, K., and Plumb, G.W. (1998): Glucosinolates and phenolics and antioxidants from plant foods. *Eur. J. Cancer Prev.*, 7(1): 17–21.
216. Wilmarth, K.R., and Froines, J.R. (1992): In vitro and in vivo inhibition of lysyl oxidase by aminopropionitriles. *J. Toxicol. Environ. Health*, 37:411–423.
217. Wolever, T.M., Hamad, S., Gittelsohn, J., Gao, J., Hanley, A.J., Harris, S.B., and Zinman, B. (1997): Low dietary fiber and high protein intake associated with newly diagnosed diabetes in a remote aboriginal community. *Am. J. Clin. Nutr.*, 66: 1470–1474.
218. Xu, R., Zhao, W., Xu, J., Shao, B., and Qin, G. (1996): Studies on bioactive saponins from Chinese medicinal plants. *Adv. Exper. Med. Biol.*, 404:371–382.
219. Yamanaka, N., Oda, O., and Nagao, S. (1997): Prooxidant activity of caffeic acid, dietary non-flabonoid phenotic acid, on Cu24-induced low density lipoprotein oxidation, *FEBS Lett.*, 405:186–190.
220. Yang, W.H., Drouin, M.A., Herbert, M., Mao, Y., and Karsh, J. (1997): The monosodium glutamate symptom complex: Assessment in double-blind placebo-controlled, randomized study. *J. Aller. Clin. Immun.*, 99:757–762.
221. Esterbauer, H. (1993): Cytotoxicity and genotoxicity of lipid peroxidation products. *Amer. J. Clin. Nutr.*, 57:779–786.
222. Yeung, J.M., Wang, H.Y., and Prelusky, D.B. (1996): Fumonisin B1 induces protein kinase C translocation via direct interaction with diacylglycerol binding site. *Toxicol. Appl. Pharmacol.*, 141(1): 178–184.
223. Zelski, R.Z., Rothwell, J.T., Moore, R.E., and Kennedy, D.J. (1995): Gossypol toxicity in preruminant calves. *Aust. Vet. J.*, 72:394–398.

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FURTHER READINGS

ICMSF (International Commission on Microbiological Specifications for Foods). (1988): *Microorganisms in Foods: 4. Application of the Hazard Analysis Critical Control Point (HACCP) System to Ensure Microbiological Safety and Quality*. Blackwell Scientific Publications, Oxford.

National Research Council (NRC) (1988): Complex mixtures: methods for in vivo toxicity testing. National Academy Press, Washington, D.C.

NRC (1996): *Carcinogens and Anticarcinogens in the Human Diet*. National Academy Press, Washington, D.C.

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Chapter **12**

Solvents and industrial Hygiene

Robert C.Spiker, Jr. and Gary B.Morris

Principles and Methods of Toxicology, Fourth Edition, edited by A.Wallace Hayes.
Taylor & Francis, Philadelphia © 2001.

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Solvent use by industry and the general public is widespread, encompassing numerous products and applications. As Table 12.1 illustrates, billions of pounds of organic solvents are produced annually (11, 12). Solvents may be utilized individually (e.g., acetone or toluene) or as mixtures containing several ingredients (e.g., paint thinners, cleaning agents). Alternative, nontraditional solvents, as described later, are also finding widespread applicability.

To better understand the hazards that these materials may present, we must first learn something

about their basic physical, chemical, and toxicological properties. Table 12.2 contains a list of physical properties of commonly encountered organic solvents (4, 101). This is followed by a brief explanation of the properties. For ease of comparison, inhalation, dermal, and oral toxicological data for solvents are often discussed in relation to the chemical groups to which they belong (aromatics, alcohols, halogenated hydrocarbons, etc.). Much of the text is devoted to a review of the basic toxicology of some widely used solvents as well as several alternative compounds.

Modern industrial hygiene practices have done much to characterize and minimize workplace solvent exposures. A number of these practices, such as workplace monitoring, local exhaust ventilation, and personal protective equipment are discussed in this chapter. Because solvent use in small businesses and the home is often not as well controlled as in major industrial settings, large populations may be at risk. A challenge to the health and

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Table 12.1 Ten-year trends in production volumes for some common solvents[a]

Name	*Production (millions of pounds)*									
	1997	1996	1995	1994	1993	1992	1991	1990	1989	1988
Acetone	NA[b]	NA	2761	2664	2430	2435	2347	2329	2524	2303
Aniline	1339	1079	1391	1263	991	1009	961	989	1016	1029
Benzene[c]	2342	2116	2168	2074	1677	1636	1569	1699	1631	1608
Cumene	6119	5879	5625	5217	4393	4666	4168	4311	4426	4455
Ethanol (Synthetic)	NA	NA	626	648	678	698	526	546	549	562
Ethylbenzene	12691	10359	13656	10758	9336	11108	8871	8369	9235	9929
Ethylene dichloride	26294	11336	17263	16762	17947	15150	13713	13849	13383	13028
Ethylene glycol	NA	NA	5230	6090	5200	5128	4809	5070	5461	5517
2-Ethylhexanol	768	760	743	732	695	692	657	650	612	743
Formaldehyde (37%)	NA	NA	8110	8165	8189	8278	6612	6720	5893	6280
Isopropyl alcohol	1478	1384	1424	1451	1272	1463	1342	1456	1474	1389
Methanol (Synthetic)	NA	NA	11292	12176	10506	8082	8704	8344	8167	8142
Methylene chloride	NA	NA	NA	403	354	362	389	461	482	504
Styrene	11366	11874	11386	11294	9594	9000	8114	8017	8337	8984
Toluene[c]	NA	NA	927	931	880	833	873	861	806	892
Xylenes[d]	8880	7054	7356	7170	6622	6574	6117	6143	6327	6572

a Data from References 11 and 12.
b NA=data not available
c Millions of gallons
d ortho- and para-isomers

safety community is to help educate these groups about the safe use of solvents.

PROPERTIES OF SOLVENTS

Vapor Pressure

Vapor pressure is the amount of pressure exerted by a saturated vapor above its own liquid in a closed container. Vapor pressure increases with increasing temperature of the solvent, slowly at lower temperatures and then more rapidly at higher temperatures (85). The higher the vapor pressure, the greater the tendency for the substance to evaporate into the atmosphere. Units of vapor pressure are usually expressed as millimeters of mercury (mm Hg) at 20°C (68°F).

The vapor pressure of a mixture of two miscible solvents differs from that of the individual constituents (85). If the mixture is left open to the environment, the more volatile component evaporates first, leaving the remaining mixture rich in the less volatile solvent. This process will continue until either the more volatile solvent is gone or an azeotropic (constant boiling) mixture is formed. If the azeotrope is formed, then both vapor constituents escape simultaneously. Examples of solvents that form azeotropic mixtures are water/ethanol (95.57% by weight ethanol, boiling at 78.15°C) and chloroform/acetone (20.0% by weight acetone, boiling at 64.7°C) (85). Vapor pressure and evaporation rate (see below) can be used to estimate how quickly a substance becomes airborne and, thus, how soon an individual may be exposed to it.

Boiling Point

The boiling point of a liquid is the temperature at which the liquid's vapor pressure equals the surrounding atmospheric pressure. This is characterized by formation of bubbles of vapor within the liquid which escape into the vapor phase. The normal boiling point is measured

in degrees centigrade (°C) or degrees Fahrenheit (°F), usually at one atmosphere pressure (760 mm Hg or 14.7 psi). Generally, the solvent with the lower boiling point will have the higher vapor pressure.

Evaporation Rate

The rate of evaporation of a solvent is also an important property to consider when selecting a material for a particular process or when evaluating its fire and health hazard potential. Since evaporation rates depend on a number of intrinsic properties as well as external conditions, evaporation rates cannot be stated in absolute numbers (120). One common way of expressing evaporation rate, however, is to compare the rate for the solvent in question to a reference material under identical conditions. Such a reference is normal butyl acetate (n-BuAc), which is given an evaporation rate of 1.0, by definition. Solvents evaporating more quickly have a value greater than 1.0 (e.g., ethyl ether and hexane) and those evaporating more slowly have a value less than 1.0 (e.g., xylene and mineral spirits).

Specific Gravity

Specific gravity is the ratio of the density of a substance to the density of a reference material at a specified temperature. Water is the reference standard for liquids and solids (density 1 g/ml at 4°C). A chemical with a specific gravity greater than 1.0 (e.g., perchloroethylene), if insoluble in water, will sink. A material having a specific gravity of less than 1.0 (e.g., toluene) is lighter than water and, if not soluble, will float on it. As can be seen, specific gravity takes on special significance in fire fighting and chemical spill cleanup operations, since many flammable liquids have a specific gravity of less than 1.0 and if insoluble, will remain on top of a pool of water.

Vapor Density

Vapor density is expressed by the ratio of the mass of a vapor or gas to the mass of an equal volume of air at the same temperature (average mass for gases in air, 29 grams per mole). The density of dry air is 0.075 1b/ft3 or 1.2 kg/m3 at 70°F and 1 atmosphere (3). Materials heavier than air (e.g., carbon disulfide, perchloroethylene, and chlorine) tend to settle in low places such as sumps, manholes, and trenches. If there is little or no air movement or temperature variability that could promote mixing, then a potentially hazardous situation exists.

Flash Point

The flash point of a solvent is the lowest temperature at which vapor is given off in sufficient quantity so that the air-vapor mixture above the surface of the solvent will ignite momentarily in a flame. The flash point is determined by heating or cooling the solvent in a closed or open cup apparatus (e.g., Setaflash, Pensky-Martens, Tag, or Cleveland) and measuring the temperature at which the flash will be obtained when a small flame is introduced into the vapor above the surface of the solvent. The Occupational Safety and Health Administration (OSHA) and the NFPA (National Fire Protection Association) define a flammable liquid as a liquid having a flash point below 100°F (37.8°C).

When vapors of a flammable solvent are mixed with air in the proper proportions, ignition is possible. This proportion, called the flammable range, is also known as the explosive range. Concentrations of vapor too lean or too rich cannot be ignited. Figure 12.1 depicts the flammability characteristics of a vapor-air mixture as concentration and temperature vary (134).

Partition Coefficient

The partition coefficient (sometimes called distribution coefficient) is the ratio of the distribution at equilibrium of a solute between two insoluble solvents. This ratio is constant at a given temperature and is valid as long as the solute does not undergo a change in solution such as dissociation. For example, the partition coefficient for iodine in water and chloroform at 25°C is 0.0117 (85). In biological systems, the partition coefficient can describe the partitioning of a toxicant between other media such as air and blood or blood and fat, and so on. Lists of partition coefficients for solvents into biological media or other compounds such as olive oil have been published (44, 51, 106, 119).

CHEMICAL CLASSIFICATION FOR SOLVENTS

Most solvents fall into one of 11 chemical groups, which are characterized by a specific chemical radical that gives the individual members the properties typical of the group. Figure 12.2 presents the chemical configurations for these classifications along with examples of common solvents belonging to each group (92). Additionally, current American Conference of Governmental Industrial Hygienists (ACGIH) eight-hour time-weighted average (TWA) threshold limit values (TLVs) are given with these examples.

OCCUPATIONAL EXPOSURE LIMITS

Various organizations and governmental agencies in the U.S. and throughout the world have issued occupational exposure limits (OELs) to help protect the health and well being of the workforce. Exposure limits com

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Table 12.2 Solvent properties*a,b*

Compound	Boiling point	Freezing point	Flash point	Vapor pressure
Acetaldehyde	20°C	−123°C	−36°F	740 mm
Acetone	56°C	−95°C	0°F	180 mm
n-Amyl acetate	149°C	−71°C	77°F	4 mm
Benzene	80°C	5.5°C	12°F	75 mm
Bromoform	149°C	8°C	None	5 mm
2-Butanone	79°C	−86°C	16°F	78 mm
n-Butyl acetate	125°C	−77°C	72° F	10 mm
n-Butyl alcohol	118°C	−90°C	84° F	6 mm
Carbon disulfide	47°C	−112°C	−22° F	297 mm
Carbon tetrachloride	77°C	−23°C	None	91 mm
Chlorobenzene	132°C	−46°C	82°F	9 mm
Chloroform	61°C	−63°C	None	160 mm
m-Cresol	203°C	12°C	187°F	0.14 mm @25°C
Crotonaldehyde	104°C	−74°C	45°F	19 mm
Cumene	152°C	−96°C	96° F	8 mm
Cyclohexane	81°C	7°C	0°F	78 mm
Cyclohexanol	161°C	25°C	154°F	1 mm
Cyclohexanone	156°C	−45°C	146°F	5 mm
Cyclohexylamine	134°C	−18°C	88°F	11 mm
1,2-Dichlorobenzene	181°C	−17°C	151°F	1 mm
Diethylamine	56°C	−50°C	−15°F	192 mm
Dimethylphthalate	284°C	6°C	295°F	0.01 mm
Dimethyl sulfate	188°C	−32°C	182°F	0.1 mm
Ethanol	78°C	−114°C	55°F	44 mm
Ethyl acetate	77°C	−83°C	24°	73 mm
Ethylene glycol	198°C	−13°C	232°F	0.06 mm
Ethylene glycol n-butyl ether	171°C	−77°C	143°F	0.8 mm
Ethylene glycol monoethyl ether	135°C	−90°C	110°F	4 mm
Ethylene glycol monethyl ether acetate	156°C	−62°C	124°F	2 mm
Ethylene glycol monomethyl ether	124°C	−85°C	102°F	6 mm
Ethylene glycol monomethyl ether acetate	145°C	−65°C	120°F	2 mm
Ethyl ether	34°C	−116°C	−49° F	440 mm
Formaldehyde (37% in water)	101°C	NA	185°F	1 mm
Formamide	211°C	2.8°C	310°F	0.1 mm @30°C
Furfural	162°C	−37°C	140°F	2 mm
Furfuryl alcohol	170°C	−14°C	149°F	0.6 mm @25°C
Heptane	98°C	−91°C	25°F	40 mm @22°C
n-Hexane	69°C	−95°C	−7°F	124 mm
Hexone	117°C	−84°C	64° F	16 mm
Isopropyl acetate	90°C	−69°C	36°F	42 mm
Isopropyl alcohol	83°C	−88°C	53°F	33 mm
Isopropyl ether	68°C	−60°C	−18°F	119 mm
Methanol	64° C	−98°C	52°F	96 mm
Methylcyclohexane	101°C	−127°C	25°F	37 mm
Methylene chloride	40° C	−95°C	None	350 mm
Nitroethane	114°C	−90°C	82° F	21 mm @25°C
Nitromethane	101°C	−29°C	95°F	28 mm
1-Nitropropane	132°C	−93°C	96°F	8 mm
n-Pentane	36°C	−130°C	−57°F	420 mm
Perchloroethylene	121°C	−19°C	None	14 mm
Propylene glycol monomethyl ether	120°C	−95°C	97°F	12 mm @25°C
Pyridine	116°C	−42°C	68°F	16 mm
Stoddard solvent	154–202°C	NA	102–110°C	NA
Styrene	145°C	−31°C	88°F	5 mm
Tetrahydrofuran	66°C	−108°C	6°F	132 mm

Toluene	111°C	−95°C	40°F	21 mm
1,1,1-Trichloroethane	74°C	−31°C	None	100 mm
Trichloroethylene	87°C	−73°C	None	58 mm
Turpentine	154–170°C	−50–−60°C	95°F	4 mm
Vinyl acetate	72°C	−93°C	18°F	83 mm
VM & P naphtha	95–160°C	NA	20–55°F	2–20 mm
o-Xylene	144°C	−25°C	63°F	7 mm

a Data from References 4 and 101.

b C, degrees centigrade; Ceiling, concentration that should not be exceeded during any part of the working exposure; F, degrees Fahrenheit; LEL, lower explosive limit; mm, mm Hg; ppm, parts per million; NA, noT available or not applicable; Specific gravity, at 20°C referenced to water at 4°C; TLV, threshold limit value; skin, potential exposure contribution due to cutaneous absorption; UEL, upper explosive limit; Vapor pressure at 20°C unless otherwise indicated.

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Specific Gravity	LEL	UEL	TLV
0.79	4.0%	60%	25 ppm (ceiling)
0.79	2.5%	12.8%	500 ppm
0.88	1.1%	7.5%	50 ppm
0.88	1.2%	7.8%	0.5 ppm (skin)
2.89	NA	NA	0.5 ppm (skin)
0.81	1.4% @93°C	11.4% @93°C	200 ppm
0.88	1.7%	7.6%	150 ppm
0.81	1.4%	11.2%	50 ppm (ceiling, skin)
1.26	1.3%	50%	10 ppm (skin)
1.59	NA	NA	5 ppm (skin)
1.11	1.3%	9.6%	10 ppm
1.48	NA	NA	10 ppm
1.03	1.1% @150°C	NA	5 ppm (skin)
0.87	2.1%	15.5%	0.3 ppm (ceiling, skin)
0.86	0.9%	6.5%	50 ppm
0.78	1.3%	8%	300 ppm
0.96	NA	NA	50 ppm (skin)
0.95	1.1% @100°C	9.4%	25 ppm (skin)
0.87	1.5%	9.4%	10 ppm
1.30	2.2%	9.2%	25 ppm
0.71	1.8%	10.1%	5 ppm (skin)
1.19	0.9%	NA	5 mg/m3
1.33	NA	NA	0.1 ppm (skin)
0.79	3.3%	19%	1000 ppm
0.90	2.0%	11.5%	400 ppm
1.11	3.2%	15.3%	100 mg/m3(ceiling)
0.90	1.1% @93°C	12.7% @135°C	20 ppm (skin)
0.93	1.7% @93°C	15.6% @93°C	5 ppm (skin)
0.98	1.7%	NA	5 ppm (skin)
0.96	1.8%	14%	5 ppm (skin)
1.01	1.7%	8.2%	5 ppm (skin)
0.71	1.9%	36%	400 ppm
1.00	7%	73%	0.3 ppm (ceiling)
1.13	NA	NA	10 ppm (skin)
1.16	2.1%	19.3%	2 ppm (skin)
1.13	1.8%	16.3%	10 ppm (skin)
0.68	1.05%	6.7%	400 ppm
0.66	1.1%	7.5%	50 ppm
0.80	1.2% @93°C	8.0% @93°C	50 ppm
0.87	1.8% @38°C	8%	250 ppm
0.79	2.0%	12.7% @93°C	400 ppm
0.73	1.4%	7.9%	250 ppm
0.79	6.0%	36%	200 ppm (skin)
0.77	1.2%	6.7%	400 ppm
1.33	13%	23%	50 ppm
1.05	3.4%	NA	100 ppm
1.14	7.3%	NA	20 ppm
1.00	2.2%	NA	25 ppm
0.63	1.5%	7.8%	600 ppm
1.62	NA	NA	25 ppm
0.96	1.6 (calc.)	13.8% (calc.)	100 ppm
0.98	1.8%	12.4%	5 ppm
0.78	NA	NA	100 ppm
0.91	0.9%	6.8%	20 ppm
0.89	2%	11.8%	200 ppm
0.87	1.1%	7.1%	50 ppm (skin)

1.34	7.5%	12.5%	350 ppm
1.46	8% @25°C	10.5% @25°C	50 ppm
0.86	0.8%	NA	100 ppm
0.93	2.6%	13.4%	10 ppm
0.73–0.76	1.2%	6.0%	300 ppm
0.88	0.9%	6.7%	100 ppm

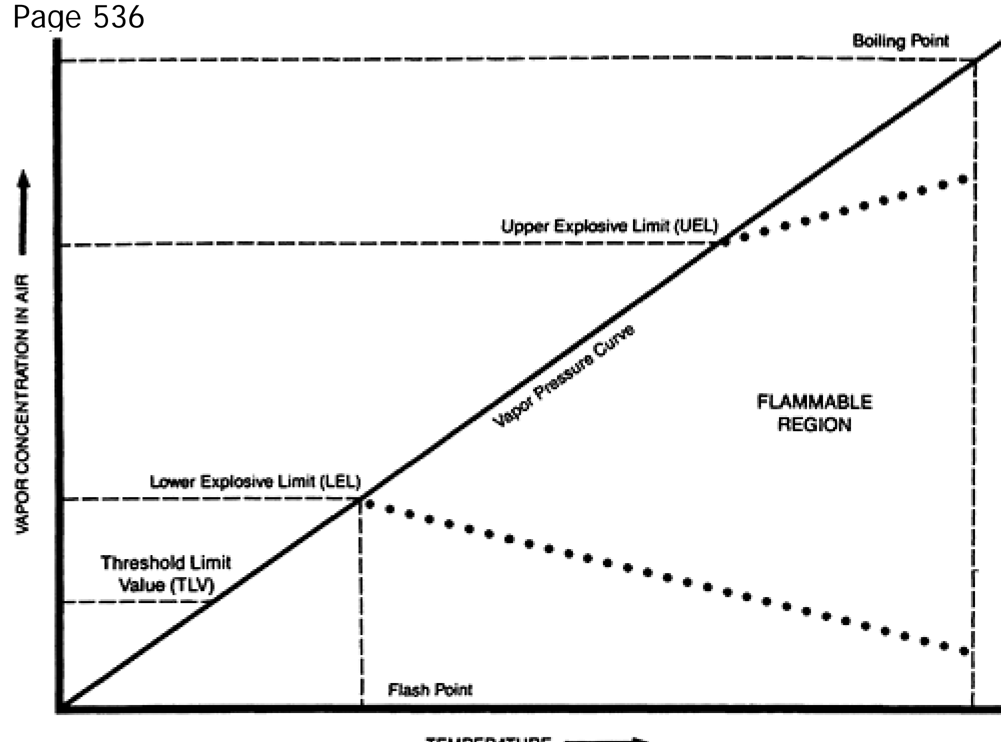

FIG. 12.1. Diagram of vapor pressure versus temperature showing relation between upper and lower flammable (explosive) limits, flammable and nonflammable regions, threshold limit value, boiling point, flash point, and vapor pressure curve. This diagram shows what happens to a vapor/air mixture as concentration and temperature vary.

monly accepted in the United States and in many parts of the world are the TLVs and the biological exposure indices (BEIs), which are updated yearly by the ACGIH. Other limits in the United States include the workplace environmental exposure level guides (WEEL) of the American Industrial Hygiene Association (AIHA), the recommended exposure limits (REL) of the National Institute for Occupational Safety and Health (NIOSH), and the permissible exposure limits (PEL) promulgated by OSHA. Exposure limits in Germany are the DFG (Deutsche Forschungsgemeinschaft) Maximum Concentration Values in the Workplace (MAKs) (2). In the United Kingdom, the COSHH (Control of Substances Hazardous to Health) Regulations mandate adherence to Maximum Exposure Limits (MEL) and Occupational Exposure Standards (OES) for protection of workers there. Additional regulations may apply for extremely hazardous substances such as carcinogens. The processes by which various occupational exposure standards are developed have been reviewed for the United States, Russia, Australia, Britain, and Norway (139). OELs are intended solely for the protection of the workforce and should not be applied to exposures unrelated to the occupational environment.

Threshold limit values are airborne concentrations of substances to which most workers may be exposed during an 8-h workday and a 40-h work week without suffering detrimental health effects (4). Most are presented as TWAs. TWAs permit excursions above the limit, provided there are compensating equivalent exposures below the limit during the workday. Importantly, TLVs are not to be considered as absolute values differentiating between hazardous and nonhazardous concentrations, nor should they be used as a relative index of toxicity. Table 12.3 presents data used by ACGIH in developing TLVs.

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Table 12.3 Data used in developing threshold limit values

Physical properties
- Lipid solubility
- Water solubility
- Vapor pressure
- Odor threshold

Acute toxicity data
- Oral toxicity, LD50
- Dermal toxicity, LD50
- Dermal and eye irritation
- Inhalation toxicity, LC50

Subchronic data (oral, dermal, or inhalation)
- 14 day, NOEL[a]
- 90 day, NOEL
- 6 month, NOEL

Other data
- Developmental (teratology and embryotoxicity)
- Mutagenicity (Ames test, *Drosophila*, etc.)
- Fertility
- Reproductive (3-generation)
- Reversibility study
- Dermal absorption tests
- Pharmacokinetics
- Cancer bioassay (2-year)

Epidemiological data
- Morbidity
- Mortality
- Case reports

Industrial hygiene exposure data
- Area samples
- Personal samples

a NOEL, no observed effect level.

To illustrate the TWA concept, consider a worker who is degreasing metal parts at two different workstations using trichloroethylene (50 ppm TLV). The employee spends 240 minutes at Station 1 with an average exposure of 30 ppm, followed by 45 minutes with no exposure (lunch), then 195 minutes at Station 2 with an exposure of 60 ppm. Calculating the 8-h TWA:

$$[30\text{ ppm}\times 240\text{ min}+(0\text{ ppm}\times 45\text{ min})+(60\text{ ppm}\times 195\text{ min})]/480\text{ min}=39\text{ ppm} \qquad (1)$$

The TLV–TWA has not been exceeded. However, one may want to evaluate the employee's work practices and other aspects of the operation to reduce the exposure as much as possible (refer to sections on Exposure Controls and Personal Protective Equipment).

The TLV of a mixture is given by:

$$C1/T1+C2/T2\ldots+Cn/Tn=1 \qquad (2)$$

provided the components have similar toxicological effects and the air is analyzed for each component. The letters C and T represent the concentration and TLV of each chemical, respectively. If the calculation gives a value greater than 1, then the TLV has been exceeded.

As an example, suppose a worker was exposed to a mixture of 25 ppm trichloroethylene and 20 ppm perchloroethylene (25 ppm TLV) during the shift. The calculated TLV is:

$$25\text{ ppm}/50\text{ ppm}+20\text{ ppm}/25\text{ ppm}=0.5+0.8=1.3 \qquad (3)$$

The threshold limit has been exceeded and action should be taken to reduce this exposure. For other examples of TLVs for mixtures, refer to the most current ACGIH TLV handbook (4).

Other categories of TLVs are short-term exposure limits (STEL) and ceilings. A STEL is a 15-minute time-weighted average exposure (above the 8-h TWA) that should not be exceeded during the workday. Allowances are made for up to four STEL excursions per day as long as there are at least 60 min between exposure periods and the 8-h TLV–TWA has not been exceeded. The STEL is supplementary to the TWA limit and should not be used exclusively as an exposure limit. Methanol is an example of a solvent having both an 8-h TLV–TWA (200 ppm) and a STEL (250 ppm). A ceiling limit is an airborne

concentration that should not be exceeded, even instantaneously. Hexylene glycol is a solvent having such a limit (25 ppm).

For certain compounds a *skin* notation has been added to indicate a possible significant contribution to overall exposure from absorption through the skin, mucous membranes, or the eyes. Both methanol and propanol have skin notations.

BEIs denote levels of determinants (primarily from exhaled air, blood, and urine) likely to be found in workers exposed to the same degree as a worker with inhalation exposure to the TLV. The determinants are primarily the chemical itself or its metabolites. BEIs are a measure of the amount of chemical in the body and may be useful when evaluating the possibility of skin absorption, effectiveness of personal protective equipment, or nonoccupational exposure. BEIs are strictly related to 8-h exposures (5 days a week) and to the specified timing for the collection of the sample. A determinant for n-hexane is 2,5-hexanedione in urine (5 mg/g creatinine), measured at the end of the shift. In the case of altered work schedules, BEIs may be

Aliphatic Hydrocarbons

Straight or branched chains of carbon and hydrogen.

*Hexane — 50 ppm
Heptane — 400 ppm
VM&P Naphtha — 300 ppm

Aromatic Hydrocarbons

Contain a 6-carbon ring structure with one hydrogen per carbon bound by energy from several resonant forms.

Benzene — 0.5 ppm
Toluene — 50 ppm
Xylene — 100 ppm

Cyclic Hydrocarbons

Ring structure saturated and unsaturated with hydrogen.

Cyclohexane — 300 ppm
Turpentine — 100 ppm

Alcohols

Contain a single hydroxyl group.

Methanol — 200 ppm
Ethanol — 1000 ppm
Isopropanol — 400 ppm

Esters

Formed by interaction of an organic acid with an alcohol.

Ethyl Acetate — 400 ppm
Isopropyl Acetate — 250 ppm

Ketones

Contain a double bonded carbonyl group, C=O, with two hydrocarbon groups on the carbon.

Methyl Ethyl Ketone — 200 ppm
Acetone — 500 ppm
Methyl Isobutyl Ketone — 50 ppm

*TLV - American Conference of Governmental Industrial Hygienists (ACGIH) Threshold Limit Value (TLV-TWA), 1998.

FIG. 12.2. Classes of organic solvents.

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Halogenated Hydrocarbon

A halogen atom has replaced one or more hydrogen atoms on the hydrocarbon.

Carbon Tetrachloride	—	5 ppm
Methyl Chloroform	—	350 ppm
Chloroform	—	10 ppm

Glycols

Contains double hydroxyl groups.

Ethylene Glycol	—	100 mg/m^3 (Ceiling)
Hexylene Glycol	—	25 ppm (Ceiling)

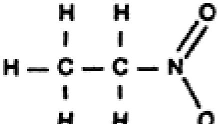
Aldehydes

Contain the double-bonded carbonyl group, C=O, with only one hydrocarbon group on the carbon.

Acetaldehyde	—	25 ppm (Ceiling)
Formaldehyde	—	0.3 ppm (Ceiling)

Nitro-Hydrocarbons

Contain an NO_2 group.



Nitroethane	—	100 ppm
Nitromethane	—	20 ppm

Ethers

Contain the C — O — C linkage.

Ethyl Ether	—	400 ppm
Isopropyl Ether	—	250 ppm

extrapolated based on pharmacokinetic and pharmacodynamic considerations (4).

The AIHA WEEL Committee develops WEELs for agents that have no current exposure guidelines established by other organizations. WEELs are expressed as TWAs; however, different time periods are specified depending on the properties of the agent. A skin notation is used in the same manner as the ACGIH TLV.

NIOSH RELs are expressed as either a TWA or a ceiling or both. These recommended limits are published as criteria documents and are periodically revised. They are established for up to a 10-h workday and are intended to provide the maximum possible protection for all workers against acute and chronic effects of exposure. Skin notations are applied.

OSHA PELs are the legal allowable concentrations of airborne contaminants. They were derived from existing standards during the enactment of the Occupational Safety and Health Act of 1970. Although the ACGIH revises some TLVs each year, the PELs remain as created unless changes are made in the law. A number of revisions

and additions have been made to the PEL list since that time. The PELs contain TWA and ceiling values and skin notations.

Immediately dangerous to life or health (IDLH) is a limit specified by NIOSH that addresses extremely hazardous conditions. It is the estimated maximum concentration of a contaminant from which a worker can escape (i.e., after failure of respiratory protection) without losing his or her life or suffering permanent health impairment. An area of concern that is becoming more and more common in today's workplace involves extended work shifts (i.e., 10-h or 12-h days). To compensate for the higher accumulated doses and reduced recovery times caused by the longer work periods, adjustments to the exposure limits need to be made. For a discussion of this subject, refer to the article by Paustenbach (104).

SAMPLING METHODOLOGY

Industrial hygiene sampling is used to characterize the concentration of a solvent in either the breathing zone of a worker or in the general work environment. There are a number of methods available to estimate solvent concentration, depending on the nature of the operation, the solvent of interest, and the objectives of the evaluation. The primary categories of industrial hygiene sampling include active sampling, direct reading, and passive dosimetry. The trend in recent years has been toward the use of direct reading instruments (e.g., colorimetric detector tubes and handheld instruments) and passive dosimetry (e.g., organic vapor badges). This is due, in large part, to the immediate feedback and/or ease of use associated with these methods. For additional information on industrial hygiene sampling, refer to the ACGIH text, *Air Sampling Instruments* (1).

Active Sampling

Active sampling involves the use of a battery-powered sampling pump to draw contaminated air onto suitable collection medium, which is then analyzed in the laboratory to determine the amount of material collected. The sampling pump requires field and/or laboratory calibration to ensure that it is pulling air at the desired flow rate. The collection medium is connected to the sampling pump via tubing and is attached to the worker in his or her breathing zone (typically the shirt collar). When completed, the medium is returned to the laboratory where the chemical of interest is extracted and analyzed. Various types of collection media are available for solvents, depending on the material being evaluated. Examples include:

(a) activated charcoal for sampling solvents such as chlorinated hydrocarbons, gasoline, many alcohols, and ketones;
(b) silica gel for amines, methanol, phenols, and aldehydes; and,
(c) chemically treated media, including filters for toluene diisocyanates, naphthylamines, and toluidines.

Direct Reading

Direct reading devices allow solvent concentrations to be measured on site with nearly instantaneous results. This approach is used in a number of applications, including identifying potential process leaks, determining peak exposure occurrences, evaluating the effectiveness of engineering controls, or in continuous monitoring applications. There are numerous direct reading devices and instruments available for measuring solvent concentrations. These include colorimetric detector (or indicator) tubes and badges, and direct reading instruments. Colorimetric detector tubes and badges contain a reagent that reacts with the solvent vapor of interest to produce a color change. This color change, or stain, is compared with a calibration scale to determine the concentration of the solvent vapor. These tubes are easy to set up, are relatively inexpensive, and may be used for short (several minutes) or longer (hours) sampling intervals. Disadvantages include possible interfering compounds, lower accuracy, and some subjectivity in the readings. Direct reading instruments are preferred over detector tubes when multiple readings are desired. Types of direct reading instruments suitable for measuring solvents include analyzers with flame ionization and infrared detectors, combustible gas/vapor meters, photoionization devices, and portable gas chromatographs. Direct reading instruments can be either hand held (for portability) or fixed (for continuous area monitoring).

Passive Dosimetry

Passive samplers (e.g., organic vapor monitors) utilize the principles of molecular diffusion rather than a sampling pump to direct samples onto a collection medium (charcoal for organic vapor monitors). This technology is particularly well suited for personal monitoring because these devices

(a) are lightweight,
(b) are unobtrusive,
(c) require no external power source,
(d) require no calibration, and

(e) can be used to obtain short-term or full-shift exposures (19).

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Organic vapor monitors are accurate and can be used to sample for many industrial solvents. Analysis is similar to charcoal tubes mentioned above.

EXPOSURE CONTROLS

Solvent overexposure may be avoided through proper planning, equipment design, and the use of process controls, where necessary. The approach to exposure control will depend on which route(s) of exposure are expected, primarily inhalation, skin contact, or a combination of both. Specific controls may be mandated by federal health and safety regulations (as in the case of benzene and vinyl chloride) or when exposure levels exceed established limits (such as permissible exposure limits or threshold limit values). The types of controls employed include engineering and administrative controls and the use of personal protective equipment. Control of solvent exposure is often achieved with a combination of these methods.

The preferred approach in controlling solvent exposure is through the use of engineering controls. Types of engineering controls (in order of preference) include change in a process to eliminate or reduce solvent usage; substitution with a less hazardous solvent; isolation of the process to minimize worker involvement; and ventilation to reduce the concentration of solvent vapor in the work environment. A discussion of each type of control follows.

Process Change/Solvent Elimination

Organic solvent-based processes can often be changed to eliminate or reduce solvent exposure. Eliminating the solvent is considered the best approach to controlling exposure and should be employed during the initial design of the process. Elimination has been used extensively in various organic solvent-based processes such as degreasing, cleaning, printing, painting, and metal treatment. These initiatives are the result of a combination of health and safety concerns, good business practices, and government regulations. In addition to minimizing worker exposure, elimination of organic solvents may have additional benefits as well, including the reduction of emissions into the environment and the cost savings associated with decreased waste disposal and personal protective equipment purchases. Two examples of process changes are replacing spray painting with paint dipping and replacing compressed air spray painting with electrostatic methods (less paint overspray). Examples of organic solvent elimination include: replacing chlorinated solvent degreasers with water-based detergent or subcritical carbon dioxide systems (discussed later); replacing solvent-based paints with water-based paints; improving flux application systems in circuit board manufacturing to eliminate the need for cleaning with chlorinated compounds; and using water-based or vegetable oil-based inks to eliminate solvent-based inks.

Substitution

In addition to directly eliminating solvents or using water-based materials, substitution of one organic solvent for another with lower toxicity or higher flash point is often employed. Substitutions may be made within a chemical series by retaining the active group. For example, substitution of butyl cellosolve for methyl cellosolve may be advantageous. The general group also can be retained such as in the substitution of aromatic naphtha for toluene, or toluene for benzene. Substituting a solvent with similar polar characteristics, but different toxicity, such as ethanol for methanol, may also be possible. Other examples include replacing perchloroethylene with citrus-based products in metal degreasing; substituting iosocyanate-containing coatings with toluene-based materials; and replacing formaldehyde used in preserving laboratory specimens with glycol-based compounds. Common solvents according to a group classification are shown in Table 12.4.

Isolation

A process can sometimes be enclosed and/or automated, to isolate the worker from the hazards of operation. When total enclosure of a solvent-based process is not possible, the operation can be separated from adjacent areas to minimize the number of workers exposed to the vapor. The isolation by enclosure of a solvent-based process usually requires the introduction of local exhaust ventilation (see below) to prevent the accumulation of vapors within the enclosure (fire/ explosion hazard). Examples of isolation are found in most manufacturing environments. For instance, manual painting in automotive assembly plants and manual metal plating operations have been replaced with robotic systems. These automated processes often can be operated and monitored from remote locations.

Ventilation

When the methods discussed above are not feasible or available, introducing mechanical ventilation to the process can control solvent exposure. For industrial situations, this involves the delivery of uncontaminated air into the work area (dilution ventilation) and/or direct removal of contaminated air (local exhaust ventilation).

Typical applications for dilution ventilation are heat control (as in foundries), or regulation of humidity

< previous page page_541 next page >

Table 12.4 Common solvents classified by group

Aliphatic Hydrocarbons	
Gasoline	Hexane
Pentane	Octane
Mineral spirits	Heptane
Aromatic Hydrocarbons	
Benzene	Ethylbenzene
Styrene	Toluene
Cumene	Xylene
Halogenated Hydrocarbons	
1,1,1,-Trichloroethane	Chlorobenzene
Ethylene dichloride	Perchloroethylene
Methylene chloride	Trichloroethylene
Alcohols	
Amyl alcohol	Methyl alcohol
Benzyl alcohol	Butyl alcohol
Ethyl alcohol	Isopropyl alcohol
Ketones	
Methyl ethyl ketone	Diacetone alcohol
Cyclohexanone	Acetone
Esters	
Acetates	Lactates
Alkyl formates	Propionates
Ethers	
Butyl ether	Isopropyl ether
Ethyl ether	Ethylene glycol monoethyl ether

and odor. If dilution ventilation is to be used to reduce the concentration of solvent vapors in the ambient air, at least four conditions must be met:

1. The quantity of solvent vapor generated must not be too great because the air volume necessary for dilution will become impractical.
2. The worker must be far enough away from the source of solvent vapor generation so that the TLV is not exceeded.
3. The solvent must have low toxicity.
4. The solvent vapor must be released into the work environment at a uniform rate (3).

In contrast to dilution ventilation, which dilutes vapors to acceptable levels by adding fresh air, local exhaust ventilation (LEV) removes the solvent vapors at the source. It is generally more effective than dilution ventilation and cheaper to operate since lower air volumes and smaller fans are required. LEV systems consist of a hood, ductwork, and fan, and an optional cleaner for contaminant removal prior to discharge to the outside environment. Figure 12.3 shows a typical local exhaust ventilation system (6).

The decision of whether or not to install LEV for solvent exposure control is based on a number of factors, including: the lack of more cost-effective controls, amount and toxicity of the solvent vapor generated by the process, governmental requirements, or simply good management practice (6).

In LEV systems, the air is exhausted to the outside environment either directly or by passing the airstream through a cleaner. There are three types of LEV hoods

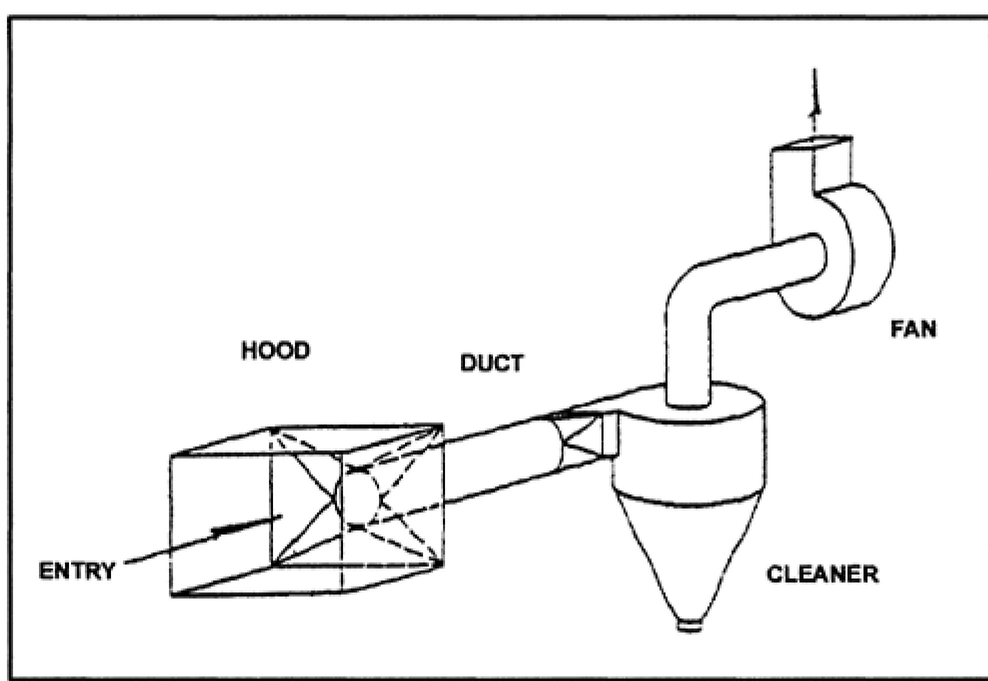


FIG. 12.3. Typical local exhaust system components. Reprinted with permission of the American Industrial Hygiene Association.

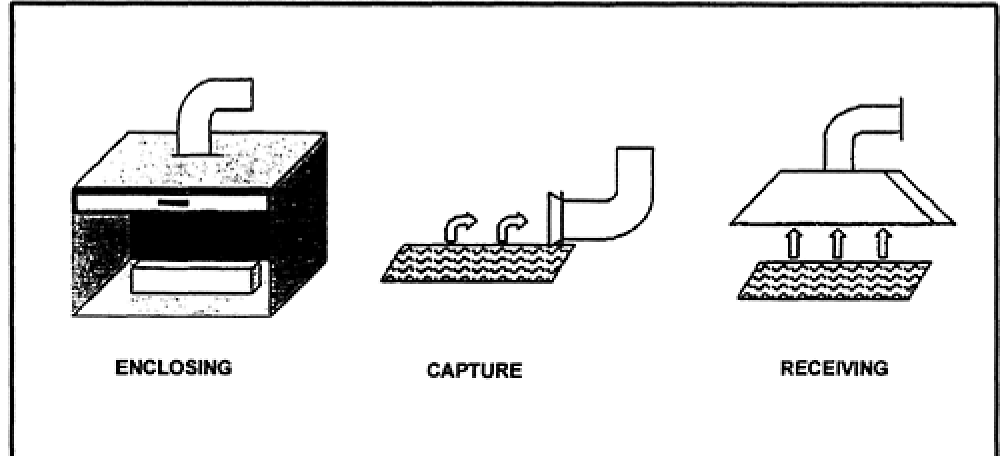


FIG. 12.4. Three types of local exhaust ventilation hoods.

used for solvent vapor control: enclosing, exterior (or capture), and receiving hoods. Figure 12.4 provides an illustration of each. Enclosing hoods are those which partially or completely enclose the process so that the point of contaminant generation is located within the hood. Enclosing the process as much as possible increases the effectiveness of LEV systems. Examples of enclosing systems include laboratory chemical fume hoods and spray paint hoods. Exterior hoods are those that are located near the point of contaminant generation, but that do not enclose it. Examples of exterior hoods are slot-type hoods used on vapor degreasing processes and flexible hoods used to exhaust solvent-based mixing processes. Receiving hoods are typically canopy-type hoods used for exhausting hot processes, for example, ovens and detergent baths. They are generally less suitable for solvent operations such as metal cleaning and degreasing.

Careful evaluation of the process should be carried out prior to selecting and designing a LEV system. Input should be provided from various disciplines, including engineering, planning, industrial hygiene, and labor. In addition to choosing the correct flow rates, designers must ensure that the arrangement of the hood and ductwork does not interfere with the work or other aspects of the facility's operation. In general, designers of LEV systems should take into account the flammability (e.g., use of approved wiring and motors) and toxicity of the solvent and the concentration generated, possible interfering air currents in the room, whether access to the work area is needed, and the amount of airflow or capture velocity required to adequately exhaust the contaminant (6).

As defined, capture velocity is the air velocity, at any point in front of the hood or at the hood opening, necessary to overcome air currents and to capture the contaminated air at that point by causing it to flow into the hood. Recommended capture velocities for solvents vary between 50 and 500 feet per minute depending on the conditions of solvent dispersion into the air (3).

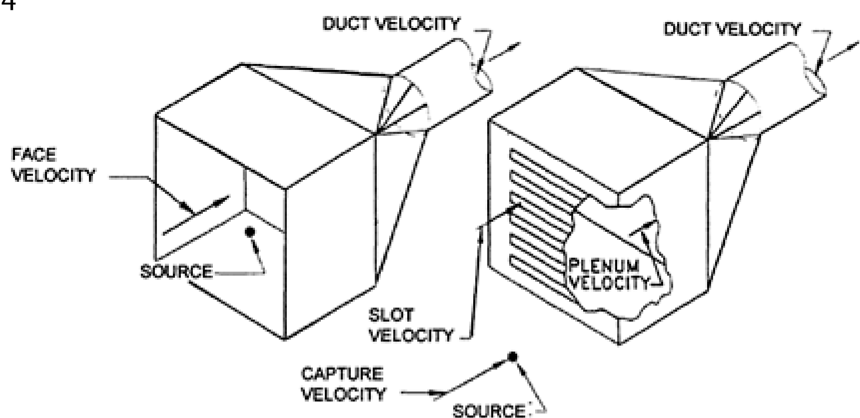
Figures 12.5–12.7 detail principles of local exhaust ventilation, including hood nomenclature and design considerations. For further reading on local exhaust ventilation systems, refer to the fundamental text, *Industrial Ventilation: A Manual of Recommended Practice*, published by the American Conference of Governmental Industrial Hygienists (3).

Administrative Controls

Although generally not the preferred approach, administrative controls may be the only feasible method to control worker exposure in certain instances or while engineering controls are being implemented. Administrative controls may include job rotation or reduction in time permitted in the area of concern. One disadvantage of limiting individual solvent exposures through job rotation is that exposures may be spread over a greater number of workers. Furthermore, administrative controls often require the continual observance of employees and additional training.

Personal Protective Equipment

If engineering or administrative controls discussed above are not feasible and/or do not provide adequate protection, personal protective equipment (PPE) must be used to minimize exposures. PPE should always be considered a last resort and managed carefully by qualified individuals. This is due to a number of limiting factors associated with PPE, which include the following:



CAPTURE VELOCITY—AIR VELOCITY AT ANY POINT IN FRONT OF THE HOOD OR AT THE HOOD OPENING NECESSARY TO OVERCOME OPPOSING AIR CURRENTS AND TO CAPTURE THE CONTAMINATED AIR AT THAT POINT BY CAUSING IT TO FLOW INTO THE HOOD.

FACE VELOCITY— AIR VELOCITY AT THE HOOD OPENING.

SLOT VELOCITY— AIR VELOCITY THROUGH THE OPENINGS IN A SLOT-TYPE HOOD. IT IS USED PRIMARILY AS A MEANS OF OBTAINING UNIFORM AIR DISTRIBUTION ACROSS THE FACE OF THE HOOD.

PLENUM VELOCITY—AIR VELOCITY IN THE PLENUM. FOR GOOD AIR DISTRIBUTION WITH SLOT-TYPES OF HOODS, THE MAXIMUM PLENUM VELOCITY SHOULD BE 1/2 OF THE SLOT VELOCITY OR LESS.

DUCT VELOCITY— AIR VELOCITY THROUGH THE DUCT CROSS SECTION. WHEN SOLID MATERIAL IS PRESENT IN THE AIR STREAM, THE DUCT VELOCITY MUST BE EQUAL TO OR GREATER THAN THE MINIMUM AIR VELOCITY REQUIRED TO MOVE THE PARTICLES IN THE AIR STREAM.

AMERICAN CONFERENCE OF GOVERNMENTAL INDUSTRIAL HYGIENISTS	HOOD NOMENCLATURE LOCAL EXHAUST			
	DATE	4–91	FIGURE	3–1

FIG. 12.5. Principles of exhaust hoods. From American Conference of Governmental Industrial Hygienists (ACGIH) (1992) (3). Reprinted with permission.

1. PPE does not eliminate the hazard. When PPE is used to control solvent exposure, the hazard continues to exist since protection is achieved by creating a barrier between the hazard and the worker. Thus, if the protection fails (such as a tear in a glove or a leak in a respirator), the worker is directly exposed to the hazard.
2. PPE requires continual worker and supervisor involvement. The effectiveness of the PPE depends not only on matching the protective device against the hazard, but, also, on the worker using the equipment properly and using it when needed. This is especially important for chemical-resistant gloves and respirator cartridges since no single glove or cartridge provides protection against every solvent.
3. Appropriate training is required. Federal regulations require that potential users be trained in the correct use and care of PPE.
4. PPE may provide a false sense of security. Some workers may believe that PPE provides complete protection, leading them to take chances when handling solvents. This is particularly true in emergency

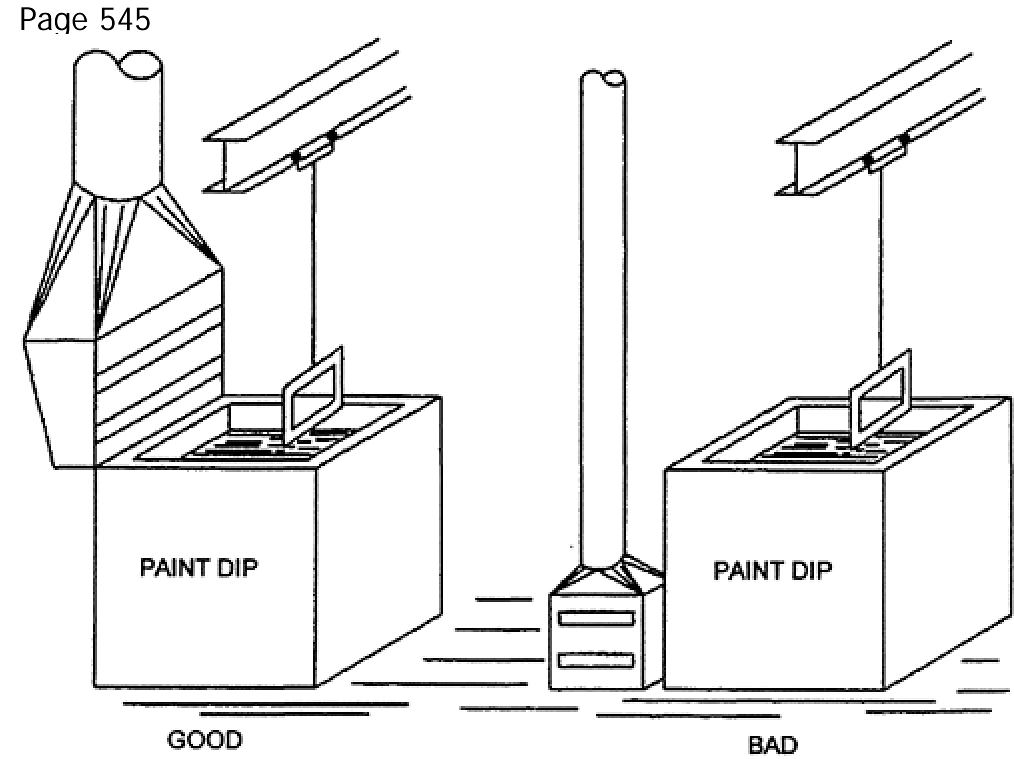


FIG. 12.6. Principles of exhaust hoods. From American Conference of Governmental Industrial Hygienists (ACGIH) (1992) (3). Reprinted with permission.

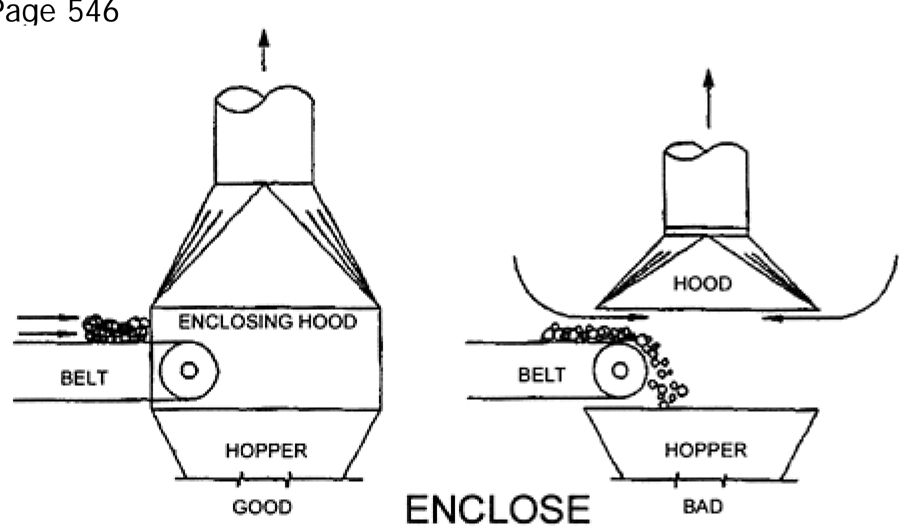
incidents where a worker may attempt to address a spill with PPE that might not be designed for the type of chemical or concentration involved.

There are various types of PPE used in the workplace, but the primary categories associated with solvent hazards are respiratory protection, protective clothing (including chemical-resistant gloves), and eye and face protection. The specific type of PPE must be carefully matched against the hazard. Often, a combination or mixture of chemicals exists, which can make PPE selection a challenge. As a general recommendation, it is always best to consult with manufacturers prior to purchasing PPE. Following is a discussion of PPE used to control solvent exposure.

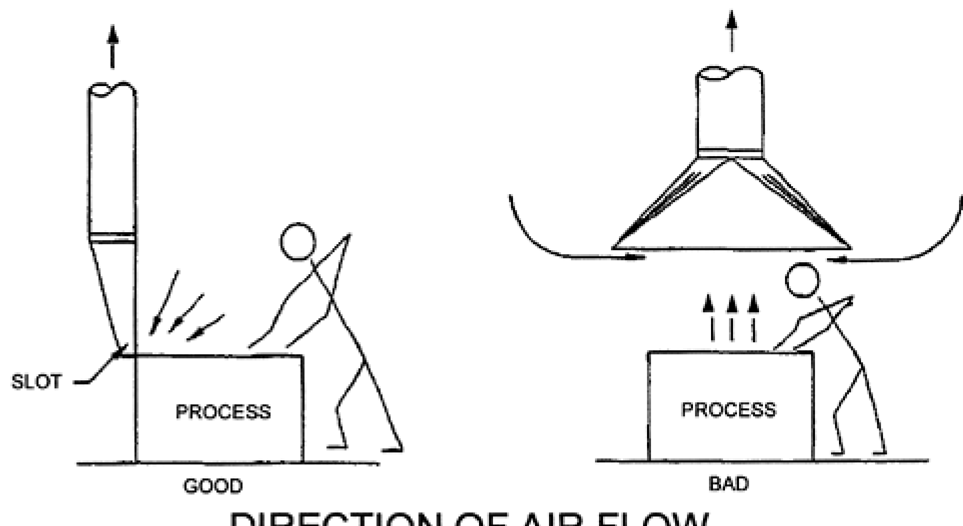
Respirators

Respiratory protection is used to prevent or reduce the level of worker exposure to airborne hazards. It is often employed to control intermittent exposures that can occur during entry into contaminated areas or during emergency repair and maintenance. However, respirators also may be the only feasible method of protection for exposures that may occur during normal work operations.

When LEV or other control methods are not feasible, the employer should provide workers with respiratory protection and implement an effective respiratory protection program. The goal is to ensure that the appropriate type of respiratory protection is selected and used correctly. OSHA has established requirements for a respiratory protection program in 29 CFR *(Code of Federal Regulations)* 1910.134. The standard instructs employers to develop written standard operating procedures to direct the respiratory protection program. Elements of the program include respirator selection, user training and fit testing, medical approval, and specific instructions for cleaning and maintenance. (See 1998 update). The two major categories of respiratory protection are air-purifying and atmosphere-supplying respirators.



ENCLOSE THE OPERATION AS MUCH AS POSSIBLE. THE MORE COMPLETELY ENCLOSED THE SOURCE, THE LESS AIR REQUIRED FOR CONTROL.



LOCATE THE HOOD SO THE CONTAMINANT IS REMOVED AWAY FROM THE BREATHING ZONE OF THE OPERATOR.

AMERICAN CONFERENCE OF GOVERNMENTAL INDUSTRIAL HYGIENISTS	*ENCLOSURE AND OPERATOR/ EQUIPMENT INTERFACE*		
	DATE *1–88*	FIGURE *3–3*	

FIG. 12.7. Principles of exhaust hoods. From American Conference of Governmental Industrial Hygienists (ACGIH) (1992) (3). Reprinted with permission.

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Air-purifying devices for solvent exposure protect the worker by removing the contaminant from the airstream prior to its reaching the worker's respiratory system. This is accomplished by passing the contaminated air through a cartridge containing a sorbent such as charcoal. Airpurifying respirators come in various models, including half-mask, full-face, and powered air-purifying devices. Atmosphere-supplying respirators provide the user with a source of clean air separate from the local environment. Examples of atmosphere-supplying respirators include air-line devices and self-contained breathing apparatus (SCBA). Only approved respirators (i.e., by the National Institute for Occupational Safety and Health) should be worn.

Other factors that must be considered when selecting respiratory protection are the type of hazard, including oxygen deficiency, concentration of the contaminant, and adequacy of warning properties. For situations involving very high solvent concentrations and/or oxygen-deficient atmospheres, air-purifying respirators do not provide adequate protection. Thus, assessment of the hazard through industrial hygiene sampling is a key component in the selection process. Warning properties, such as odor, taste, and respiratory irritation provide an indication to the user that the device is not functioning properly (e.g., the service life of the sorbent has been reached) or the device is being worn incorrectly (e.g., leak around the face seal). Air-purifying respirators should not be worn for chemicals with poor warning properties (6). Since people vary greatly in their ability to detect odors, other methods such as cartridge replacement schedules or end-of-service-life indicators are being developed by various groups to ensure greater safety when using air-purifying equipment.

Protective Clothing

Protective clothing protects against dermal exposure by forming a barrier between the skin and the solvent. Protective clothing includes gloves, laboratory coats, rubber aprons, chemical resistant suits, and boots. Various types of chemical-resistant clothing are available, depending on the solvent of concern, the work involved, and the level of protection desired. Classifications include neoprene, nitrile, natural or butyl rubber, polyvinyl chloride, and viton. Some operations may require only partial protection (such as a rubber apron) while others may need full-body enclosures (such as those used by emergency response workers). The type of material and degree of protection chosen could affect the mobility, vision, and manual dexterity of the worker. Encapsulating suits can also present potential heat stress hazards for work conducted outdoors in the summer or work conducted indoors around heat-generating operations.

The amount of protection afforded by a given type of chemical-protective clothing is related to three primary performance factors: permeation, degradation, and penetration. Permeation is the ability of a chemical to pass through the molecules of a protective film (e.g., the clothing or glove). Degradation is a reduction in one or more of the physical properties of protective clothing or gloves due to contact with a chemical. Penetration is the flow of a chemical through physical aspects of the clothing or glove, such as zippers, seams, pores, or imperfections in the material.

Manufacturers of protective clothing determine product-specific performance data via laboratory tests conducted in accordance with methods established by the American Society for Testing and Materials (ASTM). All three factors should be considered when choosing protective clothing since data for two or three of the factors may not correlate for a given type of clothing and target chemical. For example, a glove may have acceptable degradation ratings for a chemical, but the chemical may readily permeate the material. No single glove or type of protective clothing provides adequate protection against every hazard. Furthermore, a glove type from one manufacturer often has different performance data from the same glove type produced by another manufacturer. Another general source of information is *Chemical Protective Clothing*, published by the American Industrial Hygiene Association (5). This two-volume set provides data needed to select and use chemical protective clothing. Included in the document is a discussion of permeation theory, testing methods, and available vendors.

Eye and Face Protection

Eye and face protection is used to prevent injuries that may occur while handling or transporting solvents. Two types of protective eyewear typically used to prevent exposure are chemical splash goggles and face shields. Chemical splash goggles are designed to completely enclose the eyes (as opposed to safety glasses, which are designed to prevent physical injuries that may result from an object striking the eye). Some goggles may also prevent vapor exposure to the eye in addition to contact with the liquid. Face shields are often worn in conjunction with goggles to protect the face and neck. Face shields and goggles that meet recognized safety standards bear the engraving of ANSI Z-87, which indicates that the device has passed safety performance tests conducted by the American National Standards Institute.

ABSORPTION OF SOLVENTS AND INHALATION EXPOSURE

A key factor in the absorption of solvents, whether by ingestion, dermal exposure, or inhalation, is the partitioning (solubility) of the chemical into blood and tissues.

When considering solvent exposure by inhalation, the rate of solvent uptake and subsequent equilibrium concentration in tissues are also dependent on pulmonary ventilation and the minute volume of blood flow through the lung and other organs.

Solvents that are highly soluble in blood and tissues are absorbed very readily by inhalation, and blood concentrations can rise rapidly. The driving force is the difference in concentration between inspired air and blood. The amount diffusing through the alveolar capillary membrane is dependent on the air-blood partition coefficient. Tissue equilibrium concentrations with solvents such as xylene, styrene, and acetone, which are highly soluble in blood and tissues, are not limited by pulmonary ventilation because the tissues act as a sink for the inhaled solvent. As pulmonary ventilation is increased, the blood and tissue concentrations continue to rise. The limiting factor in attaining the tissue equilibrium concentration is the blood flow through the tissues and the blood-tissue partition coefficient.

Solvents such as methyl chloroform, methylene chloride, trichloroethylene, and toluene, which have lower solubilities in blood and tissues, reach equilibrium rapidly because of low solubility or low blood-air partition coefficients. Tissue concentrations also will reach equilibrium rapidly because of low tissue-blood partition coefficients. Tissue concentration is limited, then, by tissue solubility and pulmonary ventilation. To achieve a higher concentration in tissues and blood, pulmonary ventilation must increase, allowing more solvent to enter the blood and a new blood-tissue equilibrium to be obtained (13, 14).

DERMAL UPTAKE OF SOLVENTS

The opportunity for solvents to enter the body through dermal contact is enhanced, in part, due to the large surface area of the skin (18 ft2). Fortunately, the barrier properties of the skin, which are associated with filamentous proteins and lipids of the stratum corneum, naturally inhibit penetration by harmful substances. Disruption of this barrier, however, by injury, illness, or removal of lipids, can facilitate passage of these materials. For example, treatment of the skin with polar organic solvents, detergents, and some surfactants can remove the lipids, thereby increasing the skin's permeability. Penetration of the skin also depends on a number of factors such as the thickness and integrity of the skin layer, the difference in concentration of solvent on both sides of the epithelium, and a number of physical constants. In addition, hydration can increase absorption by affecting the permeability of the skin. Movement of water-soluble compounds may be impeded, however, when the stratum corneum is highly hydrated. Although hair follicles and sweat glands comprise only a small proportion of the skin's surface area, they, too, provide pathways for solvent penetration.

Solvents can damage the skin by removing lipids, resulting in irritation, cellular hyperplasia, and swelling. For example, the careless use of solvents without proper hand and arm protection frequently leads to cases of dermatitis in the workplace. In a study of skin lipid removal by solvents, it was found that the ability of a solvent to penetrate the skin depends on the polarity of the solvent and the surface charge of the skin. Results comparing penetration or removal of lipids by several solvents showed that ethanol, the solvent with the greatest polarity, extracted the most lipids, followed by acetone and then ether (17).

Treatment of the skin with solvents can also increase the penetration rate of other compounds. In a study using excised human skin, the effect of several solvents including DMSO, dimethylacetamide, formamide, and dimethylformamide on the penetration rate of sarin was examined (86). Results showed that solvent pretreatment increased the rate of sarin transport by a factor of 10–100 over that of sarin on control skin.

Penetration of solvents by the dermal route in humans was studied for toluene, xylene, and styrene vapors. Volunteers were exposed to 300 or 600 ppm for 3.5 h in a dynamic exposure situation in which the subjects wore full-face respirators to prevent pulmonary absorption of the solvents. A 10-min exercise period was sufficient to make the subjects perspire and to raise the skin temperature about 0.5°C. Perspiration and warm skin temperature enhance the hydration of the skin and subsequent percutaneous absorption. After termination of exposure, these solvents displayed biphasic elimination from the blood into exhaled air with a short half-life of about 1 h and a much longer half-life of approximately 10 hr. Xylene and styrene had slightly delayed excretion in exhaled air after percutaneous exposure when compared with inhalation exposure. Delayed excretion after dermal exposure may be accounted for by a slow release from the skin after termination of exposure.

Overall percutaneous absorption of the compounds above corresponded to only about 0.1 percent of the amount estimated to be absorbed by the pulmonary route, thus indicating a very small absorption potential by the percutaneous route. When the percutaneous absorption of xylene vapor was compared to earlier work with xylene liquid, the vapor displayed an approximately 10-fold greater efficiency in penetrating the skin than did the liquid. According to the authors, it was not uncommon to observe

greater penetration with vapor exposure because liquid solvents removed the lipids from the stratum corneum and thus interfered with absorption. Additionally, exercise promoted the absorption of solvents because of the warm hydrated skin. In general,

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under reasonable exposure conditions in the workplace, percutaneous absorption of solvent vapors would not contribute significantly to the total blood concentrations of these solvents (116).

TOXICOLOGY OF SELECTED SOLVENTS

This section deals with solvents of occupational concern that have had neurotoxic, reproductive, or carcinogenic effects in humans. Examples are provided for these as well as less toxic, alternative solvents. Generally speaking, acute exposure to high levels of solvents (i.e., greater than the TLV) results in alterations of central nervous system (CNS) function. Repeated solvent exposures give rise to the organ-specific pathology described below for individual chemicals.

Effects of Acute Solvent Exposure on the Central Nervous System

Although varying widely in chemical structure and physical properties, solvents produce a rather stereotypical set of toxicological manifestations upon acute exposure (10). Most commonly these include evidence of dysfunction of the CNS and, if exposure is sufficiently severe, narcosis. Exposure to certain solvents has been associated with alterations of cognitive and psychomotor function following short-term exposures at or near the TLV. For example, controlled studies of volunteers exposed to toluene (100 ppm for about 6 h) revealed decreased manual dexterity, visual perception, and color discrimination (15). Exposure to greater concentrations may provoke symptoms including, for instance, headache, dizziness, ataxia, a sense of euphoria, drowsiness, lightheadedness, disorientation and confusion, tremulousness, and nausea. These symptoms are readily reversible under single exposure conditions upon removal of the individual to a solvent-free environment. Exposure to potentially lethal levels of solvents can result in stupor, loss of consciousness, coma, respiratory depression, and abnormal cardiac function. Information on neurotoxic symptoms associated with acute exposure to specific solvents is found in the latter portion of this chapter.

Other Toxic Effects of Solvent Exposure

Exposure to solvents at concentrations too low to induce many of the acute symptoms cited above is of special concern with regard to neurotoxicity, because the capacity of nervous tissues for post-toxicity regeneration is limited and repeated insults may lead to cumulative damage. The subtlest symptoms of chronic solvent exposure include relatively mild alterations of mood and behavior not accompanied by quantifiable evidence of dysfunction on neurobehavioral tests (18, 57). Although dose-response and causal relationships have been difficult to study in the absence of animal models, symptoms of chronic solvent exposure may include increased irritability, decreased span of attention, and loss of interest in daily activities. More severe damage to the nervous system, both central and peripheral, occurs upon repeated exposure to certain solvents such as carbon disulfide and n-hexane (discussed later). Numerous neurobehavioral and functional tests have been used to detect such changes in both clinical and experimental settings (69, 112). Whether the acute effects of solvents play a role in determining the pathogenesis of toxic lesions observed after chronic exposure to the same solvents is uncertain. However, current thought is that the acute effects on the nervous system are mediated through nonspecific interactions of solvents with the cell membrane, that is, increases in membrane fluidity or functional alteration of cell surface receptors, while the effects of chronic exposure are mediated by specific biochemical actions of solvents.

Organs that receive a high percentage of the cardiac output are exposed to greater doses of absorbed toxicants than poorly perfused tissues. A major determinant of target organ selectivity for the toxicity of solvents is xenobiotic metabolism. While pharmacokinetics defines the quantity of solvent reaching a particular organ or tissue after absorption, metabolism may yield products with increased toxic potential relative to the parent chemical. Thus, well-perfused organs with high capacities for specific types of biotransformation reactions, mainly those catalyzed by the cytochrome P450, are common targets for solvent-induced toxicity. In particular, the liver is vulnerable to the toxicity of many solvents, owing to its high capacity for xenobiotic metabolism. Many common hepatotoxic solvents yield toxic intermediates or end products upon biotransformation, for example, carbon tetrachloride (113), chloroform (109), and trichloroethylene (16). However, some solvents, such as ethanol, may exert their hepatotoxic effects indirectly by altering cellular redox balance during metabolism and thereby deranging normal liver function and structure (117).

The kidney, as a filtering and concentrating organ of excretion, receives not only untransformed solvents but also the products of hepatic metabolism of solvents. These biotransformation products—for example, conjugates of trichloroethylene—may be more toxic than the parent chemical and produce renal-specific toxicity (81). The ion transport and solute concentrating functions of renal tubules also contribute to the vulnerability of the kidney to certain chemical toxicants (82). In addition, biochemical peculiarities of certain species and genders

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may play a major role in bringing about solvent-induced renal toxicity. A notable example is the susceptibility of the male rat to renal toxicity caused by 1,4-dichlorobenzene, Stoddard solvent, VM & P naphtha, and other hydrocarbon solvents. This has been attributed to the male rat-specific abundance of the low molecular weight protein α2u-globulin, which acts as a carrier for lipophilic molecules (128). α2u-Globulin is normally degraded in renal tubule lysosomes and binding to a solvent ligand slows degradation of the protein so that the α2u-globulin-hydrocarbon complex is sequestered by lysosomes (77). The sequestered protein apparently disrupts lysosomal function and cytotoxicity results when large amounts of α2u-globulin accumulate (121). There is apparently no counterpart to this type of nephrotoxicity in species other than the rat (7).

Organs that catalyze relatively few types of chemical biotransformation reactions or have low rates of xenobiotic metabolism, such as lung, nasal mucosa, and testes, may also be target organs for the toxicity of some solvents. For example, ethylene glycol monomethyl ether and related glycol ethers are testicular toxicants (58, 95) as are hexane and the hexane metabolite 2,5-hexanedione (22). Recently, special attention has been paid to the potential susceptibility of the tissues lining the upper airways—the nasal mucosa—to solventinduced toxicity. These tissues have high levels of certain xenobiotic-metabolizing enzymes and, in addition, are exposed to high solvent concentrations relative to the lung and other organs. In particular, esters such as propylene glycol monomethyl ether acetate, dimethylphthalate, and dimethylsuccinate are enzymatically transformed by nasal carboxylesterase to yield acidic products that may accumulate to toxic levels in the nasal mucosa (95, 126, 133). Certain solvents need no metabolism to adversely affect the tissues of the upper respiratory tract; vapors or aerosols of aldehydes cause local tissue damage to the nasal epithelium (97), presumably due to the activity of these solvents in forming protein-protein and protein-DNA crosslinks (61).

It is well known that neurotoxic chemicals can have a negative impact on the sensory function. Often, the first symptoms reported following chemical exposure are those related to the senses (48). Toluene, xylene, styrene, trichloroethylene, and carbon disulfide are examples of solvents associated with adverse affects on the auditory system (98). In the industrial environment, workers are often exposed to solvents as well as high levels of noise, which is known to damage the inner ear and cause hearing loss. In recent years, evidence has emerged from workplace studies and animal experimentation that the combined effects of noise and ototoxic solvents may increase the susceptibility to hearing loss (68, 98). In one animal study, rats were exposed to toluene or noise or toluene followed by noise and then had their auditory functions tested. Results showed that rats exposed to toluene followed by noise exhibited a decrease in auditory sensitivity greater than the sum of the effects of toluene and noise alone (98). The risk for hearing loss may be increased by factors other than noise, such as drugs or other chemicals, and can also be influenced by heredity and aging (98). It is important to take all of these factors into account when addressing hearing loss in the workplace.

Solvent Mixtures

Humans are often exposed to multiple chemicals at work or in the home. An example, as reported by Worksafe Australia (the Australian National Occupational Health and Safety Commission), involved solvent exposure and health effects in spray-painter apprentices (143). Their study identified 32 different solvents contained in 20 thinner products used by the painters. The solvents represented six classes of compounds: alcohols, aromatic hydrocarbons, esters, glycol ethers, ketones, and mixtures. Of significance was the fact that the workers commonly perceived the thinners to be equivalent and safe to use. This underscores the need for chemical communication programs to inform workers about the potential hazards of working with mixtures of chemicals.

Exposure to multiple chemicals, either simultaneously or sequentially, may alter the toxicological interactions of the individual chemicals, leading to a change in the toxicity as predicted by summing their individual effects. Thus, one chemical may alter the absorption, distribution, metabolism, and/or excretion of other chemicals in a mixture (74). The study of chemical interactions has been developed most extensively for therapeutic drugs. Although some information exists on interactions of industrial chemicals, most toxicological research to date has dealt with single, pure chemicals. These single chemical studies are important because they allow researchers to gather fundamental knowledge about the mechanisms of toxicity under conditions that are well controlled. Additional research is required to examine the potential health effects associated with exposures to multiple compounds (148).

Glycol Ethers

Glycol ethers represent an important category of solvents that are widely used in mixtures for industrial and consumer applications. They are grouped as ethylene glycol, propylene glycol, or butylene glycol ethers, with the ether portion of the molecule containing methyl, ethyl, propyl, butyl, or higher molecular

weight moieties (52). Additional members of this class of compounds

are the corresponding acetate esters. The miscibility of glycol ethers with water and many organic compounds make them ideally suited as solvents in oil-water compositions. Production capacity of the ethylene-based ethers in 1992 exceeded one billion pounds, with the coatings (paint) industry being the major consumer (52). In addition to coatings, glycol ethers are found in many household goods such as brake fluids, waxes, cleaners, dyes, detergents, degreasers, and inks. 2-Butoxyethanol has been formulated into hundreds of consumer products (27).

The current ACGIH TLV–TWAs and German MAKs for three widely used glycol ethers, 2-methoxyethanol (ME), 2-ethoxyethanol (EE), and 2-butoxyethanol (BE), are 5 ppm, 5 ppm and 20 ppm, respectively. The NIOSH RELs and proposed OSHA PELs for ME and EE are significantly lower at 0.1 ppm and 0.5 ppm, respectively. All have skin notations. ACGIH based their limit for ME on possible blood, reproductive, and CNS effects. For EE and BE, effects on reproduction and the blood were considered, respectively. The TLV and REL for propylene glycol monomethyl ether (PGME) are both 100 ppm and are based on potential irritation and CNS effects (2, 4, 101).

The commonly encountered glycol ethers are colorless liquids with mild odors. The primary routes of exposure in the industrial environment are inhalation and skin absorption (52). Some cases of accidental or intentional ingestion of products containing glycol ethers by children and adults have been reported (27). In general, the ethylene glycol ethers exhibit low acute oral toxicity (52). Experiments in rats have shown that the methyl, ethyl, and butyl ethers are readily absorbed through the skin (118). As the molecular weights of the glycol ethers increase, the potential for inhalation exposure and skin absorption decreases. Because the methyl and ethyl ethers of ethylene glycol and their acetates have demonstrated adverse reproductive, embryotoxic, teratogenic, and developmental effects in animal studies (58–60, 99, 100), their use in consumer products has declined (52).

Monoalkyl ethers of ethylene glycol are converted to their respective alkoxyacetic acids via the actions of alcohol dehydrogenase (27). Many of the observed adverse effects caused by ethylene glycol ethers in animals, such as hemotoxicity (e.g., 2-butoxyethanol) and testicular toxicity, are attributed to these toxic metabolites. Whereas rat erythrocytes have demonstrated vulnerability to the hemolytic effects of 2-butoxyacetic acid (from BE), human erythrocytes have been shown to be much less susceptible to these effects (27).

PGME and its acetate (PGMEA) are relatively innocuous compounds when compared to the ethylene glycol ethers discussed above. Overexposure to PGME has been associated only with increased liver weight and CNS depression. Studies have shown that EE and PGME are metabolized by different routes and the types of metabolites produced are responsible for the marked differences in toxicity. For example, methoxyacetic acid is the primary metabolite of EE, while propylene glycol is the main biotransformation product of PGME and PGMEA (94, 96).

Investigators have studied the potential interaction of ethanol and EE because of similar metabolic pathways and the likelihood of concomitant exposure to ethanol in some individuals (100). EE, when presented to rats alone or in combination with ethanol, seemed to increase the duration of pregnancy. Exposure to EE during gestational days 7–13 caused a decrease in certain behavioral tests such as rotorod performance. However, when EE was exposed to animals that also consumed ethanol the behavioral deficit was diminished. When EE was administered alone during late gestation, motor activity of pups was depressed and performance at avoidance conditioning trials was retarded. Combined administration of EE and ethanol seemed to exaggerate the behavioral deficits induced by EE and to depress both activity and learning.

Examination of neurotransmitters in 21-day-old pups that had been whelped by dams exposed to EE alone on gestational days 7–13 revealed an increase in several neurotransmitters such as acetylcholine, dopamine, and norepinephrine. Pups that were whelped by dams that had the combined treatment displayed a decrease in acetylcholine, dopamine, and 5-hydroxytryptamine. Thus, it was observed that ethanol during late gestation altered the neurochemical effects of EE.

In summary, concomitant exposure to ethanol can have differential effects depending on the stage of gestation. Ethanol administration during the early period of gestation ameliorated both the behavioral and neurochemical effects of EE to approximately 50 percent of the response produced by EE alone. In the late stage of gestation the combination of ethanol with EE exaggerated the effects of EE alone. This indicates that the possibility exists for ethanol-induced exaggeration of the potential toxic effects of EE exposure in pregnant workers.

Retrospective epidemiological studies of workers exposed to ME and EE have reported evidence of adverse effects on the male reproductive system, with increased frequency of reduced sperm counts (145). Evaluation of sperm production in several species has shown that the output of human sperm is

about one-fourth that of other mammals when compared on a per-gram tissue basis. This finding suggests that humans may be more susceptible to occupational toxicants than predicted by laboratory animals (130). As is the case with many widely used chemicals with potentially harmful effects, substitutes are being considered. PGMEA and ethyl-3-propionate have been identified as useful and less toxic alternatives to ethylene glycol ether solvents (23).

Benzene

Benzene has been used extensively over the years as a raw material in the manufacturing of polymers, detergents, pesticides, dyes, plastics, and resins, and as a solvent for waxes, oils, natural rubber, and other compounds (93, 123). In addition, benzene is a component of gasoline and is generally present at low levels throughout the environment (64).

Exposure to benzene in the workplace is primarily through inhalation, but skin absorption may also contribute to the overall body burden. OSHA regulates benzene as a potential occupational carcinogen with a PEL of 1 ppm and a STEL of 5 ppm (102). The ACGIH TLV–TWA and STEL for benzene are 0.5 ppm and 2.5 ppm (skin notation), respectively, and ACGIH has designated benzene as a confirmed human carcinogen (4). The NIOSH REL and STEL for benzene are 0.1 and 1 ppm, respectively. NIOSH additionally lists benzene as a potential occupational carcinogen (2). The ACGIH BEI for benzene is 25 μg of the metabolite S-phenyl-mercapturic acid per gram of creatinine in urine, measured at the end of the work shift (4).

Because of its high lipid solubility, acute exposure to benzene can cause depression of the CNS to the point of narcosis. Headache, dizziness, nausea, and vomiting are all features of benzene overexposure. Exposure to benzene at high concentrations can lead to blurring of vision, unconsciousness, convulsions, ventricular irregularities, and respiratory failure. Death as a result of exposure to extremely high concentrations of benzene may occur because of respiratory failure or cardiac arrhythmias (123, 144). Concomitant exposure to benzene and high concentrations of catecholamines can sensitize the heart and lead to ventricular fibrillation.

Benzene is hematotoxic and carcinogenic following repeated exposure to high concentrations (90). There are numerous rodent studies that also show that benzene can cause cytogenetic damage in vivo (64). In addition, examination of the chromosomes of humans exposed to high levels of benzene revealed an elevated rate of chromosomal aberrations that persisted after cessation of exposure (47). Chronic exposure to benzene leads to a progressive depression of bone marrow function (83). Epidemiological studies have demonstrated that blood dyscrasias such as pancytopenia, aplastic anemia, and acute myelogenous leukemia can develop in humans as a result of this exposure (90, 123). Furthermore, clinical investigations have shown that it may take several years after the termination of exposure for benzene-induced leukemia to appear (138).

Enzymes linked to the metabolic activation of benzene and its metabolites are the cytochrome P450 monooxygenases and myeloperoxidase (90). The major metabolic pathway for benzene appears to be oxidation to a phenol, which is then converted to a sulfate conjugate and excreted in urine. Other hydroxylated metabolites include hydroquinone and catechol. Benzene metabolism can be effected by interactions of benzene with its metabolites or other compounds. For example, experiments in mice suggest that benzene can inhibit the oxidation of phenol. Furthermore, animal and human studies have demonstrated that coexposure to toluene may significantly alter the formation of benzene metabolites. Finally, treatment with ethanol induces benzene and phenol metabolism in the liver, resulting in higher levels of active metabolites (90).

The mechanism of benzene-induced leukemia is not known. Potential mechanisms for benzene-induced bone marrow disease include metabolism of the parent compound to phenols and other metabolites, in particular, quinone-type metabolites such as catechol, quinol, and pyrogallol which could react with chromosomes and interfere with mitosis. Another possibility could be the depletion of sulfur available for glutathione detoxification, thereby leading to interaction of toxic intermediates with critical elements of the bone marrow. Another suggested mechanism involves transfer of benzene metabolites from the liver to the bone marrow (123). Researchers have investigated the metabolism and binding of radiolabeled benzene in the isolated hind limb of rats in which benzene was administered directly into the bone marrow space. Metabolites of benzene were found covalently bound to macromolecules in the bone marrow, indicating that the bone marrow has the potential of metabolizing benzene to reactive intermediates (67). The fact that benzene or benzene metabolites have been shown to inhibit the multiplication of erythrocyte precursor cells in the bone marrow may imply an additional mode of action (76).

The potential for benzene to induce leukemia in experimental animals has been difficult to demonstrate. In an inhalation study involving Sprague-Dawley rats and AKR mice, benzene vapor (300 ppm) was administered for 6 h a day, 5 days a week, for the lifetime of the animals. The rats showed signs of lymphocytopenia, mild anemia, and slightly decreased survival. The mice displayed severe lymphocytopenia and anemia with significantly decreased weight gain and survival. However, no evidence of a leukemic or pre-leukemic response was observed in either species (122). In a 2-year

carcinogenicity study, rats and mice fed benzene in corn oil developed dose-related leukopenia and tumors in multiple organs, but the study failed to show benzene-associated leukemia (64).

Toluene

Toluene is a flammable solvent that has been used extensively in the chemical, rubber, paint, and drug

industries. It is also useful as a solvent for paints, inks, lacquers, dyes, and other compounds, and as an additive for gasoline. Sources of toluene in the environment include manufacturing plants, automobile emissions, gasoline evaporation, and cigarette smoke (31, 93).

Various exposure limits and biological indicators of exposure apply to toluene. The ACGIH TLV–TWA, German MAK, and NIOSH REL for toluene are 50 ppm (skin notation), 50 ppm, and 100 ppm, respectively. NIOSH adds a 150 ppm STEL and has established an IDLH of 500 ppm. The current OSHA PEL is 200 ppm with a 300 ppm ceiling limit. The ACGIH BEIs are 0.05 mg toluene per liter of venous blood, collected before the last shift of the work week, 1.6 grams of hippuric acid per gram of creatinine in the urine, collected at the end of the shift, and 0.5 mg of o-cresol per liter of urine, collected at the end of the shift (2, 4, 101). Toluene in expired air has also been evaluated to determine its usefulness as an indicator of exposure. Analysis of expired air in toluene-exposed workers revealed that the toluene concentration was correlated to the exposure environment, representing approximately 15–20 percent of the environmental concentration (28).

The principal toxic effect of toluene is injury to the nervous system. Toluene is most rapidly absorbed by inhalation, followed by ingestion and skin contact. A substantial amount of inhaled toluene is retained in the body. The toxicity of toluene is similar to that of benzene except that it does not exhibit the hematopoietic effects characteristic of benzene. Toluene is an eye and skin irritant and animal studies indicate that its acute oral toxicity is less than that of other alkylbenzenes (31). In humans, acute effects of toluene exposure can resemble alcoholic intoxication by first stimulating and later depressing the central nervous system. Groups of volunteers exposed to 100 ppm of toluene vapor for 6 hr, complained of fatigue, sleepiness, and the feeling of intoxication. Irritation of the eyes, nose, and throat was reported as well as decreased manual dexterity and accuracy in visual perception (15).

Exposure to high concentrations of toluene, as seen in cases of solvent abuse (e.g., glue sniffing), may cause death by sensitizing the myocardium (115, 144). In chronic abusers of toluene, irreversible neurological toxicity and reversible renal damage have also been reported (129, 142). Symptoms associated with intentional inhalation of high concentrations of toluene include euphoria, mild tremors, unsteady gait, and changes in behavior. Encephalographic examination of these individuals has shown abnormalities indicative of cerebellar atrophy (71). Toluene is a lipid soluble compound that readily crosses the placenta and, as such, may pose a teratogenic risk in cases of high exposure, as with intentional abuse. A pattern of teratogenicity like that of the fetal alcohol syndrome (described in the section on ethanol) is prevalent in human studies relating to excessive in utero exposure to toluene. Coabuse of alcohol and toluene may heighten the risks (142).

Toluene is metabolized to benzoic acid, which is subsequently conjugated with glycine or glucuronic acid to form hippuric acid or benzoylglucuronates, respectively. These conjugates, as well as another metabolite, o-cresol, are excreted in the urine (75). In human studies ethanol was shown to inhibit the metabolism of toluene at blood ethanol concentrations of 21 mmol/liter (42). Results indicated that the concentration of toluene in alveolar air of the toluene/ethanol-exposed group was significantly higher than that of the toluene control group. Additionally, hippuric acid and o-cresol excretion was significantly reduced as compared to controls. During the 24 hours following the last exposure, excretion of both hippuric acid and o-cresol was about 40–50 percent of that excreted by subjects who received toluene alone. These results suggest that ethanol may alter the metabolism of inhaled toluene and prolong its elimination from the body. Therefore, the possibility of ethanol consumption should be considered during biological monitoring since ethanol intake could lead to an underestimation of the actual toluene exposure (42).

In contrast to the above, pretreatment of rats with phenobarbital (PB) showed that the metabolism of toluene could be enhanced to form benzoic acid. The pretreatment did not, however, appear to effect the rate of conjugation of benzoic acid with glycine to form hippuric acid. The hippuric acid concentration in the urine of PB pretreated rats was about three times that of rats receiving toluene only. In addition, the toluene concentration in the blood of the PB pretreated group was only about one-half that in the toluene-exposed rats. Not only did the phenobarbital pretreatment enhance metabolism of toluene to benzoic acid (with subsequent conversion to hippuric acid), it also reduced the blood concentration of toluene and thus shortened the sleeping time induced by the narcotic effect of toluene (66).

The mechanism of the neurotoxic effect of toluene is not well understood. Some experimental work with rats found that exposure to 30,000 ppm toluene for a few minutes reduced the concentration of tryptophan and tyrosine in plasma by about 50 percent and 20 percent, respectively, compared to controls. Tryptophan and tyrosine are known to be precursors of the neurotransmitters noradrenaline,

dopamine, and 5-hydroxytryptamine. The reason for the decrease in the precursors was unknown, but it was speculated to be an alteration in the hepatic uptake or utilization of these amino acids (141).

A potential factor in toluene-induced neurotoxicity is production of reactive oxygen species that can cause cell damage. Experiments using rats suggested that

benzaldehyde, a metabolite of toluene, accelerates the production of these reactive oxygen species within the nervous system, and may also contribute to the overall neurotoxicity (87).

n-Hexane

n-Hexane is a flammable liquid and one of the most toxic of the alkanes. It is an excellent organic solvent that has been used in industrial applications such as printing, low-temperature thermometers, adhesives, extractions, and cleaning processes (30, 62).

The primary routes of exposure in the industrial setting are by inhalation and skin contact. The ACGIH TLV–TWA, NIOSH REL, and German MAK are all 50 ppm. ACGIH added a skin notation and NIOSH established an IDLH value of 1100 ppm (10% of the lower explosive limit). ACGIH set the TLV based on possible neuropathy, CNS effects, and irritation (2, 4, 101).

Acute toxic responses after accidental ingestion include nausea, gastrointestinal irritation, and CNS effects. Inhalation overexposure leads to dizziness, a sense of euphoria, and numbness of the extremities. Exposure to high concentrations causes vertigo and a marked anesthetic effect. Hexane is also an irritant to the skin upon dermal exposure (30).

Many cases of polyneuropathy in workers exposed to n-hexane have been noted, with the earliest occurring in Japan (147). The severity of symptoms in the Japanese workers varied directly with degree and duration of exposure and in some cases there was incomplete recovery (62). Polyneuropathy has also been reported in cases of solvent abuse (30). The neurotoxic effect of n-hexane has characteristically been a progressive motor or sensorimotor neuropathy with symptoms usually reported after several months of exposure (62). In cases from occupational exposure, symptoms have often been sensory, with numbness and paresthesia in the distal extremities, most notably the feet or hands. Improvement of symptoms is noted after cessation of exposure, and mild cases can recover completely. Hexane is readily absorbed in laboratory animals and has an affinity for tissues high in lipid content (24). It is rapidly metabolized to hydroxylated compounds prior to being converted to a keto form (72, 84). 2,5-Hexanedione and methyl n-butyl ketone are the metabolites suspected of being responsible for the production of neurotoxicity.

The mechanism of 2,5-hexanedione-induced neuropathy is not known but several hypotheses have been presented (39). These include reduction in energy production in the axon resulting in disruption of axonal transport, alteration of protein structure, and inadequate pro teolysis of neurofilaments in the nerve terminal. 2,5-Hexanedione has been shown to interact with glyceraldehyde-3,5-dehydrogenase and phosphofructokinase, inhibiting their glycolytic properties and resulting in decreased energy production and possible disruption of axonal flow. Reaction of 2,5-hexanedione with lysine amine moieties to form pyrrole adducts and modification of neurofilament or axonal skeletal proteins is also an attractive hypothesis (40). Modification of the proteins may lead to crosslinking of the neurofilaments, which could cause difficulty in neurofilament passage through narrow regions of the axon such as the node of Ranvier, and therefore an accumulation of proteins at the site of constriction. Possible biophysical membrane changes as a result of 2,5-hexanedione may influence the degeneration of the axon. 2,5-Hexanedione binding and inactivation of calcium-dependent proteases that are important for degradation of neurofilament proteins is the last mechanism mentioned that might lead to accumulation of neurofilaments. Although none of the mechanisms mentioned fully answers all of the questions concerning n-hexane-induced neurotoxicity, these hypotheses offer some contributions to the understanding of the toxic response. It may be that several mechanisms act in parallel to produce the neurotoxic effects.

Repeated exposure of rats to n-hexane not only produces the characteristic pattern of neurotoxicity but results in testicular lesions as well (146). The testicular effects are linked to disruption of the cytoskeleton of Sertoli cells. Secondary effects, caused by a loss in functional spermatogonial cells, are seen in affected tubules. Acute exposure led to reversible effects but inhalation or oral exposures of two to five weeks led to irreversible effects. Although the neurotoxic effect of n-hexane is observed in humans, the testicular effect seen in rats has not been well documented in humans.

Methyl n-Butyl Ketone

Methyl n-butyl ketone (2-hexanone, MBK) has been used as a solvent or cosolvent (e.g., with methyl ethyl ketone) for adhesives, lacquers, vinyl coatings, printing inks, oils, varnish removers, and other materials (25, 73).

Occupationally, the principal routes of exposure to MBK are via inhalation and skin contact with the liquid. The ACGIH lowered the TLV–TWA for MBK in 1998 to 5 ppm (skin notation) to protect against possible neuropathy. The German MAK is also 5 ppm. The NIOSH REL and IDLH for MBK are 1 ppm and 1600 ppm, respectively (2, 4, 101).

Methyl n-butyl ketone has low acute oral toxicity. Inhalation of high vapor concentrations causes eye and respiratory tract irritation followed by CNS depression and narcosis (131). MBK easily penetrates the skin,

and inhalation exposure yields approximately 80–85 percent pulmonary retention. In addition, MBK is widely distributed in the tissues, the highest concentrations being found in the blood and the liver (25). Chronic exposure to low doses may produce degenerative axonal changes, primarily in the peripheral nerves and long spinal cord tracts (124, 125, 131).

Depending on the route of administration, a number of metabolites in varying amounts can be detected in the blood. The primary neurotoxic metabolite, as with n-hexane, is 2,5-hexanedione. Other metabolites identified following oral, intraperitoneal, or respiratory exposures include 2-hexanol and 5-hydroxy-2-hexanediol (25).

MBK was implicated as a neurotoxic agent in the 1970s after instances of neurotoxicity were reported in the printing and painting industries (9, 91). Inhalation was the primary route of exposure, with the severity of the developed neurotoxicity proportional to the extent of exposure. The characteristic disorder caused by methyl n-butyl ketone begins several months after chronic exposure commences. Symptoms include weight loss and distal sensory neuropathy marked by a tingling sensation in the hands or feet. The muscular weakness that develops usually involves the hands and feet, but in severe cases may extend to the legs and thighs. The sensory loss is symmetrical and may progress to the legs and thighs in severe cases. There is also a moderate reduction of nerve conduction velocity in affected peripheral nerves (8, 9).

When volunteers were given MBK by inhalation, orally, or by dermal application, 2,5-hexanedione was detected in the serum. Radioactivity associated with the radiolabeled MBK was found to be excreted slowly, indicating that repeated exposures to high concentrations of methyl n-butyl ketone may lead to prolonged exposure to its neurotoxic metabolites (41).

The relative neurotoxicity of methyl n-butyl ketone, n-hexane, and their metabolites was investigated in rats. Potency was estimated by the time required to produce evidence of severe hind limb weakness or paralysis. Results showed 2,5-hexanedione to be most toxic followed by 5-hydroxy-2-hexanone, 2,5-hexanediol, methyl n-butyl ketone, 2-hexanol, and n-hexane. An examination of the data showed that the neurotoxic potency was related to the amount of 2,5-hexanedione metabolically produced (73).

Carbon Disulfide

Carbon disulfide (CS2) is a toxic and highly flammable solvent that has found extensive use in the manufacturing of rayon, soil disinfectants, carbon tetrachloride, and electronic vacuum tubes. It is commonly used as a solvent in industrial hygiene analyses. Other applications include its use as a fumigant for grain and a corrosion inhibitor (21, 93).

Inhalation and skin contact are the main routes of occupational exposure. Because adaptation to carbon disulfide's characteristic rotten egg odor occurs rapidly, the sense of smell is not useful in judging exposure. The current ACGIH, OSHA, and NIOSH exposure limits are 10 ppm, 20 ppm, and 1 ppm, respectively, all with skin notations (2, 4). The ACGIH TLV–TWA was set to protect against cardiovascular, central nervous system, and neuropathic effects. NIOSH has established an IDLH value of 500 ppm (101). In addition to these levels, there have been proposals in the literature to lower the occupational exposure limit to 4 ppm to prevent neurological sequelae (63). The Biological Exposure Index recommended by ACGIH is 5 mg of the metabolite 2-thiothiazolidine-4-carboxylic acid (TTCA) per gram of creatinine in urine, measured at the end of the work shift (4).

Acute exposure to high concentrations of carbon disulfide can result in restlessness, euphoria, nausea, vomiting, headache, mucous membrane irritation, unconsciousness, and fatal convulsions. Chronic exposure can lead to abnormalities such as irritability, hallucinations, auditory and visual disturbances, and weight loss (21, 55, 78, 93, 137). Distal sensorimotor neuropathy is the most common chronic effect associated with CS2 exposure. This has been confirmed in experimental animals as a neurofilamentous axonopathy that effects long axons in the CNS and peripheral nervous system (36, 54). Peripheral neuropathy takes place only after frequent and prolonged exposures to CS2 and is characterized by a loss of distal sensory and motor function. The condition can progress more proximally with continued exposure. Chronic exposure to CS2, as well as hexane, 2-hexanone, and their metabolite 2,5 hexanedione, results in large swellings of the distal axons which are filled with neurofilaments. Continued exposure causes axonal degeneration distal to the axonal swellings. (34, 54). In addition to these effects, encephalopathy, detected by neurological examination and neuropsychological testing, has been reported.

There is evidence that exposure to CS2 accelerates the rate of atherosclerosis (54). In addition, an investigation to determine a possible association between CS2 exposure and ischemic heart disease mortality found that the relationship is meaningful only for workers exposed to high levels for many years. Price has suggested a safe level of between 15 and 20 ppm (111).

Approximately 70–90 percent of absorbed CS_2 is metabolized and excreted in the urine. The remaining 10–30 percent is exhaled in the breath unchanged. In addition to TTCA mentioned above, other metabolites found in workers' urine include 2-mercapto-2-thiazo-lin-5-one and thiocarbamide (63, 107, 108, 135, 136).

In a study of rayon production workers with long-term exposure to carbon disulfide at concentrations well above the TLV, evidence of neuropathy was observed in a significant number of workers and consisted of distal sensory loss, altered tendon reflexes, reduced muscle power, and reduction in nerve conduction velocity. These abnormalities persisted for up to 10 years after removal from exposure and were considered to be permanent impairments in nervous system physiology (35).

Methanol

As Table 12.1 shows, synthetic methanol (or methyl alcohol, wood alcohol) production exceeded one billion pounds in 1995. Methanol's largest use is in the production of MTBE (methyl t-butyl ether), an additive in gasoline. It is also utilized as a denaturant for ethanol, a raw material in the production of numerous other chemicals such as formaldehyde and acetic acid, and as a solvent or antifreeze in paints and strippers, cleaners, and windshield washer compounds (43).

The major routes of exposure to methanol in the industrial environment are through inhalation and dermal contact. The ACGIH TLV–TWA of 200 ppm (250 ppm STEL) is based on potential ocular toxicity and CNS effects. The OSHA PEL and NIOSH REL have also been set at 200 ppm. NIOSH has further established an IDLH value of 6000 ppm for methanol, and ACGIH and NIOSH have added skin notations as indications that skin absorption can be a contributor to the overall body burden. The ACGIH BEI is 15 mg methanol per liter of urine, collected at the end of the work shift (2, 4, 101).

Most information regarding methanol toxicity in humans is gathered from acute exposures, primarily from ingestion, but there are reports of adverse health effects from inhalation and dermal exposures (80). In one NIOSH study, teachers' aides reported headaches, blurred vision, and other symptoms following inhalation exposure to methanol used in duplicating machines. Concentrations at the site were about 2–15 times the current REL. Adverse effects have also been reported following skin applications of methanol for various purposes, although inhalation may have also contributed to these exposures (80).

Methanol is readily absorbed following oral, inhalation, or dermal exposure and is distributed throughout the body according to water content of the tissues (80). Ingestion of as little as 2 teaspoonfuls may cause toxicity, whereas the fatal dose in humans is between 2 and 8 oz (53). In the absence of medical treatment, a dose of between 4 and 10 mL can lead to blindness (114), and depending on the amount of methanol ingested, mild to severe CNS depression can occur. A latent period, commonly 12 to 24 hr, usually ensues followed by severe abdominal pain, difficult breathing, blurred vision, and pain in the eyes, among other symptoms. Visual impairment or total blindness may occur within days depending on individual susceptibility and the time when treatment began (80). Metabolic acidosis due to formic acid production is thought to be the cause of the delayed symptoms and the ocular toxicity (114).

Metabolism of methanol in the liver accounts for a high percentage of absorbed methanol in both nonhuman primates and rats. Lesser amounts are excreted unchanged in the urine and breath. Metabolism is important not only because of its primary role in clearance, but because of the connection between its metabolites and the acute toxic effects mentioned above. Methanol is oxidized by a catalase-peroxidative system in rats, rabbits, and guinea pigs and an alcohol dehydrogenase system in humans and primates. The metabolic sequence proceeds from methanol to formaldehyde to formic acid (formate) and finally to carbon dioxide and water. Formic acid is metabolized in both rats and primates via a folate-dependent pathway. Rats are able to utilize this pathway more efficiently than primates, allowing for a more rapid conversion to carbon dioxide. Because the process is slower in humans and primates, high doses of methanol cause a buildup of formate in tissues, including the eye, resulting in the observed toxicity (114).

Administration of ethanol has been used in treating methanol poisoning because ethanol inhibits the oxidation of methanol by competing for the same metabolic pathway. Prompt hemodialysis (able to remove both methanol and formate), coupled with concurrent administration of ethanol and bicarbonate, has been successful in many poisoning cases (53).

Ethanol

Ethanol (ethyl alcohol, grain alcohol) is produced in large quantities (see Table 12.1) and is utilized extensively as a solvent in industry, in numerous consumer preparations, and as an additive to gasoline (gasohol). It is used industrially as a raw material in the production of drugs, plastics, perfumes, cosmetics, and other compounds. Other applications include products such as hairsprays, mouthwashes, cleaning products, and drug formulations (53, 80). Denaturants are added to the alcohol in a number of these products to discourage ingestion. Synthesis from ethylene represents the largest source of ethanol; smaller amounts are made from fermentation of natural materials (80).

Human exposure to ethanol is primarily through ingestion of alcoholic beverages and inhalation of ethanol vapors from industrial processes and consumer products. Percutaneous absorption appears to be

much less import

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ant (80). OSHA, ACGIH, and many countries have established an exposure limit of 1000 ppm for ethanol (2, 4). The NIOSH IDLH of 3300 ppm was set because of safety concerns (10% of the lower explosive limit) rather than toxicological considerations (101).

Although there is no clear evidence that ethanol is carcinogenic in animals, it has been shown to be a tumor promoter. Additionally, the International Agency for Cancer Research (IARC) has classified alcoholic beverages as a Group 1 carcinogen based on the occurrence of a variety of tumors in humans that have been causally related to ingestion of these beverages (80).

An unfortunate occurrence associated with chronic maternal consumption of large amounts of alcohol is a pattern of congenital abnormalities commonly called fetal alcohol syndrome. Effects may include growth retardation, microcephaly, mental deficiency, facial abnormalities, and poor coordination. Children who have been effected may display a few or many of the features characteristic of the syndrome (33, 80, 110).

Ethanol is a CNS depressant that is capable of inducing all stages of anesthesia. It is readily absorbed by the GI tract and the lungs and is distributed throughout the body water (53). Absorption can be delayed, however, by food in the stomach. Subjects exposed to 5000–10,000 ppm of ethanol vapor experienced eye irritation and coughing (114). Individuals with tolerance to alcohol experienced headache, drowsiness, and stupor when exposed to concentrations of 9400–13,200 mg/m3 (5000–7000 ppm) for a period of 110 minutes (114). Ingestion of approximately one liter of an alcoholic beverage (40–55% ethanol) within several minutes can result in death (53). Individuals with blood alcohol levels of approximately 0.05–0.15 percent (50–150 mg/dl) may exhibit decreased inhibitions, poor coordination, blurred vision, and slowed reaction time. Increasing blood levels to 0.15–0.30 percent can result in slurred speech, visual impairment, hypoglycemia, and staggering. At 0.3–0.5 percent blood alcohol content (severe intoxication), symptoms can include muscular incoordination, hypothermia, vomiting and nausea, and convulsions. In adults, coma and death are typically associated with levels exceeding 0.5 percent (80, 114). The wide ranges reported above reflect the differences in tolerance and susceptibility of individuals to the effects of alcohol.

Like methanol, ethanol is metabolized primarily (about 90%) by the liver. Elimination from the body by urinary excretion and pulmonary exhalation is minimal (80). Oxidation of ethanol to acetaldehyde occurs via alcohol dehydrogenase within the cytosol. Acetaldehyde is then converted to acetic acid by action of aldehyde dehydrogenase. Both enzymes utilize oxidized nicotinamide adenine dicucleotide (NAD) as a cofactor (53). Following release to the blood, acetic acid is metabolized to carbon dioxide and water in the peripheral tissues (80). Alternative, but less active, metabolic pathways have been demonstrated in humans and other species. These include catalase and microsomal ethanol-oxidizing systems (26, 79). Adults metabolize ethanol at a rate of about 7–10 g/hr. This rate remains essentially constant for each individual within a wide range of exposure. Metabolic rates are higher for chronic alcoholics and children (80, 114).

The interaction of ethanol with other hepatotoxins is well known. Ethanol pretreatment has been shown to increase the toxicity of carbon tetrachloride, chloroform, trichloroethylene, dimethylnitrosamine, chlorpromazine, and other compounds (127). The induction of cytochrome P450 isozymes may be responsible for their metabolic effects (80).

Methylene Chloride

Methylene chloride (dichloromethane) is widely used in a number of diverse applications including manufacturing of polyurethane foams, production of pharmaceuticals, boat building, paint stripping, vapor degreasing, extraction of caffeine from coffee and tea, and various consumer products. Its high volatility, good solvent properties for fats, oils, and other compounds, and relatively good water solubility compared to other chlorinated compounds have made it quite valuable (103, 132).

Because of methylene chloride's high vapor pressure, the primary route of human exposure is through inhalation; however, dermal contact can be significant, depending on the application. The ACGIH TLV–TWA of 50 ppm was set to protect against CNS effects and anoxia. In addition, ACGIH has designated methlyene chloride as a confirmed animal carcinogen, but they also state that available epidemiological studies do not confirm an increased risk of cancer in exposed humans (4). NIOSH recommends that methylene chloride be regarded as a potential occupational carcinogen (101). OSHA considers methylene chloride a potential human carcinogen and has reduced the PEL for methylene chloride from 500 ppm to 25 ppm, with a STEL of 125 ppm (15 min) and an action level of 12.5 ppm that triggers certain requirements (103). The current German MAK is 100 ppm (2).

The primary acute hazards associated with exposure to methylene chloride are CNS depression and eye, skin, and respiratory tract irritation. In addition, one of the products of methylene chloride metabolism is

carbon monoxide, which can impair health in a manner similar to direct exposure to carbon monoxide. The resulting carboxyhemoglobin levels reduce the supply of oxygen

to the heart and may aggravate preexisting heart disease (103).

Metabolism of methylene chloride can proceed via two pathways, one by a route involving cytochrome P450 mixed-function oxidase (MFO) and the other by a route utilizing glutathione *S*-transferase (GST). Carbon dioxide is an end product in both systems, but carbon monoxide is only produced via the MFO route. At low concentrations the MFO system appears to dominate, but at higher concentrations (above 300–500 ppm) the glutathione pathway increases in a disproportionate manner (132).

Methylene chloride was shown in a 1986 National Toxicology Program inhalation study to produce lung and liver tumors in male and female mice and benign mammary tumors in male and female rats (56). Recent research has suggested that mice may be uniquely sensitive at high exposures to methylene chloride-induced lung and liver cancer (56). The tumors appear to be caused by a genotoxic mechanism involving metabolites of the GST pathway. The particular metabolites responsible are not found in high concentrations in lung or liver tissue in humans or rats.

In a study to determine the effects of alcohols and toluene upon methylene chloride-induced carboxyhemoglobin in the rat and monkey, it was shown that ethanol, methanol, isopropanol, and toluene inhibited the formation of carboxyhemoglobin. In addition, neither the rat nor the monkey demonstrated the methanol potentiation of carboxyhemoglobin that has been reported to occur in humans (32).

A study of the pharmacokinetics of [14C]methylene chloride in rats at 50, 500, and 1500 ppm for 6 h showed that metabolic processes were saturated above the 50 ppm exposure concentration. At 48 h postexposure, approximately 95 percent of the body burden attributable to the 50 ppm exposure was metabolized, in contrast to 69 percent and 45 percent at 500 and 1500 ppm, respectively (89). In addition, the production of carboxyhemoglobin reached a steady-state range of 10–13 percent regardless of the exposure concentration, suggesting that the CO metabolic pathway was saturated.

Tetrachloroethylene (perchloroethylene) is another solvent in which patterns of elimination are altered when metabolic pathways become saturated (105). In a study comparing oral and inhalation exposure of rats to [14C]tetrachloroethylene, it was found that with increasing dose, metabolism was saturated resulting in more of the parent compound being eliminated unchanged at 72 h after exposure (105). These results with methylene chloride and tetrachloroethylene indicate that just increasing the exposure concentration does not always increase the body burden in a linear manner. Such information may be useful for safety evaluations to avoid overestimation of body burden.

NONTRADITIONAL SOLVENTS

Given the negative health and environmental impacts created by some of the more widely used solvents, a great deal of effort has gone into finding suitable replacements. The following compounds are examples of nontraditional materials that show promise as replacement solvents.

d-Limonene

d-Limonene is a naturally occurring monocyclic terpene found in citrus peel oils, spices, evergreens, and human milk (140). It is considered to have low acute toxicity and is listed as GRAS (generally recognized as safe) as a food additive by the Food and Drug Administration (FDA) (21 CFR 182.60). It has found wide application as a solvent in numerous cleaning and degreasing applications, replacing more toxic and environmentally undesirable chlorinated solvents, glycol ethers, xylene, and chlorofluorocarbons (CFCs) (46). Skin contact with d-limonene may cause irritation and sensitization (attributed to the oxidation product d-limonene oxide) (140). d-Limonene has been shown to produce hyaline droplet nephropathy and renal tubular tumors in male rats. However, these effects are attributed to the unique presence of $\alpha 2u$-globulin in the male rat and are not deemed relevant to other species, including humans (45). Among the attributes of d-limonene are its antimicrobial, antiviral, antifungal, and antilarval properties (29). d-Limonene and related monoterpenes have also demonstrated chemopreventive and chemotherapeutic efficacy in experimental cancer-therapy models (37).

Based on similar metabolic pathways in rats and humans and the therapeutic successes in rodents, it has been suggested that d-limonene may be an efficacious chemotherapeutic agent for human malignancies (37).

Carbon Dioxide

Carbon dioxide is a gas under standard temperature and pressure conditions. It can be converted, however, to the liquid and supercritical phases by increasing pressure and temperature. The critical point of carbon dioxide is 31°C and 73 atm. Below this point, CO_2 can be maintained in a liquid state (e.g., 65 atm and 25°C), whereas above 31°C no amount of pressure can be applied to liquify it (supercritical phase) (65). In either of these dense phases, CO_2 exhibits good solvent properties. Beneficial characteristics include liquidlike density, gaslike diffusivity, and low surface tension. In

particular, liquid CO_2 acts like a hydrocarbon solvent, it has good homogenizing properties (i.e., immiscible liquids form a single phase when mixed with CO_2), and it is a good

solvent for many aliphatic hydrocarbons and most small aromatic hydrocarbons. Other chemical groups such as halocarbons, esters, ketones, and low molecular weight alcohols also exhibit good solvency in CO_2 (65). Since the mid-1970s, supercritical CO_2 technology has been employed in the food, beverage, pharmaceutical, and perfume industries. Applications include production of spice extracts, natural dyes, decaffeinated coffee and tea, plant extracts, active substances from drugs, and volatile oils (20, 38, 88). It has also been used in wastewater treatment, chemical analysis, and at times as an aerosol propellant. More recently, liquid CO_2 has found favor as an alternative for metal parts degreasing and as a solvent for dry-cleaning clothes (38, 70).

One such CO_2 degreasing system is being used in a pen manufacturing operation to replace perchloroethylene. It consists primarily of two separate systems, a hot oil pretreatment process, and an automated system that employs liquid carbon dioxide in a pressure vessel. The application is to degrease and remove chips from ball points after machining. The hot oil unit is used to displace fatty esters contained in machining oil and to remove chips in the point cavity. The automated unit then removes oil from the points using liquid carbon dioxide. The carbon dioxide and oil are separated in a recycling system and the carbon dioxide is used again during the next cleaning cycle.

Advantages of CO_2 usage over conventional solvents are numerous. Carbon dioxide is nonflammable, noncorrosive, nonreactive, nontoxic, inexpensive, and plentiful. Products obtained are solvent-free. Selective separations are possible. Finally, there are no environmental problems, since the gas is recovered for future use. One of the disadvantages of CO_2 systems involves the relatively high start-up costs for equipment. However, these may be recouped through improved productivity and reduced costs for waste disposal, for example.

Ionic Liquids

Ionic systems, which are made up of salts that are liquid at room temperature, are finding applications in a number of chemical processes. Ionic liquids have good solvent properties for many inorganic, organic, and, polymeric materials and, in some cases, these compounds can serve as both catalyst and solvent (49). Research has indicated that partitioning of organic solvents between an ionic liquid and water corresponds closely with that found for molecular organic solvents and water. Thus, ionic liquids have the potential to replace the toxic, flammable, and volatile organic compounds currently used in liquid-liquid separations (50). The room-temperature ionic compounds, such as 1-butyl-3-methylimidazolium hexafluorophosphate and 1-butylpyridinium nitrate, con sist of nitrogen-containing organic cations and inorganic anions. Their physical and chemical properties can be altered according to the choice of ions. Advantages compared to conventional organic solvents include low volatility and relative ease of recycling (50). Other potential uses include removal of organic contaminants from wastewater, soil cleanup, replacement of corrosive mineral acids in refinery processes, and spent nuclear fuel treatment (49). The safety and toxicological profiles of these compounds has yet to be thoroughly developed; therefore caution must be exercised before they are put into general use.

OPPORTUNITIES IN THE TOXICOLOGICAL EVALUATION OF SOLVENTS

Human exposure to solvents is quite common in today's society. These exposures frequently involve multiple chemicals that are found in numerous products such as cleaning agents, paint thinners, and fuels. Although most toxicological research to date has dealt with single chemicals, questions remain about the long-term health effects associated with low-level exposures to multiple chemicals and the sensitivity of the toxicological endpoints that are currently being relied upon. Development of innovative experimental protocols and new quantitative mechanistic approaches to the study of chemical interactions may be beneficial in this regard (74, 148).

Economic concerns and the desire for less toxic and more environmentally friendly chemicals have resulted in the introduction of numerous alternative compounds into the marketplace. In some cases, little may be known about the health and environmental impacts of these materials; examples include the ionic liquids discussed above. It is therefore essential that sufficient toxicological and environmental data be gathered before replacements are introduced on a wide scale.

Research has shown that many neurotoxic chemicals are capable of adversely affecting the sensory function. Minor changes in vision or hearing, for example, can dramatically alter job performance and the overall quality of life. While most reports to date have dealt with changes in the visual system, additional investigations into the effects of solvents on hearing, taste, and smell would provide important new information on this subject (48).

ACKNOWLEDGMENTS

The authors wish to thank Dr. Henry Ciuchta, Dr. Joseph Morton, and Mark Savell for reviewing portions of the manuscript and also Peter Kahla for his help

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with one of the figures. The authors also gratefully acknowledge the significant contributions of the previous writers Paul H.Ayres, W.David Taylor, and Michael J.Olson.

QUESTIONS

1. You are a toxicologist with industrial hygiene responsibilities in a large manufacturing company. Your boss has just told you that the solvent the factory is using to degrease metal parts will be banned by the EPA within the next six months. Your job is to lead a team of employees, who have a vested interest in the current solvent, in coming up with a suitable alternative material. What are your considerations in recommending a replacement? Explain.
2. Assume that the solvent chosen above will be used in six locations in the factory. You surmise that some sort of ventilation will be required to protect the employees. What factors must you take into account in recommending the proper system?
3. One of your employees has begun using a solvent mixture containing xylene and toluene. To ensure the safety of the worker, you have conducted personal air monitoring throughout the day and have come up with the following sampling times and monitoring results: 0800–1000:60 ppm xylene and 25 ppm toluene; 1000–1200:92 ppm xylene and 45 ppm toluene; 1200–1300: no exposure because employee left for lunch; 1300–1600:110 ppm xylene and 47 ppm toluene. Calculate the TWA exposure for each chemical. Assume that there is no dermal exposure and that the toxic effects contributed by each solvent are additive. Has the TLV–TWA been exceeded?
4. Match each solvent or metabolite with the appropriate fact listed below.

Solvent/Metabolite	*Fact*
1. d-Limonene	a. Antidotal in methanol poisonings
2. Carbon Disulfide	b. Associated with bone marrow disease in humans
3. 2,5-Hexanedione	c. Potentially useful in cancer therapy
4. Toluene	d. Teratogen and embryotoxin
5. Methanol	e. Metabolism produces carboxyhemoglobin
6. 2-Butoxyacetic Acid	f. Frequently “sniffed” to obtain euphoric effect
7. Ethylene Glycol Monomethyl Ether	g. Used in rayon production
8. Benzene	h. A few ml can lead to blindness
9. Ethanol	i. Primary causative agent in polyneuropathy
10. Methylene Chloride	j. Produces hemolytic effects in rats

REFERENCES

1. American Conference of Governmental Industrial Hygienists, Inc. (ACGIH). (1995): *Air Sampling Instruments for Evaluation of Atmospheric Contaminants* 8th ed., ACGIH, Cincinnati, OH.
2. ACGIH. (1998): *Guide to Occupational Exposure Values—1998*. ACGIH, Cincinnati, OH.
3. ACGIH. (1998): *Industrial Ventilation: A Manual of Recommended Practice*, 23rd ed., ACGIH, Inc., Cincinnati, OH.
4. ACGIH. (2000): *2000 TLVs® and BEIs®, Threshold Limit Values for Chemical Substances and Physical Agents, and Biological Exposure Indices*. ACGIH, Cincinnati, OH.
5. American Industrial Hygiene Association (AIHA). (1990): *Chemical Protective Clothing*, edited by J.S.Johnson and K.J. Anderson. AIHA, Akron, OH.
6. AIHA. (1997): *The Occupational Environment—Its Evaluation and Control*, edited by S.R.DiNardi. AIHA Press, Fairfax, VA.
7. Alden, C.L. (1986): A review of unique male rat hydrocarbon nephropathy. *Toxicol. Pathol.*, 14:109–111.
8. Allen, N. (1979): Solvents and other industrial organic compounds. In: *Handbook of Clinical Neurology Intoxications of the Nervous System*, Part 1(36), edited by P.J.Vinken and G.W.Bruyn, pp. 361–389. Elsevier/North-Holland, New York.
9. Allen, N., Mendell, J.R., Billmaier, D.J., Fontaine, R.E., and O'Neill, J. (1975): Toxic polyneuropathy due to methyl n-butyl ketone. *Arch. Neurol.*, 32:209–218.
10. Anger, W.K. (1986): Workplace exposures. In: *Neurobehavioral Toxicology*, edited by Z.Annau, pp. 331–347. Johns Hopkins University Press, Baltimore.
11. Anon. (1997): Facts and figures for the chemical industry. *Chem. Eng. News*, 75(25):38–79.
12. Anon. (1998): Facts and figures for the chemical industry. *Chem. Eng. News*, 76(26):40–81.
13. Astrand, I. (1975): Uptake of solvents in the blood and tissues of man. *Scand. J. Work Environ. Health*, 1:199–218.
14. Astrand, I. (1985): Uptake of solvents from the lungs. *Br. J. Ind. Med.*, 42:217–218.
15. Baelum, J., Anderson, I., Lundqvist, G.R., Molhave, L., Pedersen, O.F., Vaeth, M., and Wyon, D.P.

(1985): Response of solvent-exposed printers and unexposed controls to six-hour toluene exposure. *Scand. J. Work Environ. Health*, 11:271–280.
16. Baerg, R.D., and Kimberg, D.V. (1970). Centrilobular hepatic necrosis and acute renal failure in "solvent sniffers." *Ann. Intern. Med.*, 73:713–720.
17. Bahl, M.K. (1985): ESCA studies on skin lipid removal by solvents and surfactants. *J. Soc. Cosmet. Chem.*, 36:287–296.

18. Baker, E.L. (1988): Organic solvent neurotoxicity. *Annu. Rev. Public Health*, 9:223–232.
19. Bamberger, R.L., Esposito, G.G., Jacobs, B.W., Podolak, G.E., and Mazur, J.F. (1978): A new personal sampler for organic vapors. *Am. Ind. Hyg. Assoc. J.*, 39:701–708.
20. Basta, N., and McQueen, S. (1985): Supercritical fluids: Still seeking acceptance. *Chem. Eng.*, 92:14–17.
21. Beliles, R.P., and Beliles, E.M. (1993): Phosphorus, selenium, tellurium, and sulfur. In: *Patty's Industrial Hygiene and Toxicology*, Vol. IIA, 4th ed., edited by G.D.Clayton and F. E.Clayton, pp. 818–822. John Wiley, New York.
22. Boekelheide, K. (1987): 2,5-Hexanedione alters microtubule assembly. 1. Testicular atrophy, not nervous system toxicity, correlates with enhanced tubulin polymerization. *Toxicol. Appl. Pharmacol.*, 88:370–382.
23. Boggs, A. (1989): Comparative risk assessment of casting solvents for positive photo resist. *Appl. Ind. Hyg.*, 4:81–87.
24. Bohlen, P., Schlunegger, U.P., and Lauppi, E. (1973): Uptake and distribution of hexane in rat tissues. *Toxicol. Appl. Pharmacol.*, 25:242–249.
25. Bos, P.M., deMik, G., and Bragt, P.C. (1991): Critical review of the toxicity of methyl n-butyl ketone: Risk from occupational exposure. *Am. J. Ind. Med.*, 20:175–194.
26. Bradford, B.U., Seed, C.B., Handler, J.A., Forman, D.T., and Thurman, R.G. (1993): Evidence that catalase is a major pathway of ethanol oxidation in vivo: Dose-response studies in deer mice using methanol as a selective substrate. *Arch. Biochem. Biophys.*, 303:172–176.
27. Browning, R.G., and Curry, S.C. (1994): Clinical toxicology of ethylene glycol monoalkyl ethers. *Human and Exper. Toxicol.*, 13:325–335.
28. Brugnone, F., Perbellini, L., Gaffuri, E., and Apostoli, P. (1980): Biomonitoring of industrial solvent exposures in workers' alveolar air. *Int. Arch. Occup. Environ. Health*, 47:245–261.
29. Cavender, F. (1994): Alicyclic hydrocarbons: Limonene. In: *Patty 's Industrial Hygiene and Toxicology*, Vol. IIB, 4th ed., edited by G.D.Clayton and F.E.Clayton, pp. 1282–1283. John Wiley, New York.
30. Cavender, F. (1994): Aliphatic hydrocarbons: Hexanes. In: *Patty's Industrial Hygiene and Toxicology*, Vol. IIB, 4th ed., edited by G. D.Clayton and F.E.Clayton, pp. 1233–1234. John Wiley, New York.
31. Cavender, F. (1994): Aromatic hydrocarbons: Toluene. In: *Patty 's Industrial Hygiene and Toxicology*, Vol. IIB, 4th ed., edited by G. D.Clayton and F.E.Clayton, pp. 1326–1332. John Wiley, New York.
32. Ciuchta, H.P., Savell, G.M., and Spiker, R.C. (1979): The effects of alcohols and toluene upon methylene chloride-induced carboxyhemoglobin in the rat and monkey. *Toxicol. Appl. Pharmacol.*, 49:347–354.
33. Clarren, S.K., and Smith, D.W. (1978): The fetal alcohol syndrome. *N. Engl. J. Med.*, 198:1063–1067.
34. Colombi, A., Maroni, M., Picchi, O., Rota, E., Castano, P., and Foa, V. (1981): Carbon disulfide neuropathy in rats. A morphological and ultrastructural study of degeneration and regeneration. *Clin. Toxicol.*, 18:1463–1474.
35. Corsi, G., Maestrelli, P., Picotti, G., Manzoni, S., and Negrin, P. (1983): Chronic peripheral neuropathy in workers with previous exposure to carbon disulphide. *Br. J. Ind. Med.*, 40:209–211.
36. Costa, L.G., and Manzo, L. (1998): Biological monitoring of occupational neurotoxicants. In: *Occupational Neurotoxicology*, edited by L.G.Costa and L.Manzo, p. 90. CRC Press, Boca Raton, FL.
37. Crowell, P.L., Elson, C.E., Bailey, H.H., Elegbede, A., Haag, J. D., and Gould, M.N. (1994): Human metabolism of the experimental cancer therapeutic agent d-limonene. *Cancer Chemother. Pharmacol.*, 35:31–37.
38. Darvin, C.H., and Hill, E.A. (1996): Demonstration of liquid CO2 as an alternative for metal parts cleaning. *Precision Cleaning*, 4(9):25–32.
39. DeCaprio, A.P. (1985): Molecular mechanisms of diketone neurotoxicity. *Chem. Biol. Interact.*, 54:257–270.
40. DeCaprio, A.P., and O'Neill, E.A. (1985): Alterations in rat axonal cytoskeletal proteins induced by in vitro and in vivo 2,5-hexanedione exposure. *Toxicol. Appl. Pharmacol.*, 78:235–247.
41. DiVincenzo, G.D., Hamilton, M.L., Kaplan, C.J., Krasavage, W. J., and O'Donoghue, J.L. (1978): Studies on the respiratory uptake and excretion and the skin absorption of methyl n-butyl ketone in humans and dogs. *Toxicol. Appl. Pharmacol.*, 44:593–604.
42. Dossing, M., Baelum, J., Hansen, S.H., and Lundqvist, G.R. (1984): Effect of ethanol, cimetidine and

propranolol on toluene metabolism in man. *Int. Arch. Occup. Environ. Health*, 54:309–315.
43. Environmental Protection Agency (EPA), Office of Pollution Prevention and Toxics. (1994): Chemical summary for methanol. *EPA 749-F-94–013a*, pp. 1–9.
44. Fiserova-Bergerova, V., and Diaz, M.L. (1986): Determination and prediction of tissue-gas partition coefficients. *Int. Arch. Occup. Environ. Health*, 58:75–87.
45. Flamm, W.G., and Lehman-McKeeman, L.D. (1991): The human relevance of the renal tumor-inducing potential of d-limonene in male rats: Implications for risk assessment. *Reg. Toxicol. Pharm.*, 13:70–86.
46. Florida Chemical Co. Inc. (1997): *d-Limonene Product Data Sheet*. Winter Haven, FL.
47. Forni, A.M., Cappellini, A., Pacifico, E., and Vigliani, E.C. (1971): Chromosome changes and their evolution in subjects with past exposure to benzene. *Arch. Environ. Health*, 23:385–391.
48. Fox, D.S. (1998): Sensory system alterations following occupational exposure to chemicals. In: *Occupational Neurotoxicology*, edited by L.G.Costa and L.Manzo, pp. 169–184. CRC Press, Boca Raton, FL.
49. Freemantle, M. (1998): Designer solvents. *Chem. Eng. News*, 13:32–37.
50. Freemantle, M. (1998): Ionic liquids show promise for clean separation technology. *Chem. Eng. News*, 34:12.
51. Gargas, M.L., Burgess, R.J., Voisard, D.J., Cason, G.H., and Andersen, M.E. (1989): Partition coefficients of low molecular weight volatile chemicals in various liquids and tissues. *Toxicol. Appl Pharmacol.*, 98:87–99.
52. Gingell, R., Boatman, R.J., Bus, J.S., Cawley, R.J., Knaak, J.B., Krasavage, W.J., Skoulis, N.P., Stack, C.R., and Tyler, T.R. (1994): Glycol ethers and other selected glycol derivatives. In: *Patty's Industrial Hygiene and Toxicology*, Vol. IID, 4th ed., edited by G.D.Clayton and F.E.Clayton, pp. 2761–2966. John Wiley, New York.
53. Gosselin, R.E., Smith, R.P., and Hodge, H.C. (1984): Ethyl alcohol and methyl alcohol. In: *Clinical Toxicology of Commercial Products*, Section III, 5th ed., pp. 166–171, 275–279. Williams & Wilkins, Baltimore/London.
54. Graham, D.G., Amarnath, V., Valentine, W.M., Pyle, S.J., and Anthony, D.C. (1995): Pathogenic studies of hexane and carbon disulfide neurotoxicity. *Crit. Rev. Toxicol.*, 25(2):91–112.
55. Grasso, P., Sharratt, M., Davies, D.M., and Irvine, D. (1984): Neurophysiological and psychological disorders and occupational exposure to organic solvents. *Food Chem. Toxicol.*, 22:819–852.
56. Halogenated Solvents Industry Alliance (1998): Methylene chloride white paper. *HSIA*, pp. 1–6, Washington, DC.

57. Hanninen, H. (1985): Twenty-five years of behavioral toxicology within occupational medicine: A personal account. *Am. J. Ind. Med.*, 7:19–30.
58. Hardin, B.D. (1983): Reproductive toxicity of the glycol ethers. *Toxicohgy*, 27:91–102.
59. Hardin, B.D., Bond, G.P., Sikov, M.R., Andrew, F.D., Beliles, R.P., and Niemeier, R.W. (1981): Testing of selected workplace chemicals for teratogenic potential. *Scand. J. Work Environ. Health*, 7:66–75.
60. Hardin, B.D., Niemeier, R.W., Smith, R.J., Kuczuk, M.H., Mathinos, P.R., and Weaver, T.F. (1982): Teratogenicity of 2-ethoxyethanol by dermal application. *Drug Chem. Toxicol.*, 5:277–294.
61. Heck, H.d'A., Casanova, M, and Starr, T.B. (1990): Formaldehyde toxicity: New understanding. *CRC Crit. Rev. Toxicol.*, 20:397–426.
62. Herskowitz, A., Ishii, N., and Schaumburg, H. (1971): n-Hexane neuropathy. *N. Engl. J. Med.*, 285:82–85.
63. Hoet, P., and Lauwerys, R. (1998): Biological monitoring of occupational neurotoxicants. In: *Occupational Neurotoxicology*, edited by L.G.Costa and L.Manzo, pp. 57–58. CRC Press, Boca Raton, FL.
64. Huff, J.E., Haseman, J.K., DeMarini, D.M., Eustis, S., Maronpot, R.R., Peters, A.C., Persing, R.L., Chrisp, C.E., and Jacobs, A.C. (1989): Multiple-site carcinogenicity of benzene in Fischer 344 rats and B6C3F mice. *Environ. Health Perspect.*, 82:125–163.
65. Hyatt, J.A. (1984): Liquid and supercritical carbon dioxide as organic solvents. *J. Org. Chem.*, 49:5097–5101.
66. Ikeda, M., and Ohtsuji, H. (1971): Phenobarbital-induced protection against toxicity of toluene and benzene in the rat. *Toxicol. Appl. Pharmacol.*, 20:30–43.
67. Irons, R.D., Dent, J.G., Baker, T.S., and Rickert, D.E. (1980): Benzene is metabolized and covalently bound in bone marrow in situ. *Chem. Biol. Interact.*, 30:241–245.
68. Johnson, A., and Nylen, P. (1995): Effects of industrial solvents on hearing. *Occup. Med.*, 10(3):623–640.
69. Johnson, B.L., ed. (1990): *Advances in Neurobehavioral Toxicology: Applications in Environmental and Occupational Health*. Lewis, Chelsea, MI.
70. Kaplan, K. (1997): A new spin on dry cleaning. *Los Angeles Times*, September 8, 1997.
71. Knox, J.W., and Nelson, J.R. (1966): Permanent encephalopathy from toluene inhalation. *N. Engl. J. Med.*, 273:1494–1496.
72. Kramer, A., Staudinger, H., and Ullrich, V. (1974): Effect of n-hexane inhalation on the monooxygenase system in mice liver microsomes. *Chem. Biol. Interact.*, 8:11–18.
73. Krasavage, W.J., O'Donoghue, J.L., DiVincenzo, G.D., and Terhaar, C.J. (1980): The relative neurotoxicity of methyl n-butyl ketone, n-hexane and their metabolites. *Toxicol. Appl. Pharmacol.*, 52:433–441.
74. Krishnan, K., Andersen, M.E., Clewell III, H J., and Yang, R.S. H. (1994): Physiologically based pharmacokinetic modeling of chemical mixtures. In: *Toxicology of Chemical Mixtures*, edited by R.S.H.Yang, pp. 399–433. Academic Press, San Diego.
75. Laham, S. (1970): Metabolism of industrial solvents. *Ind. Med.*, 39:61–64.
76. Lee, E.W., Kocsis, J.J., and Snyder, R. (1974): Acute effects of benzene on 59-Fe incorporation into circulating erythrocytes. *Toxicol. Appl. Pharmacol.*, 27:431–436.
77. Lehman-McKeeman, L.D., Rivera-Torres, M.I., and Caudill, D. (1990): Lysosomal degradation of α2u-globulin and α2u-globulin-xenobiotic conjugates. *Toxicol. Appl. Pharmacol.*, 103: 539–548.
78. Lewey, F.H. (1941): Neurological, medical, and biochemical signs and symptoms indicating chronic industrial carbon disulphide absorption. *Ann. Intern. Med.*, 15:869–883.
79. Lieber, C.S., and DeCarli, L.M. (1970): Hepatic microsomal ethanol-oxidizing system. *J. Biol. Chem.*, 245:2505–2512.
80. Lington, A.W., and Bevan, C. (1994): Alcohols. In: *Patty's Industrial Hygiene and Toxicology*, Vol. IID, 4th ed., edited by G.D. Clayton and F.E.Clayton, pp. 2585–2622. John Wiley, New York.
81. Lock, E.A. (1988): Studies on the mechanism of nephrotoxicity and nephrocarcinogenicity of halogenated alkenes. *CRC Crit. Rev. Toxicol.*, 19:23–42.
82. Lock, E.A., and Ishmael, J. (1985): Effect of the organic acid transport inhibitor probenicid on renal cortical uptake and proximal tubular toxicity of hexachloro-1,3-butadiene and its conjugates. *Toxicol. Appl. Pharmacol.*, 81:32–42.
83. Longacre, S.L., Kocsis, J.J., and Snyder, R. (1981): Influence of strain differences in mice on the metabolism and toxicity of benzene. *Toxicol. Appl. Pharmacol.*, 60:398–409.
84. Lu, A.Y.H., Strobel, H.W., and Coon, M.J. (1970): Properties of a solubilized form of the cytochrome

P450-containing mixed-function oxidase of liver microsomes. *Mal. Pharmacol.*, 6:213–220.
85. Maron, S.H., and Prutton, C.F. (1965): *Principles of Physical Chemistry*, 4th ed., pp. 215–216, 285. Macmillan Company, New York.
86. Matheson, L.E., Jr., Wurster, D.E., and Ostrenga, J.A. (1979): Sarin transport across excised human skin. II: Effect of solvent pretreatment on permeability. *J. Pharm. Sci.*, 11:1410–1413.
87. Mattia, C.J., LeBel, C.P., and Bondy, S.C. (1991): Effects of toluene and its metabolite on cerebral reactive oxygen species generation. *Biochem. Pharmacol.*, 42:879–882.
88. McHugh, M.A. (1986): Extraction with supercritical fluids. In: *Recent Developments in Separation Science*, Vol. 9, edited by N.Li and J.Calo, pp. 75–105. CRC Press, Boca Raton, FL.
89. McKenna, M.J., Zempel, J.A., and Braun, W.H. (1982): The pharmacokinetics of inhaled methylene chloride in rats. *Toxicol. Appl. Pharmacol.*, 65:1–10.
90. Medinsky, M.A., Schlosser, P.M., and Bond, J.A. (1994): Critical issues in benzene toxicity and metabolism: The effect of interactions with other organic chemicals on risk assessment. *Environ. Health Perspect.*, 102(9): 119–124.
91. Mendell, J.R., Saida, K., Ganansia, M.F., Jackson, D.B., Weiss, H., Gardier, R.W., Chrisman, C., Allen, N., Couri, D., O'Neill, J.J., Marks, B.H., and Hetland, L.B. (1974): Toxic polyneuropathy produced by methyl n-butyl ketone. *Science*, 185:787–789.
92. Menger, F.M, Goldsmith, D.J., and Mandell, L. (1972): *Organic Chemistry—A Concise Approach*, p. 450. W.A.Benjamin, Menlo Park, CA.
93. Merck Index. (1996): 12th ed., edited by S.Budavari. Merck & Co., Inc., Whitehouse Station, NJ.
94. Miller, R.R., Hermann, E.A., Langvardt, P.W., McKenna, M.J., and Schwetz, B.A. (1983): Comparative metabolism and disposition of ethylene glycol monomethyl ether and propylene glycol monomethyl ether in male rats. *Tox. Appl. Pharmacol.*, 67:229–237.
95. Miller, R.R., Hermann, E.A., Young, J.T., Calhoun, L.L., and Kastl, P.E. (1984): Propylene glycol monomethyl ether acetate (PGMEA) metabolism, disposition, and short-term vapor inhalation toxicity studies. *Toxicol. Appl. Pharmacol.*, 75:521–530.
96. Miller, R.R., Hermann, E.A., Young, J.T., Landry, T.D., and Calhoun, L.L. (1984): Ethylene glycol monomethyl ether and propylene glycol monomethyl ether: Metabolism, disposition, and subchronic inhalation toxicity studies. *Environ. Health Persp.*, 57:233–239.

97. Monteiro-Riviere, N.A., and Popp, J.A. (1986): Ultrastructural evaluation of acute nasal toxicity in the rat respiratory epithelium in response to formaldehyde gas. *Fund. Appl. Toxicol.*, 6:251–262.
98. Morata, T.C., and Dunn, D.E. (1994): Occupational exposure to noise and ototoxic organic solvents. *Arch. Environ. Health*, 49: 359–365.
99. Nelson, B.K., Setzer, J.V., Brightwell, W.S., Mathinos, P.R., Kuczuk, M.H., Weaver, T.E., and Goad, P.T. (1984): Comparative inhalation teratogenicity of four glycol ether solvents and an amino derivative in rats. *Environ. Health Persp.*, 57:261–271.
100. Nelson, B.K., Brightwell, W.S., Setzer, J.V., and O'Donohue, T. L. (1984): Reproductive toxicity of the industrial solvent 2-ethoxyethanol in rats and interactive effects of ethanol. *Environ. Health Persp.*, 57:255–259.
101. NIOSH (1997): *Pocket Guide to Chemical Hazards.* pp. 1–341. NIOSH Publications, Cincinnati, OH.
102. Occupational Safety and Health Administration (OSHA). (1998): Benzene. *29 CFR 1910.1028*, pp. 235–253.
103. OSHA. (1997): Methylene Chloride. *29 CFR 1910.1052*, pp. 479–496.
104. Paustenbach, D.J. (1994): Occupational exposure limits, pharmacokinetics, and unusual work schedules: In: *Patty's Industrial Hygiene and Toxicology*, Vol. IIIA, 4th ed., edited by G. D.Clayton and F.E.Clayton, pp. 191–348. John Wiley, New York.
105. Pegg, D.G., Zempel, J.A., Braun, W.H., and Watanabe, P.G. (1979): Disposition of tetrachloro(14C)ethylene following oral and inhalation exposure in rats. *Toxicol. Appl. Pharmacol.*, 51:465–474.
106. Perbellini, L., Brugnone, F., Caretta, D., and Maranelli, G. (1985): Partition coefficients of some industrial aliphatic hydrocarbons (C5–C7) in blood and human tissues. *Br. J. Ind. Med.*, 42:162–167.
107. Pergal, M., Vukojevic, N., and Djuric, D. (1972): Isolation and identification of thiocarbamide. *Arch. Environ. Health*, 25:42–44.
108. Pergal, M., Vukojevic, N., Cirin-Popov, N., Djuric, D., and Bojovic, T. (1972): Carbon disulfide metabolites excreted in the urine of exposed workers. *Arch. Environ. Health*, 25:38–41.
109. Pohl, L.R. (1979): Biochemical toxicology of chloroform. In: *Reviews in Biochemical Toxicology*, Vol. 1, edited by E.Hodgson, J.R.Bend, and R.M.Philpot, pp. 79–107. Elsevier/North Holland, New York.
110. Pratt, G.E. (1982): Alcohol and the developing fetus. *Br. Med. Bull.*, 38:48–53.
111. Price, B., Bergman, T.S., Rodriquez, M., Henrich, R.T., and Moran, E.J. (1997): A review of carbon disulfide exposure data and the association between carbon disulfide exposure and ischemic heart disease mortality. *Reg. Toxicol. Pharmacol.*, pp. 119–128.
112. Rafales, L.S. (1986): Assessment of locomotor activity. In: *Neurobehavioral Toxicology*, edited by Z.Annau, pp. 54–68. Johns Hopkins University Press, Baltimore.
113. Recknagel, R.O. (1967): Carbon tetrachloride hepatotoxicity. *Pharmacol. Rev.*, 19:145–208.
114. Reese, E., and Kimbrough, R.D. (1993): Acute toxicity of gasoline and some additives. *Environ. Health Prospect. Suppl.*, 101 (Suppl. 6):115–131.
115. Reinhardt, C.F., Mullin, L.S., and Maxfield, M.E. (1973): Epinephrine-induced cardiac arrhythmia potential of some common industrial solvents. *J. Occup. Med.*, 15:953–955.
116. Riihimaki, V., and Pfaffli, P. (1978): Percutaneous absorption of solvent vapors in man. *Scand. J. Work Environ. Health*, 4:73–85.
117. Rubin, E., and Lieber, C.S. (1972): The effects of ethanol on the liver. In: *International Review of Experimental Pathology*, edited by G.W.Richter and M.A.Epstein, pp. 177–232. Academic Press, New York.
118. Sabourin, P.J., Medinsky, M.A., Thurmond, F., Birnbaum, L.S., and Henderson, R.F. (1992): Effect of dose on the disposition of methoxyethanol, ethoxyethanol, and butoxyethanol administered dermally to male F344/N rats. *Fund. Appl. Toxicol.*, 19:124–132.
119. Sato, A., and Nakajima, T. (1979): Partition coefficients of some aromatic hydrocarbons and ketones in water, blood, and oil. *Br. J. Ind. Med.*, 36:231–234.
120. Scheflan, L., and Jacobs, M.B. (1953): *The Handbook of Solvents*, p. 728. Van Nostrand Reinhold, New York.
121. Short, B.G., Burnett, V.L., Cox, M.G., Bus, J.S., and Swenberg, J.A. (1987): Site-specific renal cytotoxicity and cell proliferation in male rats exposed to petroleum hydrocarbons. *Lab. Invest.*, 57:564–577.
122. Snyder, C.A., Goldstein, B.D., Sellakumar, A., Wolman, S.R., Bromberg, L., Erlichman, M.N., and Laskin, S. (1978): Hematotoxicity of inhaled benzene to Sprague-Dawley rats and AKR mice at 300 ppm. *J. Toxicol. Environ. Health*, 4:605–618.

123. Snyder, R., and Kocsis, J.J. (1975): Current concepts of chronic benzene toxicity. *CRC Crit. Rev. Toxicol.*, 3:265–288.
124. Spencer, P.S., and Schaumburg, H.H. (1977): Ultrastructural studies of the dying-back process. IV. Differential vulnerability of PNS and CNS fibers in experimental central-peripheral distal axonopathies. *J. Neuropathol. Exp. Neurol.*, 36:300–320.
125. Spencer, P.S., Schaumburg, H.H., Raleigh, R.L., and Terhaar, C. J. (1975): Nervous system degeneration produced by the industrial solvent methyl n-butyl ketone. *Arch. Neurol.*, 32:219–222.
126. Stott, W.T., and McKenna, M.J. (1985): Hydrolysis of several glycol ether acetates and acrylate esters by nasal mucosal carboxylesterase in vitro. *Fund. Appl. Toxicol.*, 5:399–404.
127. Strubelt, O. (1980): Interaction between ethanol and other hepatotoxic agents. *Biochem. Pharmacol.*, 29:1445–1449.
128. Swenberg, J.A., Short, B., Borghoff, S., Strasser, J., and Charbormeau, M. (1989): The comparative pathobiology of α2u-globulin nephropathy. *Toxicol. Appl. Pharmacol.*, 97:35–46.
129. Taher, S.M., Anderson, R.J., McCartney, R., Popovtzer, M.M., and Schrier, R.W. (1974): Renal tubular acidosis associated with toluene “sniffing.” *N. Engl. J. Med.*, 290:765–768.
130. Thomas, J.A., and Ballantyne, B. (1990): Occupational reproductive risk: Sources, surveillance, and testing. *J. Occup. Med.*, 32:547–554.
131. Topping, D.C., Morgott, D.A., David, R.M., and O'Donoghue, J.L. (1994): Ketones. In: *Patty's Industrial Hygiene and Toxicology*, Vol. IIC, 4th ed., edited by G.D.Clayton and F. E.Clayton, pp. 1739–1787. John Wiley, New York.
132. Torkelson, T.R. (1994): Halogenated aliphatic hydrocarbons containing chlorine, bromine, and iodine. In: *Patty's Industrial Hygiene and Toxicology*, Vol. IIE, 4th ed., edited by G.D.Clayton and F.E.Clayton, pp. 4034–4045. John Wiley, New York.
133. Trela, B.A., and Bogdanffy, M.S. (1991): Carboxylesterase-dependent cytotoxicity of dibasic esters (DBE) in rat nasal explants. *Toxicol. Appl. Pharmacol.*, 107:285–301.
134. Van Dolah, R.W. (1965): Flame propagation, extinguishment, and environmental effects on combustion. *Fire Technol.*, 2:138–145.
135. van Doorn, R., Delbressine, L.P.C., Leijdekkers, C.M., Vertin, P. G., and Henderson, P.H. (1981): Identification and determination of 2-thiothiazolidine-4-carboxylic acid in urine of workers exposed to carbon disulfide. *Arch. Toxicol.*, 47:51–58.
136. van Doorn, R., Leijdekkers, C.P.M.J.M., Henderson, P.T., Vanhoome, M., and Vertin, P.G. (1981): Determination of thio compounds in urine of workers exposed to carbon disulfide. *Arch. Environ. Health*, 36:289–297.
137. Vigliani, E.C. (1950): Clinical observations on carbon disulfide intoxication in Italy. *Ind. Med. Surg.*, 19:240–242.
138. Vigliani, E.C., and Fomi, A. (1976): Benzene and leukemia. *Environ. Res.*, 11:122–127.

139. Vincent, J.H. (1998): International occupational exposure standards: A review and commentary. *Am. Ind. Hyg. Assoc. J.*, 59:729–742.
140. Von Burg, R. (1995): Toxicology update: Limonene. *J. Appl. Toxicol.*, 15(6):495–499.
141. Voog, L., and Eriksson, T. (1984): Toluene-induced decrease in rat plasma concentrations of tyrosine and tryptophan. *Acta Pharmacol. Toxicol.*, 54:151–153.
142. Wilkins-Haug, L. (1997): Teratogen update: Toluene. *Teratology*, 55:145–151.
143. Winder, C., and Ng, S.K. (1995): The problem of variable ingredients and concentration in solvent thinners. *Am. Ind. Hyg. Assoc. J.*, 56:1225–1228.
144. Winek, C.L., and Collom, W.D. (1971): Benzene and toluene fatalities. *J. Occup. Med.*, 13:259–261.
145. World Health Organization (WHO). (1990): 2-methoxyethanol, 2-ethoxyethanol, and their acetates. *Environ. Health Crit.*, 115.
146. WHO (1991): n-Hexane. *Environ. Health Crit.*, 122.
147. Yamada, S. (1964): An occurrence of polyneuritis by n-hexane in the polyethylene laminating plants. *Jpn. J. Ind. Health*, 6:192–194.
148. Yang, R.S.H. (1994): Introduction to the toxicology of chemical mixtures. In: *Toxicology of Chemical Mixtures*, edited by R.S. H.Yang, pp. 1–10. Academic Press, San Diego.

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Crop Protection Chemicals

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Acetyl-CoA Carboxylase Inhibitors, 606
Acetolactate Synthase Inhibitors, 606
Photosynthesis Inhibitors, 615
Protoporphyrinogen Inhibitors, 625
Bleaching Herbicides, 629
EPSP Synthase, Glutamine Synthase, and Dihydropteroate (DHP) Synthase Inhibitors, 629
Microtubule Assembly Inhibitors, 629
Cell Division Inhibitors, 629
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Synthetic Auxin Mimics (Phenoxy, Benzoic, and Pyridine Acids), 630
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The control of pests using chemicals dates back to more than 1000 BC by the Chinese (93). Sulfur, the first documented material, was found somewhat effective as a fumigant. However, for nearly 2500 years no additional pest control products were found. In the 16th century, the Chinese discovered arsenic could be used as an insecticide (159). Tobacco leaf (nicotine) and the seed of *Strychnos nux vomica* (strychnine) were established as possessing rodenticidal properties in 1700 (102). It was not until the mid-1800s that the insecticidally active botanicals, rotenone from the root of *Derris eliptica* and pyrethrum from the flowers of chrysanthemums, were added as insecticides. In 1880, Bordeaux mixture (copper sulfate, lime, calcium hydroxide, and water) was introduced for mildew control (93, 159). Paris

green (copper arsenite) and later calcium arsenite were used extensively at the turn of the century for Colorado potato beetle (102). The inorganic arsenicals and nature's botanicals were used for insecticides, and inorganic sulfur products for herbicides and fungicides. It was with this limited level of achievement that mankind entered the 20th century (160).

The Age of Synthetic Chemistry was ushered in with the synthetic organochlorine chemical called dichlorodiphenyltrichloroethylene or DDT (Figure 13.1). A German chemist named Zeidler (148) synthesized DDT in 1874. However, it was not until 1939 that its insecticidal properties were fully realized. DDT was developed as part of the carefully planned and targeted research of an industrial chemist, Dr. Paul Mueller (158). Despite its persistence and bioaccumulating properties, DDT has probably been responsible for saving more human lives than any other synthetic chemical in history (157).

FIG. 13.1. The structure of dichlorodiphenyltrichloroethylene (DDT).

Of all the communicable diseases, malaria has had the greatest impact on society living in tropical and subtropical areas (157). During the first half of the 20th century, every year more than 300 million people suffered from malaria. The mortality rate was estimated at 1%; 3 million people dying annually. By 1965, or less than a quarter of century after its introduction, DDT had effectively eradicated malaria as a threat to 953 million people (319). In addition to malaria, DDT controlled the outbreak of louse-borne typhus, the plague, and yellow fever, the three other most significant diseases in human history. DDT also has been credited as offering some level of control for 20 other human diseases including viral encephalitis, shigellosis, cholera, and tularemia (157).

The development and growth of the agricultural chemistry industry was rapid, particularly in regard to synthetic insect control and weed control agents, following World War II (90). Clearly, there was an intense focus on a diverse number of structures arising from the targeted application of principles of chemistry to the mechanism(s) of action toward specific and selective pest control. The use of synthetic organic crop protection chemicals grew in the United States until 1980 and then has gradually declined thereafter (98). The figures for 1992 sales were essentially the same as the 1985 number or 1.1 billion lb; 1993 sales were estimated as 1.3 billion lb (160), and 1.2 billion lb in 1995 (1).

In the United States during the last 50 years, the use of synthetic organic pesticides has enabled agriculturalists to increase crop yields as much as 50% while affording a reduction in the farm population involved by 69% (2). Despite the reduction in the number of farmers from approximately 7% to 2% of the population, the individual farmer produces enough food to feed an average of 129 people. Control of crop pests leads to healthier plants and thus reduction of mycotoxins and endotoxins, which can present significant human health risk.

Even with the extensive use of pesticides, the U.S. Food and Drug Administration (FDA) estimates that pests destroy approximately one-third of the world's food crops every year (3). The loss in the United States alone is nearly $20 billion per year. Despite the clear benefits from pesticide use, the potential hazard from their application has long been recognized and their uses are tightly regulated and monitored.

The risks associated with pesticides are evaluated by assessing the toxicity of each chemical, and estimating the magnitude of exposure from sources in the workplace, in the environment, in food, and in water. The need to balance the risk of damage to the environment and man against benefits associated with using pesticides is clear (87).

In the United States, the regulation of pesticides is covered by several legislative acts and enforced by several federal and state agencies. A brief historical review of pesticide legislation is provided as a background for current provisions for federal regulation of pesticides by the U.S. Environmental Protection Agency (EPA). Further details for the toxicology study requirements ascribed by the U.S. EPA in its guidelines (171) will be considered. In addition, guidelines, which have been generally harmonized with the U.S. EPA guidelines, are put forth by the Japanese Ministry of Agriculture, Forestry and Fisheries (MAFF), and the Organization for Economic Cooperation and Development or OECD (149, 155). Further, the U.S. EPA guidelines have been generally harmonized with those of the European Economic Community (96). This is only logical, as the United States is a member nation of the EEC and Japan belongs to the OECD. The OECD nations include Australia, Austria, Belgium, Canada, Denmark, Finland, France, Germany, Italy, Japan, the Netherlands, Norway, Spain, Sweden, Switzerland, Turkey, the United Kingdom, and the United States.

HAZARD CHARACTERIZATION OF PESTICIDES

Federal Insecticide, Fungicide, and Rodenticide Act

Several years after the introduction of DDT and other synthetic insecticides, the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) was passed in 1947 (169). The legislation was administered by the U.S. Department of Agriculture (USDA) and remained primarily a labeling requirement. FIFRA has been amended several times since that time and its registration provisions strengthened. Conner et al.

(89) provided a detailed review of FIFRA, with its history and regulations.
Pesticides are also regulated under the Federal Food, Drug, and Cosmetic Act (FFDCA). FFDCA was amended with Section 408 (the Miller Amendment), establishing pesticide tolerances on foodstuffs, in 1954, and Section 409, which established tolerances for food additives in processed foods, was added in 1958 (170).
Section 409 of FFDCA contains the Delaney Clause, which forbids use of carcinogens as food additives. Although Section 409 directly addresses food additives only, interpretations of this section by the U.S. EPA have

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determined that pesticides, which concentrate during food processing, require food additive tolerances. Thus, the Delaney Clause applies only to pesticides, which concentrate during the processing of food. In the absence of concentration, only Section 408 applies.

Public concern for environmental and health issues as well as an interest in greater efficiency in regulations led to the formation of the U.S. EPA in 1970 (89). This new creation (162, 163) did not significantly alter the process of pesticide registration, but it did centralize it in a single agency instead of the previous two (USDA and FDA).

To protect human health and the environment, it is necessary to thoroughly evaluate health and environmental effects of all new products before manufacture and distribution. Specific toxicology study requirements for registration of pesticides around the world have been harmonized (95, 96, 149, 155, 171). As testing procedures are enhanced or new guidelines developed, these are added to the specific requirements shown in Table 13.1 (243).

Acute toxicity studies are conducted by administering the chemical orally, dermally, or by inhalation to determine the dose that causes mortality in 50% of the animals tested (LD50 or LC50). Acute studies are conducted to evaluate the irritation potential of the chemical after application to skin and eyes. Finally, the potential of the chemical to cause an allergic reaction (i.e., sensitization) is determined. Because this reaction is an all-or-nothing response, chemicals are classified as either sensitizers or nonsensitizers. Acute oral and inhalation studies are usually conducted in rats, dermal and irritation studies are conducted in rabbits or rats, and the sensitization study is carried out in guinea pigs. These tests are used to establish the product labels for all crop protection chemicals (163). The criteria used are presented in Table 13.2.

Dermal toxicity is evaluated by applying the chemical to the skin for 6 h/day for 21 days (rats) to 28 days (rabbits). Feeding studies are used to evaluate the toxicological effects of the chemical when a known dose is administered orally.

The oral feeding studies involve feeding rats, mice, or dogs diets containing the chemical for various lengths of time. Rat and mouse feeding studies are conducted for 28 days, 90 days, 1 year, and for the lifetime of the animals (24 months for rats, 18 months for mice). In dogs, the studies are usually conducted for 28 days, 90 days, 1 year, or 2 years. In all cases, animals are divided into test groups, 10 to 50 rats or mice and 4 to 6 dogs. At least four test groups are used in each study, one receiving no chemical (controls) and three groups receiving low, medium, or high concentrations of chemical in their diets. In these studies, urinalysis, hematology, and clinical chemistry parameters are evaluated, and gross and microscopic pathology examinations are performed on up to 50 tissues. Maximum tolerated doses are tested in order to demonstrate toxicity (up to 1000 mg/kg/day in the diets). In this fashion, it is possible to determine whether a chemical damages or alters any organ or tissue, and to establish levels of the chemical that produce no observable effects (no-observed effect level, NOEL) and the lowest level at which effects are noted (the lowest-observed effect level, LOEL).

Hazard testing also includes the examination of the potential of a chemical to affect the development of offspring, and to identify whether it induces birth defects in either the rat or rabbit. These tests have been described as teratology studies, but are now usually referred to as developmental toxicity studies. In addition to developmental toxicity studies, a reproduction study is conducted in rats. This study involves feeding diets containing the chemical to young adult male and female rats for approximately 3 months prior to mating. The females are allowed to produce a litter of offspring that are then reared to adulthood. The animals are fed diets containing the chemical during this entire period. After reaching sexual maturity, the second-generation animals are allowed to mate.

It was noted by Weisburger (316) that certain chemical carcinogens are capable of interacting directly with genetic material such as DNA. Based upon this association, several short-term tests to identify the alteration of genetic material or mutation were introduced into hazard testing for crop protection chemicals. These include tests to examine the possible interaction with (a) genes (gene mutation tests), (b) the chromosome (clastogenic tests), and (c) directly with DNA (classified as other tests).

Since individuals may be exposed to low levels of crop protection chemicals in their diet or water over a portion of their life span, studies to evaluate lifetime exposure are conducted in animal bioassays. An important aspect of these studies is an assessment of the potential of the chemical to cause cancer. An increase in the number of tumors or the earlier onset of tumors as a result of treatment will identify a chemical as a potential carcinogen. For laboratory studies, mice and rats are divided into at least 3 treatment groups and a control group with a minimum of 50 animals/sex/group. These groups of mice and rats are fed selected concentrations of the test chemical in their diet for 18 months and 24 months, respectively. The levels of the test chemical administered in the diet are generally selected from

repeated-dose feeding studies at least 90 days in duration, and are normally used to establish the NOEL, LOEL, and the maximum tolerated dose (MTD) (97). The MTD is defined as the highest concentration of test chemical that can be administrated that can be tolerated without causing the death of the animal; often a 10% reduction in body weight gain has been used as the criterion for establishing the MTD.

Table 13.1 Series 870—Health effects test guidelines (242)

OPPTS Number	Name	Existing Numbers OPPT	OPP	OECD
	Group A—Acute Toxicity Test Guideline.			
870.1000	Acute toxicity testing—background	none	none	none
870.1100	Acute oral toxicity	798.1175	81–1	401
870.1200	Acute dermal toxicity	798.1100	81–2	402
870.1300	Acute inhalation toxicity	798.1150	81–3	403
870.2400	Acute eye irritation	798.4500	81–4	405
870.2500	Acute dermal irritation	798.4470	81–5	404
870.2600	Skin sensitization	798.4100	81–6	406
	Group B—Subchronic Toxicity Test Guidelines.			
870.3100	90-Day oral toxicity in rodents	798.2650	82–1	408
870.3150	90-Day oral toxicity in nonrodents	none	82–1	409
870.3200	21/28-Day dermal toxicity	none	82–2	410
870.3250	90-Day dermal toxicity	798.2250	82–3	411
870.3465	90-Day inhalation toxicity	798.2450	82–4	413
870.3700	Prenatal developmental toxicity study	798.4900	83–3	414
870.3800	Reproduction and fertility affects	798.4700	83–4	416
	Group C—Chronic Toxicity Test Guidelines.			
870.4100	Chronic toxicity	798.3260	83–1	452
870.4200	Carcinogenicity	798.3300	83–2	451
870.4300	Combined chronic toxicity/carcinogenicity	798.3320	83–5	453
	Group D—Genetic Toxicity Test Guidelines.			
870.5100	Bacterial reverse mutation test	798.5100, 798.5265	84–2	471, 472
870.5140	Gene mutation in Aspergillus nidulans	798.5140	84–2	none
870.5195	Mouse biochemical specific locus test	798.5195	84–2	none
870.5200	Mouse visible specific locus test	798.5200	84–2	none
870.5250	Gene mutation in Neurospora crassa	798.5250	84–2	none
870.5275	Sex-linked recessive lethal test in Drosophila	798.5275	84–2	477
870.5300	In vitro mammalian cell gene mutation test	798.5300	84–2	476
870.5375	In vitro mammalian chromosome aberration test	798.5375	84–2	473
870.5380	Mammalian spermatogonial chromosomal aberration	798.5380	84–2	483
870.5385	Mammalian bone marrow chromosomal aberration test	798.5385	84–2	475
870.5395	Mammalian erythrocyte micronucleus test	798.5395	84–2	474
870.5450	Rodent dominant lethal assay	798.5450	84–2	478
870.5460	Rodent heritable translocation assays	798.5460	84–2	none
870.5500	Bacterial DNA damage or repair tests	798.5500	84–2	none
870.5550	Unscheduled DNA synthesis in mammalian cells	798.5550	84–2	482
870.5575	Mitotic gene conversion in Saccharomyces cerevisiae	798.5575	84–2	481
870.5900	In vitro sister chromatid exchange assay	798.5900	84–2	479
870.5915	In vivo sister chromatid exchange assay	798.5915	84–2	none
	Group E—Neurotoxicity Test Guidelines.			
870.6100	Acute and 28-day delayed neurotoxicity of Organophosphorus substances	798.6450, 798.6540 798.6560	81–7, 82–5, 82–6	418, 419
870.6200	Neurotoxicity screening battery	798.6050, 798.6200, 798.6400	81–8, 82–7, 83–1	424
870.6300	Developmental neurotoxicity study	none	83–6	none
870.6500	Schedule-controlled operant behavior	798.6500	85–5	none
870.6850	Peripheral nerve function	798.6850	85–6	none
870.6855	Neurophysiology: Sensory evoked potentials	798.6855	none	none
	Group F—Special Studies Test Guidelines.			
870.7200	Companion animal safety	none	none	none
870.7485	Metabolism and pharmacokinetics	798.7485	85–1	417

870.7600	Dermal penetration	none	85–3	none
870.7800	Immunotoxicity	none	85–7	none

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Table 13.2 U.S. EPA acute toxicity classification scheme (163)

Toxicology Category	Signal Word	Oral LD50 (mg/kg)	Dermal LD50 (mg/kg)	Inhalation LC50(mg/L)	Eye Irritation	Skin Irritation
I	Danger1	Up To 50	Up To 200	Up To 0.2	Corrosive. Corneal Opacity Not Reversed In 7 Days	Corrosive
II	Warning	From 50 Through 500	From 200 Through 2000	From 0.2 Through 2.0	Corneal Opacity Reversed In 7 Days; Irritation Persisting 7 Days	Severe Irritation At 72 Hours
III	Caution	From 500 Through 5000	From 2000 Through 5000	From 2.0 Through 20	No Corneal Opacity; Irritation Reversed Within 7 Days	Moderate Irritation At 72 Hours
IV	Caution	Greater Than 5000	Greater Than 5000	Greater Than 20	No Irritation	Mild Or Slight Irritation At 72 Hours

1 The word “Poison” is used on the label if the “Danger” category is based on oral, dermal or inhalation toxicity.

Following lifetime feeding studies at the prescribed treatment levels, veterinary pathologists examine approximately 50 tissues from each animal for the presence of tumors or other evidence of tissue damage. If a statistically significant increase in the incidence of any tumor in any tissue above the incidence in the control animals is observed, then it is considered in a weight-of-evidence approach as described in the EPA cancer classification scheme (146). In a weight-of-evidence analysis, the evidence of oncogenicity in humans comes from long-term animal studies, and from epidemiology (studies of humans in exposed populations). Results from these studies are supplemented with available information from other sources that include mutagenicity and other short-term tests (for genetic effects), metabolic or kinetic studies, and other relevant toxicological studies. Using this approach, the oncogenic response is classified into one of several categories as indicated in Table 13.3.

The U.S EPA has essentially taken the approach that animal tumorigens are human carcinogens (146). This approach has been taken as a default assumption almost without regard to quality of the study, the level of test material administered, or the mechanism by which the tumor response is manifest (166). In this regard, all animal carcinogens are treated as though they have no threshold or there is a real risk at all exposure levels. The U.S. EPA has published a list identifying crop protection chemicals as known, probable, or possible human carcinogens without appropriate consideration of the mechanism by which the tumors occur or their relevance to humans (150).

However, most of the other OECD and EEC countries have long recognized that genotoxic agents exist (96, 168). For genotoxic agents, there is evidence of mutagenic and/or clastogenic responses in standard laboratory tests, and nongenotoxic chemicals are devoid of any mutagenic and/or clastogenic behavior (150). It is considered that genotoxic agents will most likely be carcinogenic in animal tests and most probably will be human carcinogens (168). These genotoxic carcinogens would be regulated as if they are devoid of a threshold, and it would be assumed that there would be a risk at all doses. On the other hand, nongenotoxic agents would be charac

Table 13.3 U.S. Environmental Protection Agency's classification of carcinogens

Carcinogen Category	Criteria for Classification	Evaluation: Uncertainty Factor	Evaluation: Mathematical Modeling
A—Human	Sufficient evidence in man		X
B—Probable Human	B1 · Limited evidence in man · Sufficient evidence in animal (two species with tumors)		X
	B2 · Inadequate human evidence · Sufficient animal evidence		X
C—Possible Human	· No evidence in man · Limited evidence in animals	X or	X
D-Not Classifiable	· Inadequate animal or human evidence	X	
E—Not a Human Carcinogen	· Sufficient animal testing with no evidence of carcinogenicity and human experience	X	

terized as having thresholds and a safety factor would be used.

Although new U.S. EPA guidelines for cancer classification based on animal data are in development (192), they have not been finalized.

Food Quality Protection Act of 1996

The Food Quality Protection Act (FQPA) amendments to the Food Drug and Cosmetic Act (170) and FIFRA (169), direct the U.S. EPA to consider a number of factors in assessing risk as part of the tolerance setting procedure (5). FQPA 1996 provides for a single, health-based standard, eliminates long-standing problems posed by multiple standards for pesticides in raw and processed foods, and requires the U.S. EPA to consider all nonoccupational sources of exposure, including drinking water, and exposure to other pesticides with a common mechanism of toxicity when setting tolerances. Most of these provisions *originally reflected* concerns that children may be especially susceptible to pesticide exposure and embodied key recommendations of a National Academy of Sciences report, "Pesticides in the Diets of Infants and Children" (153). The FQPA directs the U.S. EPA to set a tolerance for pesticide residues in food. In order to accomplish this task, the U.S. EPA uses an extra 10-fold safety factor to account for susceptibility of chil dren including effects of in utero exposure and to evaluate the cumulative effects of exposure to the pesticide. Further, the agency is directed to consider substances having a common mode of action and to consider aggregate exposure for all consumers (i.e., other routes, such as drinking water). Finally, the agency must define techniques for evaluating potential for endocrine-disrupting effects.

Unlike previous law, which contained an open-ended provision for the consideration of pesticide benefits when setting tolerances, the new law places specific limits on benefit considerations. Benefits cannot be considered for pesticides that result in reproductive or other threshold effects.

In addition, FQPA 1996 reauthorizes Federal Insecticide, Fungicide, and Rodenticide Act provisions (FIFRA) and requires tolerances to be reassessed as part of the reregistration program (5). Further, it expedites review of safer pesticides to help them reach the market sooner and replace older and potentially more risky chemicals. Minor-use pesticides and antimicrobial pesticides are given greater attention under this new law.

FUNGICIDES

The world market for agricultural and noncrop fungicides amounted to nearly $6 billion in 1995 (139). The

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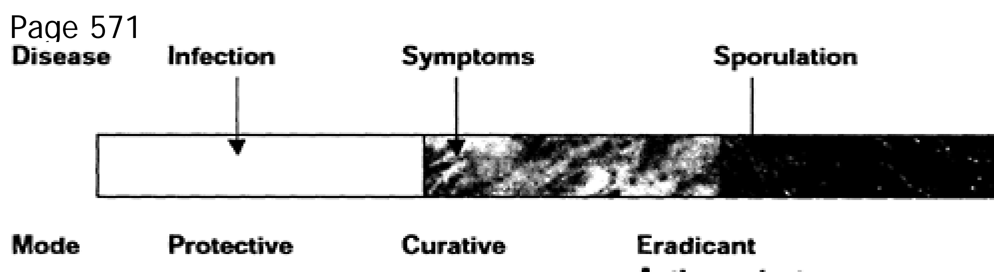

FIG. 13.2. Representation of the disease process and efficacy by mode of action.

United States, western Europe, and Japan accounted for 75% of the total world market. Tree fruits and nuts, citrus, and grapes constitute the largest market for fungicides worldwide. This sector was 28.5% of the total world market, followed by cereals, rice, vegetables, and potatoes. In western Europe, half of the $2 billion spent for fungicides was used on wheat and barley, followed by vines, tree fruits, and other crop and noncrop markets. For the Japanese market of over $1 billion, about 40% of the fungicides were applied to rice, with vegetables and tree fruits the next most important market. The U.S, market for fungicides amounted to about $800 million, with tree and vine crops accounting for 30%, followed by turf ornamentals, then vegetable crops, and finally peanuts and potatoes.

Fungicides have been classified in several different manners. Mode of action in affecting the development of fungal diseases is one of the systems used. Schematically, the disease process along with the mode of action for fungicides is presented in Figure 13.2 (91).

A *protective* or *preventative* mode of action is ascribed to fungicidal activity when the disease is prevented. *Curative* relates to activity that interrupts the development of established infection. An *eradicant,* as the name implies, stops the development of the disease after symptoms are visible in the plant, and an *antisporulant* stops or decreases spore production. Mode of action and mechanism of action determine the major classes of fungicides used in crop protection, presented in Table 13.4.

Individual fungicides representing each mode of action are discussed here. It is not feasible to discuss all fungicides within the scope of this chapter. Therefore, no attempt to consider every fungicide within a group has been made. Instead, an attempt was made to consider only those fungicides with current significant economic importance or use. Therefore, discussions focus on selected agents.

Anilinopyrimidines

The spectrum of activity for anilinopyrimidines is limited to *Ascomycetes* and *Deuteromycetes* (27). Their unique mode of action of inhibiting methionine biosynthesis as well as protease secretion necessary for infection affords both protective and curative action. The anilinopyrimidines are used to control gray mold on vines, fruits, vegetables, and ornamentals, and leaf scab on pome fruit. The anilinopyrimidines are a new class of fungicides currently represented by two commerically available products. The structures, uses, and hazard profiles of cyprodinil and pyrimethanil are presented in Table 13.5.

The profiles for the mammalian toxicity studies conducted with these fungicides suggest minimal hazard to humans from their use (224, 231).

Benzanilides

Benzanilides, which inhibit the succinate dehydrate complex in the electron transport chain and thus stop the synthesis of aspartate and glutamate, are represented by flutolanil (43). Flutolanil is a systemic fungicide with protective and curative action. It is used for control of sheath blight, white mold, and snow blight in rice, cereals, sugar beet, and other crops. The structure, uses, and hazard profile of flutolanil are given in Table 13.6.

It exhibits minimal acute toxicity and possesses a good hazard profile (186).

Benzimidazoles

The benzimidazoles have broad-spectrum activity in the inhibition of mitosis by preventing polymerization of beta-tubulin (15). Important members of this class are benomyl, thiabendazole, and thiophanate-methyl. Their structures, uses, and hazard profiles are presented in Table 13.7.

The hazards for benomyl, thiabendazole, and thio-phanate-methyl have been carefully reviewed by either the U.S. EPA or WHO (106, 132, 216). Benomyl has been classified as Category C (possible human carcinogen) based on mouse liver tumors (100).

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Table 13.4 Mode and mechanism of action for important classes of fungicides used in crop protection

Class	Mode of Action	Mechanism of Action
Anilinopyrimidines (27)	Curative and protective	Inhibition of methionine biosynthesis and protease secretion enzymes
Benzanilides (16)	Curative and protective	Inhibits fungal respiration at succinate dehydrogenase complex
Benzimidazoles (15)	Curative and protective	Inhibition of mitosis by preventing polymerization of tubulin in a broad spectrum of fungi
Phenylamides (54)	Curative and protective	Inhibition of RNA synthesis
Sterol Biosynthesis sterol Inhibitors (26)	Curative, protective, and eradicants	Interferes with biosynthesis essential for cell wall formation
Strobilurins (12)	Curative, protective, and antisporulants	Disruption of electron transport in cytochrome bc1 complex
Dicarboximides (53)	Protective, and antisporulants	Inhibition of spore germination
Dithiocarbamates (39)	Protective	Interferes with oxygen uptake and inhibits sulfur-containing enzymes
Ethylenediothio-carbamates (54)	Protective	Breaks down to cyanide which reacts with thiol compounds in the cells and interferes with sulfhydryl groups
Inorganic (23–25, 74)	Protective	Blocks enzymes and stops respiration
Organometallics (37)	Protective, some curative and antisporulant	Destroys cell membranes, and inhibits metabolism and respiration
Phenylpyrroles (35)	Protective	Interferes with membrane transport
Phthalimides (16)	Protective and curative	Inhibits enzymes by thiophosgene production
Substituted Benzenes (20)	Protective	Inhibits sulfur-containing enzymes

Phenylamides

This class of fungicides includes two products, metalaxyl and mefenoxam (55). Metalaxyl is a mixture of *R* and *S* enantiomers. Mefenoxam is the *R* enantiomer of metalaxyl. The spectrum of activity for mefenoxam is quite narrow controlling downy mildew and late blight; it is used in maize, peas, sorghum, sunflowers, and tobacco (55).

Mefenoxam is efficacious at approximately half the rate of metalaxyl; therefore, the registration of metalaxyl has been voluntarily canceled (180, 218). The structure, uses, and hazard profile for mefenoxam are presented in Table 13.8.

Table 13.5 Structures, uses, and hazard profiles for major anilinopyrimidine fungicides

Fungicide	Structure	Crops/uses	Use Rates gm (a.i.)/ha
Cyprodinil Vangard® (27)		Used on cereals, grapes, pome fruit, stone fruit, almonds, strawberries, vegetables, field crop and as a seed dressing	95–160
Pyrimethanil Mythos®, Scala ® (68, 224)		Used on pome fruit, vine crops, vegetables and ornamentals.	1000–3000

Fungicide	Irritation		LD50 (mg/kg)		LC50 (mg/L)	Sensitizing	Signal
	Eye	Skin	Oral	Dermal	Inhalation	Potential	Word
Cyprodinil (27)	Minimal Irritant	Slight Irritant	2796	>2000	>1.2	Positive	Caution
Pyrimethanil (68)	Slight Irritant	Non-irritant	>4149	>5000	>1.98	No positive	Caution

Fungicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Cyprodinil (231)	Rat/2-year	3.75	Mutagenicity	No evidence
	Dog/52-week	65.6	Developmental	Not teratogenic
	Mouse/18-month	16.1 ♂	Reproductive	No evidence
	RfD2	0.038	Oncogenicity	E(No evidence)3
Pyrimethanil (224)	Rat/2-year	20	Mutagenicity	No evidence
	Dog/52-week	30	Developmental	Not teratogenic
	Mouse/18-month	211 ♂	Reproductive	No evidence
	RfD2	0.2	Oncogenicity	C with RfD3 (thyroid tumors in rats)

1 No observable effect level
2 RfD=reference dose
3 See Table 13.3 for US EPA classification scheme

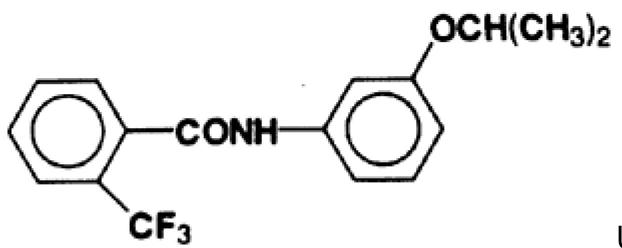
Mefenoxam is without any significant hazard to humans.

Sterol Biosynthesis Inhibitors

The sterol biosynthesis inhibitors or sterol demethylase inhibitors (DMIs) group are comprised of imidazoles, piperazines, pyridines, pyrimidines, and triazoles that produce their effect on fungi by inhibition of the synthesis of ergosterol (26). Ergosterol is essential for cell-wall integrity in fungi. The

structures and crop uses for the most prominent DMIs are given in Table 13.9.
The ability of these fungicides to inhibit cytochrome P450 demethylase required for the synthesis of ergosterol

Table 13.6 Structure, uses and hazard profile for flutolanil (Folistar®) (187)

Structure	Crops/uses	Use Rates gm (a.i.)/ha
	Used on rice, cereals, sugar beets, fruits, and vegetables	NA

Irritation		LD50(mg/kg)		LC50(mg/L)	Sensitizing Potential	Signal Word
Eye	Skin	Oral	Dermal	Inhalation		
Slight Irritant	Non-irritant	>10000	>5000	>6.0	Negative	Caution

Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Rat/2-year	87	Mutagenicity	No evidence
Dog/2-Year Oral	50	Developmental	Not teratogenic
Mouse/18-month	735	Reproductive	No evidence
RfD2	0.2 (based rat reproduction; UF3=300	Oncogenicity	E (No evidence)4

1 No observable effect level
2 RfD=reference dose
3 UF=Uncertainty factor
4 See Table 13.3 for US EPA classification scheme
NA=Not Available

in fungi also occurs in mammalian systems and often manifests itself as an induction of liver cytochrome P450 as well as inhibiting these enzymes (31). Inherent in the chemical structure of these compounds is specificity to alter the activity of cytochrome P450 isozymes responsible for the metabolism of steroids, or xenobiotics. In some instances, these liver effects are seen following chronic feeding as liver tumors in the mouse. The hazard profiles for the selected sterol biosynthesis inhibitors are presented in Table 13.10 and 13.11.

Several fungicides produce liver tumors in mice, but all are not genotoxic. Cyproconazole (177) and triadimenol (112) elicit some evidence of developmental toxicity.

Strobilurins

The strobilurins inhibit mitochondrial respiration by blocking electron transfer between cytochrome *b* and cytochrome *c*1 (12). These materials have their origins as a natural substance derived from mushrooms and are modified synthetically. Numerous chemicals in this class are under development, but only one strobilurin is available commercially, azoxystrobin. Azoxystrobin has a broad mode of action that includes protective, eradicant, and antisporant activity. The structure, uses, and hazard profile for azoxystrobin are presented in Table 13.12.

Azoxystrobin presents a rather innocuous hazard profile (196).

Inorganic Fungicides

Inorganics, such as sulfur, were used before 1000 BC as previously discussed (102). Yet elemental sulfur and forms of copper (hydroxide, oxychloride, and sulfate) are the only commercially significant fungicides in this class remaining in use. The mode of action of the inorganic fungicides is protective or preventative; they exert their effects by blockage of enzymes and inhibiting respiration (74). These materials are cheap and are applied at high use rates, from thousands to ten thousand of grams per hectare. Severe eye irritation is seen with copper hydroxide (23); copper oxychloride and copper sulfate do not exhibit this inherent hazard (24, 25).

Table 13.7 Structures, uses, and hazard profiles for important benzimidazoles

Compound	Structure	Crops/uses	Use Rates gm (a.i.)/ha
Benomyl Benlate® (15)		Used against *Ascomycetes* and *Basidomycetes* in cereals, grapes, pome and stone fruit, rice and vegetables.	Field: 140–550 Tree: 550–1100 Storage; 25–200
Thiabenazole Mertech® (77)		Used for the control of *Aspergillus, Botrytis,* and others in vegetables, bananas, cereals, cabbage, stone fruit, citrus fruit, and hops.	~250
Thiophanate-methyl Topsin-M® (78)		Used for eyespot on cereals, scab and rot on apples and pears, powdery mildew on pome fruit, stone fruit, vegetables, strawberries, and vines	30–50

Fungicide	Irritation Eye	Skin	LD50 (mg/kg) Oral	Dermal	LC50 (mg/L) Inhalation	Sensitizing Potential	Signal Word
Benomyl (15)	Moderate Irritant	Slight Irritant	>10000	>5000	>2.0	Negative	Caution
Thiabendazole (51)	Non-irritant	Non-irritant	3100	>2000	>0.4	Negative	Caution
Thiophanatemethyl (78)	Moderate Irritant	Mild Irritant	6640 ♀	>10000	>5.0	Negative	Caution

Fungicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Benomyl (132, 173)	Rat/2-year	125	Mutagenicity	No evidence
	Dog/52-week	12.5	Developmental	Inconclusive evidence
	Mouse/18-month	40	Reproductive	Decreased fertility
	RfD (based on reproduction study)2	0.05	Oncogenicity	C with RfD (liver tumors in mice)3
Thiabendazole (106)	Rat/2-year	20	Mutagenicity	No evidence
	Dog/2-Year Oral	20	Developmental	Effects only at maternally toxic doses
	Mouse/18-month	6	Reproductive	No evidence

	ADI (human study)2	0.035	Oncogenicity	Thyroid tumors in rats
ThiophanateMethyl (135)	Rat/2-year	8.0	Mutagenicity	No evidence
	Dog/52-week	50	Developmental	Minimal evidence
	Mouse/18-month	23	Reproductive	↓spermatogenesis
	RfD2	0.08	Oncogenicity	No evidence

1 No observable effect level
2 RfD=reference dose and ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme

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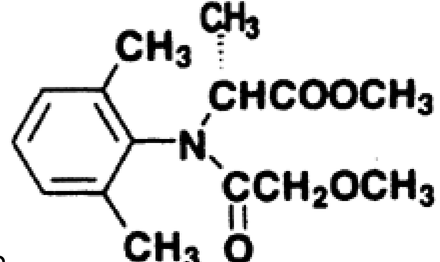
Table 13.8 Structure, uses, and hazard profile for the phenylamide fungicide mefenoxam

Fungicide	Structure	Principle Uses/Crops	Application Rate gm(a.i.)/ha
Mefenoxam (Metalaxyl-M) Ridomil Gold® (55)		Used on alfalfa, apples, asparagus, avocadoes, berries, citrus, cole crops, cotton, cucurbits, hops, peanuts, stone fruit, soybeans, sugar beets, tobacco and vegetables.	70–1680

Fungicide	Irritation Eye	Irritation Skin	LD50 (mg/kg) Oral	LD50 (mg/kg) Dermal	LC50 (mg/L) Inhalation	Sensitizing Potential	Signal Word
Mefenoxam (55)	Severe Irritant	Slight Irritant	490	>2000	>2.3	Negative	Warning

Fungicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Mefenoxam (199, 218)	Rat/2-year	13	Mutagenicity	No evidence
	Dog/52-week	8	Developmental	Not teratogenic
	Mouse/18-month	38	Reproductive	No evidence
	RfD(based on 6Month Dog)2	0.08	Oncogenicity	No evidence

1 No observable effect level

2 RfD=reference dose

Elemental sulfur is considered practically nontoxic to humans and animals (74).

Dicarboximides

The dicarboximides have a narrow spectrum of activity with strengths on *Botrytis*, *Sclerotinia*, *Monifinia*, and *Altemaria*. These fungicides appear to inhibit spore germination (51). The dicarboximides are used to treat infections in turf, strawberries, stone fruit, peanuts, and vines. Iprodione and vinclozolin represent the dicarboximide fungicides; the structures, uses, and mammalian toxicology for these agents are presented in Table 13.13.

Iprodione interferes with androgen synthesis (134); this effect results in testicular effects including interstitial-cell tumors in male rats at feeding levels of 1600 ppm and above. Vinclozolin has been shown to be metabolized to antiandrogenic metabolites, 2–1[(3,5-dichlorophenyl) carbamoyl] oxyl-2-methyl-3-butenoic acid and 3,5′-dich-loro-2-hydroxy-2-methylbut-3-enanilide, that appear to lead to infertility in male rats (140). This response is thought to be due to feminization of the outer genital organs of males exposed during development to a dietary concentration of 1000 ppm or more of vinclozolin (227).

Dithiocarbamates

The dithiocarbamates are broad-spectrum protective fungicides with multiple sites of action (39). They are use to control scab on pome fruit, blue mold on tobacco, rust on ornamentals, and diseases on vegetables. These agents interfere with oxygen uptake and inhibit sulfur-containing enzymes. The dithiocarbamates are applied at rates of 500 to over 10,000 g/hectare. Ferbam, thiram, and ziram are the commercially important chemicals in this group. Their structures, uses, and hazard profiles are given in Table 13.14.

Ferbam, thiram, and ziram have significant acute toxicity by the inhalation route. Both ferbam and ziram have been shown to alter spermatazoa in mice and thus would be placed under the endocrine disruptor category as suggested in the FQPA 1996 (5).

Table 13.9 Selected sterol synthesis inhibitors

Fungicide	Structure	Crops/uses
Cyproconazole Alto® (26)		Cereal, sugar beets, fruit trees, vines, coffee, turf, bananas, and vegetables for treatment of rust, powdery mildew, *Septoria*, *Venturia*, and others.
Difenoconazole Dividend® (31)		Seed treatment, grapes, fruit trees, potatoes, sugar beets, oilseed rape, banana, ornamentals and vegetables for treating a variety of fungal diseases.
Fenbuconazole Indar® (34)		Cereals, fruit trees, vines, beans, sugar beets, rice, bananas, ornamentals, tree nuts and vegetables
Hexaconazole Amizol® (46)		Vine, coffee, bananas, peanuts, and vegetables for treating a variety of fungal diseases.
Myclobutanil Rally®, Nova® (58)		Seed treatment, grapes, fruit trees, rice, cotton, barley, wheat, maize, grass seed, ornamentals and vegetables for treating a variety of fungal diseases
Propiconazole Tilt® (64)		Wheat, rice, coffee, bananas, peanuts, stone fruit, maize and turf for treating a variety of fungal diseases. Rates: 24 to 110 gm (a.i.)/ha
Tebuconazole Folicur® (75)		Seed treatment, cereals, coffee, fruit trees, grapes, grass seed, oilseed rape, soybeans, sugar beets, bananas, ornamentals, turf and vegetables for treating a variety of fungal diseases

Triadimefon Bayleton® (80)	Cereals, corn, fruit trees, vines, berries, sugar cane, tobacco and vegetables for treating a variety of fungal diseases
Triadimenol Baytan® (82)	Seed treatment, cereals, fruit trees, hops, vines and vegetables for treating a variety of fungal diseases
Imazalil Fungaflor® (48)	Seed, fruit trees, potatoes, bananas, vegetables, ornamentals and cereals for treating a variety of fungal diseases. Rates of 4–5 (a.i.) 100 kg seed
Prochloraz Sportak® (62)	Citrus, tropical fruit (dip), beets, oilseed rape, mushrooms, ornamentals and cereals (seed treatment). Rates: 400–600 gm (a.i.)/ha

Table 13.10 Hazard profile for sterol synthesis-inhibiting fungicides

	Irritation		LD50 (mg/kg)		LC50 (mg/L)		
Fungicide	Eye	Skin	Oral	Dermal	Inhalation	Sensitizing Potential	Signal Word
Cyproconazole (26)	Non-irritant	Non-irritant	>1020	>2000	5.7	Negative	Caution
Difenoconazole (31)	Moderate Irritant	Slight Irritant	1453	>2000	3.3	Negative	Caution
Fenbuconazole (34)	Non-irritant	Non-irritant	>2000	>5000	>2.1	Negative	Caution
Hexaconazole (46)	Mild Irritant	Non-irritant	2189	>2000	>5.9	Positive	Caution
Myclobutanil (58)	Irritant	Non-irritant	>1600	>5000	>5.0	Positive	Danger

Fungicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Cyproconazole (177)	Rat/2-year	2.2	Mutagenicity	Clastogenic (CHO)
	Dog/52-week	1.0	Developmental	Teratogenic in rabbit
	Mouse/18-month	1.8	Reproductive	No evidence
	RfD2	0.01	Oncogenicity	B2 (Mouse liver tumors in both sexes)3
Difenoconazole (204)	Rat/2-year	1.0	Mutagenicity	No evidence
	Dog/52-week	3.4 ♂	Developmental	Not Teratogenic
	Mouse/18-month	4.7 ♂	Reproductive	No evidence
	RfD2	0.01	Oncogenicity	C with RfD (Mouse liver tumors in both sexes)3
Fenbuconazole (178)	Rat/2-year	3.0	Mutagenicity	No evidence
	Dog/52-week	3.8 ♂	Developmental	Not teratogenic
	Mouse/18-month	1.4 ♂	Reproductive	(No evidence)3
	RfD2	0.03	Oncogenicity	C with RfD (Mouse liver tumors—both sexes/ thyroid tumors—male rats)3
Hexaconazole (187a)	Rat/2-year	0.5	Mutagenicity	No evidence
	Dog/52-week	2.0	Developmental	Not teratogenic
	Mouse/18-month	4.7 ♂	Reproductive	No evidence
	RfD2	0.005	Oncogenicity	C with CSF (Male rat Leydig cell tumor)3
Myclobutanil (219)	Rat/2-year	2.5	Mutagenicity	No evidence
	Dog/52-week	3.1 ♂	Developmental	Not teratogenic
	Mouse/18-month	13.7♂	Reproductive	Testicular atrophy
	RfD2	0.025	Oncogenicity	E (No evidence)3

1 No observable effect level
2 RfD=reference dose, ADI=acceptable daily intake, and CSF=cancer slope factor
3 See Table 13.3 for US EPA classification scheme

Ethylenebisdithiocarbamates

The ethylenebisdithiocarbamates (EBDCs) have a broad spectrum of activity, although their mode of action is primarily protective. Their mechanism of action is to break down to the cyanide that reacts with thiol compounds in the cell and thus interferes with sulfhydryl groups (53).

The structures of mancozeb, maneb, and zineb, the three most important members of this class, are presented

Table 13.11 Hazard profile for more sterol synthesis inhibitor fungicides

Fungicide	Irritation: Eye	Irritation: Skin	LD50 (mg/kg): Oral	LD50 (mg/kg): Dermal	LC50 (mg/L): Inhalation	Sensitizing Potential	Signal Word
Propiconazole (64, 191)	Mild Irritant	Slight Irritant	1517	>6000	>5.8	Negative	Caution
Tebuconazole (225)	Mild Irritant	Non-irritant	>3933 ♂	>5000	>0.37	Negative	Caution
Triadimefon (80)	Non-irritant	Non-irritant	>363	>2000	>3.6	Positive	Warning
Triadimenol (81)	Non-irritant	Non-irritant	>1100	>5000	>0.9	NA	Caution
Imazalil (48)	Non-irritant	Mild Irritant	>227	4200	16	Negative	Warning
Prochloraz (62)	Irritant	Mild Irritant	1600	3000	0.42	Negative	Caution

Fungicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Propiconazole (64, 191)	Rat/2-year	3.6	Mutagenicity	No evidence
	Dog/26-Week Oral	1.3	Developmental	Not teratogenic
	Mouse/18-month	15	Reproductive	No evidence
	RfD2	0.013	Oncogenicity	C with RfD (Mouse liver tumors in males)3
Tebuconazole (225)	Rat/2-year	7.4	Mutagenicity	No evidence
	Dog/52-week	3.0	Developmental	Teratogenic in rat
	Mouse/18-month	2.9	Reproductive	No evidence
	RfD2	0.03	Oncogenicity	C with RfD (Mouse liver tumors in both sexes)3
Triadimefon (111, 194)	Rat/2-year	16.4 ♂	Mutagenicity	No evidence
	Dog/2-Year Oral	11.4	Developmental	Not teratogenic
	Mouse/18-month	40	Reproductive	No evidence
	RfD (52-wk dog study with 300×UF4)2	0.04	Oncogenicity	C with RfD (Mouse liver tumors in both sexes)3
Triadimenol (112)	Rat/2-year	7.0	Mutagenicity	No evidence
	Dog/52-week	3.75	Developmental	Teratogenic in rat
	Mouse/18-month	30	Reproductive	No evidence
	ADI2	0.038	Oncogenicity	C with RfD (liver tumors in female mice)3
Imazalil (119)	Rat/2-year	5.0	Mutagenicity	No evidence
	Dog/52-week	2.5	Developmental	Not teratogenic
	Mouse/18-month	40	Reproductive	No evidence
	ADI2	0.025	Oncogenicity	C with CSF (Mouse liver)3
Prochloraz (110)	Rat/2-year	1.9	Mutagenicity	No evidence
	Dog/52-week	0.9	Developmental	Not teratogenic
	Mouse/18-month	11.7	Reproductive	Decreased litter size
	ADI2	0.009	Oncogenicity	C with CSF (Mouse liver tumors in both sexes)3

1 No observable effect level
2 RfD=reference dose, ADI=acceptable daily intake, and CSF=cancer slope factor
3 See Table 13.3 for US EPA classification scheme
4 UF=Uncertainty factor
NA=Not Available

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Table 13.12 Structure, uses, and hazard profile for azoxystrobin (Heritage®) (112, 196)

Structure	Principle Uses/Crops	Application Rate gm (a.i.)/ha
	Used on vine crops, apples, cereals, cucurbits, tomatoes, pecans, coffee, potatoes, peanuts, peaches, citrus, rice and turf	100–375

Irritation		LD50 (mg/kg)		LC50 (mg/L)		
Eye	Skin	Oral	Dermal	Inhalation	Sensitizing Potential	Signal Word
Slight Irritant	Slight Irritant	>5000	>2000	>0.7	Not positive	Caution

Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Rat/2-year	18	Mutagenicity	No evidence
Dog/1-Year Oral	25	Developmental	Not teratogenic
Mouse/18-month	38 ♂	Reproductive	No evidence
RfD2	0.18	Oncogenicity	E(No evidence)3

1 No observable effect level
2 RfD=reference dose
3 See Table 13.3 for US EPA classification scheme

with their toxicologically significant metabolite, ethylen-ethiourea, in Figure 13.3.
The hazard profiles for mancozeb, maneb, and zineb are presented in Table 13.15.
Both mancozeb and maneb are classified as B2, probable human carcinogens (100), based on the formation of mouse liver tumors and/or thyroid follicular cell tumors in rats. Although zineb was not found to be oncogenic in the rat or mouse, it was observed to produce nonneoplasic hyperplasia of the follicular cells of the thyroid in rats (217). All three fungicides are metabolized to ethylenethiourea. This is known to inhibit thyroid peroxidase and to cause progressive lesions in the thyroid follicular cells, often leading to tumor formation (126, 127, 129). The U.S. EPA has regulated the risk associated with the EBDCs using a cancer slope factor of 0.06 $(mg/kg/day)^{-1}$ (216).

Organometallic Fungicides

The organometallic fungicides are limited in spectrum of disease control, but are effective as protective, curative, and antisporulants in early and late blight, scab, leaf blotch, and powdery mildew (37).
Triphenyltin, whose structure, uses, and hazard profile are presented in Table 13.16, works through destruction of cell membranes and inhibition of respiration (37).
Triphenyltin hydroxide has been classified by the U.S. EPA as category B2, probable human carcinogen, based on mouse liver and pituitary and testicular tumors in rats (100).

Phenylpyrroles

The phenylpyrrole fungicides are a recent entry into the marketplace. They represent a new mechanism of action through interference with membrane transport (35). The structures, uses, and hazard profiles of these two new fungicides, fenpiclonil and fludioxonil, are presented in Table 13.17.
Both of these products are not acutely toxic, and do not exhibit remarkable toxicity profiles. Fludioxonil represents an exception as it has been classified as a category D or nonclassifiable in regard to carcinogenicity. This conclusion is based on the statistically significant increase in liver tumors in female rats for combined adenoma/carcinoma only. Despite the lack of a tumorigenic response in male rats or in either sex of the mouse, additional mutagenicity studies have been required (202).

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Table 13.13 Structures, uses, and hazard profiles for dicarboximide fungicides

Fungicide	Structure	Principle Uses/Crops	Application Rate gm (a.i)/ha
Iprodione Rovral® (51)	[3,5-dichlorophenyl imidazolidinedione structure; labels: Cl, Cl, O, O, N, N, $CONHCH(CH_3)_2$]	Sunflowers, cereals, fruit trees, berries, oilseed rape, rice, cotton, vegetables, vines, turf and seed treatment.	500–12000
Vinclozolin Roilan® (83)	[3,5-dichlorophenyl oxazolidinedione structure; labels: Cl, Cl, O, O, O, N, CH_3, $CH=CH_2$]	Pome and stone fruit, oilseed rape, vegetables, vines, turf and ornamentals	300–430

Fungicide	Irritation: Eye	Irritation: Skin	LD50 (mg/kg) Oral	LD50 (mg/kg) Dermal	LC50(mg/L) Inhalation	Sensitizing Potential	Signal Word
Iprodione (174)	Mild Irritant	Non-irritant	4468	>2000	>5.2	Negative	Caution
Vinclozolin (83)	Minimal Irritant	Minimal Irritant	>15000	>5000	29.1	Positive	Caution

Fungicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Iprodione	Rat/2-year	6.0	Mutagenicity	No evidence
(134, 174)	Dog/52-week	4.2	Developmental	Not teratogenic
	Mouse/18-month	1870	Reproductive	No evidence
	RfD2(300×UF)	0.04	Oncogenicity	B2 (liver, testes)3
Vinclozolin	Rat/2-year	1.2	Mutagenicity	No evidence
(136, 227)	Dog/52-week	2.4	Developmental	Not teratogenic
	Mouse/18-month	21	Reproductive	Anti-androgenic metabolite
	RfD2	0.012	Oncogenicity	B2 with RfD (multiple benign tumors in rats)3

1 No observable effect level
2 RfD=reference dose
3 See Table 13.3 for US EPA classification scheme

Phthalimides

The phthalimide fungicides represent a relatively old group of synthetic chemicals. Of this group of fungicides, only captan remains significant in regard to use. Captan has a broad spectrum of activity that owes its action to degradation to thiophosgene (16). The structure, uses, and hazard profile of captan are given in Table 13.18.

Captan has been shown to bind to DNA in vitro but not in vivo. Further, captan is classified by the U.S. EPA as category B2, probable human carcinogen, based on gastrointestinal-tract tumors in the mouse (100).

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Table 13.14 Structures, uses, and hazard profiles for dithiocarbamate fungicides

Fungicide	Structure	Principles Uses/Crops	Application Rate gm (a.i.)/ha
Ferbam (39)	$[(CH_3)_2{-}N{-}C(=S){-}S^-]_3\ Fe^{3+}$	Pome fruit, peaches, and tobacco.	300–500
Thiram Vitavax® (79)	$(CH_3)_2N{-}C(=S){-}S{-}S{-}C(=S){-}N(CH_3)_2$	Seed dressing	13–18 gm/100 lbs seed
Ziram (86)	$CH_3{-}N(CH_3){-}C(=S){-}S{-}Zn{-}S{-}C(=S){-}N(CH_3){-}CH_3$	Pome fruit, stone nuts, vines, vegetables and ornamentals.	fruit, 1550–2760

	Irritation		LD50 (mg/kg)		LC50 (mg/L)	Sensitizing	Signal
Fungicide	Eye	Skin	Oral	Dermal	Inhalation	Potential	Word
Ferbam (39)	Mild Irritant	Slight Irritant	>4000	>4000	0.4	Weak Positive	Warning
Thiram (79)	Slight Irritant	Irritant	>1800	>2000	>0.1	Positive	Warning
Ziram (86)	Severe Irritant	Non-irritant	270	>2000	0.06	Positive	Danger

Fungicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Ferbam (137)	Rat/2-year	12.0	Mutagenicity	No evidence
	Dog/52-week	5.0	Developmental	Not teratogenic
	Mouse/18-month	NA	Reproductive	Effects on sperm in mice
	ADI2	0.003 (interim)	Oncogenicity	No evidence
Thiram (121)	Rat/2-year	1.2	Mutagenicity	Positive Ames and SCE
	Dog/2-Year Oral	0.84	Developmental	Teratogenic in mice and hamster at high doses
	Mouse/18-month	3.0	Reproductive	No evidence
	ADI2	0.008	Oncogenicity	No evidence
Ziram (138)	Rat/2-year	<2.5	Mutagenicity	Clastogenic
	Dog/52-week	1.6	Developmental	Not teratogenic
	Mouse/18-month	3.0	Reproductive	Effects on sperm in mice
	ADI2 (1000×UF)	0.003	Oncogenicity	No evidence

1 No observable effect level
2 ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme
NA=Not Available

FIG. 13.3. Structures for ethylenedithiocarbamate fungicides.

Substituted Benzenes

The substituted benzene fungicides have a broad spectrum of activity and are considered protective. Chlorothalonil, in this class, controls fungal infection by inhibiting sulfur-containing enzymes (20). The structure, uses, and hazard profile of chlorothalonil are given in Table 13.19.

The U.S. EPA has classified chlorothalonil as B2, probable human carcinogen, based on kidney and forestomach tumors in both rats and mice (100, 201).

INSECTICIDES

In 1995, insecticide worldwide sales represented $3.5 billion and 13.7 million lb of product (1). Discussion of insecticides here emphasizes the major classes of commercial compounds by mode of action. Discussion of insecticide mode of action focuses on their interactions with cell-membrane proteins and the resulting expression of toxicity in the insect (8). No attempt is made to include all the important compounds within a group.

The modes and mechanisms of actions for these major groups of insecticides are included in Table 13.20.

Many of these classes are newly discovered and often originate from nature (8).

Acetylcholine Mimics

Nicotine has been used as an insecticide since the middle of the 18th century (102). Nicotine exhibits contact activity in insects and across phyla including humans (264). Nicotine mimics the action of acetylcholine, which is a major excitatory neurotransmitter in the insect central nervous system (CNS). After the presynaptic cell releases acetylcholine, it binds to the postsynaptic nicotinic acetylcholine receptor and activates an intrinsic cation channel. This results in depolarization of the postsynaptic cell due to an influx of sodium and calcium ions. The synaptic action of acetylcholine is terminated by the enzyme acetylcholinesterase, which rapidly hydrolyzes the ester linkage in acetylcholine. This activity is depicted in Figure 13.4.

A newer compound in this class is the nitroguanidine, imidacloprid. Imidacloprid generally works best as a stomach poison, and has plant systemic activity (50). Nicotine and imidacloprid activate the nicotinic acetylcholine receptors. This persistent activation leads to an overstimulation of cholinergic synapses, and results in hyperexcitation, convulsions, paralysis, and death of the insect (152). The structures, uses, and hazard profiles of these products are presented in Table 13.21.

Imidacloprid is much less toxic to mammals than nicotine (214).

Carbamates

In contrast to the nicothinoids, the carbamate insecticides inhibit acetylcholinesterase (AChE) so that acetylcholine is not destroyed, resulting in continued stimulation of cholinergic receptors. Carbamates behave in biological systems almost identically to the organophosphate insecticides. Carbamate insecticides exist as esters of carbamic acid, typically having an aryl (ring) substituent as the leaving group. The interaction of the carbamate insecticide with acetylcholinestherase (AChE) is depicted in Figure 13.5.

Carbamates react with the serine group on acetylcholinesterase to yield a carbamylation of the serine hydroxyl group. A hydoxylated leaving group is also

Table 13.15 Hazard profiles for ethylenebisdithiocarbamate fungicides

Fungicide	Structure	Principal Uses/Crops	Application Rate gm (a.i.)/ha
Mancozeb Dithane®; Manzate® (52)	$\left[S-C(=S)-N(H)-CH_2-CH_2-N(H)-C(=S)-S-Mn \right]_x (Zn)_y$	Potatoes, tomatoes, fruits, vegetables, cereals, vines, ornamentals, and tobacco.	6400–12700
Maneb Kypman® (53)	$\left[S-C(=S)-N(H)-CH_2-CH_2-N(H)-C(=S)-S-Mn \right]_x$	Potatoes, tomatoes, vegetables, apples, pears, cereals, ornamentals, vines and tobacco.	450–3600
Zineb Kypzin® (85)	$\left[S-C(=S)-N(H)-CH_2-CH_2-N(H)-C(=S)-S-Zn \right]_x$	Brassicas, lettuce, onions, oilseed rape, vegetables, berries, apples, pears, stone and citrus fruit, bananas, currants, olives, celery, potatoes, tomatoes, hops, and vines.	NA

	Irritation		LD50 (mg/kg)		LC50 (mg/L)	Sensitizing Potential	Signal Word
Fungicide	Eye	Skin	Oral	Dermal	Inhalation		
Mancozeb	Severe Irritant	Slight Irritant	>5000	>5000	5.14	Positive	Danger
Maneb	Moderate Irritant	Slight Irritant	6750	>5000	7.38	Positive	Warning
Zineb	Mild Irritant	Slight Irritant	>5200	>6000	NA	Negative	Caution

Fungicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Mancozeb (216)	Rat/2-year	4.8	Mutagenicity	Equivocal evidence
	Dog/52-week	7.0	Developmental	Teratogenic at high doses
	Mouse/18-month	17	Reproductive	No evidence
	ADI2	0.034	Oncogenicity	B2 (Thyroid tumors in rats of both sexes)3
Maneb (127, 216, 217)	Rat/2-year	5.0	Mutagenicity	No evidence
	Dog/52-week	6.4	Developmental	Not teratogenic
	Mouse/18-month	1 1	Reproductive	No evidence
	ADI2	0.034	Oncogenicity	B2 (Liver tumors in mice of both sexes; thyroid tumors in rats)3
Zineb (129)	Rat/2-year	<25	Mutagenicity	No evidence
	Dog/52-week	50	Developmental	Not teratogenic
	Mouse/18-month	No adequate study	Reproductive	No evidence
	ADI2	0.034	Oncogenicity	No evidence

1 No observable effect level
2 ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme

4 Based in a group ADI for mancozeb, alone or in combination with maneb, metiram, and/or zineb, because of similarity in structure to ethylenethiourea (216)
NA=Not Available

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Table 13.16 Hazard profile for triphenyltin (Fentin®) acetate and hydroxide (37)

Structure	Principles Uses/Crops	Application Rate gm (a.i.)/ha
R = acetate or hydroxide	Used on potatoes, celery, onions, sugar beets, peanuts, beans, wheat, coffee and pecans.	160–240

Fungicide	Irritation Eye	Skin	LD50 (mg/kg) Oral	Dermal	LC50 (mg/L) Inhalation	Sensitizing Potential	Signal Word
Triphenyltin Acetate	Severe Irritant	Non-irritant	140	450	0.044	Positive	Danger
Triphenyltin Hydroxide	Severe Irritant	Slight Irritant	110	1600	0.060	Negative	Danger

Fungicide	Species/study	NOEL[1] (mg/kg/day)	Toxicity Studies	Hazard Indicator
Triphenyltin Hydroxide[3] (37)	Rat/2-year	<0.3	Mutagenicity	No evidence
	Dog/52-week	0.2	Developmental	Not teratogenic
	Mouse/18-month	1.49	Reproductive	No evidence
	ADI[2]	0.0005	Oncogenicity	B2 (mouse liver and pituitary and testicular tumors in rats)[4]

1 No observable effect level
2 ADI=acceptable daily intake
3 Repeat dose studies conducted with triphenyltin hydroxide and not the acetate form.
4 See Table 13.3 for US EPA classification scheme (100)

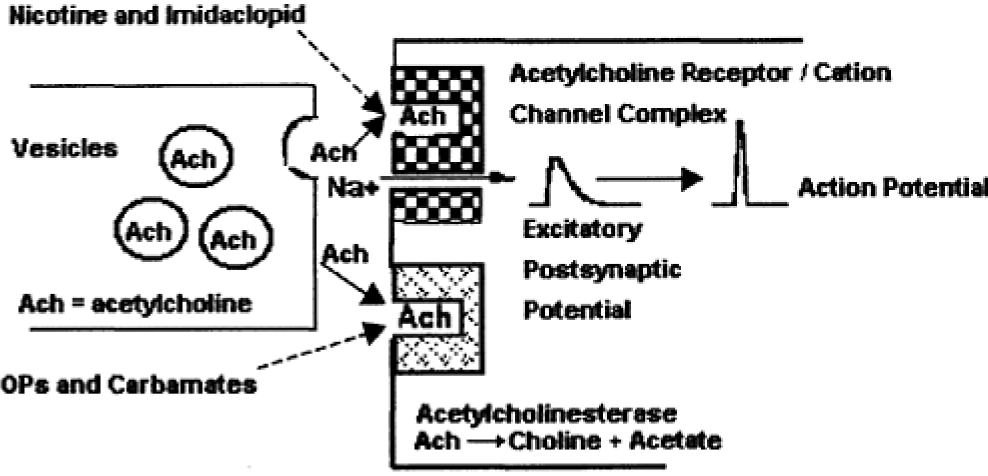

FIG. 13.4. Representation of the site of action of nicotine-type insecticides.

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Table 13.17 Structures, uses, and hazard profiles for phenylpyrrole fungicides

Fungicide	Structure	Principle Uses/Crops	Application Rate Gm (a.i.)/ha
Fenpiclonil Beret®, Gambit® (35)		Seed treatment on cereals and peas Potato seed dressing	20 gm (a.i.)/100kg 20–50 gm (a.i.)/ton
Fludioxonil Maxim® (42)		Used for seed treatment on rice, and on grapes, stone fruit, vegetables, field crops, turf and ornamentals	2.5 gm (a.i.)/100 kg

	Irritation		LD50 (mg/kg)		LC50 (mg/L)	Sensitizing	Signal
Fungicide	Eye	Skin	Oral	Dermal	Inhalation	Potential	Word
Fenpiclonil (35)	Non-irritant	Non-irritant	>5000	>2000	>1.5	Negative	Caution
Fludioxonil (42)	Slight Irritant	Non-irritant	>5000	>2000	>2.6	Negative	Caution

Fungicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Fenpiclonil (35)	Rat/2-year	1.25	Mutagenicity	No evidence
	Dog/52-week	100	Developmental	Not teratogenic
	Mouse/18-month	20	Reproductive	No evidence
	ADI2	0.013	Oncogenicity	No evidence
Fludioxonil (210, 257)	Rat/2-year	50	Mutagenicity	Clastogenic (in vitro)
	Dog/52-week	3.3	Developmental	Not teratogenic
	Mouse/18-month	143	Reproductive	No evidence
	RfD2	0.03	Oncogenicity	D with RfD3

1 No observable effect level
2 RfD=reference dose and ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme

generated. The central nervous system is the site of action of carbamates, and the signs of intoxication are similar to those of the organophosphates. The carbamylation of AChE is reversible, unlike the phosphorylation of the AChE by organophosphate insecticides. The carbamylated complex will typically hydrolyze in minutes (8).

The structures and uses of some representative members of this class are given in Table 13.22.

The carbamates are often highly toxic to mammals and must be handled carefully. The hazard profiles of these selected carbamates are presented in Table 13.23.

Aldicarb is the most acutely toxic of the selected carbamates, with an oral LD50 below 1 mg/kg as well

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Table 13.18 Hazard profile for captan (16, 133)

Structure	Principle Uses/Crops	Application Rate gm (a.i.)/ha
	Used on stone fruit, citrus, almonds, vegetables, potatoes, tomatoes, oilseed rape, berries, and ornamentals	340–5050

Irritation		LD50 (mg/kg)		LC50 (mg/L)		
Eye	Skin	Oral	Dermal	Inhalation	Sensitizing Potential	Signal Word
Corrosive	Mild Irritant	9000	>4500	5.8	Positive	Danger

Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Rat/2-year	25	Mutagenicity	Positive in vitro
Dog/66-Week Oral	60	Developmental	Positive in monkey and hamster
Mouse/18-month	NA	Reproductive	No evidence
RfD2(based on rat reproduction)	0.13	Oncogenicity	B2 (G.I. tract tumors—mouse; kidney—rat) 3

1 No observable effect level

2 ADI=acceptable daily intake

3 See Table 13.3 for US EPA classification scheme

NA=Not Available

FIG. 13.5. Representation of the interaction of carbamate insecticides with acetylcholinesterase.

as a dermal LD50 of 20 mg/kg. Aldicarb, carbofuran, methomyl, and propoxur have been classified as category C (possible human carcinogens) or D(aldicarb) by the U.S. EPA (100). These four materials elicited liver tumors in mice in the 18-month studies.

Organophosphorus Insecticides

Organophosphorus insecticides (OPs) vary tremendously in chemical structure and chemical properties (8).

These chemicals are classified into groups depending on the positioning of the central phosphorus—hence their classification as phosphates, phosphonates, phosphorothionates, phosphorodithioates, and phosphoroamidothioates. Selected examples representing these different groups are presented in Table 13.24.

The OPs react with acetylcholinesterase at the serine hydroxyl group within the enzyme active site. In this reaction, this hydroxyl group is phosphorylated, yielding a leaving group (Figure 13.6).

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Table 13.19 Hazard profile for chlorothalonil (Bravo®) (20, 120, 201)

Structure	Principle Uses/Crops	Application Rate gm (a.i.)/ha
	Used on pome fruit, stone fruit, citrus, cane fruit, vegetables, corn, ornamentals, mushrooms, tobacco, soya and turf.	1050–2190

Irritation		LD50 (mg/kg)		LC50 (mg/L)		
Eye	Skin	Oral	Dermal	Inhalation	Sensitizing Potential	Signal Word
Severe Irritant	Mild Irritant	>10000	>10000	0.093 ♀	Negative	Danger

Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Rat/2-year	2.0	Mutagenicity	No evidence
Dog/52-Week	150	Developmental	Not teratogenic
Mouse/18-month	5.35	Reproductive	No evidence
ADI2	0.03 (JMPR)	Oncogenicity	B2 with CSF of 0.0076 $(mg/kg/day)^{-1}$ (Forestomach tumors in mice and kidney tumors in rats)3
RfD (Non-cancer)	0.02		
RfD (Cancer)	0.015		

1 No observable effect level

2 RfD=reference dose and ADI=acceptable daily intake

3 See Table 13.3 for US EPA classification scheme; CSF=cancer slope factor

FIG. 13.6. Representation of the interaction of organophosphate insecticides with acetylcholinesterase. The phosphorylated acetylcholinesterase is inactivated, blocking acetacholine degradation in the synapse. This results in a buildup of this neurotransmitter and central nervous system hyperstimulation. The signs of intoxication include restlessness, hyperexcitability, tremors, convulsions, and paralysis. Reactivation of the enzyme can take many hours or even days. The toxicologic profiles for selected OPs are presented in Table 13.25.

GABA Agonists (Avermectins)

The avermectins are a group of closely related macrocyclic lactones isolated from the fungus *Streptomyces avermitilis* (Turner and Schaeffer, 1989). The structures of abamectin and emamectin-benzoate, the commercially available insecticides in this class, are shown in Figure 13.7. In addition to the avermectins,

Table 13.20 Mode and mechanism of action for important classes of insecticides used in crop protection

Class	Mode of Action	Mechanism of Action
Acetylcholine Mimics (50, 59)	Systemic acting as a contact and stomach poison	Bind to acetylcholine receptor
Cholinesterase Inhibitors (10, 11)	Systemic acting as a contact and stomach poison	React with a serine hydroxyl group within the enzyme active site and inactivates the enzyme blocking the degradation of the neurotransmitter acetylcholine.
GABA (γ-amino-butyric acid) Agonists (9)	Systemic acting as contact and stomach poison	Act by stimulating the release of GABA, an inhibitory neurotransmitter, by increasing chloride ion flux at the neuromuscular junction.
GABA Antagonists [Channel Blockers] (33)	Non-systemic acting as contact, stomach, and respiratory poison	Act antagonistically at the GABA receptor-chloride channel complex
Compounds Affecting Voltage-Dependent Sodium Channels (36)	Non-systemic acting as contact and stomach poison	Prolong the current flowing through sodium channels by slowing or preventing the shutting of the channels
Juvenile Hormone Mimics (32)	Insect growth regulator preventing metamorphosis to viable adults	Mimic the action of the juvenile hormones and disrupts molting and reproduction
Molt Inhibitors (76)	Systemic acting to inhibit molting and feeding	Inhibit chitin synthesis
Ecdysone Agonists (249)	Lethally accelerates the molting process	Act by binding to the receptor site for ecdysone
Larvicides (28)	Insect growth regulator with contact action	Inhibit embryo development interfering with molting and pupation
Pheromones (147)	Modifies the behavior of other individuals of the same species	Volatile chemicals, natural or synthetic, act for signaling and homing
Respiratory Inhibitors and Uncouplers (19)	Non-systemic acting against all stages of insect development	Inhibit mitochondrial electron transport

spinosad is derived from the fungus *Saccharopolyspora spinasa* (Figure 13.7).

The mode on action of these three products in insects is paralysis. The avermectins stimulate the release of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) at the neuromuscular junction (88, 167). The agonistic release of GABA evokes an electrical activity in vertebrate and invertebrate nerve and muscle by increasing the membrane conductance to chloride ions. The ionic concentration within the neuron increases continuously until a trigger level is reached and the action potential is produced. This activity is depicted in Figure 13.8.

Table 13.21 The hazard profile for the acetylcholine mimic insecticides

Fungicide	Structure	Principle Uses	Application Rate gm (a.i.)/ha
Imidacloprid Admire® Provado® (50)		Used to control sucking insects including asphids, thrips, and whiteflies.	290
Nicotine Nico® Soap (59)		Used to control sucking insects including ricehoppers, asphids, thrips, and whiteflies	Limited Use

	Irritation		LD50 (mg/kg)		LC50 (mg/L)		
Insecticide	Eye	Skin	Oral	Dermal	Inhalation	Sensitizing Potential	Signal Word
Imidacloprid (50)	Non-irritant	Non-irritant	424	>5000	0.07	Negative	Warning
Nicotine (59)	Irritant	Mild Irritant	50	50	NA	Negative	Danger

Insecticide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Imidacloprid	Rat/2-year	5.7	Mutagenicity	No evidence
(214)	Dog/2-Year Oral	41	Developmental	Not teratogenic
	Mouse/18-month	208	Reproductive	No evidence
	RfD2	0.057	Oncogenicity	E (No evidence)3
Nicotine	Rat/2-year	NA	Mutagenicity	No evidence
(59)	Dog/52-week	NA	Developmental	Not teratogenic
	Mouse/18-month	NA	Reproductive	NA
	RfD2	NA	Oncogenicity	NA

1 No observable effect level
2 RfD=reference dose and ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme
NA=Not Available

Avermectin intoxication in mammals begins with hyperexcitability, tremors, and incoordination, and later develops into ataxia and coma-like sedation (141). The toxicity profiles of abamectin, emamectin-benzoate, and spinosad are given in Table 13.26.

Both abamectin and emamectin benzoate have the propensity to produce neurotoxicity. This toxicity is reduced or prevented in test animals having a fully intact P-glycoprotein blood-brain barrier. Much of the early testing of the hazards of these avermectins was performed in the CF-1 mouse. The CF-1 mouse has been found to be heterozygous for P-glycoprotein and has been ruled out as an experimental model for human risk assessment (122, 142, 143, 251).

Channel Blockers

Organochlorines

The channel-blocking convulsants represent one of the oldest groups of synthetic organic insecticides, dating back to the early 1940s (148). These lipophilic compounds were found to be environmentally stable and persistent, and many, like dieldrin, endrin, and DDT, have been

Table 13.22 Structures and uses of selected carbamate insecticides

Insecticide	Structure	Principle Uses/Crops	Treatment Rate gm (a.i.)/ha
Aldicarb Temik® (11)	$H_3C{-}S(CH_3)_2{-}CH{-}N{-}C(=O){-}N(CH_3)H$	Controls chewing and sucking insects in vegetables and crops.	350–5600
Carbaryl Sevin® (17)	$OCONHCH_3$	Controls chewing and sucking insects in vegetables and various crops	250–2000
Carbofuran Furadan® (18)	$OOCNHCH_3$, O, CH_3, CH_3	Controls soil dwelling and foliar feeding insects in food crops	260–2050
Methomyl LanoxC® (56)	$CH_3C(SCH_3){=}NOC(=O)NHCH_3$	Controls chewing and sucking insects in vegetables, food crops and turf.	120–2000
Propoxur Aprocarb® (65)	$OOCNHCH_3$, $OCH(CH_3)_2$	cockroaches, flies, fleas, ants, and mosquitoes	NA

banned in the United States. However, some of the more biodegradable materials like lindane and endosulfan still find use today. Fipronil is an aryl heterocycle with a similar mode of action, but improved selective toxicity toward insects.

In both insects and mammals, chloride channelblocking insecticides cause hyperexcitability and convulsions (7). These effects occur via poisoning of the CNS through antagonism of the inhibitory neurotransmitter γ-aminobutyric acid (GABA). Normally, when GABA is released from the presynaptic nerve terminal, it binds to a postsynaptic receptor protein containing an intrinsic chloride ion channel. When GABA binds to the receptor, the channel is opened, and Cl ions flow into the postsynaptic neuron (see Figure 13.8). This chloride permeability can significantly hyperpolarize the membrane potential and has a dampening effect on nerve impulse firing. The structures, uses, and toxicology profiles for selected channel blockers are given in Table 13.27.

The organochlorine channel-blocking insecticides are generally not mutagenic, developmental, or

reproductive toxins or oncogenic.

Pyrethroids

The pyrethroid insecticides, typically esters of chrysanthemic acid, were isolated from the flowers of

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Table 13.23 Hazard profile for the carbamate insecticides

Insecticide	Irritation: Eye	Irritation: Skin	LD50 (mg/kg): Oral	LD50 (mg/kg): Dermal	LC50 (mg/L): Inhalation	Sensitizing Potential	Signal Word
Aldicarb (11)	Non-irritant	Non-irritant	0.93	20	0.2	Negative	Danger
Carbaryl (20)	Non-irritant	Non-irritant	500 ♀	>4000	206	Negative	Caution
Carbofuran (18)	Mild irritant	Mild irritant	8	>3000	0.075	Negative	Danger
Methomyl (56)	Irritant	Non-irritant	17	>5000	0.3	NA	Danger
Propoxur (65)	Slight Irritant	Non-irritant	50	>5000	0.5	Negative	Warning

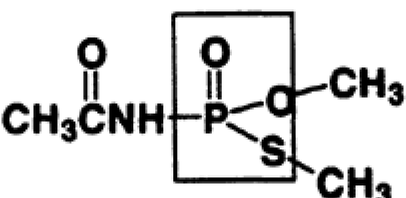
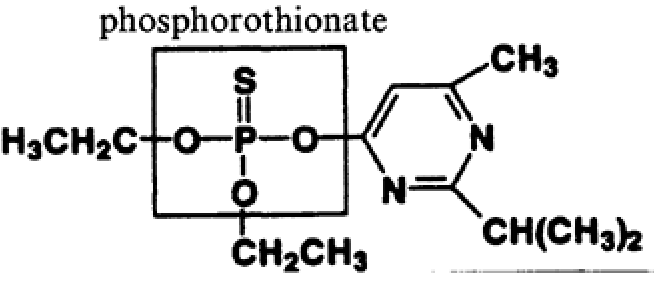
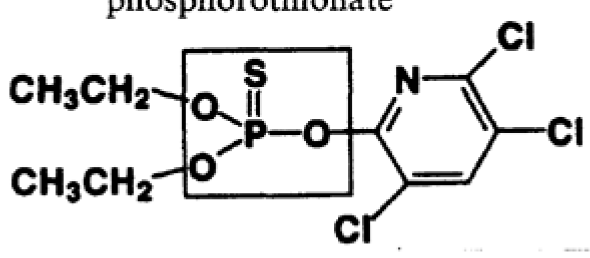
Insecticide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Aldicarb (131, 172)	Rat/2-year	0.3	Mutagenicity	No evidence
	Dog/104-week	0.1	Developmental	Not teratogenic
	Mouse/18-month	0.3	Reproductive	No evidence
	ADI2	0.003	Oncogenicity	D3
	RfD2	0.001		
Carbaryl (107)	Rat/2-year	200	Mutagenicity	No evidence
	Dog/52-week	1.43	Developmental	Not Teratogenic
	Mouse/18-month	NA	Reproductive	No evidence
	Human	0.01	Oncogenicity	E (No evidence)3
	RfD2	0.01		
Carbofuran (108)	Rat/2-year	20	Mutagenicity	No evidence
	Dog/2-Year Oral	10	Developmental	Not teratogenic
	Mouse/18-month	20	Reproductive	No evidence
	RfD2	0.002	Oncogenicity	C with RfD (Mouse liver tumors in both sexes)3
Methomyl (116)	Rat/2-year	200	Mutagenicity	No evidence
	Dog/52-week	200	Developmental	Teratogenic in mice
	Mouse/18-month	500	Reproductive	No evidence
	ADI2	0.02	Oncogenicity	C with RfD (liver tumors—female mice)3
Propoxur (117)	Rat/2-year	5.0	Mutagenicity	No evidence
	Dog/52-week	1.25	Developmental	Not teratogenic
	Mouse/18-month	40	Reproductive	No evidence
	ADI2	0.01	Oncogenicity	C with RfD3

1 No observable effect levela
2 RfD=reference dose and ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme
NA=Not Available

chrysanthemums (160). Synthetic pyrethroid chemistry and action are classified as Type 1 or Type 2, depending on the alcohol substituent (6). The members of the Type 1 group are generally unstable in the environment, which has prevented their use in row crops. The Type 2 pyrethroids are more narrowly defined in terms of their chemical structure. They specifically contain an α-cyano-3-phenoxybenzyl alcohol, which increases insecticidal activity about 10-fold.

The signs of intoxication by pyrethroids develop rapidly, and there exist different poisoning syndromes for the two types of compounds (6). Type 1 pyrethroids

Table 13.24 Structures and uses of selected organophosphate insecticides

Insecticide	Structure	Principle Uses/Crops	Treatment Rate gm (a.i.)/ha
Monocrotophos Monocron® (57)	phosphate	Control of sucking, chewing, and boring insects and spider mites.	NA
Dichlorvos Vapona® (30)	phosphonate	Control of sucking, and chewing insects and spider mites in household sprays, etc.	100
Acephate Amithene® (10)	phosphoramidothiate 	Control sucking, and chewing insects.	500
Diazinon Spectracide® (29, 123)	phosphorothionate 	Control of sucking, and chewing insects and mites.	400–800
Chlorpyrifos Lorsban® (21)	phosphorothionate 	Control of sucking, chewing, and boring insects	470–950

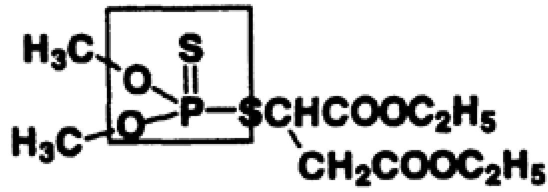
Azinphos-methyl Guthion® (118)		Control of sucking, and chewing insects.	NA
Malathion Acimal® (320)		Control of sucking, and chewing insects.	570

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Table 13.25 Hazard profiles for selected organophosphate insecticides

Insecticide	Irritation Eye	Irritation Skin	LD50 (mg/kg) Oral	LD50 (mg/kg) Dermal	LC50 (mg/L) Inhalation	Sensitizing Potential	Signal Word
Acephate	NA	Non-irritant	866	>2000	>15	Negative	Caution
Azinphos-methyl	Mild irritant	Non-irritant	6–19	150	0.15	Positive	Danger
Chlorpyrifos	Non-irritant	Non-irritant	2680	>2000	>0.67	Negative	Caution
Diazinon	Non-irritant	Non-irritant	1250	>2150	2.33	Negative	Caution
Dichlorvos	Irritant	Irritant	50	90	0.34	Negative	Danger
Malathion	NA	NA	1000 ♀	4100	>5.2	NA	Caution
Monocrotophos	Non-irritant	Non-irritant	18	130	0.08	NA	Danger

Insecticide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Acephate (113)	Rat/2-year	0.5	Mutagenicity	No evidence
	Dog/26-Week Oral	0.75	Developmental	Not teratogenic
	Mouse/18-month	NA	Reproductive	No evidence
	Human	0.3	Oncogenicity	C (Mouse liver tumor)4
	ADI2	0.03 (10×UF3)	Neurotoxicity	Not delayed neurotoxin
Azinphos-methyl (118)	Rat/2-year	0.86	Mutagencity	Effects in vitro; no in vivo
	Dog/52-Week Oral	0.74	Developmental	No evidence
	Mouse/18-month	0.88	Reproductive	Effects on fertility
	Human	0.005	Oncogenicity	E (No evidence)4
	ADI2	0.005	Neurotoxicity	Not delayed neurotoxin
Chlorpyrifos (109)	Rat/2-year	0.1	Mutagenicity	No evidence
	Dog/13-Week Oral	10	Developmental	Not Teratogenic
	Mouse/18-month	3.9	Reproductive	No evidence
	Human	0.1	Oncogenicity	No evidence
	ADI2	0.01 (10×UF3)	Neurotoxicity	Not delayed neurotoxin
Diazinon (123)	Rat/2-year	0.07	Mutagenicity	No evidence
	Dog/2-Year Oral	0.02	Developmental	Not teratogenic
	Mouse/18-month	NA	Reproductive	No evidence
	Human ADI2	0.025 0.002 (10×UF3)	Oncogenicity Neurotoxicity	No evidence Not delayed neurotoxin
Dichlorvos (124)	Rat/2-year	2.4	Mutagenicity	May be mutagenic
	Dog/52-week	NA	Developmental	Not Teratogenic
	Mouse/18-month	10	Reproductive	No evidence
	Human/21-day	0.04	Oncogenicity	No evidence
	ADI2	0.004 (10×UF3)	Neurotoxicity	Delayed neuropathy
Malathion (320)	Rat/2-year	<1.2	Mutagenicity	No evidence
	Dog/52-week	NA	Developmental	Not teratogenic
	Mouse/18-month	NA	Reproductive	Effects on litter size
	Human/56-day	0.34	Neurotoxicity	Not delayed neurotoxin
	ADI2	0.02	Oncogenicity	No evidence
Monocrotophos (130)	Rat/2-year	0.025	Mutagenicity	No evidence
	Dog/52-week	0.0125	Developmental	Not teratogenic
	Mouse/18-month	–	Reproductive	No evidence
	ADI2	0.0006	Oncogenicity	No evidence

1 No observable effect level
2 ADI=acceptable daily intake

3 Uncertainty factor of 10 used based on human cholinesterase used
4 See Table 13.3 for US EPA classification scheme
NA=Not Available

Disaccharide-oxy
Macrocycle
Spiroketal
Benzofuran

abamectin
(80% avermectin B1a, 20% avermectin B1b)
R (B1a) = CH_2CH_3
(B1b) = CH_3

emamectin benzoate
(80% avemectin benzoate B1a, 20% B1b)
R (B1a) = CH_2CH_3
(B1b) = CH_3

spinosad
spinosyn A, R=H
spinosyn D, R= CH_3

FIG. 13.7. The structures of avermectins and spinosad.

FIG. 13.8. Representation of the action of the avermectins at the presynaptic inhibitory terminal.

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Table 13.26 Hazard profile for avermectins and spinosad

Insecticide	Irritation: Eye	Irritation: Skin	LD50 (mg/kg): Oral	LD50 (mg/kg): Dermal	LC50 (mg/L): Inhalation	Sensitizing Potential	Signal Word
Abamectin (9)	Mild irritant	Non-irritant	13.6	>2000	5.73	Negative	Danger
Emamectin benzoate (256)	Severe Irritant	Non-irritant	76	>2000	2.12	Negative	Danger
Spinosad (84, 193)	Non-irritant	Non-irritant	3738	>5000	>5.18	Negative	Caution

Insecticide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Abamectin (122, 136, 251)	Rat/2-year	1.5	Mutagenicity	No evidence
	Dog/26-Week Oral	0.25	Developmental	Not teratogenic
	Mouse/18-month	4.0	Reproductive	No evidence
	RfD2 (reproduction—1000×UF 4)	0.00012	Neurotoxicity	Neurotoxicity exhibited in rodents, and dogs
			Oncogenicity	E (No evidence)3
Emamectin Benzoate (256)	Rat/2-year	0.25	Mutagenicity	No evidence
	Dog/25-Week Oral	0.25	Developmental	Not teratogenic
	Mouse/18-month	2.5	Reproductive	No evidence
	RfD2 (15-day neurotoxicity in CF-1 Mouse—900×UF4)	0.00083	Neurotoxicity	Neurotoxicity exhibited in rodents, and dogs
			Oncogenicity	E (No evidence)3
Spinosad (193)	Rat/2-year	5.0	Mutagenicity	No evidence
	Dog/26-Week Oral	2.7	Developmental	Not teratogenic
	Mouse/18-month	7.5	Reproductive	No evidence
	RfD2	0.027	Neurotoxicity	Not Neurotoxic
			Oncogenicity	E (No evidence)3

1 No observable effect level
2 RfD=reference dose and ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme
4 Uncertainty factor

cause hyperexcitability and convulsions in insects and a whole-body tremor in mammals. Type 2 pyrethroids cause ataxia and incoordination in insects, and writhing and salivation in mammals.
Pyrethroid intoxication results from their potent effects on nerve impulse generation within both the central and peripheral nervous systems. The nerve impulse or action potential consists of a transient depolarization driven by an influx of Na^+ ions, followed by the efflux of K^+ ions. This activity is depicted in Figure 13.9.

Type 1 compounds induce multiple spike discharges in peripheral sensory and motor nerves, as well as interneurons within the central nervous system (CNS). Type 2 pyrethroids depolarize the axon membrane potential, which reduces the amplitude of the action potential and eventually leads to a loss of electrical excitability. These effects occur because pyrethroids prolong the current flowing through sodium channels by slowing or preventing the shutting of the channels.

The structures and uses of some of the economically important representatives of both types of pyrethroids are given in Table 13.28.

All of these synthetic pyrethroids are generally used in the low grams per hectare range because of their toxicity to insects. The mammalian toxicity profiles of these materials are given in Table 13.29.

Both fenvalerate and lambda-cyhalothrin are acutely toxic by the oral and inhalation route. Lambda-cyhalothrin and permethrin have some potential to produce neurotoxicity, and both exhibit some weak oncogenic potential in animal models.

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Table 13.27 Hazard profiles for the organochlorine chloride channel blockers

Insecticide	Structure	Principle Uses	Application Rates gm (a.i./ha)
Endosulfan (33)		Used to control sucking, chewing and boring insects in a variety of crops including fruit, vines, vegetables, cotton and cereal	1120
Fipronil (40)		Used to control thrips, corn root worms, and termites	100–200
Gamma–HCH (44)		Used to control soilinhabiting insects, publichealth pests, and animal ectoparasites	250–750

Insecticide	Irritation Eye	Skin	LD50 (mg/kg) Oral	Dermal	LC50 (mg/L) Inhalation	Sensitizing Potential	Signal Word
Endosulfan	Non-irritant	Non-irritant	70	359	>0.034	Negative	Danger
Fipronil	Non-irritant	Non-irritant	97	>2000	0.68	Negative	Warning
Gamma–HCH	Irritant	Irritant	>88	>900	1.6	Negative	Warning

Insecticide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Endosulfan (114)	Rat/2-year	0.60	Mutagenicity	No evidence
	Dog/52-week	0.57	Developmental	Not teratogenic
	Mouse/18-month	0.84	Reproductive	No evidence
	ADI2	0.006	Oncogenicity	No evidence3
Fipronil (209)	Rat/2-year	0.20	Mutagenicity	No evidence
	Dog/52-week	0.30	Developmental	Not teratogenic
	Mouse/18-month	0.50	Reproductive	No evidence
	RfD2	0.0002	Oncogenicity	No evidence3
			Neurotoxicity	Not neurotoxic
Lindane (Gamma–HCH) (115)	Rat/2-year	0.75	Mutagenicity	No evidence
	Dog/52-week	1.6	Developmental	Not teratogenic
	Mouse/18-month	NA	Reproductive	No evidence
	ADI2	0.008	Neurotoxicity	Not neurotoxic
			Oncogenicity	No evidence3

1 No observable effect level
2 RfD=reference dose and ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme
NA=Not Available

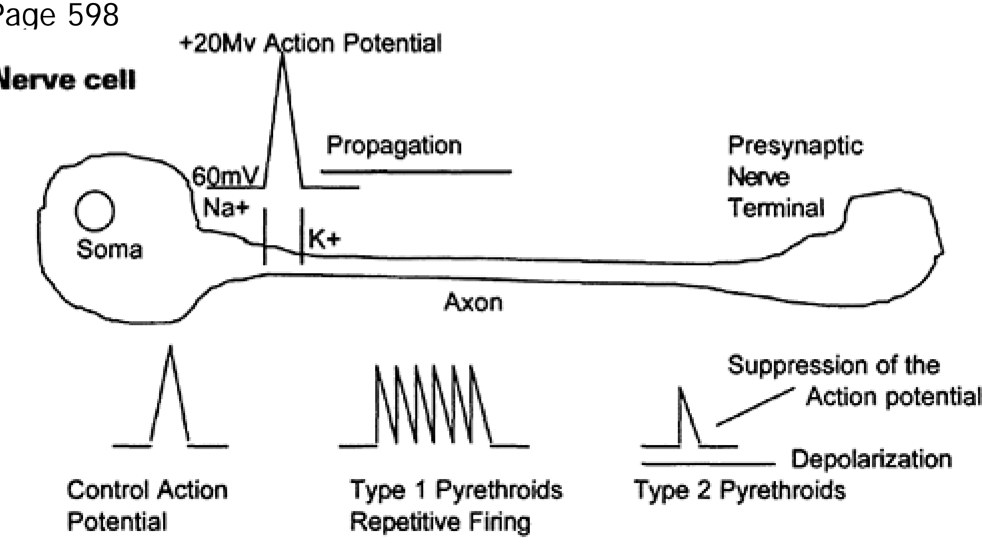

FIG. 13.9. Sites of action of the pyrethroids on nerve impulses.

Table 13.28 Structures and uses of selected pyrethroid insecticides

Insecticide	Structure	Principle Uses/Crops	Treatment Rate gm (a.i.)/ha
Fenpropathrin Danitol® (36)		Controls many species of mites and insects.	20–30
Fenvalerate Fenbaz® (38)		Controls chewing, sucking and boring insects.	20–25
Lambdacyhalothrin Karate® (51)		Controls a broad spectrum of chewing and piercing insects.	10–450
Permethrin (Type 1) Ambush® (61)		Controls leaf and fruit-eating *Lepidoptera* and *Coleoptera* in cotton.	45–65

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Table 13.29 Hazard profiles for the selected synthetic pyrethroids

	Irritation		LD50 (mg/kg)		LC50 (mg/L)		
Insecticide	Eye	Skin	Oral	Dermal	Inhalation	Sensitizing Potential	Signal Word
Fenpropathrin (36)	Not irritant	Not irritant	>870	>2000	0.096	Negative	Danger
Fenvalerate (38)	Slight irritant	Irritant	87	>2000	0.40	Negative	Warning
Lambda-cyhalothrin (52)	Not irritant	Mild irritant	56	632	0.60	Negative	Danger
Permethrin (61)	Not irritant	Not irritant	430	>2000	>0.68	Moderate positive	Warning

Insecticide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Fenpropathrin (125, 207)	Rat/2-year	7.0	Mutagenicity	No evidence
	Dog/52-week	3.0	Developmental	Not teratogenic
	Mouse/18-month	56	Reproductive	No evidence
	ADI2	0.03	Oncogenicity	E(No evidence)3
			Neurotoxicity	Not neurotoxic
Fenvalerate (208)	Rat/2-year	12.5	Mutagenicity	No evidence
	Dog/52-week	2.25	Developmental	Not Teratogenic
	Mouse/18-month	1.5	Reproductive	No evidence
	RfD2	0.02	Oncogenicity	E(No evidence)3
			Neurotoxicity	Neurotoxic
Lambda-Cyhalothrin (241)	Rat/2-year	2.5	Mutagenicity	No evidence
	Dog/2-Year Oral	0.1	Developmental	Not teratogenic
	Mouse/18-month	14.2	Reproductive	No evidence
	RfD2	0.001	Oncogenicity	D (not classifiable)3
			Neurotoxicity	Neurotoxic
Permethrin (128)	Rat/2-year	5.0	Mutagenicity	No evidence
	Dog/52-week	5.0	Developmental	Not teratogenic
	Mouse/18-month	7.1	Reproductive	No evidence
	ADI2	0.05	Oncogenicity	C/RfD (lung and liver tumors in female mice)3
			Neurotoxicity	Neurotoxic

1 No observable effect level
2 RfD=reference dose and ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme

Endotoxins

Bacillus Thuringiensis

Bacillus thuringiensis (Bt) is an aerobic spore-forming gram-positive, rod-shaped bacterium. At sporulation, Bt forms a crystalline inclusion body that contains a number of insecticidal protein toxins (13). When consumed by the insect, the inclusion is dissolved in the midgut and releases δ-endotoxins. The toxin proteins contain a few hundred to over 1000 amino acids. After they are ingested, the δ-endotoxins are cleaved to an active form by proteases within the midgut. The active toxins bind specifically to the membranes of the midgut epithelia and alter their ion permeability properties by forming a cation channel or pore. Ion movements through this pore disrupt potassium and pH gradients and lead to lysis of the epithelium, gut paralysis, and death (99).

Bt has been used directly as a preparation created from grinding up insects infected with Bt or genetically engineered into plants. In regulating this insecticide, the U.S. EPA considered that microbial preparations

of Bt have been commercially available for the last 30 years. As these bacterial strains are found in nature, the mammalian hazard test requirements are not as extensive as for synthetic pesticides (197, 229). Generally, the hazard tests performed are acute oral toxicity, in vitro digestibility under gastric conditions, and amino acid homology evaluations and comparisons.

Insect Growth Regulators

Juvenile Hormone Mimics

The juvenile hormone mimics (JHMs) are compounds bearing a structural resemblance to the juvenile hormones of insects. Juvenile hormones are lipophilic sesquiterpenoids containing epoxide and methyl ester groups. The JHMs mimic the action of the juvenile hormones on a number of physiological processes, such as molting and reproduction. Exposure to these compounds at molting results in the production of insects containing mixed larval/pupal or larval/adult morphologies. The efficacy of these compounds is greatest when normal juvenile hormone titers are low, namely, in the last larval or early pupal stages (8, 263). Thus, timing of application is important for successful control. Another useful property of these compounds is that, in adults, they disrupt normal reproductive physiology.

The structures, uses, and hazard profiles of three of these compounds are presented in Table 13.30. All three of these compounds have excellent acute and repeated-dose hazard profiles.

Molt Inhibitors

These compounds are classified as benzoylphenylureas and possess a number of halogen substituents. Insects exposed to these compounds are unable to form normal cuticle because the ability to synthesize chitin is lost. Fifty percent of the cuticle is comprised of chitin, which is a polysaccharide of *N*-acetylglucosamine. In the absence of chitin, the cuticle is unable to support the insect.

The compounds are generally not very toxic to mammals but exhibit a high degree of lipophilicity and are stored in the fat. The structures, uses, and hazard profiles of diflubenzuron and teflubenzuron are presented in Table 13.31.

Diflubenzuron and teflubenzuron have excellent hazard profiles with no evidence of significant developmental or reproductive toxicity; neither is carcinogenic.

Molt Accelerators or Ecdysone Agonists

The ecdysone agonist tebufenozide acts upon ingestion, causing the larvae to cease feeding and successfully molt. This compound is also selective, bringing a quick kill to lepidoptera pests (263). The structure, uses, and hazard profile of tebufenozide are presented in Table 13.32.

Tebufenozide is not acutely toxic and does not produce a hazard in regard to repeated-dose toxicity or developmental, reproductive, and oncogenic potential.

Other Modes of Action

Larvicides

The larvicidal agents do not all belong to the same class of chemistry as characterizes some of the previously described mode of action groups. Clofentezine is used as an acaricide/ovicide for deciduous fruits, citrus, cotton, cucurbits, vines, and ornamentals. Cyromazine, an insect growth inhibitor with contact poison features interfering with molting and pupation, is used as a foliar spray, for fly control on treated surfaces, and as a feed premix. Hexythiazox acts as an acaricide, larvicide, and nymphicide. It is used to control eggs and larvae on fruits, vines, vegetables, and cotton. The exact mechanism of action of these three chemicals is not well understood. The structures, principle uses, and hazard profiles for these novel insecticides are given in Table 13.33.

The larvicides possess limited mammalian toxicity. Although cyromazine has not been shown to have any carcinogenic potential in animal studies, both clofentezine and hexythiazox have been classified as category C carcinogens. Clofentezine is to be managed using a reference dose (RfD), whereas, hexythiazox's is regulated using a cancer slope factor value.

Pheromones

Pheromones are common chemical sex attractants secreted by special glands of one or both sexes in insects. These chemicals work to identify and/or locate insects of the opposite gender (147). A pheromone (including an identical synthetic compound) is defined by the U.S. EPA as a compound produced by an arthropod (insect, arachnid, or crustacean) that modifies the behavior of other individuals of the same species (179).

Lepidopteran pheromones, produced by a member of the order Lepidoptera, including butterflies and moths, share a physicohemical feature common to all pheromones: their volatility, which is the basis for the signaling and homing mechanism. The U.S. EPA has registered 17 arthropod pheromones active ingredients, 11 of which are lepidopteran pheromones (179). The information submitted covered compounds that were from 6- to 16-carbon unbranched alcohols, acetates, and aldehydes. The structure

for the major lepidopteran pheromone, tetradecenyl acetate, is shown in Figure 13.10.
The U.S. EPA has assumed that pheromones and other similar natural chemicals are different from conventional

Table 13.30 Hazard profile for juvenile hormone mimics

Fungicide	Structure	Principle uses	Application rates gm (a.i.)/ha
Diofenolan (32)		Used to control most scale insects and eggs in fruits and ornamentals	25–50
Pymetrozine Sterling® (67)		Used to control asphids and whiteflies in vegetables, ornamentals, cotton and field crops	25–50
Pyriproxifen Knack® (70, 260)		Used to control public health insect pests	25–50

Insecticide	Irritation		LD50 (mg/kg)		LC50 (mg/L)	Sensitizing Potential	Signal Word
	Eye	Skin	Oral	Dermal	Inhalation		
Diofenolan (32)	Not irritant	Not irritant	>5000	>2000	>3.1	Negative	Caution
Pymetrozine (67)	Not irritant	Not irritant	>5820	>2000	>1.8	Negative	Caution
Pyriproxifen (70, 260)	Not irritant	Not irritant	>5000	>2000	>3.1	Negative	Caution

Insecticide	Species/study	NOEL 1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Diofenolan (32)	Rat/13-Week Oral	12	Mutagenicity	No evidence
	Dog/13-Week Oral	12	Developmental	Not teratogenic
	Mouse/18-month	–	Reproductive	No evidence
	ADI (provisional)2	0.006(Based on 13-wk. rat with 500 UF4)	Oncogenicity	NA
Pymetrozine (245, 246)	Rat/2-year	3.7	Mutagenicity	No evidence
	Dog/52-week	0.57	Developmental	Not teratogenic
	Mouse/18-month	12	Reproductive	No evidence
	RfD2	0.0057	Oncogenicity	No evidence
Pyriproxifen (248)	Rat/2-year	35	Mutagenicity	No evidence
	Dog/52-week	100	Developmental	Not teratogenic
	Mouse/18-month	85	Reproductive	No evidence

RfD2	0.35	Oncogenicity	E(No evidence)3

1 No observable effect level
2 RfD=reference dose and ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme
4 UF=Uncertainty factor
NA=Not Available

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Table 13.31 Structures, uses, and hazard profiles for the molt inhibitors (chitin synthesis inhibitors)

Insecticide	Structure	Principle uses	Application Rates gm (a.i.)/ha
Diflubenzuron Amilin® (233, 234)		Used to control major insect pests in cotton, soya, citrus, tea, vegetables and mushrooms including larva of flies, mosquitoes, grasshoppers, and locust.	25–75
Teflubenzuron Nomolt® (76)		Used to control major insect pests in fruits, vegetables tobacco, and cotton including larva of flies, mosquitoes, grasshoppers, and locust	NA

	Irritation		LD50 (mg/kg)		LC50 (mg/L)		
Insecticide	Eye	Skin	Oral	Dermal	Inhalation	Scnsitizinc Potential	Signal Word
Diflubenzuron (234)	Not irritant	Not irritant	>4640	>10000	>35	Negative	Caution
Teflubenzuron (76)	Not irritant	Not irritant	>5000	>2000	>3.1	Negative	Caution

Insecticide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Diflubenzuron (233, 234)	Rat/2-year	2.0	Mutagenicity	No evidence
	Dog/52-week	2.0	Developmental	Not teratogenic
	Mouse/18-month	2.0	Reproductive	No evidence
	RfD2	0.02	Oncogenicity	E(No evidence)3
Teflubenzuron (76)	Rat/2-year	4.8	Mutagenicity	No evidence
	Dog/52-week	3.2	Developmental	Not teratogenic
	Mouse/18-month	2.1	Reproductive	No evidence
	ADI2	0.01 (based on the 18-month mouse with a 200×UF4)	Oncogenicity	No evidence

1 No observable effect level
2 RfD=reference dose and ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme
4 UF=Uncertainty factor
NA=Not Available

FIG. 13.10. The structure of the lepidopteran pheromone tetradecenyl acetate.

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Table 13.32 Structure, uses, and hazard profile for the ecdysone agonist tebufenozide (Confirm®) (249)

Structure	Primary Uses	Application Rates gm (a.i.)/ha
[structure]	Used for control lepidopteran larvae on rice, fruit, row crop, nut crops, vegetables, and vines.	NA

	Irritation		LD50 (mg/kg)		LC50 (mg/L)	Sensitizing	Signal
Insecticide	Eye	Skin	Oral	Dermal	Inhalation	Potential	Word
Tebufenozide	Not irritant	Not irritant	>5000	5000	4.5	Negative	Caution

Insecticide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Tebufenozide	Rat/2-year	4.8	Mutagenicity	No evidence
	Dog/52-week	1.8	Developmental	Not teratogenic
	Mouse/18-month	143	Reproductive	No evidence
	RfD2	0.018	Oncogenicity	E(No evidence)3

1 No observable effect level
2 RfD=reference dose
3 See Table 13.3 for US EPA classification scheme
NA=Not Available

synthetic pesticides. Therefore, there are fewer data requirements for registering pheromones. In fact, most of the hazard data for these pheromones are anecdotal. The data available on both lepidopteran and other arthropod pheromones, including several aromatic pheromones, have indicated no acute mammalian toxicity at the limit dose levels tested. The acute toxicity profile generally reveals oral and dermal LD50 values of greater than 5000 mg/kg and 2000 mg/kg, respectively (226). The acute inhalation LC50 value is generally >5 mg/L. Eye and skin irritation potential fall in the mild or not irritating range, with no evidence of sensitization potential. Since a miniscule amount of the pheromone will be adsorbed to the inside of the bait station, there is practically no human contact with the pheromone. Therefore, the full data package required for conventional pesticides is waived by the U.S. EPA (226).

Respiratory Inhibitors and Uncouplers

Compounds that disrupt energy metabolism have been identified from both natural and synthetic sources. An important natural product is rotenone, which is derived from cube or derris root (160). The synthetic compounds in this group include a number of nitrogen-containing heterocycles, such as pyridaben (144). Disruption of energy metabolism occurs in the mitochondria and usually takes the form of either an inhibition of the electron transport system or an uncoupling of the transport system from ATP production. Inhibition of the electron transport system blocks the production of ATP and causes a decrease in oxygen consumption by the mitochondria. These uncouplers act on coenzyme Q oxidoreductase in the electron transport chain or the cytochrome b–c1 complex (103). The electron transport system functions normally, but the production of ATP is uncoupled from the electron transport process due to a dissipation of the proton gradient across the inner mitochondrial membrane. In the presence of uncouplers, oxygen consumption increases, but no ATP is produced (104). The disruption of energy metabolism and the subsequent loss of ATP results in a slowly developing toxicity, and the effects of all these compounds include inactivity, paralysis, and death.

The structures, uses, and hazard profiles of three compounds in this group registered in the United States are given in Table 13.34.

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Table 13.33 Hazard profiles for selected larvicides

Insecticide	Structure	Principle uses	Application Rates gm (a.i.)/ha
Cyromazine Trigard® (28)		Used to control fly larvae in manure and leaf miners in vegetables.	140
Clofentezine Apollo® (252, 253)		Used to control eggs and young mobile stages of mites in vegetables and fruit.	NA
Hexythiazox Nissorun® (237)		Used to control larvae and eggs phygophagous mites in fruit, vines, cotton and vegetables.	NA

Insecticide	Irritation Eye	Skin	LD50 (mg/kg) Oral	Dermal	LC50 (mg/L) Inhalation	Sensitizing Potential	Signal Word
Cyromazine	Not irritant	Mild Irritant	2029	>1370	>2.7	Negative	Caution
Clofentezine	Not irritant	Not irritant	>5200	>2100	>2.0	Weak positive	Caution
Hexythiazox	Mild irritant	Not irritant	>5000	>5000	>2.0	Negative	Caution

Insecticide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Cyromazine	Rat/2-year	1.8	Mutagenicity	No evidence
(28, 255)	Dog/26-Week Oral	0.75	Developmental	Not teratogenic
	Mouse/18-month	6.5	Reproductive	No evidence
	RfD2	0.008	Oncogenicity	E(No evidence)3
Clofentezine	Rat/2-year	2.0	Mutagenicity	No evidence
(252, 253)	Dog/26-Week Oral	1.25	Developmental	Not teratogenic
	Mouse/18-month	7.1	Reproductive	No evidence
	RfD2	0.012	Oncogenicity	C with Q* (thyroid tumors in male rats)3
Hexythiazox	Rat/2-year	21.5	Mutagenicity	No evidence
(237, 238)	Dog/26-Week Oral	2.5	Developmental	Not teratogenic
	Mouse/18-month	37.5	Reproductive	No evidence
	RfD2		Oncogenicity	C with CSF (based on liver tumors)3

1 No observable effect level
2 RfD=reference dose, CSF=cancer slope factor
3 See Table 13.3 for US EPA classification scheme
NA=Not Available

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Table 13.34 Hazard profile for the respiration-inhibiting insecticides

Insecticide	Structure	Principle uses	Application Rates gm (a.i.)/ha
Chlorfenapyr Pirate® (19)		Used to control many insects and mites in cotton, vegetables, citrus, vines and soya beans.	NA
Hydramethylnon Amdro® (47)		Used to control agricultural and household *Formicidae*	NA
Pyridaben Poseidon® (223)		Used to control acarids on field crops, fruits, vegetables and ornamentals	100–300

	Irritation		LD50 (mg/kg)		LC50 (mg/L)		
Insecticide	Eye	Skin	Oral	Dermal	Inhalation	Scnsitizinc Potential	Signal Word
Chlorfenapyr	Moderate irritant	Not irritant	441	>2000	1.9	Negative	Warning
Hydramethylnon	Mild irritant	Not irritant	817	>2000	2.9	Negative	Caution
Pyridaben	Slight irritant	Not irritant	820	>2000	0.66	Negative	Caution

Insecticide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Chlorfenapyr (200)	Rat/2-year	2.9	Mutagenicity	No evidence
	Dog/52-week	4.0	Developmental	Not teratogenic
	Mouse/18-month	2.8	Reproductive	No evidence
	RfD2	0.03	Oncogenicity	E(No evidence)3
			Neurotoxicity	Not neurotoxic
Hydramethylnon (47, 237)	Rat/2-year	50	Mutagenicity	No evidence
	Dog/52-week	1.0	Developmental	Not teratogenic
	Mouse/18-month	25	Reproductive	No evidence
	ADI2	0.01	Oncogenicity	C-RfD (based on lung/ liver tumors in mice)3
			Neurotoxicity	Not neurotoxic
Pyridaben (223)	Rat/2-year	1.13	Mutagenicity	No evidence
	Dog/52-week	<0.5	Developmental	Not teratogenic
	Mouse/18-month	2.78	Reproductive	No evidence

ADI2	0.005	Oncogenicity Neurotoxicity	E (No evidence)3 Not neurotoxic

1 No observable effect level
2 RfD=reference dose and ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme
NA=Not Available

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All of these materials possess low to moderate toxicity to mammals. The U.S. EPA has classified hydramethylnon as category C (possible human carcinogen) based on lung tumors in mice.
HERBICIDES
Herbicides are the leading type of pesticides, in terms of both user expenditures and amount used (1). In 1995, approximately $6.25 billion (United States) and $16.25 billion (worldwide) were spent by users on herbicides; this constitutes 55% and 45% of the U.S. and worldwide markets, respectively. Quantity-wise, 556 (46%) and 2210 (39%) million lb of active ingredient were purchased in the U.S. and worldwide markets, respectively. This market has been significantly impacted with the introduction of new chemistry, such as, the sulfonylureas (active in the grams per acre range), and new technology such as corn bioengineered against the phytotoxicity of the nonspecific herbicide glyphosate (198).
As the sheer number of herbicides available is significant, the number of herbicides presented here is limited with a focus on those considered to have economic and high agronomic value. There is an attempt to cover all the different mechanisms of actions in this process. The modes and mechanisms of action for important classes of herbicides used in crop protection are presented in Table 13.35.
Acetyl-CoA Carboxylase Inhibitors
Herbicides that act via inhibition of acetyl coenzyme A (acetyl-CoA) carboxylase alter lipid biosynthesis in weeds. The lipid inhibitors include the aryloxyphenoxypropionate and cyclohexanedione herbicides. These herbicides prevent the formation of fatty acids, components essential for the production of plant lipids. Lipids are vital to the integrity of cell membranes and to new plant growth. The lipid inhibitor herbicides inhibit a single key enzyme involved in fatty acid synthesis (154). Broadleaf plants are tolerant to these herbicide families, but many of the perennial and annual grasses are susceptible. Injury symptoms are slow to develop (7 to 10 days) and appear first on new leaves emerging from the whorl of the grass plant. These herbicides are taken up by the foliage and move in the phloem to areas of new growth (92).
Aryloxyphenoxypropionates
The structures, uses, and hazard profiles for six acetyl-CoA carboxylase-inhibiting aryloxyphenoxypropionate herbicides are presented in Tables 13.36 and 13.37.
These materials are generally not acutely toxic. Clodinafop-propargyl and haloxyfop have been identified as peroxisomal proliferators (pp) in the rodent. The relevance of peroxisomal proliferation to humans is still unresolved. However, studies involving the peroxisomal proliferator response element upstream of the human acyl-CoA oxidase gene as well as in rats showed that the rat gene responded to peroxisomal proliferators; whereas, the human gene was unresponsive (318). Additional work with the new gene technologies that are currently available may afford an opportunity to put this apparent species specific phenomenon into proper perspective.
Cyclohexanediones
The cyclohexanedione herbicides inhibit acetyl-CoA carboxylase, producing the same effects on susceptible perennial and annual grasses as noted with the aryloxyphenoxypropionates. The structures, uses, and hazard profiles of clethodim and sethoxydim are presented in Table 13.38.
Both cyclohexanedione herbicides have excellent acute and repeated-dose hazard profiles, which do not trigger any mammalian toxicology concerns.
Acetolactate Synthase Inhibitors
The sulfonyureas, imidazolinones, triazolopyridimidines, and pyriidinyl thiobenzoates constitute acetolactate synthase (ALS) inhibitors. The ALS inhibitors interact with the acetolactate synthase enzyme, inhibiting biosynthesis of an essential amino acid (297).
Sulfonylureas
Sulfonylurea herbicides belong to a class of compounds comprised of three distinct components. These are an aryl group linked to a nitrogen-containing hetrocycle via a sulfonylurea bridge. Sulfonylurea herbicides inhibit root and shoot growth in rapidly growing plants by suppressing cell division (4). Initial research conducted on *Escherichia coli* and *Salmonella typhimurium* and latter confirmed in plants and yeasts indicate that the herbicidal activity is due to the inhibition of acetolactate synthase (ALS), an enzyme necessary for the biosynthesis of branched chain amino acids in bacteria, fungi, and higher plants.
A large number of sulfonylurea herbicides have been developed for commercial use in North America and Europe (Tables 13.39 and 13.40).
Sulfonylurea herbicides generally are not acutely toxic or irritating to the skin and eye, nor are they mutagenic, developmentally toxic, or oncogenic (203). Their hazard profiles are given in Tables 13.41 through 13.43.

Table 13.35 Mode and mechanism of action for important classes of herbicides used in crop protection

Class	Mode of Action	Mechanism of Action
Acetyl CoA Carboxylase (ACCase) Inhibitors (41)	Systemic grass herbicides	Inhibit ACCase lipid biosynthesis.
Acetolactate Synthase (ALS) Inhibitors (297)	Selective systemic herbicides	Normal function of the ALS enzyme is blocked, inhibiting biosynthesis of essential amino acids.
Photosynthesis Inhibitors (268)	Selective systemic herbicides	Block the photosynthetic reaction, and disrupt cellular membranes so that captured light cannot be converted to chemical energy.
Protoporphyrin Inhibitors (265)	Selective contact herbicides	Inhibits the enzyme protoporphyrinogen oxidase.
Bleaching Herbicides (301)	Selective systemic herbicides	Disrupt synthesis of carotenoid pigments which protect chlorophyll pigments in light. Lack of carotenoids lead to chlorophyll destruction and white, bleached appearance.
EPSP Synthase Inhibitors (287)	Non-selective systemic herbicides	Inhibit 5-enolpyruvlshikimate-3-phosphate synthase preventing the biosynthesis of essential amino acids.
Glutamine Synthase Inhibitors (286)	Non-selective contact herbicides	Inhibit glutamine synthase leading to accumulation of ammonium ions, and inhibition of photosynthesis.
Dihydropteroate (DHP) Synthase Inhibitors (267)	Selective systemic herbicides	Inhibit DHP synthase involved in folic acid synthesis needed for the formation of purine nucleotides required for cell division.
Microtubule Assembly Inhibitors (14)	Selective systemic herbicides	Affect seed germination and prevents weed growth.
Mitosis Inhibitors (303)	Selective systemic herbicides	Affect cell growth and cell elongation.
Inhibitors of Cellulose Synthesis (294)	Selective systemic herbicides	Inhibit cell wall biosynthesis through inhibiting the formation of cellulose.
Membrane Disruptors (302)	Non-selective contact herbicides	Disrupt internal cell membranes by formation of a superoxide preventing the cell from manufacturing energy.
Inhibitors of Lipid Synthesis (308)	Selective systemic herbicides	Block formation of lipids in the shoot (meristem) and roots of grass plants.
Synthetic Auxins (Mimic or Inhibit Indoleacetic Acid) (82)	Selective systemic herbicides	Affect growth in the meristems and leaves by affecting protein synthesis and normal cell division.

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Table 13.36 Structures, uses, and hazard profiles of acetyl-CoA carboxylase-inhibiting aryloxyphenoxypropionate herbicides

Chemical/ Common Names	Structure	Principle Uses/Crops	Application rates gm (a.i.)/ha
Clodinafop-propargyl/ Discover® (22)		Cereals	20–80
Diclofop-methyl Hoelon® (278)		Cereals	840–1680
Fenoxaprop-ethyl (206)		Cereals, soybeans, and turf	37.5–111

	Irritation		LD50 (mg/kg)		LC50 (mg/L)		
Herbicide	Eye	Skin	Oral	Dermal	Inhalation	Sensitizing Potential	Signal Word
Clodinafop-propargyl	Non-irritant	Non-irritant	1829	>2000	2.325	Positive	Caution
Diclofop-methyl	Non-irritant	Non-irritant	2020	>5000	>3.83	NA	Caution
Fenoxaprop-ethyl	Slight irritant	Slight irritant	2565	>2000	>0.511	Negative	Caution

Herbicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Clodinofop-Propargyl	Rat/2-year	0.35	Mutagenicity	Not a mutagen
	Dog/52-week	3.3	Developmental	Not a developmental toxin
	Mouse/18-month	1.2	Reproductive	Not a reproductive toxin
	ADI (2-yr. rat)2	0.004	Oncogenicity	Rodent peroxisomal proliferator (Mouse liver tumors)
Diclofop-Methyl	Rat/2-year	20	Mutagenicity	Not a mutagen
	Dog/15-Mo. Oral	8.0	Developmental	Not a developmental toxin
	Mouse/18-month	NA	Reproductive	Not a reproductive toxin
	ADI2	0.001 (proposed)	Oncogenicity	NA
Fenoxaprop-Ethyl	Rat/2-year	1.5	Mutagenicity	Not a mutagen
	Dog/15-Mo. Oral	0.375	Developmental	Not a developmental

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			toxin
Mouse/18-month	5.7	Reproductive	Not a reproductive toxin
RfD (rat reproduction)2	0.0025	Oncogenicity	C(pending)3-adrenal tumors

1 No observable effect level
2 RfD=reference dose and ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme
4 UF=Uncertainty factor
NA=Not Available

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Table 13.37 More structures, uses, and hazard profiles of acetyl-CoA carboxylase-inhibiting aryloxyphenoxypropionate herbicides

Herbicide	Structure	Principle Uses/Crops	Application rates gm (a.i.)/ha
Fluazifop-P-Butyl Fusilade® (41)		Cotton, fruit, and soybeans	53–210
Haloxyfop Galant™ (288)		Cotton, soybeans, sunflowers, oilseed rape	140–600
Propaquizafop AGIL® (63)		Soybeans, cotton, sunflower, sugar beets, potatoes, oilseed rape, vegetables, peanuts, tobacco	NA

	Irritation		LD50 (mg/kg)		LC50 (mg/L)		
Herbicide	Eye	Skin	Oral	Dermal	Inhalation	Sensitizing Potential	Signal Word
Fluazifop-PButyl	Mild Irritant	Slight Irritant	4096	>2420	>5.24	Negative	Caution
Haloxyfop	Moderate Irritant	Non-irritant	518	>5000	NA	Negative	Caution
Propaquizafop	Moderate Irritant	Non-irritant	>5000	>2000	2.5	Possibly positive	Caution

Herbicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Fluazifop-PButyl	Rat/2-year	NA	Mutagenicity	NA
	Dog/52-week	NA	Developmental	NA
	Mouse/18-month	NA	Reproductive	NA
	RfD2	0.01	Oncogenicity	NA
Haloxyfop	Rat/2-year	0.065	Mutagenicity	No evidence
	Dog/52-week	0.5	Developmental	Not a developmental toxin
	Mouse/18-month	0.6	Reproductive	No evidence
	ADI2	0.0003	Oncogenicity	Rodent peroxisomal proliferator
Propaquizafop	Rat/2-year	1.5	Mutagenicity	NA
	Dog/52-week	20	Developmental	NA
	Mouse/18-month	1.5	Reproductive	NA
	ADI2	0.015	Oncogenicity	NA

1 No observable effect level

2 RfD=reference dose and ADI=acceptable daily intake

3 See Table 13.3 for US EPA classification scheme
NA=Not Available

Table 13.38 Structures, uses, and hazard profiles of acetyl-CoA carboxylase-inhibiting cyclohexanedione herbicides

Herbicide	Structure	Principle Uses/Crops	Application Rate gm (a.i.)/ha
Clethodim Select® (275)	$CH_3CH_2SCH(CH_3)CH_2$-cyclohexenone ring with C(=NOCH$_2$CH=CHCl)CH$_2$CH$_3$ and OH	Used to control grasses in soybeans and cotton.	105–280
Sethoxydim Nabu® (308)	$CH_3CH_2SCH(CH_3)CH_2$-cyclohexenone ring with C(=NOCH$_2$CH$_3$)CH$_2$CH$_2$CH$_3$ and OH	Used to control grasses in soybean, cotton and peanut	112–560

Herbicide	Irritation: Eye	Irritation: Skin	LD50 (mg/kg) Oral	LD50 (mg/kg) Dermal	LC50 (mg/L) Inhalation	Scnsitizinc Potential	Signal Word
Clethodim	NA	Non-irritant	1360	>2000	>3.9	NA	Caution
Sethoxydim	Non-irritant	Non-irritant	2676	>5000	6.1	Negative	Caution

Herbicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Clethodim	Rat/2-year	19	Mutagenicity	Not a mutagen
	Dog/52-week	1	Developmental	Not a developmental toxin
	Mouse/18-month	28	Reproductive	Not a reproductive toxin
	ADI2	0.01	Oncogenicity	No evidence
Sethoxydim	Rat/2-year	17.2	Mutagenicity	Not a mutagen
	Dog/52-week	8.9	Developmental	Not a developmental toxin
	Mouse/18-month	14	Reproductive	Not a reproductive toxin
	ADI2	0.14	Oncogenicity	No evidence

1 No observable effect level
2 RfD=reference dose and ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme
NA=Not Available

Various target organs have been identified at high doses in chronic studies in rodents and dogs, including bone marrow, liver, kidney, testes, and the peripheral and central nervous systems. Tumor incidence was elevated above control levels in the liver (primisulfuron) and heart (oxasulfuron) at doses that exceed the maximum tolerated dose. An earlier appearance of mammary tumors has also been observed in female Sprague-Dawley rats (prosulfuron, tribenuron).

A unitary mode of action underlying effects of this class of chemical on mammalian systems is not discernable. The diversity of the effects observed in various target organs is attributed to specific functional groups and not to the defining characteristic of the class, the sulfonylurea bridge.

Imidazolinones

There are a restricted number of registered chemicals in this class of ALS-inhibiting herbicides. They include imazameth and imazamethabenzmethyl. The hazard profiles for these chemicals (Table 13.44) indicate that this class of herbicide is relatively nontoxic, even at high doses, with no evidence of mutagenic, developmental, or oncogenic effects.

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Table 13.39 Structures and uses of the ALS sulfonylurea herbicides

Chemical/ Common Names	Structure	Principle Uses/Crops	Application Rate gm (a.i.)/ha/yr.
Bensulfuron-methyl Londax® (269)		Rice	30–100
Chlorimuron-ethyl Classic® (275)		Soybeans, Peanuts	9–13
Chlorsulfuron Glean® (274)		Cereals, IWC	5–25
Halosulfuron-methyl Permit® (45)		Cereals, Corn Sorghum, Turf	NA
Imazosulfuron Sibatito®, Takeoff® (49)		Cereals, Rice, Turf	75–95
Metsulfuron-methyl Ally®, Escort® (297)		Cereals	4–7.5
Nicosulfuron Accent® (300)		Corn	35–70

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Table 13.40 More structures and uses of the ALS sulfonylurea herbicides

Chemical/ Common Names	Structure	Principle Uses/Crops	Application Rate (grams a.i./ha/yr.)
Oxasulfuron Expert® (60)		Soybeans	32
Primisulfuron-methyl Beacon® (304)		Corn	20–40
Prosulfuron Peak® (66)		Cereals, Corn, Sorghum, Pasture	10–40
Rimsulfuron Matrix® (71)		Corn, Tomatoes, and potatoes	15000
Sulfometuron-methyl Oust® (310)		IWC	NA
Sulfosulfuron (Under Development) (73)		Cereal (Wheat) IWC	10–35
Thifensulfuron-methyl Pinnacle®, Harmony® (312)		Cereals, corn, soybean, pastures	9–60

Triasulfuron Amber®, Logran® (313)		Cereal (Wheat), IWC	10
Tribenuron-methyl Express® (314)		Cereal (Wheat)	9–30

NA=Not Available

Table 13.41 Hazard profile for ALS sulfonylurea herbicides

Herbicide	Irritation		LD50 (mg/kg)		LC50 (mg/L)		
	Eye	Skin	Oral	Dermal	Inhalation	Sensitizing Potential	Signal Word
Bensulfuron (269)	Non-Irritant	Non-Irritant	>5000	>2000	>7.5	NA	Caution
Chlorimuron (276)	Non-Irritant	Non-Irritant	4102	>2000	>5.0	Negative	Caution
Chlorsulfuron (274)	Slight Irritant	Non-Irritant	5545(♂)	2500	>5.9	Negative	Caution
Halosulfuron (236)	NA	NA	8866	>2000	NA	NA	Caution
Imazosulfuron (49)	Non-Irritant	Non-Irritant	>5000	>2000	>2.4	Negative	Caution
Metsulfuron (297)	Mod. Irritant	Mild Irritant	>5000	>2000	>5.0	Negative	Caution

Herbicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Bensulfuron	Rat/2-year	37.5	Mutagenicity	No evidence
	Dog/52-week	227	Developmental	Not teratogenic
	Mouse/18-month	455	Reproductive	Not reproductive toxin
	ADI2	0.2	Oncogenicity	No evidence
Chlorimuron	Rat/2-year	12.5	Mutagenicity	No evidence
	Dog/52-week	6.25	Developmental	Not Teratogenic
	Mouse/18-month	180	Reproductive	No evidence
	ADI2	0.02	Oncogenicity	No evidence
Chlorsulfuron	Rat/2-year	5	Mutagenicity	No evidence
	Dog/52-week	50	Developmental	Not teratogenic
	Mouse/18-month	71	Reproductive	No evidence
	RfD2	0.05	Oncogenicity	No evidence
Halosulfuron	Rat/2-year	50	Mutagenicity	No evidence
	Dog/52-week	10	Developmental	Not Teratogenic
	Mouse/18-month	430	Reproductive	No evidence
	ADI2	0.1	Oncogenicity	No evidence
Imazosulfuron	Rat/2-year	106(♂)	Mutagenicity	No evidence
	Dog/52-week	75	Developmental	Teratogenic in mice
	Mouse/18-month	NA	Reproductive	No evidence
	RfD or ADI2	NA	Oncogenicity	No evidence
Metsulfuron	Rat/2-year	25	Mutagenicity	No evidence
	Dog/52-week	12.5(♀)	Developmental	Not teratogenic
	Mouse/18-month	710	Reproductive	No evidence
	ADI (Germany)2	0.0125	Oncogenicity	No evidence

1 No observable effect level; Dietary concentration (ppm) was converted to daily dose (mg/kg/day) by dividing by 20, 7, or 40 for the rat, mouse and dog, respectively.
2 RfD=reference dose and ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme
NA=Not Available

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Table 13.42 Hazard profile for more ALS sulfonylurea herbicides

Herbicide	Irritation: Eye	Irritation: Skin	LD50 (mg/kg): Oral	LD50 (mg/kg): Dermal	LC50 (mg/L): Inhalation	Sensitizing Potential	Signal Word
Nicosulfuron (300)	Mod. Irritant	NA	>5000	>2000	5.47	Negative	Caution
Oxasulfuron (60)	Non-Irritant	Non-Irritant	>5000	>2000	5.08	Negative	Caution
Primisulfuron (304)	Slight Irritant	Non-Irritant	>5050	>2010	>4.8	Negative	Caution
Prosulfuron (66)	Non-Irritant	Non-Irritant	986	>2000	>5.0	Negative	Caution
Rimsulfuron (71, 262)	Mod. irritant	Non-Irritant	>5000	>2000	>5.4	Negative	Caution
Sulfometuron (310)	Slight irritant	Slight irritant	>5000	>2000	>11	Negative	Caution

Herbicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Nicosulfuron	Rat/2-year	1000	Mutagenicity	No evidence
	Dog/52-week	125	Developmental	Not teratogenic
	Mouse/18-month	1070	Reproductive	No evidence
	ADI2	1.25	Oncogenicity	No evidence
Oxasulfuron	Rat/2-year	8.3	Mutagenicity	No evidence
	Dog/52-week	1.3	Developmental	Not teratogenic
	Mouse/18-month	1.5	Reproductive	No evidence
	ADI2	0.0026	Oncogenicity	No evidence
Primisulfuron	Rat/2-year	13	Mutagenicity	No evidence
	Dog/52-week	25	Developmental	Not teratogenic
	Mouse/18-month	45	Reproductive	Testicular degeneration
	ADI2	0.13	Oncogenicity	D (Liver tumor in ♂ mice doses>MTD)3
Prosulfuron	Rat/2-year	8.6	Mutagenicity	No evidence
	Dog/52-week	1.9	Developmental	Not teratogenic
	Mouse/18-month	80	Reproductive	No evidence
	ADI2	0.019	Oncogenicity	D (mammary tumors in ♀ rats—early onset)3
Rimsulfuron	Rat/2-year	11.8	Mutagenicity	No evidence
	Dog/52-week	1.6	Developmental	Not teratogenic
	Mouse/18-month	351	Reproductive	No evidence
	RfD2	0.016	Oncogenicity	No evidence
Sulfometuron	Rat/2-year	2.5	Mutagenicity	No evidence
	Dog/52-week	5.0	Developmental	Teratogenic: 2 species
	Mouse/18-month	140	Reproductive	No evidence
	ADI2	0.025	Oncogenicity	No evidence

1 No observable effect level; Dietary concentration (ppm) was converted to daily dose (mg/kg/day) by dividing by 20, 7, or 40 for the rat, mouse and dog, respectively.
2 RfD=reference dose and ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme
NA=Not Available

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Table 13.43 Hazard profile for other ALS sulfonylurea herbicides

Herbicide	Irritation: Eye	Irritation: Skin	LD50 (mg/kg): Oral	LD50 (mg/kg): Dermal	LC50 (mg/L): Inhalation	Sensitizing Potential	Signal Word
Sulfosulfuron (73)	Non-Irritant	Slight irritant	>5000	>5000	NA	Negative	Caution
Thifensulfuron (312)	Slight Irritant	Non-Irritant	>5000	>2000	>7.9	Negative	Caution
Trisulfuron (313)	Slight Irritant	Non-irritant	>5000	>2000	>5.1	Negative	Caution
Tribenuron (314)	Slight Irritant	Non-irritant	>5000	>2000	>5.0	Positive	Caution

Herbicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Sulfosulfuron	Rat/2-year	NA	Mutagenicity	NA
	Dog/52-week	NA	Developmental	NA
	Mouse/18-month	NA	Reproductive	NA
	ADI2	NA	Oncogenicity	NA
Thifensulfuron	Rat/2-year	2.6	Mutagenicity	No evidence
	Dog/52-week	19	Developmental	Not teratogenic
	Mouse/18-month	1070	Reproductive	No evidence
	ADI2	0.026	Oncogenicity	No evidence
Triasulfuron	Rat/2-year	32.1	Mutagenicity	No evidence
	Dog/52-week	33	Developmental	Not teratogenic
	Mouse/18-month	1.2	Reproductive	No evidence
	ADI2	0.012	Oncogenicity	No evidence
Tribenuron	Rat/2-year	1.25	Mutagenicity	No evidence
	Dog/52-week	8.2	Developmental	Not teratogenic
	Mouse/18-month	30	Reproductive	No evidence
	ADI2	0.011	Oncogenicity	C (Mammary tumors in ♀ rats—early onset)3

1 No observable effect level; Dietary concentration (ppm) was converted to daily dose (mg/kg/day) by dividing by 20, 7, or 40 for the rat, mouse and dog, respectively.
2 RfD=reference dose and ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme
NA=Not Available

Triazolopyrimidines

The triazolopyrimidine class of ALS inhibitors registered for herbicidal use includes imazamox, imazapyr, imazaquin, and imazethapyr. As with the imidazolinones, these chemicals also have excellent hazard profiles (Table 13.45). No evidence of significant target organ toxicity or mutagenic, developmental, or oncogenic potential has been realized even at doses that approximate the limit dose of 1000 mg/kg.

Pyrimidinylthiobenzoates

The members of this class of ALS-inhibiting herbicides, flumetsulam and pyriminobac-methyl (Table 13.46), are less well tolerated in mammalian systems than for other ALS inhibitors as evidence by lower no observed effect levels. However, the hazard profile for these chemicals is still favorable since no mutagenic, developmental, or oncogenic effects have been reported.

Photosynthesis Inhibitors

The photosynthesis inhibitors include triazines, phenylureas, uracils, benzothiadiazoles, nitriles, carbamate, and dicarboxylic acid. Photosynthesis inhibitors shut down the photosynthetic (food-producing) process in susceptible plants by binding to specific sites within

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Table 13.44 Structures, uses, and hazard profiles of the ALS imidazolinone herbicides

Chemical/ Common Names	Structure	Principle Uses/Crops	Application Rate (grams a.i./ha/yr.)
Imazameth Cadre® (188)		Soybeans, peanuts, sugarcane	NA
Imazamethabenz - methyl Assert® (289)		Wheat, barley Sunflower	350–530 Post 200–430 Post

Herbicide	Irritation Eye	Irritation Skin	LD50 (mg/kg) Oral	LD50 (mg/kg) Dermal	LC50 (mg/L) Inhalation	Sensitizing Potential	Signal Word
Imazameth	NA	Non-irritant	>5000	>5000	2.38	NA	Caution
Imazamethabenz - methyl	Slight Irritant	Non-irritant	>5000	>2000	>5.8	Negative	Caution

Herbicide	Species/study	NOEL1(mg/kg/day)	Toxicity Studies	Hazard Indicator
Imazameth	Rat/2-year	1029 ♂	Mutagenicity	No evidence
	Dog/52-week	>137	Developmental	Not teratogenic
	Mouse/18-month	1134 ♂	Reproductive	No evidence
	RfD2(300×UF4)	0.5	Oncogenicity	No evidence
Imazamethabenz-methyl	Rat/2-year	12.5	Mutagenicity	No evidence
	Dog/52-week	6.25	Developmental	Not teratogenic
	Mouse/18-month	19.5	Reproductive	No evidence
	ADI2	0.06	Oncogenicity	No evidence

1 No observable effect level; Dietary concentration (ppm) was converted to daily dose (mg/kg/day) by dividing by 20, 7, or 40 for the rat, mouse and dog, respectively.
2 RfD=reference dose and ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme
4 Uncertainty factor
NA=Not Available

the plant chloroplast. Inhibition of photosynthesis could result in a slow starvation of the plant; however, in many situations rapid death occurs, perhaps from the production of secondary toxic substances (101). The triazines, uracils, substituted ureas, benzothiadiazoles, and phenylpyridazines inhibit electron flow in Photosystem II, leading to destruction of cellular membranes and plant death. The bipyridyliums inhibit Photosystem I electron flow.

Injury signs include yellowing (chlorosis) of leaf tissue followed by death (necrosis) of the tissue. Three of the herbicide families (triazines, phenylureas, and uracils) are taken up into the plant via the roots or foliage and move in the xylem to plant leaves. As a result, injury signs will first appear on the older leaves, along the leaf margin. Foliar-applied photosynthetic inhibitors generally remain in the foliar portions of the treated plant, and movement from foliage to roots is negligible.

Table 13.45 Structures, uses, and hazard profiles of the ALS triazolopyrimidines

Chemical/ Common Names	Structure	Principle Uses/Crops	Application Rate gm (a.i)/ha
Imazamox Raptor®: Pending (213)		Soybeans, legumes	0.032–0.04 lbs./acre
Imazapyr Arsenal® (290)		IWC	560–1700 POST
Imazaquin Scepter® (291)		Soybeans	70–140 Pre-Plant, PPI, PRE POST
Imazethapyr Pursuit® (292)		Soybeans, Corn Legume, Peanuts	35–70 Early Pre-Plant, PPI, PRE, POST

Herbicide	Irritation Eye	Irritation Skin	LD50 (mg/kg) Oral	LD50 (mg/kg) Dermal	LC50 (mg/L) Inhalation	Sensitizing Potential	Signal Word
Imazamox	Mild irritant	Non-irritant	>5000	>4000	>6.3	Negative	Caution
Imazapyr	Irreversible	Non-irritant	>5000	>2000	>1.3	Negative	Danger
Imazaquin	Non-irritant	Slight irritant	>5000	>2000	>5.7	Negative	Caution
Imazethapyr	Slight Irritant	Slight Irritant	>5000	>2000	>2.6	Negative	Caution

Herbicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Imazamox	Rat/2-year	1068	Mutagenicity	No evidence
	Dog/52-week	1165	Developmental	Not teratogenic
	Mouse/18-month	NA	Reproductive	No evidence
	RfD2	3.0	Oncogenicity	E (No evidence)1
Imazapyr	Rat/2-year	500	Mutagenicity	No evidence
	Dog/52-week	250	Developmental	Not teratogenic
	Mouse/18-month	1500	Reproductive	No evidence
	ADI2	2.5	Oncogenicity	No evidence

Imazaquin	Rat/2-year	500	Mutagenicity	No evidence
	Dog/52-week	25	Developmental	Not teratogenic
	Mouse/18-month	150	Reproductive	No evidence
	ADI2	0.25	Oncogenicity	No evidence
Imazethapyr	Rat/2-year	500	Mutagenicity	No evidence
	Dog/52-week	25	Developmental	Not teratogenic
	Mouse/18-month	750	Reproductive	No evidence
	ADI2	0.25	Oncogenicity	No evidence

1 See Table 13.3 for US EPA classification scheme
NA=Not Available

Table 13.46 Structures, uses, and hazard profiles of the ALS pyrimidinylthiobenzoates

Chemical/ Common Names	Structure	Principle Uses/Crops	Application Rate gm (a.i.)/ha
Flumetsulam Broadstrike® (282)		Corn, Soybeans	52.5–78
Pyriminobacmethyl Prosper® (69)		Cotton	70–105

Herbicide	Irritation Eye	Skin	LD50 (mg/kg) Oral	Dermal	LC50(mg/L) Inhalation	Sensitizing Potential	Signal Word
Flumetsulam	Slight irritant	Non-irritant	>5000	>2000	>5.9	Negative	Caution
Pyriminobacmethyl	Slight irritant	Slight irritant	>5000	>2000	>5.5	NA	Caution

Herbicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Flumetsulum	Rat/2-year	35	Mutagenicity	No evidence
	Dog/52-week	100	Developmental	Not teratogenic
	Mouse/18-month	32	Reproductive	No evidence
	ADI2	0.32	Oncogenicity	No evidence
Pyriminobac-methyl	Rat/2-year	0.9	Mutagenicity	No evidence
	Dog/52-week	NA	Developmental	Not teratogenic
	Mouse/18-month	8.1	Reproductive	No evidence
	ADI2	0.009	Oncogenicity	No evidence

1 No observable effect level; Dietary concentration (ppm) was converted to daily dose (mg/kg/day) by dividing by 20, 7, or 40 for the rat, mouse and dog, respectively.
2 RfD=reference dose and ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme
NA=Not Available

Triazines and Triazinone

The triazine herbicides inhibit the Hill reaction in the process of photosynthesis (161). This unique mechanism of action is specific to photosynthesizing plants. Therefore, the triazines do not exhibit significant mammalian toxicity but rather target-species selectivity. The structures and uses of the symmetrical triazines and an asymmetrical triazine or triazinone, metribuzin, are presented in Table 13.47.

The hazard profiles of these agents are presented in Tables 13.48 and 13.49.

These triazines are generally not acutely toxic, nor do they cause a significant repeated dose toxicity. The one exception appears to be the symmetrical chlorotriazines, atrazine, cyanazine, propazine, and simazine, which in lifetime feeding studies in Sprague-Dawley female rats induce an earlier onset and/or an increase in the incidence of mammary tumors (161, 176). The relevance of these tumors to humans has been the subject of intense research and evaluation (94, 164, 317).

Recently, the International Agency for Research on Cancer reexamined atrazine and simazine (105). Both were classified as "not classifiable as to carcinogenicity

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Table 13.47 Structures and uses of selected photosynthesis-inhibiting herbicides: Triazines and triazinones

Herbicide	Structure	Principle Crops/uses	Use rates
Atrazine Aatrex® (268)		Pre- and post-emergence control of annual broadleaved and annual grasses in corn, sorghum, sugar cane, and pineapple	1.5 to 2.5 kilograms (a.i.)/hectare
Cyanazine Bladex® (276)		Pre-emergence in broad beans, corn and peas; and post-emergence in barley and wheat	1 to 3 kilograms (a.i.)/hectare 0.26 to 0.33 kilograms (a.i.)/hectare
Propazine Milo-Pro® (222)		Pre- and post-emergence control of annual broadleaved and annual grasses in sorghum, carrots, chervil, and parsley	0.5 to 3 kilograms (a.i.)/hectare
Simazine Princep® (309)		Pre- and post-emergence control of annual broadleaved and annual grasses in pome fruit, stone fruit, citrus, vines, corn, sorghum, sugar cane, and pineapple	1.5 to 3 kilograms (a.i.)/hectare
Ametryn Evik® (266)		Pre- and post-emergence control of annual broadleaved and annual grasses in bananas, citrus fruit, corn, coffee, sugar cane, and pineapple	2 to 4 kilograms (a.i.)/hectare
Prometryn Caparol® (244, 306)		Pre-emergence in vegetables, cotton, sunflower, and peanuts and post-emergence in cotton and vegetables	0.8 to 2.5 kilograms (a.i.)/hectare 0.8 to 1.5 kilograms

			(a.i.)/hectare
Prometon Pramitol® (305)		Control of most annual and many perennial broadleaved weeds, grasses and brush weeds in non-crop areas	10 to 20 kilograms (a.i.)/hectare
Metribuzin Sencor® (296)		Pre- and post-emergence control of annual broadleaved and annual grasses in soya beans, potatoes, corn, cereals, sugar cane, alfalfa, and asparagus	0.35 to 0.7 kilograms (a.i.)/hectare

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Table 13.48 Hazard profiles for selected photosynthesis-inhibiting herbicides: Triazines (165)

	Irritation		LD50 (mg/kg)		LC50 (mg/L)		
Herbicide	Eye	Skin	Oral	Dermal	Inhalation	Sensitizing Potential	Signal Word
Atrazine	Not irritant	Not irritant	3090	>3100	>5.0	Positive	Caution
Cyanazine	Not irritant	Not irritant	182	>2000	>5.3	Negative	Warning
Propazine	Mild irritant	Not irritant	>7000	>3100	>2.0	Negative	Caution
Simazine	Not irritant	Mild irritant	>5000	>3100	>5.5	Negative	Caution

Herbicide	Species/study	NOEL[1] (mg/kg/day)	Toxicity Studies	Hazard Indicator
Atrazine	Rat/2-year	0.5	Mutagenicity	No evidence
	Dog/52-week	3.75	Developmental	Not teratogenic
	Mouse/18-month	1.2	Reproductive	No evidence
	ADI[2]	0.005	Oncogenicity	Category C with CSF (based mammary tumors in female Sprague-Dawley rats)[3]
Cyanazine	Rat/2-year	12	Mutagenicity	No evidence
	Dog/52-week	25	Developmental	Teratogenic in rats and rabbits
	Mouse/18-month	1.4	Reproductive	No evidence
	ADI[2]	NA	Oncogenicity	Category C with CSF (based mammary tumors in female Sprague-Dawley rats)[3]
Propazine	Rat/2-year	5.8	Mutagenicity	No evidence
	Dog/52-week	1.3	Developmental	Not teratogenic
	Mouse/18-month	15	Reproductive	No evidence
	RfD[2]	0.02 (based on the 2-yr. rat study with 300×UF[4])	Oncogenicity	Category C with CSF (based mammary tumors in female Sprague-Dawley rats)[3]
Simazine	Rat/2-year	0.5	Mutagenicity	No evidence
	Dog/52-week	7.5	Developmental	Not teratogenic
	Mouse/18-month	5.7	Reproductive	No evidence
	ADI[2]	0.005	Oncogenicity	Category C with CSF (based mammary tumors in female Sprague-Dawley rats[3]

1 No observable effect level
2 RfD=reference dose and ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme, CSF=cancer slope factor
4 UF=Uncertainty factor

to humans" when mechanistic data were taken into account in making the overall evaluation.

Uracils and Pyridazinones

The structures, uses, and toxicity profiles for two uracils and a pyridazinone herbicide are given in Table 13.50.

The acute hazard associated with bromacil, terbacil, and norflurazon is unremarkable. None of these products have been found to represent a mutagenic, teratogenic, or reproductive hazard. However, bromacil and norflurazon have been classified as category C (possible human carcinogen), based on mouse liver tumors.

Ureas

Three ureas of this older class of photosynthesis-inhibiting herbicides are considered. The structures, uses, and toxicity for diuron, flumeturon, and linuron are provided in Table 13.51.

The acute and repeated-dose toxicity of these three ureas is nonproblematic until the oncogenic potential of these compounds is examined. Both diuron and linuron

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Table 13.49 Hazard profiles for more selected photosynthesis-inhibiting herbicides: More triazines and triazinone (165)

Herbicide	Irritation: Eye	Irritation: Skin	LD50 (mg/kg): Oral	LD50 (mg/kg): Dermal	LC50 (mg/L): Inhalation	Sensitizing Potential	Signal Word
Ametryn	Not irritant	Not irritant	1160	>2020	>5.1	Positive	Caution
Prometryn	Slight irritant	Not irritant	4550	>2020	>5.1	Negative	Caution
Prometon	Irritant	Mild irritant	1518	>2020	>3.2	Negative	Warning
Metribuzin	Not irritant	Not irritant	1090	>20000	>0.65	Negative	Caution

Herbicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Ametryn	Rat/2-year	2.5	Mutagenicity	No evidence
	Dog/52-week	10	Developmental	Not teratogenic
	Mouse/18-month	1.5	Reproductive	No evidence
	RfD2	0.025	Oncogenicity	E (No evidence)3
Prometryn	Rat/2-year	37	Mutagenicity	No evidence
	Dog/106-Week Oral	3.7	Developmental	Not teratogenic
	Mouse/102-Week Oral	1.0	Reproductive	No evidence
	RfD2	0.037 (based on the 2-yr. dog study with 100×UF4)	Oncogenicity	E (No evidence)3
Prometon	Rat/2-year	1.0	Mutagenicity	No evidence
	Dog/52-week	5.0	Developmental	Not teratogenic
	Mouse/18-month	70	Reproductive	No evidence
	RfD2	0.01	Oncogenicity	No evidence
Metribuzin	Rat/2-year	5.0	Mutagenicity	No evidence
	Dog/104-Week Oral	2.5	Developmental	Not teratogenic
	Mouse/18-month	120	Reproductive	No evidence
	RfD2	0.025 (based on 2-yr. dog—100×UF4)	Oncogenicity	No evidence

1 No observable effect level
2 RfD=reference dose and ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme
4 UF=Uncertainty factor

have been classified by the U.S. EPA with the known or likely designation as defined in the EPA 1996 classification scheme (192) or as category C (possible human carcinogen) based on the earlier scheme (146). The classification of flumeturon is still pending.

Amides and Nitriles

The structures of two nitriles, dichlobenil and ioxynil, and one amide, isoxaben, are presented along with their uses and toxicology profiles in Table 13.52.

Ioxynil's profile suggests minimal hazard. Dichlobenil is flagged for its oncogenic potential and received a category C designation based on mouse liver tumors. Isoxaben represents a bit more concern as it has been found to be weakly mutagenic and elicits liver tumors in the mouse and adrenal tumors in the rat.

Benzothiadiazoles and Phenylpyridazine

One example of a benzothiazole and a phenylpyridazine photosynthesis inhibitor are presented in Table 13.53.

Bentazon and pyridate have been found to have acute and repeated-dose profiles that suggest minimal hazard to humans.

Bipyridyliums

Diquat and paraquat, bipyridylium photosynthesis inhibitors, are unlike the triazines, uracils, substituted ureas, benzothiadiazoles, and phenylpyridazines in that they inhibit electron flow in Photosystem I. The structures, uses, and hazard profiles for diquat and paraquat are provided in Table 13.54.

Table 13.50 Structures, uses, and hazard profiles for selected photosynthesis-inhibiting herbicides: Uracils and pyridazinone

Herbicide	Structure	Principle Uses/Crops	Application Rate gm (a.i.)/ha
Bromacil Hyvar® (271)		Used to control grasses, broadleaf weeds, and brush in non-cropland area	900–7180
Terbacil Sinbar® (301)		Used to control grasses, and broadleaf weeds in nut trees, mint, alfalfa, and fruits.	450–3580 9000
Norflurazon Predict® (311)		Used to control broadleaf weeds and sedges in fruits, nuts and berries. Also used on right of ways	560–4500 9000

Herbicide	Irritation: Eye	Irritation: Skin	LD50 (mg/kg): Oral	LD50 (mg/kg): Dermal	LC50 (mg/L): Inhalation	Sensitizing Potential	Signal Word
Bromacil	Mild irritant	Mild irritant	5175	>5000	>4.8	Positive	Caution
Terbacil	Mild irritant	Not irritant	1255	>5000	>4.4	Negative	Caution
Norflurazon	Not irritant	Not irritant	9000	>20000	NA	Negative	Caution

Herbicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Bromacil	Rat/2-year	2.5	Mutagenicity	No evidence
	Dog/52-week	15.6	Developmental	Not teratogenic
	Mouse/18-month	NA	Reproductive	No evidence
	RfD2	0.1	Oncogenicity	C (liver tumors in male mice)3
Terbacil	Rat/2-year	2.5	Mutagenicity	No evidence
	Dog/104-week	1.25	Developmental	Not teratogenic
	Mouse/18-month	7.1	Reproductive	No evidence
	ADI2	0.013	Oncogenicity	E(No evidence)3
Norflurazon	Rat/2-year	19	Mutagenicity	No evidence
	Dog/26-week	1.6	Developmental	Not teratogenic
	M Mouse/18-month	41	Reproductive	No evidence
	RfD2	0.02	Oncogenicity	C (Liver tumors in mice)3

1 No observable effect level
2 RfD=reference dose
3 See Table 13.3 for US EPA classification scheme
NA=Not Available

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Table 13.51 Structures, uses, and hazard profiles for selected photosynthesis-inhibiting herbicides: Ureas

Herbicide	Structure	Principle Uses/Crops	Application Rate gm (a.i.)/ha
Diuron Diumate® (205, 281)		Used to control many annual weeds at lower rates and perennials at higher rates in nuts, berries, spices, and cereals.	200–6400
Fluometuron Cotoran® (284)		Used to control broadleaf weeds and grasses.	1120–2240
Linuron Lorox® (295)		Used to control broadleaf weeds in vegetable and cereals.	250–2240

Herbicide	Irritation Eye	Skin	LD50 (mg/kg) Oral	Dermal	LC50 (mg/L) Inhalation	Sensitizing Potential	Signal Word
Diuron	Mild irritant	Not irritant	3400	2000	>2.5	Negative	Caution
Fluometuron	Slight irritant	Not irritant	6416	>10000	>2.0	Negative	Caution
Linuron	Not irritant	Not irritant	1090	>20000	>0.65	Negative	Caution

Herbicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Diuron (205)	Rat/2-year	<1.02	Mutagenicity	No evidence
	Dog/104-week	0.625	Developmental	Not teratogenic
	Mouse/18-month	>50 (LDT)	Reproductive	No evidence
	RfD2 (300×UF5)	0.002	Oncogenicity	"Known/Likely" (liver/ mice; bladder/rats)4
Fluometuron	Rat/2-year	0.55	Mutagenicity	No evidence
	Dog/52-week	10	Developmental	Not teratogenic
	Mouse/18-month	1.3	Reproductive	No evidence
	ADI2	0.0055	Oncogenicity	Classification pending3
Linuron	Rat/2-year	2.5	Mutagenicity	No evidence
	Dog/104-Week Oral	0.77	Developmental	Not teratogenic
	Mouse/18-month	21	Reproductive	No evidence
	RfD2	0.008	Oncogenicity	C (Interstitial cell tumors in male rats)3

1 No observable effect level
2 RfD=reference dose
3 See Table 13.3 for US EPA classification scheme
4 1996 US EPA carcinogen classification system

5 UF=Uncertainty factor

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Table 13.52 Structures, uses, and hazard profile for selected photosynthesis-inhibiting herbicides: Amides and nitriles

Herbicide	Structure	Principle Uses/Crops	Application Rate gm (a.i.)/ha
Isoxaben Gallery™ (294)		Used to control annual broadleaf weeds in turf, ornamentals, nursery stock, and non-bearing fruit and nut trees.	560–1120
Dichlobenil Acme® (277)		Used to control annual, biennial broadleaf, and grasses in orchards, at industrial sites, under asphalt, and in non-crop areas.	2700–22400
Ioxynil Totril® (293)		Used for control of select weeds in fall planted small grains.	200–400

Herbicide	Irritation Eye	Skin	LD50 (mg/kg) Oral	Dermal	LC50 (mg/L) Inhalation	Sensitizing Potential	Signal Word
Isoxaben	Moderate Irritant	Slight irritant	>10000	>5000	>2.7	Negative	Caution
Dichlobenil	Non-irritant	Non-irritant	>1000	>2000	>0.25	Negative	Warning
Ioxynil	Non-irritant	Mild irritant	110	1050	>0.40	Negative	Warning

Herbicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Isoxaben	Rat/2-year	5.0	Mutagenicity	Weakly mutagenic
	Dog/52-week	10	Developmental	Not teratogenic
	Mouse/18-month	<118	Reproductive	No evidence
	RfD2	0.05	Oncogenicity	C (liver tumors in mice; adrenal in rates)3
Dichlobenil	Rat/2-year	2.5	Mutagenicity	No evidence
	Dog/52-week	1.25	Developmental	Not teratogenic
	Hamster/18-Month Oral	10	Reproductive	No evidence
	RfD2	0.013	Oncogenicity	C (liver tumors in female rats)3
Ioxynil	Rat/2-year	0.5	Mutagenicity	No evidence
	Dog/30-Week Oral	1.0	Developmental	Not teratogenic
	Mouse/18-month	<1.5	Reproductive	No evidence

ADI	0.005	Oncogenicity	No evidence

1 No observable effect level
2 RfD=reference dose and ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme

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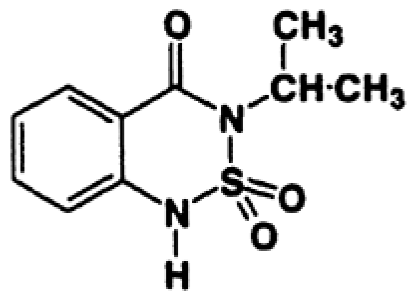
Table 13.53 Structures, uses, and hazard profiles for selected photosynthesis-inhibiting herbicides: Benzothiadiazole and phenylpyridazine

Herbicide	Structure	Principle Uses/Crops	Application Rate gm (a.i.)/ha
Bentazon Basagran® (270)		Used to control annual broadleaf weeds in soybeans, peas, peanuts and cereals.	560–2240
Pyridate (247, 307)		Used to control annual broadleaf weeds in cereals, turf and vegetables.	530–1050

	Irritation		LD50 (mg/kg)		LC50 (mg/L)		
Herbicide	Eye	Skin	Oral	Dermal	Inhalation	Sensitizing Potential	Signal Word
Bentazon	Moderate Irritant	Moderate Irritant	1100	>2500	5.1	NA	Caution
Pyridate	Non-irritant	Moderate Irritant	4690	>2000	>4.7	Positive	Caution

Herbicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Bentazon	Rat/2-year	17.5	Mutagenicity	No evidence
	Dog/52-week	3.2	Developmental	Not teratogenic
	Mouse/18-month	50	Reproductive	No evidence
	RfD2	0.03	Oncogenicity	E (No evidence)3
Pyridate	Rat/2-year	10.8	Mutagenicity	No evidence
	Dog/104-Week Oral	20	Developmental	Not teratogenic
	Mouse/18-month	<48	Reproductive	No evidence
	RfD2	0.11	Oncogenicity	E (No evidence)3

1 No observable effect level
2 RfD=reference dose
3 See Table 13.3 for US EPA classification scheme
4 UF=Uncertainty factor
NA=Not Available

Diquat is less acutely toxic than paraquat. Neither bipyridylium is mutagenic, teratogenic, or carcinogenic, nor have they been demonstrated to be reproductive toxicants.

Protoporphyrinogen Inhibitors

The mode of action for this class of herbicides is to interfere with critical enzymes needed in the biosynthesis of chlorophyll in plants. Unfortunately, mammalian systems possess a similar chromophore, namely, heme, that relies on a similar set of enzymes that are also affected by these chemicals. Therefore, it is not uncommon to find evidence of anemia in mammals exposed to protoporphyrinogen-inhibiting chemicals and other photobleaching herbicides (145, 151, 321). In addition to effects on heme synthesis, it is theorized that light(phorryhia) and oxygen-dependent peroxidation of cell membrane lipids may lead to cell lysis and death, particu

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Table 13.54 Structures, uses, and hazard profiles for selected photosynthesis-inhibiting herbicides: Bipyridyliums

Herbicide	Structure	Principle Uses/Crops	Application Rate gm (a.i)/ha
Diquat WEEDTRINE® (185, 280)	N N $2Br^-$	Used to control algae in ponds, lakes and drainage ditches	240 gm/liter of water
Paraquat Cyclone® (183, 302)	H_3C-N^+ N^+-CH_3 $2Cl^-$	Used to control existing vegetation at planting or no till.	280–1050

	Irritation		LD50 (mg/kg)	LC50 (mg/L)			
Herbicide	Eye	Skin	Oral	Dermal	Inhalation	Sensitizing Potential	Signal Word
Diquat	Non-irritant	Slight irritant	>5000	>5000	>6	Negative	Caution
Paraquat	NA	Irritant	112	240	NA	Negative	Warning

Herbicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Diquat	Rat/2-year	0.6	Mutagenicity	No evidence
	Dog/52-week	0.5	Developmental	Not teratogenic
	Mouse/18-month	3.5	Reproductive	No evidence
	RfD2	0.005	Oncogenicity	E (No evidence)3
Paraquat	Rat/2-year	1.25	Mutagenicity	No evidence
	Dog/52-week	0.45	Developmental	Not teratogenic
	Mouse/18-month	1.87	Reproductive	No evidence
	RfD2	0.0045	Oncogenicity	E (No evidence)3

1 No observable effect level
2 RfD=reference dose and ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme
NA=Not Available

larly in organs where these metabolites form or bioconcentrate. Such a theory is consistent with the experimental observation that liver damage and liver tumor formation, particularly in mice, are often a consequence of exposure to these chemicals. An alternate viewpoint is that liver damage and subsequent tumor response may result from peroxosome proliferative effects of chemicals in this class.

Diphenyl Ethers

Acifluorfen, formesafen, lactofen, and oxyfluorfen represent the diphenyl ether protoporphyrin inhibitors. The structures, uses, and toxicity profiles for the products are presented in Table 13.55.

Lactofen is a severe eye irritant; otherwise, the acute hazards associated with these protoporphyrin inhibitors are not remarkable. These diphenyl ethers are classified as B2 (probable human carcinogens) in the case of acifluorfen and lactofen (liver and stomach tumors), or C (possible human carcinogens) in the case of oxyfluorfen and formesafen (liver tumors).

N-Phenylphthalimides, Thiadiazoles, and Triazolinones

Only one representative for each of these chemical groups is featured in Table 13.56.

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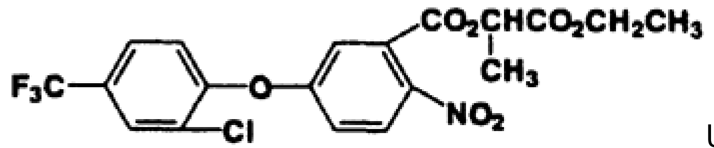
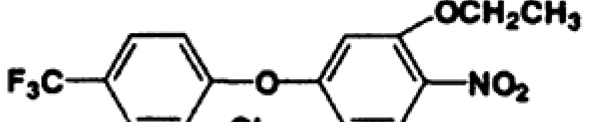
Table 13.55 Structures, uses, and hazard profiles for selected protoporphyrin inhibitors: Diphenylethers

Herbicide	Structure	Principle Uses/Crops	Application Rate gm (a.i.)/ha
Acifluorfen Scepter® (195, 265)		Used to control annual broadleaf weeds in peanuts, beans, and rice.	140–420
Fomesafen Flosil® (212)		Used to control annual broadleaf weeds in soybeans.	280–420
Lactofen Cobra® (189)		Used to control annual broadleaf weeds in cereals, potatoes, soya, and rice.	70–220
Oxyfluorfen Goal® (182)		Used to control annual broadleaf weeds in conifers, vegetables, nuts and vine crops.	250–2240

Herbicide	Irritation Eye	Irritation Skin	LD50 (mg/kg) Oral	LD50 (mg/kg) Dermal	LC50 (mg/L) Inhalation	Sensitizing Potential	Signal Word
Acifluorfen	Non-irritant	Moderate irritant	1450	>2000	>6.9	Negative	Caution
Fomesafen	Moderate irritant	Mild irritant	1250	>1000	4.97	Negative	Caution
Lactofen	Severe irritant	Non-irritant	>5000	2000	NA	NA	Danger
Oxyfluorfen	Moderate irritant	Non-irritant	>5000	>5000	NA	Negative	Caution

Herbicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Acifluorfen	Rat/2-year	25	Mutagenicity	No evidence
	Dog/52-week	NA	Developmental	Not teratogenic
	Mouse/18-month	38	Reproductive	No evidence
	RfD (rat reproduction)2	0.013	Oncogenicity	B2 (liver/stomach tumors)3
Fomesafen	Rat/2-year	0.25	Mutagenicity	No evidence
	Dog/52-week	1.0	Developmental	Not teratogenic
	Mouse/18-month	1.0	Reproductive	No evidence
	RfD2	0.0025	Oncogenicity	C with CSF (liver tumors in mice)3
Lactofen	Rat/2-year	25	Mutagenicity	No evidence
	Dog/52-week	5.0	Developmental	Not teratogenic
	Mouse/18-month	1.5	Reproductive	No evidence
	RfD2 (mouse—1000×UF 4)	0.002	Oncogenicity	B2 (liver/stomach

				tumors)3
Oxyfluorfen	Rat/2-year	2.0	Mutagenicity	No evidence
	Dog/2-Year Oral	2.5	Developmental	Not teratogenic
	Mouse/18-month	0.3	Reproductive	No evidence
	RfD2	0.003	Oncogenicity	C (liver tumors in mice)3

1 No observable effect level
2 RfD=reference dose, CSF=cancer slope factor
3 See Table 13.3 for US EPA classification scheme
NA=Not Available

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Table 13.56 Structures, uses, and hazard profiles for selected protoporphyrin Inhibitors: N-Phenylphthalimide, thiadiazole, and triazolinone

Chemical/ Common Name	Structure	Principle Uses/Crops	Application Rate gm (a.i.)/ha
Flumiclorac-pentyl Resource® (283)	$OCH_2COO(CH_2)_4CH_3$; Cl	Used to control broadleaf weeds soybeans and corn.	30–90 in
Fluthiacet-methyl Action® (211, 258)	F; Cl; SCH_2COOCH_3	Used to control annual broadleaf weeds in corn, soybeans, and cereals.	4–15
Carfentrazone-ethyl Affinity®, Aurora® (230)	F_2HC; H_3C; Cl; CH_2CHCO_2H; F; Cl	Used to control annual broadleaf weeds in cereals.	9–35

Herbicide	Irritation Eye	Irritation Skin	LD50 (mg/kg) Oral	LD50 (mg/kg) Dermal	LC50 (mg/L) Inhalation	Sensitizing Potential	Signal Word
Flumiclorac-pentyl	Slight Irritant	Non-irritant	>5000	>2000	>5.9	Negative	Caution
Fluthiacet-methyl	Slight Irritant	Non-irritant	>5000	>2000	>5.0	NA	Caution
Carfentrazone-ethyl	Minimal Irritant	Non-irritant	5143	>4000	>5.0	Negative	Caution

Herbicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Flumiclorac-Pentyl	Rat/2-year	35	Mutagenicity	No evidence
	Dog/52-week	100	Developmental	Not teratogenic
	Mouse/18-month	32	Reproductive	No evidence
	RfD2	0.32	Oncogenicity	E (No evidence)3
Fluthiacet-Methyl	Rat/2-year	2.1	Mutagenicity	No evidence
	Dog/52-week	30	Developmental	Not teratogenic
	Mouse/18-month	0.1	Reproductive	No evidence
	RfD2	0.001	Oncogenicity	Likely carcinogen (liver in

				mice/pancreas in rats)4
Carfentrazone-Ethyl	Rat/2-year	3.0	Mutagenicity	No evidence
	Dog/52-week	50	Developmental	Not teratogenic
	Mouse/18-month	10	Reproductive	No evidence
	RfD2	0.03	Oncogenicity	Not likely a carcinogen4

1 No observable effect level
2 RfD=reference dose
3 See Table 13.3 for US EPA classification scheme
4 Proposed 1996 US EPA classification scheme
NA=Not Available

Flumiclorac-pentyl is applied in the 30 to 90 g/hectare range and has an acute and repeated-dose hazard profile that should not evoke concern in regard to human health. The thiadiazole, fluthiacet-methyl, which is applied at a remarkable low rate of 4–15 g/hectare, exhibits an unremarkable acute hazard, but has been found to produce liver tumors in mice and pancreatic tumors in the rat. Carfentrazone-ethyl, which is applied at the very low rate of 9–35 g/hectare, incurs little concern based on the acute or repeated-dose hazard evaluation.

Bleaching Herbicides

The bleaching herbicides disrupt the synthesis of carotenoid pigments, which protect chlorophyll pigments in light. In the absence of carotenoids, chlorophyll is destroyed and turns white, thus having a bleached appearance. The pyridazinones, triketones, and isoxazoles bleaching herbicides are considered here. The pyridazinones, triazoles, and isoxazolidinones inhibit carotenoid biosynthesis at the phytoene desaturase step, whereas, the triketones and isoxazoles inhibit the 4-hydroxyphenylpyruvate dioxygenase enzyme (156).

Pyridazinones

The structures, uses, and hazard profiles for norflurazon and fluridone are given in Table 13.57. Neither norflurazon nor fluridone is acutely toxic; the repeated-dose profile for fluridone is unremarkable. Norflurazon is classified as category C (possible human carcinogen) based on mouse liver tumors.

Triazoles and Isoxazolidinones

Amitrole (triazole) and clomazine (isoxazolidinone) are presented with regard to their structures, uses, and toxicity profiles in Table 13.58.

These compounds are not acutely toxic. Clomazone has an unremarkable repeated-dose hazard profile. Amitrole has been classified as category B2 (probable human carcinogen) based on thyroid tumors in rats and liver tumors in mice.

Triketones and Isoxazoles

The structures, uses, and hazard profile for sulcotrione (triketone) and isoxaflutole (isoxazole) are presented in Table 13.59.

Although the acute toxicity information for sulcotrione does not suggest that it represents a hazard, the available data for its repeated-dose profile are inadequate for full hazard assessment. Isoxaflutole does not represent an acute hazard but has been shown to be a potential developmental toxin, a neurotoxin, and potential carcinogen.

EPSP Synthase, Glutamine Synthase, and Dihydropteroate (DHP) Synthase Inhibitors

A single member of each of these classes of synthase inhibitors was selected for this review. The inhibition of 5-enolpyruvlshikimate-3-phosphate (EPSP) synthase prevents the biosynthesis of essential amino acids (287). The inhibition of glutamine synthase leads to an accumulation of ammonium ions, and inhibition of photosynthesis (286). Inhibition of DHP synthase inhibits folic acid synthesis, which is needed for the formation of purine nucleotides required for cell division (267).

The structures, uses, and hazard profiles for representative ESSP synthase, glutamine synthase, and dihydropteroate synthase inhibitors are given in Table 13.60.

It can be seen that glyphosate, glufosinate-ammonium, and asulam are not acutely toxic. Both glyphosate and glufosinate-ammonium have excellent repeated-dose toxicity profiles. Asulum is also not mutagenic, teratogenic, or a reproductive toxin, but does produce tumors in male rat.

Microtubule Assembly Inhibitors

The structures, uses, and hazard profiles of three commercially important dinitroaniline microtubule assembly inhibitors are given in Table 13.61.

Benfluralin, pendimethalin, and trifluralin are not acutely toxic. There are inadequate data to access the repeated-dose hazards for benfluralin. Both pendimethalin and trifluralin were classified as category C (possible human carcinogens) by the U.S. EPA (100).

Cell Division Inhibitors

The structures, uses, and toxicology profiles for four chloracetamide inhibitors of cell division are provided in Table 13.62.

All four chloracetamide herbicides are potential sensitizers. Alachlor and acetochlor both exhibit mutagenic potential and significant oncogenic potential in both rats and mice. Dimethenamid also has exhibited weak genotoxiciy and a tumor response in the female rat. Metolachlor showed a weak oncogenic response in the liver of the female rat.

Cellulose and Lipid Synthesis Inhibitors

The structures, uses, and hazard profiles of the cellulose synthesis inhibitor isoxaben and of the lipid

synthesis inhibitors butylate and molinate are given in Table 13.63.
Isoxaben exhibits a low acute toxicity hazard. Butylate is a potential sensitizing agent, and molinate has signifi

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Table 13.57 Structures, uses, and hazard profile for selected bleaching herbicides: Pyridazinones

Chemical/ Common Names	Structure	Principle Uses/Crops	Application Rate gm (a.i.)/ha
Norflurazon Evital® (220)		Fruit Tree, Nut, & Vine crops, Soybean, Ornamentals, Peanut, Cotton, and IWC,	500–3360
Fluridone Sonar® (285)		Aquatic herbicide	2240 (0.075–0.15 mg/L)

Herbicide	Irritation Eye	Skin	LD50 (mg/kg) Oral	Dermal	LC50 (mg/L) Inhalation	Sensitizing Potential	Signal Word
Pyridazinone	Non-Irritant	Non-irritant	>9000	>20000	>0.2	Negative	Caution
Fluridone	Slight	Non-irritant	>10000	>5000	>4.12	Negative	Caution

Herbicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Pyridazinone	Rat/2-year	19	Mutagenicity	No evidence
	Dog/52-week	1.5	Developmental	Not teratogenic
	Mouse/18-month	41	Reproductive	No evidence
	RfD2	0.02	Oncogenicity	C (mouse liver tumors)3
Fluridone	Rat/2-year	8.0	Mutagenicity	No evidence
	Dog/52-week	11.4	Developmental	Not teratogenic
	Mouse/18-month	11.6	Reproductive	No evidence
	RfD2	0.08	Oncogenicity	E (No evidence)3

1 No observable effect level
2 RfD=reference dose
3 See Table 13.3 for US EPA classification scheme
NA=Not Available

cant acute inhalation toxicity. Isoxaben has some mutagenic, developmental, and oncogenic potential. The lipid synthesis inhibitor butylate appears not to exhibit significant repeated-dose toxicity. Much of the critical assessment data for molinate is not available, making full evaluation impossible; however, from the available data, molinate appears to have reproductive toxicity and oncogenic potential.

Synthetic Auxin Mimics (Phenoxy, Benzoic, and Pyridine Acids)

The synthetic auxins alter growth in the meristems and leaves by affecting protein synthesis and normal

cell division. 2,4-D (phenoxy), dicamba (benzoic), and clopyralid and picloram (pyridine acids) represent this

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Table 13.58 Structures, uses, and hazard profiles for selected bleaching herbicides: Triazole and isoxazolidinone

Herbicide	Structure	Principle Uses/Crops	Application Rate gm (a.i.)/ha
Amitrole Amizol® (261)		Fruit trees, grapes, olives Ornamentals, cereal, IWC, Aquatic plants	2000–9000
Clomazone Command® (254)		Soybeans, peas, peppers,	560–1700

Herbicide	Irritation Eye	Skin	LD50 (mg/kg) Oral	Dermal	LC50 (mg/L) Inhalation	Sensitizing Potential	Signal Word
Amitrole	Slight irritant	Slight irritant	>5000	>2000	NA	NA	Caution
Clomazone	Non-irritant	Minimal	2077	>2000	4.23 (female)	Negative	Caution

Herbicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Amitrole	Rat/2-year	0.5	Mutagenicity	No evidence
	Dog/52-week	NA	Developmental	Not teratogenic
	Mouse/18-month	1.4	Reproductive	No evidence
		CSF=1.13 (mg, kg, day)−1	Oncogenicity	B2 (thyroid tumor rats; liver tumor mice)3
Clomazone	Rat/2-year	4.3	Mutagenicity	No evidence
	Dog/52-week	12.5	Developmental	Not teratogenic
	Mouse/18-month	143	Reproductive	No evidence
	RfD2	0.043	Oncogenicity	E (No evidence)3

1 No observable effect level

2 See Table 13.3 for US EPA classification scheme

NA=Not Available

class. The structures, uses, and hazard profiles of these synthetic auxins are provided in Table 13.64. Dicamba and clopyralid were found to exhibit sensitization potential. Dicamba was shown also to have some mutagenic and weak, questionable carcinogenic potential.

Herbicides with Unknown Mechanism of Action

The structures, uses, and hazard profiles of two of these products are presented in Table 13.65. Monosodium methanearsonic acid (MSMA), an organic arsenical, has a rather unremarkable acute toxicity profile; it has, however, been shown to decrease fertility in rats and is classified as category B2 (probable human carcinogen) based on bladder tumors in the rat. Difenzoquat is labeled as a danger because of its corrosive effects on the eyes; however, it possesses a nonproblematical repeated-dose hazard profile.

CONCLUSION

Although older products, like the practically irreplaceable DDT and chlordane, have been banned because of their persistence in the environment, their replacements, the organophosphate insecticides, currently are under fire by the U.S. EPA. Therefore, this

Table 13.59 Structures, uses, and hazard profiles for selected bleaching herbicides: Triketone and isoxazole

Chemical/ Common Names	Structure	Principle Uses/Crops	Application Rate gm (a.i.)/ha
Sulcotrione Mikito® (72)	O, Cl, CO, SO_2CH_3, O	Corn, Sugar Cane	200–300
Isoxaflutole (215, 240)	O, SO_2CH_3, N, O, CF_3	Corn	75–140

	Irritation		LD50 (mg/kg)		LC50 (mg/L)		
Herbicide	Eye	Skin	Oral	Dermal	Inhalation	Sensitizing Potential	Signal Word
Sulcotrione	Mild irritant	Non-irritant	>5000	>4000	>1.6	Positive	Caution
Isoxaflutole	Mild irritant	Minimal	>5000	>2000	>5.3	Negative	Caution

Herbicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Sulcotrione	Rat/2-year	NA	Mutagenicity	No evidence
	Dog/52-week	NA	Developmental	Not teratogenic
	Mouse/18-month	NA	Reproductive	NA
	RfD or ADI2	NA	Oncogenicity	NA
Isoxaflutole	Rat/2-year	2.0	Mutagenicity	No evidence
	Dog/52-week	45	Developmental	Evidence of developmental toxicity
	Mouse/18-month	3.2	Reproductive	No evidence
	RfD2	0.002	Neurotoxicity	Evidence of neurotoxicity
			Oncogenicity	Likely to be a carcinogen4 (liver tumors in both sexes of rats and mice).

1 No observable effect level
2 RfD=reference dose, and ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme
4 Proposed 1996 US EPA carcinogen classification system
NA=Not Available

overview of crop protection chemicals and modalities has focused primarily on the newer products used for disease control, insect control, and weed control.

Besides the movement toward more natural chemistry, such as with the avermectins, the chemicals of the late 1980s and 1990s are efficacious at a low grams per acre rate with pest-specific toxicity and limited mammalian toxicity, such as for the ALS sulfonylurea herbicides. In order to reduce the area of exposure due to application, applying materials directly to the seed to be planted is

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Table 13.60 Structures, uses, and hazard profiles for selected inhibitors of EPSP: Glutamine and DHP synthase

Common Name Trade Name	Structure	Principle Uses/Crops	Application Rate gm (a.i.)/ha
Glyphosate Roundup® EPSP synthase Inhibitor (235, 287)	$HO-C(=O)-CH_2NHCH_2P(=O)(OH)-OH$	Corn, Soybeans IWC	210–4200
Glufosinateammonium Finale® Glutamine synthase inhibitor (287)	$CH_3P(=O)(O)-CH_2-CH_2-CH(NH_2)CO_2H-NH_4^+$	Fruit trees, grapes, rubber, palm ornamentals, vegetables, IWC	280–1700
Asulam Asulux® DHP synthase Inhibitor (267)	$H_2N-C_6H_4-SO_2NHCO_2CH_3$	Sugar cane, alfalfa, banana, coffee, tea, cocoa, pasture forestry	1120–4000

Herbicide	Irritation: Eye	Irritation: Skin	LD50 (mg/kg): Oral	LD50 (mg/kg): Dermal	LD50 (mg/L): Inhalation	Sensitizing Potential	Signal Word
Glyphosate	Slight	Non-irritant	5600	>5000	NA	Negative	Caution
Glufosinateammonium	Non-irritant	Non-irritant	1620 ♀	4000	1.26 ♂	NA	Caution
Asulam	Irritant	Slight Irritant	>5000	>2000	>1.8	Negative	Caution

Herbicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Glyphosate	Rat/2-year	400	Mutagenicity	No evidence
	Dog/52-week	500	Developmental	Not teratogenic
	Mouse/18-month	4500	Reproductive	No evidence
	RfD2	0.1	Oncogenicity	E (No evidence)3
Glufosinateammonium	Rat/2-year	2.1	Mutagenicity	No evidence
	Dog/52-week	NA	Developmental	Not teratogenic
	Mouse/18-month	NA	Reproductive	No evidence
	RfD2	0.02	Oncogenicity	Not oncogenic
Asulam	Rat/2-year	36	Mutagenicity	No evidence
	Dog/52-week	60	Developmental	Not teratogenic
	Mouse/18-month	713	Reproductive	No evidence
	RfD or ADI2	0.36	Oncogenicity	C (adrenal tumors in male rats)3

1 No observable effect level
2 RfD=reference dose, and ADI=acceptable daily intake

3 See Table 13.3 for US EPA classification scheme
NA=Not Available

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Table 13.61 Structures, uses, and hazard profiles for selected dinitroaniline inhibitors of microtuble assembly

Common Name Trade Name	Structure	Principle Uses/Crops	Application Rate gm (a.i.)/ha
Benfluralin Balan®, Benefin® (14)		Alfalfa, clover, lettuce and tobacco	1260–1680
Pendimethalin Prowl® (221, 303)		Corn, sorghum, rice, soybeans, cotton, potatoes, tobacco, sugarcane, beans, onions, and sunflower	560–3360
Trifluralin Treflan® (315)		Alfalfa, asparagus, beans, carrots, celery, cole crops, cucurbits, onions, okra, peas, peppers, potatoes, sunflower, tomatoes, wheat, barley, flax, soybeans, corn, sorghum, and ornamentals	340–2240

Herbicide	Irritation Eye	Skin	LD50 (mg/kg) Oral	Dermal	LD50 (mg/L) Inhalation	Scnsitizinc Potential	Signal Word
Benfluralin	Slight irritant	Slight irritant	>5000	>2000	NA	Positive	Caution
Pendimethalin	Slight irritant	Non-irritant	1050♀	>5000	320 (nominal)	Negative	Caution
Trifluralin	Slight Irritant	Non-irritant	>5000	>5000	>4.8	Positive	Caution

Herbicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Benfluralin	Rat/2-year	1000	Mutagenicity	NA
	Dog/52-week	25	Developmental	NA
	Mouse/18-month	6.5	Reproductive	NA
	RfD2	0.3	Oncogenicity	NA

Pendimethalin	Rat/2-year	10.0	Mutagenicity	No evidence
	Dog/104-week	12.5	Developmental	Not teratogenic
	Mouse/18-month	75	Reproductive	No evidence
	RfD or ADI2	0.13	Oncogenicity	C with RfD (thyroid follicular cell adenomas)3
Trifluralin	Rat/2-year	2.5	Mutagenicity	No evidence
	Dog/52-week	2.4	Developmental	Not teratogenic
	Mouse/18-month	7.5	Reproductive	No evidence
	RfD or ADI2	0.024	Oncogenicity	C with CSF (bladder, kidney, thyroid tumors)3

1 No observable effect level
2 RfD=reference dose, ADI=acceptable daily intake, and CSF=cancer slope factor
3 See Table 13.3 for US EPA classification scheme
NA=Not Available

Table 13.62 Structures, uses, and hazard profiles for selected chloroacetamide inhibitors of cell division

Common Name Trade Name	Structure	Principle Uses/Crops	Application Rate gm (a.i.)/ha
Alachlor Lassco® (228)		Corn, beans, peanuts, sorghum, soybeans, sunflowers and ornamentals	1.5–4.5 1500–4500
Acetochlor Surpass® (175)		Corn, soybeans, sorghum, and wheat	0.9–3.36 900–3360
Metolachlor Dual® (242)		Corn, soybeans, and sorghum, cucurbits, onions, peas, pecans, peppers, potatoes, sugar beets	1.25–6.2 1250–6200
Dimethenamid Frontier® (184)		Corn and soybeans	590

Herbicide	Irritation Eye	Skin	Oral	LD50 (mg/kg) Dermal	LD50 (mg/L) Inhalation	Sensitizing Potential	Signal Word
Alachlor	Non-irritant	Non-irritant	930	13, 300	>1.04	Positive	Caution
Acetochlor	Slight Irritant	Non-irritant	2148	4166	>3.0	Positive	Caution
Metolachlor	Non-irritant	Minimal Irritant	>2780	>10000	>1.75	Positive	Caution
Dimethenamid	Slight Irritant	Non-irritant	1570	>2000	>5.0	Positive	Caution

Herbicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Alachlor	Rat/2-year	2.5	Mutagenicity	Positive (UDS)
	Dog/52-week	1.0	Developmental	Not teratogenic
	Mouse/18-month	16.6 ♂	Reproductive	No evidence
	RfD2	0.01	Oncogenicity	C/RfD (nasal; rats; lung; mice)3
Acetochlor	Rat/2-year	8.0	Mutagenicity	Positive (CHO, UDS, mouse lymphoma)
	Dog/52-week	2.0	Developmental	Not teratogenic
	Mouse/18-	13	Reproductive	No evidence

	month			
	RfD2	0.02	Oncogenicity	B2 (liver, thyroid, nasal, rats; lung tumors in mice)3
Metolachlor	Rat/2-year	15	Mutagenicity	No evidence
	Dog/52-week	10	Developmental	Not teratogenic
	Mouse/18-month	120	Reproductive	No evidence
	RfD2	0.1	Oncogenicity	C/RfD (liver tumors; ♀ rats)3
Dimethenamid	Rat/2-year	5.0	Mutagenicity	Weak positive (CHO: UDS)
	Dog/52-week	9.6	Developmental	Not teratogenic
	Mouse/18-month	40 ♀	Reproductive	No evidence
	RfD2	0.05	Oncogenicity	C/RfD (liver, ovary; ♀ rats)3

1 No observable effect level
2 RfD=reference dose and ADI=acceptable daily intake
3 See Table 13.3 for US EPA classification scheme

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Table 13.63 Structures, uses, and hazard profiles for selected benzamide and thiocarbamate inhibitors of cellulose or lipid synthesis

Herbicide	Structure	Principle Uses/Crops	Application Rate gm (a.i.)/ha
Isoxaben Gallery® (294)		Turf, ornamentals non-bearing fruit and nut trees and conifers	560–1120
Butylate Sutan® (272)		Corn	3500–6900
Molinate Ordram® (298)		Rice	2240–5600

Herbicide	Irritation		LD50 (mg/kg)		LD50 (mg/L)	Sensitizing Potential	Signal Word
	Eye	Skin	Oral	Dermal	Inhalation		
Isoxaben	Moderate Irritant	Slight Irritant	>10000	>2000	>2.68	Negative	Caution
Butylate	Non-irritant	Mild Irritant	4659 ♂	1659	4.64	Positive	Caution
Molinate	Moderate Irritant	Mild Irritant	720	~4000	0.003	Negative	Danger

Herbicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
Isoxaben	Rat/2-year	5.0	Mutagenicity	Positive micronucleus test
	Dog/52-week	10.0	Developmental	Positive only at maternal toxic doses
	Mouse/18-month	NA	Reproductive	No evidence
	RfD or ADI2	0.05	Oncogenicity	C (adrenal and liver tumors)3
Butylate	Rat/2-year	50	Mutagenicity	No evidence
	Dog/52-week	5.0	Developmental	Not teratogenic
	Mouse/18-month	NA	Reproductive	NA
	RfD or ADI2	0.05	Oncogenicity	E (No evidence)3
Molinate	Rat/2-year	NA	Mutagenicity	NA
	Dog/52-week	NA	Developmental	NA
	Mouse/18-month	NA	Reproductive	Effect on sperm
	RfD or ADI2	0.002	Oncogenicity	C with CSF (kidney tumors in rats)3

1 No observable effect level
2 RfD=reference dose and CSF=cancer slope factor

3 See Table 13.3 for US EPA classification scheme
NA=Not Available

Table 13.64 Structures, uses, and hazard profiles for selected synthetic auxin mimics (phenoxy, benzoic, and pyridine acids)

Chemical Class Common Name	Structure	Principle Uses/Crops	Application Rate gm (a.i.)/ha
Wedare® (250)		Turf, cereals, sorghum, corn, soybeans, asparagus, and fruit trees	280–2240
Dicamba Banvel® (232)		Corn, turf, sorghum, cereals, pastures, and asparagus	70–2240
Clopyralid Reclam® (202)		Sugar beets, corn, grass seed, conifers, and pasture	105–560
Picloram Tordon® (259)		Industrial Weed Control, forestry, pasture, and range land.	35–1120

Herbicide	Irritation Eye	Skin	LD50 (mg/kg) Oral	Dermal	LD50 (mg/L) Inhalation	Sensitizing Potential	Signal Word
2,4-D	Severe Irritant	Moderate Irritant	639 ♂	>2000	1.8	Negative	Warning
Dicamba	Corrosive	Non-irritant	1851 ♀	>2000	>9.6	Positive	Danger
Clopyralid	Severe Irritant	Slight Irritant	4300	>2000	1.3	Negative	Warning
Picloram	Moderate Irritant	Non-irritant	4012 ♀	>2000	>0.035	Positive	Danger

Herbicide	Species/study	NOEL[1] (mg/kg/day)	Toxicity Studies	Hazard Indicator
2,4-D	Rat/2-year	5.0	Mutagenicity	No evidence
	Dog/52-week	1.0	Developmental	Not teratogenic
	Mouse/18-month	1.0	Reproductive	No evidence
	RfD or ADI[2]	0.01	Oncogenicity	D (Not classifiable)[3]
Dicamba	Rat/2-year	125	Mutagenicity	Positive (*B. subtilis;* UDS)
	Dog/52-week	60	Developmental	Not teratogenic
	Mouse/18-month	108 ♂	Reproductive	No evidence

	RfD or ADI[2]	0.6	Oncogenicity	D (Not classifiable)[3]
Clopyralid	Rat/2-year	50	Mutagenicity	No evidence
	Dog/52-week	100	Developmental	Not teratogenic
	Mouse/18-month	500	Reproductive	No evidence
	RfD or ADI[2]	0.5	Oncogenicity	E (No evidence)[3]
Picloram	Rat/2-year	20	Mutagenicity	No evidence
	Dog/52-week	35	Developmental	Not teratogenic
	Mouse/18-month	500	Reproductive	No evidence
	RfD or ADI[2]	0.2	Oncogenicity	E (No evidence)[3]

1 No observable effect level
2 RfD=reference dose
3 See Table 13.3 for US EPA classification scheme

Table 13.65 Structures, uses, and hazard profiles for selected herbicides whose mode of action is unknown

Chemicals Class Common Name	Structure	Principle Uses/Crops	Application Rate gm (a.i.)/ha
MSMA Drexar® (181, 299)		Controls broadleaf weeds in noncrop areas, cotton and turf.	2220–2770
Difenzoquat Avenge® (279)		Barley and wheat	700–1120

Herbicide	Irritation		LD50 (mg/kg)		LC50 (mg/L)	Sensitizing Potential	Signal Word
	Eye	Skin	Oral	Dermal	Inhalation		
MSMA	Mild Irritant	Mild Irritant	1059E	>2000	>6.0	NA	Caution
Difenzoquat	Corrosive	Moderate Irritant	373E	>2000	0.5	Negative	Danger

Herbicide	Species/study	NOEL1 (mg/kg/day)	Toxicity Studies	Hazard Indicator
MSMA	Rat/2-year	3.2	Mutagenicity	NA
	Dog/52-week	NA	Developmental	Not teratogenic
	Mouse/18-month	NA	Reproductive	Decreased fertility
	RfD2	0.01	Oncogenicity	B2 (Bladder fibrosarcomas)3
Difenzoquat	Rat/2-year	25	Mutagenicity	NA
	Dog/52-week	20	Developmental	Not teratogenic
	Mouse/18-month	75	Reproductive	No evidence
	RfD2	0.20	Oncogenicity	E (No evidence)3

1 No observable effect level
2 RfD=reference dose (190)
3 See Table 13.3 for US EPA classification scheme
NA=Not Available

being used extensively. More recently, seeds have been genetically engineered to incorporate the natural insecticide control stock of *Bacillus thuringiensis*. Most recently, a gene has been installed in crop seed to protect the crop from a nonspecific herbicide. It can be seen that crop protection chemicals are evolving to superefficacious agents. In most cases, this enhanced efficacy for the target pest did not come at the expense of human or environmental safety.

The crop protection industry has become a significant business during the last 50 years. However, with the harvest reaped by this industry, there have been substantial benefits provided to society. Looking beyond the lives saved by DDT from its use in disease control, the agriculturist's ability to produce food and fiber have significantly increased and labor costs have significantly decreased. Despite a 69% decrease in the number of U.S. farmers from the 1930s to the 1990s, the individual farmer can now feed an average of 129 people; this is up from 19 in the 1930s. Currently less than 2% of our population now grows enough food to feed the entire U.S. population, with surplus.

The average life expectancy has increased from 60 years in 1930 to in excess of 75 years today, primarily due to the availability of an adequate and healthy supply of food. It has been less than 60 years since DDT was first used as an insecticide, and we have learned a lot about the need to develop chemicals that do not disturb our environment.

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We are also learning that nature itself may provide us with better ways of achieving that objective. The challenge is ours. In the next 60 years, world agriculture must be able to provide food for more than 11 billion people. This means that we must triple our output over the next six decades while eliminating any impact on the environment.

QUESTIONS

1. Farmers must contend with some 80,000 plant diseases, 30,000 species of weeds, and 1000 species of nematodes and more than 10,000 species of insects. Today, national and international agricultural organizations estimate that as much as 45% of the world's crops continue to be lost to these types of hazards. In the United States alone, about $20 billion worth of crops (one-tenth of production) is lost each year. What do you think would be the status of our national food production capacity without the use of pesticides?
2. Who assures that pesticides can be used without unacceptable hazard to the consumer to protect food crops and maximize yields?
3. How stringent are the testing requirements for the registration of a pesticide when compared to those for products used in the household and yard, industrial chemicals, or even pharmaceuticals?
4. Has the introduction of pesticides into your food supply had a positive or negative impact on the quality of your life?

REFERENCES

1. Aspelin, A.L. (1996): *Pesticide Industry Sales and Useage 1994 and 1995 Market Estimates Reports.* Economic Analysis Branch, Biological and Economic Analysis Division. http://www.epa.gov/oppbead1/95pestsales/Intro.html (accessed 5/99).
2. Avery, D. (1993): Environmental agriculture. 60 Years of inspiration. *National Agricultural Chemicals Association and Farm Chemicals Magazine*, 1:1–13.
3. Ballantine, L.G. (1992): An overview of the U.S. pesticide registration guidelines. *Agric. Newsletter*, 3(2):1–6.
4. Beyer, E.M., Jr., Duffy, M.J., Hay, J.V., and Schlueter, D.D. (1988): Sulfonylureas. In: *Herbicides; Chemistry, Degradation and Mode of Action,* edited by P.C.Kearney and D.D.Kaufman, pp. 117–189. Marcel Dekker, New York.
5. Bliley, R. (1996): *Food Quality Protection Act of 1996.* 104 Congress, 2nd Session. Report 104–669, part 2, pp. 1–89. Government Printing Office, Washington, DC.
6. Bloomquist, J.R. (1993a): Neuroreceptor mechanisms in pyrethroid mode of action and resistance. *Rev. Pestic. Toxicol.,* 2:185–226.
7. Bloomquist, J.R. (1993b): Toxicology, mode of action, and target site-mediated resistance to insecticides acting on chloride channels. *Mini Rev. Comp. Biochem. Physiol.,* 106C:301–314
8. Bloomquist, J.R. (1999): Insecticides: Chemistries and characteristics. At *Radcliffe's IPM World Textbook Home Page.* http://ipmworld.umn.edu/chapters/bloomq.htm (Accessed 4/99).
9. British Crop Protection Council (BCPC). (1997): Abamectin. In: *A World Compendium: The Pesticide Manual,* 11th ed., edited by C. D.S.Tomlin, pp. 3–5. British Crop Protection Council, Farnham, Surrey.
10. British Crop Protection Council (BCPC). (1997): Acephate. In: *A World Compendium: The Pesticide Manual,* 11th ed., edited by C.D.S.Tomlin, pp. 7–9. British Crop Protection Council, Farnham, Surrey.
11. British Crop Protection Council (BCPC). (1997): Aldicarb. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 26–28. British Crop Protection Council, Farnham, Surrey.
12. British Crop Protection Council (BCPC). (1997): Azoxystrobin. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 70–72. British Crop Protection Council, Farnham, Surrey.
13. British Crop Protection Council (BCPC). (1997): *Bacillus thuringiensis.* In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 73–78. British Crop Protection Council, Farnham, Surrey.
14. British Crop Protection Council (BCPC). (1997): Benfluralin. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C. D.S.Tomlin, pp. 94–95. British Crop Protection Council, Farnham, Surrey.
15. British Crop Protection Council (BCPC). (1997): Benomyl. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 100–102. British Crop Protection Council, Farnham, Surrey.
16. British Crop Protection Council (BCPC). (1997): Captan. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 177–179. British Crop Protection Council, Farnham, Surrey.
17. British Crop Protection Council (BCPC). (1997): Carbaryl. In: *A World Compendium: The Pesticide*

Manual, 11th ed., edited by C.D.S.Tomlin, pp. 180–182. British Crop Protection Council, Farnham, Surrey.
18. British Crop Protection Council (BCPC). (1997): Carbofuran. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 186–188. British Crop Protection Council, Farnham, Surrey.
19. British Crop Protection Council (BCPC). (1997): Chlorfenapyr. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 209–211. British Crop Protection Council, Farnham, Surrey.
20. British Crop Protection Council (BCPC). (1997): Chlorothalonil. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 227–229. British Crop Protection Council, Farnham, Surrey.
21. British Crop Protection Council (BCPC). (1997): Chlorpyrifos. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 235–237. British Crop Protection Council, Farnham, Surrey.
22. British Crop Protection Council (BCPC). (1997): Clodinafop-propargyl. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 251–253. British Crop Protection Council, Farnham, Surrey.
23. British Crop Protection Council (BCPC). (1997): Copper Hydroxide. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, p. 268. British Crop Protection Council, Farnham, Surrey.
24. British Crop Protection Council (BCPC). (1997): Copper oxychloride. In: *A World Compendium: The Pesticide Manual*,

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_639.html[2010/10/28 18:46:19]

11th ed., edited by C.D.S.Tomlin, pp. 269–270. British Crop Protection Council, Farnham, Surrey.
25. British Crop Protection Council (BCPC). (1997): Copper sulfate. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 270–272. British Crop Protection Council, Farnham, Surrey.
26. British Crop Protection Council (BCPC). (1997): Cyproconazole. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 317–318. British Crop Protection Council, Farnham, Surrey.
27. British Crop Protection Council (BCPC). (1997): Cyprodinil. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C. D.S.Tomlin, pp. 319–321. British Crop Protection Council, Farnham, Surrey.
28. British Crop Protection Council (BCPC). (1997): Cyromazine. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 321–322. British Crop Protection Council, Farnham, Surrey.
29. British Crop Protection Council (BCPC). (1997): Diazinon. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 354–356. British Crop Protection Council, Farnham, Surrey.
30. British Crop Protection Council (BCPC). (1997): Dichlorvos. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C. D.S.Tomlin, pp. 849–852. British Crop Protection Council, Farnham, Surrey.
31. British Crop Protection Council (BCPC). (1997): Difenoconazole. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 389–390. British Crop Protection Council, Farnham, Surrey.
32. British Crop Protection Council (BCPC). (1997): Diofenolan. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C. D.S.Tomlin, pp. 430–431. British Crop Protection Council, Farnham, Surrey.
33. British Crop Protection Council (BCPC). (1997): Endosulfan. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C. D.S.Tomlin, pp. 459–461. British Crop Protection Council, Farnham, Surrey.
34. British Crop Protection Council (BCPC). (1997): Fenbuconazole. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 508–509. British Crop Protection Council, Farnham, Surrey.
35. British Crop Protection Council (BCPC). (1997): Fenpiclonil. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C. D.S.Tomlin, pp. 522–523. British Crop Protection Council, Farnham, Surrey.
36. British Crop Protection Council (BCPC). (1997): Fenpropathrin. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 524–525. British Crop Protection Council, Farnham, Surrey.
37. British Crop Protection Council (BCPC). (1997): Fentin. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 533–537. British Crop Protection Council, Farnham, Surrey.
38. British Crop Protection Council (BCPC). (1997): Fenvalerate. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 539–541. British Crop Protection Council, Farnham, Surrey.
39. British Crop Protection Council (BCPC). (1997): Ferbam. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 541–543. British Crop Protection Council, Farnham, Surrey.
40. British Crop Protection Council (BCPC). (1997): Fipronil. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 545–547. British Crop Protection Council, Farnham, Surrey.
41. British Crop Protection Council (BCPC). (1997): Fluazifop-P-butyl. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 547–549. British Crop Protection Council, Farnham, Surrey.
42. British Crop Protection Council (BCPC). (1997): Fludioxonil. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 566–568. British Crop Protection Council, Farnham, Surrey.
43. British Crop Protection Council (BCPC). (1997): Flutolanil. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 608–609. British Crop Protection Council, Farnham,

Surrey.
44. British Crop Protection Council (BCPC). (1997): Gamma-HCH. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 664–666. British Crop Protection Council, Farnham, Surrey.
45. British Crop Protection Council (BCPC). (1997): Halosulfuron-methyl. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 657–659. British Crop Protection Council, Farnham, Surrey.
46. British Crop Protection Council (BCPC). (1997): Hexaconazole. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 674–675. British Crop Protection Council, Farnham, Surrey.
47. British Crop Protection Council (BCPC). (1997): Hydramethylnon. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 681–683. British Crop Protection Council, Farnham, Surrey.
48. British Crop Protection Council (BCPC). (1997): Imazalil. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 691–694. British Crop Protection Council, Farnham, Surrey.
49. British Crop Protection Council (BCPC). (1997): Imazosulfuron. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 703–706. British Crop Protection Council, Farnham, Surrey.
50. British Crop Protection Council (BCPC). (1997): Imidacloprid. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 706–708. British Crop Protection Council, Farnham, Surrey.
51. British Crop Protection Council (BCPC). (1997): Iprodione. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 724–726. British Crop Protection Council, Farnham, Surrey.
52. British Crop Protection Council (BCPC). (1997): Lambda-cyhalothrin. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 300–302. British Crop Protection Council, Farnham, Surrey.
53. British Crop Protection Council. (1997): Mancozeb. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D. S.Tomlin, pp. 761–763. British Crop Protection Council, Farnham, Surrey.
54. British Crop Protection Council. (1997): Maneb. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D. S.Tomlin, pp. 764–766. British Crop Protection Council, Farnham, Surrey.
55. British Crop Protection Council (BCPC). (1997): Metalaxyl-M. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 794–795. British Crop Protection Council, Farnham, Surrey.
56. British Crop Protection Council (BCPC). (1997): Methomyl. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by

C.D.S.Tomlin, pp. 815–817. British Crop Protection Council, Farnham, Surrey.
57. British Crop Protection Council (BCPC). (1997): Monocrotophos. In: *A World Compendium: The Pesticide Manual*. 11th ed., edited by C.D.S.Tomlin, pp. 849–852. British Crop Protection Council, Farnham, Surrey.
58. British Crop Protection Council (BCPC). (1997): Myclobutanil. In: *A World Compendium: The Pesticide Manual.*, 11th Ed., edited by C.D.S.Tomlin, pp. 854–855. British Crop Protection Council, Farnham, Surrey.
59. British Crop Protection Council (BCPC). (1997): Nicotine. *A World Compendium: The Pesticide Manual.*, 11th ed., edited by C.D.S.Tomlin, pp. 879–880. British Crop Protection Council, Farnham, Surrey.
60. British Crop Protection Council (BCPC). (1997): Oxasulfuron. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 911–914. British Crop Protection Council, Farnham, Surrey.
61. British Crop Protection Council (BCPC). (1997): Permethrin. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C. D.S.Tomlin, pp. 944–946. British Crop Protection Council, Farnham, Surrey.
62. British Crop Protection Council (BCPC). (1997): Prochloraz. In: *A World Compendium: The Pesticide Manual*. 11th ed., edited by C. D.S.Tomlin, pp. 1000–1002. British Crop Protection Council, Farnham, Surrey.
63. British Crop Protection Council (BCPC). (1997). Propaquizafop. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 1021–1024. British Crop Protection Council, Farnham, Surrey.
64. British Crop Protection Council (BCPC). (1997): Propiconazole. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 1030–1032. British Crop Protection Council, Farnham, Surrey.
65. British Crop Protection Council (BCPC). (1997): Propoxur. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 1036–1039. British Crop Protection Council, Farnham, Surrey.
66. British Crop Protection Council (BCPC). (1997): Prosulfuron. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 1041–1043. British Crop Protection Council, Farnham, Surrey.
67. British Crop Protection Council (BCPC). (1997): Pymetrozine. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 1045–1046. British Crop Protection Council, Farnham, Surrey.
68. British Crop Protection Council (BCPC). (1997): Pyrimethanil. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 1068–1069. British Crop Protection Council, Farnham, Surrey.
69. British Crop Protection Council (BCPC). (1997): Pyriminobac-methyl. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 1071–1072. British Crop Protection Council, Farnham, Surrey.
70. British Crop Protection Council (BCPC). (1997): Pyriproxyfen. In: *A World Compendium: The Pesticide Manual*, 11th ed., C.D.S. Tomlin, pp. 1072–1073. British Crop Protection Council, Farnham, Surrey.
71. British Crop Protection Council (BCPC). (1997): Rimsulfuron. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 1095–1097. British Crop Protection Council, Farnham, Surrey.
72. British Crop Protection Council (BCPC). (1997): Sulcotrione. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C. D.S.Tomlin, pp. 1124–1125. British Crop Protection Council, Farnham, Surrey.
73. British Crop Protection Council (BCPC). (1997): Sulfosulfuron. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 1130–1131. British Crop Protection Council, Farnham, Surrey.
74. British Crop Protection Council (BCPC). (1997): Sulfur. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 1133–1134. British Crop Protection Council, Farnham, Surrey.
75. British Crop Protection Council (BCPC). (1997): Tebuconazole. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 1144–1146. British Crop Protection Council,

Farnham, Surrey.
76. British Crop Protection Council (BCPC). (1997): Teflubenzuron. In: *A World Compendium: The Pesticide Manual* 11th ed., edited by C.D.S.Tomlin, pp. 1158–1159. British Crop Protection Council, Farnham, Surrey.
77. British Crop Protection Council (BCPC). (1997): Thiabendazole. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 1183–1185. British Crop Protection Council, Farnham, Surrey.
78. British Crop Protection Council (BCPC). (1997): Thiophanate-methyl. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 1201–1203. British Crop Protection Council, Farnham, Surrey.
79. British Crop Protection Council (BCPC). (1997): Thiram. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 1203–1205. British Crop Protection Council, Farnham, Surrey.
80. British Crop Protection Council (BCPC). (1997): Triadimefon. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 1216–1218. British Crop Protection Council, Farnham, Surrey.
81. British Crop Protection Council (BCPC). (1997): Triadimenol. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 1218–1220. British Crop Protection Council, Farnham, Surrey.
82. British Crop Protection Council (BCPC). (1997): Trichlopyr. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C. D.S.Tomlin, pp. 1237–1239. British Crop Protection Council, Farnham, Surrey.
83. British Crop Protection Council (BCPC). (1997): Vinclozolin. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C. D.S.Tomlin, pp. 1267–1268. British Crop Protection Council, Farnham, Surrey.
84. British Crop Protection Council (BCPC). (1997): XDE-105 (Spinosad). In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 1272–1273. British Crop Protection Council, Farnham, Surrey.
85. British Crop Protection Council (BCPC). (1997): Zineb. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 1276–1277. British Crop Protection Council, Farnham, Surrey.
86. British Crop Protection Council (BCPC). (1997): Ziram. In: *A World Compendium: The Pesticide Manual*, 11th ed., edited by C.D.S.Tomlin, pp. 1277–1279. British Crop Protection Council, Farnham, Surrey.
87. British Industrial Biological Research Association (BIBRA). (1988): *Screening for Safety: Pesticides*, p. 3. Liebling Stewart Design Associates, London.
88. Clark, J.M., Scott, J.G., Campos, F., and Bloomquist, J.R. (1995): Resistance to avermectins: Extent, mechanisms, and management implications. *Annu. Rev. Entomol.*, 40:1–30.

89. Conner, J.D., Jr., Ebner, L.S., Landfair, S.W., O'Connor, C. III, Weinstein, K.W., and Jovanovich, A.P. (1991): *Pesticide Regulations Handbook*, 3rd ed., p. 1. Executive Enterprises, New York.
90. Cremlyn, R. (1978): *Pesticides. Preparation and Mode of Action*. John Wiley and Sons, New York.
91. Delp, C.P. (1985): *Fungicide Resistance in North America*, edited by C.P.Delp, pp. 1–20. American Phytopathology Society Press, St. Paul, MN.
92. Duke, S.O., and Kenyon, W.H. (1988): Polycyclic alkanoic acids. In: *Herbicides; Chemistry, Degradation and Mode of Action*, edited by P.C.Kearney and D.D.Kaufman, pp. 71–116. Marcel Dekker, New York.
93. Ecobichon, D.J. (1993): Toxic effects of pesticides. In: *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 4th ed., edited by J.Doull, C.D.Klaassen, and M.O.Amdur, pp. 565–621. Macmillan, New York.
94. Eldridge, J.C., Stevens, J.T., Wetzel, L.T., Tisdel, M.O., Breckenridge, C.B., McConnell, R.F., and Simpkins, J.W. (1996): Atrazine: Mechanisms of hormonal imbalance in female SD rats. *Fundam. Appl. Toxicol.* 24(12):2–5.
95. European Economic Community (ECC). (1993): Commission Directive 93/67/EEC: 1993. Laying Down the Principles for Assessment of Risks to Man and the Environment of Substances Notified in accordance with Council Directive 67/548/EEC. July 20, 1993.
96. European Economic Community (EEC). (1994): Commission Directive 94/79/EC of December 1994 amending Council Directive 91/414/EEC concerning the placing of plant protection products on the market. *Off. J. Euro. Communities*, December 31:L354/16.
97. Farber, T.M. (1987): Pesticide assessment guidelines, Subdivision F. Position Document: Selection of a Maximum Tolerated Dose (MTD) in oncogenicity studies. Toxicology Branch, Hazard Evaluation Division, Office of Pesticides Programs, U.S. Environmental Protection Agency, Washington, DC. NTIS PB88–116736.
98. Gianessi, L.P. (1986): *A National Pesticide Useage Data Base*, pp. 1–14. Resources for the Future, Washington, DC.
99. Gill, S.S., Cowles, E.A., and Pietrantonio, P.V. (1992): The mode of action of *Bacillus thuringiensis* endotoxins. *Ann. Rev. Entomol.*, 37:615–36.
100. Goldman, L.R. (1998): Chemicals and children's environment: What we don't know about risk. *Environ. Health Perspect.*, 106(suppl. 3):875–880.
101. Gunsolus, G.L., and Curran, W.S. (1999): Herbicide Mode of Action and Injury Symptoms. http://www.mes.umn.edu/Documents/D/C/DC3832.htm (Accessed 6/99).
102. Hayes, W.J., Jr. (1991): Introduction. In *Handbook of Pesticide Toxicology. Volume 1. General Principles*, pp. 1–37. Academic Press, San Diego.
103. Hollingshaus, J. (1987): Inhibition of mitochondrial electron transport by hydramethylnon: A new amidinohyrazone insecticide. *Pestic. Biochem. Physiol.*, 27:61–70.
104. Hollingworth, R.Ahmmadsahib, K.Gedelhak, G., and McLaughlin, J. (1994): New inhibitors of complex I of the mitochondrial electron transport chain with activity as pesticides. *Biochem. Soc. Trans. (Lond.)*, 22:230–233.
105. International Agency for Research on Cancer (IARC). (1998): Monographs working group—Volume 73: Evaluation or re-evaluation of some agents which target specific organs in rodent bioassays (in preparation). http://193.51.164.11/past&future/OCT98.html (Accessed 11/98).
106. Joint Expert Committee on Food Additives (JECFA). (1997): Tiabendazole (Thiabendazole). *JECFA Monograph Series 31*. World Health Organization, WHO/JECMONO.31.4, pp. 1–23.
107. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues in Food (JMPR). (1974): Carbaryl. FAO Agricultural Studies., No. 92: WHO Technical Report Series, No. 545, 1974. 1973 Evaluation of Some Pesticide Residues in Food. FAO/AGP/1974/M/11.
108. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues (JMPR). (1983): Carbofuran. FAO Plant Production and Protection Paper 46. 1982. FAO Plant Production and Protection Paper 46.
109. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues (JMPR). (1983): Chlorpyrifos. Pesticide Residues in Food—1982 Evaluations. FAO Plant Production and Protection Paper 46.
110. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues (JMPR). (1985): Prochloraz. FAO Plant Production and Protection Paper 56. 1984. Pesticide residues in food—1984 Evaluations. FAO Plant Production and Protection Paper 61.
111. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues (JMPR). (1985): Triadimefon. FAO Plant Production and Protection Paper 68, Pesticide Residues in Food—1985 evaluations; Part II—Toxicology. FAO Plant Production and Protection Paper 72/2.

112. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues (JMPR). (1989): Triadimenol. FAO Plant Production and Protection Paper 99. Pesticide Residues in Food—1989 Evaluations; Part II—Toxicology. FAO Plant Production and Protection Paper 100/2.
113. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues in Food (JMPR). (1990): Acephate. FAO Plant Production and Protection Paper 103. Pesticide Residues in Food—1990 Evaluations; Part II—Toxicology. World Health Organisation, WHO/PCS/92.47.
114. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues (JMPR). (1990): Endosulfan. FAO Plant Production and Protection Paper 99. Pesticide Residues in Food—1989 Evaluations; Part II—Toxicology. FAO Plant Production and Protection Paper 100/2.
115. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues (JMPR). (1990): Lindane. FAO Plant Production and Protection Paper 99. Pesticide Residues in Food—1989 Evaluations; Part II—Toxicology. FAO Plant Production and Protection Paper 100/2.
116. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues (JMPR). (1990): Methomyl. FAO Plant Production and Protection Paper 99. Pesticide Residues in Food—1989 Evaluations; Part II—Toxicology. FAO Plant Production and Protection Paper 100/2.
117. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues (JMPR). (1990): Propoxur. FAO Plant Production and Protection Paper 99. Pesticide Residues in Food—1989 Evaluations; Part II—Toxicology. FAO Plant Production and Protection Paper 100/2.
118. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues in Food (JMPR). (1991): Azinphos-methyl. FAO Plant Production and Protection Paper 111. Pesticide Residues in Food—1991 Evaluations; Part II—Toxicology. http://www.inchem.org/documents/jmpr/jmpmonno/v91pr02.htm (Accessed 6/99).
119. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues (JMPR): (1992) Imazalil. FAO Plant Production and Protection Paper 111. Pesticide Residues in Food—1991 Evaluations; Part II—Toxicology & Environment. World Health Organization, WHO/PCS/92.52.
120. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues in Food (JMPR). (1993): Chlorothalonil. FAO Plant Pro-

duction and Protection Paper 116. Pesticide Residues in Food—1992 Evaluations; Part II—Toxicology. World Health Organization, WHO/PCS/93.34.
121. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues in Food (JMPR). (1993): Thiram. FAO Plant Production and Protection Paper 116. Pesticide Residues in Food—1992 Evaluations; Part II—Toxicology. World Health Organization, WHO/PCS/93.34.
122. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues (JMPR). (1994): Abamectin. Pesticide Residues in Food—1993. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 127, pp. 15–17.
123. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues in Food (JMPR). (1994): Diazinon. Pesticide Residues in Food—1993 Evaluations; Part II—Toxicology. FAO Plant Production and Protection Paper 122. World Health Organization, WHO/PCS/94.4.
124. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues in Food (JMPR). (1994): Dichlorvos. FAO Plant Production and Protection Paper 122. Pesticide Residues in Food—1993 Evaluations; Part II—Toxicology. World Health Organization, WHO/PCS/94.4.
125. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues (JMPR). (1994): Fenpropathrin. FAO Plant Production and Protection Paper 122. Pesticide Residues in Food—1993 Evaluations; Part II—Toxicology. WHO/PCS/94.4.
126. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues in Food (JMPR). (1994): Mancozeb. FAO Plant Production and Protection Paper 122. Pesticide Residues in Food—1993 Evaluations; Part II—Toxicology. World Health Organization, WHO/PCS/94.4.
127. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues in Food (JMPR). (1994): Maneb. FAO Plant Production and Protection Paper 122. Pesticide Residues in Food—1993 Evaluations; Part II—Toxicology. World Health Organization, WHO/PCS/94.4.
128. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues (JMPR). (1994): Permethrin. FAO Plant Production and Protection Paper 122. Pesticide Residues in Food—1993 Evaluations; Part II—Toxicology. WHO/PCS/94.4.
129. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues in Food (JMPR). (1994) Zineb. FAO Plant Production and Protection Paper 122. Pesticide Residues in Food—1993 Evaluations; Part II—Toxicology. World Health Organization, WHO/PCS/94.4.
130. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues in Food (JMPR). (1995): Monocrotophos. FAO Plant Production and Protection Paper 133. Pesticide Residues in Food—1994 Evaluations; Part II—Toxicology & Environment. World Health Organization, WHO/PCS/96.48.
131. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues in Food (JMPR). (1996): Aldicarb. FAO Plant Production and Protection Paper 133. Pesticide Residues in Food—1995 Evaluations; Part II—Toxicology & Environment. World Health Organization, WHO/PCS/96.48.
132. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues in Food (JMPR). (1996): Benomyl. FAO Plant Production and Protection Paper 133. Pesticide Residues in Food—1995 Evaluations; Part II—Toxicology & Environment. World Health Organization, WHO/PCS/96.48, pp. 3–32.
133. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues in Food (JMPR). (1996): Captan. FAO Plant Production and Protection Paper 133. Pesticide Residues in Food—1995 Evaluations; Part II—Toxicology & Environment. World Health Organization, WHO/PCS/96.48.
134. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues in Food (JMPR). (1996): Iprodione (addendum). FAO Plant Production and Protection Paper 133. Pesticide Residues in Food—1995 Evaluations; Part II—Toxicology & Environment. World Health Organization, WHO/PCS/96.48, pp. 231–237.
135. Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food (JMPR). (1996): Thiophanate-methyl. FAO Plant Production and Protection Paper 133. Pesticide Residues in Food—1995 Evaluations; Part II—Toxicology & Environment. World Health Organization, WHO/PCS/96.48, pp. 351–374.
136. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues (JMPR). (1996): Vinclozolin. FAO Plant Production and Protection Paper 133. Pesticide Residues in Food—1995 Evaluations; Part II—Toxicology & Environment. World Health Organization, WHO/PCS/96.48, pp. 375–404.
137. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues in Food (JMPR). (1997): Ferbam. FAO Plant Production and Protection Paper 140. Pesticide Residues in Food—1996 Evaluations; Part II—Toxicology & Environment. World Health Organization, WHO/PCS/97.06.
138. Joint Meeting of the FAO/WHO Panel of Experts on Pesticide Residues in Food (JMPR). (1997):

Ziram. FAO Plant Production and Protection Paper 140. Pesticide Residues in Food—1995 Evaluations; Part II—Toxicology & Environment. World Health Organization, WHO/PCS/97.12.
139. Kaufman, S. (1997): Fungicides. In: *SRI International's Chemical Economics Handbook*, pp. 1–144. SRI Press, Menlo Park, CA.
140. Kelce, W.R., Monosson, E., Gamcsik, M.P., Laws, S.C., and Gray, L.E., Jr. (1994): Environmental hormone disruptors: Evidence that vinclozolin developmental toxicity is mediated by antiandrogenic metabolites. *Toxicol. Appl. Pharmacol.*, 126:276–285.
141. Lankas, G.R., and Gordon, L.R. (1989): Toxicology. In: *Ivermectin and Abamectin*, edited by W.R.Campbell, pp. 89–112. Springer-Verlag, New York.
142. Lankas, G.R., Minsker, D.H., and Robertson, R.T. (1989): Effects of ivermectin on reproduction and neonatal toxicity in rats. *Food Chem. Toxicol.*, 27:523–529.
143. Lankas, G.R., Cartwright, M.E., and Umbenhauer, D. (1997): P-glycoprotein deficiency in a subpopulation of CF-1 mice enhances avermectin-induced neurotoxicity. *Toxicol. Appl. Pharmacol.*, 143:357–365.
144. Larson, L.L. (1999): Novel organic and natural product insect management tools. *National IPM Network*. hppt://ipmworld.umn.edu/chapters/larson.htm (Accessed 1/99).
145. Matringe, M., Clair, D., and Scala, R. (1990): Effects of peroxidizing herbicides on protoporphyrin IX levels in non-chlorophyllous soybean cell culture. *Pestic. Biochem. Physiol.*, 36:300–307.
146. McGaughy, R. (1986): Guidelines for carcinogen risk assessment. *Fed. Reg.*, 51(185):33992–34003.
147. Meister, R.T. (1997): Pheromone. In: *Farm Chemical Handbook '97*, p. C286. Meister, Willoughby, OH.
148. Mellanby, K. (1992): *The DDT Story*, pp.6–7. British Crop Protection Council, Farnham, Surrey.
149. Ministry of Agriculture, Forestry and Fisheries (MAFF). (1985): Notification of the Director-General. Requirements for Safety Evaluation of Agricultural Chemicals, Agricultural Production Bureau, Ministry of Agriculture, Forestry and Fisheries, Japan, 59 NohSan No. 4200, January 28.
150. Moolenaar, R.J. (1994): Default assumptions in carcinogenic risk assessment used by regulatory agencies. *Regul. Toxicol. Pharmacol.*, 20:S135–S141.

151. Morishima, Y., Osabe, H., and Goto, Y. (1990): Action mechanism of DLH-1777, a novel 4-pyridone-3-carboxamide herbicide: Peroxidizing activity and accumulation of porphyrins. *J. Pestic. Sci.*, 15:553–559.
152. Mullins, J.W. (1993): Imidacloprid: A new nitroguanidine insecticide. ACS Symp. Ser. 524: Newer Pest Control Agents and Technology with Reduced Environmental Impact. ACS, Washington, DC.
153. National Research Council (NRC). (1993): *Pesticides in the Diets of Infants and Children,* National Academy Press, Washington, DC.
154. Nikolau, B.J., Wurtele, E.S., Caffrey, J., Chen, Y., Crane, V., Diez, T., Huang, J.-Y., Mc Dowell, M.T., Shang, X.-M., Song, J., Wang, X. and Weaver, L.M. (1993): The biochemistry and molecular biology of acetyl-CoA carboxylase and other biotin enzymes. In: *Biochemistry and Molecular Biology of Membrane and Storage Lipids of Plants,* edited by N.Murata and C. Somerville, pp. 138–149. American Society of Plant Physiologists Press. Brentwood, TN.
155. Organization for Economic Cooperation and Development. (1981): OECD Guideline for Testing of Chemicals, Section 4, Health Effects, adopted May 12, 1981. OECD. Paris, France.
156. Retzinger, J.E., Jr., and Mallory-Smith, C. (1999): Classification of Herbicides by Site of Action for Weed Resistance Management Strategies. http://www.css.orst.edu/weeds/Publications/site_action.htm (Accessed 5/99).
157. Simmons, S.W. (1959): The use of DDT insecticides in human medicine. In: *DDT: The Insecticide Dichlorodiphenyl-Trichloroethane and Its Significance,* edited by P.Miller, Vol. 2, pp. 251–502. Birkhaeriger, Basel.
158. Spindler, M. (1990): DDT: Health aspects in relation to man and risk/benefit assessment based thereupon. *Residue Rev.,* 90:1–34.
159. Stetter, J. (1993): Trends in the future development of pest and weed control—An industrial point of view. *Regul. Toxicol. Pharmacol.,* 17:346–370.
160. Stevens, J.T. (1994): Aspects related to uses of organic and inorganic chlorine compounds: Pesticides. In: *Toxicology Forum. Chlorinated Organic Chemicals. Their Effects on Human Health and the Environment*, pp. 567–580. Berlin.
161. Stevens, J.T., Wetzel, L.T., Breckenridge, C.B., Gillis, J.H., Luempert, L.G. III and Eldridge, J.C. (1994): Hypothesis for mammary tumorigenesis in female Sprague-Dawley rats exposed to chloro-s-triazine herbicides. *J. Toxicol. Environ. Health*, 43(2): 139–154.
162. Stevens, J.T. (1997): Risk assessment of pesticides. In: *Comprehensive Toxicology. Vol. 2. Toxicological Testing and Evaluation,* edited by I.G.Sipes, C.A.McQueen, and A.J.Gandolfi, pp. 17–26. Elsevier Science, Oxford.
163. Stevens, J.T., Sumner, D.D., and Luempert, L. (1995): Agricultural chemicals: The impact of regulations under FIFRA on science and economics. In: *Primer on Regulatory Toxicology,* edited by C. Chenzelis, J.Holson, and S.Gad, pp. 133–163. Raven Press, New York.
164. Stevens, J.T., Breckenridge, C.B., Wetzel, L.T., Thakur, A.K., Liu, C., Werner, C., Luempert, L.C. III, and Eldridge, J.C. (1999). A risk characterization for atrazine: Oncogenicity profile. *J. Toxicol. Environ. Health, A,* 56:69–109.
165. Stevens, J.T., Werner, C., Breckenridge, C.B., and Sumner, D.D. (1999): Hazard assessment for selected symmetrical and asymmetrical triazine herbicide. In: *The Triazine Herbicides,* edited by H.M.LeBaron, J.McFarland, O.Burnside, and R.Clark. In press.
166. Sumner, D.D., and Stevens, J.T. (1994): Pharmacokinetic factors influencing risk assessment: Saturation of biochemical processes and co-factor depletion. In: *Pharmacokinetics: Defining Dosimetry for Risk Assessment. Environ. Health Perspect.,* 102(suppl. 11):13–22.
167. Turner, M.J., and Schaeffer, J.M. (1989): Chapter 5: Mode of Action of Ivermectin. In: *Ivermectin and Abamectin*, edited by W.R.Campbell, pp. 73–88. Springer-Verlag, New York.
168. U.K. Department of Health. (1991): Guidelines for the Evaluation of Chemicals for Carcinogenicity, p. 1. Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment, UK Department of Health, London.
169. U.S.Congress. (1947): Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). Pub. L. No. 80–104, 61 Stat. 163, 1947, p.1.
170. U.S. Congress. (1958): Food additive amendments to the Federal Food, Drug, and Cosmetic Act (FFDCA) 409. Pub. L. No. 85–929, 72 Stat., 1785, 1958, p. 1.
171. U.S. Environmental Protection Agency (EPA). (1982): Pesticide Assessment Guidelines, Subdivision F.Hazard Evaluation: Human and Domestic Animals. Environmental Protection Agency, 540/9–82–025. Available from NTIS, Springfield, VA.

172. U.S. Environmental Protection Agency (EPA). (1983): Aldicarb. CASRN 116–06–3. Integrated Risk Information System. http://www.epa.gov/iris/subst/0003.htm (Accessed 3/99).
173. U.S. Environmental Protection Agency (EPA). (1987): Benomyl. CASRN 17804–35–2. Integrated Risk Information System. http://www.epa.gov.ngispgm3/Iris/subst/0011.htm (Accessed 3/99).
174. U.S. Environmental Protection Agency (EPA). (1988): Iprodione. CASRN 36734–19–7. Integrated Risk Information System. http://www.epa.gov.ngispgm3/Iris/subst./0291.htm (Accessed 3/99).
175. U.S. Environmental Protection Agency (EPA). (1994): Acetochlor; Pesticide tolerance. *Fed. Reg.*, 59(56): 13654–13558.
176. U.S. Environmental Protection Agency (EPA). (1994): Atrazine, simazine and cyanazine; Notice of initiation of special review. http://www.epa.gov/fedrgstr/EPA-PEST/1994/November/Day-23/pr-54.html (accessed 3/99).
177. U.S. Environmental Protection Agency (EPA). (1995): Cyproconazole; Pesticide tolerance. *Fed. Reg.*, 60(153):40545–40548.
178. U.S. Environmental Protection Agency (EPA). (1995): Fenbuconazole: Pesticide tolerances. *Fed. Reg.*, 60(100):27419–27421.
179. U.S. Environmental Protection Agency (EPA). (1995): Lepidopteran pheromones: Tolerance exemption. Final rule. *Fed. Reg.*, 60(168):45060–45062.
180. U.S. Environmental Protection Agency (EPA). (1995): Metalaxyl: Pesticide tolerance. *Fed. Reg.*, 60(244):65579–65581.
181. U.S. Environmental Protection Agency (EPA). (1995): Monosodium methanearsonate and disodium methanearsonate; Toxic chemical release reporting; Community right to know. *Fed. Reg.* http://www.epa.gov?fedrgstr/EPA-TRI/April/Day-20/pr13.html (Accessed 6/99).
182. U.S. Environmental Protection Agency (EPA). (1995): Oxyfluorfen: Pesticide tolerance. *Fed. Reg.*, 60(187):49816–49818.
183. U.S. Environmental Protection Agency (EPA). (1995): Paraquat: Pesticide tolerance. *Fed. Reg.*, http://www.epa.gov/fedrgstr/EPAPest/1995/March/Day-15/pr-178.html (Accessed 6/99).
184. U.S. Environmental Protection Agency (EPA). (1996): Dimethenamid. Pesticide tolerance petition: Notice of filing. *Fed. Reg.*, 61(62): 10681–10684.
185. U.S. Environmental Protection Agency (EPA). (1996): Diquat. Pesticide tolerance. *Fed. Reg.*, 61(60):13474–13476.
186. U.S. Environmental Protection Agency (EPA). (1996): Flutolanil. Pesticide tolerance. *Fed. Reg.*, 61(124):33041–33044.

187. U.S. Environmental Protection Agency (EPA). (1996): Glufosinate-ammonium. Pesticide tolerance petition: Notice of filing. *Fed. Reg.*, 61(223):58684–58688.
187a. U.S. Environmental Protection Agency (EPA). (1996): Hexaconazole: Pesticide tolerance. *Fed. Reg.*, 61(70):15895–15896.
188. U.S. Environmental Protection Agency (EPA). (1996): Imazameth. Pesticide tolerance for cadre. *Fed. Reg.*, 61(55):11311–11313.
189. U.S. Environmental Protection Agency (EPA). (1996): Lactofen: Pesticide tolerance. *Fed. Reg.*, 61(47):9399–9401.
190. U.S. Environmental Protection Agency (EPA). (1996): Office of Pesticide Programs Reference Dose Tracking Report, pp. 1–77. Office of Prevention, Pesticides, and Toxic Substances.
191. U.S. Environmental Protection Agency (EPA). (1996): Propiconazole: Pesticide tolerances for emergency exemptions. *Fed. Reg.*, 61(220):58135–58140.
192. U.S. Environmental Protection Agency (EPA). (1996): Proposed Guidelines for Carcinogen Risk Assessment. Office of Research and Development. Washington, DC. EPA/600/p-92/003c.
193. U.S. Environmental Protection Agency (EPA). (1996): Spinosad; Pesticide tolerance petition. Notice of filing. *Fed. Reg.*, 61(227): 59437–59440.
194. U.S. Environmental Protection Agency (EPA). (1996): Triadimefon: Pesticide tolerances for emergency exemptions. *Fed. Reg.*, 61(232):63726–63726.
195. U.S. Environmental Protection Agency (EPA). (1997): Acifluorfen. Notice of filing of pesticide petitions. *Fed. Reg.*, 62(143):39967–39974.
196. U.S. Environmental Protection Agency (EPA). (1997): Azoxystrobin. Notice of filing of pesticide petitions. *Fed. Reg.*, 62(48): 11441–11447.
197. U.S. Environmental Protection Agency (EPA). (1997): *Bacillus thuringiensis* subspecies tolworthi Cry9C. Notice of filing of pesticide petitions. *Fed. Reg.*, 62(182):49224–49226.
198. U.S. Environmental Protection Agency (EPA). (1997): BASF Monsanto and Dekalb Genetics Corporation; Receipt of petition for determination of nonregulated status for genetically engineered corn. *Fed. Reg.*, 62(156):43311–43312.
199. U.S. Environmental Protection Agency (EPA). (1997): CGA329351. Notice of filing of pesticide petitions. *Fed. Reg.*, 62(143):40080–40086.
200. U.S. Environmental Protection Agency (EPA). (1997): Chlorfenapyr. American Cyanamid Company. Pesticide tolerance petition filing. *Fed. Reg.*, 62(24):5399–5403.
201. U.S. Environmental Protection Agency (EPA). (1997): Chorothalonil; ISK Biosciences Corporation; Pesticide tolerance petition filing. *Fed. Reg.*, 62(63): 15700–15704.
202. U.S. Environmental Protection Agency (EPA). (1997): Clopyralid. Pesticide tolerance for emergency exemption. *Fed. Reg.*, 62(48): 11360–11364.
203. U.S. Environmental Protection Agency (EPA). (1997): Cloransulam-methyl. Pesticide tolerance for emergency exemption. *Fed. Reg.*, 62(48): 11360–11364.
204. U.S. Environmental Protection Agency (EPA). (1997): Difenoconazole. Notice of filing of pesticide petitions. *Fed. Reg.*, 62(143):40075–40080.
205. U.S. Environmental Protection Agency (EPA). (1997): Diuron. Drexel Chemical Company; Pesticide tolerance petition filing. *Fed. Reg.*, 62(16):3685–3688.
206. U.S. Environmental Protection Agency (EPA). (1997): Fenoxaprop-ethyl; Notice of filing of pesticide petitions. *Fed. Reg.*, 62(180):48837–48842.
207. U.S. Environmental Protection Agency (EPA). (1997): Fenpropathrin. Pesticide tolerances for emergency exemptions. *Fed. Reg.*, 62(134):37516–37522.
208. U.S. Environmental Protection Agency (EPA). (1997): Fenvalerate; Pesticide tolerances. *Fed. Reg.*, 62(228):63019–63037.
209. U.S. Environmental Protection Agency (EPA). (1997): Fipronil. Notice of filing of pesticide petitions. *Fed. Reg.*, 62(119): 33641–33647.
210. U.S. Environmental Protection Agency (EPA). (1997): Fludioxonil: Pesticide tolerance petition filing. *Fed. Reg.*, 62(24): 5403–5406.
211. U.S. Environmental Protection Agency (EPA). (1997): Fluthiacet-methyl: Pesticide tolerance petition filing. *Fed. Reg.*, 63(193):53660–53662.
212. U.S. Environmental Protection Agency (EPA). (1997): Fomesafen. Pesticide tolerance for emergency exemption. Final Rule. *Fed. Reg.*, 62(223):61639–61645.
213. U.S. Environmental Protection Agency (EPA). (1997): Imazamox. Pesticide tolerance. Final rule. *Fed, Reg.*, 62(105):29669–29673.

214. U.S. Environmental Protection Agency (EPA). (1997): Imidacloprid. Bayer Corporation: Pesticide tolerance petition filing. *Fed. Reg.*, 62(38):8734–8734.
215. U.S. Environmental Protection Agency (EPA). (1997): Isoxaflutole; Pesticide tolerance petition filing. *Fed. Reg*,, 62(38):8737–8740.
216. U.S. Environmental Protection Agency (EPA). (1997): Mancozeb, maneb, and ethylenethiourea tolerances. Notice of filing of pesticide petitions. *Fed. Reg.*, 62(148):41383–41386.
217. U.S. Environmental Protection Agency (EPA). (1997): Maneb: Pesticide tolerances for emergency exemptions. *Fed. Reg.*, 62(185):49918–49925.
218. U.S. Environmental Protection Agency (EPA). (1997): Mefenoxam: Pesticide tolerance for emergency exemptions. *Fed. Reg.*, 62(149):42019–42030.
219. U.S. Environmental Protection Agency (EPA). (1997): Myclobutanil: Pesticide tolerance for emergency exemptions. *Fed. Reg.*, 62(6): 1284–1288.
220. U.S. Environmental Protection Agency (EPA). (1997): Norflurazon. BASF Corporation. Pesticide tolerance petition filing. *Fed. Reg.*, 62(58): 14423–14426.
221. U.S. Environmental Protection Agency (EPA). (1997): Pendimethalin: Pesticide tolerance for emergency exemptions. *Fed. Reg.*, 62(100):28355–28361.
222. U.S. Environmental Protection Agency (EPA). (1997): Propazine. Pesticide tolerance petition filing. *Fed. Reg.*, 63(193):53657–53660.
223. U.S. Environmental Protection Agency (EPA). (1997): Pyridaben. BASF Corporation. Pesticide tolerance petition filing. *Fed. Reg.*, 62(48): 11450–11453.
224. U.S. Environmental Protection Agency (EPA). (1997): Pyrimethanil. Pesticide tolerance. *Fed. Reg.*, 62(231):63662–63669.
225. U.S. Environmental Protection Agency (EPA). (1997): Tebuconazole: Pesticide tolerance petition filing. *Fed. Reg.*, 62(43):10047–10050.
226. U.S. Environmental Protection Agency (EPA). (1997): *trans*-11-Tetradecenyl Acetate Technical Pheromone Pesticide Fact sheet. Unconditional Registration. February 1997. http://www.epa.gov/fedrgstr/EPA-PEST/1997/rJune/Day-13/f-pl5562.htm (Accessed 6/99).
227. U.S. Environmental Protection Agency (EPA). (1997): Vinclozolin: Pesticide tolerance petition filing. *Fed. Reg.*, 62(53): 13000–13005.
228. U.S. Environmental Protection Agency (EPA). (1998): Alachlor. Registration Eligibility Decision for Alachlor. http://www.epa.gov/docs/oppsrrd1/REDs/index.html (Accessed 6/99)
229. U.S. Environmental Protection Agency (EPA). (1998): *Bacillus thuringiensis* variety kurstaki. Notice of filing of pesticide petitions. *Fed. Reg.*, 63(67):17174–17176.

230. U.S. Environmental Protection Agency (EPA). (1998): Carfentrazone-ethyl; Pesticide tolerances. *Fed. Reg.*, 63(189):52174–52180.
231. U.S. Environmental Protection Agency (EPA). (1998): Cyprodinil. Novartis Crop Protection Inc., Approval of a pesticide product. *Fed. Reg.*, 63(108):30749–3070.
232. U.S. Environmental Protection Agency (EPA). (1998): Dicamba. Notice of filing of pesticide petitions. *Fed. Reg.*, 63(2240): 64481–64484.
233. U.S. Environmental Protection Agency (EPA). (1998): Diflubenzuron. Notice of filing of pesticide petitions. *Fed. Reg.*, 63(37):9528–9532.
234. U.S. Environmental Protection Agency (EPA). (1998): Diflubenzuron. Temporary pesticide tolerance. *Fed. Reg.*, 63(92): 26481–26488.
235. U.S. Environmental Protection Agency (EPA). (1998): Glyphosate; Pesticide tolerance. Final rule. *Fed. Reg.*, 63(195): 54058–54066
236. U.S. Environmental Protection Agency (EPA). (1998): Halosulfuron-methyl; Pesticide tolerance petition filing. *Fed. Reg.*, 63(103):29401–29409.
237. U.S. Environmental Protection Agency (EPA). (1998): Hexythiazox. Notice of filing of pesticide tolerance. Notice. *Fed. Reg.*, 63(18):4252–4255.
238. U.S. Environmental Protection Agency (EPA). (1998): Hexythiazox. BASF Corporation. Pesticide tolerance petition filing. *Fed. Reg.*, 63(137):38644–38646.
239. U.S. Environmental Protection Agency (EPA). (1998): Hydramethylnon. American Cyanamid Company. Pesticide tolerance petition filing. *Fed. Reg.*, 63(157):43702–43705.
240. U.S. Environmental Protection Agency (EPA). (1998): Isoxaflutole; Pesticide tolerances. *Fed. Reg.*, 63(184):50773–50784.
241. U.S. Environmental Protection Agency (EPA). (1998): Lambda-cyhalothrin; Pesticide tolerances. *Fed. Reg.*, 63(30): 7291–7299.
242. U.S. Environmental Protection Agency (EPA). (1998): Metolachor; Pesticide tolerances for emergency exemptions. *Fed. Reg.*, 63(176):48586–48594.
243. U.S. Environmental Protection Agency (EPA). (1998): Pesticide Assessment Guidelines. http://www.epa.gov/docs/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Series (Accessed 3/99).
244. U.S. Environmental Protection Agency (EPA). (1998): Prometryn; Pesticide tolerances. *Fed. Reg.*, 63(37):9494–9499.
245. U.S. Environmental Protection Agency (EPA). (1998): Pymetrozine. Notice of filing of pesticide petitions. *Fed. Reg.*, 63(97):27723–27727.
246. U.S. Environmental Protection Agency (EPA). (1998): Pymetrozine. Notice of filing of pesticide petitions. *Fed. Reg.*, 63(194):53906–53909.
247. U.S. Environmental Protection Agency (EPA). (1998): Pyridate; Pesticide tolerances. Final rule. *Fed. Reg.*, 63(194):53837–53844.
248. U.S. Environmental Protection Agency (EPA). (1998): Pyriproxyfen; Pesticide tolerances. Final rule. *Fed. Reg.*, 63(128):33366–36373.
249. U.S. Environmental Protection Agency (EPA). (1998): Tebufenozide. Rohm and Haas Company; Notice of filing of pesticide tolerance. Notice. *Fed. Reg.*, 63(160):44439–44456.
250. U.S. Environmental Protection Agency (EPA). (1999): 2,4-D; Time-limited pesticide tolerances. Final rule. *Fed. Reg.*, 64(46):11792–11799.
251. U.S. Environmental Protection Agency (EPA) (1999): Avermectin; Pesticide tolerances for emergency exemptions; Final rule. *Fed. Reg.*, 64(66):16843–16850 (Appendix 3).
252. U.S. Environmental Protection Agency (EPA). (1999): Clofentezine. Pesticide tolerance. Petition filing. *Fed. Reg.*, 64(18):4414–4418.
253. U.S. Environmental Protection Agency (EPA). (1999): Clofentezine. Pesticide tolerance. Petition filing. *Fed. Reg.*, 64(74):19042–19050.
254. U.S. Environmental Protection Agency (EPA). (1999): Clomazone. Pesticide tolerance. Petition filing. *Fed. Reg.*, 64(32):8087–8090.
255. U.S. Environmental Protection Agency (EPA). (1999): Cyromazine; Pesticide tolerances for emergency exemptions. Final rule. *Fed. Reg.*, 62(168):45735–45741.
256. U.S. Environmental Protection Agency (EPA). (1999): Emamectin benzoate; Pesticide tolerance: Final rule. *Fed. Reg.*, 64(96):27192–27200.
257. U.S. Environmental Protection Agency (EPA). (1999): Fludioxonil; Pesticide tolerance for emergency exemptions: Final rule. *Fed. Reg.*, 64(76):19484–19489.

258. U.S. Environmental Protection Agency (EPA). (1999): Fluthiacet-methyl; Pesticide tolerance: Final rule. *Fed. Reg.*, 64(7):18351–18357.
259. U.S. Environmental Protection Agency (EPA). (1999): Picloram. Time-limited pesticide tolerances. Final rule. *Fed. Reg.*, 64(2):418–425.
260. U.S. Environmental Protection Agency (EPA). (1999): Pyriproxyfen; Notice of filing of pesticide petitions. *Fed. Reg.*, 64(34):8638–8641.
261. U.S. Environmental Protection Agency (EPA) (1999): Reregistration Eligibility Document. Amitrole. http://www.epa.gov/docs/oppsrrdl/REDs/0095/html (Accessed June 1999).
262. U.S. Environmental Protection Agency (EPA). (1999): Rimsulfuron; Pesticide tolerances for emergency exemptions. Final rule. *Fed. Reg.*, 64(41):10227–10233.
263. Valentine, B.J., Gurr, G.M. and Thwaite, W.G. (1996): Efficacy of the insect growth regulators tebufenozide and fenoxycarb on lepidopteran pest control in apples, and their compatibility with biological control for integrated pest management. *Austr. J. Exp. Agric.*, 36:501–506.
264. Ware, G.W. (1999): An introduction to insecticides. Radcliffe's IPM World Textbook Home Page. http://ipmworld.umn.edu/chapters/bloomq.htm (Accessed 4/99).
265. Weed Science Society of America (WSSA). (1994): Acifluorfen. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 5–7. Weed Science Society of America, Champaign, IL.
266. Weed Science Society of America (WSSA). (1994): Ametryn. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 12–14. Weed Science Society of America, Champaign, IL.
267. Weed Science Society of America (WSSA). (1994): Asulum. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 18–19. Weed Science Society of America, Champaign, IL.
268. Weed Science Society of America (WSSA). (1994): Atrazine. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 20–23. Weed Science Society of America, Champaign, IL.
269. Weed Science Society of America (WSSA). (1994): Bensulfuron. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 28–30. Weed Science Society of America, Champaign, IL.
270. Weed Science Society of America (WSSA). (1994): Bentazon. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 32–34. Weed Science Society of America, Champaign, IL.
271. Weed Science Society of America (WSSA). (1994): Bromacil. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 37–39. Weed Science Society of America, Champaign, IL.
272. Weed Science Society of America (WSSA). (1994): Butylate. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 43–45. Weed Science Society of America, Champaign, IL.

273. Weed Science Society of America (WSSA). (1994): Chlorimuron. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 56–58. Weed Science Society of America, Champaign, IL.
274. Weed Science Society of America (WSSA). (1994): Chlorsulfuron. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 58–60. Weed Science Society of America, Champaign, IL.
275. Weed Science Society of America (WSSA). (1994): Clethodim. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 62–64. Weed Science Society of America, Champaign, IL.
276. Weed Science Society of America (WSSA). (1994): Cyanazine. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 72–74. Weed Science Society of America, Champaign, IL.
277. Weed Science Society of America (WSSA). (1994): Dichlobenil. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 94–96. Weed Science Society of America, Champaign, IL.
278. Weed Science Society of America (WSSA). (1994): Diclofop. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 101–103. Weed Science Society of America, Champaign, IL.
279. Weed Science Society of America (WSSA). (1994): Difenzoquat. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 106–108. Weed Science Society of America, Champaign, IL.
280. Weed Science Society of America (WSSA). (1994): Diquat. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 108–110. Weed Science Society of America, Champaign, IL.
281. Weed Science Society of America (WSSA). (1994): Diuron. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 113–115. Weed Science Society of America, Champaign, IL.
282. Weed Science Society of America (WSSA). (1994): Flumetsulam. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 131–133. Weed Science Society of America, Champaign, IL.
283. Weed Science Society of America (WSSA). (1994): Flumiclorac-pentyl. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 133–135. Weed Science Society of America, Champaign, IL.
284. Weed Science Society of America (WSSA). (1994): Fluometuron. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 135–137. Weed Science Society of America, Champaign, IL.
285. Weed Science Society of America (WSSA). (1994): Fluridone. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 141–143. Weed Science Society of America, Champaign, IL.
286. Weed Science Society of America (WSSA). (1994): Glufosinate-ammonium. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 147–149. Weed Science Society of America, Champaign, IL.
287. Weed Science Society of America (WSSA). (1994): Glyphosate. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 149–152. Weed Science Society of America, Champaign, IL.
288. Weed Science Society of America (WSSA). (1994): Haloxyfop. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 153–156. Weed Science Society of America, Champaign, IL.
289. Weed Science Society of America (WSSA). (1994): Imazamethabenz. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 159–161. Weed Science Society of America, Champaign, IL.
290. Weed Science Society of America (WSSA). (1994): Imazapyr. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 161–163. Weed Science Society of America, Champaign, IL.
291. Weed Science Society of America (WSSA). (1994): Imazaquin. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 163–166. Weed Science Society of America, Champaign, IL.
292. Weed Science Society of America (WSSA). (1994): Imazethapyr. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 166–168. Weed Science Society of America, Champaign, IL.
293. Weed Science Society of America (WSSA). (1994): Ioxynil. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 168–171. Weed Science Society of America, Champaign, IL.
294. Weed Science Society of America (WSSA). (1994): Isoxaben. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 173–175. Weed Science Society of America, Champaign, IL.
295. Weed Science Society of America (WSSA). (1994): Linuron. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 177–179. Weed Science Society of America, Champaign, IL.
296. Weed Science Society of America (WSSA). (1994): Metribuzin. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 200–203. Weed Science Society of America, Champaign, IL.
297. Weed Science Society of America (WSSA). (1994): Metsulfuron. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 203–205. Weed Science Society of America, Champaign, IL.
298. Weed Science Society of America (WSSA). (1994): Molinate. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 205–206. Weed Science Society of America, Champaign, IL.
299. Weed Science Society of America (WSSA). (1994): MSMA. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 209–211. Weed Science Society of America, Champaign, IL.
300. Weed Science Society of America (WSSA). (1994): Nicosulfuron. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 216–217. Weed Science Society of America, Champaign, IL.
301. Weed Science Society of America (WSSA). (1994): Norflurazon. In: *Herbicide Handbook*, 7th ed.,

edited by W.H.Ahrens, pp. 218–220. Weed Science Society of America, Champaign, IL.
302. Weed Science Society of America (WSSA). (1994): Paraquat. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 226–228. Weed Science Society of America, Champaign, IL.
303. Weed Science Society of America (WSSA). (1994): Pendimethalin. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 230–233. Weed Science Society of America, Champaign, IL.
304. Weed Science Society of America (WSSA). (1994): Primisulfuron. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 238–240. Weed Science Society of America, Champaign, IL.
305. Weed Science Society of America (WSSA). (1994): Prometon. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 243–244. Weed Science Society of America, Champaign, IL.
306. Weed Science Society of America (WSSA). (1994): Prometryn. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 245–247. Weed Science Society of America, Champaign, IL.
307. Weed Science Society of America (WSSA). (1994): Pyridate. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 256–258. Weed Science Society of America, Champaign, IL.
308. Weed Science Society of America (WSSA). (1994): Sethoxydim. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 266–267. Weed Science Society of America, Champaign, IL.
309. Weed Science Society of America (WSSA). (1994): Simazine. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 270–272. Weed Science Society of America, Champaign, IL.
310. Weed Science Society of America (WSSA). (1994): Sulfometuron. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 274–276. Weed Science Society of America, Champaign, IL.
311. Weed Science Society of America (WSSA). (1994): Terbacil. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 278–280. Weed Science Society of America, Champaign, IL.
312. Weed Science Society of America (WSSA). (1994): Thifensulfuron. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 282–283. Weed Science Society of America, Champaign, IL.
313. Weed Science Society of America (WSSA). (1994): Triasulfuron. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 287–289. Weed Science Society of America, Champaign, IL.

314. Weed Science Society of America (WSSA). (1994): Tribenuron. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 290–291. Weed Science Society of America, Champaign, IL.
315. Weed Science Society of America (WSSA). (1994): Trifluralin. In: *Herbicide Handbook*, 7th ed., edited by W.H.Ahrens, pp. 296–299. Weed Science Society of America, Champaign, IL.
315a. WSSA (1994).
315b. WSSA (1994).
316. Weisburger, J.H. (1975): In: *Toxicology, The Basic Science of Poisons*, edited by L.J.Casarett and J.Doull, pp. 333–378. Macmillan, New York.
317. Wetzel, L.T., Luempert, L.C. III, Breckenridge, C.B., Tisdel, M. O., Stevens, J.T., Thakur, A.K., Extrom, P.J. and Eldridge, J.C. (1994): Chronic effects of atrazine on estrus and mammary tumor formation in female Sprague-Dawley and Fischer 344 rats. *J. Toxicol. Environ. Health*, 43(2): 182–196.
318. Woodyatt, N.J., Lambe, K.G., Myers, K.A., Tugwood, J.D., and Roberts, R.A. (1999): The peroxisome proliferator (PP) response element upstream of the human acyl CoA oxidase gene is inactive among a sample human population: Significance for species differences in response to PPs. *Carcinogenesis*, 20(3):369–372.
319. World Health Organization (WHO). (1967): WHO Expert Committee on Malaria. Thirteenth Report. WHO Tech. Rep. Serv. No. 357. World Health Organization, Geneva.
320. World Health Organization (WHO). (1977): Malathion. Data Sheets on Pesticides No. 29. http://www.inchem.org/documents/jmpr/jmpmono/v91pr02.htm (Accessed 5/99).
321. Yanase, D., and Andoh, A. (1989): Porphyrin synthesis involvement in diphenyl ether-like mode of action of TNPP-ethyl, a novel phenylpyrazole herbicide. *Pestic. Biochem. Physiol.* 35:70–80.

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Chapter **14**

Metals

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Principles and Methods of Toxicology,
Fourth Edition, edited by A.Wallace Hayes.
Taylor & Francis, Philadelphia © 2001.

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Metals are elements generally characterized by ductility, luster, being electropositive with a tendency to lose electrons, and having the property of conducting heat and electricity. However, a number of the elements individually discussed in the body of the chapter are not true metals (e.g., arsenic, fluorine). The attempt was made to include elements that have physiological actions (both beneficial and toxic) by virtue of their chemical ionic form. Elements such as oxygen and sulfur, which are essential to life in some forms (e.g., water,

amino acids) but which also exist in forms that are chemically reactive and hence toxic (e.g., hydroperoxide, sulfuric acid), are not covered in this chapter.

Metals can have a variety of physiological effects, and it is often possible to demonstrate the toxicity of any given metal in any given organ, provided that the dose is both high and prolonged (but not so high and prolonged that the primary target organ receives a fatal dose). Essential elements may be toxic at a dose that overwhelms homeostatic controls on absorption and excretion, and the mechanism of toxicity is commonly related to an essential physiological role of the metal (e.g., control of osmolarity for sodium consumption in excess of water intake, neurotransmission for potassium consumption in excess of water intake; redox reactions for iron intake in excess of protein binding capacity). Physiological actions of nonessential elements include substituting for essential elements in enzymatic reactions, energy metabolism, neurotransmission, structural components (bone), reacting covalently or noncovalently with enzymes, membranes, DNA, and stimulating the production of active oxygen species (223). The variety of physiological effects makes it difficult to determine which action is responsible for toxicity in the most sensitive target organ. In some cases, organs are most sensitive for a biochemical reason (e.g., thallium interferes with energy metabolism, and target organs are those with the highest energy requirement); in other cases, the most sensitive organ is simply the organ in which the accumulation is greatest (e.g., cadmium and uranium accumulate in the kidneys, which are the target organs). Metals can interact with each other either to enhance toxicity (e.g., by affecting the same target organ) or to reduce toxicity (e.g., by stimulating defense mechanisms); this must be particularly kept in mind for the interpretation of animal experiments (e.g., levels of calcium, iron, and zinc should be controlled in investigations of cadmium toxicity) and epidemiological studies (e.g., fluoride reduces the incidence of dental caries: therefore, a population with the lowest fluoride exposure is likely to have the highest exposure to mercury and other metals used in dental restorations). The number of combinations of metals that could potentially be investigated is huge, and such studies are most useful either when a sensitive subpopulation is identified (e.g., individuals with insufficient intake of specific nutrients) or when a specific mechanism is revealed. Few treatments for metal toxicity are based on interfering with the mechanism of action; rather, measures are designed to reduce gastrointestinal absorption (from acute poisoning) by removing or binding the metal or are designed to speed elimination from the body (e.g., chelation therapy) (172). Prevention of excessive exposure is generally the best way to reduce the potential for metal toxicity.

The variety of physiological effects that metals can have is also the reason that adverse effects can often be demonstrated in most organ systems. Reproductive, developmental, immunological, and neurological toxicity, which are often not investigated in routine bioassays, are endpoints of increasing concern. For metals in particular, which on general principles would be expected to at least have the potential for these types of toxicity, toxicological understanding should not be considered complete without some information on whether these systems might be the most sensitive.

QUANTIFICATION OF TOXICOLOGICAL EFFECTS OF METALS

Consideration of the toxicity of metals must be quantitative because of the need to identify the most sensitive organ among all the systems that can be affected by the metal, and also because metals are naturally occurring and ubiquitous. Exposure to any metal cannot be banned the way exposure to, for example, an organic pesticide or food additive can be banned; some elements are essential to life, and even for those that are not, with sufficiently sensitive analytic techniques their presence can be demonstrated in any given sample of food, water, soil, or air. The quantification of the toxic effects of metals must attempt to precisely identify the highest level that is not expected to cause undue adverse effects because in many cases the traditional approach of using a safety/uncertainty factor of 10 would quickly lead to calculated levels that are below those essential for health (e.g., zinc, molybdenum) or levels that are below background exposures from food or water and hence extremely costly to achieve (e.g., cadmium, arsenic).

An example of the need to quantify toxicity is in the U.S. Environmental Protection Agency (U.S. EPA) program to address abandoned hazardous waste sites. For each site, a quantitative risk assessment is performed to determine the need for and extent of remediation (583). Essentially this risk assessment calculates doses of contaminants based on the concentration in a medium (air, soil, food, water) and the intake of that medium (e.g., adults are assumed to ingest 100 mg of soil per day). This dose to the maximally exposed person is then compared to two quantitative toxicological values. The first is the reference dose (RfD) or reference concentration (RfC), which is the highest dose or concentration not thought to be associated with adverse noncancer health effects (the "threshold"). The derivation of RfDs and RfCs is described in the chapter by Beck et al. (chapter 2) in this volume. RfDs quantify oral (and

potentially dermal) toxicity, and RfCs quantify inhalation toxicity; separate values may be derived for acute, intermediate, and chronic exposure duration. The second toxicological

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value is the slope factor, which quantifies the cancer risk corresponding to a given lifetime dose (see Chapter 2). There are commonly separate slope factors for the inhalation and oral routes; risks from less-than-lifetime exposure are evaluated by dividing the duration of exposure by an (assumed) 70-year lifetime. Cleanup standards for a site are commonly set as the concentrations that would deliver a dose to the most exposed individual that is less than the RfD for each chemical, and that results in an "acceptable" cancer risk (e.g., 10^{-4} to 10^{-6} incremental lifetime cancer risk). For metals, one important issue is whether they are present at the hazardous-waste site at levels exceeding the natural background level (which are not necessarily below what would pose an unacceptable risk to the most exposed individual). Another issue that arises is incorporation of uncertainty into derivation of cleanup standards. One example is chromium, commonly measured as total chromium, which leads to uncertainty because only the rarer hexavalent form, not the more common trivalent form, is considered a carcinogen (589); considering all chromium detected at a hazardous to be hexavalent will lead to an overestimate of the risk and the need for cleanup, by an unknown amount. Another example is antimony, which has an oral RfD derived with a safety factor of 1000 (589); it is likely that this safety factor is too conservative and that cleanup standards will be more stringent, and therefore more expensive, than needed. For both chromium and antimony, additional information (speciation at the site for chromium; better toxicology data for antimony) would allow risk assessments to determine more precisely acceptable levels, which would prevent the setting of potentially unnecessarily strict standards. On the other hand, there may be situations where current standards of exposure are not strict enough. Human activities such as mining and smelting, fossil fuel burning and incineration, fertilizer-intensive agriculture, and other industrial processes have increased human exposure to many elements to levels far above those of the preindustrial environment. Lead and cadmium are two examples of metals for which the level of exposure deemed acceptable has dropped many times over the years, as concern about frank toxicity among workers was replaced by concern about more subtle signs of toxicity in workers, which was in turn replaced by concern about even more subtle adverse effects in the general population exposed through environmental (including dietary) routes (94, 108). There are numerous examples of metals for which our knowledge of toxicology primarily consists of information on frank toxicity in exposed workers and a few animals studies, very similar to the extent of information that was used to derive standards for lead and cadmium that we now know could cause substantial toxicity in the general population. One of the major reasons for the advances in knowledge about lead, cadmium, and a few other metals was the development of biomarkers of exposure (blood lead levels and urinary cadmium levels) that provide a way to quantify environmental exposure and thus allow studies linking exposure to health effects in the general population. Biomarkers of exposure commonly provide much more precise quantification of exposure than is possible by traditional means, particularly for the general population that may be exposed by several routes (food, air, water), all of which are variable in time and location. One important future direction for investigation of metals toxicology is developing and validating biomarkers of exposure, and using these biomarkers to investigate potential adverse effects in the general population.

For most metals, quantification of toxicological effects has not been done using human studies with validated biomarkers. Instead, the traditional methods are used (discussed in more detail in Chapter 2): assembling the entire data set, surveying the data to determine the most sensitive target organ (the organ exhibiting an adverse effect at the lowest dose), identifying the no-observed-adverse-effect level (NOAEL) or the lowestobserved-adverse-effect level (LOAEL), and applying safety or uncertainty factors to derive a threshold below which no noncancer effects are expected to occur. For cancer risk assessment, the process involves determining a weight-of-evidence judgment as to whether the element has the potential to cause cancers in humans (the U.S. EPA uses classifications of group A, known human carcinogen; group B, probable human carcinogen; group C, possible human carcinogen; and group D, not classifiable as to human carcinogenicity); a separate step is to quantify the cancer risk associated with a given dose, on the no-threshold assumption that any exposure carries some cancer risk, with a safety margin built in by using the most sensitive sex/species/organ carcinogenic response and by using the upper 95th percentile confidence limit of the slope (583).

Several issues for the qualitative and quantitative evaluation of toxicity pertain particularly to metals.

Essentiality

Recommended dietary allowances (RDA) are defined as "the levels of intake of essential nutrients that, on the basis of scientific knowledge, are judged by the National Research Council's (NRC) Food and Nutrition Board to be adequate to meet the known nutrient needs of practically all healthy persons" (420). They are revised and published periodically by the NRC, which convenes expert committees to

estimate the mean dietary requirement for the population based on deficiency studies, balance studies, nutritional intakes, bioavailability, interactions, and homeostatic regulatory mechanisms

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(446). A normal Gaussian distribution for the range of requirements within the population and a coefficient of variation of 15% are generally assumed. The RDA is then set at two standard deviations above the mean. Statistically, the RDA represents the 97.5th percentile of the nutrient requirement in the healthy population (86). The first RDAs were set during World War II, when food was rationed, and it was important to set minimum requirements to prevent frank deficiency diseases. Today, although preventing nutrient-deficient diseases is still important, public health concerns are directed toward defining the amounts of nutrients needed to ensure optimum health, provide excellent physiological and mental function, and prevent degenerative diseases (291). For example, the 1989 recommendation for selenium is based on the amount needed to support maximal activity of the selenium-dependent enzyme glutathione peroxidase and prevent cardiomyopathy (420); however, recent epidemiological studies suggest selenium has cancer preventive activity at levels significantly higher than that needed to support maximal activity of glutathione peroxidase (125, 126, 627). A higher recommendation for selenium might be set if a reduction in cancer risk were chosen instead of preventing the disease process associated with frank selenium deficiency, cardiomyopathy (135). This will require a "reconstructed," trilevel RDA, tentatively named the dietary reference intake (DRI) (397), which will address (a) the amount needed to prevent the deficiency disease, (b) the amount needed to provide specific health benefits, and (c) the amount associated with health hazards. The Food and Nutrition Board of the Institute of Medicine, National Academy of Sciences, has formed the Committee on the Scientific Evaluation of Dietary Reference Intakes to address these issues.

Route of Exposure

Two major routes of exposure to metals are by inhalation and oral exposure. Inhalation of metals, particularly as fumes or dusts, commonly causes systematic effects on the lung, ranging from mild, self-limiting metal fume fever from acute exposure and benign pneumoconiosis from chronic exposure for some metals to severe chronic obstructive lung disease for others (254, 430). Standards for inhalation exposure to metals are developed by the American Conference of Governmental Industrial Hygienists (ACGIH), based primarily on occupational data; the highest allowable standard, 10 mg/m3, pertains to dusts that are not chemically reactive but present a cumulative, physical burden on the lung that can be harmful from long-term exposure (31). Oral exposure to many metals with known lung toxicity often has no adverse effects (although at high enough oral doses, most metals cause acute gastrointestinal irritation and distress) (172), possibly due in part to the faster turnover of gastrointestinal versus lung cells. Another important route distinction has to do with carcinogenicity. Several metals are considered to be carcinogenic by the inhalation route, but not by the oral route (e.g., cadmium, chromium, nickel). This classification is based on the observation of an increased rate of lung but not other forms of cancer among workers and experimental animals exposed by inhalation, and on no observed increased rate among experimental animals exposed orally. However, these metals could have weak rather than no oral carcinogenicity; on theoretical grounds, it could be argued that most mechanisms by which an element is carcinogenic to lung tissue could operate in other tissues as well, and certain metals such as arsenic are known to be both lung carcinogens and systemic carcinogens. The potential human oral carcinogenicity of metals, particularly those known to be inhalation carcinogens, is an area deserving further study, and again, valid biomarkers of exposure would be very valuable for such studies. Another point concerning route of exposure is that some metals, such as cadmium, are known to have very different toxicokinetic and toxicological properties by parenteral routes than by oral or inhalation routes. For cadmium at least, this is most likely due to the binding to metallothionein as a required step in oral or inhalation absorption, which is bypassed by parenteral exposure (16). Cancers can be induced in experimental animals by implantation of solids (metals as well as other solids); this "solid-state" carcinogenicity is typically considered to be only marginally relevant to human exposures. However, solid-state carcinogenesis may be relevant in humans with implanted metal-containing prosthetic devices (605). In general, studies using parenteral routes of exposure are often of limited use, unless the goal is to evaluate human parenteral exposure from medical procedures. A final point with respect to route of exposure is that most metals are considered not to be absorbed through the skin (with certain exceptions such as mercury and thallium); however, few data actually exist to substantiate this assumption, and further studies would be useful to quantify the dermal absorption of metals.

Form

Some metals, such as mercury, exist in elemental, ionic, and organic forms, and each of these forms has a unique toxicity. Other metals, such as chromium, may exist in two or more valence states with different effects. Still others, such as nickel, may be primarily protein bound in food sources but primarily free ions in water, which may affect absorption. Finally, there are some metals, such as

cadmium, that appear to have similar toxicological effects regardless of their form. Ideally, in

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formation would be available to do a separate quantification for each toxicologically distinct form; this would only be useful, of course, in situations where the form to which humans are to be exposed is actually known. Failing this ideal, the attempt is made to derive a standard for the most toxic form of a given metal; however, in many cases this leads to overly stringent standards, and in some cases lack of information on the most toxic form of a metal may lead to standards that are too lax.

Duration of Exposure

The influence of duration of exposure on the quantitative, and even qualitative, toxicology of a metal depends on its toxicokinetics. Cadmium is an example of a cumulative toxin. To a relatively good approximation, the same total dose given over a week, a month, or a year will accumulate in the kidneys to the same extent and have the same physiological effect (16). Other metals, particularly essential elements, are excreted so efficiently that any dose that can be tolerated for a day can also be tolerated for a lifetime (420). For well-studied metals, information is generally available to account for duration of exposure. One example is that ACGIH commonly derives threshold limit values (TLVs) as time-weighted averages (TWAs), but may also derive a short-term exposure limit (STEL) or a TLV ceiling (TLV-C) for substances that have acute as well as chronic effects (31). For less studied metals, many standards are derived based on animal data with very little information on toxicokinetics, which means that extrapolation to durations of exposure other than those used in the study at hand are quite uncertain.

Age at Exposure

Infants and young children may be particularly sensitive to toxic effects of metals both because they often absorb a greater fraction of ingested metals than older children or adults and because some developing systems (particularly the nervous system) are more sensitive to toxic effects than mature systems. Lead is an example of a metal that is known to be most deleterious to fetuses, infants, and toddlers (108, 109). The elderly are another group that may be more sensitive than healthy adults to the toxic effects of metals due to diminution of homeostatic and adaptive mechanisms. Definition of a safe level of exposure has an inherent uncertainty for metals lacking data on effects on infants and the elderly.

Animal Versus Human Data

For well-studied metals, animal and human toxicity appear to be in general qualitative agreement, although there are some exceptions, such as the difficulty in demonstrating that arsenic is carcinogenic to experimental animals. However, quantitative differences do occur; for example, gastrointestinal absorption of cadmium is about two to three times lower in experimental animals than humans (16). Use of a 10-fold safety factor for animal-to-human extrapolation in this case would yield a toxicological value about 3 times more stringent than necessary. This emphasizes the importance of using human data whenever possible for quantification of toxicity.

Toxicokinetic Modeling

Toxicokinetic modeling is very useful for evaluating the toxicity of well-studied metals. Good models can integrate information on the effects of routes of exposure, chemical forms, age at exposure, duration of exposure, and interindividual variation on absorption, distribution, excretion, and target-organ sensitivity. The toxicokinetic model developed for quantifying the systemic toxicity of cadmium is discussed later in this chapter. In addition, toxicological modeling is used in the quantification of the cancer risk from exposure to radioactive elements. Principles of radiological toxicity are covered in detail in the chapter on radioactivity (Chapter 15); the following is a brief description highlighting the use of toxicokinetic modeling for radioactive elements. Radioactive elements can cause damage at levels of exposure many orders of magnitude below those at which their nonradioactive forms cause chemical damage because radioactive decay involves the release of a large amount of energy in the form of alpha particles, beta particles, and/or gamma rays. A single radioactive decay can initiate a cascade of events that creates a huge number of active oxygen species, which are thought to be the ultimate cause of radioactive damage. This damage can cause cell death at high levels of exposure; of greater concern is the possibility of mutations that can initiate or promote cancer or result in hereditary defects (582).

Quantification issues are addressed for individual metals in the remainder of this chapter. The reader should recognize that the U.S. EPA continually reviews and revises toxicity values, and that the numbers presented here are simply the values that were specified in December 2000.

Sources of Information

There are numerous sources of information on toxicity of metals, many of which are updated on a regular basis. The U.S. EPA maintains the Integrated Risk Information System (IRIS), which has a summary of information (including RfD and RfC values) on numerous toxic

chemicals, including a number of metals (589). This database is regularly updated, and is available on the EPA web site (http://www.epa.gov). The American Conference of Governmental Industrial Hygienists (ACGIH) publishes annually a listing of all chemicals for which threshold limit values (TLV) and biological exposure indices (BEI) exists (32). The documentation for the development of these values is also available, with the latest update of this publication occurring in 1996 (31). The U.S. Agency of Toxic Substances Disease Registry has published many documents that summarize the toxicological effects of elements and chemicals; documents exist for many of the elements discussed in this chapter. Finally, the series *Patty's Industrial Hygiene and Toxicology* contains a vast amount of information on the toxicity of metals and other elements and compounds, with the major emphasis being on industrial exposures and effects (63, 64, 142, 460). Treatments for exposures to metals and other compounds are described in detail in *Clinical Toxicology of Commercial Products* (222) and in *Diagnosis and Treatment of Human Poisoning* (172).

ESSENTIAL ELEMENTS

Calcium

Calcium is essential both for the physical structure of bone and for normal physiological function (e.g., nerve conduction, muscle contraction, blood clotting, membrane permeability, enzyme activation, acetylcholine synthesis) (27). The average healthy adult body contains about 1200 g of calcium, 99% of which is found in bone and teeth, with the remaining 1% in extracellular fluids, intracellular structures, and cell membranes. The average calcium content of the blood ranges from 9.0 to 10.5 mg/dl with tight physiological controls. Decreased body calcium leads to loss of bone mineral, reduction of bone strength, increased susceptibility to fractures (479), and may increase blood pressure (382), particularly among pregnant women (65). Calcium deficiency is also associated with convulsions and tetany. The RDA for calcium is derived from the need to maintain skeletal calcium, using an estimated 200–250 mg/day obligatory loss and an oral absorption fraction of 30–40%, leading to a recommendation of 1200 mg/day for ages 11–24 years and 800 mg/day for older age groups, except that 1200 mg/day is recommended throughout pregnancy and lactation (420). In 1994 a National Institutes of Health Consensus Conference on Optimal Calcium Intakes recommended all Americans over 5 years of age consume levels of calcium higher than the current RDA, with the greatest change in calcium intake being for elderly persons, who should consume 1500 mg/day (419).

Calcium is not a very toxic metal, but adverse effects may occur at intakes greater than 2000 mg/day (419). Intestinal absorption of calcium decreases as intake increases; however, very large intakes of calcium can increase the calcium body burden (420) as well as interfere with the absorption of magnesium (515), zinc (195), and iron (240). Very large chronic intakes are associated with hypercalcemia and/or hypercalciuria. Other symptoms of calcium excess include renal failure and soft tissue calcification. High-calcium diets could increase the risk of kidney stones in susceptible individuals and reduce the biovailability of zinc and iron. Although excessive calcium intake from food and municipal water was previously seen mainly in individuals with conditions predisposing them to increased calcium absorption, such as parathyroidectomy (379), the consumption of calcium-fortified foodstuffs (e.g., sparkling water, breakfast cereal, orange juice) in addition to a diet containing generous amounts of dairy products could theoretically reach levels of concern (615). Education of health-care professionals and the general public is needed to prevent both overconsumption of calcium in one population and the risk of calcium deficiency in another. A potential adverse effect associated with habitual intake of calcium supplements is ingestion of heavy metals, such as arsenic, cadmium, and lead, which have been found to contaminate some calcium supplements (84, 614). With the increased interest in daily calcium supplementation as a preventive measure for colon cancer, osteoporosis, and hypertension, the possible contaminants of these supplements warrant further investigation. The Food and Drug Administration (FDA) does not regulate nutritional supplements; the amount of trace metal contaminants is variable.

Chlorine

Chloride is the principal extracellular inorganic ion. It is required for maintenance of fluid and electrolyte balance and for the production of gastric acid (420). Dietary chloride deficiency is rare, but prolonged loss of electrolytes from vomiting, diarrhea, heavy sweating, and so forth can lead to hypochloremic metabolic alkalosis. The minimum requirement of chloride, based on its close association with sodium in both dietary sources and physiological losses, is 750 mg/day (420).

Reactive chlorine compounds (chlorine gas, hydrochloric acid, hypochlorite, chlorine dioxide, etc.) are irritating to the tissues they contact, but neutral chloride solutions are nontoxic (179). Habitual excess intake of table salt may contribute to hypertension in susceptible individuals, and animal data suggest that the chloride ion may play a role as well as the sodium ion (410). This question has more than

theoretical implications because

potassium chloride is widely used as a salt substitute by individuals seeking to restrict their sodium intake. However, the sparse human data on the association between chloride intake and blood pressure are generally negative, and more studies are needed before restriction of chloride intake, independent of sodium intake, could be suggested to have a beneficial effect in the general population. The U.S. EPA has not derived any toxicity values for chloride (589).

Chromium

Chromium is a first series transition metal, with its name derived from the Greek word for color, because most chromium compounds are brightly colored. The only important chromium ore is chromite. Chromium is used as an alloy with other metals, and is also used for plating of metals (63). Although chromium can have valences from −2 to +6, the most important valences are +3 and +6 (31). Trivalent chromium is the most abundant form of chromium in the environment. Chromium(III) is an essential nutrient that plays a role in glucose metabolism (35, 63). Although Cr3+ is poorly absorbed orally (158), absorption is greatly enhanced by the presence of the "glucose tolerance factor," which forms a complex with Cr3+ (513). Chromium(III) is considered to be relatively nontoxic in vivo (63). Mice exposed to chromium(III) acetate in drinking water for over 2 years did not show an increased incidence of tumors (502).

Hexavalent chromium is the most important valence from a toxicity standpoint. Unlike chromium(III), chromium(VI) is readily absorbed by all tissues. Because chromate (CrO_4^{2-}) is structurally similar to phosphate and sulfate (139), it readily enters all cells via the general anion channel protein. Chromium(VI) is acutely toxic, with most reports of human toxicity occurring as a result of accidental or intentional ingestion. The lethal oral dose of soluble chromates in humans is estimated to be in the range of 50–70 mg/kg. Symptoms of acute toxicity include vomiting and generalized gastrointestinal tract damage, with gastrointestinal bleeding leading to cardiovascular shock. If the victim survives the initial toxic effects, liver necrosis, tubular necrosis of the kidney, and damage to the blood-forming tissues can occur (63). Long-term occupational exposure to chromium has been associated either with low-molecular-weight proteinuria, or with elevated levels of proteins normally found in the urine (63, 139). Although animal studies have shown that parenteral administration of 15 mg/kg potassium chromate (+6) is nephrotoxic, chronic renal disease due to occupational or environmental exposure has not yet been reported (63).

Dermal exposure to potassium dichromate and other chromium compounds can lead to the development of a sensitization reaction. The resulting hypersensitivity results from chromium binding to proteins and becoming a hapten (151). Prior to the implementation of appropriate industrial hygiene precautions, occupational inhalation exposure to Cr6+ was associated with changes in the septal mucosa, ranging from irritation to septal perforation (63). However, inhalation exposure rarely causes asthma (430).

The carcinogenicity of chromium in the respiratory tract has been well established, beginning when the first nasal tumors were described among Scottish chrome pigment workers in the late 19th century (432), and has been reviewed in the recent literature (63, 130, 139). The mechanism of action believed to be from a direct modification of DNA (444). After hexavalent chromate enters a cell, it is rapidly reduced to Cr3+. During the reduction process, unstable and reactive intermediates, including Cr(IV), Cr(V), hydroxide, thiyl and organic (RS and R) radicals, and active oxygen radicals are formed, and it is believed that these moieties are responsible for chromium carcinogenicity (130). Because Cr6+ is readily absorbed by all tissues, one could postulate that chromium-induced cancers should be noted in other organs. Although the evidence is not as strong, exposure to hexavalent chromium is associated with an increased incidence of may types of cancers (139).

The U.S. EPA has established an oral RfD for chromium(III) of 1.5 mg/kg/day, an oral RfD for chromium(VI) of 5 μg/kg/day, an inhalation RfC for chromic acid mists and dissolved Cr(VI) aerosols of $8.6\times10-3\mu$g/m3, and an inhalation RfC for Cr(VI) particulates of $1\times10-1\mu$g/m3. The U.S. EPA has classified chromium(III) as a group D (not classifiable) carcinogen and chromium(VI) as a group A (human) carcinogen.

Cobalt

Cobalt is an essential component of vitamin B12, which is involved in intermediary metabolism, nucleic acid synthesis, and single-carbon metabolism, and is required to prevent macrocytic megaloblastic anemia, atrophic gastritis, achlorhydria, neurologic degeneration, and dementia (27). Vitamin B12 is synthesized by bacteria, fungi, and algae, but not by yeasts, plants, or animals (420). Cobalt deficiency may develop in animals dependent on gut microflora for their vitamin B12, such as ruminants, and in strict vegetarians consuming no animal products. The RDA for vitamin B12 is 2 μg/day, and although

cobalt is known to activate the enzyme arginase (554), the only recognized requirement for cobalt is as a component of vitamin B12. Cobalt is a hard, silvery metal widely distributed in rocks and soils and always occurs with nickel and usually with arsenic (13). It is primarily used in the production of superalloys, as a drier in paints,

and in magnets, and in the production of prosthetic devices. Occupational exposure occurs in the hard metal industry, among cobalt blue dye plate painters, and in coal miners, and this exposure is reflected in elevated levels of cobalt in tissues and body fluids.

Cobalt can be toxic. For the general population, ingestion is the primary route of exposure (13). Oral exposure to cobalt caused cardiomyopathy among individuals who drank excessive amounts of beer (8–25 pints/day) containing cobalt as a foam stabilizer (405). This effect may have been potentiated by a combination of alcohol, preexisting heart damage, and/or poor diets associated with heavy alcohol consumption, because anemic individuals have been exposed to higher levels of cobalt without a similar effect (405, 516). Cobalt can cause allergic dermatitis (eczema and urticaria, mainly of the hands) (28, 598), and cross-reaction with nickel is frequent (595, 493). Inhalation exposure to cobalt alloyed to tungsten carbide (hard metal) is associated with hard metal disease, which is characterized by interstitial fibrosis and restrictive respiratory impairment (352). The toxic mechanism of hard metal particles is thought to involve both cobalt sensitivity and the generation of oxygen radicals by the carbide particles (352, 431). Cobalt by itself has caused occupational asthma in diamond polishers, and the effect has been attributed to an immunologic mechanism with cobalt acting as a hapten (207).

The carcinogenicity of cobalt is uncertain. Animal studies are positive only for subcutaneous, intramuscular, or intratracheal administration, but not for inhalation, and the excess rates of lung cancer observed in men occupationally exposed to cobalt dust could be explained by simultaneous exposure to nickel, arsenic, and/or tobacco (338, 439).

The U.S. EPA has not derived toxicity values for cobalt. The ACGIH has adopted TLV-TWA values for cobalt carbonyl and cobalt hydrocarbonyl of 0.1 mg Co/m3 (32).

Copper

Copper occurs naturally as the free metal and occurs in compounds in +1 or +2 valence state. Copper is incorporated into several enzymes involved in hemoglobin formation, carbohydrate metabolism, catecholamine biosynthesis, and cross-linking of collagen, elastin, and hair keratin (6). These enzymes include cytochrome *c* oxidase, dopamine β-hydroxylase, ascorbic acid oxidase, and superoxide dismutase, as well as interaction with ceruloplasmin and metallothionein. Copper deficiency causes anemia, neutropenia, and impaired growth, particularly in children (420). The ingestion of copper in foods is the primary source for copper intake. The intake from copper plumbing and unpolluted fresh water is not significant. The estimated safe and adequate daily dietary adult intake is 1.5 to 3.0 mg/day (420). The U.S. EPA action level for copper in tap water is 1.3 mg/L (129).

Copper is readily absorbed following oral ingestion, but homeostatic mechanisms limit further intake once requirements are met. Copper overload is normally further controlled by binding to metallothionein. Copper is either active or in transit, with little or no excess copper being normally stored (350). Following absorption, copper is bound to albumin and transcuprein, and is mainly deposited in liver hepatocytes with lesser amounts in the kidney. Biliary excretion is the major route with small amounts secreted in the urine. Considering these homeostatic mechanisms following oral intake, absorption through the inhalation or dermal routes may allow toxic levels to pass unimpeded into the blood.

The consumption of water containing high levels of copper or suicide attempts with copper sulfate can result in vomiting, diarrhea, nausea, abdominal pain, hemolytic anemia, hepatic and renal neurosis, and death. Industrial exposure may occur to copper fumes resulting in metal fume fever with dyspnea, chills, headache, and nausea (63). The ACGIH has adopted TLV-TWA values for copper of 1 mg Cu/m3 for dusts and mists and 0.2 mg Cu/m3 for fumes (32). The OSHA PEL differs with 0.1 mg/m3 for copper fume. Copper can be dermally absorbed from copper-containing topical products (335, 465, 466). Dermal irritation and contact allergic dermatitis have been associated with copper jewelry, intrauterine contraceptive devices, and through occupational exposure to electroplating and copper containing agricultural products (335).

Wilson's disease is one of several examples of toxicity involving copper in humans. This disease is due to an autosomal recessive disorder that affects normal copper homeostasis. There is an excessive retention of hepatic copper, decreased concentration of plasma ceruloplasmin, impaired biliary copper excretion, and hypercupremia, resulting in hepatic and renal lesions and hemolytic anemia (6). Menkes's disease is a multisystemic lethal disorder characterized by neurodegenerative symptoms and connective tissue manifestations. The disease is attributable to a deficiency of one or more copper-dependent enzymes (571).

Fluorine

Fluorine, the most reactive of the elements, is a pale yellow gas with a pungent odor. The chief fluoride sources are fluorspar (CaF2) and cryolite (Na3AlF6). Fluorine, hydrogen fluoride, and other fluorine

compounds are used in a wide number of applications in the nuclear (in the

synthesis of uranium hexafluoride), agrochemical (pesticides), drug (anticaries agents), and other industries (31, 63). Fluorine gas is a severe eye, mucosal, and skin irritant (31).

Hydrogen fluoride (HF) is a weak acid that causes severe burns on the skin and in the eye, either in aqueous solution or as the anhydrous acid (63). In addition to causing dermal and ocular damage, hydrogen fluoride is readily absorbed through the skin. Once absorbed, fluoride complexes with calcium and causes hypocalcemia. If the hypocalcemia is severe, death can occur via cardiac arrhythmia. Hydrogen fluoride burns over as little as 2.5% of the body surface have caused fatalities, depending on the concentration of HF (113, 118, 318).

Fluoride is incorporated into bones and tooth enamel, making teeth more resistant to caries, but fluoride deficiency has never been conclusively demonstrated in humans or animals, although goats fed <1 mg F/kg dry ration had reduced growth and survival (40). The NRC classifies fluoride as a beneficial but not an essential element (420). Fluoride replaces hydroxyl ions in enamel, yielding an apatite crystal that is more resistant to acid. Some studies suggest that fluoride supplements may also increase bone strength (452). Fluoride in aqueous solutions is virtually 100% absorbed, while absorption of fluoride in bone meal may be as low as 40% (420). The estimated safe and adequate daily dietary intake for fluoride from both food and water is set equal at 1.5–4 mg/day, based on the range of fluoride composition of diets in the United States (420). Although serious complications are rare (because of limitations set by the U.S. Food and Drug Administration on the total amount of fluoride in an over-the-counter anticaries drug product), acute fluoride toxicity can occur from accidental ingestion of fluoride containing products. In his review of reported accidental fluoride poisoning cases, Whitford (612, 613) proposed a "probably toxic dose" of 5 mg/kg, although toxicity has been reported at doses as low as 0.1 mg/kg (23).

Doses of fluoride above 2 mg/day can cause mottled teeth in children, doses over 8 mg/day can cause osteosclerosis, and doses of 20 mg/day for 10–20 years can cause hypermineralization of bone leading to crippling skeletal fluorosis and renal toxicity (378, 420). Fluoride increases bone mass but decreases its tensile strength, and is apparently not a treatment for osteoporosis (55). Case reports indicate that administration of sodium fluoride for treatment of osteoporosis can exacerbate rheumatoid arthritis, possibly by stimulating leukocytes and other mediators of the acute inflammatory response (163).

Human epidemiological studies have found no evidence that fluoride causes gastrointestinal, respiratory, reproductive, or developmental toxicity (92). Skeletal and dental changes can be seen in rodents exposed to fluoride, as well as chronic stomach inflammation and ulcers (92, 378). The U.S. EPA has derived an oral RfD for fluorine based on a no-observed-effect level (NOEL) for objectionable mottling of the teeth (dental fluorosis), which may occur in children drinking water with more than 1 ppm fluoride, leading to a NOEL of 0.06 mg/kg/day in a 20-kg child drinking 1 L/day and ingesting 0.1 mg/kg/day of dietary fluoride (589). The endpoint of dental fluorosis is not considered toxic or adverse. The ACGIH has adopted a TLV-TWA value for fluorides of 2.5 mg F/m3, with carcinogenicity classification A4 (not classifiable as a human carcinogen) (32).

The potential carcinogenicity of fluoride is debatable. No increase in tumors was found among mice exposed to 1.75 mg/kg/day of sodium fluoride in water for 30 months (297). Sprague-Dawley rats had no statistically significant increase in tumors following 2 years of exposure to doses up to 25 mg/kg/day (378). The National Toxicology Program 2-year drinking water study of sodium fluoride at doses up to 10 mg/kg/day found no evidence for carcinogenicity in female rats, male mice, or female mice, and equivocal evidence of carcinogenicity in male rats (92). The evidence in male rats consisted of an increase in bone osteosarcomas with a dose-response trend that was statistically significant but an incidence in the highest dose group that was not significantly elevated compared to controls (92). Also, no osteosarcomas were found in female rats even though they accumulated fluoride in bones to the same extent as the male rats and they exhibited fluoride-induced osteosclerosis (92). The U.S. EPA has not yet evaluated fluoride for potential human carcinogenicity (589). While human epidemiology studies have generally been negative, the question of whether fluoride is a potential human carcinogen is still open, and more studies are needed to resolve this question of some public health importance (55, 92, 378).

Iodine

Iodine is the heaviest of the halogens that are of industrial interest. In solid form, iodine takes the form of gray-black plates or granules. It volatilizes at room temperature, yielding a violet vapor (460). The major sources of iodine are oil and natural gas brines, with Japan's natural gas-well brines being credited with as much as four-fifths of the world's iodine reserve (460). Topical iodine solutions (2% iodine and 2% NaI in 50% alcohol, USP) have been used for decades as germicides and antiseptics (31). When inhaled, iodine vapor can be intensely irritating to mucous membranes and affects the upper

and lower portions of the pulmonary tract (30). Flury and Zernik (194) reported that humans could work undisturbed at 0.1 ppm, work with

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difficulty at 0.2 ppm, and could not work at 0.3 ppm. Topical application of iodine solutions can cause irritation, and strong solutions can cause burns (31).

Iodide is required for the synthesis of the thyroid hormones thyroxine and triiodothyronine. Iodide is efficiently absorbed, and excess iodide is excreted in the urine (420). Deficiency of iodide causes hypothyroidism and goiter, and severe deficiency in the newborn may cause cretinism and mental retardation (183). The RDA for iodine is 150 μg/day for adults, with an extra 25 μg/day during pregnancy and 50 μg/day during lactation (420). Chronic absorption of high levels of iodide can lead to a condition known as iodism. This condition is characterized by sleeplessness, tremor, rapid heart rate, diarrhea, weight loss, conjunctivitis, rhinitis, and bronchitis. This syndrome is usually associated with long-term ingestion of iodide containing medications (31). The U.S. EPA and ACGIH have not derived toxicity values for iodide (32, 589).

Iron

Iron is a silver-white solid metal found mainly in combination with other elements as oxides, carbonates, sulfides, and silicates (63). It exists in two stable oxidation states, oxidized ferric (Fe^{3+}) and reduced ferrous (Fe^{2+}), which accounts for its essentiality as a trace element and its crucial role in the oxygen and electron transport reactions of all living cells. Dietary iron is available as either heme or nonheme (27). Heme iron is found in meats and is relatively well absorbed compared with nonheme iron, which is also found in meats, grains, and vegetables. Intestinal absorption of iron depends on iron status, with 10% of the total (heme plus nonheme) being absorbed when iron status is normal, but up to 20% in deficiency states. Adequate intakes of vitamin C increase the intestinal absorption of nonheme iron by two- to fourfold (138), which may be of significance to the iron status of vegetarians. Iron is lost through the shedding of cells, sweat, nails, hair, blood loss, menstruation, and in the urine. Early symptoms of iron deficiency are nonspecific and include fatigue and weakness. This progresses to iron-deficiency anemia, which is characterized by small red blood cells with low hemoglobin content (microcytic hypochromic anemia). These symptoms resolve after administration of iron. The RDA for iron is derived using an adequate body store of 300 mg, estimated losses of 1 mg/day in men and 1.5 mg/day in women, and an oral absorption fraction of 10–15%, leading to a recommendation of 10 mg/day for adults males and 15 mg/day for adults females, with an additional 15 mg/day recommended during pregnancy (420).

Free iron is an oxygen-reactive substance, highly toxic to cells, and will enhance the formation of free radicals and peroxidation of membrane lipids (49, 50, 491). Humans are unable to eliminate excess iron and regulate body iron stores by limiting absorption (381). Divalent iron is taken up by intestinal mucosa and converted to the trivalent form. The trivalent form is bound to transferrin (63, 247), a glycoprotein with two iron-binding sites (363). Iron is transported as transferrin to the liver or spleen, where it is stored as ferritin, which has a high iron storage capacity and prevents iron from participating in the Fenton reaction (59, 389). Of the typical 4-g body iron stores found in adults, 66% is bound as hemoglobin, 10% as the protein myoglobin, with a minute amount in iron-containing enzymes, and the rest as intracellular storage proteins. The physiological controls on this essential but potentially toxic metal can be overwhelmed, either by an acute large intake (accidental ingestion of dietary supplements by children) or by chronic excessive intake (endogenous sub-Saharan African populations with a probable genetic defect who consume beverages brewed in steel drums may develop pancreatic, hepatic, and/or renal toxicity from their accumulation of excessive iron) (221). Inhalation exposure to iron dust or fumes has resulted in pulmonary siderosis; fibrosis does not develop and the clinical course is benign (430). Hepatotoxicity is typically seen in patients with iron overload and can progress from portal fibrosis to cirrhosis (541). A gray-bronze hyperpigmentation of the skin caused by increased melanin and iron deposition usually resolves after iron removal. Free radical stress and lipid peroxidation have both been suggested as factors in the etiology of diabetes (443), and increased iron stores have been reported to contribute to the development of non-insulin-dependent diabetes (495). An increased risk of infection by a number of microorganisms, including *Vibrio vulnificus, Listeria monocytogenes, Yersinia enterocoloitica, Eschericia coli,* and *Candida* species, may result from excessive iron intake, due to direct effects on the immune system and/or enhanced bacterial growth due to the increased availability of iron (220, 249). Epidemiologic evidence suggests excess dietary iron is a coronary risk factor (494, 573, 574) and regular blood donation in middle-aged males is associated with a reduced risk of myocardial infarction (496).

Iron poisoning is the most common fatal poisoning in children reported to poison control centers in the United States (354, 610). Despite supplements being packaged in child-resistant packages and carrying warning labels, the public perception of their potential danger is low (274) and fatalities in children have

recently increased (34, 68, 610). Iron poisoning is characterized by four distinct clinical stages, but individual patients do not always demonstrate each stage (172, 228, 385): Stage I (initial period) occurs 0.5–2 h postingestion and is characterized by the onset of acute gastrointestinal symptoms (vomiting and diarrhea), but central nervous system (CNS) symptoms (lethargy and coma) may be present in severe cases;

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stage II (quiescent period) occurs 6–24 h postingestion and the victim may be asymptomatic or appear to have improved; stage III (recurrent period) occurs 12–48 h postingestion and is characterized by gastrointestinal perforation, coma, convulsions, cardiovascular collapse, hepatic and renal failure, and metabolic acidosis; and stage IV (late period) occurs 3 to 4 weeks postingestion with the appearance of gastrointestinal scarring. The failure to recognize a patient in stage II iron poisoning has been noted as a "pitfall" in the medical management of iron poisoning (396) and has resulted in premature discharge (172). Gastrointestinal symptoms typically occur following the ingestion of 20 mg elemental iron/kg body weight, and doses greater than 60 mg/kg are often lethal. Treatment involves stabilizing vital functions, removing unabsorbed iron from the gastrointestinal tract, and intravenous administration of deferoxamine if symptoms are severe (34, 385, 396). Deferoxamine is an iron chelator produced by *Streptomyces pilosus* and removes iron from transferrin and ferritin, but not hemoglobin (359, 555). Current research is directed toward the design of an orally active, nontoxic, selective iron chelator (258, 320). The FDA recently issued regulations requiring all iron-containing products to carry a label stating the dangers of iron overdosage and unit-dose packaging for products containing 30 mg or more per dosage unit (591). The U.S. EPA has not derived any toxicity values for iron (589); the ACGIH has adopted TLV-TWA values for iron of 5 mg Fe/m3 for iron oxide dust and fume and 1 mg Fe/m3 for soluble iron salts (32).

Magnesium

Magnesium is essential to a large number of biochemical and physiological processes including neuromuscular conduction in skeletal and cardiac muscle (63). It is also an important structural component of bone (420). Plasma concentrations of magnesium are regulated within a narrow range (0.65–1.0 m*M*), primarily by adjustments in the reabsorption of filterable magnesium in the loop of Henle, and also by the passive buffering by bone magnesium (420). Magnesium deficiency can occur secondarily to general malnutrition, alcoholism (480), or other disease states that affect gastrointestinal electrolyte absorption or excretion or renal cation reabsorption. Magnesium deficiency results in reduced levels of potassium and calcium, as well as symptoms of nausea, muscle weakness, irritability, and mental derangement (525). The RDA for magnesium is derived from balance studies indicating that magnesium balance can be maintained in healthy men at intakes of 3.0–4.5 mg/kg/day, leading to a recommendation of 350 mg/day for men and 280 mg/day for women, with an extra 20 mg/day during pregnancy and 75 mg/day during lactation (420). Average intakes of U.S. adults are not much above the RDAs; however, there is no definitive evidence of effects attributable to magnesium deficiency (420). Vitamin D facilitates the absorption of magnesium (398).

Oral exposure to magnesium is not toxic except in individuals with impaired renal function, who may experience nausea, vomiting, and hypotension, followed by CNS depression accompanied by a sharp drop in blood pressure and respiratory paralysis (63, 420). Magnesium salts are poorly absorbed orally and are commonly used as antacids or cathartics. Inhalation exposure to magnesium oxide can cause metal fume fever (19, 63, 430). The U.S. EPA has not derived toxicity values for magnesium; the ACGIH has adopted a TLV-TWA value for magnesium oxide fumes of 10 mg/m3 (nuisance dust) (32).

Manganese

Manganese is a silver-gray soft metal and occurs in ores mainly as oxides (63). Manganese and its compounds are used in numerous products and applications including iron and steel alloys, dry cell batteries, paints, inks, fertilizers, and fungicides (208). Manganese is an essential trace metal that is a component of several mitochondrial enzymes, pyruvate carboxylase and superoxide dismutase, and activates a wide variety of enzymes (decarboxylases, transferases, hydrolases). It occurs in meats, poultry, nuts, grains, green leafy vegetables, and tea. Although outright manganese deficiency has not been observed in the human population, suboptimal manganese intake may be a concern (589). In animals, manganese deficiency can cause impaired growth, skeletal abnormalities, and altered metabolism of carbohydrates and lipids (22). The estimated safe and adequate daily dietary intake for manganese is set equal to current U.S. dietary intakes, based on a lack of evidence for human manganese deficiency, yielding a range of 2–5 mg/day (420). The U.S. EPA has reviewed numerous human and animal studies and related information and concluded that an appropriate chronic oral reference dose for manganese is 10 mg/day (0.14 mg/kg/day) (589). Only between 3 and 10% of dietary manganese is absorbed in normal adults, and total body stores are controlled by a complex homeostatic mechanism regulating absorption and excretion. Calcium, iron deficiencies, age, and other factors may increase manganese absorption (22, 589).

Occupational inhalation exposure is the primary route for manganese toxicity. The primary toxic effect of occupational inhalation exposure is neurological damage (483); however, inhalation exposure to

manganese can also affect the lung directly, causing metal fume fever, pneumonitis, chronic obstructive lung disease, and pneumonia (22, 430). Occupational exposure to manganese

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at levels of about 1 mg/m3 may decrease male fertility (205, 337). The neurological effects of inhalation of manganese dusts, termed manganism, typically begin with weakness and lethargy, and may progress to disturbances in speech and gait, a mask-like face, tremor, and possibly hallucinations and psychosis (483). Symptoms may resemble Parkinson's disease, but there is only minimal response to L-dopa therapy. The pathobiochemical aspects of manganism involve the striatum and globus pallidus. Cell damage may be due to the auto-oxidation of dopamine with the formation of free radicals (596). Manganese applied to the nasal cavity in rats is taken up in the olfactory receptor cells and transported along the primary neurons to the olfactory bulbs, with subsequent migration into most parts of the brain. This route circumvents the blood-brain barrier (559). More subtle nonclinical neurological damage can be identified by neurobehavioral tests (reaction time, finger tapping, hand steadiness, etc.) in men chronically exposed to levels as low as 0.14mg/m3 for 1–35 years (283). Impairment of speed and coordination of motor function are noted.

The U.S. EPA has derived a recent inhalation RfC of 0.05 μg/m3 based on studies of Roels et al. (483, 589). The previous RfC was 0.4 μg/m3. The ACGIH has also lowered the TLV-TWA value to 0.2 mg Mn/m3 for elemental and inorganic compounds (32). Manganese cyclopentadienyl tricarbonyl (MMT) is a gasoline octane enhancer in use since 1970. The major combustion products of MMT are manganese particulates of manganese phosphate with some sulfates and a small amount of oxides. The TWA-TLV for MMT is 0.1 mg Mn/m3, with a notation noting the potential for dermal absorption (32). Little evidence exists to suggest that manganese has carcinogenic potential. A 2-year bioassay of manganese sulfate monohydrate in the diet found no evidence of carcinogenicity to rats and equivocal evidence of carcinogenicity to mice (22). The U.S. EPA has classified manganese as a group D carcinogen (not classifiable as to human carcinogenicity), based on inadequate evidence in humans and animals (589).

Molybdenum

Molybdenum is a silver-white metal of the second transition series. The primary molybdenum-containing ore is molybdenite (MoS_2), with minor ores being powellite ($CaMoO_4$) and wulfenite ($PbMoO_4$). Metallic molybdenum is used in a number of important applications, such as in high temperature and tool steel alloys, and in missile and aircraft parts. Molybdenum disulfide is used as a dry lubricant or as a component in lubricants (31).

Molybdenum is a constituent of several enzymes, including aldehyde oxidase, xanthine oxidase, and sulfide oxidase (420). Deficiency is extremely rare; one patient on total parenteral nutrition had disturbed sulfur and uric acid metabolism that resolved after molybdenum supplementation (420). The estimated safe and adequate daily dietary intake for molybdenum is set equal to the current U.S. dietary intake for molybdenum, based on the lack of evidence for human molybdenum deficiency, yielding a range of 75–250 μg/day (420). High levels of molybdenum in herbage eaten by cattle caused diarrhea in cattle (186), which could be alleviated by the administration of copper salts (429). Further study has shown an inverse relationship between molybdenum and copper. When molybdenum intake in cattle is increased, the concentration of utilizable copper in the liver decreases (63).

The acute oral toxicity of molybdenum compounds is related to their solubility. Molybdenum trioxide, calcium molybdate, and ammonium molybdate caused fatalities in rats when administered at doses from 1.2 to 6.0 g Mo/kg. Conversely, administration of insoluble molybdenum disulfide to rats at concentrations as great as 6.0 g Mo/kg did not cause any fatalities (182). The U.S. EPA derived an oral RfD for molybdenum of 5 μg/kg/day (250 μg/day for a 70-kg adult) based on an increase in urinary uric acid levels in humans exposed to 10 mg Mo/day in the diet with an uncertainty factor of 30 (589). Rodent bioassays of molybdenum trioxide indicate that this compound is carcinogenic in rats and mice, causing an increased incidence of alveolar/bronchiolar adenoma or carcinoma (combined). Male rats and mice appear to be more sensitive to the carcinogenic effects of molybdenum trioxide (114).

Dental technicians exposed to dust of vitallium alloy, which contains chromium, cobalt, and molybdenum, can develop pneumoconiosis that is clearly different from hard-metal lung disease associated with cobalt exposure (430), and there are some data to suggest that molybdenum inhalation can cause pneumoconoiosis (31). The ACGIH has adopted TLV-TWA values of 10 mg Mo/m3 for insoluble compounds and 5 mg Mo/m3 for soluble compounds (32).

Phosphorus

Phosphorus is an essential component of bone and also participates in many important biochemical reactions (420). Approximately 85% of the body store of phosphorus is in bone, with the rest as soluble phosphate ion and a component of a variety of biomolecules. Absorption of phosphate ranges from 50–70% when intake is adequate, to 90% when intake is low (420). Dietary phosphorus deficiency is rare but can occur following prolonged use of the antacid aluminium hydroxide, which binds phosphorus into

an unavailable form. Symptoms of

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phosphorus deficiency include bone loss, weakness, anorexia, and pain. The RDA for phosphorus is set equal to the RDA for calcium, based on a lack of evidence for either phosphorus deficiency or toxicity at usual U.S. intakes, yielding 1200 mg/day for ages 11–24 years, 800 mg/day for older age groups, with an extra 400 mg/day throughout pregnancy and lactation (420).

High-level phosphate intake in the forms of phosphate-fortified infant formulas, phosphoric acid in carbonated beverages, or purified amino acids may cause calcium loss, which can be adverse in situations of inadequate calcium intake; however, phosphorous in the form of complex proteins does not seem to have this effect (420, 537). Certain reactive forms of phosphate may be chemically irritating, but neutral phosphate solutions are essentially nontoxic (304). Phosphorus as the free element does not occur in nature. It exists either as relatively nontoxic red phosphorus or toxic yellow (or white) phosphorus (150). Toxic exposure to yellow phosphorus can occur through the oral, dermal, or respiratory routes. Rodenticides and insecticides containing yellow phosphorus have accounted for poisonings characterized initially by gastrointestinal burning and severe abdominal pain, vomiting, and diarrhea. Acute cardiovascular collapse may occur (276, 463). If survived, a second stage of symptoms may occur up to several weeks later, resulting in systemic toxic effects on the liver, heart, kidneys, or central nervous system. Phosphorus can cause necrotic skin burns. The fumes are irritating to the respiratory tract, eyes, and skin. Phosphorous is converted to phosphates and excreted in the urine. The U.S. EPA has derived a chronic oral RfD for elemental phosphorus based on studies of Condray (137), which found increased mortality in pregnant rats near the end of gestation at a dose of 0.075 mg/kg/day for 80 days prior to mating and during gestation. A NOAEL of 0.015 mg/kg/day was converted to an RfD of 0.02 μg/kg/day (1.4 μg/day for a 70 kg adult) with low confidence, using an uncertainty factor of 1000 (a factor of 10 for interspecies variation, 10 for intraspecies variation, and 10 for incomplete reproductive/developmental data and a less-than-adequate lifetime study), and a modifying factor of 1 (589). The ACGIH has adopted TLV-TWA values for phosphorus of 0.2 ppm for phosphorus trichloride, 1 mg/m3 for phosphorus pentasulfide, 0.1 ppm for phosphorus pentachloride, 0.1 ppm for phosphorus oxychloride, and 0.02 ppm for yellow phosphorus (32). No data exist to suggest that phosphorus may have carcinogenic potential.

Potassium

Elemental potassium is a highly reactive soft metal with a silver-colored appearance and is not found in nature. Potassium compounds are common. Elemental potassium is even more reactive than sodium and must be stored under airtight anhydrous conditions, such as under xylene. Oxidation on the surface of the metal may form highly reactive superoxides, which can detonate the bulk, causing spattering and skin and eye penetration (326). Autoignition can occur at room temperature. Dermal and ocular thermal burns and liquefaction necrosis due to the formation of potassium hydroxide are the primary effects following exposure. Imbedded particles require surgical debridement. Water irrigation is contraindicated.

Potassium is the principal cation of intracellular fluid, accumulating to a concentration about 30 times higher than in plasma. Potassium in plasma is involved in nerve transmission, muscle contraction, and blood-pressure homeostasis. The gastrointestinal absorption of potassium is nearly complete; plasma concentrations are kept within a narrow range by regulation of urinary excretion, and by depletion of body stores in cases of low potassium intake (420). Dietary potassium deficiency is rare, but prolonged vomiting, diarrhea, or diuretic use may deplete potassium enough to cause weakness, anorexia, nausea, drowsiness, irrational behavior, and, in severe cases, potentially fatal cardiac arrhythmias (420). Potassium appears to moderate the effect of increased sodium intake on elevating blood pressure, probably by affecting renal sodium excretion (410). The minimum requirement for potassium is based on the need for 1600 mg/day to maintain normal body stores and plasma levels (420). Dietary potassium is not toxic if sufficient water is ingested and renal function is adequate to maintain homeostasis; symptoms of hyperkalemia from dehydration or acute renal failure are similar to those of hypokalemia, including muscle weakness, fatigue, and paralysis (74). The U.S. EPA has not derived any toxicity values for potassium.

Selenium

Selenium is widely distributed in nature and found in combination with sulfides and other minerals (20, 64). It has semiconducting properties and is used in photocopying machines, light meters, and rectifiers; cadmium selenide is a pigment used for car taillights; and it is used in agriculture and personal care as a component of fertilizers, pesticides, animal feeds, and antidandruff shampoo (20, 31, 196). Although selenium has long been known to protect vitamin E-deficient rats from liver necrosis (512) a specific biochemical role was not elucidated until Rotruck et al. (487) demonstrated it to be an essential constituent of glutathione peroxidase. This enzyme protects polyunsaturated fatty acids in the cell

membrane from oxidative damage caused by free radicals. Its identification in human erythrocytes established selenium as an essential trace element in human nutrition (46). Selenium deficiency has been ident

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ified as the major causal factor in the potentially fatal cardiomyopathy affecting young children and women of child-bearing age in the Keshan region of the People's Republic of China (303). A diet based primarily on local produce grown in the selenium-poor soil resulted in a selenium deficiency, alleviated by supplementing the diet with selenium-fortified table salt (123). Additional evidence for essentiality in humans is provided by the observed cardiomyopathy seen in patients maintained on long-term total parenteral nutrition (292, 355, 594). Selenium also plays an important role in the control of thyroid hormone (70), which is essential for normal growth, development, and metabolism. The selenoenzymes, iodothyronine deiodinases, are responsible for the activation of thyroxine (T4) to triiodothyronine (T3), and a selenium deficiency may cause reduced growth rates. The RDA for selenium was derived from the intake associated with a plateauing of plasma glutathione peroxidase activity in Chinese adult males (40 μg/day) (625), adjusted for differences in body weight between the reference Chinese and North American male, with an additional safety factor of 1.3 to account for individual variation (346, 420). The RDA for selenium is 70 and 55 μg/day in males and females, respectively with additional recommendations of 10 μg/day during pregnancy and 20 μg/day during lactation. Selenium is readily absorbed from the gastrointestinal tract, and the average U.S. diet typically provides 60–150 μg/day (539), which should be adequate to prevent cardiomyopathy in the general population.

Selenium toxicity has long been observed in cattle grazing on milk vetch (legumes of *Astragalus* species) grown in the seleniferous soils of Wyoming and South Dakota (246, 299, 374, 408). Acute intoxication in livestock is known as "blind staggers" and is characterized by signs of CNS impairment (ataxia, impaired vision, disorientation), and respiratory distress. Chronic exposure to moderately toxic selenium levels is known as "alkali disease" and results in skin lesions with alopecia, hoof necrosis and loss, growth retardation, anemia, and cardiac atrophy. In humans, chronic sublethal selenium toxicity has been observed in individuals living in seleniferous areas and is characterized by hair or nail loss, thickened or brittle nails, garlicky breath, tooth decay (235, 236), skin lesions, gastrointestinal disorders, and CNS abnormalities including peripheral anesthesia, acroparesthesia, and pain in the extremities (503, 534, 625). It has also been reported following the ingestion of superpotent selenium dietary supplements, and consumers need to be aware of its potential for toxicity (253, 289). The deterioration of keratinized tissue is thought to result from the replacement of sulfur with selenium in methionine, cystine, and other sulfurcontaining amino acids. Acute selenium intoxication resulting from ingestion is rare in humans (204), but has been reported following suicidal, accidental, and homicidal exposure (102, 323, 364, 490). Symptoms include gastrointestinal disturbances due to the irritative properties of selenium, a characteristic garlicky breath from the exhalation of dimethyl selenide (384), formication of the nose, signs of rhinitis, neurological symptoms ranging from mild tremors to myoclonic jerks, and cardiovascular shock. Acute inhalation of hydrogen selenide has been reported to cause severe dyspnea with abnormal pulmonary function tests (501), and chronic inhalation of the gas leads to garlicky breath, gastrointestinal disturbances, dental caries, nail deformities, and conjunctivitis (24). Chronic overexposure to selenium has been associated with the motor neuron disease amyotrophic lateral sclerosis (329, 599). Although selenium is known to be an avian teratogen (198, 264, 408) there is inconclusive evidence linking it to mammalian teratogenesis (287, 521); Yang et al. (624, 625) did not observe teratogenesis in babies during epidemiological studies in seleniferous regions where malformed chicks hatched from local eggs.

The U.S. EPA has established a chronic oral RfD for selenium using the study of Yang et al. (624) and corroborated by Longnecker et al. (357). The NOAEL of 0.85 mg Se/day was converted to a dose of 0.015 mg/ kg/day (based on an average adult body weight of 55 kg), and an RfD of 5 μg/kg/day was derived using an uncertainty factor of 3 (less than a full factor of 10 was used to account for sensitive individuals because of the availability of epidemiological data from two independent studies of moderate size) (589). Confidence in this RfD is considered high (589). The U.S. EPA has not derived RfCs for selenium. The ACGIH has adopted a TLV-TWA value for selenium of 0.2 mg Se/m3 (32).

Various animal models report a protective effect of pharmacologic levels of selenium against chemical carcinogenesis (127, 282, 473, 557). In 1969, Shamberger and Frost (520) reported an inverse relationship between cancer mortality rates in the United States and plant selenium levels as mapped by Kubota et al. (328). Subsequent epidemiological studies have reported promising but inconclusive findings (136, 273, 319). A recent randomized cancer prevention trial reports that 200 μg selenium daily did not protect against the development of recurrent skin cancers, but was inversely associated with mortality from total prostrate, lung, and colorectal cancers (125, 126). Another study reports an inverse relationship between advanced prostrate cancer and toenail selenium concentration (627), an indicator of past selenium intake. One of the authors, Gerald Combs, pointed out that "the greatest value of

epidemiology is in generating hypotheses, not testing them" (184), and stressed the requirement for further research before a beneficial effect of increased selenium intake can be established.

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Selenium sulfide has been shown to be a rodent carcinogen by the oral (417) but not dermal (416, 418) route. A 2-year gavage bioassay of selenium sulfide by the National Toxicology Program produced evidence of carcinogenicity in male rats (liver), female rats (liver), and female mice (liver and lung), but not in male mice (417). The U.S. EPA classifies selenium sulfide as a group B2 carcinogen (probable human carcinogen), based on inadequate data from human studies and sufficient evidence from rodent studies; no quantitative risk assessment was performed (589). Other selenium compounds are classified as group D carcinogens (not classifiable as to carcinogenicity in humans) based on inadequate evidence in both humans and animals (589). The suggested beneficial antioxidant effects of selenium and the potential widespread use of selenium supplements make it important to gain a fuller understanding of selenium toxicology.

Sodium

Sodium is a highly reactive soft metal with a silver appearance that is not found in the elemental form in nature (326). Sodium compounds are ubiquitous in nature. Elemental sodium must be stored under airtight anhydrous conditions, such as under oil, to prevent oxidation, which can produce autoignition at room temperature. Superoxides may form resulting in a violent explosion. Dermal and ocular thermal burns and liquefaction necrosis due to the formation of sodium hydroxide are the primary effects following sodium exposure. Explosion may cause particles to imbed in the skin and eye requiring surgical debridement. Water irrigation is contraindicated.

Sodium is the principal cation of extracellular fluid and the primary regulator of extracellular fluid volume. Sodium also regulates osmolarity, acid-base balance, and membrane potential, and participates in active transport across cell membranes. Renal excretion of sodium maintains homeostasis over a wide range of intakes and losses, via aldosterone control of tubular excretion. Sodium deficiency is very uncommon but may occur after heavy and prolonged sweating, chronic diarrhea, or renal disease, and constitutes a medical emergency. Dietary sodium is not toxic if sufficient water is ingested and renal function is adequate to maintain homeostasis (420). Lifelong excess intake of sodium may predispose sensitive individuals to hypertension, and individuals diagnosed with high blood pressure are commonly advised to limit sodium intake to 1–2 g/day or less (410). At present, the public health benefit of restricting sodium intake in the general population is not firmly established (420). The U.S. EPA has not derived any toxicity values for sodium.

Zinc

Zinc is a bluish-white, soft metal extracted from ore and is used in alloys, for galvanizing iron to prevent corrosion and oxidation, and in numerous compounds including use in cosmetics, pharmaceuticals, and dry-cell batteries (190). At temperatures approaching its boiling point, zinc volatilizes and oxidizes to the white fume of zinc oxide (31). Zinc is an essential trace element and is a required component of many enzymes (420). Zinc is stored in bone and muscle, but is not readily released from these stores during deficiency. Gastrointestinal absorption of zinc is higher when body stores are lower, and is also higher from more refined diets. Zinc deficiency causes loss of appetite, growth retardation, and slow wound healing; no single enzyme function has been identified as associated with these signs of zinc deficiency. Severe zinc deficiency causes hypogonadism and dwarfism, which are alleviated with zinc supplementation. The RDA for zinc is derived from an estimated 2.2–2.8 mg daily loss and an oral absorption fraction of 20%, plus a 20% safety factor, leading to a recommendation of 15 mg/day for adult men and 12 mg/day for adult women, with an additional 3 mg/day during pregnancy and 7 mg/day during lactation (420).

Inhalation exposure to zinc oxide fume can cause metal fume fever (31). Zinc chloride fume is a corrosive material that has caused chemical pneumonitis, alveolar and bronchial obliteration, and death (21). Zinc compounds are absorbed orally and excreted primarily in the feces. Zinc has low human toxicity by the oral route, but high levels can cause gastrointestinal (GI) distress (21). Long term oral intakes of zinc at levels of 18.5–25 mg/day can interfere with copper absorption, and intakes 10–30 times the RDA can impair immune responses and decrease serum high-density lipoprotein (420). The U.S. EPA has derived an oral RfD for zinc of 0.3 mg/kg/day (589). No inhalation RfC has been derived for zinc. The U.S. EPA has classified zinc as a group D carcinogen (not classifiable as to human carcinogenicity) based on inadequate evidence in humans and animals. The ACGIH has adopted TLV-TWA values for zinc of 10 mg/m3 for zinc dust, 5 mg/m3 for zinc oxide fume, and 1 mg/m3 for zinc chloride fume (32).

A summary of quantitative values for essential elements is given in Table 14.1. Elements are listed in the order of highest to lowest dietary requirement.

MAJOR TOXIC METALS

Arsenic
Arsenic is a Group VA element of the periodic table, the 52nd most abundant element in the earth's crust.

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Table 14.1 Essential elements

Element	Recommended intake (mg/kg/day)*a*	Chronic oral toxicity RfD*b* (mg/kg/day)	Confidence	Chronic inhalation toxicity RfC*c* μg/m3)	Confidence	Carcinogenicity Inhalation slope factor [risk/(μg/m3)]	Classification*d*
Potassium	30	–	–	–	–	–	–
Calcium	20	–	–	–	–	–	–
Phosphate	20	–	–	–	–	–	–
Chloride	10	–	–	–	–	–	–
Sodium	7	–	–	–	–	–	–
Magnesium	5	–	–	–	–	–	–
Iron	2×10^{-1}	–	–	–	–	–	–
Zinc	2×10^{-1}	3×10^{-1}	Medium	–	–	–	D
Manganese	$3–7\times10^{-2}$	1.4×10^{-1}	Medium	5×10^{-2}	Medium	–	D
Fluoride	$2–6\times10^{-2}$	6×10^{-2} (Cosmetic)	High	–	–	–	–
	–	12×10^{-2} (Adverse)	High	–	–	–	–
Copper	$2–4\times10^{-2}$	–	–	–	–	–	D
Iodide	2×10^{-3}	–	–	–	–	–	–
Molybdenum	$1–4\times10^{-3}$	5×10^{-3}	Medium	–	–	–	–
Chromium	$0.7–3\times10^{-3}$	1.5 (Cr^{3+})	Low	–	–	(Cr^{3+}	D(Cr^{3+}
	–	3×10^{-3} (Cr^{6+})	Low	8×10^{-3}*e*	Low	1.2×10^{-2} (Cr^{6+})	A(Cr^{6+})
	–	–	–	1×10^{-1}*f*	Medium	–	–
Selenium	1×10^{-3}	5×10^{-3}	High	–	–	–	B2 (SeS_2) D (all)
Cobalt	1×10^{-6}*g*	–	–	–	–	–	–

a For a 70-kg adult; see text for actual values.
b RfD, reference dose.
c RfC, reference concentration.
d Group A, known human carcinogen; group B2, probable human carcinogen; group D, not classification as to human carcinogenicity.
e Chromic acid mists and soluble Cr(VI) aerosols
f Chromium(VI) particulates
g Based on cobalt content of vitamin B12.

Arsenic is refined from the minerals arsenopyrite and loellingite, or it can be prepared from the reduction of arsenic trioxide. The main use of arsenic in the United States is in the production of herbicides and other agricultural chemicals. Arsenic is also used in the semiconductor industry (32). Although arsenic can exist in several valence states, the +3 and +5 states are the most prevalent, with arsenic (+3) being more toxic than arsenate (+5) (172). Dietary consumption of arsenic is generally low; the typical daily American intake is 145 μg/day from both food and water (60). However, consumption of seafood can increase the amount of arsenic ingested (278, 570).

Arsenic is readily absorbed via the gut (63), and excretion occurs primarily in the urine (93, 141, 369, 549). Two processes are involved in the metabolism of arsenate and arsenite; the interconversion of arsenate and arsenite, and the conversion of these moieties to monomethyl arsenic acid and dimethyl arsenic acid. Because the meth ylated forms of arsenic are less toxic and because methylation results in lower tissue retention of inorganic arsenic, the methylation process is viewed as a detoxification mechanism (15).

Arsenic is believed to exert its toxic effects through at least two mechanisms, depending on its valence state. Arsenate inhibits ATP synthesis by uncoupling oxidative phosphorylation, whereas arsenite reacts with thiol groups on the active sites of many enzymes and tissue proteins, such as keratin (i.e., skin, nails, and hair) (550). Because of this reactivity with thiol groups, arsenic concentrates in the skin, hair, and nails. Mee's lines (horizontal white lines on the fingernails) appear in exposed individuals after the exposed nail bed grows to the exterior (390). At one time, inorganic arsenic was widely used as a "criminal poison" because it was odorless and nearly tasteless. The lethal dose of arsenic trioxide can be as low as 0.2 g (222). Acute toxicity is characterized by severe gastrointestinal symptoms, which

occur from 30 min to several hours after ingestion. Eventually, severe gastrointestinal hemorrhaging occurs, leading to profound losses of fluid and electrolytes, resulting in collapse, shock, and death (222). If the victim survives the initial toxic sequelae, jaundice, renal failure, and peripheral neuropathology can develop (172, 222).

In cases of acute intoxication, chelation therapy can be very effective in reducing or preventing symptoms. The agent of choice is British anti-lewisite (BAL), which is dimercaptopropanol (620). D-Penicillamine is also effective as a chelating agent, but nephrotoxicity and optic neuritis can result from long-term use (172). Therefore, BAL remains the treatment of choice in arsenic poisoning (222).

Chronic ingestion of arsenic can be difficult to diagnose. Diarrhea and abdominal pain can occur, as well as hyperpigmentation, hyperkeratosis, and numerous other skin- and hair-related disorders (48, 101, 172, 267, 553). Peripheral vascular occlusive disease has also been linked to chronic exposure to high levels of arsenic in drinking water in Chile (Raynaud's phenomenon) (83) and in Taiwan (blackfoot disease) (566). Neurological changes have been associated with occupational inhalation exposure to inorganic arsenic by smelter workers (81, 185, 257). Neurologic changes included peripheral neuropathy of sensory and motor neurons, as measured by motor and sensory deficits (406) and encephalopathy, as evidenced by hallucinations and other psychological disturbances (61).

Chronic exposure to arsenic in drinking water is associated with an increased incidence of cancer. Numerous studies have been conducted in Taiwan, comparing residents in the blackfoot disease endemic area with residents in areas with low levels of arsenic in drinking water. These studies have consistently shown an increase in the incidence of skin cancer and several internal cancers in areas with high arsenic consumption (119–122, 566, 567, 621). A similar study was conducted in Japan, which showed an association between high levels of ingested arsenic and lung and urinary-tract cancer (569). Chronic exposure to arsenic via the inhalation route is also associated with the development of tumors. Studies of smelter worker populations have shown strong associations between exposure and an increased incidence of lung cancer (47, 175, 341, 476, 560), as have studies of pesticide manufacturing workers (362, 451) case reports of lung cancer in arsenical pesticide applicators (486). Although the carcinogenicity of arsenic is well established in humans, carcinogenicity in animal models has been more difficult to establish. Of the many animal carcinogenicity studies reviewed by the IARC in 1980 (570), only two gave positive results: one with subcutaneous/intravenous administration of sodium arsenite in mice in a multigenerational study (450), and one with intratracheal installation of copper and calcium arsenate in rats (285). Later studies showed that both calcium arsenate and arsenic trioxide are carcinogenic when administered intratracheally to Syrian golden hamsters (284, 461, 462). The mechanism of carcinogenicity is postulated to be related to the multistep metabolism of pentavalent arsenic to dimethyl arsenic acid, during which free radicals are produced (63, 550).

Arsine, AsH3, is a gaseous form to arsenic that is formed whenever arsenic is in the presence of hydrogen (222) and as such can be generated in metal tanks storing acids that contain arsenic impurities (63). The toxicity profile of arsine is different from all other arsenic compounds. The hallmark of arsine toxicity is hemolysis, sometimes followed by acute renal failure (222). BAL and D-penicillamine are not effective treatments for arsine poisoning (222).

The U.S. EPA has established the following RfDs for arsenic compounds: inorganic arsenic, 0.3 μg/kg/day (group A (human) carcinogen); arsine 0.05 μg/m3 (589). The ACGIH has adopted the following TLVs for arsenic compounds: arsenic, elemental arsenic, and inorganic compounds, as As, 0.01 mg/m3 (A1 carcinogenicity notation; confirmed human carcinogen); arsine, 0.05 ppm (32).

Cadmium

Cadmium is a soft silver-white metal, often found in association with zinc and obtained primarily as a by-product of zinc preparation (63). It is used primarily in the production of nickel-cadmium batteries (35%), but also for metal plating (30%), pigments (15%), plastics and synthetics (10%), and miscellaneous uses (10%) (16). The toxicity of cadmium has been widely investigated, and cadmium has been shown to affect nearly every organ system if the dose is high enough (71). Acute effects of cadmium depend on the route of exposure. Symptoms of acute inhalation exposure to cadmium develop 4–10 h postexposure and initially simulate metal fume fever (fever, nausea, vomiting, headache, cough, dyspnea, nasopharyngeal irritation), but with progression to chemical pneumonitis and a potentially fatal pulmonary edema (71, 76, 79, 164). A fatal dose can be inhaled by exposed individuals who are unaware of either the presence of cadmium or its inhalation hazard (71, 360). Cadmium absorption following inhalation exposure is dependent on particle size and solubility and ranges from 20 to 50% of the amount inhaled (200). Fatal doses have been estimated at 50 mg/m3 for 1 h (57, 96) and 9 mg/m3 for 5 h (71). Recovery following acute high-level exposure or chronic exposure at lower levels may be

accompanied by pulmonary fibrosis (56, 144, 562). Oral exposure to cadmium is rarely fatal because the gastrointestinal irritation leads to vomiting, eliminating most of the dose before absorption (52, 95, 361, 440, 526). Gastrointestinal

absorption is about 5%, but can reach 20% with concurrent calcium or iron deficiency (192, 200). Rats exposed by intravenous injection to 4 mg Cd/kg developed a potentially lethal hepatic necrosis (162, 213).

Chronic inhalation or ingestion of cadmium results in kidney damage, characterized by tubular and/or glomerular dysfunction with proteinuria, low concentration capacity, and decreased inulin clearance (200). Increased urinary excretion of β2-microglobulin, a low-molecularweight protein normally reabsorbed in the proximal tubule, is an early indicator of renal dysfunction and should be regarded as an adverse effect because it is predictive of an increase in the age-related decline in the glomerular filtration rate (484). Absorbed cadmium is first transported to the liver, where it stimulates the synthesis of metallothionein and is sequestered as cadmium-metallothionien. Small amounts of liver cadmium-metallothionein are released into the plasma following normal cell turnover, filtered with the primary urine, and reabsorbed into the proximal tubular cells, where lysosomes degrade the metallothionein portion, with the release of cadmium, which then induces renal metallothionein synthesis. Renal damage results when the kidneys can no longer produce sufficient metallothionein to sequester the cadmium ion and prevent its interaction with critical macromolecules (224). Free cadmium may inactivate metalloenzymes, activate calmodulin, and/or damage cell membranes through activation of oxygen (602). This threshold level is commonly called the *critical concentration*. Kjellström et al. (314) proposed the term *population critical concentration* (PCC), where a PCC-10 indicates the cadmium concentration in the renal cortex that is likely to result in renal dysfunction in 10% of an exposed population—that is, 10% of the exposed population will have exceeded their individual critical concentrations. They estimated the PCC-10 to be 180–220 μg/g and the PCC-50 to be 25% higher. Excess inhalation or ingestion exposure to cadmium leads to abnormalities of calcium metabolism, and susceptible individuals may develop a painful bone disease as first discovered in a cadmium-contaminated area in Japan (Toyama Prefecture) and termed itai-itai (ouch-ouch) disease (412, 568). The disease is characterized by osteomalacia and osteoporosis with an increased tendency to spontaneous fracture and is associated with bone pain and renal tubular dysfunction. Cadmium has been shown to increase bone resorption and inhibit bone formation in both in vivo and in vitro systems (73). Current knowledge suggests that bone changes associated with pregnancy, lactation, and menopause may enhance cadmium's effect on bone. There is conflicting evidence concerning cadmium exposure and the risk of developing hypertension, with human studies reporting either positive, negative, or no effect (16, 413). This suggests that a cadmium effect on blood pressure is small to nonexistent compared to other established risk factors. Cadmium-exposed populations are not reported to have elevated death rates associated with cardiovascular disease. Maternal and fetal toxicity of cadmium is well documented in rodents (16, 453). Elevated levels of cadmium in neonates are associated with a decreased birth weight (271), but further research is required to determine if developmental effects of cadmium are of concern at environmental levels. Tobacco plants are known to concentrate soil cadmium, and it is estimated 1-pack/day smokers can absorb 1–3 μg cadmium/day (347). Pregnant smokers have an increased cadmium concentration in both maternal and cord blood (117), and cadmium-metallothionein mobilized into the serum has been suggested to be the toxic serum factor associated with preeclampsia (124). High doses of parenteral cadmium will induce testicular necrosis in male rodents, and this effect is thought to be related to cadmium inhibition of a testes-specific enzyme (16). Pretreatment or concurrent treatment with various substances, including zinc and selenium, will inhibit the acute toxic effect of cadmium, but the precise protective mechanism is unknown (527).

The U.S. EPA has derived a chronic oral RfD for cadmium based on an early version of the toxicokinetic model for cadmium (201). Separate values were derived for food and water exposure, assuming 2.5% absorption of cadmium from food and 5% from water with a 0.01% per day excretion, and a kidney concentration of 200 μg Cd/g wet human renal cortex is considered the NOAEL (589). An uncertainty factor of 10 was used for intrahuman variability, and the resulting RfD values are 0.001 mg Cd/kg/day (food) and 0.0005 mg Cd/kg/day (water); confidence in these values is considered high. No reference concentration values for chronic cadmium inhalation exposure were calculated. The Agency for Toxic Substances and Disease Registry (ATSDR) has calculated chronic minimal risk levels (MRLs) for cadmium based on human studies with measured exposures (16). The inhalation MRL was calculated from a NOAEL for renal effects in workers exposed to 0.0016 mg/m3 (288); adjusting for continuous lifetime exposure and using an uncertainty factor of 10 to account for sensitive members of the population, the chronic inhalation MRL is 0.0002 mg/m3. A chronic oral MRL of 0.0007 mg/kg/day was calculated from a study in a Japanese population exposed to cadmium in rice (438). The average nonsmoking American absorbs approximately 1–3 μg Cd/day from the diet (16), which is only 2 to 4 times lower than the oral

MRL, indicating that there is not a large margin of safety with respect to cadmium toxicity, particularly given evidence that postmenopausal women and diabetics may be more sensitive to cadmium toxicity than members of the general population (94).

The carcinogenicity of cadmium is the subject of much discussion. In animals, inhalation exposure causes lung cancer in rats but not in mice or hamsters (211, 252, 547). In humans, increases in lung cancer rates were associated with cadmium exposure in one cohort (558), but not in three others (4, 170, 535); in these same cohorts, early indications of an increase in prostate tumors were not borne out by longer term follow-up. The role of arsenic and/or smoking in the cohort with the increased rate of lung cancer is controversial (156, 331, 542). Studies investigating environmental cadmium and its role in prostate cancer have been hampered by a lack of dose quantification (2, 53, 611), and results from studies with occupational exposure have been mixed (315, 536). In animals, older studies found no significant increase in tumors following lifetime oral exposure to cadmium, but the doses in these studies were relatively low and histological examination was limited compared to present standards (133, 358, 502). A more recent study reports prostate tumors in male rats after oral cadmium exposure (603). Further research is required to establish the role of cadmium in human prostate cancer (604). Cadmium is genotoxic (248); it has been reported to induce apoptosis, which may serve to remove critically damaged cells (234, 241). The International Agency for Research on Cancer (IARC) accepts cadmium as a category 1 (human) carcinogen (281) based primarily on its role in lung cancer (542). The U.S. EPA classifies cadmium as a group B1 carcinogen (probable human carcinogen) (589) based on evidence in humans and animals (547, 558). An inhalation unit risk of 1.8×10^{-3} per ($\mu g/m^3$) was calculated from the study of Thun et al. (558).

If future research were to establish that present levels of exposure to cadmium carry an unacceptable risk of either noncancer or cancer effects, difficult questions would arise. The primary difficulty would be how to reduce exposure; the main source of cadmium exposure is the diet, but reducing this exposure, by removing cadmium from fertilizer, would be costly and difficult. Another policy issue is whether cigarette smokers should be considered a sensitive subpopulation deserving extra protection, so that exposure must be controlled to levels that would be safe for smokers, or whether smokers should be considered individuals with a demonstrated lack of concern for, and need for protection from, adverse health effects.

Lead

Lead is a heavy, bluish-gray metal and, although it serves no biological purpose, is the most widely used nonferrous metal (17, 187). Lead and/or lead compounds have been used in many industrial applications, including batteries, ammunition, paints and varnishes, gasoline, pigments, radiation shields, medical equipment, solder, glass, and ceramic glazes (17). Inhalation and ingestion are the main routes of exposure for inorganic lead (261). Adults are primarily exposed occupationally (17), and this occurs by inhalation with 35–40% of inhaled lead dust or fumes deposited in the lungs with extensive (95%) blood absorption (342). Children are primarily exposed by ingestion and absorb 50% of an ingested dose through the gastrointestinal tract. In contrast, adults absorb 10% of an ingested dose, but gastrointestinal absorption will vary with particle size (inverse proportion), solubility, nutritional status, and fasting. Excretion is mainly in the urine, with lesser amounts in the feces, sweat, hair, and nails.

In adults, early symptoms are often nonspecific (fatigue, depression, sleep disturbance, anorexia, intermittent abdominal pain, nausea, constipation, diarrhea, and myalgia) (261). Blood lead level (PbB) is the single best diagnostic test for lead exposure (261). Epidemiologic studies and animal experimentation suggest PbB levels as low as 14 μg/dl may elevate blood pressure (245, 510), but the results are not definitive (256). A recent study (269) suggests that long-term lead accumulation—measured as bone lead, as opposed to PbB, which reflects recent exposure—is associated with developing hypertension. However, other researchers report no consistent effect (540), and the subject remains controversial. Reversible slowing of nerve conduction velocity has been observed at PbB as low as 30 μg/dl (519), and adverse effects on reaction time, mood, and visual-motor coordination at 30–50 μg/dl (51, 544). Anemia is not seen until PbB are in excess of 50 μg/dl (270). Overt neurotoxicity (wrist drop) is reported at levels in excess of 80 μg/dl (219). Chronic irreversible nephropathy requires high and sustained PbB exposure (225), but recent evidence suggests low-level lead exposure (PbB<10 μg/dl) is associated with renal impairment, as measured by an increase in serum creatinine (308). Morphological alterations and decreases in sperm count, density, and motility have all been reported in heavily exposed males (PbB>40 μg/dl) (42, 45, 345, 572). Paternal occupational lead exposure has been reported to increase the risk of low birth weight and prematurity (349). Lead readily crosses the placenta to the fetus (305, 482), and maternal PbBs in excess of 15 μg/dl are associated with low birth weights and preterm delivery, and PbB in excess of 30 μg/dl with spontaneous abortions (176, 180, 485).

Lead poisoning in children caused by ingestion of lead paint was first noted in Australia and became

recognized as a public health problem in the United States in the 1920s (351). Early symptoms of chronic poisoning in children are often nonspecific, including headaches, anorexia, vomiting, and constipation, progressing to anemia with

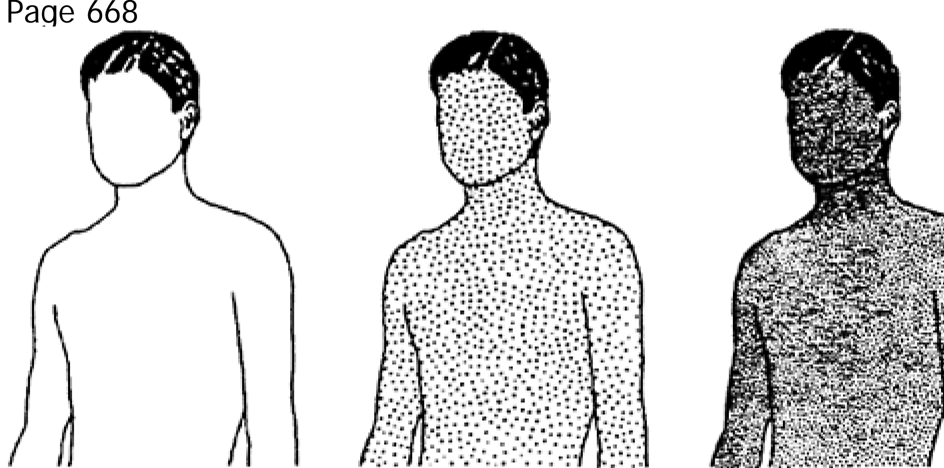

FIG. 14.1. Comparison of lead body burden (from left to right): ancient people uncontaminated by industrial lead (1 dot); typical American (1000 dots); level associated with clinical lead poisoning (4000 dots). Each dot represents 40 μg Pb/70 kg person. Reprinted from Patterson et al. (455), *Sci. Total Environ.*, copyright 1991, pp. 205–236, with permission from Elsevier Science.

basophilic stippling of red cells, Burton's line, chronic nephritis, peripheral neuropathy (manifested as wrist and/or foot drop), and radiographs of long bones revealing lead deposits (442, 459). Frank encephalopathy (PbB>80 μg/dl) is characterized by ataxia, coma, convulsions, cerebral edema, and even death. The long-term neurologic consequences of childhood lead poisoning were recognized in 1943 when Byers and Lord follow up 20 "cured" cases and found poor academic performance in all but 1 (98). In 1975, de la Burde and Choate (147) reported school failure due to learning and behavioral problems in asymptomatic lead-exposed children. Asymptomatic children with elevated dentine lead levels in first and second grade scored lower on standardized tests, especially in areas measuring verbal performance and auditory processing, and were more likely to exhibit disruptive behavior relative to controls (152, 427). Reexamined 11 years later as adolescents, those with greater lead exposure were more at risk for dropping out of school, reading disability, absenteeism, poor hand-eye coordination, and low scholastic class standing (66, 428, 511, 561).

Since 1970, the CDC has repeatedly lowered the level of concern for PbB from 70 to 10 μg/dl, and the following federal laws have been enacted to reduce lead exposure: the 1971 Lead-Based Paint Poisoning Prevention Act (575); the U.S. EPA phaseout of lead in gasoline, starting in 1973 (581) with completion in 1995 (587); the U.S. EPA ban on lead in plumbing, fixtures, fittings, and solder (576); the Consumer Product Safety Commission (CPSC) 1978 ban on the use of paint containing more than 0.06% lead by weight for interior/exterior residential surfaces, toys, and furniture (579); the FDA ruling to eliminate lead-solder in food cans by December 1995 (590); and the Residential Lead-Based Paint Hazard Reduction Act of 1992 (577). Results of two National Health and Nutrition Examination Surveys, NHANES II (1976 to 1980) and NHANES III (phase I, 1988 to 1991; phase II, 1991 to 1994), indicate a substantial decline in PbB (111, 216, 464). Since the late 1970s, the average PbB in children 1–5 years of age has declined from 15 μg/dl to 2.7 μg/dl, and the reduction of lead in gasoline and dietary sources (primarily through the former use of lead-soldered cans for food and beverages) is believed responsible for this effect (88, 464). Although this decline is substantial, Patterson et al. (455) estimate today's average American has a mean body burden of 40 mg industrial Pb/70 kg, whereas analysis of pre-Columbian American Indian skeletons indicates their mean body burden was 40 μg Pb/70 kg (Figure 14.1). Based on bone: blood lead ratios, this equates with a PbB of 0.016 μg/dl, which is 600-fold lower than the current level considered acceptable for children (i.e., 10 μg/dl) (193) and places current guidelines much closer to lethal Pb levels than natural (529). It is estimated that 890,000 (4.4%) U.S. preschool children have a PbB of 10 μg/dl or higher (111). Further reductions in PbB will require primary prevention efforts to reduce exposure to lead remaining in housing and soil (334, 464, 530). The Centers for Disease Control and Prevention (CDC) 1997 lead poisoning prevention program recommends targeted screening and follow-up care for high-risk children (i.e., children who live in older-homes, children from low-income families) (111). Although controversial (367), it reserves universal screening of young children to those meeting at least one of the following criteria: (a) child resides in ZIP code where at least 27% of the housing predates 1950; (b) child receives public assistance for the poor; or (c) caretaker's response to a risk assessment questionnaire suggests child is at risk.

The use of car radiators containing lead solder for the illegal distillation of alcohol (moonshine) has long been associated with lead poisoning (178), and recent reports suggest middle-aged men in rural settings continue to be at risk (173, 456). Excessive PbB (>40 μg/dl) have been reported in automobile radiator repair mechanics, and "take-home" lead is a potential source of elevated PbB in their children (217), as has been reported in other lead-related industries (300). Apart from congenital intoxication, lead poisoning in infants has been reported from the use of traditional folk remedies, which are often known by their common names of azarcon, greta, and ghasard (104–107); the use of lead-contaminated water to prepare formula (523); use of a lead-soldered samovar (urn) for formula preparation (522); and household renovation (110). Lead as galena (PbS) was the main constituent of eye cosmetics (kohls) in Oman (243) and may contribute to lead poisoning in households with children practicing pica. Lead poisoning in children with pica has been reported following the ingestion of foreign objects including an imported clothing accessory (177), fishing sinkers (407), and curtain weights (77). The dissolution of retained lead gunshot has resulted in lead poisoning (306, 368), with rapid onset when the bullet lodges in contact with synovial fluid (392). Lead in crystal leaches into alcoholic beverages, and lead contents as high as 21.5 mg/L have been reported in beverages stored in crystal decanters (227). The lead content of various calcium supplements (bonemeal, dolomite, calcium carbonate) has been tested, and levels of supplementation providing 800 mg calcium would also contain over 6 μg lead in over one-quarter of the 70 different brands tested (85, 614). Although cases of lead intoxication by this route have not been reported, pregnant women and children are the populations most at risk from this source. Lead-contaminated heroin has been reported as a source of lead intoxication, and physicians need to be aware of this possibility (454).

The primary treatment for lead poisoning is to identify and eliminate exposure. For children with venous PbB >45 μg/dl the CDC (108) currently recommends chelation therapy. In 1991 the U.S. FDA approved the use of *meso*-2,3-dimercaptosuccinic acid (DMSA, Succimer), which is an effective oral chelating agent and is more specific for lead than CaNa2EDTA, the use of which is associated with urinary loss of essential trace elements. PbB ranging from 20 to 44 μg/dl is treated with chelation therapy if a provocative test indicates substantial lead can be mobilized. Pharmacological treatments are not currently available for PbBs<20 μg/dl. Iron deficiency is associated with increased lead absorption (365) and should be treated in all cases (36).

The adverse health effects associated with lead occur at PbBs so low as to be without a threshold, and the U.S. EPA considers it inappropriate to derive RfD values for lead (589). The U.S. EPA classifies lead as a group B2 carcinogen (probable human carcinogen) based on sufficient evidence in animals with dietary and subcutaneous exposure and inadequate evidence in humans. The U.S. EPA considers it inappropriate to quantify the carcinogenic risk from oral exposure because toxicokinetic differences between humans and animals cannot be taken into account using the standard procedures.

Mercury

Mercury is a silver-white fluid trace metal found in igneous and sedimentary rocks and in the form of the ore cinnabar (mercury sulfide) (63). It is biologically nonessential and toxic to all organisms. Mercury may occur in the elemental form, or as inorganic and organic compounds. Mercury and its compounds are used in electrical meters, chloralkali production, thermometers, and as antimicorbial preservatives in paints, cosmetics, and pharmaceuticals. Use in dry-cell batteries is now restricted due to environmental toxicity concerns following disposal (578, 588). Use in interior latex paints is prohibited (584). Toxicity is related to the covalent binding of mercury to sulfhydryl groups, as well as to carboxy, amide, amine, and phosphoryl groups, thereby inactivating cellular functions (100).

Acute elemental mercury ingestion is usually of no significance due to poor absorption from the GI tract. Acute exposure to high concentrations of elemental mercury vapors are irritating to the respiratory tract. Chronic exposure to the vapors produces CNS toxicity, which includes muscle weakness and tremors, nervousness, memory loss, and anorexia. Inorganic mercury compounds generally demonstrate local irritant or corrosive activity. Acute ingestion may result in necrosis to the GI tract and renal tubular necrosis. Chronic effects produce CNS toxicity similar to that noted for elemental mercury. Organic mercury compounds have been used for treatment of syphillis and as diuretics, but have been replaced by less toxic drugs. Organic mercury compounds, such as phenylmercuric acetate, thimerosal, and mercurochrome, are primarily used as antimicrobial preservatives in ophthalmic preparations, vaccines, and nasal sprays (100). Contact dermatitis may occur to both inorganic and organic mercurials, with cross-sensitivity to each being reported (18, 188). Elemental mercury and its compounds are excreted in urine, feces, and through respiration.

Dietary intake, from agricultural products treated with mercurial fungicides and fish from mercury-

polluted water, is the major route for toxicity of organic mercury compounds. Metallic and inorganic mercury can enter the air and water from rock and ore deposits, burning of fossil fuels, industrial and agricultural emissions,

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Table 14.2 Major toxic metals

Metal	Chronic oral toxicity		Chronic inhalation toxicity		Carcinogenicity		
	RfD[a] (mg/kg/day)	Confidence	RfC[b] (μg/m3)	Confidence	Oral slope factor [risk/(mg/kg/day)]	Inhalation slope factor [risk/(μg/m3)]	Classification[c]
Lead	–[d]	–	–	–	–[e]	–	B2
Cadmium	1×10^{-3} (food) 5×10^{-4} (water)	High	–	–	–	1.8×10^{-3}	B1
Mercury	–	–	3×10^{-1}	Medium	–	–	D
Arsenic	3×10^{-4}	Medium	–	–	1.5	4.3×10^{-3}	A

a RfD, reference dose.
b RfC, reference concentration.
c Group A, known human carcinogen; group B1, B2, probable human carcinogen; group D, not classifiable as to human carcinogenicity.
d U.S. EPA quantifies lead toxicity using a biokinetic model; threshold is equivalent to intake of about 5×10^{-3} mg/kg/day.
e U.S. EPA has not quantified lead carcinogenicity; slope factor would be in the range of $1–4\times10^{-3}$/(mg/kg/day).

and trash disposal and incineration. Atmospheric fallout adds to water pollution. Inorganic mercury compounds may be methylated by the microflora of soil and water to form methylmercury. Through the food chain, edible fish can concentrate methylmercury to levels a thousands times greater than in the environment (100). Methylmercury is neurotoxic, and the effects are both dose and time dependent (63). Ataxia is an early symptom, followed by slurred speech, weakness, vision and hearing loss, tremors, coma, and death. Well-documented poisonings from contaminated fish and grains occurred in Japan and Iraq (63, 100). Additionally, methylmercury is a well-known neuroteratogen (18).

Recently, the use of dental amalgam fillings has generated concern, both because exposure of dental workers may exceed occupational standards and because a variety of illnesses (multiple sclerosis, rheumatoid arthritis, leukemia, etc.) have been attributed to dental mercury exposure in the general population (41, 203, 330, 601). Contact dermatitis is experienced by some dental patients, and for those in which symptoms do not quickly subside with antihistamine treatment, replacement of fillings with nonmercury materials may alleviate immunological and dermatological symptoms (203, 330). However, other diseases have not been firmly linked to mercury exposure, and at present replacement of mercury-containing fillings cannot be justified in nonallergic individuals, while the issue of whether mercury should continue to be used for new dental fillings is more controversial (41, 203, 330). Additional research is needed before the potential for dental amalgams to cause harm, and the benefit of substituting more costly or less durable materials, can be reasonably evaluated. Concern must include the potential risk of effects on the fetus (18).

The U.S. EPA has derived a chronic inhalation exposure RfC for elemental mercury (vapor) of 0.3 μg/m3 based on critical effects of hand tremor, memory disturbances, and autonomic dysfunction (acrodynia) with a medium level of confidence (589). There is no current chronic oral exposure RfD pending further review (589). The ACGIH has adopted TLV-TWA values for inorganic forms and metallic mercury of 0.025 mg/m3, for aryl mercury compounds of 0.1 mg/m3, and 0.01 mg/m3 for alkyl compounds (32). These TLV-TWA values carry a skin notation that points out the potential for dermal absorption. The ACGIH and EPA consider mercury as not classifiable as to human carcinogenicity, based on no evidence of carcinogenicity in humans and inadequate evidence in animals (32, 589).

A summary of some quantitative toxicity values for these four major toxic metals is given in Table 14.2. Metals are listed in approximate reverse order of toxicity (least toxic first).

MINOR TOXIC METALS WITH RfDs

Antimony

Antimony is a brittle silver-colored metal extracted from ores (63). Compounds of antimony cover the full range of toxicity. Less toxic compounds have found use in cosmetic pigments (antimony sulfide) and medicinals (antimony potassium tartrate; tartar emetic). Stibine, the metal hydride of antimony, is a colorless

highly toxic gas used in the manufacture of semiconductors (63). Ingestion of antimony compounds can cause gastrointestinal, cardiac, dermatological, hepatic, and neurological toxicity in humans and animals (9). Mechanisms for these effects include binding to sulfhydryl groups and inhibiting protein and carbohydrate metabolism (380). Acute inhalation exposure to antimony trichloride or antimony pentachloride may cause pneumonitis, but the injury may be caused by the chloride rather than by the antimony itself; acute exposure to antimony hydride can cause hemolysis, but, again, the antimony itself may not be responsible (430). Long-term inhalation exposure to antimony can cause benign pneumoconiosis (430) and may raise blood pressure (9). Dermatological reactions to antimony (eczema, pustules) exhibit signs of an acute inflammatory response but do not appear to be an allergic reaction (380). The U.S. EPA has derived a chronic oral RfD for antimony based on the study of Schroeder et al. (508), which found changes in blood glucose and cholesterol levels in rats exposed to antimony in drinking water at a dose of 0.35 mg/kg/day. This LOAEL was converted to an RfD of 0.4 μg/kg/day using an uncertainty factor of 1000 (10 for interindividual variation, 10 for interspecies variation, and 10 for use of a LOAEL rather than a NOAEL). Confidence in the RfD was considered low (589). However, a recent study of potassium antimony tartrate in rats following 90-day exposure via drinking water gave a NOAEL level of 0.06 mg/kg/day (467). Using a 100-fold safety factor, as just described, 0.6 μg/kg/day approximates the RfD. The U.S. EPA has not derived an inhalation RfC for antimony (589). The ACGIH has adopted a TLV-TWA value for antimony of 0.5 mg Sb/m3 (32).

The carcinogenicity of antimony is uncertain. Mice given antimony potassium tartrate in drinking water at a dose of 0.88 mg/kg/day for 33 months had no increased incidence of lung, liver, or total tumors (297). Workers exposed to antimony concentrations well over 5 mg Sb/m3 had an increased risk of lung cancer (155); however, exposure to arsenic may have caused the excess (380). Female rats exposed to >30 mg Sb/m3 had an increased incidence of lung tumors (433). A chronic inhalation oncogenicity study in rats of antimony trioxide dust at doses less than 30 mg Sb/m3 did not show carcinogenicity (233). Antimony has not been evaluated for human carcinogenic potential by the U.S. EPA (589). The ACGIH has classified antimony trioxide production as a suspect human carcinogen for which exposure levels should be as low as reasonably achievable (31, 32).

Barium

Barium is a silvery-white alkaline earth metal and is found in nature in combination with other elements (10). The barium ion is highly reactive and its toxicity is dependent on the solubility of the specific compound, with water-soluble forms (i.e., chloride, hydroxide, nitrate) being more toxic than insoluble forms (i.e., sulfate, carbonate). Barium compounds are used primarily as lubricating agents in drilling muds, but also in the manufacture of paints, bricks, tiles, glass, rubber, and pesticides. Barium sulfate is used medically as a contrast agent in x-ray diagnosis. Hospital staff familiar with this use may fail to recognize barium as a potential toxic agent, and this has contributed to at least one fatality (159). The general population is exposed by ingestion (i.e., food, drinking water) and inhalation. Some plants bioconcentrate barium from the soil, with brazil nuts having very high concentrations (3000–4000 ppm) (62).

Occupational exposure to inhaled barium sulfate can cause a benign pneumoconiosis (baritosis) (154), which resolves with cessation of exposure. Acute ingestion of soluble Ba2+ salts acts as a muscle poison characterized by stimulation followed by paralysis (475). Symptoms of poisoning start with the gastrointestinal muscles (gastric pain, vomiting, diarrhea) and progress to skeletal and cardiac muscle with ventricular fibrillation followed by death due to respiratory muscle paralysis (5, 153, 475, 488). The barium ion is thought to act as a potassium antagonist, producing an extracellular hypokalemia (488) relieved by intravenous infusion of potassium salts (5, 153, 475). However, potassium infusion does not relieve the hypertension (153, 488, 589). Prompt oral administration of sodium sulfate to form the highly insoluble barium sulfate (1 g dissolves in 400,000 parts water) has been used to prevent absorption (5, 475). The U.S. EPA has derived an oral RfD for barium based on two studies involving humans, one experimental (618) and one epidemiological (87), and the subchronic and chronic rodent studies performed by the National Toxicology Program (NTP) (423). Wones et al. (618) found a NOAEL of 0.21 mg Ba/kg/day (the highest dose tested) in healthy male volunteers exposed to barium in drinking water. Brenniman and Levy (87) found no convincing evidence of a difference in hypertension or other effects between 2 communities, one exposed to <0.2 mg Ba/L and the other to a mean of 7.3 mg Ba/L (0.20 mg Ba/kg/day). These very similar NOAELs were converted to an RfD of 0.07 mg/kg/day using an uncertainty factor of 3 (to account for database deficiencies and to protect sensitive individuals; a factor lower than 10 was considered appropriate because the supporting studies considered adult males, those likely to be most sensitive to barium's hypertensive effects) (589). Confidence in the RfD was considered

medium. The ACGIH has adopted a TLV-TWA value of 0.5 mg Ba/m3 for soluble barium compounds and 10 mg Ba/m3 for barium sulfate (32).

The National Toxicology Program (NTP) performed a 2-year rodent bioassay with barium chloride dihydrate in drinking water and found no carcinogenic effects in either rats or mice (423). The U.S. EPA classifies barium as group D (not classifiable as to human carcinogenicity) (589). The carcinogenic potential of inhaled barium cannot be determined due to the lack of adequate animal inhalation studies.

Beryllium

Beryllium is an alkaline earth metal that is the lightest of the structural metals. Beryllium is a rare metal, and is extracted primarily from bertrandite (beryllium-silicate ore) and beryl (beryllium-aluminum oxide-silicate ore). The primary uses of beryllium are as a structural metal in lightweight applications, in metal alloys, and in nuclear reactor technology, as beryllium is an excellent neutron reflector and moderator (31, 63).

As beryllium is a rare metal, its toxic effects were not completely recognized until it became widely used in the 1940s. Although beryllium and its compounds can cause contact dermatitis, the primary target organ is the lung. Two types of beryllium-induced lung injury can occur, acute and chronic. The acute and frequently fatal syndrome resembles chemical pneumonitis and is associated with exposure to soluble forms of beryllium (e.g., beryllium sulfate and beryllium fluoride), where the concentration of airborne beryllium is greater than 0.1 mg/m3 (168). First reported in the early 1930s (63, 593), this syndrome has been virtually eliminated in the workplace after 1950, because of controls limiting the concentration of these beryllium compounds in the air (11, 169).

Chronic beryllium disease was first reported in 1946 as a delayed pneumonitis (244). The disease (also known as berylliosis) is characterized by granuloma formation, fibrosis, emphysema, and reduction in vital capacity of the lung, and total lung capacity (11). The chronic disease has two forms, one that occurs during exposure, and a second where the disease becomes evident 10 or more years after the cessation of exposure (63). The mechanism of the delayed onset of the condition is not known. The disease has a strong immunological component; chelation treatment has little effect on the course of the disease, whereas corticosteroid treatment has been effective in disease suppression (63).

Beryllium dermatitis is a hypersensitivity reaction that is usually noted 1 to 2 weeks after exposure to soluble beryllium salts. Patch tests of individuals with soluble beryllium salts provoke a positive response. Beryllium can also induce dermal ulceration if particles of beryllium salts become imbedded in the skin (63). The ulceration can be long-lasting, and surgical intervention can be required to resolve the condition (556).

Beryllium compounds are also carcinogenic. The IARC reviewed the available literature and in 1993 published its findings that there was sufficient evidence in humans and animals for the carcinogenicity of beryllium (281). In several retrospective epidemiologic studies conducted in the 1970s and 1980s, a consistent (but small) increase has been noted in the incidence of lung cancer in workers exposed to beryllium. The decade of hire was one of the strongest correlates of lung cancer mortality.

The U.S. EPA has established the following toxicity values for beryllium: oral, RfD, 2 μg/kg/day; inhalation, RfC, 0.02 μg/m3 and has classified it as a group B1, probable human carcinogen (589). The ACGIH has adopted TLV values for beryllium of 0.002 mg Be/m3 as an 8-h TWA and 0.01 mg Be/m3 as a STEL/C. An A1 carcinogenicity notation (confirmed human carcinogen) is present (32).

Boron

Boron is a metalloid. It is a solid element and, because of its high affinity for oxygen, always occurs in nature bound to oxygen in the form of inorganic borates (12, 167, 400). Boron and associated compounds have many industrial applications, including the production of borosilicate glass, laundry bleaches (sodium perborate), wood preservatives, fire retardants, pesticides (cockroach control), cosmetics, and pharmaceuticals (12). In 1875, Lister used boric acid as an antiseptic (353), but its effectiveness has since been discredited. The world's two largest borate deposits occur in the Mojave Desert (near Boron, California) and in Western Turkey (167). Borates have long been known to be essential for plants, but a specific biochemical role remains to be determined (80). Although boron deficiency has been reported in rats, chickens, and humans, as yet no requirement has been established in humans (420). Nielsen in 1996 (436, 437) classified it as an ultratrace element, and the World Health Organization (WHO) Expert Committee on Trace Elements in Human Nutrition came to the conclusion that it is "probably essential" in human nutrition (140). Biochemical and physiological consequences of boron deprivation in humans suggest it affects calcium and magnesium metabolism (435). Inadequate dietary boron (<0.2 mg B/day) has been suggested as a factor contributing to osteoporotic bone loss (434).

Inorganic borates exhibit a low order of acute toxicity in mammals, with the oral LD50 for boric acid in male rats being 4.5 g/kg body weight (142). However, inadvertent use of a 2.5% boric acid solution in

preparation of infant formula has resulted in toxicity and death (619). The oral lethal dose in adults has been reported as 15–20 g (528), but 80–297 g has been tolerated in a single ingestion (600). Nausea with vomiting and diarrhea, both

a characteristic blue-green color, are common. Frequently the skin shows signs of erythema, desquamation (boiled lobster appearance), and exfoliation. Death generally occurs several days after ingestion and results from renal injury, circulatory collapse, and shock. Although the use of boric acid solutions as an antiseptic is now obsolete, concentrated boric acid is still readily available as a household pesticide, and householders, particularly those with young children, need to be familiar with symptoms of poisoning (528, 532). Borates are not absorbed through intact skin (166), including that of newborns (202), unless the skin is damaged, abraded, or otherwise compromised (161). The use of boric acid as a dusting power during diapering has resulted in fatalities (215). The Cosmetic Ingredient Review Expert Panel (72) reviewed the use of borates in cosmetics and concluded that "cosmetic formulations containing free sodium borate or boric acid at this concentration (5%) should not be used on infant or injured skin". Animal experiments indicate that chronic oral exposure to boric acid or borax is toxic to the male reproductive system, with testicular lesions being observed in rats, dogs, and mice (181, 422, 609), and boric acid is a developmental toxicant in all three mammalian species tested (rat, mouse, rabbit) (251, 469, 470). The rat was the most sensitive species for developmental effects, with a NOAEL of 9.6 mg B/kg/day (469). The effect of boron on male fertility has been investigated indirectly in an occupationally exposed population using a questionnaire to determine the standardized birth ratio (SBR; ratio of the observed number of births to the expected); no adverse reproductive effects were reported (617).

The U.S. EPA has derived a chronic oral RfD for boron (589) using the study of Weir and Fisher where dogs were exposed to boric acid in the diet for 2 years (609) as the pivotal study. Testicular atrophy occurred at a dose of 29 mg/kg/day, whereas no effects were noted at 8.8 mg/kg/day. This NOAEL was converted to an RfD of 90 μg/kg/day using an uncertainty factor of 100 (a factor of 10 each for both inter- and intraspecies variation); confidence in this RfD was considered medium. Other risk assessments of boron (411, 616) considered the 1972 Weir and Fisher dog study (609), which predated Good Laboratory Practices, to be of insufficient power and design for use as a pivotal study, using instead the rat developmental study of Price et al. (469), where decreased fetal body weight occurred in a dose-dependent manner. No inhalation RfC has been derived for boron. The ACGIH has adopted several TLV values for boron compounds: TWA values of 10 mg/m3 for boron oxide, 5 mg/m3 for sodium tetraborate decahydrate, and 1 mg/m3 for sodium tetraborate pentahydrate and anhydrous, and ceiling values of 1 ppm for boron tribromide and boron trifluoride (32). NTP conducted a 2-year carcinogenesis biosassay in male and female B6C3F1 mice and reported testicular atrophy and interstitial-cell hyperplasia in males receiving 201 mg B/kg/day, but found no evidence of carcinogenicity (422). The U.S. EPA has not evaluated the potential human carcinogenicity of boron, but classifies it as group D (not classifiable as to human carcinogenicity) (589) based on lifetime studies conducted in mice (422) and rats (609).

Nickel

Nickel is member of the Group VIIB series of transition metals. There are three principal classes of nickel ores: sulfide, silicate, and arsenide. Nickel is used in a wide variety of applications, with 80% of the nickel in the United States being used in the production of nickel metal and alloys (63). The essentiality of nickel in humans is debatable. Nickel deficiency can be experimentally induced in rats and larger mammals (38, 420). There is some evidence that nickel is essential for methyl metabolism and iron, calcium, and zinc absorption (38, 457), but that if essential, the amount of nickel required would be amply met by the amount of nickel in a typical American diet (457).

Animal experiments have indicated that nickel compounds can be nephrotoxic, hepatotoxic, immunotoxic, and teratogenic (546). In humans, nickel can cause allergic contact dermatitis, particularly in young women using nickel-containing earrings in pierced ears (97), and is the most frequent disease among nickel workers. Statistical evaluations showed that up to 17% of all occupational allergies may be related to nickel occupational exposure (63). Allergic asthma is rare (430), but case reports have been published (63). Acute inhalation exposure to metallic nickel can cause metal fume fever (430). Nickel carbonyl is a colorless, volatile liquid that is particularly hazardous. It has been estimated that exposure to 30 ppm nickel carbonyl for 30 min may be lethal in humans (33). Acute inhalation exposure to this material can cause immediate and delayed toxic effects. Headache, dizziness, and nausea are the immediate manifestations. Ten to 36 h after exposure, substernal pain, coughing, and dyspnea, consistent with chemical pneumonitis, are observed (31, 63). Sodium diethyl-dithiocarbamate (dithiocarb—a chelating agent) has been employed in the therapy of nickel carbonyl-exposed workers (33). Recovery is protracted and is characterized by fatigue upon slight exertion (63). Short-term exposure to 150 ppb Ni(CO)4 can cause immediate but not delayed symptoms, whereas short-term exposure to

concentrations on the order of a few parts per million can cause the more severe, delayed-type reactions (63). The U.S. EPA has derived a chronic oral RfD for soluble salts of nickel of 20 μg/kg/day. Neither oral nor inhalation

RfDs have been established for nickel subsulfide, nickel refinery dust, nor nickel carbonyl (589). Inhalation of nickel compounds can cause lung cancer. The initial observation of excessive lung cancer and nasal tumors among nickel refinery workers was made as early as 1932 (63, 404). Since the initial observation, numerous epidemiologic studies have been conducted that show conclusively the association between occupational exposure to nickel refinery dust and nickel subsulfide and lung and nasal cancer (63). The latency period for nickel-induced lung cancer was 13 to 14 years and that for nasal cancer was 15 to 24 years after first employment (31). Nickel exposure has not been clearly associated with respiratory cancer in any of the industries using nickel (31). The U.S. EPA has classified nickel subsulfide and nickel refinery dust as group A carcinogens (human carcinogen) on the basis of animal and epidemiologic carcinogenicity data; nickel carbonyl has been classified as a group B2 carcinogen (probable human carcinogen) (589). IARC has classified nickel compounds as carcinogenic to humans (280). The National Toxicology Program published results on the carcinogenicity of inhaled nickel oxide (424), nickel subsulfide (425), and nickel sulfate hexahydrate (426) in rats and mice. There was no evidence of carcinogenicity of nickel sulfate hexahydrate in either species, clear evidence of carcinogenicity of nickel subsulfide in both species, and either no, equivocal, or some evidence of carcinogenicity of nickel oxide, depending on species and sex. The ACGIH has adopted the following TLVs for nickel compounds: elemental/ metal, 1.5 mg/m3; soluble compounds, 0.1 mg/m3; insoluble compounds, 0.2 mg/m3; nickel carbonyl, 0.05 ppm; nickel subsulfide, 0.1 mg/m3 (32).

Silver

Alloys of silver are used in jewelry, tableware, photographic materials, electronics, dental products, and as topical antibacterial agents for the treatment of burn wounds (63, 266). Silver is generally low in toxicity. It is absorbed following inhalation, ingestion, or topical application (31). Accumulation of silver results in argyria, a blue-gray discoloration of the skin, mucous membranes, and eyes. Silver sulfadiazine used in the management of burn wound sepsis has resulted in argyria, ocular injury, leukopenia, and toxicity in kidney, liver, and neurological tissues. Silver may affect the immune system, and contact dermatitis has been observed following exposure to various silver compounds (7). Toxicity has been attributed to the free silver ion released into solution and interaction with sulfhydryl, amino, carboxyl, and other groups on membrane or enzyme proteins (266). Excretion from oral, respiratory, or topical exposure is primarily through the GI tract (7). Mucociliary escalator activity accounts for removal of silver following respiratory exposure. Silver is not considered to be a carcinogen or a reproductive or developmental toxicant (7, 589). The U.S. EPA has derived a chronic oral RfD for silver of 5 μg/kg/day (589). No inhalation RfC has been derived for silver. The ACGIH has adopted TLV-TWA values for silver of 0.1 mg Ag/m3 for the metal and 0.01 mg Ag/m3 for soluble compounds (32). The ionic form is highly toxic to fish, but is found at extremely low concentrations in the aquatic environment, and other more common forms of silver show only low to moderate toxicity (265).

Strontium

Radiotoxicity is beyond the scope of this chapter and the following discussion pertains to stable strontium. Strontium is a soft silvery metal that turns yellow upon formation of the oxide (63). Its salts are used in the manufacture of color television screens, pyrotechnics (metal and salts impart a characteristic red color to flames), and electrical materials (586). Over 99% of the typical body burden of 320 mg is found in bone, and there is no conclusive evidence it is an essential trace element in mammals (63). Epidemiologic data suggest drinking water containing strontium in the presence of fluoride (5–6 mg Sr^{2+} and 1 mg F^{-}/L) decreases the incidence of dental caries in children (143). The gastrointestinal absorption of the strontium ion is poor (586) and acute strontium toxicity is low, with an oral LD50 of 2250 mg/kg body weight reported for strontium chloride in rats (99). Acute lethality is due to respiratory failure (132). In contrast, bone is the target organ following chronic strontium exposure. High doses inhibit calcification of the epiphyseal cartilage and cause deformities of long bones (533). Strontium causes these effects by substituting for calcium in the hydroxyapatite crystal during calcification or displacing calcium from existing calcified bone. The metabolic basis of strontium's effect on calcium metabolism is thought to be inhibition of the renal synthesis of 1,25-dihydroxyvitamin D3 (447). Young animals (still growing) are more susceptible to the toxic effects of strontium than adults, with widening of the epiphyseal cartilage being observed at lower levels of dietary strontium (545). Dietary calcium plays a protective role in strontium toxicity. Weanling rats maintained on diets containing 950 mg Sr/kg and 0.69% calcium for 4 weeks exhibited rachitic changes that were not seen in rats supplemented with 1.6% calcium (174). In contrast to these toxic effects, pharmacologic treatment with low doses of strontium suppresses bone resorption (372), and low-dose strontium lactate has been used to treat osteoporosis (383). The U.S. EPA has derived an oral RfD for strontium of 0.6

mg/kg/day, and confidence in this value is medium (589). Stable

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strontium has not been adequately evaluated for carcinogenic potential.

Thallium

Thallium is a soft bluish-white metal, widely but sparingly distributed in the earth (63). Historically it has been used to treat gput, venereal disease, dysentery, ringworm, and tuberculosis (115). Thallium sulfate was widely used as a rodenticide, but its use was banned in the United States in 1972 (63). It is still used as a rodenticide in other parts of the world, and rodent resistance to warfarin may cause this use to increase (301). Thallium intoxication from contaminated heroin, presumably imported from areas where thallium is still used as a rodenticide, has been reported (472). It is currently used in the electronics industry and in the manufacture of prisms, imitation jewelry, low-temperature thermometers, and infrared spectrometers. Thallium is well absorbed following oral ingestion and causes severe gastrointestinal symptoms followed by painful paresthesia of the extremities, motor paralysis, and death from respiratory failure (255, 316, 366, 393, 409). Individuals surviving the acute phase suffer characteristic scalp alopecia about 10 days postingestion (255, 409, 592). For an adult the LD50 has been calculated to be 8 to 12 mg Tl/kg (409). Treatment involves the use of activated charcoal and oral Prussian blue (ferric ferrocyanide, C.I. 77510), which prevents enterohepatic recirculation and enhances fecal elimination, hemodialysis, and forced diuresis (316, 391, 409, 592). The FDA has yet to approve the human use of Prussian blue (391, 409), making suitably pure material difficult to obtain, although it is used as a diagnostic agent in pathology laboratories. The effectiveness of Prussian blue in thallotoxicosis is dependent on the size of the crystal lattice (325). Chelating agents (dithiocarb) have caused a redistribution of thallium to target organs, with an increase in toxicity (172, 295). The precise mechanism of toxicity is unknown, but likely involves the substitution of the thallous ion for potassium in the sodium/ potassium ATPase pump and/or interference with sulfhydryl enzymes (409). Interference with tissue riboflavin with subsequent effects on metabolic pathways has also been suggested (103). The U.S. EPA has derived a chronic oral RfD for thallium based on its own 90-day study of rats exposed to aqueous thallium sulfate by gavage at doses up to 0.20 mg Tl/kg/day (589). Treatment-related effects of serum chemistry changes, alopecia, and lacrimation without histopathological changes were not considered adverse, and 0.2 mg/kg/day was considered the NOAEL (589). This NOAEL was converted to an RfD of 0.09 μg/kg/day using an uncertainty factor of 3000 (10 for intraspecies extrapolation, 10 for interspecies variation, 10 for less than chronic exposure duration, and 3 for lack of reproductive and chronic toxicity data). Confidence in the RfD was considered low. No inhalation RfC has been derived for thallium. The ACGIH has adopted a TLV-TWA of 0.1 mg Tl/m3 for elemental and soluble thallium compounds (32). Thallium is classified as group D (not classifiable as to human carcinogenicity), based on two inadequate negative studies in humans and a lack of animal studies designed to examine carcinogenic endpoints (589). Existing data do not indicate thallium is mutagenic (343).

Uranium

Uranium is a soft, malleable metal of the actinide series in the periodic table. The primary uranium ores are pitchblende (uranium oxide) and carnotite (uranium/ vanadium-containing mineral). Uranium is primarily used as nuclear fuel, but there are some minor uses, such as a colorant in ceramics or glass, or in armor-piercing projectiles (31). Occupational exposure occurs in mining operations and in uranium enrichment (uranium hexafluoride).

Acute inhalation exposure to uranium hexafluoride can cause pneumonitis, but the injury may be caused by the fluoride rather than the uranium itself (430). Chronic inhalation exposure of uranium dioxide dust at concentration of 5 mg U/m3 produced no observable adverse effects in rats, dogs, or monkeys (339). The kidney is the main target of uranium's chemical toxicity, with the targets being the pars recta of the proximal tubules, the ascending limb of the loop of Henle, and collecting tubules (31). Attempts have been made to define a "critical concentration" of uranium in the kidney that constitutes a threshold for renal damage (197). Experimental evidence clearly indicates that the "consensus value" of 3 μg/g is above the level shown to produce toxic effects by a factor of 5–10; however, studies of workers exposed to uranium at levels derived from this consensus value have shown no evidence of chemical toxicity (197). This may reflect differences between animal and human metabolism (e.g., binding of uranium to metallothionein or other detoxifying proteins) or differences between the route and duration of the animal and human exposures. The U.S. EPA developed a chronic RfD of soluble salts of uranium of 3 μg U/kg/day; no inhalation RfC has been derived for uranium (589).

Uranium isotopes are radioactive, but only weakly due to their extremely long half-lives. Natural uranium has a radioactivity of about 0.7 pCi/μg (585). No direct evidence exists that uranium is carcinogenic to humans or animals. However, based on the fact that uranium does emit ionizing radiation as it decays, the U.S. EPA has classified uranium as a group A carcinogen (known

human carcinogen) and has proposed to quantify the cancer risk of uranium in drinking water using toxicokinetic modeling (585).

Vanadium

Vanadium is a white to gray common trace metal that does not occur in nature but occurs in combination with oxygen, sodium, sulfur, and chloride. Vanadium deficiency can occur in laboratory animals on a very strict diet (39, 420), and there is evidence that vanadium helps regulate some phosphoryl transfer enzymes (457). A requirement for vanadium extrapolated from animal experiments is 10–25 μg/day, whereas typical intake is 8–18 μg/day (457); however, the National Research Council believes that there is only weak evidence that vanadium is essential and that any vanadium requirement would be met by naturally occurring levels (420).

Inhalation exposure to vanadium pentoxide can cause tracheobronchitis with persistent bronchial hyperreactivity and inflammation (14, 31, 430). A greenish-black discoloration of the tongue, gastrointestinal symptoms, neurotoxicity, and renal toxicity have also been reported in workers exposed to vanadium pentoxide. The short term repeated inhalation in rats of vanadium metavanadate (8 h/day for 4 days) at a concentration encountered by humans over a typical work week altered pulmonary immune cell function and produced significant changes in the lungs themselves (131). Oral exposure to vanadium can be toxic to the gastrointestinal, renal, and neurological systems. Vanadium is rapidly excreted in feces and urine following termination of exposure. The U.S. EPA has derived a chronic oral RfD for vanadium pentoxide of 5 μg V/kg/day (9 μg VO5/kg/day), which was derived using an uncertainty factor of 100 (10 for interindividual variation and 10 for interspecies variation); confidence in this RfD was considered low (589). The U.S. EPA has not derived an inhalation RfC for vanadium compounds. The ACGIH has adopted TLV-TWA values for vanadium of 1 mg/m3 for ferrovanadium dust and 0.05 mg/m3 for vanadium pentoxide (32).

The results of behavior testing show that oral sodium metavanadate in rats resulted in significant reductions in both general activity and learning (498). Mice given vanadyl sulfate in drinking water at doses up to 1 mg/kg/day for up to 33 months had no significant increase in tumor incidence (296, 505). The U.S. EPA has not evaluated the potential human carcinogenicity of vanadium (589).

Table 14.3 presents the quantitative toxicity values derived by the U.S. EPA for the metals listed in this

Table 14.3 Minor toxic metals with RfDs

	Chronic oral toxicity		Chronic inhalation toxicity		Carcinogenicity		
Metal	RfD*a* (mg/kg/day)	Confidence	RfC*b* (μg/m3)	Confidence	Oral slope factor [risk/(mg/kg/day)]	Inhalation slope factor [risk/(μg/m3)]	Classification
Boron	9×10−2	Medium	–	–	–	–	–
Barium	7×10−2	Medium	–	–	–	–	D
Nickel	2×10−2	Medium	–	–	–	2.4×10−4	A
Ni refinery dust					–	4.8×10−4	A
Ni3S2					–		B2
Ni(CO)4					–	–	–
Soluble Ni							
Vanadium	9×10−3	Low	–	–	–	–	–
Silver	5×10−3	Low	–	–	–	–	D
Beryllium	2×10−3	Low-medium	2×10−2	Medium	–	2.4×10−3	B1
Uranium	3×10−3	Medium	–	–	–	–	A
Antimony	4×10−4	Low	–	–	–	–	–
Thallium	9×10−5	Low	–	–	–	–	D

a RfD, reference dose.
b RfC, reference concentration.
c Group A, known human carcinogen; group B1, probable human carcinogen; group D, not classifiable as to human carcinogenicity.

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section, with metals listed in reverse order of toxicity (least to most toxic).

MINOR TOXIC METALS WITHOUT RfDs

Aluminum

Aluminum is the third most abundant element in the earth's crust, and is extracted from bauxite ore. Although aluminum is not an essential element, humans consume a substantial amount in the diet and are exposed to aluminum from a number of nondietary sources. On average, American adults consume 2 to 25 mg A1 daily from food and beverages (230), with average amounts being 8.2 mg/day for males and 7.1 mg/day for females (458). Aluminum is naturally present at low levels in most foods, but the primary source of dietary aluminum is from food additives. Over-the-counter antacids contain large amounts of aluminum hydroxide, and millions of consumers are dermally exposed to aluminum salts from the use of antiperspirants and deodorants (230).

Aluminum is generally considered to have a low order of toxicity, but it can cause reproductive toxicity when administered in high doses to experimental animals (218). The potential role of aluminum in either causing Alzheimer's disease or speeding its progression is highly controversial. Aluminum is certainly neurotoxic. In renal dialysis patients, excessive parenteral exposure to aluminum can cause a progressive, fatal neurological syndrome known as dialysis dementia (26). In addition, injection of aluminum salts into the brain of rabbits leads to the development of neurofibrillary tangles, but not *β*-amyloid plaques, which are also indicators of Alzheimer's disease (317). Some studies have found elevated levels of aluminum in some regions of the brain (327, 564, 606, 622, 626), whereas others have found no difference in aluminum levels between Alzheimer's and control brain tissue (286, 373, 565).

Epidemiologic studies do not show an association between aluminum exposure and the incidence of Alzheimer's disease. Canadian miners, between 1944 and 1979, were exposed to high concentrations of aluminum and aluminum oxide powder (McIntyre Powder) preceeding each shift as a prophylactic treatment against silicotic lung disease. In an initial study of this population, there was no increased incidence of neurological disorders in exposed miners, but there was an increase in neurological impairment as measured by cognitive testing (478). A follow-up study was conducted to address several methodological weaknesses in the initial study. No statistically significant differences were noted between exposed and nonexposed miners in either neurological disease or cognitive impairment incidence (477). Similarly, an association was noted with aluminum in drinking water and the incidence of Alzhemier's disease (376). A follow-up study (with methodological improvements) found no evidence of such an association (375). Another recent study has shown no association between occupational exposure to aluminium and the incidence of Alzheimer's disease (226).

The ACGIH has adopted TLV-TWA values for aluminum of 10 mg Al/m3 for metal dust and aluminium oxide, 5 mg Al/m3 for pyro powders and welding fumes, and 2 mg Al/m3 for soluble salts and alkyls (32).

Bismuth

Elemental bismuth is a soft lustrous metal and can occur naturally or in combined forms in ores. Bismuth is used in low-melting alloys. Insoluble bismuth salts are poorly absorbed orally or dermally. Excretion is primarily through the GI tract. Bismuth compounds demonstrate a low order of toxicity (63). They are used as coloring agents in cosmetics, and in pharmaceuticals, including use for diarrhea, gastroesophageal reflux, and in ulcer therapy. Bismuth subsalicylate used in ulcer therapy has no substantial capacity to neutralize gastric acid, but rather provides cytoprotection involving enhanced secretion of mucous and HCO_3^-, inhibition of pepsin activity, and the formation of bismuth protein complexes that may afford a protective barrier against peptic digestion. Primary activity may be due to the antibacterial effect of bismuth compounds against the bacteria *H. pylori* in the gastrointestinal mucosa (90). Toxic bismuth levels are not reached with normal use, although salicylism has been reported following use of bismuth subsalicylate (597). Rats exposed to bismuth oxychloride in the diet for 2 years at doses up to 2.0 mg/kg/day were found to have no increased incidence of tumors (468). The ACGIH has adopted a TLV-TWA value for bismuth telluride of 10 mg/m3 (32).

Bromine

Bromine is a reddish-brown, noncombustible liquid. Although the earth's crust contains a vast amount of bromine, the most readily recoverable sources of bromine are in salt lakes and brines. The largest use of bromine is in the production of fire retardants, gasoline antiknock agents, and in the agricultural chemical industry (460). Bromine vapors are highly toxic; exposure to 1000 ppm bromine is rapidly fatal in humans, and exposure to 40–60 ppm is dangerous for brief exposures (460). The symptoms of

inhalation exposure include coughing, nosebleed, dizziness and headache, and abdominal pain and diarrhea; sometimes a measles-like eruption on the trunk and extremities can occur (460). Bromine vapor is extremely irritating to the eyes, skin, and mucous

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membranes and produces inflammatory lesions in the upper respiratory tract (25). Prolonged contact with the skin causes ulceration (460).

Bromine may be an essential element. A brominedeficient diet impaired the growth and reproductive success of goats; however, the evidence for the essentiality of bromine is weak, and no cases of human deficiency are likely to occur, due to the widespread distribution of bromine in foods (37).

Excessive oral intake of bromide can cause neurological symptoms in humans (headache, lethargy, ataxia, disorientation, etc.), and high levels of bromine in the diet (400–1200 mg/kg) can cause CNS depression in mice (543). Potassium bromate is a widely used form of bromine. Its primary use is as a conditioner for flour and dough (460). Potassium bromate has been investigated for possible carcinogenic activity. Renal and other tumors were induced in male and female Fischer 344 rats exposed to potassium bromate in drinking water in a 2-year bioassay; tumor formation was dose dependent (279). The IARC has classified potassium bromate as a group 2B (possibly carcinogenic to humans) carcinogen. Conversely, mice and rats fed diets high in bread containing up to 75 mg/kg potassium bromate showed no increase in tumor incidence (191, 210). The lack of a carcinogenic effect could be attributable to the degradation of potassium bromate during the baking process (279).

The ACGIH has adopted TLV values for bromine gas of 0.1 ppm as an 8-h TWA and 0.2 ppm as a STEL/C (32). Although the U.S. EPA has established a number of toxicity values for bromine-containing compounds, the U.S. EPA has not derived any toxicity values for bromine (589).

Cerium

Cerium is a lanthanous rare earth metal and is used in fireworks and cigarette lighter flints, self-cleaning ovens, and as an abrasive for polishing glass (63). Occupational inhalation exposure has been reported to cause a pneumoconiosis without pulmonary functional impairment (275). Intravenous administration of cerium chloride produces severe hepatotoxicity in rats (414). The U.S. EPA has not derived toxicity values for cerium (589).

Gallium

Gallium is a relatively rare metal that has found uses in diagnostic radiology (63), as an antineoplastic agent (82), and for the control of cancer-related hypercalcemia (157, 607). It is used in the manufacture of alloys and semiconductor electronic devices. There are limited indications of occupationally related toxicity. Occupational exposure to GaF3 fumes resulted in a rash with sub sequent reversible neurological effects consisting of muscular weakness (63). Gallium is excreted in urine. Renal toxicity is noted in rats with the formation of precipitates of gallium complexed with calcium and phosphate (63). No adverse effects were noted in a reproduction study conducted in male mice (134). There are no occupational health standards for gallium or its compounds (63). The U.S. EPA has not derived toxicity values for gallium.

Germanium

Germanium is a Group IVA semiconducting metal. The pure metal has a metallic appearance, but is very brittle, much like glass. Germanium is not found in the free state, but always in combination with other elements, such as silver, copper, and arsenic. Germanium is used in the semiconductor industry (germanium was used in the first transistor), and it is often used in combination with other materials, such as arsenic and antimony, and alloyed with aluminum, gallium, and indium. It is also used in certain optic applications, as the pure metal is transparent to infrared radiation. Industrial exposures are to the dusts and fumes of germanium metal during extraction from ore and metal fumes from welding operations (63). Germanium oxide and germanium sesquioxide have been used in "elixirs" for the treatment of cancer and AIDS (552).

In longer term oral animal studies, germanium and germanium oxide have been shown to be nephrotoxic (497), neurotoxic (309, 377), and myotoxic (377). The potential for germanium to induce lung injury is unclear; in one 4-week inhalation toxicity study of germanium powder in rats, histopathologic changes consistent with pulmonary toxicity were present (43), but a follow-up study using germanium dioxide showed no treatment-related histopathologic effects (44). Germanium does not appear to be carcinogenic; in fact, certain germanium compounds appear to have antineoplastic activity (206). In a lifetime feeding study in rats, animals receiving 5 ppm sodium germanate in water had a significantly lower incident of tumors than the control animals (297).

Because of this anticancer activity, germanium-containing elixirs have been sold, first in Japan, and then in other countries as a treatment for cancer and other diseases. To date, there have been at least 31 reported cases of toxicity associated with oral intake of germanium compounds, of which 9 were fatal (552). Nephrotoxicity is the primary manifestation of germanium intoxication, although neurotoxicity and myotoxicity have been reported (548, 552).

No TLV has been set for germanium or germanium oxide, but a TLV-TWA of 0.2 ppm has been set for germanium tetrahydride (32).

Gold

Gold is a soft yellowish metal and belongs to Group IB of the periodic table. Its excellent heat and electrical conductivity and malleability have made it important in industrial applications (62). Medically, it is used either orally or by intramuscular injection to slow the progression of rheumatoid arthritis, but treatment is associated with a high incidence of toxicity (277). Adverse skin and mucous membrane effects (dermatitis, stomatitis, pruritus) are most frequent, with incidence and severity less for oral as opposed to parenteral treatment (563). A mild proteinuria is the most common renal effect, but gold-induced nephrosis may occur. Aplastic anemia is relatively rare and has been associated with poor prognosis (623), which may improve with bone marrow transplantation (293). Although traditionally regarded as inert, gold is being recognized as a common contact allergen (268). In Sweden it is second only to nickel (91), and results from the North American Contact Dermatitis Group rank it among the 10 most common allergens in the United States (386). Gold hypersensitivity is characterized by late reactions, and failure to monitor the test site for a minimum of 3 weeks may result in false negatives. The U.S. EPA has not derived toxicity values for gold (589).

Hafnium

Hafnium is a gray metallic element having a silverlike luster and is found in association with zirconium ores (31, 171). It has outstanding corrosion resistance and is used for this characteristic in atomic reactors, in electronic components, and in alloys. Hafnium compounds show moderate toxicity in acute animal tests by several routes of administration (239). Studies indicate concentration in the liver and skeleton. Hafnium is poorly absorbed orally (313) and the dust is considered to have relatively low toxicity. Workers exposed to 150 mg/m3 of hafnium- and zirconium-containing dusts showed no adverse effects after 2–6 years (165). The ACGIH has adopted a TLV-TWA value for hafnium of 0.5 mg/m3 (32).

Indium

Indium is a Group IIIA metal that is widely distributed in the earth's crust. It is not found in the free state, but most commonly in association with copper, zinc, and sulfur. Indium is used in surface protection of metals, and in many alloys because of its ability to increase hardness. Indium compounds are also used in the photovoltaic and semiconductor industry. Industrial exposures to indium occur during extraction and purification, and in plating and the manufacture of cer tain electronic instruments (63). Absorption of indium compounds is highly dependent on form; insoluble indium compounds are poorly absorbed and distributed, whereas soluble compounds, such as InCl3 and In2(SO4)3, are rapidly absorbed and distributed (63, 628). Consistent with these findings, soluble indium compounds are also more toxic than their insoluble counterparts. The acute lethal dose range for the soluble compound indium chloride in rabbits, rats, and dogs was 0.33 to 3.6 mg/kg (160), whereas the minimum lethal dose for insoluble indium oxide in rats was 955 mg/kg (3), and the oral and intraperitoneal LD50 for insoluble indium phosphide was greater than 5 g/kg (294).

Indium compounds are toxic when inhaled. Copper indium diselenide and indium trichloride, when acutely administered intratracheally to rats at high doses (higher than would be expected in an industrial exposure), induced a persistent inflammatory response (78, 402). Copper indium diselenide was only slightly fibrogenic to the lung, and this corresponds with the limited solubility of this compound (403). Subchronic inhalation of indium sesquioxide in rats induced a persistent inflammatory response; no fibrosis was noted (31). Hamsters were treated once per week for 15 weeks with either indium arsenide or indium phosphide (dose=7.5 mg arsenic or phosphorus) by intratracheal installation and were examined at the end of their lifespan. Adverse histopathologic findings were significantly higher in the treated groups (551).

Several studies have investigated the reproductive and developmental toxicity of indium compounds. Indium arsenide, administered intratracheally, reduced epididymal sperm counts in rats (449) but not hamsters (448); intratracheal instillation of indium chloride in mice did not affect reproductive performance of either males or females, but it was fetotoxic (116).

The ACGIH TLV-TWA for indium and its compounds is 0.1 mg In/m3 (32).

Lithium

Lithium is a silvery-white metal and the lightest solid element. Although it is used in batteries, organic synthesis (Grignard reagent), the space industry, as a swimming pool sanitizer, and in air conditioners, industrial intoxication has not been reported (344). Lithium hydride in contact with water releases hydrogen gas (flammable), and it must be stored under air-tight anhydrous conditions (63). Inhalation exposure to lithium hydride can cause pulmonary edema, but the hydride rather than the lithium is likely responsible (430). The ACGIH has adopted a TLV-TWA value for lithium hydride of 0.025 mg/m3 (32).

Oral lithium salts are widely used in the treatment of manic-depressive disorders, but fre

quent individual monitoring of serum concentrations is required because of the narrow therapeutic index (232, 371). The same levels of lithium are devoid of psychotropic effects in individuals not suffering manic-depressive disorders (371). Effective treatment generally requires levels between 0.8 and 1.2 mEq/L, and toxic effects have been seen at serum levels above 1.5 mEq/L. Signs of lithium toxicity are primarily neurologic and range from fine tremors and muscle weakness in mild cases, to dysarthria, hyperreflexia, coma, and collapse. Lithium therapy may produce lasting neurologic consequences (231). Lithium has properties similar to sodium, and substitution for body cations (sodium, potassium) may account for these effects (592). There is no specific antidote. Renal symptoms of intoxication include polyuria, polydipsia, and renal failure (445). Lithium therapy during pregnancy has been associated with an increase risk of cardiac anomalies, and there are sufficient animal and human data to indicate lithium can cause developmental toxicity (399). The U.S. EPA has not derived toxicity values for lithium (589).

Niobium

Niobium is a white, soft metal found in ores in combination with tantalum and other elements (63). Niobium is used in alloys and may find use in surgical implants and dental applications (63, 69, 518). Organometallic niobium compounds have shown antitumor and anti-HIV activity in vitro and in mice (321, 322, 507). Acute and chronic animal tests have been conducted on several niobium compounds (238, 307, 508). Niobium is poorly absorbed from the GI tract. Parenteral administration of niobium pentachloride results in decreased respiration, lethargy, and death. The compound is a moderate to severe skin irritant, with less irritation noted in rabbit eyes. Life-term studies of sodium niobate in mice and rats did not show carcinogenicity (297). Occupational or general health standards have not been established for niobium in the United States. No reports of occupational health hazards from dust or fumes associated with forging or other fabrication techniques of niobium metal and alloys have been reported (63).

Osmium

Osmium is a platinum-group metal. Osmium tetroxide is a noncombustible, colorless to pale yellow solid, with a disagreeable chlorine-like odor. Osmium tetroxide is apparently formed quite readily from finely divided osmium metal by heating in air, or even at room temperature (242). Osmium is found in combination with platinum and nickel-bearing ores. The major use of osmium is as osmium tetroxide, which is used as a biological stain for adipose tissues (31).

Metallic osmium and most of its other compounds are not considered highly toxic (31); however, osmium tetroxide has been shown to be toxic in animals and in man. The oral LD50 for osmium tetroxide has been reported to be 14 mg/kg in the rat and 162 mg/kg in the mouse; the intraperitoneal LD50 for the mouse was 14 mg/kg (63). The reported LC50 for the rat and mouse is 400 mg/m3 (31). Additionally, rabbits exposed for 30 minutes to osmium tetroxide at a concentration of 130 mg/m3 died after 4 days from pulmonary edema (89). Application of a drop of a 1% solution of osmium tetroxide to the rabbit eye caused severe corneal damage, permanent opacity, and superficial vascularization (89). Toxic effects have also been reported on guinea pig bone marrow, although the route of administration, dose, and duration were not reported (242).

Osmium tetroxide-induced toxicity in humans has been reported in the early toxicology literature. Inhalation exposure to OsO_4 can cause irritation of the nose and throat, which can persist for at least 12 h (242). Industrial exposure to osmium tetroxide concentrations ranging from 0.1 to 0.6 mg/m3 induced lacrimation and disturbances in vision (i.e., the appearance of rings around lights). Other complaints included conjunctivitis, cough, and headache. Recovery usually occurred within a few days (387). One human fatality has been reported, resulting from inhalation of osmium tetroxide (387). The exposure concentration was not reported; death was attributed to capillary bronchitis and pulmonary edema.

The ACGIH has adopted a TLV-TWA of 0.0002 ppm and a STEL/C of 0.0006 ppm for osmium tetroxide, both measured as osmium (32).

Platinum

Although platinum is relatively rare, it is found both as the pure metal and in combination with nickel, copper, and gold (63). Platinum is used as a catalyst in the automotive, chemical, and pharmaceutical industries, and its nobility (resistance to oxidation) makes it important in the manufacture of laboratory equipment (481). Metallic platinum is relatively inert, but the complex salts are frequent sensitizers, producing conjunctivitis, urticaria, dermatitis, and eczema following inhalation and/or dermal exposure (212). A syndrome formerly known by the misnomer "platinosis" is characterized by lacrimation, sneezing, rhinorrhea, cough, dyspnea, bronchial asthma (from chloroplatinates), and cyanosis (212). This term suggests a pneumoconiosis and fibrosis, which are not a part of platinum allergy syndrome,

and the condition is more accurately known as "allergy to platinum compounds containing reactive halogen

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ligands" (272). The mentioned symptoms are elicited by either an immediate (type I) or delayed (type II, within 24 h) hypersensitivity reaction (212). The platinum analogue cisplatin has been used as a chemotherapeutic agent against various cancers, especially testicular and ovarian tumors, despite nephrotoxicity at therapeutic doses (212). More recently, carboplatin has been used with comparable efficacy (for many types of cancer) and less toxicity, with thrombocytopenia being the major side effect (212, 356). The ACGIH has adopted TLV-TWA values of 1 mg/m3 for platinum metal and 0.002 mg/m3 for soluble salts (32). The TLV for platinum salts protects against sensitization, but does not offer protection to a previously sensitized individual. No increased risk of cancer has been reported from occupational exposure to platinum (212). The U.S. EPA has not evaluated the toxicity of platinum (589).

Rhodium

Rhodium is a silver-white, hard metal that can form highly corrosive resistant alloys and coatings used in electrical contacts, reflectors, and jewelry (31, 212). Unlike the related platinum compounds, rhodium compounds have not been found to be clinically active in cancer therapy. Antimalarial activity is reported for a rhodiumchloroquine complex (499). Only a limited toxicity profile has been developed for rhodium and its compounds. Intravenously administered rhodium trichloride was moderately low in acute toxicity in rats and rabbits (~200 mg/kg), with death possibly due to central nervous system depression (333). Oral rhodium trichloride was low in toxicity (LD50>500 mg/kg) (212). A chronic feeding study showed slight carcinogenic activity in mice (504). Rhodium has been reported to cause allergic contact urticaria (189). The ACGIH has adopted a TLV-TWA of 1 mg Rh/m3 for elemental and insoluble compounds, and 0.01 mg Rh/m3 for soluble rhodium compounds, concurrent with the determination that elemental and rhodium compounds are not classifiable as human carcinogens (31, 32). The OSHA PEL values for elemental, insoluble, and soluble rhodium compounds are one-tenth these levels (31).

Tantalum

Tantalum is a gray, hard metal found in ores in combination with niobium and other metals. It is used in electric capacitors, as the carbide for tools, and has found a wide range of use in medical diagnostic and surgical implant applications (31, 63, 75). Elemental tantalum and its principal oxide are essentially nontoxic in vitro and in vivo. Occupational exposure to tantalum and its oxide has shown no overt adverse health effects (31). Medical uses include tantalum gauze in the repair of hernias, implant plates and screws, and radiographic lung and bone markers (75). The TLV-TWA for tantalum metal and oxide as dust is 5 mg Ta/m3 (32).

Tellurium

Tellurium is placed in Group IVA of the periodic table. Tellurium has a number of industrial uses and is also found in a variety of food products (e.g., condiments, dairy products, nuts, fish) in high concentration. Pneumonitis and hemolytic anemia are prominent features of acute tellurium intoxication (29). Tellurium hydride has been shown to be highly toxic, causing pulmonary irritation and intravascular hemolysis (608). Acute oral or parenteral tellurium intoxication resulted in numerous symptoms, with hematuria noted in all animals treated (29, 388). Weanling rats fed 1% tellurium in the diet developed a peripheral neuropathy characterized by a transient demyelinating/remyelinating event (67, 332).

There have been no reports of serious illness or death in workers exposed to tellurium and its compounds. However, absorbed tellurium is slowly metabolized to dimethyl telluride and is excreted in urine, sweat, and breath (149). It is dimethyl telluride that is responsible for the "garlic breath" that is associated with tellurium exposure (336). Two fatalities occurred after unintentional treatment with 2 g sodium tellurite by ureteral catheter (302). Autopsy revealed acute fatty degeneration and edema of the liver. The ACGIH has adopted a TLV-TWA value of 0.1 mg Te/m3 for tellurium and compounds (32).

Tin

Tin is a soft, white metal that occurs in combination with other chemicals (e.g., chlorine, oxygen) (8). It is alloyed with other metals to make pewter, solder, bronze, and a special cast bronze termed bell metal (up to 24% tin), which is noted for its tonal quality (63). Most of the tin used in the United States is for plating steel cans. The fluoride is used in toothpaste, and the chloride is used to make frost-free windshields (500). Organotins function as antimicrobials in agriculture and industry, as stabilizers in polyvinyl chloride (PVC) plastics, and as marine antifouling agents (63). Although Schwarz et al. (514) in 1970 reported a significant growth effect of dietary tin in weanling rats maintained on purified diets, this has not since been independently confirmed and tin is not considered to be essential. Inorganic tin compounds are poorly absorbed from the gastrointestinal tract [i.e., rats dosed orally absorbed 2.8% of Sn(II) and less than 1% of Sn(IV) (260)] and are therefore relatively nontoxic; acute oral LD50 for SnCl2 in rats is

700 mg/kg (99), but after intravenous administration it is reported as 100 mg Sn/kg (517). Soluble salts of inorganic tin are gastric irritants producing nonspecific signs of nausea, vomiting, and diarrhea. Rats maintained on diets containing 0.3% or more as soluble inorganic tin salts (i.e., stannous chloride) experienced growth retardation and anemia (146). Injected stannous chloride is a potent inducer of rat renal microsomal heme oxygenase, enhancing heme breakdown (298). Diets supplemented with high levels of iron and copper protected rats from the anemia, but did not alleviate growth depression (145). Tin has adverse effects on the absorption and metabolism of the essential elements iron, copper, and zinc (145, 146, 229). In contrast, organotins, especially the trialkyl derivatives, are highly toxic [i.e., rat acute oral LD50 of 10 mg/kg (310)]. Triethyltin compounds are skin irritants and potent neurotoxins, producing a decrease in myelin content of the CNS and edema of the white matter (492). Uncoupling of oxidative phosphorylation has been proposed as the mechanism of action (401). Acute inhalation exposure to tin can cause metal fume fever and chronic exposure can cause a benign pneumoconiosis, stannosis (430). The ACGIH has adopted TLV-TWA values of 2 mg/m3 for the inorganic compounds (except tin hydride, SnH_4) and a TLV-TWA of 0.1 mg/m3 for organic tin compounds (32). Although the U.S. EPA has not evaluated tin and tin-containing compounds for carcinogenicity, the NTP has performed a 2-year bioassay for stannous chloride in rats and mice with negative results in all but male rats, where the results were equivocal for thyroid C-cell tumors (421).

Titanium

Titanium is a silver-gray metal that can occur naturally in several forms including titanium dioxide. Titanium is a component of several alloys, and is used in surgical implants where it is considered nontoxic (250). Titanium dioxide, the most common oxide of titanium, is extensively used as a white pigment in paints, plastics, inks, and cosmetics (63, 209). Titanium dioxide is generally considered to be essentially nontoxic by the oral, dermal, and inhalation routes. A 2-year feeding study of titanium dioxide at maximum doses of 2.5 g/kg/day in rats and 6.4 g/kg/day in mice found no evidence of carcinogenicity (415), although the dose-response relationship was statistically significant for thyroid tumors in female rats and keratoacanthomas in male rats (214). Toxicity from the more prevalent respiratory exposure to titanium dioxide has been investigated. A 2-year inhalation study was conducted in rats with acceptable results at a level of 10 mg/m3; high levels produced squamous-cell carcinomas, which are postulated to be the result of saturation of normal pulmonary clearance mechanisms (31, 340). Epidemiological findings and related information do not conclusively support a relationship between occupational exposure to titanium dioxide and pulmonary fibrosis, cancer, or other adverse health effects (31). The ACGIH has adopted a TLV-TWA value of 10 mg/m3 for titanium dioxide (32).

Tungsten

Tungsten is a member of the third series of transition metals and occurs in nature in combination with iron, manganese, and calcium. The major use of tungsten is in cutting and wear resistant materials (63). The bulk of inhaled tungsten oxide is rapidly excreted in dogs (1). Although exposure to soluble tungsten compounds can be toxic in experimental animals (311, 531), insoluble tungsten compounds have a low order of toxicity (148, 199, 312, 395). Male and female rats given sodium tungstenate in water for $2\frac{1}{2}$ years at doses of 0.25 and 0.29 mg/kg/day had no significant increase in tumor incidence (506).

Pulmonary fibrosis observed in men with inhalation exposure to cobalt-cemented tungsten carbide (394) has been attributed to cobalt (63). Evaluation of workers with long-term exposure to tungsten or its insoluble compounds showed no development of pneumoconioses (31).

The tungsten ion antagonized the normal metabolic action of the molybdate ion, and therefore can inhibit molybdate-dependent enzymes (259, 290, 441). The ACGIH has adopted TLV-TWA values of 5 mg W/m3 for insoluble compounds and 1 mg W/m3 for soluble compounds (32).

Yttrium

Yttrium has a silvery luster and is used primarily as the matrix producing the red color in television tubes (63). Yttrium chloride has been reported to cause granulomatous changes in the rat lung following intratracheal instillation (262), and the liver and spleen are reported to be the primary target organs following intravenous injection (263). Despite a long history of industrial use, there are no definitive reports of adverse effects in workers (63). The LD50 for yttrium chloride following intraperitoneal injection in rats is 132 mg Y/kg body weight (128). The U.S. EPA has not derived toxicity values for yttrium. The ACGIH had adopted a TLV-TWA value of 5 mg Y/m3 but reduced this to 1 mg Y/m3 (31, 32) based on a report that yttrium inhalation caused severe lung damage (63).

Zirconium

Zirconium is a grayish-white element of the second series of transition metals. The metal is produced from

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two main sources of ore, zircon ($ZrO SiO_2$), and baddeleyite (ZrO_2); hafnium is always associated with zirconium (32). Zirconium is used for the cladding in nuclear fuel rods, and zirconium compounds are also used in foundry and sandblasting applications. Industrial exposure occurs during mining and purification operations, and in foundry and other industries. A significant percentage of the general population is exposed (dermally) to aluminum zirconium chlorohydrate complexes in commercially marketed antiperspirant products.

Zirconium oxide has a low order of toxicity via the inhalation route in animals (538); slight toxicity was noted in dogs when exposed to an airborne mist of zirconium chloride at 6 mg Zr/m^3 for 2 months. Zirconium oxide and zirconium chloride exposure at 3.5 mg Zr/m^3 for 1 year had no measurable adverse effect on the animals exposed (63). Similarly, in most studies of industrially exposed workers, no adverse effects have been associated with inhalation exposure to zirconium fumes or other zirconium compounds (237, 370, 474). However, several cases of either fibrotic (58) or granulomatous (324, 348) changes in the lung associated with inhalation exposure to zirconium compounds have been reported. Long-term exposure of mice to zirconium sulfate was not associated with increased tumor incidence (297).

Certain zirconium compounds, such as zirconium lactate, when applied to human skin (54, 63, 489, 524) or the skin of experimental animals (471), can produce dermal granulomas of allergic origin. Aluminum zirconium chlorohydrate complexes, used as active ingredients in antiperspirants, do not appear to cause these granulomatous reactions, but because of risk/benefit considerations, the U.S. Food and Drug Administration banned the use of these materials in aerosolized drug and cosmetic products (580). The ACGIH has adopted a TLV-TWA value of 5 mg Zr/m^3 and a STEL/C value of 10 mg Zr/m^3 for zirconium compounds (32).

ACKNOWLEDGMENTS

The authors gratefully acknowledge the significant contributions of the previous author, Fanny K.Ennever.

QUESTIONS

1. Margins of safety are normally calculated by determining a no-observable-adverse-effect level (NOAEL) in an animal species and modifying the value by factors to account for inter- and intraspecies variability. U.S. EPA reference doses (RfD) are calculated using a similar approach. How does the oral RfD for the essential metal mol ybdenum show the limitations of this approach for calculating margins of safety?

Answer: The estimated safe and adequate daily dietary intake for molybdenum is set at the current U.S. dietary intake of 75–250 μg/day, whereas the oral RfD for molybdenum is 350 μg/day, a value very close to the current dietary intake. In fact, the U.S. EPA had initially derived an RfD of 280 μg/day, essentially the same as current U.S. dietary intake levels. These calculations show that the indiscriminate use of safety factors (without consideration of other relevant information) can lead to overestimation of the toxicity of a substance.

2. Your 15-kg patient has ingested 10 tablets, each containing 324 mg ferrous sulfate. How much elemental iron did the child ingest? (Ferrous sulfate contains 20% elemental iron.)

Answer: $10\times324\times0.20=648$ mg elemental iron. Then $648/15=43.2$ mg Fe/kg body weight. Inaccurate calculation of the dose of elemental iron ingested is a known pitfall in the management of iron poisoning.

3. Why is toenail selenium used instead of blood or hair selenium concentrations for estimating past selenium intake?

Answer: Hair selenium content is difficult to accurately measure because of the common use of selenium-containing antidandruff shampoos. Blood selenium is not a good indicator of past selenium intake because the life of an erythrocyte is only 120 days.

4. Some metals elicit clinical neurotoxicity at certain workplace exposure levels. For example, manganism is often characterized by Parkinson-like symptoms. Describe tests useful in determining neurotoxicity prior to clinical symptoms in order that safe exposure levels may be established and corrective action taken.

Answer: Nonclinical neurological injury can be evaluated by controlled neurobehavioral tests that evaluate reaction time, eye-hand coordination, and hand steadiness.

REFERENCES

1. Aamodt, R.L. (1975): Inhalation of181 W labeled tungstic oxide by six beagle dogs. *Health Phys.*, 28:733–742.

2. Abd Elghany, A., Schumacher, M.C., Slattery, M.L., et al. (1990): Occuaption, cadmium exposure, and

prostate cancer. *Epidemiology*, 1:107–115.
3. Adamson, R.H., Canellos, G.P., and Sieber, S.M. (1975): Studies on the antitumor activity of gallium nitrate and other group IIIa metal salts. *Cancer Chemother. Rep.*, 59:599–610.
4. Ades, A.E., and Kazantis, G. (1988): Lung cancer in a nonferrous smelter: The role of cadmium. *Br. J. Ind. Med.*, 45:435–442.

5. Agarwal, A.K., Ahlawat, S.K., Gupta, S., et al. (1995): Hypokalemic paralysis secondary to acute barium carbonate poisoning. *Tropical Doctor*, 25:101–103.
6. Agency for Toxic Substances and Disease Registry (ATSDR). (1990): *Toxicological Profile for Copper.* Atlanta, GA.
7. Agency for Toxic Substances and Disease Registry (ATSDR). (1990): *Toxicological Profile for Silver.* Atlanta, GA.
8. Agency for Toxic Substances and Disease Registry (ATSDR). (1990): *Toxicological Profile for Tin.* Atlanta, GA.
9. Agency for Toxic Substances and Disease Registry (ATSDR). (1991): *Toxicological Profile for Antimony*. Atlanta, GA.
10. Agency for Toxic Substances and Disease Registry (ATSDR). (1992): *Toxicological Profile for Barium.* Atlanta, GA.
11. Agency for Toxic Substances and Disease Registry (ATSDR). (1992): *Toxicological Profile for Beryllium.* Atlanta, GA.
12. Agency for Toxic Substances and Disease Registry (ATSDR). (1992): *Toxicological Profile for Boron.* Atlanta, GA.
13. Agency for Toxic Substances and Disease Registry (ATSDR). (1992): *Toxicological Profile for Cobalt.* Atlanta, GA.
14. Agency for Toxic Substances and Disease Registry (ATSDR). (1992): *Toxicological Profile for Vanadium.* Atlanta, GA.
15. Agency for Toxic Substances and Disease Registry (ATSDR). (1993): *Toxicological Profile for Arsenic.* Atlanta, GA.
16. Agency for Toxic Substances and Disease Registry (ATSDR). (1993): *Toxicological Profile for Cadmium.* Atlanta, GA.
17. Agency for Toxic Substances and Disease Registry (ATSDR). (1993): *Toxicological Profile for Lead.* Atlanta, GA.
18. Agency for Toxic Substances and Disease Registry (ATSDR). (1993): *Toxicological Profile for Mercury.* Atlanta, GA.
19. Agency for Toxic Substances and Disease Registry (ATSDR). (1994): *Toxicological Profile for Magnesium.* Atlanta, GA.
20. Agency for Toxic Substances and Disease Registry (ATSDR). (1994): *Toxicological Profile for Selenium.* Atlanta, GA.
21. Agency for Toxic Substances and Disease Registry (ATSDR). (1994): *Toxicological Profile for Zinc.* Atlanta, GA.
22. Agency for Toxic Substances and Disease Registry (ATSDR). (1998): *Toxicological Profile for Manganese.* Draft for Public Comment (Update). Atlanta, GA.
23. Akiniwa, K. (1997): Re-examination of acute toxicity of fluoride. *Fluoride*, 30:89–104.
24. Alderman, L.C., and Bergin, J.J. (1986): Hydrogen selenide poisoning: An illustrative case with review of the literature. *Arch. Environ. Health*, 41:354–358.
25. Alexandrov, D.D. (1983): Bromine and Compounds. In: *Encylopaedia of Occupational Health and Safety*, 3rd ed., Volume 1, edited by L.Parmeggiani, pp. 326–329. International Labour Organization, Geneva.
26. Alfrey, A.C. (1993): Aluminum toxicity in patients with chronic renal disease. *Ther. Drug Metab.*, 15:593–597.
27. Allen, L.A. (1996): Nutritional products. In: *Handbook of Nonprescription Drugs*, pp. 361–392. American Pharmaceutical Association, Washington, DC.
28. Alomar, A., Conde-Salazar, L., and Romaguera, C. (1985): Occupational dermatoses from cutting oils. *Contact Dermatitis*, 12:129–138.
29. Amdur, M.L. (1958): Tellurium oxide, An animal study in acute toxicity. *AMA Arch. Ind. Health*, 17:665–667.
30. Amdur, M.O. (1978): Respiratory response to iodine vapor alone and with sodium chloride aerosol. *J. Toxicol. Environ. Health*, 4:619–630.
31. American Conference of Governmental and Industrial Hygienists (ACGIH). (1996): *Documentation of the threshold limit values and biological exposure indicies*, 6th ed. and suppl. ACGIH, Cincinnati, OH.
32. American Conference of Governmental Industrial Hygienists (ACGIH). (2000): *2000 TLV's and BEI's.* ACGIH, Cincinnati, OH.
33. American Industrial Hygiene Association (AIHA). (1968): *Hygienic Guide Series—Nickel Carbonyl*

(rev. 1968), pp. 304–307. AIHA, Fairfax, VA.
34. Anderson, A.C. (1994): Iron poisoning in children. *Curr. Opin. Pediatr.*, 6:289–294.
35. Anderson, R.A. (1997): Chromium as an essential nutrient for humans. *Regul. Toxicol. Pharmacol.*, 26:S35–S41.
36. Angle, C.R. (1993): Childhood lead poisoning and its treatment. *Annu. Rev. Pharmacol. Toxicol.*, 32:409–434.
37. Anke, M, Groppel, G., and Arnhold, W. (1990): Essentiality of the trace element bromine. *Acta Agronom. Hung.*, 39:297–303.
38. Anke, M., Groppel, G., and Krause, U. (1991): Essentiallity of the toxic elements cadmium, arsenic, and nickel. In: *Trace Elements in Man and Animals,* Vol. 7. edited by B.Momcilovic, pp. 11–6 to 11–8. IMI, Zagreb, Croatia.
39. Anke, M., Groppel, G., and Krause, U. (1991): Essentiality of the toxic elements aluminum and vanadium. In: *Trace Elements in Man and Animals,* Vol. 7, edited by B.Momcilovic, pp. 11–9 to 11–11. IMI, Zagreb, Croatia.
40. Anke, M., Groppel. G., and Krause, U. (1991): Fluorine deficiency in goats. In: *Trace Elements in Man and Animals,* Vol. 7, edited by B.Momcilovic, pp. 26–28 to 26–27 . IMI, Zagreb, Croatia.
41. Anneroth, G., Ericson, T., Johansson, I., et al. (1992): Comprehensive medical examination of a group of patients with alleged adverse effects from dental amalgams. *Acta Odontolog. Scand.*, 50:101–111.
42. Apostoli, P., Kiss, P., Porru, S., Bonde, J.P., Vanhoorne, M., and ASCLEPIOS. (1998): Male reproductive toxicity of lead in animals and humans. *Occup. Environ. Med.*, 55:364–374.
43. Arts, J.H.E., Reuzel, P.G.J., Falke, H.E., and Beems, R.B. (1990): Acute and sub-acute inhalation toxicity of germanium metal powder in rats. *Food Chem. Toxicol.*, 28:571–579.
44. Arts, J.H.E., Til, H.P., Kuper, R., and Swennen, B. (1994): Acute and subacute inhalation toxicity of germanium dioxide in rats. *Food Chem. Toxicol.*, 32:1037–1046.
45. Assennato, G., Paci, C., Baser, M.E., Molinini, R., Candela, R. G., Altamura, B.M., and Giorgino, R. (1987): Sperm count suppression without endocrine dysfunction in lead-exposed men. *Arch. Environ. Health*, 42:123–127.
46. Awasthi, Y.C.. Beutler, E., and Srivastava, S.K. (1975): Purification and properties of human erythrocyte glutathione peroxidase. *J. Biol. Chem.*, 250:5144–5149.
47. Axelson, O., Dahlgren, E., Jansson, C.-D., and Rehnlund, S.O. (1978): Arsenic exposure and mortality: A case referent study from a Swedish copper smelter. *Br. J. Ind. Med.*, 35:8–15.
48. Ayres, S., Jr., and Anderson, N.P. (1934): Cutaneous manifestations of arsenic poisoning. *Arch. Dermatol. Syphil.*, 30:33–43.
49. Bacon, B.R., and Britton, R.S. (1990): The pathology of hepatic iron overload: A free radical mediated process? *Hepatology*, 11:127–137.
50. Bacon, B.R., Tavill, A.S., Brittenham, G.M., et al. (1983): Hepatic lipid peroxidation in vivo in rats with chronic iron overload. *J. Clin. Invest.*, 71:429–439.
51. Baker, E.L., White, R.F., Pothier, L.J., et al. (1985): Occupational lead neurotoxicity: Improvement in behavioral effects after reduction of exposure. *Br. J. Ind. Med.*, 42:507–516.
52. Baker, T.D., and Hafner, W.G. (1961): Cadmium poisoning from a refrigerator shelf used as an improvised barbecue grill. *Public Health Rep.*, 76:543–544.
53. Bako, G., Smith, E.S., Hanson, J., et al. (1982): The geographical distribution of high cadmium concentrations in the environment and prostate cancer in Alberta. *Can. J. Pub. Health*, 73:92–94.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_684.html[2010/10/28 18:46:47]

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54. Baler, G.R. (1965): Granulomas from topical zirconium in poison ivy dermatitis. *Arch. Dermatol.*, 91:145–148.
55. Banting, D.W. (1991): The future of fluoride. An update one year after the National Toxicology Program study. *J. Am. Dental Assoc.*, 123:86–91.
56. Barnhart, S., and Rosenstock, L. (1984): Cadmium chemical pneumonitis. *Chest*, 86:789–791.
57. Barret, H.M., and Card, B.Y. (1947): Studies on the toxicity of inhaled cadmium. II. The acute lethal dose cadmium oxide for man. *J. Ind. Hyg. Toxicol.*, 29:286–293.
58. Bartter, T., Irwin, R.S., Abraham, J.L., Dascal, A., Nash, G., Himmelstein, J.S., and Jederlinic, P.J. (1991): Zirconium compound-induced pulmonary fibrosis. *Arch. Intern. Med.*, 151:1197–1201.
59. Bast, A., Haenen, G.R., and Doelman, C.J. (1991): Oxidants and antioxidants: State of the art. *Am. J. Med.*, 91(suppl. 3C):2–13.
60. Bates, M.N., Smith, A.H., and Hopenhayn-Rich, C. (1992): Arsenic ingestion and internal cancers: A review. *Am. J. Epidemiol.*, 135:462–476.
61. Beckett, W.S., Moore, J.L., Keogh, J.P., and Bleecker, M.L. (1986): Acute encephalopathy due to occupational exposure to arsenic. *Br. J. Ind. Med.*, 43:66–67.
62. Beliles, R.P. (1979): The lesser metals. In: *Toxicity of Heavy Metals in the Environment*, edited by F.W.Oehme, pp. 547–615. Marcel Dekker, New York.
63. Beliles, R.P. (1994): The metals. In: *Patty 's Industrial Hygiene and Toxicology*, edited by G.D.Clayton and F.E.Clayton, pp. 1879–2352. John Wiley & Sons, New York.
64. Beliles, R.P., and Beliles, E.M. (1994): Phosphorus, selenium, tellurium, and sulfur. In: *Patty's Industrial Hygiene and Toxicology*, edited by G.D.Clayton and F.E.Clayton, pp. 783–829. John Wiley & Sons, New York.
65. Belizan, J.M., Villar, J., Zalazar, A., et al. (1983): Preliminary evidence of the effect of calcium supplementation on blood pressure in normal pregnant women. *Am. J. Obstet. Gynecol.*, 146:175–180.
66. Bellinger, D., Leviton, A., Waternaux, C., Needleman, H., and Rabinowitz, M. (1987): Longitudinal analyses of prenatal and postnatal lead exposure and early cognitive development. *N. Engl. J. Med.*, 316:1037–1043.
67. Berciano, M.T., Calle, E., Fernández, R., and Lafarga, M. (1998): Regulation of Schwann cell numbers in tellurium-induced neuropathy: Apoptosis, supernumerary cells and internodal shortening. *Acta Neuropathol.*, 95:269–279.
68. Berkovitch, M., Matsui, D., Lamm, S.H., et al. (1994): Recent increases in numbers and risk of fatalities in young children ingesting iron preparations. *Vet. Hum. Toxicol.*, 36:53–55.
69. Berry, J.P., Bertrand, F., and Galle, P. (1993): Selective intra-lysosomal concentration of niobium in kidney and bone marrow cells: A microanalytical study. *BioMetals*, 6:17–23.
70. Berry, M.J., and Larsen, P.R. (1992): The role of selenium in thyroid hormone action. *Endocr. Rev.*, 13:207–219.
71. Beton, D.C., Andrews, G.S., Davies, H.J., et al. (1966): Acute cadmium fume poisoning: Five cases with one death from renal necrosis. *Br. J. Ind. Med.*, 23:292–301.
72. Beyer, K.H., Bergfeld, W.F., and Berndt, W.O. (1983): Final report on the safety assessment of sodium borate and boric acid. *J. Am. Coll Toxicol.*, 2:87–125.
73. Bhattacharyya, M.H., Jeffery, E., and Silbergeld, E.K. (1996): Bone metabolism: Effects of essential and toxic trace metals. In: *Toxicology of Metals*, edited by L.W.Chang, pp. 959–971. CRC Press, New York.
74. Birch, N.J., and Karim, A.R., (1988): Potassium. In: *Handbook on Toxicology of Inorganic Compounds*, edited by N.G.Sieler and H. Sigel, pp. 543–553. Marcel Dekker, New York.
75. Black, J. (1994): Biological performance of tantalum. *Clin. Materials*, 16:167–173.
76. Blanc, P., and Boushey, H.A. (1993): The lung in metal fume fever. *Semin. Resp. Med.*, 14:212–225.
77. Blank, E., and Howieson, J. (1983): Lead poisoning from a curtain weight. *J. Am. Med. Assoc., 249:2176–2177.*
78. Blazka, M.E., Dixon, D., Haskins, E., and Rosenthal, G.J. (1994): Pulmonary toxicity to intratracheally administered indium trichloride in Fischer 344 rats. *Fundam. Appl. Toxicol.*, 22:231–239.
79. Blejer, H.P. (1966): Death due to cadmium oxide fumes. *Ind. Med. Surg.*, 35:363–364.
80. Blevins, D.G. and Lukaszewski, K.M. (1994): Proposed physiologic functions of boron in plants pertinent to animal and human metabolism. *Environ. Health Persp.*, 102(suppl. 7):31–33.
81. Blom, S., Lagerkvist, B., and Linderholm, H. (1985): Arsenic exposure to smelter workers: Clinical and neurophysiological studies. *Scand. J. Work Environ. Health*, 11:265–269.
82. Bockman, R.S., Wilhelm, F., Siris, E., Singer, F., Chausmer, A., Britton, R., Kotler, J., Bosco, J., Eyre,

D.R., and Levenson, D. (1995): A multicenter trial of low dose gallium nitrate in patients with advanced Paget's disease of bone. *J. Clin. Endocrinol. Metab.* 80:595–602.
83. Borgoño, J.M., Vicent, P., Venturino, H., and Infante, A. (1977): Arsenic in the drinking water of the city of Antofagasta: Epidemiological and clinical study before and after the installation of a treatment plant. *Environ. Health Perspect.,* 19:103–105.
84. Boulos, B.M., and von Smolinski, A. (1988): Alert to users of calcium supplements as antihypertensive agents due to trace metal contaminants. *Am. J. Hypertens.*, 1:137S–142S.
85. Bourgoin, B.P., Evans, D.R., Cornett, J.R., et al. (1993): Lead content in 70 brands of dietary calcium supplements. *Am. J. Public Health,* 83:1155–1160.
86. Bowman B.A., and Rishert, J.F. (1994): Comparison of the methodological approaches used in the derivation of recommended dietary allowances and oral reference doses for nutritionally essential elements. In: *Risk Assessment of Essential Elements*, edited by W.Mertz, C.O.Abernathy, S.S.Olin, pp. 63–73. ILSI Press, Washington, DC.
87. Brenniman, G.R., and Levy, P.S. (1984): Epidemiological study of barium in Illinois drinking water supplies. In: *Advances in Modern Toxicology*, Vol. 9, edited by E.J.Calabrese, pp. 231–249. Princeton Scientific, Princeton, NJ.
88. Brody, D.J., Pirkle, J.L., Kramer, R.A., et al. (1994): Blood lead levels in the US population. Phase 1 of the 3rd National health and Nutrition Examination Survey. *J. Am. Med. Assoc.*, 272:277–283.
89. Brunot, F.R. (1933): The toxicity of osmium tetroxide (osmic acid). *J. Ind. Hyg.*, 15:136–143.
90. Brunton, L.L. (1996): Bismuth Compounds. In: *Agents for Control of Gastric Acidity and Treatment of Peptic Ulcers,* edited by J.G. Hardman, L.E.Limberd, P.B.Molinoff, and R.W.Ruddon, pp. 901–915. McGraw-Hill, New York.
91. Bruze, M., Hedman, H., Bjorkner, B., and Moller, H. (1995): The development and course of test reactions to gold sodium thiosulfate. *Contact Dermatitis*, 33:386–391.
92. Bucher, J.R., Hejtmanick, M.R., Toft, J.D., et al. (1991): Results and conclusions of the National Toxicology Program's rodent carcinogenicity studies with sodium fluoride. *Int. J. Cancer,* 48:733–737.
93. Buchet, J.P., Lauwerys, R., and Roels, H. (1981): Urinary excretion of inorganic arsenic and its metabolites after repeated ingestion of sodium metaarsenite by volunteers. *Int. Arch. Occup. Environ. Health*, 48:111–118.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_685.html[2010/10/28 18:46:47]

94. Buchet, J.P., Lauwerys, R., Roels, H., et al. (1990): Renal effects of cadmium body burden of the general population. *Lancet*, 336:699–702.
95. Buckler, H.M., Smith, W.D., and Rees, W.D. (1986): Self poisoning with oral cadmium chloride. *Br. Med. J.*, 292:1559–1560.
96. Bulmer, F.M.R., Rothwell, N.F., and Frankish, E.R. (1938): Industrial cadmium poisoning, a report of fifteen cases, including two deaths. *Can. Public Health J.*, 29:19–26.
97. Burrows, D. (1988): Mischievous metals—Chromate, cobalt, nickel and mercury. *Clin. Exp. Dermatol.*, 14:266–272.
98. Byers, R.K., and Lord, E.E. (1943): Late effects of lead poisoning on mental development. *Am. J. Dis. Child.*, 66:471–494.
99. Calvery, H.O. (1942): Trace elements in food. *Food Res.*, 7:313–331.
100. Campbell, D., Gonzales, M., and Sullivan, J.B., Jr. (1992): Mercury. In: *Hazardous Materials Toxicology*, edited by J.B. Sullivan, Jr., and G.R.Krieger, pp. 824–833. Williams & Wilkins, Baltimore, MD.
101. Carleton, A.B., Peters, R.A., and Thompson, R.H.S. (1948): The treatment of arsenical dermatitis with dimercaptopropanol (BAL). *Q.J. Med.*, 17:49–79.
102. Carter, R.F. (1966): Acute selenium poisoning. *Med. J. Aust.*, 1:525–528.
103. Cavanagh, J.B. (1991): What have we learnt from Graham Frederick Young? Reflections on the mechanism of thallium neurotoxicity. *Neuropathol. Appl. Neurobiol.*, 17:3–9.
104. Centers for Disease Control (CDC). (1981): Use of lead tetroxide as a folk remedy for gastrointestinal illness. *Morbid. Mortal. Weekly Rep.*, 30:546–547.
105. Centers for Disease Control (CDC). (1983): Folk remedy-associated lead poisoning in Hmong children—Minnesota. *Morbid. Mortal. Weekly Rep.*, 32:555–556
106. Centers for Disease Control (CDC). (1983): Lead poisoning from Mexican folk remedies—California. *Morbid. Mortal. Weekly. Rep.*, 32:554–555.
107. Centers for Disease Control (CDC). (1984): Lead poisoning-associated death from Asian Indian folk remedies—Florida. *Morbid. Mortal Weekly Rep.*, 33:638, 643–645.
108. Centers for Disease Control (CDC). (1991): *Preventing lead poisoning in young children: A statement by the Centers for Disease Control—October 1991.* Centers for Disease Control, U.S. Department of Health and Human Services, Atlanta, GA.
109. Centers for Disease Control (CDC). (1991): *Strategic plan for the elimination of childhood lead poisoning.* Centers for Disease Control, U.S. Department of Health and Human Services, Atlanta, GA.
110. Centers for Disease Control (CDC). (1996): Children with elevated blood lead levels attributed to home renovation and remodeling activities—New York, 1993–1994. *Morbid. Mortal. Weekly Rep.*, 45:1120–1123.
111. Centers for Disease Control (CDC). (1997): *Screening young children for lead poisoning: Guidance for state and local public health officials*. Centers for Disease Control, U.S. Department of Health and Human Services, Atlanta, GA.
112. Centers for Disease Control (CDC). (1997): Update: Blood lead levels—United States, 1991–1994. *Morbid. Mortal. Weekly Rep.*, 46:141–146.
113. Chan, K.-M., Svancarek, W.P., and Creer, M. (1987): Fatality due to acute hydrofluoric acid exposure. *J. Toxicol. Clin. Toxicol.*, 25:333–339.
114. Chan, P.C., Herbert, R.A., Roycroft, J.H., Haseman, J.K., Grumbein, S.L., Miller, R.A., and Chou, B.J. (1998): Lung tumor induction by inhalation exposure to molybdenum trioxide in rats and mice. *Toxicol. Sci.*, 45:58–65.
115. Chandler, H.A., and Scott, M. (1986): A review of thallium toxicology. *J. Roy. Nav. Med. Serv.*, 72:75–79
116. Chapin, R.E., Harris, M.W., Hunter, S., Davis, B.J., Collins, B. J., and Lockhart, A.C. (1995): The reproductive and developmental toxicity of indium in the Swiss mouse. *Fundam. Appl. Toxicol.*, 27:140–148.
117. Chatterjee, M.S., Abdel-Rahman, M., Bhandal, A., et al. (1988): Amniotic fluid cadmium and thiocyanate in pregnant women who smoke. *J. Reprod. Med.*, 33:417–420.
118. Chela, A., Reig, R., Sanz, P., Huguet, E., and Corbella, J. (1989): Death due to hydrofluoric acid. *Am. J. Forens. Med. Pathol.*, 10:47–48.
119. Chen, C.-J., Chuang, Y.-C., Lin, T.-M., and Wu, H.-Y. (1985): Malignant neoplasms among residents of a blackfoot disease-endemic area in Taiwan: High-arsenic artesian well water and cancers. *Cancer Res.*, 45:5895–5899.
120. Chen, C.-J., Chuang, Y.-C., You, S.-L., Lin, T.-M., and Wu, H.-Y. (1986): A retrospective study on

malignant neoplasms of bladder, lung and liver in blackfoot disease endemic area in Taiwan. *Br. J. Cancer*, 53:399–405.
121. Chen, C.-J., and Wang, C.-J. (1990): Ecological correlation between arsenic level in well water and age-adjusted mortality from malignant neoplasms. *Cancer Res.*, 50:5470–5474.
122. Chen, C.-J., Wu, M.-M., Lee, S.-S., Wang, J.-D., Cheng, S.-H., and Wu, H.-Y. (1988): Atherogenicity and carcinogenicity of high-arsenic artesian well water. Multiple risk factors and related malignant neoplasms of blackfoot disease. *Arteriosclerosis*, 8:452–460.
123. Cheng, Y.-Y., and Qian, P.-C. (1990): The effect of selenium-fortified table salt in the prevention of Keshan disease on a population of 1.05 million. *Biomed. Environ. Sci.*, 3:422–428.
124. Chisolm, J.C., and Handorf, C.R. (1996): Further observations on the etiology of pre-eclampsia: Mobilization of toxic cadmium-metallothionein into the serum during pregnancy. *Med. Hypoth.*, 47:123–128.
125. Clark, L.C., Combs, G.F., Jr., Turnbull, B.W., et al. (1996): Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. *J. Am. Med. Assoc.*, 276:1957–1963. [Published erratum appears in *J. Am. Med. Assoc.*, 1997, 277:1520].
126. Clark, L.C., Dalkin, B., Krongrad, A., et al. (1998): Decreased incidence of prostate cancer with selenium supplementation: Results of a double-blind cancer prevention trial. *Br. J. Urol.*, 81:730–734.
127. Clayton, C.C., and Bauman, C.A. (1949): Diet and azo dye tumors: Effect of diet during a period when the dye is not fed. *Cancer Res.*, 9:575–582.
128. Cochran, K.W., Doull, J., Mazur, M., and DuBois, K.P. (1950): Acute toxicity of zirconium, columbium, strontium, lanthanum, cesium, tantalum and yttrium. *Arch. Ind. Health*, 1:637–650.
129. *Code of Federal Regulations.* Protection of the Environment. (1997): National primary drinking water standards. Volume 40: Part 141.80. U.S. Government Printing Office, Washington, DC.
130. Cohen, M.D., Kargacin, B., Klein, C.B., and Costa M. (1993): Mechanisms of chromium carcinogenicity and toxicity. *Crit. Rev. Toxicol.*, 23:255–281.
131. Cohen, M.D., Yang, Z., Zelikoff, J.T., and Schlesinger, R.B. (1996): Pulmonary immunotoxicity of inhaled ammonium metavanadate in Fisher 344 rats. *Fundam. Appl. Toxicol.*, 33:254–263.
132. Cole, V.V., Harned, B.K., and Hafkesbring, R. (1941): The toxicity of strontium and calcium. *J. Pharmacol. Exp. Ther.*, 71:1–5.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_686.html[2010/10/28 18:46:48]

133. Collins, J.F., Brown, J.P., Painter, P.R., et al. (1996): On the oral carcinogenicity of cadmium. *Regul. Toxicol. Pharmacol.*, 23:298–299.
134. Colomina, J.M., Llobtet, J.M., Sirvent, J.J., Domingo, J.L., and Corbella, J. (1993): Evaluation of the reproductive toxicity of gallium nitrate in mice. *Food Chem. Toxicol.*, 31:847–851.
135. Combs, G.F. (1996): Should intakes with beneficial actions, often requiring supplementation, be considered for RDAs? *J. Nutr.*, 126:2373S–2376S.
136. Comstock, G.W., Bush, T.L., and Helzlsouer, K. (1992): Serum retinol, beta-carotene, vitamin E, and selenium as related to subsequent cancer of specific sites. *Am. J. Epidemiol.*, 135:115–121.
137. Condray, J.R. (1985): *Elemental yellow phosphorus one-generation reproduction study in rats.* IR-82–215, IRD No. 401–189. Monsanto Company, St. Louis, MO.
138. Cook, J.D., and Monsen, E.R. (1977): Vitamin C, the common cold, and iron absorption. *Am. J. Clin. Nutr.*, 30:235–241.
139. Costa, M. (1997): Toxicity and carcinogenicity of Cr(VI) in animal models and humans. *Crit. Rev. Toxicol.*, 27:431–442.
140. Coughlin, J.R. (1996): Inorganic borates: Chemistry, human exposure, and health and regulatory guidelines. *J. Trace Elem. Exp. Med.*, 9:137–151.
141. Crecelius, E.A. (1977): Changes in the chemical speciation of arsenic following ingestion by man. *Environ. Health Perspect.*, 19:147–150.
142. Culver, B.D., Smith, R.G., Brotherton, R.J., et al. (1994): Boron. In: *Patty's Industrial Hygiene and Toxicology*, edited by G.D. Clayton and F.E.Clayton, pp. 4411–4448. John Wiley & Sons, New York.
143. Curzon, M.E.J., Spector, P.C., and Iker, H.P. (1978): An association between strontium in drinking water supplies and low caries prevalence in man. *Arch. Oral Biol.*, 23:317–321.
144. Davison, A.G., Fayers, P.M.. Taylor, A.J., et al. (1988): Cadmium fume inhalation and emphysema. *Lancet*, 1(8587):663–667.
145. De Groot, A.P. (1973): Subacute toxicity of inorganic tin as influenced by dietary levels of iron and copper. *Food Cosmetic. Toxicol.*, 11:955–962.
146. De Groot, A.P., Feron, V.J., and Til, H.P. (1973): Short-term toxicity studies on some salts and oxides of tin in rats. *Food Cosmetic. Toxicol.*, 11:19–30.
147. de la Burde, B., and Choate, M.S. (1975): Early asymptomatic lead exposure and development at school age. *J. Pediatr.*, 87:638–642.
148. Delahant, A.B. (1955): An experimental study of the effects of rare metals on animal lungs. *AMA Arch. Ind. Health*, 12:116–120.
149. DeMeio, R.H. (1947): Tellurium. II. Effect of ascorbic acid on the tellurium breath. *J. Ind. Hyg. Toxicol.*, 29:393–395.
150. Desai, H. (1992): Phosphorus and phosphorus compounds. In: *Hazardous Materials Toxicology*, edited by J.B.Sullivan, Jr., and G.R.Krieger, pp. 937–939. Williams & Wilkins, Baltimore, MD.
151. Descotes, J. (1989): *Immunotoxicology of Drugs and Chemicals.* Elsevier, Amsterdam.
152. Dietrich, K.N. (1991): Human fetal lead exposure: Intrauterine growth, maturation and postnatal neurobehavioral development. *Fundam. Appl. Toxicol.*, 16:17–19.
153. Digenott, D., Rozsa, O., Levy, N., and Muammar, S. (1964): Hypokalemia in barium poisoning. *Lancet*, 2:343–344.
154. Doig, A.T. (1976): Baritosis: A benign pneumoconiosis. *Thorax*, 31:30–39.
155. Doll, R. (1985): Relevance of epidemiology to policies for the prevention of cancer. *Hum. Toxicol.*, 4:81–96.
156. Doll, R. (1992): Is cadmium a human carcinogen? *Ann. Epidemiol.*, 2:335–337.
157. Domingo, J.L., and Corbella, J. (1991): A review of the health hazards from gallium exposure. *Trace Elem. Med.*, 8:56–64.
158. Donaldson, R.M., and Barreras, R.F. (1966): Intestinal absorption of trace quantities of chromium. *J. Lab. Clin. Med.*, 68:484–493.
159. Downs, J.C., Milling, D., and Nichols, C.A. (1995): Suicidal ingestion of barium-sulfide-containing shaving powder. *Am. J. Forens. Med. Pathol.*, 16:56–61.
160. Downs, W.L., Scott, J.K., Steadman, L.T., and Maynard, E.A. (1959): *The toxicity of indium.* Univ. Rochester At. Energy Rep. UR-588.
161. Draize, J.H., and Kelley, E.A. (1959): The urinary excretion of boric acid preparations following oral administration and topical applications to intact and damaged skin of rabbits. *Toxicol. Appl. Pharmacol.*, 1:267–276.
162. Dudley, R.E., Svoboda, D.J., and Klaassen, C.D. (1982): Acute exposure to cadmium causes severe

liver injury in rats. *Toxicol. Appl. Pharmacol.*, 65:302–313.
163. Duell, P.B., and Chesnut, C.H. III. (1991): Exacerbation of rheumatoid arthritis by sodium fluoride treatment of osteoporosis. *Arch. Intern. Med.*, 151:783–784.
164. Dunphy, B. (1967): Acute occupational cadmium poisoning: A critical review of the literature. *J. Occup. Med.*, 9:22–26.
165. Duverger-van Bogaert, and Lambotte-Vandepaer M. (1988): Hafnium. In: *Handbook on Toxicity of Inorganic Compounds*, edited by H.G.Sieler and H.Sigel, pp. 313–318. Marcel Dekker, New York.
166. ECETOC. (1995): Reproductive and general toxicology of some inorganic borates and risks assessment for human beings. *Tech. Rep.* No. 63, Brussels, Belgium.
167. ECETOC. (1997): *Ecotoxicology of some inorganic borates.* Special Rep. No. 11, Brussels, Belgium.
168. Eisenbud, M., Berghout, C.F., and Steadman, L.T. (1948): Environmental studies in plants and laboratories using beryllium: The acute disease. *J. Ind. Hyg. Toxicol.*, 30:282–285.
169. Eisenbud, M., and Lisson, J. (1983): Epidemiological aspects of beryllium-induced nonmalignant lung disease: A 30-year update. *J. Occup. Med.*, 25:196–202.
170. Elinder, C.G., Kjellström, T., Hogstedt, C., et al. (1985): Cancer mortality of cadmium workers. *Br. J. Ind. Med.*, 42:651–655.
171. Elinder, C.G., and Zenz, C. (1994): Other metals and their compounds: Hafnium and its compounds. In: *Occupational Medicine*, edited by C.Zenz, O.B.Dickerson, and E.P.Horvath, Jr., pp. 595–616. Mosby-YearBook, St. Louis, MO.
172. Ellenhorn, M.J., and Barceloux, D.G. (1988). *Medical Toxicology: Diagnosis and Treatment of Human Poisoning*, Elsevier, New York.
173. Ellis, T., and Lacy, R. (1998): Illicit alcohol (moonshine) consumption in West Alabama revisited. *S. Med. J.*, 91:858–860.
174. Engfeldt, B., and Hjertquist, S.O. (1969): Effect of strontium administration on bones and teeth of rats maintained on diets with different calcium contents. *Virchows Arch. Abt. A Pathol. Anat.*, 346:330–344.
175. Enterline, P.E., and Marsh, G.M. (1982): Cancer among workers exposed to arsenic and other substances in a copper smelter. *Am. J. Epidemiol.*, 116:895–911.
176. Ernhart, C.B. (1992): A critical review of low-level prenatal lead exposure in the human: Effects on the fetus and newborn. *Reprod. Toxicol.*, 6:9–19.
177. Esernio-Jenssen, D., Donatelli-Guagenti, A., and Mofenson, H.C. (1996): Severe lead poisoning from an imported clothing accessory: “Watch” out for lead. *Clin. Toxicol.*, 34:329–333.
178. Eskew, A.E., Crutcher, J.C., Zimmerman, S.L., et al. (1961): Lead poisoning resulting from illicit alcohol consumption. *J. Forens. Sci.*, 6:337–350.
179. Ewers, U., Manojilovic, N., Hadnagy, W., and Grover, Y.P. (1988): Chlorine. In: *Handbook on Toxicity of Inorganic Compo-*

unds, edited by H.G.Sieler and H.Sigel, pp. 223–237. Marcel Dekker, New York.
180. Fahim, M.S., Fahim, Z., and Hall, D.G. (1976): Effects of subtoxic lead levels on pregnant women in the State of Missouri. *Res. Commun. Chem. Pathol. Pharmacol.*, 13:309–331.
181. Fail, P.A., George, J.D., Seely, J.C., Grizzle, T.B., and Heindel, J.J. (1991): Reproductive toxicity of boric acid in Swiss (CD-1) mice: Assessment using the continuous breeding protocol. *Fundam. Appl. Toxicol.*, 17:225–239.
182. Fairhall, L.T., Dunn, R.C., Sharpless, N.E., and Pritchard, E.A. (1945): *The toxicity of molybdenum.* Public Health Bull. No. 293, U.S. Government Printing Office, Washington, D.C.
183. Farwell, A.P. and Braverman, L.E. (1996): Thyroid and antithyroid drugs. In: *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, edited by J.G.Hardman, L.E.Limbird, and A.G.Gilman, p. 1392. McGraw-Hill, New York.
184. FDC Reports. (1997): Selenium cancer reduction health claim needs further clinical evidence. *Tan Sheet*, 5(39):8–10.
185. Feldman, R.G., Niles, C.A., Kelly-Hayes, M., Sax, D.S., Dixon, W.J., Thompson, D.J., and Landau, E. (1979): Peripheral neuropathy in arsenic smelter workers. *Neurology*, 29:939–944.
186. Ferguson, W.S., Lewis, A.H., and Watson, S.J. (1938): Action of molybdenum in nutrition of milking cows. *Nature*, 141:553.
187. Fischbein, A. (1992): Occupational and environmental lead exposure. In: *Environmental and Occupational Medicine*, edited by W.N.Rom, pp. 735–758. Little, Brown, Boston.
188. Fisher, A.A. (1986): Antiseptics and disinfectants. In: *Contact Dermatitis*, pp. 178–194. Lea and Febiger, Philadelphia.
189. Fisher, A.A. (1986): Contact urticaria. In: *Contact Dermatitis*, p. 698. Lea and Febiger, Philadelphia.
190. Fisher, D. (1992): Zinc. In: *Hazardous Materials Toxicology*, edited by J.B.Sullivan, Jr., and G.R.Krieger, pp. 865–868. Williams & Wilkins, Baltimore, MD.
191. Fisher, N., Hutchinson, J.B., Berry, R., Hardy, J., Ginocchio, A. V., and Waite, V. (1979): Long-term toxicity and carcinogenicity studies of the bread improver potassium bromate. I. Studies in rats. *Food Cosmet. Toxicol.*, 17:33–39.
192. Flanagan, P.R., McLellan, J.S., Haist, J., et al. (1978): Increased dietary cadmium absorption in mice and human subjects with iron deficiency. *Gastroenterology*, 74:841–846.
193. Flegal, A.R., and Smith, D.R. (1992): Lead levels in preindustrial humans. *N. Engl J. Med.*, 326:1293–1294.
194. Flury, F., and Zernik, F. (1931): *Schädliche gase däpfe, nebel, rauch- und staubarten*, p. 309. Verlag von Julius Springer, Berlin.
195. Forbes, R.M. (1960): Nutritional interactions of zinc and calcium. *Fed. Proc. FASEB*, 19:643–647.
196. Foster, L.H., and Sumar, S. (1997): Selenium in health and disease: A review. *Crit. Rev. Food Sci. Nutr.*, 37:211–228.
197. Foulkes, E.C. (1990): The concept of critical levels of toxic heavy metals in target tissues. *CRC Crit. Rev. Toxicol.*, 20:327–339.
198. Franke, K.W., Moxon, A.L., Poley, W.E., and Tully, W.C. (1936): Monstrosities produced by the injection of selenium salts into hens' eggs. *Anat. Rec.*, 65:15–22.
199. Fredrick, W.G., and Bradley, W.R. (1946): Toxicity of some materials used in the manufacture of cemented tungsten carbide tools. *Ind. Med.*, 15:482–483.
200. Friberg, L. (1984): Cadmium and the kidney. *Environ. Health Perspect.*, 54:1–11.
201. Friberg, L., Piscator, M., Nordberg, G.F., et al. (1974): *Cadmium in the Environment.* CRC Press, Boca Raton, FL.
202. Friis-Hansen, B., Aggerbeck, B., and Jansen, J.A. (1982): Unaffected blood boron levels in newborn infants treated with a boric acid ointment. *Food Chem. Toxicol.*, 20:451–454.
203. Fung, Y.K., and Molvar, M.P. (1992): Toxicity of mercury from dental environment and from amalgam restorations. *Clin. Toxicol.*, 30:49–61.
204. Gasmi, A., Garnier, R., Galliot-Guilley, M., et al. (1997): Acute selenium poisoning. *Vet Hum. Toxicol.*, 39:304–308.
205. Gennart, J.-P., Buchet, J.-P., Roels, H., Ghyselen, P., Ceulemans, E., and Lauwerys, R. (1992): Fertility of male workers exposed to cadmium, lead, or manganese. *Am. J. Epidemiol.*, 135:1208–1219.
206. Gerber, G.B., and Leonard, A. (1997): Mutagenicity, carcinogenicity and teratogenicity of germanium compounds. *Mutat. Res.*, 387:141–146.
207. Gheysens, B., Auwerx, J., Van den Eeckhout, A., and Demedts, M. (1985): Cobalt-induced bronchial asthma in diamond polishers. *Chest*, 88:740–744.

208. Gilmore, D.A., Jr., and Bronstein, A.C. (1992): Manganese and magnesium. In: *Hazardous Materials Toxicology*, edited by J. B.Sullivan, Jr., and G.R.Krieger, pp. 896–901. Williams & Wilkins, Baltimore, MD.
209. Gilmore, D.A., Jr., and Bronstein, A.C. (1992): Titanium. In: *Hazardous Materials Toxicology*, edited by J.B.Sullivan, Jr., and G.R.Krieger, pp. 904–905. Williams & Wilkins, Baltimore, MD.
210. Ginocchio, A.V., Waite, V., Hardy, J., Fisher N., Hutchinson, J. B., and Berry, R. (1979): Long-term toxicity and carcinogenicity studies of the bread improver potassium bromate. 2. Studies in mice. *Food Cosmet. Toxicol.*, 17:41–47.
211. Glaser, U., Hochrainer, D., Otto, F.J., and Oldiges, H. (1989): Carcinogenicity and toxicity of four cadmium compounds inhaled by rats. *Toxicol. Environ. Chem.*, 27:153–162.
212. Goering, P.L. (1992): Platinum and related metals: Palladium, indium, osmium, rhodium, and ruthenium. In: *Hazardous Materials Toxicology*, edited by J.B.Sullivan, Jr., and G.R. Krieger, pp. 874–881. Williams & Wilkins, Baltimore, MD.
213. Goering, P.L., and Klaassen, C.D. (1984): Zinc-induced tolerance to cadmium hepatotoxicity. *Toxicol. Appl. Pharmacol.*, 74:299–307.
214 Gold, L.S., Sawyer, C.B., Magaw, R., et al. (1984): A carcinogenic potency database of the standardized results of animal bioassays. *Environ. Health Perspect.*, 58:9–319.
215. Goldbloom, R.B., and Goldbloom, A. (1953): Boric acid poisoning: Report of four cases and a review of 109 cases from the world literature. *J. Pediatr.*, 43:631–643.
216. Goldman, L.R. (1998): Linking research and policy to ensure children's environmental health. *Environ. Health Perspect.*, 106:S857–S862.
217. Goldman, R.H., Baker, E.L., Hannan, M., and Kamerow, D.B. (1987): Lead poisoning in automobile radiator repair mechanics. *N. Engl. J. Med.*, 317:214–218.
218. Golub, M.S., and Domingo, J.L. (1996): What we know and what we need to know about developmental aluminum toxicity. *J. Toxicol. Environ. Health*, 48:585–597.
219. Gompertz, D. (1981): Assessment of risk by biological monitoring. *Br. J. Ind. Med.*, 38:198–201.
220. Gordeuk, V.R., McLaren, G.D., and Samowitz, W. (1994): Etiologies, consequences, and treatment of iron overload. *Crit. Rev. Clin. Lab. Sci.*, 31:89–133.
221. Gordeuk, V.R., Mukiibi, J., Hasstedt, S.J., et al. (1992): Iron overload in Africa. Interaction between a gene and dietary iron content. *N. Engl. J. Med.*, 326:95–100.
222. Gosselin, R.E., Smith, R.P., and Hodge, H.C. (1984): Arsenic. In: *Clinical Toxicology of Commercial Products*, edited by R.E. Gosselin, R.P.Smith, and H.C.Hodge, pp. III-42 to III-47. Williams & Wilkins, Baltimore, MD.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_688.html[2010/10/28 18:46:49]

223. Goyer, R.A. (1986): Toxic effects of metals. In: *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 3rd ed., edited by C.D.Klaassen, M.O.Amdur, and J.Doull, pp. 582–635. Macmillan, New York.
224. Goyer, R.A., Miller, C.R., Zhu, S.-Y., and Victery, W. (1989): Non-metallothionein-bound cadmium in the pathogenesis of cadmium nephrotoxicity in the rat. *Toxicol. Appl. Pharmacol.*, 101:232–244.
225. Goyer, R.A., and Rhyne, B.C. (1973): Pathological effects of lead. *Int. Rev. Exp. Pathol.*, 12:1–77.
226. Graves, A.B., Rosner, D., Echeverria, D., Mortimer, J.A., and Larson, E.B. (1998): Occupational exposures to solvents and aluminum and estimated risk of Alzheimer's disease. *Occup. Environ. Med.*, 55:627–633.
227. Graziano, J.H., and Blum, C. (1991): Lead exposure from lead crystal. *Lancet*, 337:141–142.
228. Greengard, J. (1975): Iron poisoning in children. *Clin. Toxicol.*, 8:575–597.
229. Greger, J.L., and Johnson, M.A. (1981): Effect of dietary tin on zinc, copper, and iron utilization by rats. *Food Cosmet. Toxicol.*, 19:163–166.
230. Greger, J.L., and Sutherland, J.E. (1997): Aluminum exposure and metabolism. *Crit. Rev. Clin. Lab. Sci.*, 34:439–474.
231. Grignon, S., and Bruguerolle, B. (1996): Cerebellar lithium toxicity: A review of recent literature and tentative pathophysiology. *Therapie*, 51:101–106.
232. Groleau, G. (1994): Lithium toxicity. *Conc. Controv. Toxicol.*, 12:511–531.
233. Groth, D.H., Stettler, L.E., and Burg, J.R. (1986): Carcinogenic effects of antimony trioxide and antimony ore concentrate in rats. *J. Toxicol. Environ. Health*, 18:607–626.
234. Habeebu, S.S.M., Liu, J., and Klaassen, C.D. (1998): Cadmium-induced apoptosis in mouse liver. *Toxicol. Appl. Pharmacol.*, 149:203–209.
235. Hadjimarkos, D.M. (1965): Effect of selenium on dental caries. *Arch. Environ. Health*, 10:893–899.
236. Hadjimarkos, D.M., Storvick, C.A., and Remmert, L.F. (1952): Selenium and dental caries. *J. Pediatr.*, 40:451–455.
237. Hadjimichael, O.C., and Brubaker, R.E. (1981): Evaluation of an occupational respiratory exposure to a zirconium-containing dust. *J. Occup. Med.*, 23:543–547.
238. Haley, T.J., Komesu, N., and Raymond, K. (1962): Pharmacology and toxicology of niobium chloride. *Toxicol. Appl. Pharmacol.*, 4:385–392.
239. Haley, T.J., Raymond, K., Komesu, N., and Upham, H.C. (1962): The toxicologic and pharmacologic effects of hafnium salts. *Toxicol. Appl. Pharmacol.*, 4:238–246.
240. Hallberg, L., Brune, M., Erlandsson, M., et al. (1991): Calcium: Effect of different amounts of nonheme- and heme-iron absorption in humans. *Am. J. Clin. Nutr.*, 53:112–119.
241 Hamada, T., Tanimoto, A., and Sasguri, Y. (1997): Apoptosis induced by cadmium. *Apoptosis*, 2:359–367.
242. Hamilton, A., and Hardy, H. (1974): Osmium. In: *Industrial Toxicology*, pp. 155–156. Publishing Sciences Group, Acton, MA.
243. Hardy, A.D., Vaishnav, R., Al-Kharusi, S.S.Z., Sutherland, H. H., and Worthing, M.A. (1998): Composition of eye cosmetics (kohls) used in Oman. *J. Ethnopharmacol.*, 60:223–234.
244. Hardy, H.L., and Tabershaw, I.R. (1946): Delayed chemical pneumonitis occurring in workers exposed to beryllium compounds. *J. Ind. Hyg. Toxicol.*, 28:197–211.
245. Harlan, W.R. (1988): The relationship of blood lead levels to blood pressure in the U.S. population. *Environ. Health Perspect.*, 78:9–13.
246. Harr, J.R., and Muth, O.H. (1972): Selenium poisoning in domestic animals and its relationship to man. *Clin. Toxicol.*, 5:175–186.
247. Hartman, R.S., Conrad, M.E., Hartman, R.E., et al. (1963): Ferritin-containing bodies in human small intestinal epithelium. *Blood*, 22:397–405.
248. Hartwig, A. (1994): Role of DNA repair inhibition in lead- and cadmium-induced genotoxicity: A review. *Environ. Health Perspect.*, 102(suppl. 3):45–50.
249. Hatchcock, J.N., and Rader, J.I. (1990): Macronutrient safety. *Ann. NY Acad. Sci.*, 587:257–266.
250. Haug, R.H. (1996): Retention of asymptomatic bone plates used for orthognathic surgery and facial fractures. *J. Oral Maxillofac. Surg.*, 54:611–617.
251. Heindel, J.J., Price, C.J., Field, E.A., et al. (1992): Developmental toxicity of boric acid in mice and rats. *Fundam. Appl. Toxicol.*, 18:266–277.
252. Heinrich, U., Peters, L., Ernst, H., et al. (1989): Investigation on the carcinogenic effects of various cadmium compounds after inhalation exposure in hamsters and mice. *Exp. Pathol.*, 37:253–258.
253. Helzlsouer, K., Jacobs, R., and Morris, S. (1985): Acute selenium poisoning in the United States.

Fed. Proc. FASEB, 44:1670.
254.. Henderson, Y., and Haggard, H.W. (1943): *Noxious Gases*, p. 133. Reinhold, New York.
255. Herrero, F., Fernandez, E., Gomez, J., et al. (1995): Thallium poisoning presenting with abdominal colic, paresthesia, and irritability. *Clin. Toxicol.*, 33:261–264.
256. Hertz-Picciotto, I., and Croft, J. (1993): Review of the relation between blood lead and blood pressure. *Epidemiol. Rev.*, 15:352–373.
257. Heyman, A., Pfeifer, J.B., Willett, R.W., and Taylor, H.M. (1956): Peripheral neuropathy caused by arsenical intoxication. *N. Engl. J. Med.*, 254:401–408.
258. Hider, R.C., Choudhury, R., Rai, B.J., et al. (1996): Design of orally active iron chelators. *Acta Haematol.*, 95:6–12.
259. Higgins, E.S., Richert, D.A., and Westerfeld, W.W. (1956): Molybdenum deficiency and tungstate inhibition studies. *J. Nutr.*, 59:539–559.
260. Hiles, R.A. (1974): Absorption, distribution, and excretion of inorganic tin in rats. *Toxicol. Appl. Pharmacol.*, 27:366–379.
261. Hipkins, K.L., Materna, B.L., Kosnett, M.J., et al. (1998): Medical surveillance of the lead exposed worker. *AAOHN J.*, 46:330–339.
262. Hirano, S., Kodama, N., Shibata, K., and Suzuki, K.T. (1990): Distribution, localization, and pulmonary effects of yttrium chloride following intratracheal instillation into the rat. *Toxicol. Appl. Pharmacol.*, 104:301–311.
263. Hirano, S., Kodama, N., Shibata, K., and Suzuki, K.T. (1993): Metabolism and toxicity of intravenously injected yttrium chloride in rats. *Toxicol. Appl. Pharmacol.*, 121:224–232.
264. Hoffman, D.J., Ohlendorf, H.M., and Aldrich, T.W. (1988): Selenium teratogenesis in natural populations of aquatic birds in central California. *Arch. Environ. Contam. Toxicol.*, 17:519–525.
265. Hogstrand, C., and Wood, C.M. (1998). Toward a better understanding of the bioavailability, physiology, and toxicity of silver in fish: Implications for water quality criteria. *Environ. Toxicol. Chem.*, 17:547–561.
266. Hollinger, M.A. (1996): Toxicological aspects of topical silver Pharmaceuticals. *Crit. Rev. Toxicol.*, 26:255–260.
267. Holmquist, I. (1951): Occupational arsenical dermatitis. A study among employees at a copper ore smelting work including investigations of skin reactions to contact with arsenic compounds. *Acta Dermatol. Venereol.*, 31(suppl. 26).
268. Hostynek, J.J. (1997): Gold: An allergen of growing significance. *Food Chem. Toxicol.*, 35:839–844.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_689.html[2010/10/28 18:46:50]

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269. Hu, H., Aro, A., Payton, M., Korrick, S., Sparrow, D., Weiss, S. T., and Rotnitzky, A. (1996): The relationship of bone and blood lead to hypertension. *J. Am. Med. Assoc.*, 275:1171–1176.
270. Hu, H., Watanabe, H., Payton, M., Korrick, S., and Rotnitzky, A. (1994): The relationship between bone lead and hemoglobin. *J. Am. Med. Assoc.*, 272:1512–1517.
271. Huel, G., Boudene, C., and Ibrahim, M.A. (1981): Cadmium and lead content of maternal and newborn hair: Relationship to parity, birth weight and hypertension. *Arch. Environ. Health*, 36:221–227.
272. Hughes, E.G. (1980): Medical surveillance of platinum refinery workers. *J. Soc. Occup. Med.*, 30:27–30.
273. Hunter, D.J., Morris, J.S., Stampfer, M.J., et al. (1990): A prospective study of selenium status and breast cancer risk. *J. Am. Med. Assoc.*, 264:1128–1131.
274. Huott, M.A., and Storrow, A.B. (1997): A survey of adolescents' knowledge regarding toxicity of over-the-counter medications. *Acad. Emerg. Med.*, 4:214–218.
275. Husain, M.H., Dick, J.A., and Kaplan, Y.S. (1980): Rare earth pneumoconiosis. *J. Soc. Occup. Med.*, 30:15–19.
276. Hussey, H.H. (1976): Phosphorus poisoning in children. *J. Am. Med. Assoc.*, 235:1366.
277. Insel, P.A., (1995): Analgesic-antipyretic and antiinflammatory agents and drugs employed in the treatment of gout. In: *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, edited by J.G.Hardman, L.E.Limbird, and A.G.Gilman, pp. 617–657. McGraw-Hill, New York.
278. International Agency for Research on Cancer. (1980): Some metals and metallic compounds, *IARC Monogr. Eval. of the Carcinogen. Risk of Chem. Hum.*, 23:39–141.
279. International Agency for Research on Cancer. (1986): Potassium bromate. *IARC Monogr. Eval. Carcinogen. Risks Hum.*, 40.
280. International Agency for Research on Cancer. (1990): Chromium, nickel and welding. *IARC Monogr. Eval. Carcinogen. Risks Hum.*, 49:257–445.
281. International Agency for Research on Cancer. (1993): Beryllium, cadmium, mercury, and exposures in the glass manufacturing industry. *IARC Monogr. Eval. Carcinogen. Risks Hum.*, 58:41–237.
282. Ip, C. (1985): Selenium inhibition of chemical carcinogenesis. *Fed. Proc. FASEB*, 44:2573–2578.
283. Iregren, A. (1990): Psychological test performance in foundry workers exposed to low levels of manganese. *Neurotoxicol. Teratol.*, 12:673–675.
284. Ishinishi, N., Yamamoto, A., Hisanaga, A., and Inamasu, T. (1983): Tumorigenicity of arsenic trioxide to the lung in Syrian golden hamsters by intermittent instillations. *Cancer Lett.*, 21:141–147.
285. Ivankovic, S., Eisenbrand, G., and Preussmann, R. (1979): Lung carcinoma induction in BD rats after a single intratracheal instillation of an arsenic-containing pesticide mixture formerly used in vineyards. *Int. J. Cancer*, 24:786–788.
286. Jacobs, R.W., Duong, T., Jones, R.E., Trapp, G.A., and Scheibel, A.B. (1989): A reexamination of aluminum in Alzheimer's disease: Analysis by energy dispersive x-ray microprobe and flameless atomic absorption spectrophotometry. *Can J. Neurol. Sci.*, 16:498–503.
287. Jaffe, W.G., and Velez, F.B. (1973): Selenium intake and congenital malformations in humans. *Arch. Latinoam. Nutr.*, 23:515–517.
288. Jarup, L., Elinder, C.G., and Spang, G. (1988): Cumulative blood-cadmium and tubular proteinuria: A dose-response relationship. *Int. Arch. Occup. Environ. Health*, 60:223–229.
289. Jensen, R., Closson, W., and Rothenberg, R. (1984): Selenium intoxication—New York. *Morbid. Mortal. Weekly Rep.*, 33:157–158.
290. Johnson, J.L., and Rajagopalan, K.V. (1974): Molecular basis of the biological function of molybdenum. *J. Biol. Chem.*, 249:859–866.
291. Johnson, P.E. (1996): New approaches to establish mineral element requirements and recommendations: An introduction. *J. Nutr.*, 126:2309S–2311S.
292. Johnson, R.A., Baker, S.S., Fallon, J.T., et al. (1981): An occidental case of cardiomyopathy and selenium deficiency. *N. Engl. J. Med.*, 304:1210–1212.
293. Jones, G., and Brooks, P.M. (1996): Injectable gold compounds: An overview. *Br. J. Rheumatol.*, 35:1154–1158.
294. Kabe, I., Omae, K., Nakashima, H., Nomiyama, T., Uemura, T., Hosoda, K., Ishizuka, C., Yamazaki, K., and Sakurai, H. (1996): In vitro solubility and in vivo toxicity of indium phosphide. *J. Occup. Health*, 38:6–12.
295. Kamerbeek, H.H., Rauws, A.G., Ham, M.T., et al. (1971): Redistribution of thallium by treatment with sodium diethyldithiocarbamate. *Acta Med. Scand.*, 189:149–154.
296. Kanisawa, M., and Schroeder, H.A. (1967): Life term studies on the effect of arsenic, germanium,

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_690.html[2010/10/28 18:46:51]

tin and vanadium on spontaneous tumors in mice. *Cancer Res.*, 27:1192–1195.
297. Kanisawa, M., and Schroeder, H.A. (1969): Life term studies on the effect of trace elements on spontaneous tumors in mice and rats. *Cancer Res.*, 29:892–895.
298. Kappas, A., and Maines, M.D. (1976): Tin: A potent inducer of heme oxygenase in kidney. *Science*, 192:60–62.
299. Katz, S.A. (1995): The toxicity/essentiality of dietary minerals: A review on some micronutrients prepared in honor of the award for life achievement to Doctor Krist Kostial. *Arh. Hig. Rada. Toksikol.*, 46:333–345.
300. Kaye, W.E., Novotny, T.E., and Tucker, M. (1987): New ceramics-related industry implicated in elevated blood lead levels in children. *Arch. Environ. Health*, 42:161–164.
301. Kazantzis, G. (1979): Thallium. In: *Handbook on the Toxicology of Metals*, edited by L.Friberg, G.F.Nordberg, and V.B.Vouk, pp. 599–612. Elsevier/North Holland, New York.
302. Keall, J.H.H., Martin, N.H., and Tunbridge, R.E. (1946): A report of three cases of accidental poisoning by sodium tellurite. *Br. J. Ind. Med.*, 3:175–176.
303. Keshan Disease Research Group. (1979): Epidemiologic studies on the etiologic relationship of selenium and Keshan isease. *Chin. Med. J.*, 92:477–482.
304. Kettrup, A., and Hüppe, U. (1988): Phosphorus. In: *Handbook on Toxicity of Inorganic Compounds*, edited by H.G.Sieler and H.Siegel, pp. 521–532. Marcel Dekker, New York.
305. Khera, A.K., Wibberley, D.G., and Dathan, J.G. (1980): Placental and stillbirth tissue lead concentrations in occupationally exposed women. *Br. J. Ind. Med.*, 37:394–396.
306. Kikano, G.E., and Stange, K.C. (1992): Lead poisoning in a child after a gunshot injury. *J. Fam. Pract.*, 34:498–504.
307. Kim, G.-S., Judd, D.A., Hill, C.L., and Schinazi, R.F. (1994): Synthesis, characterization, and biological activity of a new potent class of anti-HIV agents, the peroxoniobium-substituted heteropolytungstates. *J. Med. Chem.*, 37:816–820.
308. Kim, R., Rotnitzky, A., Sparrow, D., Weiss, S.T., Wager, C., and Hu, H. (1996): A longitudinal study of low-level lead exposure and impairment of renal function. *J. Am. Med. Assoc.*, 275:1177–1181.
309. Kim, T.S., and Yim, S.Y. (1997): Peripheral nerve and muscle diseases I. *Brain Pathol.*, 7:1117–1121.
310. Kimbrough, R.D. (1976): Toxicity and health effects of selected organotin compounds: A review. *Environ. Health Perspect.*, 14:51–56.
311. Kinard, F.W., and van de Erve, J. (1940): Rat mortality following sodium tungstate injection. *Am. J. Med. Sci.*, 199:668–670.

312. Kinard, F.W., and van de Erve, J. (1943): Effect of tungsten metal diets in the rat. *J. Lab. Clin. Med.*, 28:1541–1543.
313. Kittle, C.F., King, E.R., and Brucer, M. (1951): The tissue distribution and excretion of radioactive hafnium mandelate in the rat. *J. Pharmacol. Exp. Ther.*, 101:21.
314. Kjellström, T., Elinder, C.-G., and Friberg, L. (1984): Conceptual problems in establishing the critical concentration of cadmium in human kidney cortex. *Environ. Res.*, 33:284–295.
315. Kjellström, T., Friberg, L., and Rahnster, B. (1979): Mortality and cancer morbidity among cadmium-exposed workers. *Environ. Health Perspect.*, 28:199–204.
316. Klaassen, C.D. (1995): Nonmetallic environmental toxicants, In: *Goodman & Gilman's The pharmacological basis of therapeutics*, 9th ed., edited by J.G.Hardman, L.E.Limbird, and A.G.Gilman, pp. 1673–1696. McGraw-Hill, New York.
317. Klatzo, I., Wesniewski, H., and Streicher, E. (1965): Experimental production of neurofibrillary degeneration. *J. Neuropathol. Exp. Neurol.*, 24:187–199.
318. Kleinfeld, M. (1965): Acute pulmonary edema of chemical origin. *Arch, Environ. Health*, 10:942–946.
319. Kok, F.J., de Bruijn, A.M., Hofman, A., et al. (1987): Is serum selenium a risk factor for cancer in men only? *Am. J. Epidemiol.*, 125:12–16.
320. Kontoghiorghes, G.J. (1995): Comparative efficacy and toxicity of desferrioxamine, deferiprone and other iron and aluminum chelating agents. *Toxicol. Lett.*, 80:1–18.
321. Köpf-Maier, P., and Klapötke, T. (1992): Antitumor activity of ionic mobecene and molybdenocene complexes in high oxidation states. *J. Cancer Res. Clin. Oncol.*, 118:216–221.
322. Köpf-Maier, P., and Köpf, H. (1994): Organometallic titanium, vandadium, niobium, molybdenum and rhenium complexes-early transition metal antitumour drugs. In: *Metal Compounds in Cancer Therapy*, edited by S.P.Fricker, pp. 109–146. Chapman and Hall, London.
323. Koppel, C., Baudisch, H., Beyer, K.-H., et al. (1986): Fatal poisoning with selenium dioxide. *Clin. Toxicol.*, 24:21–35.
324. Kotter, J.M., and Zieger, G. (1992): Sarkoidale Granulomatose nach mehrjáhriger zircokoniumexposition, eine "zirkoniumlunge." *Pathologe*, 13:104–109.
325. Kravzov, J., Rios, C., Altagracia, M., Monroy-Noyola, A., and Lopez, F. (1993): Relationship between physiochemical properties of Prussian blue and its efficacy as antidote against thallium poisoning. *J. Appl. Toxicol.*, 13:213–216.
326. Krenzelok, E.P. (1992): Sodium and potassium. In: *Hazardous Materials Toxicology*, edited by J.B.Sullivan, Jr., and G.R. Krieger, pp. 797–799. Williams & Wilkins, Baltimore, MD.
327. Krishnan, S.S., Harrison, J.E., and Crapper McLachlan, D.R. (1987): Origin and resolution of the aluminum controversy concerning Alzheimer's neurofibrillary degeneration. *Biol. Trace Element Res.*, 13:35–42.
328. Kubota, J., Allaway, W.H., Carter, D.L., et al. (1967): Selenium in crops in the United States in relation to selenium-responsive diseases of animals. *J. Agric. Food Chem.*, 15:448–453.
329. Kurtzke, J.F. (1991): Risk factors in amyotrophic lateral sclerosis. *Adv. Neurol.*, 56:245–270.
330. Laine, J., Kalimo, K., Forssell, H., and Happonen, R.P. (1992): Resolution of oral lichenoid lesions after replacement of amalgam restorations in patients allergic to mercury compounds. *Br. J. Dermatol.*, 126:10–15.
331. Lamm, S.H., Parkinson, M., Anderson, M., et al. (1992): Determinants of lung cancer risk among cadmium-exposed workers. *Ann. Epidemiol.*, 2:195–211.
332. Lampert, P.W., and Garret, R.S. (1971): Mechanism of demyelination in tellurium neuropathy. Electron microscope observations. *Lab. Invest.*, 25:380–388.
333. Landolt, R.R., Berk, H.W., and Russell, H.T. (1972): Studies on the toxicity of rhodium trichloride in rats and rabbits. *Toxicol. Appl. Pharmacol.*, 21:589–590.
334. Lanphear, B.P. (1998): The paradox of lead poisoning prevention. *Science*, 281:1617–1618.
335. Lansdown, A.B.G. (1995): Physiological and toxicological changes in the skin resulting from the action and interaction of metal ions. *Crit. Rev. Toxicol.*, 25:397–462.
336. Larner, A.J. (1995): Biological effects of tellurium: A review. *Trace Elem. Electrol.*, 12:26–31.
337. Lauwerys, R., Roels, H., Benet, P., et al. (1985): Fertility of male workers exposed to mercury vapor or to manganese dust: A questionnaire study. *Am. J. Ind. Med.*, 7:171–176.
338. Lauwerys, R.R. (1989): Metals—Epidemiological and experimental evidence for carcinogenicity. *Arch. Toxicol. (Suppl)*, 13:21–27.
339. Leach, L.J., Maynard, E.A., Hodge, H.C., Scott, J.K., Yuile, C. L., Sylvester, G.E., and Wilson, H.B.

(1970): A five year inhalation study with uranium dioxide (UO_2) dust. I. Retention and biologic effect in the monkey, dog and rat. *Health Phys.*, 18:599–612.
340. Lee, K.P., Henry, N.W., III, Trochimowicz, H.J., and Reinhardt, C.F. (1986): Pulmonary response to impaired lung clearance in rats following excessive TiO_2 dust deposition. *Environ. Res.*, 44:144–167.
341. Lee-Feldstein, A. (1983): Arsenic and respiratory cancer in man: Follow-up of an occupational study, In: *Arsenic: Industrial, Biomedical, and Environmental Perspectives*, edited by W.H. Lederer and R.J.Fensterheim, pp. 245–265. Van Nostrand Reinhold, New York.
342. Leggett, R.W. (1993): An age-specific kinetic model of lead metabolism in humans. *Environ. Health Perspect.*, 101:598–616.
343. Leonard, A., and Gerber, G.B. (1997): Mutagenicity, carcinogenicity and teratogenicity of thallium compounds. *Mutat. Res.*, 387:47–53.
344. Leonard, A., Hantson, P., and Gerber, G.B. (1995): Mutagenicity, carcinogenicity and teratogenicity of lithium compounds. *Mutat. Res.*, 339:131–137.
345. Lerda, D. (1992): Study of sperm characteristics in persons occupationally exposed to lead. *Am. J. Ind. Med.*, 22:567–571.
346. Levander, O.A. (1991): Scientific rationale for the 1989 Recommended Dietary Allowance for selenium. *Perspect. Pract.*, 91:1572–1576.
347. Lewis, G.P., Coughlin, L., Jusko, W., et al. (1972): Contribution of cigarette smoking to cadmium accumulation in man. *Lancet*, 1:291–292.
348. Liipo, K.K., Anttila, S.L., Taikina-aho, O., Ruodonen, E.-L., Toivonen, S.T., and Tuomi, T. (1993): Hypersensitivity pneumonitis and exposure to zirconium silicate in a young ceramic tile worker. *Am. Rev. Respir. Dis.*, 148:1089–1092.
349. Lin, S., Hwang, S.A., Marshall, E.G., and Marion, D. (1998): Does paternal occupational lead exposure increase the risks of low birth weight or prematurity? *Am. J. Epidemiol.*, 148:173–181.
350. Linder, M.C., and Hazegh, M. (1996): Copper biochemistry and molecular biology. *Am. J. Clin. Nutr.*, 63:797S–811S.
351. Lin-Fu, J.S. (1980): Lead poisoning and undue lead exposure in children: History and current status. In: *Low Level Lead Exposure: The Clinical Implications of Current Research*, edited by H.L. Needleman, pp. 5–16. Raven Press, New York.
352. Lison, D. (1996): Human toxicity of cobalt-containing dust and experimental studies on the mechanism of interstitial lung disease (hard metal disease). *Crit. Rev. Toxicol.*, 26:585–616.
353. Lister, J. (1875): Recent improvements in the details of antiseptic surgery. *Lancet*, 603–605.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_691.html[2010/10/28 18:46:51]

354. Litovitz, T.L., Holm, K.C., Bailey, K.M., and Schmitz, B.F. (1992): 1991 Annual report of the American Association of Poison Control Centers National Data Collection System. *Am. J. Emerg. Med.*, 10:452–505.
355. Lockitch, G., Taylor, G.P., Wong, L.T.K., et al. (1990): Cardiomyopathy associated with nonendemic selenium deficiency in a caucasian adolescent. *Am. J. Clin. Nutr.*, 52:572–577.
356. Lokich, J., and Anderson, N. (1998): Carboplatin versus cisplatin in solid tumors: An analysis of the literature. *Ann. Oncol.*, 9:13–21.
357. Longnecker, M.P., Taylor, P.R., Levander, O.A., et al. (1991): Selenium in diet, blood, and toenails in relation to human health in a seleniferous area. *Am. J. Clin. Nutr.*, 53:1288–1294.
358. Loser, E., (1980): A two year oral carcinogenicity study with cadmium on rats. *Cancer Lett.*, 9:191–198.
359. Lovejoy, F.H. (1982): Chelation therapy in iron poisoning. *J. Toxicol. Clin. Toxicol.*, 19:871–874.
360. Lucas, P.A., Jariwalla, A.G., Jones, J.H., Gough, J., and Vale, P. T. (1980): Fatal cadmium fume poisoning. *Lancet*, 2(8187):205.
361. Lufkin, N.H., and Hodges, F.T. (1944): Cadmium poisoning, report of outbreak. *U.S. Nav. Med. Bull.*, 43:1273–1276.
362. Mabuchi, K., Lilienfeld, A.M., and Snell, L.M. (1979): Lung cancer among pesticide workers exposed to inorganic arsenicals. *Arch. Environ. Health*, 34:312–320.
363. MacGillivray, R.T., Mendez, E., Sinha, S.K., et al. (1982): The complete amino acid sequence of human serum transferrin. *Proc. Natl. Acad. Sci. USA*, 79:2504–2508.
364. Mack, R.B. (1990): The fat lady enters stage left. *N. Carol. Med. J.*, 51:636–638.
365. Mahaffey-Six, K., and Goyer, R.A. (1972): The influence of iron deficiency on tissue content and toxicity of ingested lead in the rat. *J. Lab. Clin. Med.*, 79:128–136.
366. Malbrain, M.L., Lambrecht, G.L., Zandijk, E., et al. (1997): Treatment of severe thallium intoxication. *Clin. Toxicol.*, 35:97–100.
367. Manheimer, E.W., and Silbergeld, E.K. (1998): Critique of CDC's retreat from recommending universal lead screening for children. *Public Health Rep.*, 113:38–46.
368. Manton, W.I. (1994): Lead poisoning from gunshots—A five century heritage. *Clin. Toxicol.*, 32:387–389.
369. Mappes, R. (1977): Versuche zur ausscheidung von arsen im urin. *Int. Arch. Occup. Environ. Health*, 40:267–272.
370. Marcus, R.L., Turner, S., and Cherry, N.M. (1996): A study of lung function and chest radiograms in men exposed to zirconium compounds. *Occup. Med.*, 46:109–113.
371. Marcus, W.L. (1994): Lithium: A review of its pharmacokinetics, health effects, and toxicology. *J. Environ. Path. Toxicol. Oncol.*, 13:73–79.
372. Marie, P.J., Gabra, M.-T., Hott, M., and Miravet, L. (1985): Effect of low doses of stable strontium on bone metabolism in rats. *Miner. Electrolyte Metab.*, 11:5–13.
373. Markesbery, W.R., Ehmann, W.D., Hossain, T.I.M., Aluaddin, M., and Goodin, D.T. (1981): Instrumental neutron activation analysis of brain aluminum in Alzheimer disease and aging. *Ann. Neurol.*, 10:511–516.
374. Martin, J.L., and Gerlach, M.L. (1972): Selenium metabolism in animals. *Ann. NY Acad. Sci.*, 192:193–199.
375. Martyn, C.N., Coggon, D.N., Inskip, H., Lacey, R.F., and Young, W.F. (1997): Aluminum concentrations in drinking water and risk of Alzheimer's disease. *Epidemiology*, 8:281–286.
376. Martyn, C.N., Osmond, C., Edwardson, J.A., Barker, D.J.P., Harris, E.C., and Lacey, R.F. (1989): Geographical relation between Alzheimer's disease and aluminum in drinking water. *Lancet*, 1:59–62.
377. Matsumuro, K., Izumo, S., Higuchi, I., Ronquillo, A.T., Takahashi, K., and Osame, M. (1993): Experimental germanium dioxide-induced neuropathy in rats. *Acta Neuropathol.*, 86:547–553.
378. Maurer, J.K., Cheng, M.C., Boysen, B.G., and Anderson, R.L. (1990): Two-year carcinogenicity study of sodium fluoride in rats. *JNCI*, 82:1118–1126.
379. McAlister, N.H., Abrams, H.B., Schlosser, R., and Sturtridge, W. (1990): Unintentional self-intoxication with inorganic calcium. *J. Intern. Med.*, 228:193–195.
380. McCallum, R.I. (1989): The industrial toxicology of antimony. The Ernestine Henry Lecture 1987. *J. R. Coll. Physicians Lond.*, 23:28–32.
381. McCance R.A., and Widdowson, E.M. (1937): Absorption and excretion of iron. *Lancet*, 2:680.
382. McCarron, D.A., Morris, C.D., and Cole, C. (1982): Dietary calcium in human hypertension. *Science*, 217:267–269.

383. McCaslin, F.E., and Janes, J.M. (1959): The effect of strontium lactate in the treatment of osteoporosis. *Mayo Clin. Proc.*, 34:329–334.
384. McConnell, K.P., and Portman, O.W. (1952): Excretion of dimethyl selenide by the rat. *J. Biol. Chem.*, 195:277–282.
385. McGuigan, M.A. (1996): Acute iron poisoning. *Pediatr. Ann.*, 25:33–38.
386. McKenna, K.E., Dolan, O., Walsh, M.Y., et al. (1995): Contact allergy to gold sodium thiosulfate. *Contact Dermatitis*, 32:143–146.
387. McLaughlin, A.I.G., Milton, R., and Perry, K.M.A. (1946): Toxic manifestations of osmium tetroxide. *Br. J. Ind. Med.*, 3:183–186.
388. Mead, L.D., and Geis, W.J. (1901): Physiological and toxicological effects of tellurium compounds, with a special study of their influence on nutrition. *Am. J. Physiol.*, 5:104–149.
389. Medeiros, D.M., Wildman, R., and Liebes, R. (1997): Metal metabolism and toxicities. In: *Handbook of Human Toxicology*, edited by E.J.Massaro, pp. 149–188. CRC Press, New York.
390. Mees, R.A. (1919): The nails with arsenical polyneuritis. *J. Am. Med. Assoc.*, 72:1337.
391. Meggs, W.J., Cahill-Morasco, R., Shih, R.D., et al. (1997): Effects of Prussian blue and N-acetylcysteine on thallium toxicity in mice. *Clin. Toxicol.*, 35:163–166.
392. Meggs, W.J., Gerr, F., Aly, M.H., et al. (1994): The treatment of lead poisoning from gunshot wounds with succimer (DMSA). *Clin. Toxicol.*, 32:377–385.
393. Meggs, W.J., Hoffman, R.S., Shih, R.D., et al. (1994): Thallium poisoning from maliciously contaminated food. *Clin. Toxicol.*, 32:723–730.
394. Miller, C.W., Davis, M.W., Goldman, A., and Wyatt, J.P. (1953): Pneumoconiosis in the tungsten-carbide tool industry. *AMA Arch. Ind. Hyg. Occup. Med.*, 8:453–465.
395. Miller, J.W., and Sayers, R.R. (1941): The response of peritoneal tissue to industrial dusts. *U.S. Public Health Serv. Rep.*, 56(1):264–272.
396. Mills, K.C., and Curry, S.C. (1994): Acute iron poisoning. *Conc. Controv. Toxicol.*, 12:397–413.
397. Monsen, E.R. (1996): New Dietary Reference Intakes proposed to replace the Recommended Dietary Allowances. *J. Am. Diet. Assoc.*, 96:754–755.
398. Moon, J. (1994): The role of vitamin D in toxic metal absorption: A review. *J. Am. Coll. Nutr.*, 13:559–569.
399. Moore, J.A. (1995): An assessment of lithium using the IEHR evaluative process for assessing human developmental and reproductive toxicity of agents. *Reprod. Toxicol.*, 9:175–210.
400. Moore, J.A. (1997): An assessment of boric acid and borax using the IEHR Evaluative process for assessing human developmental and reproductive toxicity of agents. *Reprod. Toxicol.*, 11:123–160.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_692.html[2010/10/28 18:46:52]

401. Moore, K.E., and Brody, T.M. (1961): Effect of triethyl tin on mitochondrial swelling. *Biochem. Pharmacol.*, 6:134–142.
402. Morgan, D.L., Shines, C.J., Jeter, S.P., et al. (1997): Comparative pulmonary absorption, distribution, and toxicity of copper gallium diselenide, copper indium diselenide, and cadmium telluride in sprague-dawley rats. *Toxicol. Appl. Pharmacol.*, 147:399–410.
403. Morgan, D.L., Shines, C.J., Jeter, S.P., Wilson, R.E., Elwell, M. P., Price, H.C., and Moskowitz, P.D. (1995): Acute plumonary toxicity of copper gallium diselenide, copper indium diselenide, and cadmium telluride intratracheally instilled into rats. *Environ. Res.*, 71:16–24.
404. Morgan, J.G. (1958): Some observations on the incidence of respiratory cancer in nickel workers. *Br. J. Ind. Med.*, 15:224–234.
405. Morin, Y., and Daniel, P. (1967): Quebec beer-drinkers' cardiomyopathy: Etiological considerations. *Can. Med. Ass. J.*, 97:926–928.
406. Morton, W.E., and Caron, G.A. (1989): Encephalopathy: An uncommon manifestation of workplace arsenic poisoning? *Am. J. Ind. Med.*, 15:1–5.
407. Mowad, E., Haddad, I., and Gemmel, D.J. (1998): Management of lead poisoning from ingested fishing sinkers. *Arch. Pediatr. Adolesc. Med.*, 152:485–488.
408. Moxon, A.L., and Rhian, M. (1943): Selenium poisoning. *Physiol. Rev.*, 23:305–337.
409. Mulkey, J.P., and Oehme, F.W. (1993): A review of thallium toxicity. *Vet. Hum. Toxicol.*, 35:445–453.
410. Muntzel, M., and Drüeke, T. (1992): A comprehensive review of the salt and blood pressure relationship. *Am. J. Hypertens.*, 5:1S–42S.
411. Murray, F.J. (1995): A human health risk assessment of boron (boric acid and borax) in drinking water. *Regul. Toxicol. Pharmacol.*, 22:221–230.
412. Nakada, T., Furuta, H., Koike, H., et al. (1989): Impaired urine concentrating ability in itai-itai (ouch-ouch) disease. *Int. J. Urol. Nephrol.*, 21:201–209.
413. Nakagawa, H., and Nishijo, M. (1996): Environmental cadmium exposure, hypertension and cardiovascular risk. *J. Cardiovasc. Risk*, 3:11–17.
414. Nakamura, Y., Tsumura, Y., Tonogai, Y., et al. (1997): Differences in behavior among the chlorides of seven rare earth elements administered intravenously to rats. *Fundam. Appl. Toxicol.*, 37:106–116.
415. National Cancer Institute. (1978): *Bioassay of titanium dioxide for possible carcinogenicity.* Techn. Rep. No. 97. NCI, Bethesda, MD.
416. National Cancer Institute. (1980): *Bioassay of selenium sulfide (dermal study) for possible carcinogenicity*. NCI Tech. Rep. Ser. No. 197, NTP No. 80–18.
417. National Cancer Institute. (1980): *Bioassay of selenium sulfide (gavage) for possible carcinogenicity*. NCI Tech. Rep. Ser. No. 194, NTP No. 80–17.
418. National Cancer Institute. (1980): *Bioassay of Selsun (trade name) for possible carcinogenicity*. NCI Tech. Rep. Ser. No. 199, NTP No. 80–19.
419. National Institutes of Health. (1994): Optimal calcium intake. *NIH Consens. Stat.*, 12:1–31.
420. National Research Council. (1989): *Recommended Dietary Allowances*, 10th ed. National Academy Press, Washington, DC.
421. National Toxicology Program. (1982): *Bioassay of stannous chloride for possible carcinogenicity*. Tech. Rep. No. 231. NTP, Research Triangle Park, NC.
422. National Toxicology Program. (1987): *Toxicology and carcinogenesis studies of boric acid (CAS No. 10043–35–3) in B6C3F1 mice.* U.S. Department of health and Human Services, National Institute of Health, Techn. Rep. Ser. 324, October.
423. National Toxicology Program. (1994): Toxicology and carcinogenesis studies of barium chloride dihydrate (CAS no. 10326–27–9) in F344/N rats and B6C3F1 mice (drinking water studies). Tech. Rep. No. 432. NTP, Research Triangle Park, NC.
424. National Toxicology Program. (1996): Tech. Rep. Ser. No. 451: *Toxicology and carcinogenesis studies of nickel oxide in F344/N rats and B6C3F*1 *Mice.* NIH Publication No. 96–3367. National Institute of Environmental Health Sciences, Research Triangle Park, NC.
425. National Toxicology Program. (1996): Tech. Rep. Ser. No. 453: *Toxicology and carcinogenesis studies of nickel subsulfide in F344/N rats and B6C3F*1*Mice.* NIH Publication No. 96–3369. National Institute of Environmental Health Sciences, Research Triangle Park, NC.
426. National Toxicology Program. (1996): Tech. Rep. Ser. No. 454: *Toxicology and carcinogenesis studies of nickel sulfate hexahydrate in F344/N rats and B6C3F*1*Mice.* NIH Publication No. 96–3370. National Institute of Environmental Health Sciences, Research Triangle Park, NC.

427. Needleman, H.L., Gunnoe, C., Leviton, A., et al. (1979): Deficits of psychologic and classroom performance of children with elevated dentine lead levels. *N. Engl J. Med.*, 300:689–695.
428. Needleman, H.L., Schell, A., Bellinger, D., et al. (1990): The long-term effects of exposure to low doses of lead in childhood. An 11-year follow-up report. *N. Engl. J. Med.*, 322:83–88.
429. Neilands, J.B., Strong, F.M., and Elvehjem, C.A. (1948): Molybdenum in the nutrition of the rat. *J. Biol. Chem.*, 172:431–439.
430. Nemery, B. (1990): Metal toxicity and the respiratory tract. *Eur. Respir. J.*, 3:202–219.
431. Nemery, B., Lewis, C.P.L., and Demerts, M. (1994): Cobalt and possible oxidant-mediated toxicity. *Sci. Total Environ.*, 150:57–64.
432. Newman, D. (1890): A case of adeno-carcinoma of the left inferior turbinated body, and perforation of the nasal septum, in the person of a worker in chrome pigments. *Glasgow Med. J.*, 33:469.
433. Newton, P.E., Bolte, H.F., Daly, I.W., Pillsbury, B.D., Terrill, J. B., Drew, R.T., Ben-Dyke, R., Sheldon, A.W., and Rubin, L.F. (1994): Subchronic and chronic inhalation toxicity of antimony trioxide in the rat. *Fundam. Appl. Toxicol.*, 22:561–576.
434. Nielsen, F.H. (1992): Facts and fallacies about boron. *Nutr. Today*, 27:6–12.
435. Nielsen, F.H. (1994): Biochemical and physiologic consequences of boron deprivation in humans. *Environ. Health Perspect.*, 102(suppl. 7):59–63.
436. Nielsen, F.H. (1996): Evidence for the nutritional essentiality of boron. *Trace Elem. Exp. Med.*, 9:215–229.
437. Nielsen, F.H. (1996): How should dietary guidance be given for mineral elements with beneficial actions suspected of being essential? *J. Nutr.*, 126:2377S–2385S.
438. Nogawa, K., Honda, R., Kido, T., et al. (1989): A dose-response analysis of cadmium in the general environment with special reference to total cadmium intake limit. *Environ. Res.*, 48:7–16.
439. Nordberg, G. (1994): Assessment of risks in occupational cobalt exposures. *Sci. Total Environ.*, 150:201–207.
440. Nordberg, G., Stenstrom, T., and Slorach, S. (1973): [Cadmium poisoning caused by a cooled-soft-drink machine.] *Lakartidningen*, 70:601–604. [Swedish, English translation]
441. Notton, B.A., and Hewitt, E.J. (1971): The role of tungsten in the inhibition of nitrate reductase in spinach (*Spinacea oleracea* L.) leaves. *Biochem. Biophys. Res. Comm.*, 44:702–710.
442. Nye, L.J.J. (1929): An investigation of the extraordinary incidence of chronic nephritis in young people in Queensland. *Med. J. Austr.*, 2:145–169.
443. Oberley, L. (1988): Free radicals and diabetes. *Free Radical Biol. Med.*, 5:113–124.

444. O'Brien, P., and Kortenkamp, A. (1995): The chemistry underlying chromate toxicity. *Transition Met. Chem.*, 20:636–642.
445. Okusa, M.D., and Crystal, L.J.T. (1994): Clinical manifestations and management of acute lithium intoxication. *Am. J. Med.*, 97:383–389.
446. Olin, S.S. (1998): Between a rock and a hard place: Methods for setting dietary allowances and exposure limits for essential metals. *J. Nutr.*, 128:364S–367S.
447. Omdahl, J.L., and DeLuca, H.F. (1971): Strontium induced rickets: Metabolic basis. *Science*, 174:949–951.
448. Omura, M., Hirata, M., Tanaka, A., Zhao, M., Makita, Y., Inoue, N., Gotoh, K., and Ishinishi, N. (1996): Testicular toxicity evaluation of arsenic-containing binary compound semi-conductors, gallium arsenide and indium arsenide, in hamsters. *Toxicol. Lett.*, 89:123–129.
449. Omura, M., Tanaka, A., Hirata, M., Zhao, M., Marita, Y., Gotoh, K., and Ishinishi, N. (1996): Testicular toxicity of gallium arsenide, indium arsenide, and arsenic oxide in rats by repetitive intratracheal instillation. *Fundam. Appl. Toxicol.*, 32:72–78.
450. Osswald, H., and Goerttler, K. (1971): Arsenic-induced leucoses in mice after diaplacental and postnatal application. *Verh. Dtsch. Ges. Pathol.*, 55:289–293.
451. Ott, M.G., Holder, B.B., and Gordon, H.L. (1974): Respiratory cancer and occupational exposure to arsenicals. *Arch. Environ. Health*, 29:250–255.
452. Pak, C.Y.C., Sakhafe, K., Zerwekh, J.E., Parcel, C., Peterson, R., and Johnson, K. (1989): Safe and effective treatment of osteoporosis with intermittent slow release sodium fluoride: augmentation of vertebral bone. *J. Clin. Endocrinol. Metab.*, 68:150–159.
453. Parizek, J. (1965): The peculiar toxicity of cadmium during pregnancy. An experimental "toxaemia of pregnancy" induced by cadmium salts. *J. Reprod. Fertil.*, 9:111–112.
454. Parras, F., Patier, J.L., and Ezpeleta, C. (1987): Lead-contaminated heroin as a source of inorganic-lead intoxication. *N. Engl. J. Med.*, 316:755.
455. Patterson, C., Ericson, J., Manea-Krichten, M., and Shirahata, H. (1991): Natural skeletal levels of lead in *Homo sapiens sapiens* uncontaminated by technological lead. *Sci. Total Environ.*, 107:205–236.
456. Pegues, D.A., Hughes, B.J., and Woernle, C.H. (1993): Elevated blood lead levels associated with illegally distilled alcohol. *Arch. Intern. Med.*, 153:1501–1504.
457. Pennington, J.A., and Jones, J.W. (1987): Molybdenum, nickel, cobalt, vanadium, and strontium in total diets. *J. Am. Diet. Assoc.*, 87:1644–1650.
458. Pennington, J.A., and Shoen, S.A. (1995): Estimates of dietary exposure to aluminum. *Food Addit. Contam.*, 12:119–128.
459. Perlstein, M.A., and Attala, R. (1966): Neurologic sequelae of plumbism in children. *Clin. Pediatr.*, 5:292–298.
460. Perry, W.G., Smith, F.A., and Kent, M.B. (1994): The halogens. In: *Patty's Industrial Hygiene and Toxicology*, edited by G.D. Clayton and F.E.Clayton, pp. 4449–4521. John Wiley & Sons, New York.
461. Pershagen, G., and Bjorklund, N.-E. (1985): On the pulmonary tumorigenicity of arsenic trisulfide and calcium arsenate in hamsters. *Cancer Lett.*, 27:99–104.
462. Pershagen, G., Nordberg, G., and Björklund, N.-E. (1984): Carcinomas of the respiratory tract in hamsters given arsenic trioxide and/or benzo[a]pyrene by the pulmonary route. *Environ. Res.*, 34:227–241.
463. Pietras, R., Stavrakos, C., Gunnar, R.M., Tobin, J.R., Jr., (1968): Phosphorus poisoning simulating acute myocardial infarction. *Arch. Intern. Med.*, 122:430–434.
464. Pirkle, J.L., Brody, D.J., Gunter, E.W., et al. (1994): The decline in blood lead levels in the United States. The National Health and Nutrition Examination Surveys (NHANES). *J. Am. Med. Assoc.*, 272:284–291.
465. Pirot, F., Millet, J., Kalia, Y.N., and Humbert, P. (1996): In vitro study of percutaneous absorption, cutaneous bioavailability and bioequivalence of zinc and copper from five topical formulations. *Skin Pharmacol.*, 9:259–269.
466. Pirot, F., Panisset, F., Agache, P., and Humbert, P. (1996): Simultaneous absorption of copper and zinc through human skin in vitro. *Skin Pharmacol.*, 9:43–52.
467. Poon, R., Chu, I., Lecavalier, P., Valli, V.E., Foster, W., Gupta, S., and Thomas, B. (1998). Effects of antimony on rats following 90-day exposure via drinking water. *Food Cosme. Toxicol.*, 36:21–35.
468. Preussmann, R., and Ivankovic, S. (1975): Absence of carcinogenic activity in BD rats after oral administration of high doses of bismuth oxychloride. *Food Cosmet. Toxicol.*, 13:503–508.
469. Price, C.J., Marr, M.C., and Myers, C.B. (1994): Determination of the no-observable-adverse-effect-

level (NOAEL) for developmental toxicity in Sprague-Dawley (CD) rats exposed to boric acid in feed on gestational days 0 to 20, and evaluation of postnatal recovery through postnatal day 21. Report 65C–5657–200. Research Triangle Institute, Research Triangle Park, NC.
470. Price, C.J., Marr, M.C., Myers, C.B., et al. (1991): Final report on the developmental toxicity of boric acid (CAS No. 10043–35–3) in New Zealand white rabbits. NIEHS/NTP Order PB92–129550.
471. Prior, J.T., Rustad, H., and Cronk, G.A. (1957): Pathological changes associated with deodorant preparations containing sodium zirconium lactate: An experimental study. *J. Invest. Dermatol.*, 29:449–463.
472. Questel, F., Dugarin, J., and Dally, S. (1996): Thallium-contaminated heroin. *Ann. Intern. Med.*, 124:616.
473. Reddy, B.S., Rivenson, A., El-Bayoumy, K., et al. (1997): Chemoprevention of colon cancer by organoselenium compounds and impact of high- or low-fat diets. *JNCI*, 89:506–512.
474. Reed, C.E. (1956): A study of the effects on the lung of industrial exposure to zirconium dusts. *AMA Arch. Ind. Health*, 13:578–580.
475. Reeves, A.L. (1979): Barium. In: *Handbook on the Toxicology of Metals*, edited by L.Friberg, G.F.Nordberg, and V.B.Vouk, pp. 321–328. Elsevier, New York.
476. Rencher, A.C., Carter, M.W., and McKee, D.W. (1977). A retrospective epidemiological study of mortality at a large western copper smelter. *J. Occup. Med.*, 19:754–758.
477. Rifat, S.L., Corey, P.N., and McLachlan, D.R.C. (1997): Neuropsychiatric disorders in a follow-up study of northern Ontario miners. *Am. J. Epidemiol.*, 145:S16.
478. Rifat, S.L., Eastwood, M.R., Crapper McLachlan, D.R., and Corey, P.N. (1990): Effect of exposure of miners to aluminum powder. *Lancet*, 336:1162–1165.
479. Riggs, B.L., and Melton, L J. (1983): Evidence for two distinct syndromes of involutional osteoporosis. *Am. J. Med.*, 75:899–901.
480. Rivlin, R.S. (1994): Magnesium deficiency and alcohol intake: Mechanisms, clinical significance and possible relation to cancer development (a review). *J. Am. Coll. Nutr.*, 13:416–423.
481. Rodgers, K. (1998): Platinum. In: *Immunotoxicology of Environmental and Occupational Metals*, edited by J.T.Zelikoff and P. T.Thomas, pp. 195–206. Taylor & Francis, Bristol, PA.
482. Roels, H.A., Hubermont, G., Buchet, J.P., and Lauwerys, R. (1978): Placental transfer of lead, mercury, cadmium, and carbon monoxide in women. *Environ. Res.*, 16:236–247.
483. Roels, H.A., Lauwerys, R., Buchet, J.P., et al. (1987): Epidemiological survey among workers exposed to manganese: Effects on lung, central nervous system, and some biological indices. *Am. J. Ind. Med.*, 11:307–327.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_694.html[2010/10/28 18:46:53]

484. Roels, H.A., Lauwerys, R.R., Buchet, J.P., et al. (1989): Health significance of cadmium induced renal dysfunction: A five year follow up. *Br. J. Ind. Med.*, 46:755–764.
485. Rom, W.N. (1976): Effects of lead on female reproduction: A review. *Mt. Sinai J. Med.*, 43:542–552.
486. Roth, F. (1958): Über den Brochialkrebs Arsengeschädigter Winzer. *Virchows Arch.*, 331:119–137.
487. Rotruck, J.T., Pope, A.L., Ganther, H.E., et al. (1973): Selenium: Biochemical role as a component of glutathione peroxidase. *Science*, 179:588–590.
488. Roza, O., and Berman, L.B. (1971): The pathophysiology of barium: Hypokalemic and cardiovascular effects. *J. Pharmacol. Exp. Ther.*, 177:433–439.
489. Rubin, L.Slepyan, A.H., Weber, L.F., and Neuhauser, I. (1956): Granulomas of the axillas caused by deodorants. *J. Am. Med. Assoc.*, 162:953–955.
490. Ruta, D.A., and Haider, S. (1989): Attempted murder by selenium poisoning. *Br. Med. J.*, 299:316–317.
491. Ryan, T.P., and Aust, S.D. (1992): The role of iron in oxygen-mediated toxicities. *CRC Crit. Rev. Toxicol.*, 22:119–141.
492. Rybak, L.P. (1992): Hearing: The effects of chemicals. *Otolaryngol. Head Neck Surg.*, 106:677–686.
493. Rystedt, I., and Fischer, T. (1983): Relationship between nickel and cobalt sensitization in hard metal workers. *Contact Dermatitis.*, 9:195–200.
494. Salonen, J.T., Nyyssonen, K., Korpela, H., et al. (1992): High stored iron levels are associated with excess risk of myocardial infarction in eastern Finnish men. *Circulation*, 86:803–811.
495. Salonen, J.T., Tuomainen, T.P., Nyyssonen, K., et al. (1998): Relation between iron stores and non-insulin dependent diabetes in men: Case-control study. *Br. Med. J.*, 317:727.
496. Salonen, J.T., Tuomainen, T.P., Salonen, R., et al. (1998): Donation of blood is associated with reduced risk of myocardial infarction. The Kuopio Ischaemic Heart Disease Risk Factor Study. *Am. J. Epidemiol.*, 148:445–451.
497. Sanai, T., Okuda, S., Onoyama, K., Oochi, N., Takaichi, S., Mizuhira, V., and Fujishima, M. (1991): Chronic tubulointerstitial changes induced by germanium dioxide in comparison with carboxyethylgermainum sesquioxide. *Kidney Int.*, 40:882–890.
498. Sanchez, D.J., Colomina, M.T., and Domingo, J.L. (1998): Effects of vanadium on activity and learning in rats. *Phys. Behav.*, 63:345–350.
499. Sanchez-Delgado, R.A., Navarro, M., Perez, H., and Urbina, J.A. (1996): Toward a novel metal-based chemotherapy against tropical diseases. 2. Synthesis and antimalarial activity in vitro and in vivo of new ruthenium and rhodium-chloroquine complexes. *J. Med. Chem.*, 39:1095–1099.
500. Schafer, S.G., and Femfert, U. (1984): Tin—A toxic heavy metal? A review of the literature. *Regul. Toxicol. Pharmacol.*, 4:57–69.
501. Schecter, A., Shanske, W., Stenzler, A., et al. (1980): Acute hydrogen selenide inhalation. *Chest*, 77:554–555.
502. Schroeder, H.A., Balassa, J.J., and Vinton, W.H., Jr. (1964): Chromium, lead, cadmium, nickel and titanium in mice: Effect on mortality, tumors and tissue levels. *J. Nutr.*, 83:239–250.
503. Schroeder, H.A., Frost, D.V., and Balassa, J.J. (1970): Essential trace elements in man: Selenium. *J. Chron. Dis.*, 23:227–243.
504. Schroeder, H.A., and Mitchener, M. (1971): Scandium, chromium(VI), gallium, yttrium, rhodium, palladium, indium in mice: Effects on growth and life span. *J. Nutr.*, 101:1431–1437.
505. Schroeder, H.A., and Mitchener, M. (1975): Life-term effects of mercury, methyl mercury, and nine other trace metals on mice. *J. Nutr.*, 105:452–458.
506. Schroeder, H.A., and Mitchener, M. (1975): Life-term studies in rats: Effects of aluminum, barium, beryllium, and tungsten. *J. Nutr.*, 105:421–427.
507. Schroeder, H.A., Mitchener, M., Balassa, J.J., Kanisawa, M., and Naron, A.P. (1968): Zirconium, niobium, antimony and fluorine in mice: Effects on growth, survival and tissue levels. *J. Nutr.*, 95:95–101.
508. Schroeder, H.A., Mitchener, M., and Nason, A.P. (1970): Zirconium, niobium, antimony, vanadium and lead in rats: Life term studies. *J. Nutr.*, 100:59–68.
509. Schroeder, H.A., Mitchner, M., and Nason, A.P. (1970): Zirconium, niobium, indium, antimony, vanadium and lead in rats: Life term studies. *J. Nutr.*, 100:59–68.
510. Schwartz, J. (1988): The relationship between blood lead and blood pressure in NHANES II survey. *Environ. Health Perspect.*, 78:15–22.
511. Schwartz, J. (1994): Societal benefits of reducing lead exposure. *Environ. Res.*, 66:105–124.

512. Schwarz, K., and Foltz, C.M. (1957): Selenium as an integral part of factor 3 against dietary necrotic liver degeneration. *J. Am. Chem. Soc.*, 79:3292–3293.
513. Schwarz, K., and Mertz, W. (1959): Chromium(III) and the glucose tolerance factor. *Arch. Biochem. Biophys.*, 85:292–295.
514. Schwarz, K., Milne, D.B., and Vinyard, E. (1970): Growth effects of tin compounds in rats maintained in a trace element-controlled environment. *Biochem. Biophys. Res. Comm.*, 40:22–29.
515. Seelig, M.S., and Master, A.C.N. (1994): Consequences of magnesium deficiency on the enhancement of stress reactions: Preventive and therapeutic implications (a review). *J. Am. Coll. Nutr.*, 13:429–446.
516. Seghizzi, P., D'Adda, F., Borleri, D., Barbic, F., and Mosconi., G. (1994): Cobalt cardiomyopathy. A critical review of literature. *Sci. Total Environ.*, 150:105–109.
517. Seifert, J. (1943): Intravenous injections of soluble tin compounds. *J. Lab. Clin. Med.*, 28:1344–1348.
518. Semlitsch, M., Staub, F., and Weber, H. (1985): Titanium-aluminum-niobium alloy, development for biocompatible, high strength surgical implants. *Biomed. Tech.*, 30:334–339.
519. Seppalainen, A.M., Hernberg, S., Vesanto, R., and Kock, B. (1983): Early neurotoxic effects of occupational lead exposure: A prospective study. *Neurotoxicology*, 4:181–192.
520. Shamberger, R.J., and Frost, D.V. (1969): Possible protective effect of selenium against human cancer. *Can. Med. Assoc. J.*, 100:682.
521. Shamberger, R.J. (1971): Is selenium a teratogen? *Lancet*, 1316.
522. Shannon, M. (1998): Lead poisoning from an unexpected source in a 4-month old infant. *Environ. Health Perspect.*, 106:313–316.
523. Shannon, M., and Graef, J.W. (1989): Lead intoxication from lead-contaminated water used to reconstitute infant formula. *Clin. Pediatr.*, 28:380–382.
524. Shelley, W.B., and Hurley, H. (1958): The allergic origin of zirconium deodorant granulomas. *Br. J. Dermatol.*, 70:75–101.
525. Shils, M.E. (1988): Magnesium in health and disease. *Annu. Rev. Nutr.*, 8:429–460.
526. Shipman, D.L. (1986): Cadmium food poisoning in a Missouri school. *J. Environ. Health*, 49:89.
527. Shiraishi, N., and Waalkes, M.P. (1996): Acquired tolerance to cadmium-induced toxicity in rodent testes. *Toxic Subst. Mechan.*, 15:27–42.
528. Siegel, E., and Wason, S. (1986): Boric acid toxicity. *Pediatr. Clin. North Am.*, 33:363–367.
529. Silbergeld, E.K. (1996): Lead poisoning: the implications of current biomedical knowledge for public policy. *Maryland Med. J.*, 45:209–217.
530. Silbergeld, E.K. (1997): Preventing lead poisoning in children. *Ann. Rev. Public Health*, 18:187–210.

531. Sivjakov, K.I., and Braun, H.A. (1959): The treatment of acute selenium, cadmium, and tungsten intoxication in rats with calcium disodium ethylenediaminetetraacetate. *Toxicol. Appl. Pharmacol.*, 1:602–608.
532. Skipworth, G.B., Goldstein, N., and McBride, W.P. (1967): Boric acid intoxication from "medicated talcum powder." *Arch. Dermatol.*, 95:83–86.
533. Skoryna, S.C. (1984): Metabolic aspects of the pharmacologic use of trace elements in human subjects with specific reference to stable strontium. In: *Trace Substances in Environmental Health*, edited by D.D.Hemphill, pp. 3–20. University of Misssouri, Columbia.
534. Smith, M.I., Franke, K.W., and Westfall, B.B. (1936): The selenium problem in relation to public health. *Public Health Rep.*, 51:1496–1505.
535. Sorahan, T. (1987): Mortality from lung cancer among a cohort of nickel cadmium battery workers: 1946–1984. *Br. J. Ind. Med.*, 44:803–809.
536. Sorahan, T., and Waterhouse, J.A.H. (1983): Mortality study of nickel-cadmium battery workers by the method of regression models in life tables. *Br. J. Ind. Med.*, 40:293–300.
537. Spencer, H., Kramer, L., and Osis, D. (1988): Do protein and phosphorus cause calcium loss? *J. Nutr.*, 118:657–660.
538. Spiegl, C.J., Calkins, M.C., DeVoidre, J.J., et al. (1956): Inhalation toxicity of zirconium compounds. I. Short-term studies. Atomic Energy Commission Project, Report No. UR-460. University of Rochester, Rochester, NY.
539. Stadtman, T.C. (1977): Biological function of selenium. *Nutr. Rev.*, 35:161–166.
540. Staessen, J.A., Roels, H., and Fagard, R. (1996): Lead exposure and conventional and ambulatory blood pressure. *J. Am. Med. Assoc.*, 275:1563–1570.
541. Stal, P. (1995): Iron as a hepatotoxin. *Dig. Dis.*, 13:205–222.
542. Stayner, L., Smith, R., Thun, M., et al. (1992): A dose-response analysis and quantitative assessment of lung cancer risk and occupational cadmium exposure. *Ann. Epidemiol.*, 2:177–194.
543. Sticht, G., and Käferstein, H. (1988): Bromine. In: *Handbook on Toxicity of Inorganic Compounds*, edited by H.G.Sieler and H.Siegel, pp. 143–154. Marcel Dekker, New York.
544. Stollery, B.T. (1996): Reaction time changes in workers exposed to lead. *Neurotoxicol. Teratol.*, 18:477–483.
545. Storey, E. (1961): Strontium "rickets": Bone, calcium and strontium changes. *Aust. Ann. Med.*, 10:213–222.
546. Sunderman, F.W., Jr. (1988): Nickel. In: *Handbook on Toxicity of Inorganic Compounds*, edited by H.G.Sieler and H.Sigel, pp. 454–468. Marcel Dekker, New York.
547. Takenaka, S., Oldiges, H., Konig, H., et al. (1983): Carcinogenicity of cadmium chloride aerosols in W rats. *J. Natl. Cancer Inst.*, 70:367–373.
548. Takeuchi, A., Yoshizawa, N., Oshima, S., Kubota, T., Oshikawa, Y., Akashi, Y., Oda., T., Niwa, H., Imazeki, N., Seno, A., and Fuse, Y. (1992): Nephrotoxicity of germanium compounds: Report of a case and review of the literature. *Nephron*, 60:436–442.
549. Tam, G.K., Charbonneau, S.M., Bryce, F., Pomroy, C., and Sandi, E. (1979): Metabolism of inorganic arsenic (74As) in humans following oral ingestion. *Toxicol. Appl. Pharmacol.*, 50:319–322.
550. Tamaki, S., and Frankenberger, W.T., Jr. (1992): Environmental biochemistry of arsenic. *Rev. Environ. Contam. Toxicol.*, 124:79–110.
551. Tanaka, A., Hisanaga, A., Hirata, M., Omura, M., Makita, Y., Inoue, N., and Ishinishi, N. (1996): Chronic toxicity of indium arsenide and indium phosphide to the lungs of hamsters. *Fukuoka Acta Med.*, 87:108–115.
552. Tao, S.-H., and Bolger, P.M. (1997): Hazard assessment of germanium supplements. *Regul. Toxicol. Pharmacol.*, 25:211–219.
553. Tay, C.-H., and Seah, C.-S. (1975): Arsenic poisoning from anti-asthmatic herbal preparations. *Med. J. Aust.*, 2:424–428.
554. Taylor, A., and Marks, V. (1978): Cobalt: A review. *J. Hum. Nutr.*, 32:165–177.
555. Tenenbein, M. (1996): Benefits of parenteral deferoxamine for acute iron poisoning. *Clin. Toxicol.*, 34:485–489.
556. Tepper, L.B., Hardy, H.L., and Chamberlin, R.I. (1961): *Toxicity of Beryllium Compounds* Elsevier, New York.
557. Thompson, H.J., and Becci, P.J. (1980): Selenium inhibition of N-methyl-N-nitrosourea-induced mammary carcinogenesis in the rat. *J. Natl. Cancer Inst.*, 1299–1301.
558. Thun, M.J., Schnorr, T.M., Smith., A.B., et al. (1985): Mortality among a cohort of U.S. cadmium

production workers—An update. *J. Natl. Cancer Inst.*, 74:325–333.
559. Tjalve, H., and Henriksson, J. (1997): Manganese uptake in the brain via olfactory pathways. Abstract. Fifteenth International Neurotoxicology Conference, Little Rock, AR.
560. Tokudome, S., and Kuratsune, M. (1976): A cohort study on mortality from cancer and other causes among workers at a metal refinery. *Int. J. Cancer*, 17:310–317.
561. Tong, S., Baghurst, P., McMichael, A., et al. (1996): Lifetime exposure to environmental lead and children's intelligence at 11–13 years: The Port Pirie cohort study. *Br. Med. J.*, 312:1569–1575.
562. Townshend, R.H. (1982): Acute cadmium pneumonitis: A 17-year follow-up. *Br. J. Ind. Med.*, 39:411–412.
563. Tozman, E.C.S., and Gottlieb, N.L. (1987): Adverse reactions with oral and parenteral gold preparations. *Med. Toxicol.*, 2:177–189.
564. Trapp, G.A., Miner, G.D., Zimmerman, R.L., Mastri, A.R., and Heston, L.L. (1978): Aluminum levels in brain in Alzheimer's disease. *Biol. Psychiatry*, 13:709–718.
565. Traub, R.D., Rains, T.C., Garruto, R.M., Gadjusek, D.C, and Gibbs, C.J. (1981): Brain destruction alone does not elevate brain aluminum. *Neurology*, 31:986–990.
566. Tseng, W.P. (1977): Effects and dose-response relationships of skin cancer and blackfoot disease with arsenic. *Environ. Health Perspect.*, 19:109–119.
567. Tseng, W.P., Chu, H.M., How, S.W., Fong, J.M., Lin, C.S., and Yeh, S. (1968): Prevalence of skin cancer in an endemic area of chronic arsenicism in Taiwan. *J. Natl. Cancer Inst.*, 40:453–463.
568. Tsuchiya, K. (1969): Causation of ouch-ouch disease (itai-itai byo): An introductory review. Part 1. Nature of the disease. *Keio J. Med.*, 18:181–194.
569. Tsuda, T., Babazono, A., Yamamoto, E., et al. (1995): Ingested arsenic and internal cancer: A historical cohort study followed for 33 years. *Am. J. Epidemiol.*, 141:198–209.
570. Tsuda, T., Inoue, I., Kojima, M., and Aoki, S. (1995): Market basket and duplicate portion estimation of dietary intakes of cadmium, mercury, arsenic, copper, manganese, and zinc by Japanese adults. *J. Assoc. Off. Anal. Chem. Intl.*, 78:1363–1368.
571. Tümer, Z., and Horn, N. (1996): Menkes disease: Recent advances and new insights into copper metabolism. *Ann. Med.*, 28:121–129.
572. Tuohimaa, P., and Wickmann, L. (1985): Sperm production of men working under heavy-metal or organic solvent exposure. In: *Occupational Hazards and Reproduction*, edited by K.Hemminki, M.Sorsa, and H.Vanio, pp. 73–80. Hemisphere, New York.
573. Tuomainen, T.P., Punnonen, K., Nyyssonen, K., and Salonen, J. T. (1998): Association between body iron stores and the risk of acute myocardial infarction in men. *Circulation*, 97:1461–1466.
574. Tzonou, A., Lagiou, P., Trichopoulou, A., et al. (1998): Dietary iron and coronary heart disease risk: A study from Greece. *Am. J. Epidemiol.*, 147:161–166.
575. U.S. Congress. (1971): Lead-Based Paint Poisoning Prevention Act. Public Law 91–695. U.S. Government Printing Office, Washington, DC.

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576. U.S. Congress. (1986): Amendments to the Safe Drinking Water Act. Public Law 99–339. U.S. Government Printing Office, Washington, DC.
577. U.S. Congress. (1992): Residential Lead-Based Paint Hazard Reduction Act of 1992. Public Law 102–550, Title X.U.S. Government Printing Office, Washington, DC.
578. U.S. Congress. (1996): Mercury-Containing and Rechargeable Battery Management Act. Public Law 104–142. U.S. Government Printing Office, Washington, DC.
579. U.S. Consumer Product Safety Commission. (1977): Lead-containing paint and certain consumer products bearing lead-containing paint. *Fed. Reg.*, 42:44199–44201.
580. U.S. Department of Health, Education, and Welfare, Food and Drug Administration. (1977): 21 CFR Parts 310.510 and 700.16, Final Rule. *Fed. Reg.*, 42:41374–41376.
581. U.S. Environmental Protection Agency. (1973): Control of lead additives in gasoline. *Fed. Reg.*, 38:33734–33741.
582. U.S. Environmental Protection Agency. (1989): *Office of Radiation Programs. Risk Assessment Methodology, Environmental Impact Statement for NESHAPS Radionuclides. Vol. I. Background Information Document.* U.S. Environmental Protection Agency, Washington, DC. EPA 520/1–89–005.
583. U.S. Environmental Protection Agency. (1990): *Office of Emergency and Remedial Response. Risk Assessment Guidance for Superfund, Vol. I. Human Health Evaluation Manual (Part A).* Interim final. U.S. Environmental Protection Agency, Washington, DC. EPA/540/1–89/002.
584. U.S. Environmental Protection Agency. (1990): Pesticide products containing phenylmercury and other mercury compounds; Receipt of requests for voluntary cancellation and amendments to delete uses. *Fed. Reg.*, 55:26754–26756.
585. U.S. Environmental Protection Agency. (1991): National primary drinking water regulations: Radionuclides; proposed rule. *Fed. Reg.*, 56:33050–33127.
586. U.S.Environmental Protection Agency. (1992): Health and environmental effects document for stable strontium. Office of Solid Waste and Emergency Response, Washington, DC (ECAO-CIN-G111).
587. U.S. Environmental Protection Agency. (1996): Prohibition on gasoline containing lead or lead additives for highway use. *Fed. Reg.*, 61:3832–3838.
588. U.S. Environmental Protection Agency. (1997): Implementation of the Mercury-Containing and Rechargeable Battery Management Act. Washington, DC.
589. U.S. Environmental Protection Agency. (1998): Integrated Risk Information System (IRIS). http://www.epa.gov/ngispgm3/iris/subst-fl.htm (As of July, 2000).
590. U.S. Food and Drug Administration. (1995): Lead-soldered food cans. *Fed. Reg.*, 60:33106–33109.
591. U.S. Food and Drug Administration. (1997): Iron-containing supplements and drugs: Label warning statements and unit-dose packaging requirements; Final rule. *Fed. Reg.*, 62:2217–2250.
592. van der Voet, G.B., and de Wolff, F.A. (1996): Human exposure to lithium, thallium, antimony, gold, and platinum. In: *Toxicology of Metals*, edited by L.Magos and T.Suzuki, pp. 455–460. CRC Press, New York.
593. Van Ordstrand, H.S., Hughes, R., and Carmody, M.G. (1943): Chemical pneumonia in workers extracting beryllium oxide. *Cleve. Clin. Q.*, 10:10–18.
594. Van Rij, A.M., Thomson, C.D., McKenzie, J.M., and Robinson, M.F. (1979): Selenium deficiency in total parenteral nutrition. *Am. J. Clin. Nutr.*, 32:2076–2085.
595. Veien, N.K., Hattel, T., Justesen., O., and Norholm, A. (1987): Oral challenge with nickel and cobalt in patients with positive patch tests to nickel and/or cobalt. *Acta Dermato-Venereol.*, 67:321–325.
596. Verity, M.A. (1997): Manganese neurotoxicity: Pathobiochemical aspects. Abstract. Fifteenth International Neurotoxicology Conference, Little Rock, AR.
597. Vernace, M.A., Bellucci, A.G., and Wilkes, B.M. (1994): Chronic salicylate toxicity due to consumption of over-the-counter bismuth subsalicylate. *Am. J. Med.*, 97:308–309.
598. Vilaplana, J., Grimalt, F., Romaguera, C., and Mascaro, J.M. (1987): Cobalt content of household cleaning products. *Contact Dermatitis*, 16:139–141.
599. Vinceti, M., Guidetti, D., Pinotti, M., et al. (1996): Amyotrophic lateral sclerosis after long-term exposure to drinking water with high selenium content. *Epidemiology*, 7:529–532.
600. Von Burg, R. (1992): Boron, boric acid, borates and boron oxide. *J. Appl. Toxicol.*, 12:149–152.
601. Votaw, A.L., and Zey, J. (1991): Vacuuming a mercury-contaminated dental office may be hazardous to your health. *Dent. Assist.*, 60:27–29.
602. Waalkes, M.P., and Goering, P.L. (1990): Metallothionein and other cadmium-binding proteins: Recent developments. *Chem. Res. Toxicol.*, 3:281–288.
603. Waalkes, M.P., and Rehm, S. (1992): Carcinogenicity of oral cadmium in the male Wistar (WF/NCr)

rat: Effect of chronic dietary zinc deficiency. *Fundam. Appl. Toxicol.*, 19:512–520.
604. Waalkes, M.P., and Rehm, S. (1994): Cadmium and prostate cancer. *J. Toxicol. Environ. Health*, 43:251–169.
605. Ward, J.J., Thronbury, D.D., Lemons, J.E., and Dunham, W.K. (1990): Metal-induced sarcoma: A case report and literature review. *Clin. Orthopaed. Related Res.*, 252:299–306.
606. Ward, N.I., and Mason, J.A. (1987): Neutron activation analysis techniques for identifying elemental status in Alzheimer's disease. *J. Radioanal. Nuclear Chem.*, 113:515–526.
607. Warrell, R.P. (1997): Gallium nitrate for the treatment of bone metastases. *Cancer (Suppl.)*, 80:1680–1685.
608. Webster, S.H. (1946): Volatile hydrides of toxicological importance. *J. Ind. Hyg. Toxicol.*, 28:167–182.
609. Weir, R.J., and Fisher, R.S. (1972): Toxicologic studies on borax and boric acid. *Toxicol. Appl. Pharmacol.* 23:351–364.
610. Weiss, B., Alkon, E., Weindlar, F., et al. (1993): Toddler deaths resulting from ingestion of iron supplements-Los Angeles. *Morbid. Mortal. Weekly Ref.*, 42:111–113.
611. West, D.W., Slattery, M.L., Robison, L.M., et al. (1991): Adult dietary intake and prostate cancer risk in Utah: A case-control study with special emphasis on aggressive tumors. *Cancer Causes Control*, 2:85–94.
612. Whitford, G.M. (1987): Fluorides in dental products: Safety considerations. *J. Dent. Res.*, 66:1056–1060.
613. Whitford, G.M. (1992): Acute and chronic fluoride toxicity. *J. Dent. Res.*, 71:1249–1254.
614. Whiting, S.J. (1994): Safety of some calcium supplements questioned. *Nutr. Rev.*, 52:95–97.
615. Whiting, S.J., and Wood, R.J. (1997): Adverse effects of high-calcium diets in humans. *Nutr. Rev.*, 55:1–9.
616. WHO (1998): Boron, Environmental Health Criteria 204, World Health Organization, Geneva.
617. Whorton, M.D., Haas, J.L., Trent, L., and Wong, O. (1994): Reproductive effects of sodium borates on male employees: Birth rate assessment. *Occup. Environ. Med.*, 51:761–767.
618. Wones, R.G., Stadler, B.L., and Frohman, L.A. (1990): Lack of effect of drinking water barium on cardiovascular risk factors. *Environ. Health Perspect.*, 85:355–359.
619. Wong, L.C., Heimbach, M.D., Truscott, D.R., and Duncan, B. D. (1964): Boric acid poisoning: Report of 11 cases. *Can. Med. Assoc. J.*, 90:1018–1023.

620. Woody, N.C., and Kometani, J.T. (1948): BAL in the treatment of arsenic ingestion of children. *Pediatrics*, 1:372–378.
621. Wu, M.-M., Kuo, T.-L., Hwang, Y.-H., and Chen, C.-J. (1989): Dose-response relation between arsenic concentration in well water and mortality from cancers and vascular diseases. *Am. J. Epidemiol.*, 130:1123–1132.
622. Xu, N., Majidi, V., Markesbery, W.R., and Ehmann, W.D. (1992): Brain aluminum in Alzheimer's disease using an improved GFAAS method. *Neurotoxicology*, 13:735–744.
623. Yan, A., and Davis, P. (1990): Gold induced marrow suppression: A review of 10 cases. *J. Rheumatol.*, 17:47–51.
624. Yang, G., Yin, S., Zhou, R., et al. (1989): Studies of safe maximal daily dietary Se-intake in a seleniferous area in China. II. Relation between Se-intake and the manifestation of clinical signs and certain biochemical alterations in blood and urine. *J. Trace Elem. Electrolytes Health Dis.*, 3:123–130.
625. Yang, G.-Q., Wang, S.Z., Zhou, R.H., and Sun, S.Z. (1983): Endemic selenium intoxication of humans in China. *Am. J. Clin. Nutr.*, 37:872–881.
626. Yoshimasu, F., Yasui, M., Yoshiro, Y., Iwata, S., Gajdusek, C., Gibbs, C.J., and Chen, K.-M. (1980): Studies on amyotrophic lateral sclerosis by neutron activiation analysis—2. Comparative study of analytical results on Guam PD, Japanese ALS and Alzheimer disease cases. *Folia Psychiat. Neurolog. Jpn.*, 34:75–82.
627. Yoshizawa, K., Willett, W.C., Morris, S.J., et al. (1998): Study of prediagnostic selenium level in toenails and the risk of advanced prostate cancer. *J. Natl. Cancer. Inst.*, 90:1219–1224.
628. Zheng, W., Winter, S.M., Kattnig, M.J., Carter, D.E., and Sipes, I.G. (1994): Tissue distribution and elimination of indium in male Fisher 344 rats following oral and intratracheal administration of indium phosphide. *J. Toxicol. Environ. Health*, 43:483–494.

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Chapter **15**

Ionizing Radiation

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Principles and Methods of Toxicology, Fourth Edition, edited by A.Wallace Hayes.
Taylor & Francis, Philadelphia © 2001.

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The increasing use of radiation in the modern world and recent incidents of massive radiation exposure dictate that certain basic elements of radiation toxicity be addressed.

Radiation toxicology is the study of the adverse effects of radiation on living organisms. It is a multidisciplinary science, borrowing freely from several of the basic sciences. The cytopathological consequences of radiation exposure are similar to those induced in other types of cellular injury. Radiation-induced cell changes may result in death of the organism, death of the cells, modulation of physiological activity, or cancers that have no features distinguishing them from those induced by other types of cell injury.

Electromagnetic radiation is divided into nonionizing and ionizing radiation according to the energy required to eject electrons from molecules (575). Ionizing radiation, which may exhibit the properties of both waves and particles, has sufficient energy to produce ionization in matter. The ionizing radiations

that exhibit corpuscular properties include alpha and beta particles whereas those that behave more like waves of energy include x-rays and gamma rays.

Radiation exposure comes from many sources and may be *directly ionizing* or *indirectly ionizing*. Directly ionizing radiation carries an electric charge that directly interacts, by electrostatic attraction or repulsion, with atoms in the tissue or medium exposed. Indirectly ionizing radiation is not electrically charged, but results in production of charged particles by which its energy is absorbed. A characteristic of charged particles produced directly or indirectly is *linear energy transfer* (LET), the energy loss per unit of distance traveled, usually expressed in kiloelectron volts (keV) per

micrometer (μm). The LET, depending on the velocity and charge of the particle, may vary from about 0.2 to more than 1000 keV/μm.

DOSIMETRY AND EXPOSURE

Système Internationale

The International Commission on Radiological Units and Measurement (ICRU) introduced the "Système Internationale" or SI units in 1980 to express radiation dose (268). The gray (Gy), the SI unit for absorbed dose, corresponds to an energy absorption of 1 joule/kg or 100 rads. This concept of energy absorption is useful for determining absorbed doses of x-rays and gamma rays. However, determination of the absorbed dose in tissues exposed to fast neutron radiation involves more elaborate calculations. The absorbed dose of neutron radiation depends on the transfer of energy from neutrons to directly ionizing particles in the tissue and is described by the kinetic energy released in the material.

For general use, a quantity different from the rad or gray has been introduced, the dose equivalent. The dose equivalent allows for the relative effectiveness of a particular type of radiation. Gamma rays and x-rays are regarded as the standard and a quality factor of 1 is multiplied by the dose to compute the dose equivalent. Therefore, the dose equivalent (Seiverts, Sv) for x-rays and gamma rays is equal to the dose (grays). However, neutrons are thought to be roughly 10 times more effective in producing tissue damage than x-rays and therefore are assigned a quality factor of 10.

Exposure Factors

Before discussing the effects of ionizing radiation, some of the factors that influence the toxicity of radiation should be reviewed. One of the major factors related to the exposure is the dose or total amount of radiation received (Table 15.1). The absorbed dose of radiation is the quotient dE/dm where dE is the differential energy deposited into a differential mass, dm (268). The unit of absorbed dose, in the CGS (centimeter-gram-second) system, is the rad (radiation-absorbed dose) and 1 rad=100 ergs/g (a dose of 1 rad of ionizing radiation has been absorbed when 100 ergs of energy have been deposited in each gram of material) (555). Another term commonly used, particularly in the field of radiation protection, is the rem (roentgen equivalent man). This unit was developed to enable radiation protection personnel to set standards of exposure (rem=rad×quality factor×distribution factor). The quality factor is a unit to equate the relative biological effectiveness (RBE) of one radiation to another and the distribution factor

Table 15.1 Radiation quantities and units used in radiobiology[a]

Unit or quantity	Symbol	Application
Becquerel	Bq	SI quantity of radioactivity Bq=1 disintegration/s Bq=2.7×10^{-11} Ci
Curie	Ci	Quantity of radioactivity 1 Ci=3.7×10^{10} dps 1 Ci=3.7×10^{10} Bq
Gray	Gy	SI unit of absorbed dose 1 Gy=100 rad=1 J/kg
Rad	rad	Unit of absorbed dose 1 rad=0.01 Gy=100erg/g
Rem	rem	Unit of dose equivalent rad×Q×other modifying factor 1 rem=0.01 Sv
Sievert	Sv	SI unit of dose equivalent rad×Q×other modifying factor 1 Sv=100 rem
Linear energy transfer	LET	Energy deposition per unit of path length Usually in eV/micron
Relative biological effectiveness	RBE	Same effect from same dose of reference radiation Used in radiobiology
Quality factor	Q	Biological effectiveness of radiations
Working level	WL	1.3×10^{-5} MeV α-energy/L air
Working level month	WLM	1 WL×170 h
Electron volt	eV	Unit of energy; 1 eV=1.6×10^{-12} ergs 1 eV=1.6×10^{-19} J

a From References 50 and 437.

attempts to compensate for the varying sensitivity of the different parts of the body. The roentgen, the amount of radiation required to produce one electrostatic unit of charge per cubic centimeter of air, is an older radiation exposure term that may still be seen in literature. This is a measure of only the actual ionizations produced by x-ray or gamma ray irradiation in air.

A second factor influencing the toxicity of radiation is the dose rate, $D = dD/dt$, the differential dose with respect to time; when there is no variability in dose dE/dm, $D = D/t$. When reviewing experiments in radiation toxicology, the variables, total dose, dose rate, type of radiation, and variability of the model, must be considered.

The exposure to radon (^{222}Rn) and radon daughters may be expressed, by convention, as the concentration of radon daughters measured in working levels (WL), and cumulative exposures over time are measured in working level months (WLM) (49). The WL is defined as any combination of radon daughters in 1 liter of air that results in the ultimate release of 1.3×10^5 MeV of potential alpha energy. This is approximately the alpha

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Table 15.2 Average annual effective dose equivalent of ionizing radiations[a]

Source	Dose equivalent		Effective dose equivalent	
	mSv	mrem	mSv	%
Natural				
Radon	24.0	2400	2.0	55.0
Cosmic	0.27	27	0.27	8.0
Terrestrial	0.28	28	0.28	8.0
Internal	0.39	39	0.39	11.0
Total natural	–	–	3.0	82.0
Artificial				
Medical				
X-ray diagnosis	0.39	39	0.39	11
Nuclear medicine	0.14	14	0.14	4.0
Consumer products	0.10	10	0.10	3.0
Occupational	0.009	0.9	<0.01	<0.3
Nuclear fuel cycle	<0.01	<1.0	<0.01	<0.03
Fallout	<0.01	<1.0	<0.01	<0.03
Miscellaneous	<0.01	<1.0	<0.01	<0.03
Total artificial	–	–	0.63	18
Total natural and artificial	–	–	3.6	100

a From Reference 50. Reprinted with permission from *Health Effects of Exposure to Low Levels of Ionizing Radiation (BEIR V)*. Copyright 1990 by the National Academy of Sciences. Courtesy of the National Academy Press, Washington, DC.

energy emitted by the radon daughters in equilibrium with 100 pCi of radon. The WLM is defined as exposure to this concentration for a working month of 170 h.

SOURCES

The quantity of radiation present could range from the irreducible natural background levels to large scale releases such as occurred at Chernobyl and Chelyabinsk in Eastern Europe. Biological damage may be detected at levels only slightly above the former, whereas the latter would result in extreme biological toxicity of unknown proportions. The sources of radiation may be broken into two major components: technologically induced or man-made radiation and natural radiation (Table 15.2).

Technologic or Man-Made Radiation

Health Sciences

The use of man-made radiation in the health sciences is normally divided into three areas: (i) diagnostic x-ray examinations, (ii) nuclear medicine, and (iii) therapeutic radiation. In the more highly developed countries, exposure from medical sources may equal or exceed natural background radiation, but in undeveloped countries, the relative contribution of medical irradiation may be only about 5% of the total exposure (437).

The use of x-rays in diagnostic examinations, including dental, represents the single largest man-made source of radiation exposure in the U.S. population. The Bureau of Radiological Health, Food and Drug Administration (FDA), estimated that approximately 65% of the people in the United States were exposed to x-rays for medical and dental diagnostic examinations in 1970. The mean active bone marrow dose to adults was 103 mrads with the 65 and older age group receiving the highest per capita dose (48). Dental x-ray examinations are the most common of the diagnostic examinations and approximately 30% of the total diagnostic examinations are received on an outpatient basis (437).

The use of radiopharmaceuticals in nuclear medicine has almost doubled over a 10-year period. It is estimated that up to 12 million doses of radiopharmaceuticals are given each year in the U.S. for diagnostic purposes (48). However, the per capita effective dose equivalent from these procedures in the United States is only about 140 μSv (437).

Radiation therapy has been used almost exclusively for the treatment of malignant neoplasms. The high absorbed dose, 50–70 Gy, required in most malignant conditions, leads to *nonstochastic* or direct effects such as cell death.

Therefore, some of the normal tissue surrounding the neoplasm may be exposed and incur some long-range risk. The risk, however, is usually eclipsed by the immediate benefits normally associated with increased life expectancy.

Nuclear Weapons

The first atomic weapon was detonated in 1945 on a New Mexico desert north of Alamagordo. Since that day hundreds of test explosions have been conducted by the United States, the Soviet Union, the United Kingdom, India, France, and the People's Republic of China. Between 1945 and 1984, the total estimated yield of all atmospheric nuclear explosions was approximately 546 megatons (437). A one-megaton (MT) explosion equals the explosive force of 1 million tons of 2,4, 6-trinitrotoluene (TNT).

The radioactive fallout from nuclear explosions may be divided into three portions depending on the yield and height of the burst. The larger, intensively radioactive particles fall out close to the site within hours. Slightly smaller particles behave somewhat like aerosols and are dispersed into the troposphere where they will stay for a matter of months. The fallout from this portion remains in bands around the earth at the latitude of the detonation. The third portion penetrates the strato sphere and its particles are deposited worldwide over a period of months to years (196). Most of the radioactive fallout is downwind from the explosion and up to 70% is in the larger particle portion, returning to the earth close to the detonation site within hours. The intensity of the radioactivity varies inversely with distance from the site of explosion. With a steady wind, the pattern of accumulated dose of radioactivity assumes nested cigar-shaped contours, each contour denoting a particular dose.

A one-MT thermonuclear weapon detonating at ground level with a steady wind of approximately 15 miles per hour would produce a fallout radioactivity dose rate of 400 rem in 24 h in an area of approximately 400 square miles. At a dose rate of 2 rem per year, more than 20 times the maximum recommended by the U.S. Environmental Protection Agency (EPA), an area of 1200 square miles would remain unfit for use for a year and more than 20,000 square miles would be uninhabitable for a month (201).

In a nuclear explosion over 400 radioactive isotopes are released into the biosphere. Among these, about 40 radionuclides are considered potentially hazardous. Of particular interest are those isotopes whose organ specificity and long half-lives present a danger of irreversible damage or induction of malignant alterations. Both early and delayed fallout result in the deposition of radioactive material in the environment (102). The annual average whole-body fallout rate in the United States is now approximately 45 μSv (4.5 mrem) and is projected to stay at this level through the year 2000 (48, 437).

Nuclear Power Production

When radiation exposure from nuclear power production is mentioned, most persons immediately think of nuclear power reactors and the environmental dispersion of radionuclides, particularly krypton 85, tritium, carbon 14, and iodine 129. However, exposure from nuclear power production should also include mining, uranium fuel fabrication, and waste storage and disposal (437).

Although uranium mines increase the amount of uranium and its decay products, along with radon and its daughters, the environmental risks from the radioactive emissions from uranium mines is insignificant (196, 290). However, mill tailings may represent a significant source of environmental radiation due to the emanation of ^{222}Rn, dispersion of the tailings by wind and water, and by the use of mill tailings in building construction.

About 1000 land-based nuclear reactors have been constructed and operated at some time throughout the world. Some of the reactors were built for research, or the production of radioisotopes and plutonium. Approximately 200 naval vessels throughout the world are powered by nuclear reactors. Yet, the environmental release from nuclear operations in the United States results in a dose rate for the average person of less than 1 mrem/year (48).

Accidents

Although the environmental release of radionuclides from nuclear reactor operations is approximately 1 mrem/year per person, malfunctions can develop and accidents can happen (48). The contents of the reactor at the time of an accident, the amount of contaminant, including its physical and chemical properties, depend on the reactor type, its application, and the duration of operation (596). Not all of the nearly 800 nuclides produced in reactors are radioactive and of these only 54 are considered significant in risk assessment (196). With core damage, the severity of the accident and therefore the risk, depends on the radioactivity, mainly as ^{131}I and ^{137}Cs, being released to the environment.

Since 1952, there have been 14 reactor accidents that involved core damage. One, the Windscale, U.K. Atomic Energy Works accident, was the first time radioactive material was released from a reactor accident. In October 1957, a plutonium production reactor located on the coast of Cumbria in northwest

England released approximately 740 TBq ^{131}I, 22 TBq ^{137}Cs, 8.8 TBq ^{210}Po, and 3 TBq ^{89}Sr (122, 196). The core in the No. 1 Pile of the two air-cooled, graphite-moderated, natural uranium reactors was partially consumed by fire, releasing the fission products onto the seashore and foothills southwest

of the Cumbrian Mountains, over much of England, and parts of northern Europe. As an aftermath of this accident, the village of Seascale has had four fatal leukemia cases in children who were under 20 years of age between 1950 and 1980. Based on statistics, only 0.5 cases would have been expected (617).

On March 28, 1979, the worst accident in the history of U.S. commercial nuclear power generation occurred on Three Mile Island (TMI) in Pennsylvania (290). Even though the accident at TMI-2 released the radionuclides ^{131}I, ^{133}Xe, and ^{135}Xe into the environment, the collective dose equivalent to the population from the release was less than 1% of the dose accrued from natural background radiation in a year (596). As with the Windscale accident, the radionuclide identified as principal concern was ^{131}I, but in this case, only 1 TBq ^{131}I was released and the fission products at TMI were retained within the vessel (121, 122). Although radiation exposure to the plant workers and the public was insignificant, the nuclear power industry was set back almost a decade. Even orders for the construction of new nuclear plants were canceled (196).

The largest airborne dispersion of radionuclides thus far occurred from the explosion and ensuing 10-day fire of the graphite-moderated reactor of Unit No. 4 of the Chernobyl nuclear power station in the former Soviet Union (now Ukraine) on April 26, 1986. It has been estimated that this has been the single most costly industrial accident in history (122, 196, 310, 596). One revealing evaluation of the economic loss from this accident has been the estimate that from 8 to 10 annual budgets for the Republic of Belarus will be consumed in order to appropriately address the effects of this disaster, just for the needs generated in that country alone (91). Another more conservative estimate puts the costs of modestly dealing with the Chernobyl nuclear accident at 45 billion dollars, not including, of course, the human and ecological toll (594). The health care needs, for instance, are only now becoming apparent (154).

Fallout from the Chernobyl nuclear disaster was very widespread, with a large amount of a variety of radionuclides distributed throughout the northern hemisphere (20, 214, 355, 661). In Eastern Europe and Scandinavia, it has been estimated that approximately 4×10^{18} Becquerels (Bq) were dispersed by the accident (315, 534, 608). The radioactive cloud moved outside of the area of the Soviet Union in the first few days after the accident, and the event only became publicized when radioactive levels in Sweden became elevated, reaching 14 times background levels (121). The importance of meteorological conditions at the time and place of nuclear accidents or thermonuclear detonations was clearly evidenced in the dispersion patterns of the fallout. The majority of the ensuing environmental radioactivity now resides in the newly created nation of Belarus (immediately north of the Chernobyl nuclear complex), which now has the distinction of having approximately 30% of its territory with a significant contamination from the accident, including 20% of the forests and 18% of the farmland (315). Ukraine and Russia have most of the remainder of the inventory that was released. The most prominent radionuclides released, in terms of quantity and widespread geographic dispersion, were ^{137}Cs, ^{90}Sr, and ^{131}I. Although less widespread, significant levels of ^{239}Pu and various transuranics were also distributed in the areas around the reactor complex.

The International Atomic Energy Agency (IAEA) report published by the Soviet Union in 1987 determined a radiation dose for the two most highly contaminated areas as (i) a 106,340-man-Sv collective 50 year dose for a population of 10.1 million in Belarus, and (ii) a 80,660-man-Sv collective 50 year dose for a population of 29.8 million in Ukraine (664). Although many east European scientists in post-Soviet scientific circles challenge these estimates as too low, they do indicate a significant radiation dose to a large number of people in the contaminated areas of these two countries. Indeed, newer estimates are that the one million people in the most contaminated areas of Belarus and Ukraine will accumulate between 150,000 and 200,000 Sv (as much as the total dose for both countries in the 1987 report), with the total population dose approaching 1,000,000 Sv (429). Many villages surrounding the Chernobyl reactor area had to be abandoned, along with the entire city of Pripyat, formerly housing over 50,000 people. This made Pripyat the first sizable city in history to be abandoned solely on the basis of radioactive fallout. Some of the inhabitants of the abandoned villages were calculated to have been expected to accumulate at least 35 rem per person (429). In Belarus alone, approximately 2.2 million people live in the areas significantly contaminated by the accident, including 800,000 children (154). At least 135,000 people were evacuated just from the Chernobyl exclusion zone, now established in the areas immediately around the reactor complex (613, 699).

One result of the Chernobyl accident has been that the people and the ecosystem of the contaminated areas of Belarus, Ukraine, and Russia have become a living laboratory of the consequences of widespread radioactive contamination. In the ecosystem, deposition of the radionuclides from the reactor

fire and dispersion tended to be very "patchy," with variation of over 100% in soil and sediment samples even taken only meters apart (318). Uptake of radionuclides into wildlife around the reactor was very high, with radiocesium concentrations averaging 18,000 Bq/g in one rodent species (363) and up to 200 Bq/g in fish (318). Food contamination from environmental radioactivity resulted in leafy vegetables reaching 10 μCi/kg and iodine levels in milk commonly measured at 1 μCi/L (196).

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The ecotoxicological effects from the Chernobyl accident have also provided an unprecedented observation recently of the widespread dispersion of radionuclides in the environment. Evaluations of the blood cell DNA of fish from the radioactively contaminated aquatic habitats near the reactor revealed abnormal DNA distributions, hyperdiploidy, and cell cycle perturbations, though there were no gross physical malformations (155, 156, 386, 628). An even more extensive evaluation of rodents in Chernobyl-contaminated areas has been published in the last few years. A reduction in fertility and various other physiological disorders were reported in some mammalian species in the years immediately following the accident (223, 365). Cytogenetic and other mutagenic effects were observed in rodents from sites ranging from the Chernobyl power plant vicinity to Sweden (142, 143, 250, 603). At least some of the variation in response observed can be attributed to a species difference in sensitivity, such as the high radioresistance reported for *Clezhrionomys glareolus* from Chernobylcontaminated areas (308, 363). Species differences in oxidative stress enzyme response were also found in rodents from these areas, with *C. glareolus* showing radioresistance relative to another species (298), despite a much higher deposition of radionuclides measured internally (114). There was sufficient radioactivity released in the accident to kill about 400 hectares of pine forest, and more than one million square meters of ground was bulldozed and buried (430).
The human health effects of the Chernobyl nuclear accident are only now becoming evident, with additional reports appearing each year. A relatively high incidence of thyroid cancer has been documented in Belarus, Ukraine, and Russia (66, 232, 517). The incidence of leukemia is still under investigation, but a statistically relevant increase related to the accident has not yet been substantiated (249, 334, 604, 703, 712). An increased frequency of chromosomal aberrations have been detected in the blood cells of people living in the contaminated areas, or those who worked as liquidators in the cleanup after the accident (582, 618). An increased mutation rate at human minisatellites was found in children born in a contaminated region of Belarus relative to a control population (187, 188). These minisatellites provided perhaps the only currently available system for the efficient monitoring of germline mutation in humans, and it was concluded that the damage was probably not due to DNA damage induced directly at the minisatellites, but from radiation-induced damage at other sites in the genome (188). Somatic minisatellite mutation events were present in a subset of radiation-induced, but not sporadic, thyroid cancers, suggesting that this type of genomic instability may play a role in radiation-induced tumorigenesis in the thyroid gland (496). There was an increased frequency in both lymphocyte micronuclei (an assay of chromosomal integrity) and somatic mutations in erythrocytes at the glycophorin A locus of residents of Chernobyl-contaminated cities in Belarus, and these effects were significantly correlated with radiocesium content (391, 537). Somatic mutation responses were also found in the glycophorin A locus of erythrocytes from Chernobyl workers from the Baltic countries (61, 62) and in immigrants to Israel (707). There has been some indication that children exposed to low LET radiation due to Chernobyl may have an increase in cataract formation (166). Among children in Belarus, it was also reported that after the accident there were increases in endocrine and dermatologic diseases, digestive organ diseases, autoimmune thyroiditis, and chronic tonsillitis and adenoiditis (393).
Another serious radiation accident occurred in the central Brazilian plateau state of Goias and went virtually unnoticed by most of the world (144). The Instituto Goiano de Radioterapia, a private radiotherapy clinic in Goiânia, Brazil, ceased operation in 1985, leaving a 137Cs radiotherapy unit in an insecure situation in an abandoned treatment room. In September 1987, the 50.9-TBq 137Cs source was removed from the protective housing of the therapy unit. With the later rupture of the container, the 137Cs became widely dispersed throughout the city's one million population. Exposure to the 137CsCl resulted in 4 deaths, 28 other cases of acute radiation sickness, and 3500 m3 of radioactive waste. The four persons who died received estimated doses ranging from 4.5 to 6.0 Gy. Other than massive accidents with widespread contamination such as occurred at Chernobyl, this is one of the most serious radiation accidents that has ever occurred.
Nuclear Waste Management
The disposal of radioactive waste is part of a dilemma facing a technologically advanced society. One of the basic demands of such a growing society is availability of convenient and inexpensive sources of energy. As the demand for energy increases, the reliance on nuclear power will increase, as will the production of radioactive waste.
Radioactive waste is classified by its physical and chemical properties as well as its source (196, 236). Three general categories of radioactive waste are: (i) low level, (ii) transuranic, and (iii) high level. Low-level radioactive waste (LLRW) includes residues from laboratory research, medical institutions, uranium mill tailings, and waste generated in the cleanup of uranium, radium, and thorium processing plants.

LLRW is further subdivided into classes A, B, and C, depending on the concentration, energy levels, half-life and the sources of the radionuclides in the waste. Radionuclides found in LLRW include 241Am, 14C, 242Cm, 60Co, 137Cs, 129I, 241Pu, 226Ra 90Sr, 99Tc, 230Th, and 235U. Because the

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national inventory of LLRW waste is growing at 105 m3/year (30% from medical institutions), management of the waste is facing a crisis in storage and disposal.
Transuranic (TRU) wastes are materials containing radionuclides with atomic numbers greater than uranium, such as americium, curium, and plutonium. These wastes originate mainly as by-products in the production and fabrication of plutonium for military purposes. Resulting from an industrial process involving transuranic materials, the TRU wastes are predominantly contaminated with 238Pu and 239Pu. These wastes tend to be water-soluble and pose a distinct health hazard because they can contaminate a variety of physical forms ranging from absorbent papers and rubber to discarded tools.
The most radioactivity and the highest concentration of radionuclides associated with nuclear wastes is found in spent fuel from civilian nuclear power reactors, and the reprocessing of civilian and military spent fuel. Typical radionuclides found in this high-level radioactive waste (HLRW) are: 60Co, 137Cs, 239−242Pu, 106Ru, and 90Sr. Because of the high hazard duration (>105 years) associated with these nuclides, large quantities (80 million gallons in 1982) (196, 236) of highly toxic liquid and solid HLRW must be isolated from the environment for thousands of years.
At present much of the HLRW is stored at temporary sites in concrete-encased steel tanks a few meters below the surface of the ground. Considering the finite lifetime of the steel tanks (15–40 years), it becomes clear that these wastes must be transferred to other containers or sites in the future. Several options for the permanent repository of radioactive waste have been considered (196, 251) and the method currently in favor is in deep underground mined cavities.
The worst example of nuclear waste management has been the experience of the Soviet nuclear weapons production complex, MAYAK, which started in 1948 on the Techa River in the Ural mountains of the Soviet Union (about 60 miles from the city of Chelyabinsk). The city constructed nearby to support the facility was originally called Chelyabinsk-65 (it is now called Ozersk), and this nuclear contamination area is now usually referred to simply as Chelyabinsk. Over 2×107 Ci were released over time into the surrounding area, making this the largest release of radionuclides at a single site in history (428, 652). Most of this inventory was released in 1957 in an explosion in a fuel reprocessing plant, referred to afterwards as the Kyshtym accident. The workers in the MAYAK facility were reported to have had high levels of radioactive exposure, exceeding 1 Gy annually for 25% of the radiochemical workers in the first 5 years of operation, with 11% of the workers overall receiving 6.3 Gy over the first decade (249). This resulted in some deaths from chronic radiation lung injury (359) and elevated lung cancer deaths and leukemia in the workers (360). In the people living in the villages along the Techa River and in the areas contaminated by the Kyshtym accident, there were also reports of high internal radionuclide doses (249) and an increased incidence of leukemia (361).
Natural Environmental Radiation
Natural background radiation is the greatest contributor to radiation exposure in the world. In most countries natural background radiation contributes slightly more than half of the absorbed radiation dose (437). Relative contributions to the total absorbed dose may range from 42% in highly developed countries to 94% in most developing countries. Exposure to natural sources of irradiation is unavoidable and life has evolved under a continuous exposure of ionizing radiation. This background radiation has three components: (a) cosmic radiation (external), (b) terrestrial radiation (external), and (c) naturally occurring radionuclides (internal).
Cosmic and Solar Radiation
Cosmic and solar radiation originate predominately from galactic sources and consist mostly of high-energy protons and alpha particles (48, 437, 555). Cosmic radiation at the earth's surface varies with altitude, geomagnetic latitude, and solar modulation (214, 290). For instance, in the United States, 48% of the population lives at sea level to 152.5 m and receives a dose rate of approximately 27 mrem/year (0.27 μSv/year), whereas in Leadville, Colorado (altitude 3200 m), the residents receive about 125 mrem/year. This effect of altitude becomes increasingly important to passengers and crews of high-flying aircraft. It is estimated that cabin attendants and crew members receive approximately 160 mrem/year above that received at sea level (196). The cosmic rays are reduced by the earth's atmosphere, resulting in a shielding effect. This shielding effect decreases with altitude, with cosmic ray exposure doubling every 1500 m above the earth's surface (437).
Above the earth's atmosphere the radiation consists of two main components. One is the dose from highly energetic cosmic radiation geomagnetically trapped in the earth's magnetic field. The second component is received beyond the earth's magnetic field and is due to background cosmic radiation of about 85% protons and 14% alpha particles. Astronauts traveling into outer space must traverse two belts of geomagnetically trapped radiation, the primary cosmic radiation, radiation from solar flares, and

directed beams of gamma rays emitted by certain quasars and pointed directly at Earth (206).
Within the United States the effect of latitude on cosmic radiation dose rate is less than 10%, with an average dose rate at sea level of about 270 μSv/year (437).

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However, in the United Kingdom the annual dose rate varies from about 280 μSv a year in the south of England to 310 μSv a year in the north of Scotland (214). The dose rate variation with latitude depends primarily upon the variations in the earth's magnetic field, with which cosmic radiation interacts (48).

Terrestrial Radiation

Terrestrial radiation levels and rates from natural background sources are functions of geographic location and living habits. In most areas on earth the terrestrial radiation level varies within relatively narrow limits, but in certain regions of Brazil, China, France, Italy, Madagascar, and Nigeria, the terrestrial radiation substantially exceeds the normal range (196, 398, 437). For instance, a person suntanning on some beaches along the Atlantic coast of Brazil may receive as much as 17.5 cGy/year from the sand alone (437). Meanwhile, the exposure from the fine monazite particles of the soil in the Donganling and Tongyou regions of China would run between 18 cGy and 20 cGy per year (398).

The conterminous United States may be divided into three general radiation regions (48). The Atlantic and gulf costal plains receive an average of 23 mrems/year whereas the range in the Colorado plateau area may be as high as 140 mrems/year. The average terrestrial level for the remainder of the United States is only 46 mrems/year, with an estimated national average of 40 mrems/year.

The terrestrial radiation rate varies with the type of soil in the area and the naturally occurring radionuclide content of the soil. Approximately 70 of the 340 nuclides found in nature are radioactive (196). These radionuclides have existed on the earth's crust since its formation and are known as *primordial radionuclides*. These primordial radionuclides have half-lives comparable to the age of the universe and are the source of terrestrial radiation (437).

Three distinct chains of primordial radioactive elements are found in the earth's crust and account for much of the terrestrial radiation exposure (437). These are: (i) the uranium series, (ii) the thorium series, and (iii) the actinium series. Uranium, the origin of the actinium series, is found in various quantities in rocks and soils. The uranium isotopes are alpha emitters and therefore do not contribute to the gamma background radiation. The presence of uranium in soils and in fertilizers leads to its presence, via the food chain, in plant and animal tissues. At equilibrium, an adult human male may be expected to have a uranium content of 100–125 μg. The thorium (232Th) decay series may also move through the food chain, but, due to its relative insolubility and low specific gravity, it is present in biological materials only in insignificant amounts (196). Thorium may be found in silty clay and peaty soils, and in such vegetables as potatoes, corn, carrots, beans, and squash. However, the principal source of human exposure is inhalation of soil particles. Thorium is removed very slowly from bone and its concentration increases with age.

Radium 226 (226Ra), an alpha emitter originating in the uranium decay series, is present in varying amounts in all rocks, soils, and water, and is of special importance, along with its daughter products (437). 226Ra, with a half-life of 1622 years, decays to radon (222Rn), a noble gas radio-nuclide with a half-life of 3.8 days. Radon, to be discussed later, also emits alpha particles but adds to the gamma radiation level of the environment through its gamma-emitting descendants.

Radium is very similar to calcium and is absorbed by plants from the soil like calcium. It then passes through the food chain to humans, where 70–90% is concentrated in bone. The amount of 226Ra moving through the food chain depends upon its content in the soils and its rate of absorption by plants. This rate of absorption by plants is related to the amount of exchangeable calcium in the soil. Brazil nuts, because of their tendency to concentrate barium, another chemical very similar to radium, may have a 226Ra content approximately 1000 times greater than the average diet.

Radionuclides

Internal radiation results from naturally occurring radionuclides contained within the body, and contributes approximately 11–17% of the average radiation exposure of the population (50, 614). Although some of the radio-active emitters may be freely dispersed throughout the body, others are concentrated in specific organs and all of the emitted decay energy is absorbed locally (555, 716). The deposition of naturally occurring radionuclides such as bismuth, carbon, hydrogen, lead, polonium, potassium, radium, radon, thorium, and uranium results primarily from the inhalation and ingestion of these materials in air, food, and water (48).

In a terrestrial ecosystem, radionuclides such as 210Ra, 226Ra, and 222Rn that occur in the soil, or are deposited in the soil, are incorporated metabolically into plants (196). In addition to root absorption, plants are contaminated by direct foliar deposition. Foliar deposition is potentially a major source of food chain radionuclide contamination because the radionuclide may be absorbed metabolically by the plant or transferred directly to animals consuming or coming in direct contact with the foliage. Individual radionuclides pass from the roots or the leaves to the remainder of the plant. Mean 232Th

concentrations of 0.018±0.022 Pci/kg have been found in the edible portions of 25 vegetables, including beans, carrots, corn, potatoes, and squash. Flora near the summit of the Morro do Ferro, a hill in the state of Minas Gerais, Brazil, have absorbed so much ^{228}Ra that they can easily be autoradiographed (196).

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Atmospheric radionuclides are eventually deposited on surface waters as well as on the soil. Therefore, the atmosphere is coupled to soils, surface waters, and subsurface aquifers. Radionuclides are eventually transported into streams or subsurface aquifers. Those that appear in deep underground aquifers may eventually reach surface waters and become incorporated into the biosphere again.

Rivers, estuaries, and coastal waters are major receptors of effluent radionuclides from industrial plants and cities. These waters are of special importance because of their high biological activity and productivity. Phytoplankton in these relatively shallow waters convert mineral resources in the aquatic environment into food for higher organisms. Zooplankton, the basic food of several higher trophic levels, use phytoplankton as their source of nourishment. Certain bottom-dwelling fish and animals also use phytoplankton as a source of nourishment.

Once the radionuclides have settled in an aquatic system over time, they tend to accumulate in the bottom sediments. In a collection pond built to contain a significant radioactive spill, 85% of the primary radionuclide present (radiocesium) was irreversibly bound up in the sediments, with some of the rest of the radionuclides available for remobilization from the sediment and subsequent exchange with the water column (700, 701). This accumulation in the sediments is an important factor in determining uptake and deposition in various aquatic species, with bottom-dwellers tending to accumulate more radionuclides than some other organisms in the water column.

The importance of radionuclides in marine and fresh water foods depends, in part, on where the radionuclide is located in the organism. A radionuclide is a higher risk if it concentrates in an organ consumed by higher organisms, such as humans, than if it is deposited in a portion that is not eaten. The radionuclides of cobalt (^{60}Co) and zinc (^{65}Zn) concentrate in edible tissues whereas those of radium (^{226}Ra) and strontium (^{90}Sr), although concentrated by clams, oysters, scallops, and certain crabs, are stored in the shell, which is not ordinarily consumed (196).

Uptake and retention of radionuclides is influenced by the portal of entry, chemistry and solubility, metabolism, and particle size. Internal contamination normally occurs via three principal routes of entry: inhalation, ingestion, and skin absorption. Of the three, inhalation is the biggest problem.

Direct ingestion from contaminated food is also a problem. Gastrointestinal exposure depends upon transit time through the gut, whereas absorption depends upon the solubility of the radionuclide.

Contamination of skin with radionuclides is of less consequence because the skin forms a formidable barrier. However, contamination of an open wound may result not only in continuous radiation of the surrounding tissue, but in the introduction of the radionuclide into the rest of the body.

Regardless of the portal of entry, the radionuclide passes throughout the body of the animal and into the milk, flesh, internal organs, and eggs. When the radio-active material enters the body it becomes an internal emitter. It will continue to radiate the body until it is excreted by some physiological process, mainly through urine and feces, or until its radioactivity decays (102). The time it takes an organism to eliminate half of the radionuclide is known as the *biologic half-life* and the time necessary for a radionuclide to decay to half of its activity is the *physical half-life* (Table 15.3). If the biologic and physical half-lives are known for a particular radionuclide, the *effective half-life* may be calculated (437).

Radon

Radon (^{222}Rn), the short-lived radionuclide decay product of ^{226}Ra, accounts for approximately 60% of the effective dose equivalent from internal emitters (437). As seen in Table 15.2, radon and its decay products or progeny (^{222}Rn, ^{214}Bi, ^{214}Pb, ^{214}Po, ^{218}Po) contribute 55% of the total average annual effective dose equivalent of 3.6 mSv.

Since 1974, international concern has centered upon radon, with radon progeny as indoor air pollutants that concentrate in nearly airtight homes and office buildings, resulting from efforts directed toward energy conservation. These energy-efficient homes cause exposure to all segments of the population in which much lower air concentrations of radon progeny may be inhaled during life-span exposures (that is, at much lower dose

Table 15.3 Half-lives of some biologically significant radionuclides[a]

Nuclide	Half-life		
	Physical	Biological[b]	Effective
^{241}Am	458 y	100 y	100 y
^{14}C	5730 y	40 d	40 d
^{137}Cs	30 y	70 d	70 d
^{131}I	8 d	138 d	8 d
^{55}Fe	657 d	2000 d	494 d
^{32}P	14 d	260 d	14 d

^{239}Pu	24,000 y	180 y	180 y
^{24}Na	15 h	11 d	14 h
^{90}Sr	28 y	36 y	16 y
^{3}H	12 y	12 d	12 d
^{235}U	7.1×10^{8} y	20 d	15 d
^{65}Zn	245 d	400 d	152 d

a From References 102, 196, 437, and 575.
b Whole body.

rates than those that have occurred in uranium mining populations). In addition, the low dust concentrations in such buildings with very low air changeover rates result in greatly increased fractions of radon progeny that are unattached to air carrier aerosols, causing proportionately higher radiological doses in the basal-cell epithelium of the conducting airways of the lungs. Measurements of air concentrations in Colorado Plateau uranium miners showed less than 2% unattached radon progeny, but in regions of quiet air such as in private homes, levels became an order of magnitude higher; 81% of the attached RaA may become unattached upon decay (275). During the years 1940 to 1988, many models were developed to calculate the radiation dose to the lungs as a whole or to selected regions of the respiratory tract. Dose conversion factors of rad/WLM show considerable increases above unity as the unattached fraction of radon progeny increases.

In order to define the role of attachment of radon progeny to aerosols and the incidence and site of radon progeny-induced respiratory carcinoma, a series of experimental studies used specific pathogen-free Wistar rats (627). Groups of 32 rats received inhalation exposure during 84 hours per week to 900 WL radon progeny attached to 15 mg/m3 carnotite uranium ore dust. Exposures lasted for 150 days and the animals were then held for life-span carcinogenesis studies. These animals showed 60% incidence of squamous carcinoma or adenocarcinoma in the periphery of the respiratory tract following these prolonged inhalation exposures to radon progeny that were 98–99% attached to uranium ore dust aerosol, but the animals showed no tumors of the nasal pharynx. In marked contrast, matched groups of rodents that received exposure to radon progeny with only room-air aerosol (from 10–25% unattached, as likely to occur in private homes) displayed less than 6% squamous carcinoma in the peripheral lung but 100% nasal squamous metaplasia and several cases of squamous carcinoma in the nasal pharynx, plus 22% squamous metaplasia in the major conducting airways such as the bronchi and first generations of secondary bronchi (627). Additional studies in the same laboratory involved rats exposed to several concentrations of radon progeny attached to uranium ore dust, designed to determine the effect of exposure rate and unattached fraction of radon progeny on the nature and incidence of pulmonary carcinoma (147). Groups of 32 or 48 male specific pathogen-free Wistar rats received inhalation exposures to radon progeny in groups having unattached percentages of 1.6 and 10, the latter representing exposure conditions that might occur in minimally ventilated dwellings. Pulmonary neoplasms included epidermoid carcinoma, adenocarcinoma, adenosquamous carcinoma, mesothelioma, and adenoma. Exposure regimes included background level, 250, 500, and 1000 WL, with total exposures of 640 or 2560 WLM. Percentages of lung tumors resulting from these exposures ranged from none in the case of animals receiving background exposures to laboratory air to 47% in those animals at the 500 WL dose rate, with the total received exposure of 2560 WLM. Pathological evaluation resulting from the dose rate exposure study indicated an increase in the risk of pulmonary lung tumors as the exposure rate *decreased*.

Epidemiological studies of the incidence of bronchio genic carcinoma among uranium miners in the Colorado Plateau, as well as in several European uranium mining studies, show considerable consistency in the relationship of the risk of bronchial carcinoma *per* WLM (275). Studies using human volunteers that inhaled significant concentrations of radon progeny attached to uranium mine aerosols in the Colorado Plateau underground mines showed 90–100% attached radon progeny that were deposited in the regions of bronchi and subsegmental bronchi (146). Because of the efficient deposition of unattached RaA in the brachia and major bronchi, the dose to bronchial epithelium from unattached RaA can be greater than three times that of unattached progeny per unit concentration in the atmosphere. Values for the dose conversion factor of rad/WLM in this region of the respiratory tract are 0.5 for underground miners. Analyses of exposures of individuals in the general population indicate values of 0.7 rad/WLM for men, 0.6 for women, 1.2 for children, and 0.6 for infants (275). The unit WLM is defined only in terms of potential alpha energy from radon progeny per liter of air. There can be very significant changes in the magnitude of this dose, with factors of up to twofold higher, due to differing characteristics of inhaled atmospheres. These significant differences are not accounted for when using WLM alone as a unit or exposure.

In 1984, the focus of attention of carcinogenesis resulting from radon progeny inhalation by humans shifted from the miners of the Colorado Plateau to the discovery of unusually high radon levels in a home built upon a geological formation called the Reading Prong in Pennsylvania. It soon became evident that each state had different problems associated with radon exposure to the general population. Whereas Pennsylvania had about 22,000 homes on its section of the Reading Prong, more than 250,000 homes were located on the New Jersey Reading Prong (495).

In order to explain the current concern concerning radon/radon progeny levels in the home, a further

definition of the working levels is necessary. Because it is far simpler to measure radon, the parent, rather than the individual radon progeny, even though the latter contribute 95–98% of the dose to the respiratory epithelium, the "working level" can be defined according to its original estimate, that is, 100 pCi of radon per liter of air, which at 100% equilibrium with its progeny will give,

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by definition, 1.0 working level. In 1984, the National Council on Radiation Protection and Measurement Report No. 74 recommended an action level of 8 pCi per liter environmental exposures, that is, exposures to the general population. Because only 50% of equilibrium is generally assumed for radon progeny 218Po, 214Pb, 214Bi, and 214Po (i.e., one-half the concentration of the parent 222Rn), this corresponds to an equivalent of 0.04 working level.

In 1986, in order to provide a more conservative position, the EPA issued a citizen's guide to radon recommending 4 pCi/L as an action level (663). The recommended caution level by the EPA is based upon linear extrapolations of high-dose level exposure over 5–30 years of a small mining population on the Colorado Plateau (663); the equating of lung cancer risk from a specified level of radon progeny to a given level of cigarette smoking is particularly difficult in that there is a close synergism between incidence of bronchogenic carcinoma in the uranium miner population and high levels of cigarette smoking by these men. The wide limits of uncertainty found in the lung cancer risk estimate associated with a given total exposure level and the importance of dose rate factors, demonstrated by recent animal and epidemiological studies, have resulted in risk estimate calculations by agencies within the United States as well as in Canada and Europe, that suggest possible lower risk per WLM or pCi/L exposure level. The action level for homes recommended by the EPA of 4 pCi/L is low when compared with that recommended by Canada and Finland (110). It is likely that countries of the European Union will follow the example of these two countries with action levels at 8 or 20 pCi/L.

RADIATION BIOCHEMISTRY

Radiation toxicity represents a dynamic interaction between radiation physical constraints and molecular damage, which are further amplified by biological processes resulting in injury (Figure 15.1). Biochemistry provides this important link through which a quantity of energy sufficient to raise the temperature of a liter of water by 0.1°C, an amount less than the caloric energy of a candy bar, can elicit toxicity resulting in death, or the beneficial therapy of a malignant tumor, diverse extremes, and yet mechanistically similar at the molecular level, the initial injurious events that occur in 10−17–10−5 s, may be irreversible and may require seconds to years for expression. The expression of injury for some cancers is so slow that it may not be observed over the individual's lifetime. The four processes mediating and amplifying radiation toxicity, ionization and excitation, molecular injury, biochemical damage, and amplification and expression of injury, are illustrated in Figure 15.1. The first three will be discussed in this section, followed by amplification and expression of biological injury in the remaining sections. The latter sections emphasize the important roles of biological mediators and hormones in this process, as well as delineating specific risk concerns such as cancer.

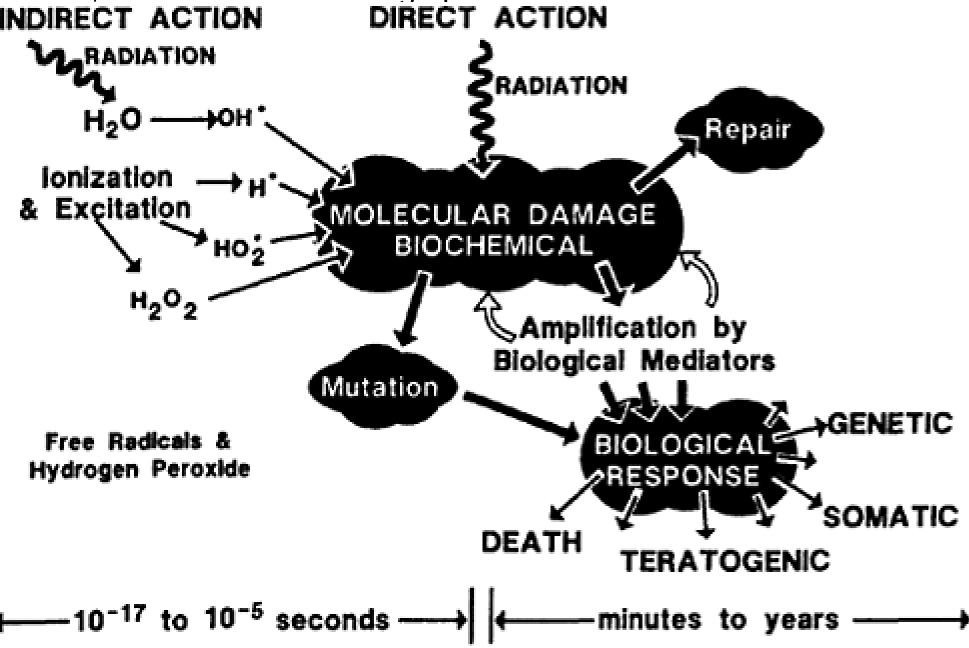

FIG. 15.1. Schematic for radiation injury to biological systems. The small quantity of radiation interacts with matter by ionization and excitation, producing both direct and indirect damage; the latter occurs primarily through free-radical attack. Because free radicals induce chain reaction of free-radical injury, the initial event is magnified. The molecular and biochemical damages in turn stimulate further

amplification through the release of biological mediators and hormones. At each of these points, actions can be taken (i.e., radioprotection, radiosensitization) that will enhance or suppress the biological expression of injury. (From References 296 and 685. Used with permission.)

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There are four basic possibilities for the interaction of ionizing radiation with an atom: direct and indirect ionization, excitation, and pair production. Ionization occurs through the impartation of sufficient energy to eject an electron out of its orbit, resulting in formation of an ion pair, the positively charge atom and the negatively charged electron. If the ejected electron is also imparted with additional energy, then it too may interact with other atoms to produce additional ion pairs, and this is an indirect ionization process. With excitation, the energy imparted to the electron is sufficient to raise it to a higher electron orbital, but not to escape. Ionization does not occur, but the energy may be sufficient to break chemical bonds. Between 20 to 35 electron volts (eV) are required to produce an ion pair, whereas hydrogen bonds can be broken by 5 eV (686, 692). Lastly, photon radiation with energies greater than 1.02 MeV are capable of interacting with the electromagnetic field of the nucleus to convert the energy into the formation of positron and an electron, *pair production*. Neutrons, alpha particles, and other particulate radiations also produce ionization and excitation. Further, in the process of nuclear activation, the neutron is absorbed by the nucleus, forming a radioactive isotope.

Although differing types of radiation may be equal in their initial energy, they differ in the pattern in which the radiation is imparted to matter and, as a result, differ in their biological responsiveness. These differences are quantified for their *relative biological effectiveness* (RBE), determined as the dose of a given type of radiation (neutron, gamma, x-ray, etc.) to produce a reference biological effect divided by the dose of a standard radiation, usually 250 kilovolt potential (kVP) x-rays, to produce the same effect. This is illustrated by the threshold for cataract formation in humans of 2 Gy for x-rays and 0.2 Gy for neutron exposures, resulting in a RBE of 10 (48). This RBE applies only to this particular effect; for a different response the RBE might be 1 or even 50. Differences in effectiveness arise because the amount of energy transferred by a unit dose of radiation per unit pathway traveled through matter, the LET, varies with the type of radiation. Neutrons, protons, alpha and beta particles, and atomic nuclei are high LET radiations, whereas gamma and x-rays are low LET radiations. The depth of penetration is determined by the total energy and the LET factor. High LET radiations produce many ionizations per distance traveled, dissipate energy quickly, and have low depths of penetration. In fact, alpha particles dissipate all of their energy before they can transverse through a sheet of paper, a layer of paint, or the stratum corneum of the skin. The consequence of such a concentrated delivery to a biological system is that more ionizations and therefore more damage is concentrated in a smaller area, making it more difficult to repair the damage (49, 686, 687, 692). Higher RBEs for neutrons (up to 46 for life shorting and 80 for cancer transformation) are elicited when lower doses and dose rates are used because of the better ability of cells to repair injury due to low LET radiation (115, 269, 493). Low dose neutron exposure is more carcinogenic for mammary tissue than x-rays, and a low environmental or accidental exposure to neutron could therefore be more damaging than the total exposure dose alone might indicate (50). In general, the LET increases from electrons to neutrons to alpha particles. The RBE increases with increasing LET up to 100–125 keV/μ (50, 269). Of particular concern, low dose and dose rate exposures to high LET neutron radiation may be more effective at causing cancers than similar exposures to low LET radiation.

Radiation interacts with matter by direct and indirect processes to form ion pairs, some of which may be free radicals. These ion pairs rapidly interact with themselves and other surrounding molecules to produce free radicals. Both the indirect and direct activities of ionizing radiation lead to molecular damage which is then translated to biochemical damage. Biochemical damage may then be amplified and expressed as biological injury in one of three basic processes, including damage to the DNA which may become expressed, stimulation and release of biological mediators, and alteration of nutritional vascular support.

Radiolysis of Water

Radiation may impart energy to matter in one of two primary ways, either directly through ionizations or indirectly by transfer of energy and formation of free radicals. The most abundant molecule in living systems is water. It accounts for about 55% of the mass in humans. Ionizing radiation interacts with water molecules to form an ionized pair consisting of a free electron (e−) and an ionized water molecule (H2O+) in a process termed radiolysis. The free electron rapidly interacts with water to form the hydrated electron (H2O−) which decomposes to OH− and H· The dot (·) designates a free radical, a molecule having an unpaired electron in the outer electron shell. The free radical may also be electrically neutral but remains highly reactive because of the unpaired electron. The second ion from the ion pair, (H2O+), decomposes to H+ (hydrogen ion) and OH· (hydroxyl radical). The ionic designation depends on the molecular charge, so it is possible for a molecule to be a free radical and an ion. The hydroxyl radical contains nine protons and nine electrons, and is electrically neutral.

The end products of the radiolysis of water without oxygen are H·, OH·, H+, and OH−. Of these, H· and OH· are the most important, and comprise 55% of the initial relative yield (49). Both are highly reactive and have half-lives of 10^{-11} s. This allows for the initial

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impact of the radiation ionization event, which may have missed the biological target, membrane or DNA, etc., to diffuse away from the initial site and produce damage by free-radical attack of a nearby molecule. The two primary factors influencing the formation of free radicals are the presence of oxygen and the LET. Radiolysis in the presence of oxygen produces the hydroperoxy radical ($HO_2\cdot$), the hydroperoxy ion (HO_2^-), and hydrogen peroxide (H_2O_2). These chemical entities are powerful oxidizing agents with longer half-lives on the order of 10^{-10} and may diffuse even farther from the initial site of ionization (49, 296, 692). The type of radical species formed is also governed by how closely or how rapidly radicals are formed, because they may interact to form H_2, H_2O, and H_2O_2, neutralizing the radical attack before reaching the biological target. For example, H· reacts with OH· to form H_2O. High LET radiations produce more ionizing events closer together, with a greater chance of free-radical neutralization. Hence, direct effects tend to predominate with high LET. The net effect of the above processes is that biological material irradiated in a dry state and in the presence of oxygen, is more resistant to injury than when the reverse conditions are present. Interaction of a free radical with a biological molecule (RH) results in the formation of an organic free radical and stabilization of the initial radical:

$$OH\cdot + RH \rightarrow H_2O + R\cdot$$

The Oxygen Effect

The most effective sensitizer of biological tissues is oxygen (269). Biological material and living systems irradiated in the presence of oxygen are more susceptible to injury than when irradiation occurs without oxygen. This response, known as the *oxygen effect* was first observed in 1909 by Gottwald Schwartz who noted that pressure applied to the forearm during irradiation reduced the resulting erythema (686). He was not aware that lack of oxygen per se was responsible. The effectiveness of oxygen in modifying the response to radiation is expressed as the *oxygen enhancement ratio* (OER), the radiation dose in the absence of oxygen to observe a given response divided by the radiation dose required to observe the response in the presence of oxygen. OERs for low LET radiations are generally 2.8 to 3, and tend to decrease as the RBE increases, up to a point, between 100 to 200 keV/μ. The radiosensitizing effects of oxygen are particularly important in radiotherapy because tumors may have hypoxic centers either because of poor vascular supply, tumor compression from the outer cells, or altered metabolism. Therapeutic attempts to address this issue have tried to increase tumor oxygenation (through increased vascularization, vasodilation, hyper baric oxygen, or increased oxygen delivery to tissues using intravascular perfluorocarbons) or to use electronegative compounds that mimic oxygen (269). Hypoxia decreases the radiosensitivity of tissues, but increases the sensitivity to hyperthermic treatments. The oxygen concentration must be reduced below 2.0% to see any appreciable protection, which increases rapidly with decreasing oxygen concentrations below 0.5% (3 mm partial pressure of oxygen, compared to 40 mm in venous blood and 60–80 mm in oxygenated arterial blood), at which point the OER is about 2 (269). Oxygen results in the formation of hydroperoxy and hydrogen peroxide radicals that are more damaging because of their longer half-life. Free radical damage is "fixed" through reaction of oxygen leading to formation of peroxy and hydroperoxy organic products that are more resistant to biochemical repair processes (263, 269). The presence of oxygen can also enhance the therapeutic efficacy of some chemotherapeutic agents, whereas some agents including misonidazole, mitomycin C, doxorubicin, and tirapazamine are more effective in hypoxic environments (269, 640, 641). Ethanol, narcotics, leukotrienes, and some thiols are radioprotective in part through their ability to induce hypoxia (238, 686). Ethanol and narcotics suppress the respiratory center in the central nervous system, resulting in hypoxia.

Effects of Radiation on Macromolecules

Radiation-induced modification of biomolecules can be divided into structural degradation and decomposition, cross-linking of molecules, and breakage of chemical bonds (12, 263, 296, 680, 685, 686, 692). The individual responses for the different classes of macromolecules are presented in Table 15.4. Bond breakage occurs through energy transfer, ionization transfer, or electron transfer. Cross-linking can occur between similar and also different classes of molecules: protein-protein, lipid-lipid, protein-DNA, etc. Structural and conformational changes may alter or eliminate biochemical activity directly and expose internal sites to radical attack (117, 233, 692). The DNA bases are protected from free-radical attack because of their position in the center of the helix, but are exposed by strand breaks, breakage of hydrogen bonds, and unwinding processes. Conformation is also critical for enzyme activity and structural proteins. The response of macromolecules in vitro may vary from the effects in vivo due to molecular interaction and biological repair processes.

Lipid Peroxidation

Radiation injury of lipids in vitro and in vivo occurs primarily through peroxidation by free radical attack at the double bonds and carbonyls (543, 685, 686). Lipid

Table 15.4 Effects of ionizing radiation on macromolecules

Amino acids	Liberation of ammonia, hydrogen sulfide, pyruvic acid, carbon dioxide, and hydrogen.
Carbohydrates	Cleaveage of glycosidic bonds, depolymerization of individual monomers, oxidation of terminal alcohols to aldehydes.
DNA	Degradation with base loss or modification; breakage of hydrogen bonds or sugar-phosphate bonds; cross-linking of DNA-DNA or DNA-protein; strand breakage; formation of guanyl, thymidyl, and sugar radicals.
Lipids	Peroxidation; bond rearrangement—conjugated diene formation, aldehyde formation, β-scission, cross-linking; increased microviscosity.
Proteins	Degradation and modification of amino acids, chain scission, and cross-linkage. Denaturation and changes in molecular weight, and solubility.
Thiols	Oxidation, reduction, radical formation, cross-linkage.

Table 15.5 Steps in lipid peroxidation*a*

Initiation	
	Enzymatic
	LH+Ionizing Radiation→LH· +H·
	LH+OH·→L· +H2O
	LH+R·→L· +RH
Propagation	
	L· +O2→LOO·
	LOO· +LH (or RH)→→L· (or R·)+LO2H
Termination	
	LOO· +LOO· (or ROO·)
	L· +LOO·
	LO· +LO·
	L· +L·
	L· +R·
	L· +H·
	L· +free radical scavenger
	(L=lipid and R=other organic molecules)

a From References 357 and 543.

peroxidation, as illustrated in Table 15.5, consists of three phases: initiation, propagation, and termination. Lipid peroxidation is important to homeostasis and is associated with the formation of lipid mediators, including the prostaglandins and leukotrienes, as well as lipid degradation (Figure 15.2). Peroxidation may be mediated by three possible initial events (543, 686) (Table 15.5). Initiation may occur enzymatically, as with cyclo-oxygenase and lipoxygenase enzymes, and requires molecular oxygen and ferric cofactors. Radiation-induced lipid peroxidation is initiated by direct or indirect ionization, or by free-radical attack. The primary radical species involved are the hydroxyl radical and superoxide (193, 543).

Lipid peroxidation is affected by the lipid structure and composition, the presence of oxygen and antioxidants, pH, temperature, and the conditions of irradiation (193, 543, 686). Increasing the number of double bonds in the lipid carbon backbone enhances its susceptibility to free-radical oxidation in solution. For example, arachidonic acid possesses four double bonds and is more sensitive than linoleic acid which has three double bonds (477). The greater the lipid concentration, the greater the likelihood that the free radicals propagate the chain reaction by attacking another lipid molecule. On the other hand, with increased rate of radical formation, the risk of radical interaction and neutralization increases. Therefore, unlike other radiation-induced molecular injury processes, greater lipid peroxidation product yield is obtained with lower radiation doses and exposure rates.

Lipid peroxidation is a chain reaction in which interaction of the lipid radical with another organic molecule results in conversion of that organic molecule to the free-radical state and propagation of damage. Alternatively, the lipid radical may terminate the reaction by one of several different processes as outlined in Table 15.5. It may react with another free radical or with a free-radical scavenger. The primary free-radical scavengers in biological systems are vitamins A and E and the thiols, and their membrane concentrations influence cellular radiosensitivity (357, 686, 687). In addition, at least eight enzyme systems are associated with detoxification and repair of free-radical injury, not including DNA-specific repair enzymes (368, 686). These include glutathione transferase, NADPH-dependent glutathione reductase, selenium-dependent glutathione peroxidase, selenium independent glutathione

peroxi

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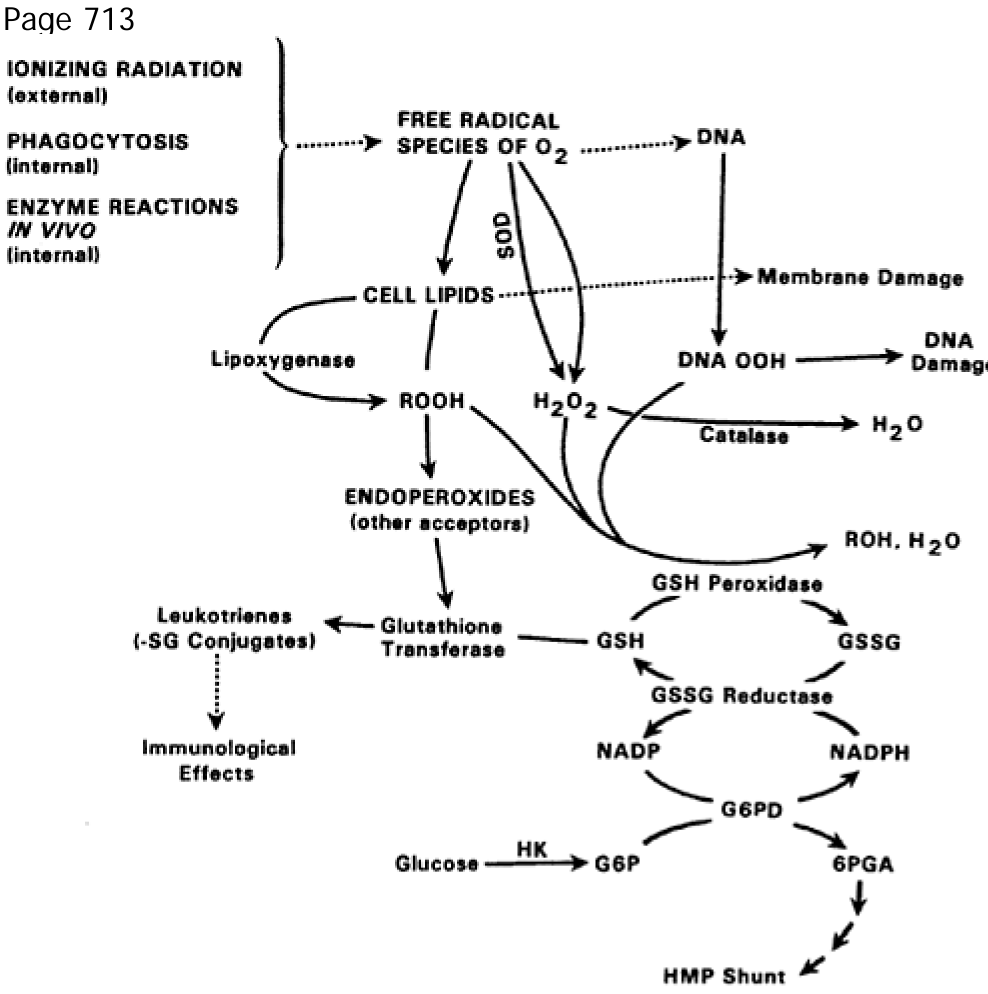

FIG. 15.2. Enzymatic repair of peroxide damage. Reactive oxygen species formed by enzymatic and nonenzymatic processes may induce oxidative attack of DNA, lipids, and other biomolecules. Repair of this damage can be mediated by glutathione (GSH-reduced glutathione) or glutathione dependent peroxidases. As shown, oxidized glutathione (GSSH-glutathione disulfide) can be reduced/renewed, and most of the system can be maintained as long as a sufficient supply of reducing power (NADPH-nicotinamide adenosine diphosphate) generated by the metabolism of glucose is available. DNA-deoxyribonucleic acid; G6PD-glucose 6-phosphate dehydrogenase; HK-Hexokinase; HMP-hexose monophosphate shunt; ROOH-organic peroxide; SOD-superoxide dismutase. (From Reference 368. Used with permission.)

dase, ferric superoxide dismutase, manganese superoxide dismutase, copper-zinc superoxide dismutase, and catalase. Metallothionein is another protein with free-radical scavenging ability, because one third of its amino acids are cysteine residues. Synthesis of this protein is enhanced under stress situations, including lipid peroxidation and radiation injury (686), and can be induced by zinc supplementation. Another thiol-containing protein, thioredoxin, participates in the reduction of a number of important DNA enzymes and transcription factors, including ribonucleotide reductase (225), and is a radioprotectant. It is itself reduced by NADPH-dependent thioredoxin reductase, a selenoprotein. Vitamin E is an important free-radical scavenger that is located primarily in the cell membrane. Once modified or activated by free-radical attack, it is detoxified and renewed by specific enzymatic pathways. Vitamin E interacts with organic radicals to form a stable organic alcohol (357). In this process, vitamin E is con verted to an excited state that is restored by interaction with vitamin C. Vitamin C, in turn, is renewed through the action of an NADH-dependent enzyme. Interestingly, melatonin, the pineal gland hormone, has been demonstrated to scavenge free radicals (546), and pretreatment of human lymphocytes irradiated in vitro reduced subsequent micronuclei damage (678).

Thiols are molecules containing free or potential sulfhydryl groups (SH−) in their structure. Examples include the amino acids methionine and cysteine, and the complex lipid thiol ether, leukotriene C4. The most abundant nonprotein thiol, the tripeptide glutathione (GSH), is present intracellularly at 1–3 mM. Thiols act as cofactors for some enzymatic processes and participate in radical scavenging and

detoxification processes. Their activities are dependent on cellular concentration, location, synthesis, and catabolism. During the reaction of a thiol with a free radical, donation of hydrogen by the reduced sulfhydryl of the thiol to the organic radical

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results in a form of chemical repair:

$$GSH+R\cdot \rightarrow RH+GS\cdot$$

Two glutathione radicals (GS·) may then react to form a disulfide (GSSG), terminating the radical chain reaction. This disulfide, or oxidized glutathione, is regenerated to yield two GSH molecules by the action of NAPDH-dependent glutathione reductase (Figure 15.2). Thiols may also affect responses to irradiation through formation and destruction of disulfide bridges. Glutathione may form a disulfide bridge with the free sulfhydryl group of another molecule, perhaps in an enzyme active site, masking and protecting the sulfhydryl from radical injury. Organic peroxides are repaired through the actions of selenium-dependent and -independent glutathione peroxidases (368, 686).

Hydrogen donation reducing power for these processes is provided by NADPH generated through the hexose monophosphate shunt. Therefore, anything interfering with the hexose monophosphate shunt, glucose utilization, or synthesis of NAD+, NADP+, or glutathione may ultimately influence free-radical scavenging and tissue injury (59, 368). In fact, hypoxic cells depleted of glutathione become more sensitive to radiation (59, 463, 686). This has implications for the radiotherapy of tumor cells, although sensitization has not been consistently observed for aerobic cells (686). The difficulty arises in that glutathione concentrations must be reduced below 5% for sensitization to be observed (463, 686). Yet, even at these low levels, glutathione-dependent enzymes usually maintain activity because they retain glutathione. The depletion of glutathione levels by use of specific glutathione synthetase inhibitors, such as buthionine sulfoximine, may have a greater effect on DNA damage, because glutathione synthesis does not take place in the nucleus (29). Glutathione must diffuse into the nucleus. As a result, glutathione depletion in the nucleus would subject the DNA to greater free-radical damage. The participation of other non-glutathione-dependent enzyme systems for modification of oxidative damage also limits the significance of glutathione during irradiation under aerobic conditions. Under aerobic conditions, the electron transport chain ensures a steady supply of NADPH to drive the glutathione-dependent repair enzymes. Because of their roles in free-radical scavenging, chemical repair, and, in some instances, the induction of hypoxia, thiol compounds have been studied extensively as potential agents for radioprotection.

Amifostine (WR-2721) is an organothiophosphate initially developed through the Antioxidation Drug Development Program of the Walter Reed Army Institute of Research, and is approved by the Food and Drug Administration for chemoprotection of the kidneys in patients receiving cisplatin therapy. Amifostine is such a potent radioprotective agent when given prior to radiation exposure that it raises (protects) the necessary hematopoietically lethal dose of radiation by 2.5- to 2.7-fold (724). This compound has also been used in clinical trials to minimize radiation injury to normal tissues of patients receiving radiation therapy (115, 178, 269, 353). It is administered intravenously; potential side effects include hypotension, nausea, vomiting, and hypocalcemia. Studies demonstrate that amifostine is preferentially taken up by and therefore protects normal tissues, as opposed to tumor tissues (725). There is minimal central nervous system protection, because it does not cross the blood-brain barrier.

Superoxide is produced physiologically by several enzyme systems, is released by activated neutrophils, and is formed during the radiolysis of water in the presence of oxygen (686). It is removed through conversion to hydrogen peroxide by the action of superoxide dismutase (SOD):

$$2H^{+}+2O_2\rightarrow H_2O_2+O_2$$

Treatment with SOD has been shown to protect macromolecules, cells in vitro, and animals. Hydrogen peroxide is itself a potent oxidizing agent that can also be converted, in the presence of ferric compounds, to yield hydroxyl radicals. Hydrogen peroxide is decomposed by catalase:

$$2H_2O_2\rightarrow 2H_2O+O_2$$

Nitric oxide, NO, is a neurotransmitter and also acts as endothelial cell relaxation factor, a vasodilator. Superoxide interacts with nitric oxide to form NO_2 and NO_3, processes that are inhibited by SOD. As such, SOD potentiates the response to nitric oxide. Peroxynitrite ($ONOO^-$) is a toxic oxidant product from superoxide and nitric oxide interaction that can breakdown to hydroxyl and nitrous oxide radicals that participate in lipid peroxidation (203). There is much interest in the biological roles of nitric oxide, with as yet little information on modification by ionizing radiation. There are three isozymes of nitrite synthetase, one of which is an inducible form, which has been shown to stimulated in the rate colon and ileum (401). The increase in nitric oxide is thought to be associated with radiation-induced ileal dysfunction (401). Exposure of mice to 7 Gy of gamma irradiation has been shown to stimulate the L-arginine-dependent production of nitric oxide from the terminal guanidine group in mouse liver, lung, brain, and spleen (679). Treatment of mice with a specific inhibitor of nitric oxide synthetase, or with DEA/NO, a nitric oxide-releasing agent, results in increased animal survival of the irradiated mice.

Interestingly, both agents have

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been shown to increase the hypoxic fraction of the mouse bone marrow, and this may be related to alterations in regional blood flow (induction of acute hypoxia). Protection is most effective when these compounds are given close to and before irradiation.

Because of its similarities to oxygen, nitric oxide has also been evaluated as a radiosensitizing agent to hypoxic cells in vitro, with a sensitization enhancement ratio of 2.4 (464). It is thought that under hypoxic conditions, the free radical, nitric oxide, interacts with carbon-based free radicals to "fix" radiation-induced free-radical injury in a manner similar to oxygen.

Effects on Amino Acids, Peptides, and Proteins

The primary effects of radiation on amino acids and proteins in solution are provided in Table 15.4 (12, 384, 686). Radiation-induced breakage of hydrogen bonds and disulfide bridges, or cross-linkage formation, can affect conformation and therefore activity/function. The radiation dose to inactivate proteins in the dried state is proportional to the molecular weight, that is, a larger target requires more radiation. This relationship has established radiation-inactivation of proteins as an accepted method of determining molecular weights. Further alterations may occur from moderation of radiation synthesis, although, in general, protein synthesis is not affected by radiation doses within the lethal range for humans (12, 507). RNA synthesis usually decreases following irradiation of radiosensitive tissues (12, 686). Radiation effects on inducible enzyme systems depend on the particular system and vary between species and sexes. Some drug detoxification enzymes are reduced in males in association with decreased testosterone synthesis. Chronic radiation exposure has been shown to induce the hsp 70 heat shock protein (hsp) in mouse lung (436), whereas 0.25 Gy to cells in vitro induces PBP74/mortalin/Grp75, another member of the heat shock protein family (571). Induction of heat shock proteins have been related to increased radioresistance.

Effects on Carbohydrates

The basic effects of carbohydrates in solution are shown in Table 15.4. Radiation may cause depolymerization of glycogen, because the α-glycosidic linkages found in glycogen and cellulose are more radiosensitive than *β*-glycosidic linkages that might be found in bacterial cell walls (686). In vivo, the effects of radiation on carbohydrates are dominated by alterations in metabolic processes. Radiation promotes glycogenesis and gluconeogenesis during the first several days post-irradiation primarily as a result of hormonal influences and alterations in metabolic enzymes (12, 113, 686). Insulin and adrenocorticoid release stimulates an increase in blood glucose, providing a source for the glycogen synthesis which is further supplemented by shunting of amino acids released by tissue injury. Decreases in hexokinase, aldolase, and pyruvate kinase and an increase in transketolase are observed (113, 686). Additional NAPDH reducing power is obtained by shunting of carbohydrates through the pentose phosphate pathway.

Effects on Nucleic Acids and DNA

Ionizing radiation exposure of DNA may cause degradation of bases and sugars, breakage of hydrogen or sugar phosphate bonds or cross-linking (Table 15.4, Figure 15.3) (117, 233, 263, 296, 680, 692). The incidences for the individual types of damage is base damage >single-strand breaks >DNA-protein cross-links >double-strand breaks (692). In base damage, sensitivity is thymine >cytosine > adenine >guanine (263). At radiation doses of 200 kGy, it is proposed that the two primary radicals formed in DNA are the thymine anion and the guanine cation (295, 680, 681). Radicals may be of a charged anion,

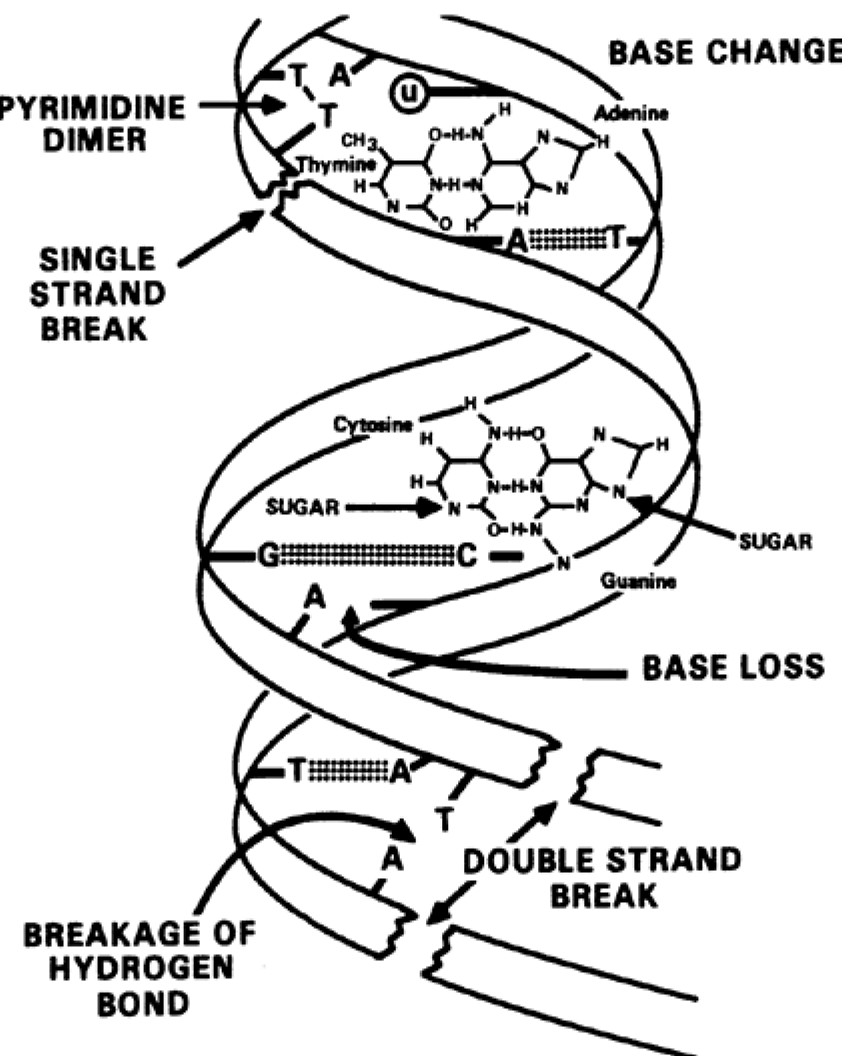

FIG. 15.3. Radiation damage to deoxyribonucleic acid. The basic categories of molecular damage, degradation, bond breakage, and cross-linking that occur in biomolecules following radiation exposure are illustrated for this double-stranded segment of DNA. The dotted lines represent hydrogen bonding between the bases, three between cytosine (C) and guanine (G), and two between adenine (A) and thymine (T). (From Reference 685. Used with permission.)

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deprotonated cation, H-addition radicals, H-abstraction radical, and opened sugar ring nature (295). DNA damage to the carbohydrate produces strand breakage, glycosylic bond breakage, and formation of precursors to malonaldehyde (263, 681, 692). DNA cross-linkages occur between DNA and DNA, DNA and proteins, or DNA and lipids. DNA to DNA cross-links may form between adjacent bases on the same strand or between different strands. The most common DNA-protein adducts would be between DNA and the associated histones and are more likely to involve tyrosine or lysine residues (508). DNA-protein cross-links formation is more common in expanded chromatin than in compressed chromatin (117). Intracellularly, DNA-protein crosslinks are enhanced by glutathione depletion but are reduced in the presence of oxygen. Important factors controlling injury are the medium composition, DNA conformation, and presence of repair enzymes (686, 692). For example, metal ions are thought to induce structural changes inhibiting free radical access to key sites and to stabilize the double-strand helix (615).

Thymine glycol is one of the oxidative DNA base damage products that can result from radiation exposure (380). Using a sensitive assay with 10–15 micromole detection limits, thymine glycol has been identified in the DNA of A549 human lung carcinoma cells irradiated in vitro with doses less than 5 cGy. The sensitivity at this low radiation dose was sufficient to detect 4.3 molecules of thymine glycol per one billion DNA bases. The responses to this assay, although linear, are dependent on the source of DNA, and irradiation of the DNA inside the cell (lower response rate, protective of DNA), as opposed to naked DNA in solution. The A549 cells were able to remove 50% of the thymidine glycol from their DNA within 2 hours and 80% by 4 hours (380).

Breakage of sugar phosphate bonds leads to single-strand breaks (SSB) and double-strand breaks (DSB). There are two hypotheses regarding DSB formation (692). In the first, hydroxyl radical attack on one strand produces a strand break through radical formation that attacks the opposite strand producing a DSB. According to the second hypothesis, DSB are produced by local multiply damaged sites to regional areas of both strands. Cell lethality is directly related to DSB. A 1-Gy dose of ionizing radiation results in 63 to 70 DSB per cell (49). The importance of DSB over SSB in relation to radiation-induced lethality is illustrated by the fact that a radiation dose that kills 63% of the exposed cells, produced 1000 SSB, but only 40 DSB (692). There would also be an estimated 440 locally multiply damaged sites. Exposure to hydrogen peroxide, by comparison, would require production of over 2.5 million SSB per cell to kill 63% of the cells. SSBs are chemical assay phenomena that do not lead to mutational events or expression of injury because the remaining strand is intact, holding the two ends together for repair by DNA ligase. With DSBs, there is no stable end point for repair, and the chromosomal material may be lost during the subsequent cell division or rejoined to a different chromosome, forming a chromosomal aberration.

Several types of chromosomal aberrations are observed following irradiation. These include rejoining to the original chromosome—normal, inversions, and translocations; terminal or interstitial deletions; and ring and dicentric formation. Irradiation of the chromosomes in G1 results in chromosomal aberrations, whereas exposure after DNA synthesis in G2 results in chromatid aberrations. Chromosomal exchanges occurring during G1 irradiation may either be reciprocal (two chromosomes) or nonreciprocal (three chromosomes). In human fibroblasts irradiated in vitro, 50% of the chromosomal exchanges were nonreciprocal (80). The rate of chromosomal aberrations is directly related to the radiation dose and is higher for higher LET irradiation and higher dose rates (49, 685, 686). Chromosomal aberrations were still evident in the lymphocyte chromosomes of the Japanese atomic bomb survivors 35 years after the bombing (544), and elevations have been identified in radiotherapy patients and in persons receiving occupational radiation exposures such as the uranium miners (49, 50, 685). Chromosomal aberrations have also been demonstrated to be elevated in the lymphocytes of astronauts who completed long-term space flights on the Russian MIR space station (502). They represent a reasonable means of biological dosimetry and can be detected by several methods including phytohemagglutinin-stimulated peripheral blood lymphocytes, premature chromosomal condensation, micronuclei technique, and fluorescent in situ hybridization (FISH; "chromosome painting"), and the comet assay (192, 339, 395, 685, 686). The results are influenced by the scoring criteria, type of irradiation, percent of body irradiated, degree of time following radiation exposure, culture time, and age of the individual, as well as exposure to other environmental toxins or agents that might also produce aberrations. Assays also exist for detection of somatic cell mutations including the hypoxenthine-guanine phosphoribosyl-transferase (HPRT) locus and the glycophorin A locus. The latter codes for a glycoprotein found on the surface of red blood cells (similar to and in conjunction with the ABO blood grouping) and expressed by alleles M and N (can also have "null," O). Increases in glycophorin A variants are dose dependent and have been elevated in the

atomic bomb survivors, and in patients from the radiation accidents in Goiana, Brazil, and Chernobyl (377). The assay required 0.1 ml of blood and can be completed in less than 1 day. An example of a variant of somatic mutation would be the detection of an MO allele expression in a person who was genetically MN (or MM).

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The final expression of DNA damage is modified by repair processes. Repair may occur at several levels: nonspecific suicide enzymatic repair, excision/ligation repair, SOS repair (mismatch repair), single base repair as with DNA glycosylases and methylation. Depending on the process, more than one enzyme may be required. Excision/ligation repair involves several enzymes, one to make a nick on the DNA strand containing the damaged site, presuming radiation has not already provided that nick, an excinuclease that excises damaged bases, synthesis of the new sequences based on the complimentary strand by the action of a DNA polymerase, and, finally, rejoining of the two loose ends by a DNA ligase (263, 381, 685, 686, 692). The main repair enzyme in bacteria is DNA polymerase I and, in mammals, DNA polymerase II. In addition, bacteria have a second category of repair enzymes called SOS-repair, or error-prone repair. As the name implies, they play a major role in repair of DSBs and are associated with a high error rate (263). Similar mismatch-repair genes have been identified in humans, and are designated hMSH1, hMSH2, hMLH1, and PMS2 (213). Cells with functional mismatch repair genes are more sensitive to ionizing radiation (cell survival) than mutants lacking active forms of these repair genes (213). The hardest break to repair without errors is the DSB; the repair depends on whether the two breaks are coincident, with possible loss of intervening information, or if the two breaks are separated with overlapping sequences to ensure proper rejoining and repair (49, 692). The closer the approximation of the two breaks, the greater the chance of loss.

Mechanisms

The biochemical response of a biological system to ionizing radiation is influenced by eight major groups of factors as outlined in Table 15.6. Repair, reoxygenation, redistribution, and regeneration have been called the "four Rs of radiotherapy" (708). It is appropriate to continue the "R" series. The first group of factors relates to the radiation itself (49, 686). Most biochemical responses are dose dependent or at least may have a damage threshold (12, 686). In addition, the rate at which the radiation dose is delivered and whether the dose is delivered in fractions or in a single exposure affect the biological response, and depend on the process and cell type in question. In general, increasing the dose rate over the range of 1 to 100 cGy/min results in an enhancement of the effectiveness of the radiation in producing injury secondary to overcoming compensating mechanisms of repair of sublethal damage and cellular proliferation (46, 269). Cells have specific repair processes for DNA and the ability to repair small amounts of damage to cell membranes. Therefore, damage that occurs

Table 15.6 Eight "Rs" influencing biochemical reactions to ionizing radiation exposure[a]

Radiation factors
- Dose
- Dose rate
- Dose fractionation
- Radiation quality (LET)
- Internal versus external irradiation

Radioprotectors/radiosensitizers

Reoxygenation

Resilience
- Age/health/individual
- Vascular integrity

Response
- Nutritional/homeostasis
- Biological mediators
- Mutational
- Circadian

Repopulation

Recovery

Repair

a From References 12, 48, 49, 269, 686, 687, and 708.

at rates below the cellular capacity will not be as cumulatively injurious. LET is another factor influencing damage. High LET radiation produces more ionizations per unit of matter and more localized damage, making repair difficult. Larger radiation doses given in single treatments tend to cause more injury than the same dose given in fractions or over a more prolonged period. Injury to early responding tissues (hematopoietic stem cells, intestinal crypt stem cells, and many tumors) is reduced by prolonging the duration of the exposure (or in the case of cancer therapy, by extending the treatment over days or weeks), whereas the total dose and fraction size are important to the degree of an effect on late

responding tissues (muscle, connective tissue, and nerve tissue) (269). Internal exposure is also a consequence for alpha and beta emitters; for example, 214Po, 218Po, 222Rn, or 236U cause alpha exposure to lung alveoli (49, 50). Transmutation produces both radiation exposure and chemical instability. The transmutation of carbon 14 in a key structural position to nitrogen causes mutations in *Drosophila* and in mice (48). Transmutation of tritium also causes mutations. However, the major effect of a transmutation is the radiation exposure (48, 50). The ability of beta-emitters to produce localized irradiation has been successfully applied to the use of 89Sr and samarium 153 to treat painful bone metastases in patients with breast cancer, prostate cancer, and multiple myeloma. These compounds are incorporated into bone similar

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to calcium, and concentrated at sites of increased bone remodeling/growth activity, such as a fracture, or in a medical use, a cancerous lesion in bone. The localized radionuclide then releases radiation to the surrounding area and tumor as it radioactively decays. Similar therapies are under development to bring radioactive nuclides in contact with soft tissue tumors by attaching the radionuclide to a monoclonal antibody directed to the tumor. The incorporation of 90Sr and 131I through the food chain represents a concern for human exposure from nuclear weapon fallout and nuclear power plant accidents, like Chernobyl.

Biochemical responses may be modified by the presence of radioprotective or radiosensitizing agents (238, 686, 688). Substances may induce radioprotection by several mechanisms, including hypoxia, free-radical scavenging, immunomodulation, hematopoietic and intestinal stem cell recovery, and modulation of the cell cycle (239). Reversal of these same processes is radiosensitizing. Cells exposed to ultraviolet radiation or hyperthermia are more sensitive to ionizing radiation, whereas animals exposed to hypothermia are more resistant to ionizing radiation. Radiation may affect core body temperature through prostaglandin synthesis and histamine release (332). Many biological processes, enzymatic rates, and fidelity are temperature dependent. Even the normal spontaneous depurination of DNA can be accelerated by elevations in temperature and this could affect mutations. Irradiated tissues remain sensitive to extreme temperature variations. Cold temperatures can accelerate the degeneration of irradiated skin (624).

An interesting observation that may ultimately prove useful in the development of a radioprotective agent for the medical/civil defense/space environments is the paradoxical nature of many of the biological mediators, such as the cytokines, prostaglandins, leukotrienes, histamines, and serotonin (494, 685, 687, 688). Their release following radiation exposure plays a key role in the biological amplification of injury, yet when administered before radiation, they are the most potent of the naturally occurring biological substances. The degree of protection afforded by a radioprotective agent is described by its dose reduction factor (DRF) or dose modifying factor (DMF). Both values are ratios greater than 1 for protection, and represent the radiation exposure in the presence of a radioprotective agent to produce a given biological response divided by the radiation dose required without the agent. Radiosensitizing substances are described by the sensitizer enhancement ratio (SER). It is a positive value representing the dose of radiation required to produce a given biological endpoint without a protective agent divided by the radiation dose in the presence of the radioprotective agent. Many of the chemotherapeutic drugs (e.g., Adriamycin, bischloroethylnitrosourea (BCNU), bleomycin, cisplatin, fludarabine, gemcitabine, hydroxyurea, cyclophosphamide, cytosine arabinoside, doxorubicin, 5-fluorouracil, interferon, taxol, and topotecan) have additive or synergistic responses when given with radiation exposures (112, 267, 269). These may be beneficial for tumor therapy but may also adversely affect normal tissues such as heart (Adraimycin), lung (bleomycin), bone marrow, and mucosal tissue (such as the inner lining of the mouth and rectum). Significant advances in oncology have been made using combined modality therapy (chemotherapy and radiotherapy) to improve local control, disease-free survival, and even overall survival. Some patients with laryngeal, bladder, or rectal cancer are able to receive organ preservation therapies involving chemo- and radiation therapy without compromising survival. Patients with nasopharyngeal carcinomas who are treated by concomitant cisplatin/5-fluorouracil chemotherapy with radiation therapy have a greater overall survival than patients who receive an identical course of radio-therapy alone, 78% versus 47% 3-year overall survival, respectively (9). The chemotherapy is thought to reduce the tumor volume, radiosensitize the tumor, and to provide systemic treatment to prevent distant metastases.

For some drugs, because the mode of injury is similar, there may be a memory response equivalent to, or resembling a fractionated radiation exposure, rather than drug injury at one time and radiation injury at another. Caffeine and the cardiac glycosides, digoxin and ouabain, as well as metoclopramide, an antiemetic agent, are also radiosensitizers. Drugs that produce free-radical damage and/or alkylation of DNA, or produce biological responses similar to ionizing radiation, are described as *radiomimetic* (30, 269). Examples include azaserine, benzene, bleomycin, BCNU, cyclophosphamide, diethylthiocarbamate, furazolidone, hydrogen peroxide, mitomycin C, neocarzinostatin, nitrofurantoin, nitrosoureas, nitrosoguanidine, ozone, superoxide, streptonigrin, sulfur mustard, tetranodecanoylphorbor acetate, thiotepa, and the trichothecene mycotoxin T-2 (30, 269).

Alterations in cell populations influence biochemical responses by several mechanisms. Radiation may alter the population of the cell type responsible for producing a particular product, as in the reduction of estrogen which may occur following ovarian irradiation, or it may alter the population of a controlling or modifying cell. These processes may have dire consequences such as decrease in prostaglandin (PG)

production by the bone marrow stromal support cells responsible for maintaining a microenvironment suitable for hematopoietic stem cell development. Studies of bone marrow populations in culture demonstrate that radiation doses that do not kill the hematopoietic stem cells may still halt stem cell progression by injuring the stromal support cells. When stromal synthesis of prostaglandins was inhibited by ionizing radiation treatment, the hematopoietic stem cell

development stopped (239). It resumed when the marrow stromal cells were treated with a 100-Gy dose killing the stromal cells, but stimulating endogenous prostaglandin release. Repopulation kinetics and cell-cell influences play important roles in some forms of radiation-induced congenital anomalies where loss, reduction, or inhibition of one cell or tissue type influences the development of surrounding tissues and organs.

Hormonal control of homeostasis is affected by ionizing radiation and at the same time may affect biochemical responses and even survival to ionizing radiation. Physiological processes may be influenced by electrolyte imbalances, polydipsia, and polyuria following irradiation (686, 722). As mentioned later in the chapter, levels of the stress hormone adrenocorticotropin hormone (ACTH), glucagon, cortisol, insulin, and growth hormone are altered by ionizing radiation exposure. Radiation acts as a general stressing agent on the body (12, 685, 686). Estrogen and testosterone both induce radioprotection when administered to animals, and radiosensitivity has been shown to vary throughout the estrous cycle (685, 686). Correspondingly, testosterone acts as a growth stimulus for prostate cancer cells. The use of lutenizing hormone-releasing hormone agonists that reduce testosterone levels, in combination with radiation therapy can improve the success of therapy in some stages of prostate cancer (531). Circadian rhythm and season variations also modify responses. The spontaneous induction rate for congenital anomalies and the ability of radiation to induce anomalies are both enhanced during the winter months (685). The sensitivity of the individual to ionizing radiation is modified by the state of health, individual genetic and physiological variations, and the presence of combined injuries. Patients with medical histories of scleroderma and xeroderma pigmentosum have worse skin reactions to ionizing radiation. Although the lethal radiation dose for humans is approximately 4.5 Gy, a 5% lethality is estimated for a 2-Gy dose (722). In large radiation exposures, the presence of combined injuries such as infections or pathophysiological injuries, including burns, wounds, or other trauma, plays an important factor in survival (139, 197). Other factors are more difficult to describe. For example, a study on the ability of 210Po to induce lung cancers in hamsters found a 0% incidence of lung cancers when 40 nCi of 210Po was introduced by intratracheal administration, but a 5% incidence of lung tumors if the same administration was followed by an injection of saline (387).

Radiation induces a block in the G2 phase of the cell cycle and a prolongation of S phase (12, 269, 270, 566, 686). Cells are more sensitive when irradiated during the mitosis and G2 phases of the cell cycle and progressively less sensitive during early and late S phases, where preparation for and synthesis of DNA occurs. The G2 delay is probably related to repair processes. Studies with inhibitors of protein synthesis indicate that radiation inhibits the synthesis of a protein or key proteins necessary to proceed through the cell cycle (686). Decreased synthesis and phosphorylation of histones is observed after irradiation (511). When mitosis resumes, there is usually an increase in the percentage of cells undergoing mitosis compared to the unirradiated population due to a partial synchronization and abortive mitoses. There may also be an overcompensation from stimulation of cells normally in the G0 stage back into active cycling. Increasing the radiation dose proportionally increases the mitotic delay time up to a point at which the possibility of radiation-induced cell death becomes a more likely outcome. This delay in resumption of mitosis has significant consequences for repopulation kinetics and potential lethality. There are four major acute radiation lethality syndromes: instantaneous, hematopoietic, intestinal, and central nervous system death (722). Of these, the hematopoietic and intestinal cell deaths are governed by repopulation of the respective stem cell compartments. Hematopoietic death results in humans from doses between 2 and 7 Gy, with lethality occurring between 2 weeks to 2 months postirradiation. Death is directly attributable to the mitotic inhibition/ death of two stem cell populations, one responsible for hematopoiesis and one for marrow support. The formed components of blood, red and white blood cells, and platelets have finite lifetimes, 4.5 days for platelets to 120 days for red blood cells. They are continually replaced by the bone marrow; but, if marrow production is halted, maturation depletion occurs with hematopoietic progenitor cells in various stages of development. An acute aplastic anemia develops without the continued formation of new progenitors. The duration of mitotic delay is crucial in the recovery process because a point might be reached at which the pathological consequences of the loss of platelets and white blood cells cannot be reversed by the onset of new hematopoiesis in time to avert death. In theory, survival or transplantation of a single hematopoietic stem cell (with proper stromal support) can repopulate the entire hematopoietic system, resulting in survival. Bone marrow transplantation has been used to treat victims of radiation accidents. It is not necessary for the entire marrow to be mitotically inhibited for the acute hematopoietic syndrome to be initiated. Production can be suppressed below a threshold. With a loss of platelets and white blood cells, hemorrhaging and infectious processes lead to death. A similar inhibition

of the intestinal crypts of Lieberkuhn by radiation doses greater than 12 Gy progresses to acute intestinal syndrome death between 3 to 7 days post-irradiation. Intestinal villi cells continue to be sloughed off or die without replacement. This produces a decrease in villi height, with an associated decrease in absorptive

area, followed by eventual denuding of the villi surface, loss of electrolytes, and loss of the protective barrier permitting infection and hemorrhaging. Attempts to transplant intestinal crypt cells have not been successful. A person may survive a hematopoietically lethal dose of radiation only to die instead, and at an earlier period, from the acute intestinal syndrome. Agents that are radioprotective for the hematopoietic system may not necessarily affect the intestinal crypt cells in the same manner.
Radiation is unique among environmental mutagens in that it may affect any of the three stages of tumor development: initiation, promotion, and latency (48, 49, 269, 685, 686). There are several chromosomal breakage syndromes, such as xeroderma pigmentosum and ataxia telangiectasia, that are associated with deficiencies in DNA repair and are, therefore, more susceptible to radiation-induced mutational injury. Cancer induction has been related to the activation of proto-oncogenes to oncogenic status. Oncogenes may influence the development of cancer by direct stimulatory effects through altered or amplified gene products or by suppressive activity. In the latter case, the normal function of the gene acts to suppress cancer, and inactivation or loss leads to expression of transformation of cells and carcinogenesis. The most studied human suppressor oncogene, p53, is associated with the induction of retinoblastoma, colon cancer, and lung cancers in humans. Deletions and point mutations in the p53 gene have been observed in some radiation-induced cancers, including, in one study, 7 of 19 radon-associated lung cancers from uranium miners (666), and 16 of 52 in another study (639). Interestingly, 31% of the miners with lung cancer in the second study had a specific transversion of A<u>G</u>G to A<u>T</u>G (arginine to methionine) at codon 249 (639). This codon was not mutated in miners from the first study.
Radiation-induced genetic injury may arise from chromosomal loss of a gene or an entire chromosome, deletions resulting in codon loss, or frameshifts and point mutations involving base substitutions that produce amino acid substitutions or stop codons. Radiation-induced mutations have been observed in the p53, c-*myc,* c-***abl, β*** 2M, c-*fms,* K-*ras*, and H-*ras* oncogenes. Analyses of radiation induced gene inactivation in vitro suggest that induced mutations are more likely to be expressed through DNA deletions or rearrangements than as point mutations (643). Interestingly, the types of activated K-*ras* point mutations observed in neutron radiation-induced murine thymic lymphomas were lower in yield and differed from the spectrum of *ras* point mutations induced by gamma irradiation (612). They also lacked a characteristic point mutation of adenine for guanine in codon 12 that was present in 87% of the gamma-radiation induced *ras* mutations. Characteristic deletions have been observed for other radiation-induced cancers, such as the deletions on chromosome 2 in low LET radiation-induced murine myeloid leukemias (653).
Radiation exposure may alter both enzyme levels and the availability of substrates and cofactors. For example, the increase of PGs in mouse spleens following exposure to 2 Gy was shown to be related to a decrease in the activity of the enzyme associated with PG degradation, 15-hydroxy-PG dehydrogenase (689). This effect was not observed in irradiated human colon tissues (224). Prostaglandin synthesis has also been studied in the pig skin following X-irradiation (731). There was an increase in PGE2 levels within 12 hours after 10-Gy irradiation followed by decreases 24 to 48 hours postirradiation (731). Interestingly, during this same period PGF2α increased. Both changes were related to an increase in NADPH-dependent PGE2 9-keto reductase, an enzyme that converts PGE2 to PGF2α. In general, PGE2 antagonizes the actions of PGF2α, much like prostacyclin (PGI2) antagonizes the actions of platelet-clotting activity of thromboxane.
Exposure to ionizing radiation results in induction of a number of "immediate early genes" and early response genes (47, 60, 195, 207, 270, 490, 509, 535, 566, 571, 573). There are more than 100 stress-inducible genes, or "immediate early genes" induced by ionizing radiation (535). Several of these genes have been detected within 15 min postirradiation and have been elicited with doses as low as 10–50 cGy of ionizing radiation. Many of these can also be induced by oxidative stress and free-radical production (270, 619), and are associated with transcription (including *c-fos, c-jun,* and NF-*κ*B), translation, cell cycle regulation, carcinogenesis, and immunosuppression (270). Several are proto-oncogenes, including c-*fos,* c-Ha-*ras,* c-*jun* and c-*myc* (270, 535). In addition, radiation exposure produces biological mediators and second messengers, such as the cytokines (α-interferon, interleukin [IL]-1, interleukin-6, tumor necrosis factor [TNF]-α, and transforming growth factor-*β*) (18, 39, 270, 565), which in turn can induce specific gene transduction (270). Some genes are down-regulated producing postirradiation consequences. The decreased production of cyclin B following radiation exposure is an underlying factor in radiation-induced mitotic delay (173, 270, 305). Cyclin A and topoisomerase II-alpha also decrease following radiation exposure (173).
There are a number of important biological mediators that amplify and/or elicit inflammation, fibrosis, or biological end points. Among these mediators are hormones, histamine, serotonin, leukotrienes, PGs,

phospholipids, cyclic nucleotides, and cytokines. Each of these may have unique or cooperative interactions resulting in injury, death, or may even be necessary for recovery processes. Histamines and prostaglandins are mediators of radiation-induced skin erythema (85, 686). The cytokines

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are a diverse class of proteins produced by lymphoid cells and other tissues that mediate cell-cell interactions in a hormonal manner. These proteins include IL-1 and IL-12, TNF, granulocyte/macrophage colony stimulating factor (GM-CSF), stem cell factor (SCF), transforming growth factor-*β*, and the interferons to name a few. These molecules act through specific receptors, and have important physiological and pathological functions/responses. Lung pneumonitis and fibrosis postirradiation are in part related to elevation of transforming growth factor-*β* and to interleukin-1α, both of which are capable of stimulating collagen gene expression and fibrosis. Prolonged plasma elevation of transforming growth factor-*β* in patients receiving radiotherapy has been associated with increased risk of developing pulmonary toxicity (18, 88, 494). Depending on the situation and the cytokine interactions, many of these compounds are able to elicit radioprotective properties or radiosensitization (494). TNF, SCF, and IL-1 and IL-12 are radioprotective in mice when administered individually 18–24 hours prior to irradiation, whereas IL-6 interferons α and *β* and transforming growth factor-*β* act as radiosensitizers (494). The end point of study is important, because administration of IL-12 to mice results in radioprotection of hematopoietic tissue, but gastrointestinal radiosensitization. Other cytokines including granulocyte colony-stimulating factor, keratinocyte growth factor, and transforming growth factor-*β*, are being examined in terms of their wound-healing effects and are undergoing clinical trial tests in patients receiving radio-therapy in order to minimize the injury to normal tissues, while not affecting the tumor tissues (657). Other agents that modify cytokine activity, such as lisofylline reduction of inflammatory cytokines, are also under evaluation to minimize radiation toxicity (657). Granulocyte colony-stimulating factor has been used to increase white blood cell production in patients who have become neutropenic to combinations of ionizing radiotherapy and chemotherapy. Thrombopoietin has been isolated, and is being investigated in clinical trials. It too should have advantages in those patients with radiation-induced bone marrow suppression (but not ablation), but its use will have to await appropriate clinical trials and FDA approval.

Biological processes may also be altered through changes in receptor expression (686). PG, leukotriene, *β*-adrenergic, histamine, serotonin, alpha IIb *β* 3 integrin, and 3H-corticosterone receptors have been studied in irradiated cells or animals. Radiation exposure had no effect on the specific binding of leukotriene C4 at doses up to 20 Gy (688). Adrenergic receptors play important roles in homeostasis by the autonomic nervous system. Radiation induces a decrease in adrenergic receptors in both the rat and rabbit during the first week postirradiation (378, 647). In rats, this is followed by an increase in *β*-adrenergic receptors apparently associated with a late developing radiation-induced congestive heart failure (378). Changes in receptor expression can be related to increased production or to increased use and destruction. An exposure of 2 Gy (a common daily fraction size in clinical radiation oncology) has been shown to increase the mRNA synthesis of the epidermal growth factor receptor in cells irradiated in vitro within the first 24 hours (584). The serotonin3, or 5HT3, receptor is active in radiation-induced emesis, and specific receptor antagonists (ondansetron or granisetron) have been successful in reducing emesis in radiotherapy patients. Administration of 5-HT3 antagonists to ferrets reportedly stopped radiation-induced emesis within 30 s of administration (58). Some radiation-induced receptor changes may have dire consequences because the increased expression of integrin receptors by B16 murine melanoma cells following irradiation is associated with increased lung colony forming ability, or metastasis (510). Expression of the integrin receptor was detected within 15 min postirradiation with downregulation occurring by 4 hours postirradiation.

There are several important signal transduction pathways and cascades that are variously activated by ionizing radiation with important resultant modifications of cellular physiology (329, 341, 583, 619). Among these are the stress-activated protein kinase pathway (SAPK), mitotic-activated protein kinase pathway (MAPK), protein kinase C, modifications of intracellular calcium with corresponding activation of calcium-dependent enzymes, bioactive lipids synthesized or released by phospholipases, and the nuclear transcription factor NF-*κ*B (341, 583). Activation of the MAPK pathway, with corresponding stimulation of mitogenic proliferation is thought to account for the accelerated repopulation of irradiated cells that affects the sensitivity and response of cells (including tumor cells) to chronic low doses as might occur environmentally or accidentally (e.g., Chernobyl), or fractionated doses of ionizing radiation (that are routinely used clinically) (583). The steps involved in the activation of this pathway by ionizing radiation are illustrated in Figure 15.4. This pathway can be activated by binding of the receptors on the membrane cell surface to their ligands (including TNF and other cytokines released or elevated by ionizing radiation) (341), or intracellularly through reactive oxygen intermediates (341, 619), changes in intracellular calcium, and phosphorylated activation of the epidermal growth factor receptor (583). Inhibition of epidermal growth factor receptor phosphorylation in vitro has been shown to block ionizing

radiation-induced cellular proliferation (583). In addition, use of specific inhibitors of MAPK in vitro, resulted in an enhancement of double-stranded DNA breaks and reproductive cell death (95). NF-κB, a nuclear transcription factor, is one of the

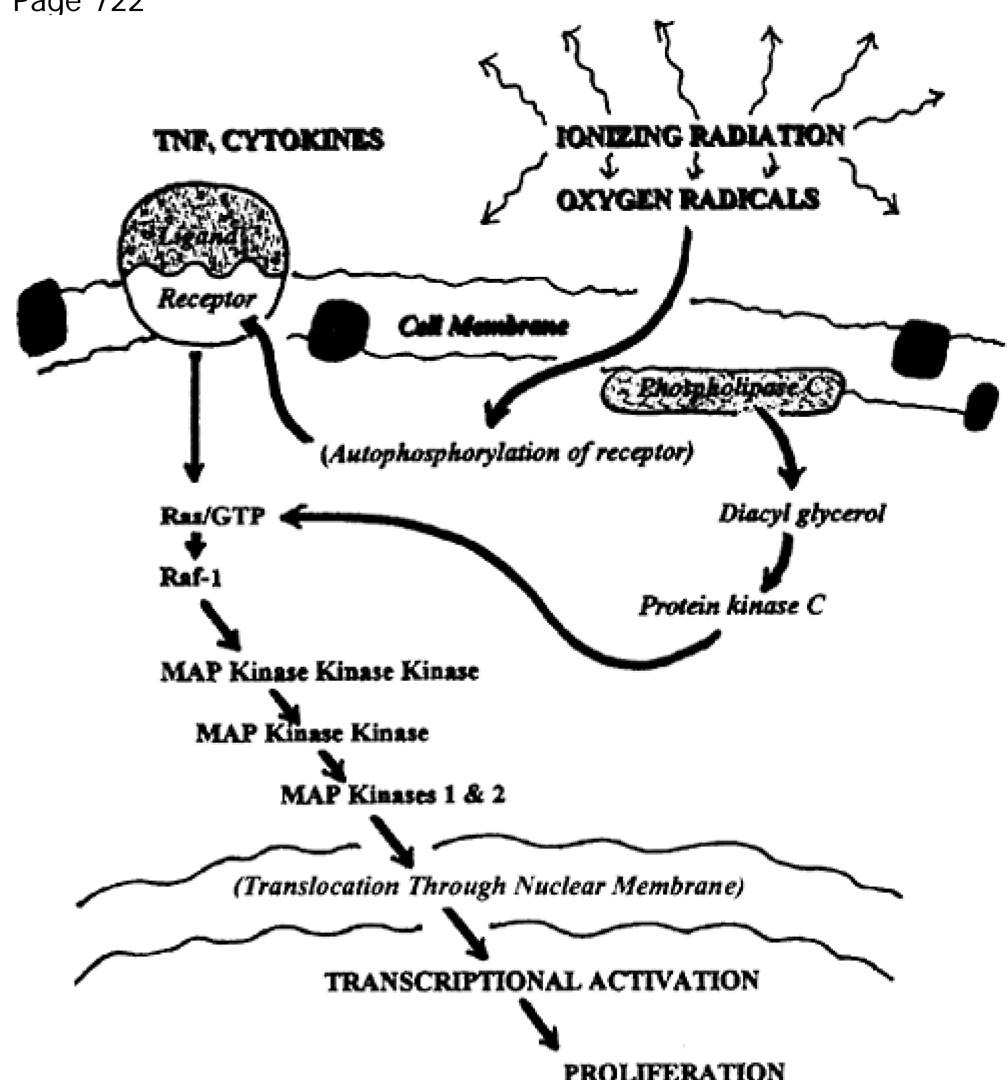

FIG. 15.4. Mitogen-activated protein (MAP) kinase signal transduction pathways can be stimulated in response to ionizing radiation. Radiation exposure results in signal transduction activation of the MAP kinase pathway. The MAP kinase pathway can normally be elicited by cytokines including tumor necrosis factor (TNF). The best characterized receptor-mediated initiation has been the radiation-induced activation through the epidermal growth factor (EGF) receptor by autophosphorylation. The exact mechanism is under investigation, and may involve reactive oxygen intermediates, intracellular calcium mobilization, or indirectly through other biological mediators (diacyl glycerol is released from the phospholipid membrane through the action of phospholipase C). The pathway involves activation of several kinases (phosphorylation activation), and can also be inhibited by phosphatase activities. The resultant pathway action is cell proliferation and differentiation. (Based on References 329, 583, 619.)

early response genes induced by ionizing radiation exposure (270, 341). Its regulation is dysfunctional in people with the genetic disease, ataxia telangiectasia, and is thought to account for their increased sensitivity to ionizing radiation (341). There is also an ataxia telangiectasia-mutated gene (ATM) that participates in DNA repair and recombination, suppression of apoptosis, and induction of p53 (566). The mutated gene is deficient in these functions.

MOLECULAR AND CELLULAR EFFECTS

Effects on Energy Systems

Radiation exposure affects biological energy processes by specific and nonspecific interactions. Nonspecifically, absorptive functions in the stomach and colon may be altered and lead to prolonged gastric emptying times (185), induction of nausea and vomiting, increased stool frequency, loose stools or diarrhea, fatigue, and/or loss of appetite. Radiation affects carbohydrate metabolism by the release of stress-related hormones and by reducing the levels of the thyroid hormones triiodothyronine and thyroxine (686). The reduced thyroid activity is also reflected by an increase in thyroid-stimulating

hormone (TSH). In humans receiving radiotherapy, a single dose of 7.5 Gy results in a 35% incidence of elevated TSH (610). Subclinical hypothyroidism is more common (normal thyroid hormone level, elevated TSH), and like clinical hypothyroidism, responds to thyroid hormone (levothyroxine) supplementation.

Specifically, radiation exposure causes an uncoupling of nuclear and oxidative phosphorylation with corresponding decreases in oxygen-dependent ATP synthesis

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(12, 686). It has been suggested as a mechanism for radiation-induced interphase death (354). This effect has been observed in the rat spleen with doses as low as 1 Gy (668) and as early as 15 min postirradiation (37). It is observed in the spleen, liver, and thymus but is not present in all tissues. The decrease in oxidative phosphorylation is preceded by a decrease in NAD+ levels, and the decreases in ATP are preceded by reductions in AMP and ADP. The losses are attributable to decreased synthesis, use of remaining stores, and increased leakage through the cell membrane. In nuclei, macrophages, and neutrophils, a specific membrane NADPH oxidase consumes molecular oxygen to produce superoxide but depletes NADPH in the process. The depletion of NADPH, coupled with radiation-induced inhibition of glucose-6-phosphate dehydrogenase, results in the uncoupling of oxidative phosphorylation.
NAD concentrations also decrease following irradiation. Decreases are observed within 15 min postirradiation and have been elicited with doses as low as 0.25 Gy, although doses of 9 Gy or higher are usually required to observe changes (686). This decrease is related to the activation of adenosine 5′-diphospho-5-β-D-ribosyl transferase (ADPRT) by double-stranded DNA breaks. ADPRT uses the ADP contained in NAD to form a poly (ADP-ribosyl) chain that attaches to histones and to several DNA-repair enzymes, including DNA ligase II, Ca2+- and Mg2+ -dependent endonucleases, and to protein elongation factor 2. Although its specific function is not known, poly (ADP-ribosyl) affects many biological processes, including DNA repair and cell growth and differentiation. It is also important in some infectious processes because cholera and diphtheria toxins have ADPRT activity. The Poly (ADP-ribosyl) binds to the histones initiating a relaxation of chromatin, facilitating DNA repair. As long as double-stranded DNA damage is present, ADPRT will remain activated and the poly (ADP-ribosyl) chain will remain attached to the histones, preventing condensation of DNA necessary for metaphase. A suicide model of cell death has been proposed in which extensive DNA damage leads to activation of ADPRT, with the subsequent use and depletion of NAD (56). The decrease in NAD for electron transport results in decrease in ATP synthesis, leading to a decrease in other biosynthetic and active transport processes, with a consequential interphase cell death. Most cells can maintain sufficient NAD concentrations to avoid this complication with radiation doses under 20 Gy. Because poly (ADP-ribosyl) participates in both DNA repair, where inhibition would be radiosensitive, and in this cell death model, where inhibition would be protective, the use of ADPRT inhibitors such as caffeine, nicotinamide, and 3-aminobenzamine have produced mixed results (686).
Human immunodeficiency virus (HIV) activation is not a major response to ionizing radiation exposure; however, both ultraviolet (UV) radiation (732) and X-irradiation can stimulate the in vitro replieation of the HIV responsible for the acquired immunodeficiency syndrome (AIDS) in humans (497). A 200% increase in viral replication was observed following a 1.5-Gy X-irradiation dose. The increase was thought to be mediated by a cAMP-dependent process. Much larger doses of either UV- or X-irradiation are required for inactivation (282). The LD37 dose was 4.5 kGy. In another study, transcription of the HIV promoter could be activated in cells in vivo by doses as low as 25 cGy (198). Radiotherapy remains an effective means of treating Kaposi's sarcomas, lymphomas, and other radiosensitive tumors in AIDS patients (140). Radiation can also cause activation of the herpes zoster virus responsible for chicken pox and shingles (zoster) in humans. Reactivation of the virus (shingles) has been reported in 16% (up to 50% in some studies) of patients receiving radiation therapy for Hodgkin's Disease (257). Most cases occurred within 18 months following completion of radiation therapy and was most common in patients receiving chemotherapy and radiotherapy, and in children.
The Target
The "target" for radiation-induced cellular injury depends on the end point examined. The membrane appears to be the target for some of the behavioral alterations following ionizing radiation, while the chromosome material is thought to be the target for cell lethality. This is supported by the results of microsurgical techniques on irradiated amoeba to transfer the nucleus to unirradiated amoeba and to transfer an unirradiated nucleus to the irradiated cytoplasm. Significantly greater lethality followed the irradiated nucleus. The unirradiated amoeba that contained the irradiated nucleus behaved as if the entire nucleus had been irradiated, whereas the amoeba with the irradiated cytoplasm containing the unirradiated nucleus required much higher doses to the cytoplasm for lethality (255).
In a classic set of experiments, Munro (486) irradiated either the cytoplasm or the nucleus of Chinese hamster fibroblast cells using a needle made of 210Po, an alpha emitter. When the needle remained in the cytoplasm, away from the nucleus, the alpha radiation doses of 250 Gy were dissipated in the cytoplasm with no effect. However, when the needle was placed close to the nucleus, cell lethality resulted from much smaller doses. Finally, cell lethality is directly proportional to the number of chromosomal aberrations observed after irradiation. They may be detected by several methods including

phytohemagglutinin (PHG)-stimulated peripheral blood

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lymphocytes, premature chromosomal condensation, micronuclei technique, and FISH (686). The result is influenced by radiation factors, the degree of time following exposure to ionizing radiation, culture time, and age of the individual. The premature chromosomal condensation technique can be assayed in 2 h, whereas the other assays require 48–52 h to allow the stimulated lymphocytes to grow sufficiently for assay. The basic technique permits growth of the blood lymphocytes to permit sufficient numbers of cells to conduct the assay. The complete assay time including culturing the blood lymphocytes takes 3–5 days.

Other experiments that indicate that the DNA is the main target for radiation injury are those conducted with the halogenated pyrimidines, 5-iododeoxyuridine and 5-bromodeoxyuridine (269, 309). These compounds are incorporated into the DNA and increase cellular radio-sensitivity by increasing susceptibility to free-radical attack (including the hydrated electron) and damage and by modification of DNA repair (269, 309, 325). Cells in exponential growth are more sensitive than cells in plateau growth containing equivalent amounts of base substitution. Experiments incorporating 125I-iododeoxyuridine into cellular DNA, or iodinated proteins into cell membranes, further demonstrated that the nucleus, rather than the plasma membrane, is the target for radiation-induced chromosomal instability that occurs after several cell divisions as well as later after radiation exposure (333).

Cellular Responses

There are three basic categories of cellular damage and eight basic responses of cells to ionizing radiation. The basic categories of damage are sublethal damage, potentially lethal damage, and lethal damage. Potentially lethal damage is radiation-induced damage that would result in cell death if not repaired (63), and is affected by postradiation therapy or environment (269). The basic responses of cells to ionizing radiation include no visible response, mitotic delay, transformation, hyperplasia without accompanying cell division leading to giant cells, instant cell death, interphase cell death, reproductive cell death, and apoptosis.

The cell cycle describes the process of cells preparing for and undergoing cell division. Some cells are in a resting phase, termed G0, and are not actively dividing or preparing for division. Cells go through a G1 (Gap 1) phase incurring growth prior to initiating the S phase (synthesis), during which the chromosomes are replicated. This is followed by a second gap (G2), in between DNA synthesis and cell division (mitosis). Classical radiobiology studies of synchronized cells in culture have shown that cells tend to be most sensitive to ionizing radiation when irradiated in G2 and mitosis, and to be less sensitive when irradiated in S phase. Because cells in mitosis and G2 are more sensitive than the other stages of the cell cycle, cells that are rapidly cycling have more likelihood to be in a radiosensitive phase of the cell cycle than cells that are slowly dividing or cells that are removed from the cell cycle (G0, or resting phase). This can be particularly important in radiation therapy where differences in radiosensitivity are exploited to maximize tumor kill and patient cure. In fact, agents that affect cell cycle progression, or synchronize cells (for example hydroxyurea), can be used for radiosensitization. Molecular and cellular biology studies are providing insight both on cell cycle regulation and how radiation is able to affect the cell cycle (270, 487, 566). There are two key check points in the cell cycle which can be inhibited by ionizing radiation, the first is at G1/S, and the second is at G2/M. Several sets of key proteins are involved. The first set of proteins, called cyclins, are specific for particular phases of the cell cycle. They bind to and activate phosphorylating enzymes called cyclin-dependent kinases (566). There may be several cyclins within a particular class, and there are specific inhibitors for the activated cyclin/cyclin-dependent kinase complexes. Seven different cyclins, A-E, G, and H, have been identified (Table 15.7).

Radiation results in stimulation/accumulation of p53 (Figure 15.5), a tumor suppressor protein with DNA transcriptional and other regulatory roles, which in turn induces transcription of the protein, p21 (Cip1/waf1). Increased levels of p21 inhibits both cyclin E cyclin-dependent kinase 2 (cdk2) and cyclin A-cdk2 kinase with a resultant arrest in G1 (189, 270, 566). The cdk4/cyclin D kinase activity is also inhibited. In the presence of p21, the cyclin E-cdk2 complex remains in the unphosphorylated and inactivated form. Cells with normal expression of the p53 protein are called "wild type" (normal). Cells with p53 deficiencies or mutations are unable to elicit the inhibition of cyclin E-cdk2 activation, or cell cycle inhibition at G1 (269, 270, 566). Irradiated mice with deficiencies in p21 (p21 −/−) are still able to elicit G1 cell cycle inhibition, but to a lesser degree (169). This indicates that dual pathways can result

Table 15.7 Role of cyclins in radiation-induced cell cycle response

Cyclin	*Dependent kinase*	*Cell cycle phase*	*Radiation Effect*
Cyclin A	cdk2	S	G1/S Block
Cyclin B	cdk1	G2/M	*G2/M Block*

Cyclin C	cdk, cdk9	G1/2, G2/M	?
Cyclin D	cdk4, cdk6	G0, G1	G1 Block
Cyclin E	cdk2	G1/S	*G1 Block*
Cyclin G	cdk5	G1/S, G2/M	G2/M Block
Cyclin H	cdk7	G2/M	?

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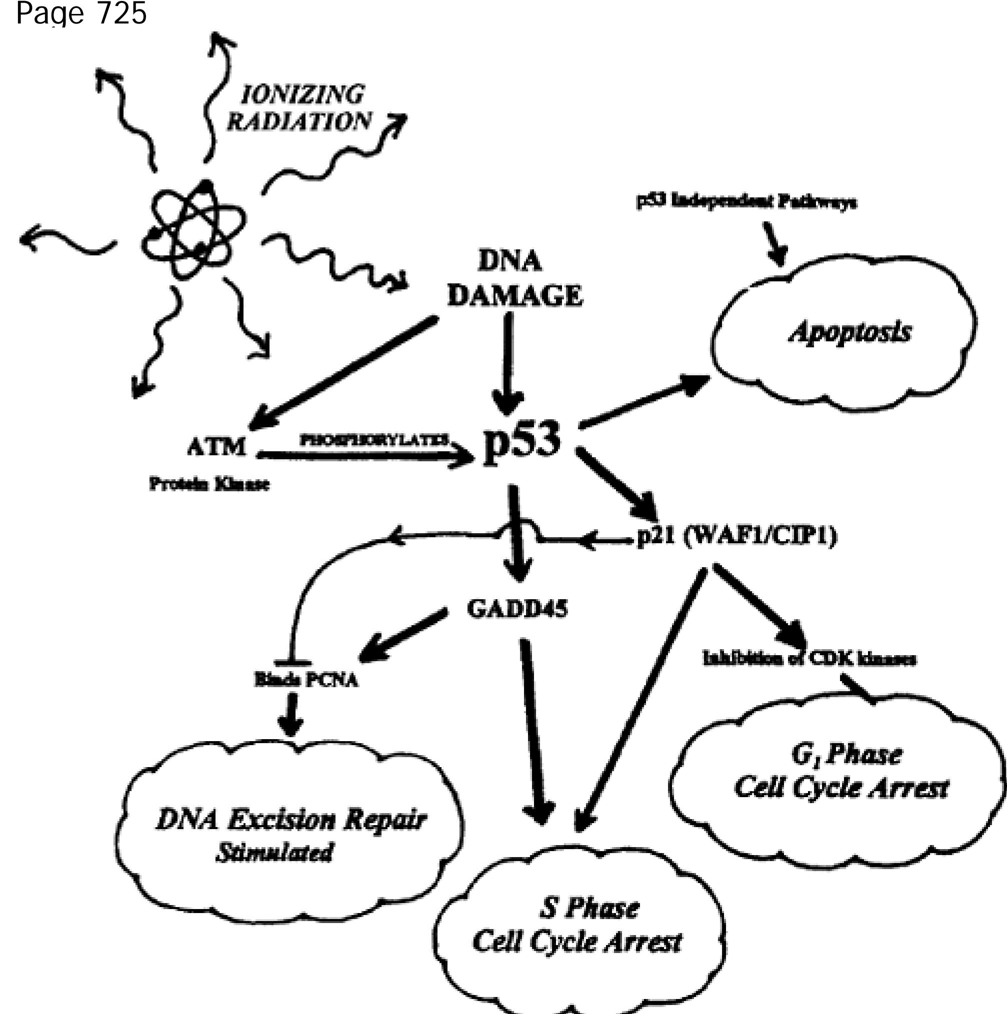

FIG. 15.5. Roles of p53 in response to ionizing radiation. Radiation or DNA damage results in activation of the ataxia telangectasia gene (ATM), a protein kinase that phosphorylates and activates the tumor suppressor/transcriptional activator protein, p53. The actions controlled by p53 are shown. p53 induces transcription of p21, a protein that subsequently inhibits kinases, regulating cell cycle control. p53 increases GADD45 protein levels, which can interact with PCNA (proliferating cell nuclear antigen) to stimulate DNA repair (as shown, p21 can inhibit PCNA binding and activity). p53, in turn, is important in mediating apoptosis, but there are also p53-independent pathways (ceramide signalling) that result in apoptosis.

in G1 inhibition, but that the major pathway is through p53 induction of p21. In general, cells with deficient p53 genes do not get blocked at the G1 check point after exposure to ionizing radiation. Radiation also produces a delay in the cell cycle by blocking the G2 check point. Combination of the cyclin B with cdk1 forms a phosphorylated complex called the *mitotic proliferating factor*. Ionizing radiation results in a decrease in the cdc25 protein that normally dephosphorylates the threonine 14 and tyrosine 15 position of the mitotic proliferating factor, resulting in its activation, and allowing the cell to proceed through mitosis (270, 305, 566). The radiation-induced decrease in cdc25 results in an unactivated/phosphorylated mitotic proliferating factor and, in turn, inhibition at the G2 check point. Reduction in cyclin B transcription, decreased stability of the cyclin B mRNA, and increases in elongation factor 1-delta following irradiation have also been implicated in cell cycle blockage at G2 (270, 305, 487, 566). This a simplified description of a more complicated interaction involving over- and under-expression of cyclin-dependent kinase inhibitors, upstream regulators, and other integrated pathways. Interestingly, overexpression of cyclins have been identified in several cancers (566).

If the reproductive potential of a cell has been eliminated but other functional components of a cell remain intact, the cell may continue to grow without dividing, forming a giant cell. In interphase, death occurs after an individual/organism receives a dose of radiation with so much resulting damage that the cell dies before it can undergo mitosis and potentially gets “stuck” in mitosis. The lymphocyte is considered a very radiosensitive cell in humans where doses of 0.05 Gy have been shown to kill

lymphocytes (722). Reproductive death refers to the cellular processes by which a cell dies by inhibition in metaphase within several cell divisions after radiation exposure. Apoptosis, characterized by nuclear pyknosis, cytoplasmic condensation, and cellular phagocytosis with appearance of apoptotic bodies,

requires the synthesis of specific proteins and has been observed in rat thymocytes following exposure to ionizing radiation (715).

Following radiation exposure, some cells die by apoptotic death, through activation of a genetically preprogrammed cascade. The apoptotic cascade is not specific to ionizing radiation, but has been reported in cells exposed to other cellular stresses, including oxidative stress, hyperthermia, viruses, microbiological toxins, cytotoxic chemotherapeutic agents, hormone deprivation, steroid therapy, cytokine-stimulated stress, antibody/immune-related stress, photodynamic therapy, and UV light (65, 264, 342, 489). It has been estimated that apoptotic death may be a factor in up to 25% of cells dying from radiation (174, 264), although radiation-induced apoptosis is not found in some cell types (264). Apoptosis functions normally in embryos to reduce cell numbers, and also functions in the adult organism. It differs from necrosis in the nuclear pyknosis, with organized degradation of the DNA into nucleosomal units, and condensation of the cytoplasm with packaging of apoptotic products into membrane bound apoptotic bodies (65, 340). This organized process can involve isolated cells, without inflammation (65).

There are at least two mechanistic routes by which ionizing radiation initiates apoptosis. The first is by initiation of DNA damage (65, 264, 342) and the second is mediated through the cell membrane action of sphingomyelinase, releasing the second messenger ceramide (118, 264). The pathway mediated through DNA damage requires nonmutated/functional (wild type) expression of the p53 protein, which controls production of bax, an apoptotic inducing protein. Cells that have a mutated p53 protein (as many cancer cells have) do not undergo apoptotic death in response to radiation-induced DNA damage and p53 activation, but may still be induced to undergo apoptosis through the membrane-induced (ceramide) pathway (118, 264). Bcl-2 and bcl-x1 are suppressor proteins of apoptosis that are thought to bind to each other and to bax (65, 264), forming either homodimers or heterodimers. The key events in apoptosis are speculated to occur either from homodimerization of bax, and/or from decreased availability of the bcl-2 and bcl-xL suppressors (65). Membrane receptor mediated events can inactivate bad, another apoptotic promoter that would otherwise bind to and inactivate bcl-xL. Bcl-2 and bcl-xL prevent the translocation of cytochrome c from the mitochondria (209). In the cytoplasm, cytochrome c acts as an apoptotic protease-activating factor, leading to the activation of cysteinyl-containing aspartate-specific proteases (caspaces, there are at least nine types) that mediate the apoptotic cascade, including a caspace activated DNase (209). Activation of caspaces 3, 8, and 9 are considered important in the apoptotic cascade, and further differences in activation pathways can be delineated based on whether the initiating processes are caspace 9 dependent, caspace 3 dependent, or caspaces 3 and 9 independent (caspace 8 dependent) (265). Among the ligands that can bind to receptors and activate the apoptosis pathway are the *fas* ligand and TNF. The membrane-mediated pathway for radiation-induced apoptosis is inhibited by protein kinase C activation (264). There is interest in tumor therapy to enhance the radiosensitivity of tumor cells through stimulation of apoptosis, either by restoring a normal (wild type) p53 protein, or by inhibiting protein kinase C.

The sensitivity of cells to ionizing radiation is influenced by several factors, but the general principles describing cellular radiosensitivity were described for cancer cells in 1906 (57), and are referred to as the Law of Bergonie and Triblondeau. The radiosensitivity of cells and tissues is related to the rate of cell division and the reproductive potential. Cells that are more rapidly dividing and have the potential for a number of successive generations will be more radiosensitive. There are some exceptions to these principles, such as the terminally differentiated lymphocytes, but it may be used to explain why rapidly dividing cells such as hematopoietic stem cells and intestinal crypt cells are more sensitive than nondividing tissues such as heart, skeletal muscle, and nerve cells. The law is usually presented as an explanation for the radiosensitivity of cancer tissues that are rapidly dividing and have unstable chromosome compositions. There may not be sufficient time to repair the chromosomal damage before the next cell division. The expression of injury in some biological systems may require long periods simply because the rate of cell division in a particular tissue is very slow. Even if radiation exposure produced so great a damage as to modify or prevent cell division, it would take years for the cell to reach the point where the division would occur and injury is expressed. The cell cycle time of some ocular cells is 3 years, explaining in part the long latency period for expression of radiation-induced cataract formation (48).

Prokaryotes and insects also represent contradictions to the Law of Bergonie and Triblondeau. Mammals are much more sensitive, with the LD50s for pigs, mice, and men being 2.5 Gy, 6–8 Gy and 4.5 Gy, respectively (23). The LD50 for *Escherichia coli* is between 20–50 Gy, compared to 100–1000 Gy for insects and 1000 Gy for amoeba (23). Plant sensitivities range from 4–400 Gy, with 75–90 Gy being

lethal for the common chrysanthemum (36). These differences are explained in part by application of the target theory, in that cells with large chromosome numbers and large nuclear to cytoplasmic ratios tend to be more radiosensitive in part because they have larger critical target sizes (36). The chrysanthemum has a polyploidy of 22. Some plant species such as pine

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Table 15.8 Radiation dose ranges and associated pathophysiological events*a*

Dose range (cGy)	Pathophysiological events		
	Prodromal effects	Manifest-illness effects	Survival
75–150	Mild	Slight decrease in blood cell count	Virtually certain
150–300	Mild to moderate	Beginning symptoms of bone marrow damage	Probable (>90%)
300–530	Moderate	Moderate to severe bone marrow damage	Possible bottom third of range: LD5/60 Middle third: LD10/60 Top third: LD50/60
530–830	Severe	Severe bone marrow damage	Death within $3\frac{1}{2}$–6 weeks Bottom half: LD90/60 Top half: LD99/60
830–1100	Severe	Bone marrow pancytopenia and moderate intestinal damage	Death within 2–3 weeks
1100–1500	Severe	Combined gastrointestinal and bone marrow damage; hypotension	Death within 1–$2\frac{1}{2}$ weeks
1500–3000	Severe	Severe gastrointestinal damage with upper half of range: early transient incapacitation (ETI); gastrointestinal death	Death within 5–12 days
3000–4500		Gastrointestinal and cardiovascular damage	Death within 2–5 days

a From Reference 42.

trees have radiosensitivities similar to humans, making them ideal biological dosimeters surrounding nuclear facilities (255).

One last interesting aspect of radiobiology is that radiation exposure does not always result in harm. Hormesis describes the beneficial responses to biologically harmful agents such as poison and even radiation, Radiation has been used to improve the growth of seeds, separate from production of genetic mutations. It has even been reported to extend the life span of female mice, although it did so by sterilization, which eliminated the repeated stress of pregnancy.

SOMATIC EFFECTS OF RADIATION

In higher animals there is no simple, direct relationship between nuclear chromosome volume and sensitivity to ionizing radiation. Rather, the effects of irradiation on specific organ systems are more critical (504).

Acute Radiation Syndrome

There have been less than 25 documented fatalities worldwide, between 1946 and 1985, that may be attributed to radiation accidents (437). Although exposure of the whole body to lethal amounts of ionizing radiation is very rare, any discussion of the biological effects of ionizing radiation would be incomplete without mentioning acute radiation sickness and the acute radiation syndrome (ARS).

Acute radiation sickness is manifest in characteristic clinical sequelae known as the ARS, a combination of syndromes determined primarily by the total radiation dose received, the rate the radiation is delivered, and how the radiation is distributed in the body (722). Signs and symptoms of the ARS result from injury to bone marrow, gastrointestinal system, cardiovascular system, central nervous system, gonads, and skin. The variation in radiation sensitivity of these tissues causes the signs and symptoms of the ARS to occur in three successive phases: an initial prodromal phase, a later latent period, and the manifest illness phase. The length of each phase may vary directly with the radiation dose and the time between each phase may vary indirectly with the dose, so at an extremely high dose of radiation, the phases will blend, with the latent period disappearing completely (Table 15.8).

The prodromal phase may begin about 2–4 h after doses of 3–5 Gy or within minutes after exposure to

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45 Gy or higher. The initial prodromal phase is characterized by a combination of gastrointestinal and neuromuscular symptoms such as anorexia, nausea, vomiting, diarrhea, apathy, tachycardia, fever, headaches, insomnia, dizziness, and vertigo. The pathogenesis of the prodromal phase is not known, but several causal factors have been suggested, including direct radiation effects on the central and autonomic nervous systems, disturbance of the endocrine balance, and the production and release of various chemical mediators (176, 687). The latent period, which follows the prodromal phase, is relatively asymptomatic and is believed to be the time between initial cell damage and the interference of radiation with cell renewal in the affected organs (722).
The manifest illness phase of the ARS is classically divided into three major syndromes, traditionally known as the hemopoietic, gastrointestinal, and central nervous system (CNS) syndromes (410). However, the current view replaces the CNS syndrome with the neurovascular syndrome (722).
The hemopoietic syndrome may be encountered after exposure to 2–7 Gy, and 1 Gy or more can significantly damage the blood-forming capability of the body. Radiation kills the mitotically active hemopoietic precursors of red cells, white cells, and platelets. Pathophysiological consequences include increased susceptibility to infection, bleeding, anemia, and lowered immunity. Death usually results from hemorrhage and infection (410, 437, 722).
Radiation exposure above 7 Gy contributes to the gastrointestinal syndrome by inhibiting the renewal of the cells lining the digestive tract. At doses of 3–8 Gy, tight junctions between the epithelial cells are disrupted, allowing increased fluid and electrolyte loss and permitting the movement of bacterial endotoxins into the blood. At higher doses (10–15 Gy), denudation of the mucosa occurs and death results from dehydration, electrolyte imbalance, and septicemia (437, 722).
The neurovascular syndrome is the least understood of the radiation-induced deaths. The syndrome is unique in that death occurs very quickly before damage to the gastrointestinal and hemopoietic systems becomes apparent. Readily obvious CNS signs and symptoms include disorientation, loss of muscular coordination, respiratory distress, apathy, prostration, convulsive seizures, and coma associated with death. Some researchers believe that 50 Gy is necessary for the neurovascular syndrome and doses above 100 Gy are required for direct damage of the nervous system. However, ionizing radiation exposure modified electroencephalographic activity (649) and in vivo exposure decreased hippocampal synaptic transmission and spike generation (297) at lower doses than anticipated. Details of nervous system effects are given later.
Radiation-Induced Developmental Effects
Preimplantation in utero is never succeeded directly by postimplantation, the two being separated by the time it takes the placenta to develop into a functioning organ (472). This interval of development takes 1–2 days in rodents and 10–14 days in humans. Therefore, the traditional categories of prenatal development known as preimplantation, postimplantation, and fetal are replaced by conceptus, embryo, and fetus. The term conceptus is used for the stage of development lasting from ovulation until the placenta becomes a functioning organ. Even though there is some controversy about the maturation of an embryo to a fetus, most classifications of human developmental stages agree that this metamorphosis occurs in the last portion of the first trimester of gestation (289).
The developing conceptus and early embryo have a variety of rapidly dividing progenitor cells. These proliferating cells are much more sensitive to irradiation than differentiated, nondividing cells, making an organism more radiosensitive during its early stages of development than at any other stage of its life. The conceptus exhibits the lowest LD50 of any stage of development and is easily killed by doses of ionizing radiation that would cause abnormalities at later stages of development (77, 555). Rather than a malformed organism developing to term, it dies in the conceptus stage due to irradiation-induced chromosomal damage (77). Thus, it is believed by some that the highest risk of irradiation during this stage is the death of the developing organism rather than teratogenesis (472).
In some animals, exposure to ionizing radiation in utero can result in anomalies in every organ system and the concept has been formulated that irradiation, however small, can inflict damage to the embryo or fetus (555, 558). Irradiation-induced anomalies occurring during the middle stages of development may result in death of the organism or abnormal development of one or more organ systems. However, instead of lethality, morphological abnormalities are associated with irradiation during this time. Exposure during this period may result in gross malformations, growth retardation at term or as an adult, and structural pathology (437, 726). In the human, most major organogenesis occurs during the first trimester of pregnancy with embryonic death and congenital abnormalities resulting from irradiation exposure during this period (555).
During late organogenesis and in the perinatal period, just before and just after birth, radiation damage

tends to be functional rather than structural. Perinatal irradiation with x-rays and gamma rays (140–180 cGy) induces changes in tissue enzyme activity (14), hormone production (190, 311), and hemopoiesis (247, 471). The major effects of perinatal in utero exposure in humans

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is seen in the developing CNS with neurological damage and behavioral changes not always obvious in histological examination (77, 133, 458, 470, 499, 555).
Susceptibility to radiation carcinogenesis is relatively high during prenatal development (440, 556, 684). During the last four decades, a major concern has been the risk of childhood leukemia and other neoplasms following irradiation in utero (50, 55, 77, 247, 437, 527, 685). Studies of the effects of ionizing radiation on the fetus are extremely important because there seems to be no biological reason to expect the fetus to be resistant (160). The embryo may be 50 times more vulnerable than the adult to irradiation-induced leukemia (77) and the risk of a child dying of cancer before the 10th birthday may be increased 40–60% by in utero irradiation (476).
The developing conceptus, embryo, and fetus show high susceptibility to ionizing radiation and the extent of injuries depends on the stage of development as well as the dose of radiation. Developmental anomalies are induced with doses much lower than previously used to demonstrate anomalies in adults (317, 440, 458, 471).
The risk of leukemia in children with Down syndrome (trisomy 21) is estimated to be about 20 times higher than in the normal population and there has been some evidence that preconceptional ionizing radiation is linked to the nondisjunction of chromosome 21 (638). Clusters of cases of Down syndrome have been observed in Germany following the passage of the radioactive cloud from Chernobyl and experimental results have demonstrated that ionizing radiation may induce nondisjunction in oogenesis and spermatogenesis (674).
Even though there is a long latency period of radiation-induced cancer, a dramatic increase of up to 100-fold in the number of childhood thyroid cancers have been observed in the heavy radiation contaminated areas of Belarus and Ukraine and the Bryansk regions of Russia following the Chernobyl accident (28, 569). A strong relationship is indicated between the thyroid cancer and radiation from the Chernobyl cloud.
The in utero developing nervous system is particularly vulnerable to ionizing radiation with defects of the eye and of spinal development among some of the more common malformations encountered following early gestational exposure (83, 84, 243). Later prenatal radiation exposure may result in dose related abnormalities of the hippocampus with disorganized and loosely scattered neurons in the CA-1 and CA-3 regions and agenesis of the corpus callosum (455). Perinatal irradiation, during neuronal migration and differentiation, resulted in delay of migration and severe reduction of neuronal tissue in the cerebral and cerebellar cortex and in the hippocampus (220, 221, 321). Postnatal cephalic irradiation of newborn rat pups produced an increase (122%) in noradrenaline activity and a marked decrease (38%) in monoamine oxidase activity in the cer ebellum (177). The irradiation-induced 60% reduction in cerebellar weight may account for some of the marked increase (223%) in noradrenaline concentration and increase (206%) in tyrosine hydroxylase activity.

Direct Effects of Radiation on Reproductive Organs

During a period of approximately 20 years in the early part of this century, radiation was used in an attempt to increase fertility. Exposure normally was to 1.5–2.5 Gy over a period of 3 weeks. These levels apparently had little effect on fertility or on any later conceived children (437). However, neonatal irradiation of rats (211, 212) and hamsters (637) demonstrated impaired fertility in mature male and female animals. In utero irradiation of male rat pups resulted in atrophy of the testes, ventral prostates, and seminal vesicles with a complete disappearance of germinal cells from the testes (629). Irradiation treatment of children 15 years of age and younger with Hodgkin's disease resulted in azoospermia in many of the mature males and ovarian injury in some of the mature females (512).
The safety of radiation levels is often questioned because the threshold for the effects of ionizing radiation on male reproduction is difficult to predict (588). Although fully developed sperm cells and primary spermatocytes are relatively radioresistant, the quiescent and proliferating spermatogonial cells of the testis are highly sensitive to ionizing radiation (437, 555, 670, 671). Germ cell dysfunction is common following testicular irradiation and a dose-dependent impairment of spermatogenesis with gradual recovery may be seen following doses of up to 6 Gy (274, 351, 611). The effects of ionizing radiation on spermatogenesis are normally reversible with the recovery of fertility predictable. Although an acute irradiation dose of 6 Gy to the testis is likely to produce permanent sterility, conception has occurred for males after years of either aspermic or hypospermic conditions following absorbed doses between 2.3 and 3.7 Gy (437, 555). However, following the Chernobyl nuclear reactor disaster there has been a widespread fear of damage to the reproductive system, with implications for fertility problems and adverse effects on offspring. A pilot study of 18 salvage workers (liquidators) revealed incomplete genesis of sperm characterized by certain ultramorphological parameters of the sperm head (205). The

frequency of amorphous sperm head shape in the study group was significantly higher than in the local Ukrainian control group of 18 men.

Although the human testis is considered relatively resistant to the carcinogenic effects of radiation (50), occupational radiation exposure of the testis produces

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significant changes in serum gonadotropins and semen parameters. Testicular irradiation may be associated with elevated plasma levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), reduced levels of androgen-binding protein (ABP) and testosterone, and reduced prostate and seminal vesicle weights (246, 330, 351, 432, 529, 686). The decreased ABP levels and increased FSH levels are associated with Sertoli cell dysfunction, although the FSH increase may be a secondary result of germ cell depletion rather than a direct effect of irradiation. The changes in LH and testosterone levels and the decrease in prostate and seminal vesicle weights are indicative of Leydig cell function impairment.

As a direct effect of ionizing radiation, the human prostate epithelial cells may also display malignant transformation after multiple exposure (366). After a cumulative x-ray dose of 30 Gy, tumors characterized as poorly differentiated adenocarcinomas developed from prostate epithelial cells. This report may provide the first evidence of malignant transformation of human prostate epithelial cells resulting from direct exposure to ionizing radiation.

Although there are no proliferating stem cells in the mature female reproductive system, the oocytes are in follicles in various stages of development. Animal experiments indicate that radiosensitivity of the ova depends on the maturity of the follicle (555). Irradiation depletion of the radiosensitive mature and intermediate follicles will result in periods of temporary sterility followed by fertility due to maturation of surviving immature follicles. Sensitivity varies among species and temporary sterility can be produced in humans with doses as low as 1.5 Gy (686). Cases have been reported of irradiated women receiving as much as 6.4 Gy becoming pregnant as long as 2 years later and delivering normal children (437). However, the estimated dose required to produce permanent sterility in the female ranges between 6.25 and 30 Gy, depending on age of the subject (555), sensitivity increasing with the approach of menopause (686).

Ovarian failure is associated with whole body irradiation (149), abdominal irradiation (690, 691), or radiation therapy for cervical carcinoma (425). Pubertal failure or premature menopause was common in females irradiated in childhood (690, 691). Premenopausal women receiving radiation therapy may produce sign and symptoms associated with menopause, such as amenorrhea, dyspareunia, hot flashes, irritability, and loss of libido (199).

Breast cancer, the most frequent spontaneous malignancy diagnosed in women in the western world is increasing in incidence (568). Exposure of the breast to ionizing radiation is now known to increase the risk of breast cancer, especially for younger women (420, 421). The risk of developing breast cancer is very high in women exposed to ionizing radiation before or during puberty, when the differentiation of terminal mammary end buds and alveolar structures is occurring (605). In fact, the carcinogenic effects of ionizing radiation on the vestigial male breast may be quite similar to that seen in the prepubertal female breast (644). The male breast displays an increase in risk for breast cancer with three or more radiographic examinations.

The Lymphohematopoietic System and Immune Competency

One of the systems most sensitive to irradiation is the hematopoietic system. Because of the rapidly proliferating hematopoietic elements of the bone marrow, the hemopoietic syndrome may be encountered following irradiation of 100 cGy or more (475, 555). Approximately 50% of individuals exposed to 300 cGy (LD50/60) will die within 2 months. The signs and symptoms result from radiation damage to the bone marrow, lymphatic organs, and immune system. The hematopoietic syndrome is characterized by a depression in the peripheral blood levels of mature erythrocytes, granulocytes, lymphocytes, monocytes, and platelets. Except for lymphocytes, the mature blood cells are relatively radioresistant and function normally in the peripheral blood after irradiation levels that will produce bone marrow damage.

Mature cells in peripheral blood have limited life spans and the replacement of their functional cell types is dependent on the proliferation of the hematopoietic elements of the bone marrow. One type of stem cell, a pluripotent stem cell (PPSC), has the dual capability of self-perpetuation and differentiation, and can meet the demands of the lymphohematopoietic and reticuloendothelial systems.

The progeny of the PPSC have specific functions in the body. Granulocytes are involved in activities against invasive bacteria and are related to the nonspecific immune response, whereas the cell-mediated and humoral responses of the lymphocytes are related to the specific immune response. The monocyte migrates into specific tissues and differentiates into a macrophage of the reticuloendothelial system. The platelet is a critical element of hemostasis and thrombocytopenia may result in hemorrhage and purpura. Thus, radiation damage to the PPSC may seriously compromise all of these systems, resulting in hemorrhage, infection, and death.

Depression of the mature cells in circulating blood is dose dependent (437, 475). At radiation doses near the LD50 changes in the small lymphocytes can be seen in 1 h and lymphocytes may totally disappear from peripheral blood in 2–3 days (245, 409, 418, 437, 555). Although lymphocyte depletion may be measured in hours,

granulocytes and platelets are depleted over days, and depletion of erythrocytes may be measured in weeks.

The small lymphocytes in lymphoid tissue are some of the most radiosensitive cells in the body and the small lymphocyte is the earliest to decrease in the peripheral blood following irradiation of man or animals. The lymphocyte not only shows the most rapid reduction in number, but it is the slowest to return to normal (418). The reduction in lymphocytes leads to impaired specific immune responses and immunosuppression. Regeneration of both the T- and B-lymphocyte levels depends on the lymphoid stem cells, which in turn depend on the PPSC of the bone marrow. Radiation-induced alterations in the nuclear material of lymphocytes might also have a significant impact on the function of this cell type. Chromosomal aberrations have been found in individuals exposed to radiation from both the Chernobyl accident-related cleanup activities (618) and to the subsequent fallout afterward (582).

Radiation depresses nonspecific immune responses by reducing the levels of circulating monocytes and granulocytes (475). Granulocytes serve as the first line of bactericidal defense at wounds whereas the macrophage, a progeny of the monocyte, can phagocytize and catabolize foreign substances such as microorganisms and toxins. The macrophage is also involved in the humoral specific immune response by processing foreign substances and presenting them as antigens for recognition. Radiation-induced depression of both specific and nonspecific immune responses through the reduction of monocytes, granulocytes, and lymphocytes is potentially life-threatening because of the enhanced susceptibility to opportunistic infections (197, 521).

Ionizing radiation induces functional and quantitative abnormalities in the lymphoid cells of both man and experimental animals (6, 252). Although ionizing radiation was found to deplete both T and B murine cells in equal proportions in the spleen (252), studies of late effects of atomic-bomb radiation on the immune system showed alteration of the balance and interaction between T and B cells, with a decrease in the T-cell population and an increase in the B-cell population in the periphery (6). Autoimmune deviations, both humoral and cellular, were observed in residents of areas contaminated from the Chernobyl accident and subjects participating in the clean-up of the accident (27, 159, 473, 607). Structural and functional changes seen in the kidney, thyroid, and crystalline lens of the eye well may be associated with significant changes in the humoral immunity systems related to ionizing radiation exposure.

Irradiation usually does not depress the platelet (thrombocyte) count significantly in a healthy individual (437, 475). The platelet's life span is 9–12 days and depression in circulating levels reach an initial nadir 10 days after irradiation. The individual will face problems similar to those of a patient with aplastic anemia: thrombocytopenia, capillary fragility, abnormal bleeding, and purpura. When the platelets reach a critical level, hemorrhage is likely to occur and may result in death of the individual. Therefore, platelet transfusion becomes critical for the irradiated individual.

Because of the relative radioresistance and long life span of erythrocytes (120 days), circulating blood levels fall slowly without complicating hemorrhage or infection (475). In the presence of thrombocytopenia and hemorrhage, transfusions may be required. Erythrocyte recovery after irradiation normally follows granulocyte and platelet recovery.

Radiation at the LD50/30 dose level (350–400 cGy in humans) may kill more than 99% of the critical cells in the hematopoietic tissue (437, 475). At this dose level cytological restoration of the bone marrow begins about the 25th day postirradiation in man. The PPSC will self-replicate and differentiate to produce hematopoietic progenitor cells. The risk of death from hematopoietic radiation injury depends on the regeneration rate of bone marrow stem cells (306), and the level of medical treatment provided (593). With bone marrow transplantation and heroic supportive therapy, survival is possible following whole-body irradiation as high as 1200 cGy (105, 293, 437). Although bone marrow transplantation certainly may be indicated, other clinical support regimens will stimulate hematopoietic regeneration, accelerate recovery, increase the LD50/30, and enhance survival (405, 406, 518–520, 592).

The most common neoplastic disorder of the hematopoietic system are the leukemias and several different types of leukemia have been observed in a variety of experimental animals following exposure to ionizing radiation (550, 713). Leukemias account for about 32% of all cancer diagnosed in children, with about 85% of leukemias classified as acute (728). The acute leukemias in children may be related to parental occupational exposures to a number of carcinogenic substances or with prenatal and postnatal exposures to ionizing radiation.

Clinical evidence leads to the concept that ionizing radiation can cause leukemia by inducing DNA damage. Two hematopoietic cell lines were subjected to gamma irradiation to investigate the susceptibility of human cells to irradiation at the genetic recombination stage of leukemogenesis (168).

The irradiation induced the formation of fusion genes characteristic of leukemia in both cell lines. The cell lines studied showed differences in susceptibility and frequency at which the different fusion genes were formed. These differences in selectivity may help to explain the differences in risk development of some types of leukemia that have been observed following high doses of irradiation.

Digestive Tract Dysfunction

The digestive tract includes the esophagus, small and large intestine, and rectum. The mucosa of the digestive tract undergoes continuous stress and in order for its functions to remain unimpaired, it must renew itself rapidly to replace lost cells. This fast turnover supported by a marked mitotic activity makes the digestive tract mucosa extremely radiosensitive. The duodenum is the most radiosensitive region of the digestive tract, followed by jejunum, ileum, esophagus, stomach, colon, and rectum, in order of decreasing radiosensitivity (45).

Surprisingly little has been written about radiation damage to the esophagus (665). The mucosal cells are characterized by a rapid proliferation rate and a relatively high degree of radiosensitivity. Acute radiation injuries are quite symptomatic with submucosal congestion and leukocytic infiltration, followed by mucosal necrosis and sloughing. Healing occurs rapidly and in animals with esophageal irradiation, the mucosa appeared completely normal 1 year following 2.5 Gy exposure. Humans who received 7.3–7.6-Gy irradiation exhibited narrowing of the esophageal lumen, partial loss of the mucosa and muscularis, and widening of the submucosa for 2–8 months following exposure. In spite of the initial radiation injury, radiation therapy for esophageal carcinoma results in very low mortality.

At radiation doses of 7–50 Gy, injury to the gastrointestinal tract inhibits the renewal of the cell lining. The intestinal epithelial stem cell is the target of radiation damage, and the resulting decrease in mitotic activity leads to denudation of the intestinal mucosa, fluid and electrolyte imbalance, and bacteremia (258). The symptoms of the gastrointestinal syndrome include lethargy, emesis, diarrhea, dehydration, and sepsis. At doses of 3–8 Gy, temporary injury to the tight junctions between epithelial cells of the mucosal lining permit the escape of bacterial endotoxins into the bloodstream. As dose increases, the epithelial lining is more extensively depleted. With doses of 10–15 Gy, denudation of the mucosa exacerbates the loss of fluid and electrolytes. Beginning at about 12.5 Gy, early mortality occurs due to dehydration and electrolyte imbalance, with death occurring 4–5 days postexposure.

At doses of 1 Gy or more irradiation of many mammals produces nausea and vomiting, signifying the prodromal phase of the acute radiation syndrome (45, 186, 258, 349). Radiation-induced emesis, often accompanied by delayed gastric emptying, may be associated with areas of the brain known as the area postrema and the vomiting center, both located in the medulla (258). Ablation of the area postrema has been observed to abolish radiation-induced emesis in some mammals (258) and zacopride, an antiemetic, inhibited radiation-induced emesis and suppression of gastric emptying in the monkey (186) and abolished radiation-induced emesis in ferrets (350). Inhibition of radiation-induced emesis has been achieved also through the use of the selective 5-hydroxytryptamine (5-HT3) receptor antagonists granisetron (58, 305), ondansetron (284, 536), and Y-25130 (216).

Functional alteration of the stomach by radiation included a decrease in the production and secretion of HCl, pepsinogen, and mucus (45), and an increase is serum levels of pepsinogen and gastrin (696). Histopathological alterations included vasodilation and edema indicative of increased microvascular permeability (86), and marked degenerative features including atrophic mucosa and ulceration (45, 75).

Intestinal mucosa cells originate from a single stem cell type located at the base of the intestinal crypts. As the cells proliferate and differentiate they move to the tips of the villi, a journey of 3–5 days. The differentiated cells are shed continuously from the tips of the villi. Radiation damage to the cells of the crypt leads to the death of some cells and an arrest of mitotic activity of others (15, 45, 491). The severely damaged crypt stem cells do not divide and replace the cells lost from the tips of the villi. This results in decreased absorption and allows a ready entry for intestinal flora into the systemic circulation (103, 259, 515). Electrolyte transport is also altered in the jejunum and ilium following exposure to ionizing radiation and this functional change may be related to the decreased mast cells and histamine (402, 403).

Intestinal absorption may also be decreased due to vascular concrescence. In examinations of irradiated animals the villous capillaries showed initial marked vasodilation followed by constriction, with many capillaries becoming totally nonpatent while the endothelial cells showed changes consistent with vascular damage (2, 3, 402). These findings were consistent with functional changes seen in several studies reviewed by Cockerham and Hawkins (131).

The colon has a relatively high radiotolerance, possibly due to the long turnover of its cells, and the rectum may tolerate more than 50 Gy irradiation (45). Postirradiation pathological events are essentially the same as in the small intestine with inhibition of mitosis, changes in cell morphology, and edema and vascular changes in the submucosal and serosal layers. An inflammatory response occurs within 24 h and a progressive degeneration leading to ulcerations occurs after high doses (86). A late development of colorectal irradiation seen in murine studies was a dose-dependent decrease in compliance (417),

possibly due to altered ratios of collagen isotypes, especially in the circular muscle layer and villi (416). As with the other portions of the gastrointestinal tract, the loss of water and electrolyte imbalance due to diarrhea and the development of bacteremia are considered the most important

factors in the gastrointestinal syndrome (258). The rat colon also becomes unresponsive to neurally evoked electrolyte transport following exposure to ionizing radiation of 10 Gy (208). This response also correlates with decreased mast cells and histamine.

Cardiovascular Dysfunction

Cardiovascular dysfunction (CVD) has been defined as the inability of any element of the cardiovascular system to perform adequately upon demand. The maintenance of cardiovascular integrity is determined by the changes in (i) the pumping action of the heart, (ii) compliance of the vascular beds, (iii) resistance of the peripheral circulation, (iv) quantity of blood in the vascular system, and (v) viscosity of the blood. Failure of any of the mechanisms to respond properly may compromise the integrity of the entire cardiovascular system (131, 272, 280). Exposure to supralethal doses of radiation has been shown to induce alterations in cardiovascular function in many species, including human. The extent of the radiation-induced CVD and its etiology may vary with the species, level of exposure, and dose rate. Radiation-induced CVD is surprisingly common (26) and may be manifest as circulatory shock. Although postirradiation hypotension does not occur with equal frequency in all species, it has been reported in rats, monkeys, and dogs (124, 130, 242, 441). However, evidence indicates than even if sublethal doses of radiation induce a functional cardiovascular deficiency that manifests itself as early hypotension, the lesion may be masked during a period of circulatory deterioration because of cardiovascular reserve (230, 488). When the cardiovascular reserve is no longer capable of maintaining homeostasis the damage may then be recognized as radiation-induced shock (278).

Irradiated rats displayed compromised myocardial function with a decline in cardiac output and an increased left ventricular end-diastolic volume (591, 719), which correlated with a decline in capillary density and focal degeneration of the myocardium. Cardiac performance after irradiation using an isolated working rat heart preparation showed a dose-dependent decrease in cardiac function and Frank-Starling curves, suggesting a loss of contractile function of the myocardium (712). Ultrastructural findings in the irradiated rat heart included intercalated disc damage and mitochondrial damage of the myocytes and swelling of the capillary endothelial cells and collapse of the capillaries (120). Mediastinal irradiation of human patients damages endothelial cells with loss of capillaries and ischemia, leading to increase in collagen and fibrous tissue throughout the heart (26). Long-term side effects of mediastinal irradiation include pericarditis, accelerated coronary artery disease, myocardial fibrosis, and valvular injury (26, 93).

The response of the gastrointestinal microcirculation to radiation has received little attention, although this may be an important factor in the development of both the cardiovascular and gastrointestinal subsyndromes. Here, as in other parts of the vascular system, the endothelial cell is one of the most radiosensitive cells (545). The initial expression of radiation injury to the endothelial cells is an increased vascular permeability leading to changes in extracellular environment (24). Following a single irradiation of 10–20 Gy, acute damage to endothelial cells may be detected in 1–5 days. Although not identical, radiation-induced endothelial damage in the lungs, kidney, myocardium, and intestine are similar and characterized by the plasma membrane becoming irregular with projections into the vascular lumen, followed by focal or generalized cytoplasmic swelling that narrows the lumen and may obstruct it completely. Damaged endothelial cells may retract from the basement membrane, causing exposure of the membrane. This results in platelet adhesion, followed by aggregation and the development of thrombosis and vascular occlusion. Damaged capillaries may be manifest by telangiectasia or be replaced by a collagen scar formation (38, 545). When reviewing the response of any microcirculation to irradiation, the variables of total dose, dose rate, type of radiation, variability of the animal model, and postirradiation time of observation must be considered. Irradiation damage to the microcirculation may cause dilation or constriction and either an increase or decrease in blood flow, depending on the above factors (131).

A complication involving the endothelium of intracerebral vessels is the impaired integrity of the blood-brain barrier (BBB) following irradiation (279). Functional alterations of the BBB are manifest in the endothelium by the activation of pinocytotic vesicular transport (162, 655) and in astrocytes by glycogen deposition. The changes in BBB permeability seem to be the result of the intense vesicular response of the endothelium rather than opening of endothelial tight junctions or altered regional blood flow (162, 656). The opening of the BBB has been associated with cerebral vasogenic edema and ischemia (352, 630). However, Gobbel et al. (248) suggest that postirradiation edema-induced vascular compression was not responsible for changes in regional cerebral blood flow observed in dogs.

A reduction in systemic blood pressure can reduce the driving force required to maintain cerebral blood flow and result in cerebral ischemia. The acute irradiationinduced hypotension in the monkey has a

temporal, if not causal, relationship with observed postirradiation reduction in regional cerebral blood flow (134). However,

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the reduced cerebral blood flow seen in hippocampi and cortices of rat brains and the hypothalamus of humans 10–24 weeks postirradiation was probably due to the telangiectatic vessels, spreading edema, focal regions of necrosis, and hemorrhage observed in the brains (31, 115, 358, 392).
Several biochemical mediators have been implicated in postirradiation CVD and reduced cerebral blood flow, including histamine, serotonin, opiate peptides, platelet-activating factor, eicosanoids, cyclic nucleotides, and catecholamines (176, 279, 686). Evidence that further implicates histamine includes the finding that plasma histamine increases precipitously in dogs and monkeys after exposure to radiation (126, 132). Infusing histamine into humans resulted in decreased blood pressure and altered cerebral blood flow (8, 602). Pretreatment with antihistamines diminished the cardiovascular effects of irradiation in dogs and monkeys (125, 132), further implicating histamine in postirradiation CVD. However, in another study infusing histamine into humans, Krabbe and Olesen (362) were unable to alter either blood pressure or cerebral blood flow. Likewise, the "histamine hypothesis" does not explain the postirradiation response of the rat (279). Considering that the etiology of postirradiation CVD may vary with the species, perhaps the radiation-induced production or release of other intermediates such as serotonin (129) or free radicals (123) may account for the CVD.

Radiation Effects on Bone, Cartilage, and Muscle

Mature bone is relatively radioresistant and radionecrosis of bone is very rare (437, 585). Many of the effects from irradiation seen in bone may be attributed to a reduction in the number of blood vessels supplying the bone and a decreased blood flow. Radiation damage to bone may be apparent only after months or years following irradiation because of impaired progenitor cell proliferation (161, 702). If the vascular support of the bone can recover, mitotic activity may reappear within 2 weeks after a dose of less than 17.5 Gy. However, osteoradionecrosis, the characteristic late bone injury, often accompanied by osteomyelitis, occurs at a minimum dose of 50 Gy (585).
Radiation-damaged adult bone characteristically displays a decreased ability to resist infection, increased susceptibility to fractures, and poor healing after damage (148, 437). The most common site for osteoradionecrosis and postirradiation complications is the mandible, probably due to its less than abundant blood supply. Clavicles and ribs have an increased incidence of fractures after radiation therapy for breast cancer and tumor-induced fractures of long bones do not heal following radio therapy of approximately 30 Gy (585). Following surgical trauma of rat femur, a dose-dependent radiationinduced delay is seen in new bone formation (25).
Radiation effects on bone are age dependent, with developing bone more sensitive to irradiation than adult bone, with the most pronounced effects seen during organogenesis (437, 585). However, a reduction in number of blood vessels and decreased blood flow are not thought to be the primary causes of reduction in growth. Impaired progenitor cell proliferation may be the reason, because a single dose of 6 Gy has been demonstrated to produce a decrease in mitotic activity.
Internal irradiation of bone by radionuclides may occur through occupational exposure or therapeutic administration (437). A famous case of iatrogenic poisoning was the use of Radithor, a patent medicine used as a metabolic stimulant and aphrodisiac (400). Unfortunately, the victim died of radium poisoning after his skeleton accumulated a dose that may have been greater than 350 Sv.
Probably a more famous case of radioisotope poisoning involved the manufacture of watch dials painted with luminous compounds containing radium (437, 564). The radioisotopes 226Ra and 228Ra were ingested by the women workers when they tipped the brushes with their lips. Isotopes of radium, strontium, and calcium are considered *volume seekers* and ultimately are included in the matrix of bone, whereas plutonium and thorium isotopes are considered *surface seekers* and accumulate on the periosteum and endosteal surfaces of the bone. The accumulation of radium in the bones of the female dial painters correlated with fractures of long bones, coarsening of the trabecular pattern, bone infarcts, and aseptic osteonecrosis. Bone sarcomas and head carcinomas have occurred at a higher than normal rate.
More than 3000 children of the 1495 women dial painters in the basic group were exposed continuously to an alpha and gamma-enhanced radiation environment during their entire period of gestation (564), but no evidence exists to suggest that any effects have occurred.
Growing cartilage is more radiosensitive than growing bone (585). Radiation doses exceeding 18 Gy cause permanent cessation of growth but chondrocytes recover from irradiation less than 10 Gy. Children aged 6 years and under and during puberty are the most vulnerable to irradiation-induced growth depression. Uneven irradiation of the spine results in scoliosis and doses up to 20 Gy result in major deformities. Pronounced growth retardation occurs above 35 Gy.
Mature cartilage, like adult bone, is fairly radioresistant (437, 585). Doses of 60–70 Gy may be tolerated

by mature cartilage if the irradiation is applied over 6–7 weeks. However, if the same dose is given in less than 6 weeks, radionecrosis of the cartilage is to be expected.

Although atrophy of muscle fibers may be seen following fractionated irradiation of 22–54 Gy, doses more than 500 Gy are required to produce acute radionecrosis of skeletal muscle (437). Recently, destructive alterations in muscle proteins were observed after 60Co gamma irradiation as low as 1.0 kGy, with a three-fold decrease in elasticity with doses of 15 kGy (356). Ischemia from a radiation-damaged vascular supply may result in fibrosis, but the extent of muscle damage depends, in part, on whether the entire muscle was exposed, or only a portion.

Radiation Dermatosis

The effects of ionizing radiation on the skin range from erythema to necrosis. The regular sequence of change progresses, as the dose is increased, over two periods, one occurring within 70–120 days and the other from 4 months to years later (22). The first period is characterized by erythema, pigmentation, epilation, dry desquamation, and moist desquamation, whereas the second period is characterized by atrophy, telangiectasia, fibrosis, and necrosis. Erythema, associated with an increased vascular permeability, appears after a single dose of 500 cGy or more and after multiple-dose fractions when the total dose is 1200 cGy or more (22). The first phase of erythema, presumably due to release of vasoactive amines, usually occurs within the first 1–2 days and lasts for a week. The second phase begins at about 10–12 days, reaches a maximum at about 20 days, and lasts for 30–40 days. This second phase is due to vascular damage and increased blood flow (437). The fading of erythema merges with increased pigmentation that may be permanent or fade for days or weeks as dry desquamation proceeds (22, 437). This pigmentation is associated with an increased melanin content of the basal layer.

Some epilation may be noted at 10 days following irradiation with a single dose of 300–600 cGy (22, 437). The evolution and time course of epilation are not dose dependent but epilation may be complete at 4 weeks with hair beginning to return in the second month and continuing for up to a year. However, a single dose of 700 cGy may cause permanent epilation (437). Radiation epilation sensitivity varies with body area; the scalp and beard are the most sensitive, followed by chest, axillary, abdominal, eyebrow, eyelash, and pubic hair.

Dry desquamation, preceded by decreasing erythema and an increasing pigmentation, is characterized by a loss of epidermal cells accompanied by replacement. The cells may scale off or peel off in a sheet, leaving an intact, erythematous epidermal surface. The regenerative capacity usually exceeds the destructive capacity as long as the single dose does not exceed 2000 cGy, or the mul tiple fraction total does not exceed 4500 cGy (22, 437). The reduced proliferative potential and regenerative capacity of irradiated skin cells may be manifest also in an interference with wound healing at doses of 400 cGy or greater (141).

If the dose exceeds the levels allowing regeneration to occur, 2400 cGy for single dose and 5000 cGy for multiple fraction total, the epidermal cell population becomes depleted and the loss of epidermis allows serum leakage and a moist desquamation (22). A bullous-type, moist desquamation may occur with the small blisters tending to coalesce and rupture (437). Blisters may even form beneath the basal layer and the lesion may appear, similar to a second- or third-degree thermal burn. The ruptured blisters may become infected and ulceration may occur. The ulceration is usually associated with a reduction in circulation due to obliterative arteriolar and small artery changes.

Late skin damage may follow the early reactions by week to years or not at all. Alternatively, late skin damage may be manifest without an earlier reaction. The late reaction is, in part, dose dependent and may progress or remain static (22).

In the weeks or months following irradiation at a single dose level of 1700–2400 cGy or a multiple fraction total of 4500–5000 cGy, telangiectasia manifest as superficial, elongated, and dilated blood vessels (22). Radiation-damaged endothelial cells are lost and the microvessels shorten, uncoil, and dilate. A loss of total microvasculature occurs as well as a decrease in functional vessels. The formation of telangiectatic vessels is dose dependent and if the epidermal response is severe, focal keratosis and dysplasia may be present.

Skin changes occurring months to years following irradiation may include increased induration, stiffening, and thickening of the dermis associated with increasing fibrosis (22, 437). Although the onset and formation of fibrosis are dose dependent, once started, fibrosis is progressive with a characteristic proliferation of the small arteries and arterioles. As the degree of fibrosis increases, so does the probability that necrosis will be the result.

Following a single dose of radiation that exceeds 2700 cGy or a multiple fraction total dose greater than 6000 cGy, the end stage of radiation dermatosis is a nonhealing necrosis (22). Radiation-induced necrosis is associated with the progressive loss of the dermal microvasculature and is the end stage to

progressive fibrosis.

Chronic exposure to low-dose (0.015 cGy/s) x-ray for 9–18 months (total doses equal 2.025 and 4.05 cGy) will produce a hyperkeratinization in the rat, along with a decrease in skin concentration of zinc and an increased concentration of iron (111). The chronic sequelae following cutaneous radiation may include telangiectases, radiation keratoses, radiation ulcers, hemangiomas,

splinter hemorrhages in the distal nail bed, lentiginous hyperpigmentation, and severe subcutaneous fibrosis (525). This predominant involvement of the skin is sometimes described as the cutaneous radiation syndrome and can become the characteristic feature of chronic cutaneous irradiation.

The Urinary System

The kidney is relatively radiosensitive compared to other abdominal organs and has a definite but low sensitivity to radiation carcinogenesis (48, 50, 437, 714). The time course for pathophysiological and histopathological changes are dose dependent with a fractionated tolerance dose (TD) of 20–23 Gy for the human kidney (437, 714). The kidneys are considered to be late-reacting organs with the effects of radiation nephropathy appearing months to years after exposure. However, pathological changes in the endothelial cells of the renal microvasculature seen soon after exposure may have long-lasting effects and later tubule and glomeruli degeneration may be secondary to renal ischemia (714). Functional changes seen in mice following fractionated irradiation with x-rays include decreased EDTA clearance, increased urine output, and reduced hematocrit (623).

Reports of patients dying of renal failure and hypertension following therapeutic radiation include subacute changes of intimal necrosis, subendothelial thickening, fibrinoid thrombosis, atrophy of tubules, and replacement with collagen (437). In some instances myointimal proliferation with "foamy cells" and sclerosis of the glomeruli may be seen as well.

There are no human data on acute radiation effects following exposure to single doses (437). However, acute pathological changes seen in animals are hyperemia, increased capillary permeability, interstitial edema, and microvascular endothelial degeneration. These changes are usually followed by occlusive changes in the interlobular arteries and afferent arterioles, reducing blood flow to the nephron. Although in many cases the changes are transient, they may progress to severe diffuse endarteritis and necrotizing vasculitis, which may result in malignant hypertension (714).

Irradiation of pigs with a single 60Co gamma ray dose of 7.8 Gy or higher resulted in a dose-dependent reduction in effective renal plasma flow (ERPF) and glomerular filtration rate (GFR) (553, 554). A normochromic normocytic anemia, with a significant reduction in erythrocyte count, hematocrit, and hemoglobin levels, developed within 6–8 weeks following irradiation. Studies in pigs (552) and mice (620) indicate that the kidney fails to exhibit complete recovery in function following irradiation and that irradiation of a pre viously irradiated kidney is likely to lead to severe renal damage.

Chronic renal dysfunction develops 1–5 years after irradiation and involves a slow evolution of anemia, hypertension, and impairment of renal function (437). The changes are progressive and irreversible, with the treatment usually symptomatic. The pathology is an extension of that seen in short term radiation-induced renal dysfunction. Chronic glomerulonephritis has been reported in subjects participating in the clean-up after the Chernobyl accident (159). The renal pathology was associated with significant changes in the humoral immunity system manifested by increased serum levels of immunoglobulin M, immunoglobulin G, and circulating immune complexes. However, chronic ingestion of drinking water containing uranium (0.004–9 μg/kg body weight) indicated that the proximal tubule, rather than the glomerulus, was the site of injury (727).

The urinary bladder is relatively more radioresistant than the kidney and can usually tolerate 55–60 Gy if the dose is fractionated (437). Bladder complications are seen most often in humans following radiotherapy for cancer of the cervix, prostrate, or bladder (626). The symptoms of acute radiation cystitis appear 4–6 weeks following treatment and include dysuria, nocturia, and increased frequency. Edema, hyperemia, and partial desquamation of the mucosa may be seen. Acute pathological changes in humans are less well documented than those in animals.

Acute changes in the bladders of dogs following irradiation are more pronounced than those seen in rodents (626). Acute and short term pathophysiological changes in the rodent bladder include an increase in urination frequency, reduced bladder volume, decreased compliance of the bladder wall, and diminished pressure during micturition (179, 396, 439, 620). Reirradiation tolerance for late bladder damage was inversely related to the first dose and independent of the interval between treatments (622).

Late-occurring or chronic pathological alterations seen in the urinary bladders of dogs following irradiation are similar to those observed in humans. These include a small, shrunken, and contracted bladder with thick and fibrotic walls (437, 626). There may be multiple areas of edema and telangiectasia and collagen may replace muscular tissue. Squamous metaplasia is common and extensive mucosal ulceration may extend into and beyond the muscle layers. In women treated for cervical carcinoma, this ulceration can lead to vesicovaginal fistulas.

The most radioresistant portion of the urinary system is said to be the ureter (437). However, others

classify the ureter as radiosensitive, with ureteral fibrosis, stenosis, and obstruction following doses of 12.5 Gy (292, 600).

Dogs have been shown to tolerate 17.5-Gy irradiation of the ureter with early injury due to ulceration of the epithelium seen at 25 Gy or above (241). However, histological evidence suggested that chronic injury of the canine ureter seen after 5 years was of vascular etiology.

Radiation-Induced Hepatic Dysfunction

Early literature describing hepatic irradiation contained many contradictory reports concerning the radiosensitivity of the liver (322, 437). The hepatic cells are relatively radioresistant and there is a marked capacity for regeneration following destruction of a large portion of the liver. The liver is able to tolerate fractionated doses of 30–35 Gy over 3–4 weeks, but 35 Gy should not be exceeded (322). Following liver damage the regenerating portion is more radiosensitive (437).

Radiation-induced hepatic injury represents a continuum of clinical, pathological, and radiographic findings, ranging from asymptomatic biochemical changes to fulminant, fatal hepatic failure (322). Radiolesions in the liver are dose dependent in animals (74, 260, 261) and are primarily due to damage to the fine vasculature and connective tissue (234, 235, 322). Characteristic changes following liver irradiation portray a nonspecific form of venous occlusive disease (VOD) resembling pathologically the Budd-Chiari syndrome (322, 437).

Pathological changes that occur following irradiation of the liver to doses greater than 35 Gy may be divided into two stages. The acute phase may begin 2–6 weeks postirradiation and continue for 3–6 months. Clinical signs include hepatomegaly, ascites, jaundice, and elevated serum alkaline phosphatase and serum transaminases (SGOT and SGPT) (74, 234, 322, 437). A distinct decrease in hepatic biotransformation and tolerance to a wide variety of drugs may be seen within a few days postirradiation (480, 711). These clinical manifestations are usually associated with pathological changes that include sinusoidal congestion, occlusion of the central vein, disrupted intrahepatic blood flow, and parenchymal cell damage, atrophy, and necrosis (235, 322, 437).

The late phase of pathological changes and hepatic dysfunction manifests itself more than 6 months postirradiation (322, 437). There is less hepatic congestion, but the signs and symptoms of portal hypertension and right-sided congestive heart failure are manifest. Pathological findings are those of a veno-occlusive process leading to obstruction of hepatic outflow. The centrilobular veins may be obliterated with dense collagen and periportal fibrosis is extensive. The liver appears shrunken and pale, and atrophy due to cell loss is evident. Serum phosphatase and transaminase levels may be slightly elevated or normal but the serum albumin levels are decreased (322, 480). The postirradiation VOD seen in the liver is unique but is similar to conditions caused by various drugs (437).

Radiation Pneumonitis and Pulmonary Fibrosis

The lung is relatively radiosensitive and lesions in the lung are common after any irradiation. Involvement of even a small portion of the thorax results in some degree of pulmonary damage (437, 654).

The lung's response to irradiation is biphasic (231). The first phase, *radiation pneumonitis,* may vary in postirradiation time of onset depending on species, type of radiation, and dose (231, 379, 526, 646). A clinical threshold of 6–7 Gy and a maximum of 8 Gy in a single dose has been suggested for the development of radiation pneumonitis (437).

During the pneumonitis phase functional changes are prominent, including respiratory distress, hypoxemia, increased bronchoalveolar lavage protein, impaired surfactant function, decrease in pulmonary blood flow, and pulmonary hypertension (231, 526, 646). Alveolar epithelium and endothelial damage (379, 526) may be associated with a radiation-induced production of free radicals, release of histamine, leukotriene, and prostaglandin, and the acceleration of lipid peroxidation (78, 253, 501). Pulmonary blood flow problems are indicative of loss of fine vasculature, pulmonary hypertension, right ventricular hypertrophy, and radiation-induced heart failure (54, 599).

The second phase of pulmonary injury, *pulmonary fibrosis,* is seen with or without the presence of pneumonitis (654). Although many patients are asymptomatic during the fibrosis phase, functional changes include arterial hypoxia, a decreased lung volume, decreased compliance, and a reduced maximum breathing capacity. Endothelial and epithelial cell damage may contribute to the development of postirradiation pulmonary fibrosis (526), but the final picture includes replacement of septa by collagen, decreased total alveolar volume, reduced functional microvasculature, and atelectasis (437, 654).

Radiation Effects on Endocrine Function

Many discussions of radiation effects of the endocrine system are limited to the pituitary, thyroid, parathyroid, and adrenal glands (437). Other endocrine glands less often considered are the pineal, pancreas, ovary, and testis. Although most endocrine glands are relatively radioresistant with direct

effects of radiation resulting from injury to the fine vasculature, endocrine abnor-

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malities are relatively common following irradiation of the head and neck (437).

Pineal Gland (Epiphysis)

Melatonin, a hormone that inhibits ovarian and testicular function, is synthesized and secreted by the pineal gland (148, 686). In several species, including human, melatonin synthesis and secretion increase during the dark period of the day and is at a lower level during the daylight hours (228). Pineal synthesis of melatonin in response to the light cycle is altered by radiation, with a decreased synthesis seen in rats following 3.5-Gy exposure (686). However, melatonin has been shown to be radioprotective, with cellular destruction occurring postirradiation in other glands without melatonin (369, 686). Even so, the function of melatonin and the pineal in the radiation response of human remains relatively obscure.

Pituitary Gland (Hypophysis)

Physiologically, the pituitary gland is divisible into two distinct portions: the *anterior pituitary* or the *adenohypophysis,* and the *posterior pituitary* or the *neurohypophysis*. Most control of secretion by the pituitary comes from the *hypothalamus* by either nervous or hormonal signals. Secretion by the anterior pituitary is controlled by hormones secreted within the hypothalamus and transported to the anterior pituitary through the *hypothalamic-hypophysial portal system* of blood vessels. Secretion from the posterior pituitary is controlled by nerve fibers originating in the hypothalamus and terminating in the posterior pituitary. The hypothalamus receives signals from sources throughout the nervous system and uses this information to control secretion of the pituitary hormones (228, 262).

Six important hormones are secreted by the anterior pituitary and play major roles in the control of metabolic functions throughout the body. These six hormones are (i) *growth hormone* (GH), (ii) *adrenocorticotropic hormone* (ACTH, corticotropin), (iii) *thyroid-stimulating hormone* (TSH, thyrotropin), (iv) *prolactin* (luteotropic hormone, LTH), (v) *follicle-stimulating hormone* (FSH), and (vi) *luteinizing hormone* (LH). The two hormones secreted in the posterior pituitary are, (i) *antidiuretic hormone* (vasopressin, ADH), which controls water excretion, and (ii) *oxytocin,* which helps deliver milk from the glands to the nipple and may help in delivery at the end of gestation. ADH and oxytocin are formed in the supraoptic and paraventricular nuclei of the hypothalamus and released from nerve endings in the posterior pituitary (228, 262).

Hypothalamic-pituitary failure is a common complication of cranial and neck irradiation and signs of endocrine deficiency may appear from 1–15 years postirradiation. Some researchers propose that radiation-induced alterations in pituitary function can be attributed to effects of radiation on the hypothalamus (574). There is good evidence that the earliest irradiation damage to the hypothalamic-pituitary axis is at the level of the hypothalamus and that any subject receiving a total irradiation dose of 20 Gy or more to the axis is at risk of hypopituitarism (302, 370, 388). In general, the direct effects of radiation on the hypothalamic-pituitary axis result in hypopituitarism manifested through alterations of the direct actions of the pituitary hormones or through their influence on other endocrine glands (389, 437). Radiation-induced pituitary dysfunction may result in loss of weight, loss of body hair, dry skin, slow pulse, low body temperature, dwarfism in children, genital atrophy, primary amenorrhea in females, failure of sexual development in males, and other dysfunctions associated with the endocrine system (437, 574).

Thyroid

Thyrotropin-releasing hormone (TRH) is secreted by the hypothalamus, and stimulates the release of TSH by pituitary thyrotrophs and the release of *calcitonin* from the C cells of the thyroid. TSH stimulates all thyroidal functions associated with the production and release of *triiodothyronine* (T3) and *tetraiodothyronine* (thyroxine, T4). Through a classic feedback loop, excessive amounts of T3 and T4 suppress the release of TSH by the pituitary (343). Calcitonin, from the C cells or parafollicular cells in the thyroid gland, serves to lower the serum calcium and phosphate levels (228).

Radiation-induced injury of the hypothalamic-pituitary axis may be manifest in the development of hypothyroidism (370, 388). However, direct irradiation of the thyroid gland results in decreased production of T3 and T4 (686). Estimates of irradiation doses required to produce hypothyroidism vary from 2–50 Gy, with thyroid ablation a possible result of the larger doses. Data confirm the high incidence of thyroid dysfunction when the gland is included in the radiation field (19, 202, 273, 505, 718).

Radiation hypothyroidism, through the feedback loop, will result in an increase in TSH secretion by the pituitary. This increase in TSH has been associated with increases in radiation-induced thyroid cancer (686). TSH has also been studied in relation to radiation exposure in children, particularly after the Chernobyl nuclear disaster (249, 538).

The latent period for radiation-induced thyroid cancer was in excess of 15 years following the exposure of the Japanese to the atomic bomb explosions at Hiroshima and Nagasaki. Increased numbers of thyroid cancer cases became evident within just 4 or 5 years following the Chernobyl nuclear accident, however, and continued to increase substantially over the next decade (66, 232). Another interesting aspect of the dynamics of thyroid

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cancer incidence in that accident was that geographic correlation of thyroid cancers was more closely related to the transportation corridors than to the isopleths of 131I distribution or to population density (517).

Hypothyroidism following 10-Gy irradiation has been associated with a shift in the ratio of the alpha-myosin heavy chain to the beta-myosin heavy chain in the rat heart, a shift usually associated with overload of the heart or aging (686). This change may correspond with a low resting ejection fraction and decreased response to exercise seen in cardiac scans following therapy for Hodgkin's disease (431). Therapy for hyperthyroidism using 131I decreases basal calcitonin levels and may cause C cell deficiency (43). However, this may not be of extreme consequence because total thyroidectomy does not reduce the circulating level of the hormone to zero (228).

The thyroid gland of children is especially vulnerable to the carcinogenic action of ionizing radiation, with one of the highest risk coefficients of any organ and may be the only tissue with convincing evidence for risk at about 0.10 Gy (559). This vulnerability is dramatically illustrated in the 100-fold increase in the number of childhood thyroid cancers observed in heavily contaminated areas of Belarus, Ukraine, and the Bryansk regions of Russia following the accident at the Chernobyl nuclear power plant in 1986 (28, 569).

Parathyroid

In humans there are usually four parathyroid glands embedded in the poles of the thyroid gland. Chief cells in the parathyroid are sensitive to circulating levels of ionized calcium and act to secrete parathyroid hormone (PTH) in response to decreased Ca^{2+} levels. The PTH acts directly on bone to increase bone resorption and mobilize Ca^{2+}. PTH also acts to depress plasma phosphate by increasing phosphate excretion (228).

Although parathyroid cells are relatively resistant to radiation, data confirm an association between hyperparathyroidism and radiation exposure (44, 215, 437). The hyperparathyroidism may be secondary to a depletion of calcium that occurs in mammals following exposure to greater than 3 Gy radiation (686, 687). Other biological responses occurring as a result of irradiation-induced calcium loss include increased blood clotting, bone damage, and convulsions (686).

Adrenal Glands

The endocrine functions of the adrenal glands are associated with the adrenal cortex that produces over 30 different steroids. Only two of these corticosteroids are of exceptional importance to the endocrine function of the adrenal cortex: *aldosterone,* the principal mineralocorticoid and *cortisol,* the principal glucocorticoid. The mineralocorticoids affect the levels of the electrolytes of the extracellular fluids and the glucocorticoids serve to increase blood glucose concentration and affect protein and fat metabolism. Aldosterone secretion is regulated by: (i) potassium ion concentration in extracellular fluid, (ii) renin-angiotensin system, (iii) quantity of sodium in the body, and (iv) ACTH. Secretion of cortisol is controlled almost entirely by ACTH (262).

Radiation-induced changes in the endocrine functions of the adrenal glands are difficult to document because stress factors, including radiation, result in increased release of ACTH from the pituitary (437, 686). ACTH acts on the adrenal cortex stimulating the synthesis and release of aldosterone and cortisol. Even though irradiation of the hypothalamic-pituitary axis produces a defect in ACTH release, adrenal corticosterone levels may be normal or elevated following irradiation, indicating a possible hypersensitivity to the ACTH present or the presence of some other controlling factor (686).

The direct effects of radiation on the adrenal glands are manifest in three phases of activity, each associated with increases in plasma and adrenal corticosterone levels (686). The first phase occurs early postirradiation and the second peak of activity is associated with gastrointestinal damage. The third phase of activity is associated with hematopoietic injury. The increase in activity may be seen following absorbed doses of 15–35 Gy (437, 686). However, if the dose exceeds 35 Gy, normal steroidogenesis may occur under nonstress conditions, but the ability to respond to stress is impaired. Hypertrophy of the adrenal cortex has been demonstrated following irradiation with 15 Gy and higher (172, 686).

Pancreas

The pancreas has both endocrine and exocrine functions. The islets of Langerhans in the pancreas are associated with its endocrine function and act to secrete *insulin* and *glucagon*. Insulin is produced by the beta cells of the islets and glucagon by the alpha cells. The secretion of insulin and glucagon is controlled by the blood glucose concentration. An increase in blood glucose concentration stimulates insulin secretion and inhibits glucagon secretion. A decrease in blood glucose concentration has the opposite effect on both hormones. Insulin acts to increase glucose uptake by most tissues of the body and to stimulate glycogen synthesis. Glucagon stimulates breakdown of hepatic glycogen and adipose

tissue, and also stimulates gluconeogenesis from amino acids. All its actions increase blood glucose concentration (228).

The pancreas is relatively radioresistant compared to the surrounding structures such as the liver and small intestine (437). The islet cells show more postirradiation changes than do the acinous cells, with the beta cells of the islets being more radioresistant than the alpha cells (172, 477). Decreases in insulin and glucagon have been observed following radiotherapy and impaired insulin

secretion and hypoglycemia were seen in rats 4 days after 10-Gy irradiation (576, 686). One month postirradiation the insulin secretion impairment persisted and was accompanied by a reduced number of beta cells.

Ovaries

The two types of ovarian hormones are the *estrogens* and *progestins* and are secreted by the ovaries in response to FSH and LH from the anterior pituitary. FSH and LH, in turn, are secreted by the anterior pituitary in response to luteinizing hormone-releasing hormone (LHRH) from the hypothalamus. By far the most important of the estrogens is *estradiol,* secreted by the theca interna and granulosa cells of the ovarian follicles and by the corpus luteum. The most important progestin is *progesterone,* secreted by the corpus luteum (228, 262).

Ovarian radiation severely reduces the formation of ovarian steroid hormones, even to the point of gonadal failure (389, 691). Estrogen decreases have been seen in humans following radiation doses of 6–100 Gy (389, 686, 691), with large doses producing premature menopause (686). Persistently elevated gonadotrophin levels (FSH and LH) and amenorrhea were associated with the reduced ovarian hormones (389, 691). Abdominal radiation of females in childhood resulted in pubertal failure or premature menopause (690). Neonatal irradiation of rats with 15 cGy produced a decrease in progesterone levels, but not estradiol levels, in adult animals (211).

Testes

The testes secrete several hormones that are called *androgens* because of their masculinizing effects. *Testosterone,* considered to be the most significant testicular androgen, is formed by the interstitial cells of Leydig. As in the female, LHRH from the hypothalamus stimulates secretion of LH by the anterior pituitary. LH in turn stimulates hyperplasia of the Leydig cells and the production of testosterone by these cells (228, 262).

The most dramatic endocrine effect of irradiation of the testis is the increase in FSH and LH secretion from the anterior pituitary (432). FSH levels have been used as an indication of damage to the germinal epithelium. Elevated LH levels associated with normal testosterone levels are indicative of Leydig cell damage (97, 351, 390, 432, 530). This condition may occur at irradiation doses of 2–12 Gy, because damage to the Leydig cells may be compensated by an increase in the number of cells (hyperplasia) in response to the elevated LH (686). Higher doses of irradiation (20–30 Gy) have been shown to produce Leydig cell damage, decrease testosterone production, and increase secretion of LH (246, 432, 597). Of course, sperm cells and the less differentiated cells giving rise to sperm cells are also known to be affected by radiation exposure. For example, ultramorphological sperm characteristics have been altered in workers cleaning up materials containing relatively high levels of radioactivity following the Chernobyl nuclear accident (205).

Nervous System

Radiogenic Effects on Sensory Functioning

Ionizing radiation can be sensed at extremely low levels (698). For instance, the olfactory response threshold to radiation is less than 1.0×10^{-4} Gy and the visual system is sensitive to levels below 5.0×10^{-6} Gy. Ionizing radiation has been shown to be as efficient as light in producing retinal activity (as assessed by the electroretinogram), and the visibility of ionizing radiation is now firmly established (344).

Whereas visual system pathomorphology occurs only at high doses (219), this is not true of visual function disruption. Rats trained to a brightness discrimination task were unable to differentiate shades of gray after 3.6 Gy or to make sensitivity changes after 6 Gy of whole-body x-rays (for review, see 344). Chimpanzees showed impaired accuracy and visual acuity on visual discrimination tests after about 4 Gy of gamma irradiation (551). Kekcheyev (337) reported that 1 day after exposure to 0.3–1.0 Gy of x-rays, temporary decrements in scotopic visual sensitivity were observed in humans. Further, Lenoir (383) found long-term delays (20–36 days) in dark adaptation in patients exposed to 4–62 Gy of x-rays.

Most of the literature suggests that significant hearing changes, unlike vision changes, require massive doses (e.g., 10–70 Gy) of radiation (for review, see 217). Heinz (285), using fractionated head-only exposures of baboons to x-rays, found that 10, 12, and 15 Gy caused a long-term hearing deficit. The highest exposure produced a hearing loss of >90 dB that was not frequency specific. Lower doses of x-rays caused slowly developing, transient elevations in auditory reaction times. Vestibular function may be more radiosensitive than audition. Depression in vestibular function may exist at doses close to the LD_{50}, with higher doses producing longer lasting disruptions than low doses (21).

Although not much literature is available, several reports exist of olfactory, gustatory, and cutaneous sensory changes in patients exposed to therapeutic irradiation (217). Altered taste perception was found

in patients exposed to 36 Gy of x-rays, with a metallic taste being the most common report. Transient changes in taste and olfactory sensitivity were also reported in radiotherapy patients and rats (see review in 344). There is empirical evidence of radiogenic changes in pain perception. While gamma photons produce a dosedependent analgesia in mice (642), data also suggest that X- or gamma rays do not alter the analgesic effects of

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morphine or the anesthetic effects of halothane in rats, except under a narrow set of experimental conditions (89, 181). Miyachi and colleagues reported that the olfactory system of mice is important in detecting radiation (467) as well as modulating radiation-induced analgesia (465).

Radiogenic Pathology of the Adult Nervous System

A review of standard radiobiology textbooks revealed the common belief that the adult CNS is relatively resistant to damage from ionizing radiation exposure (96). This conclusion was derived, in part, from early clinical reports suggesting that radiation exposures, given to produce some degree of tumor control, produced no immediate morphological effects on the CNS (299). However, this view was changed when it was later shown that the latency period for the appearance of radiation damage in the CNS is simply longer than it is in other organ systems (399). Later interest in the pathogenesis of delayed radiation necrosis in clinical medicine has produced a significant body of literature. Studies of radiation-induced brain damage in patients used computed axial tomography (CAT) technology to confirm CNS abnormalities that are not associated with tumor treatment but that occur because of the radiotherapy (294).

General, although not universal, agreement exists that there is a threshold dose below which no late radiation-induced morphological sequelae in the CNS occur. In laboratory animals, single doses of radiation up to 10 Gy produced no late morphological changes in the brain or spinal cord (281, 385). Necrotic lesions were observed in the forebrain white matter from doses of 15 Gy (92, 100, 338). In human, the "safe" dose has been a topic of considerable debate. Depending on the radiation field size, the threshold for CNS damage was estimated to be 30–40 Gy if the radiation was given in fractions (516); spinal cord damage occurred with fractionated doses as low as 25 Gy (191). The difference between a safe and a pathogenic radiation dose to the brain may be as small as 4.3 Gy (414).

Different topographical regions of the brain may vary in susceptibility to ionizing radiation (667). The most sensitive area is the brain stem (24). The cerebral cortex may be less sensitive than the subcortical structures (385), such as the hypothalamus (720), the optic chiasm, and the dorsal medulla (562). Although radiation lesions occur more frequently in brain white matter (307, 557, 669), the radiosensitivity of white matter also appears to vary from region to region (385). It may be that selective necrosis of white matter is due to the slow reproductive loss of glia or their precursors. The radiosensitivity of certain types of glial cells (beta astrocyte) is well recognized (547, 548). The earliest sign of their damage is widening of the nodes of Ranvier and segmental demyelination as early as 2 weeks after a dose of 5–60 Gy (419).

The technique and end points selected to assess neuropathology can profoundly influence its detection. In proton-irradiated brain tissue stained with silver to detect degenerating neural elements, punctate brain lesions were found within 3 days after as little as 2 Gy (631). The lesions were not detectable with standard hematoxylin and eosin stain. These effects are similar to a multi-infarction syndrome in which the effects of small infarctions accumulate and may become symptomatic. Similarly, Philpott and his associates (528) found that both the synaptic density and the spine length in area CA1 of the hippocampus were lower in mice irradiated with 0.005 or 0.5 Gy of 40Ar. Because this pathology was observed at doses of radiation previously believed to be completely safe, confirmation of these data may profoundly influence our view of the radiosensitivity of brain tissue.

The phenomenon of latent CNS radiation damage with doses above threshold has been well documented (55, 96, 567). The long latent period has led to considerable speculation on the likely pathogenesis of late radiation lesions: (a) radiation may act primarily on the vascular system, with necrosis secondary to edema and ischemia; and (b) radiation may have a primary effect on cells of the neural parenchyma, with vascular lesions exerting a minor influence (299).

The first evidence in support of a vascular hypothesis was obtained when canine brains that had been exposed to x-rays were examined (399). It was suggested that delayed damage of capillary endothelial cells may occur, leading to a breakdown of the blood-brain barrier. This would result in vasogenic edema (163), the elevated pressure-impaired circulation of cerebral spinal fluid, and eventually neuronal and myelin degeneration (100, 101). The finding that hypertension accelerated the appearance of vascular lesions in the brain after irradiation with 10–30 Gy also supports a hypothesis of vascular pathogenesis (300). The occlusive effects of radiation on arterial walls may cause a transient cerebral ischemia (288). Sequential monkey-brain CAT scans revealed brain edema and hydrocephalus that accompanied hypoactivity and the animal's loss of alertness following 20 Gy of radiation (271). Head-only exposure of rabbits to 4, 6, or 8 Gy of x-rays disturbed the blood-brain barrier permeability, which returned to normal after only 6 days (704). The transient nature of the vascular phenomena may partially explain some of the behavioral deficits observed after exposure to intermediate or large doses

of ionizing radiation (426, 601).

Evidence of the direct action of radiation on the parenchymal cells of the nervous system, rather than the indirect effect through the vascular bed, was first provided when brain tissue in irradiated human patients was examined (503). None of the brain lesions could be attributed to vascular damage because they were (a) predominantly

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in white matter and not codistributed with blood vessels, (b) not morphologically typical of ischemic necrosis, and (c) often found without any vascular effects (146, 301, 312, 533, 729). Thus, it appears that direct neuronal or glial mechanisms caused at least some of the observed radiogenic brain lesions.

Alterations in Nervous System Physiology and Functioning

In addition to radiogenic changes in CNS morphology, a variety of changes in parameters of brain function were reported. For example, changes in brain metabolism were reported after very low (0.11–0.24 Gy) doses of ionizing radiation (194). In a more detailed analysis with the 14C-2-deoxyglucose method of measuring local cerebral glucose utilization, a dose of 15 Gy of x-rays was administered to the rat brain (313). Significantly lower rates of glucose use were found in 16 different rat brain structures at 4 days after irradiation and in 25 structures at 4 weeks. Although large radiogenic changes existed in the metabolism of particular brain nuclei, a weighted average rate for the irradiated brains was approximately 15% below that for the controls.

Researchers measured the functional sensitivity of some brain areas and the insensitivity of others (4, 444). The activation of behaviors through electrical stimulation of the lateral hypothalamus (but not of the sepal nucleus or substantia nigra) is still possible after 100 Gy (119, 452). However, years after clinical irradiations, dysfunctions of the hypothalamus are prominent even without evidence of hypothalamic necrosis (427). Local subcortical changes may exist in the reticular formation and account for radiation-induced convulsability of the brain (560, 561). Similarly, postirradiation spike discharges are more likely to be observed in the hippocampal electroencephalograph (EEG) than in the cortical EEG (226). This idea of selective neurosensitivity is further supported by experiments in which electrical recordings were made from individual nerve fibers after irradiation (237). These data reveal a hierarchy of radiosensitivity in which gamma nerve fibers are more sensitive than beta fibers, and alpha nerve fibers are the least sensitive.

Electrophysiology

Measures of electrophysiology illustrate changes in brain function after exposure to ionizing radiation. Several studies were reported in which cortical EEG changes were observed in humans and in animals following doses as low as 0.05 Gy (382). Typically, an initial temporary increase in bioelectric amplitude was followed, within minutes, by a depression. Other investigations frequently needed higher doses of radiation to observe changes in EEG. For example, changes were not seen in EEGs after 0.03–0.04 Gy x-rays, but significant alterations were observed after 2 Gy (266). At a higher dose (15 Gy), monkey cortical EEG abnormalities consisted of the slowing of activity, with an increase in amplitude (562). Spiking and patterns of *grand mal* seizure also occurred. A rapid onset of high-amplitude slow waves (delta waves) seemed to relate to periods of behavioral incapacitation (424). Exposures to 4–6 Gy 60Co gamma radiation appeared to stimulate spontaneous activity in the neocortex, whereas exposures of higher than 9 Gy inhibited all brain activities (474). Many of the liquidators involved in the cleanup of the Chernobyl nuclear accident were reported to have abnormal EEGs (682, 730).

The hippocampus shows significant changes in physiological activities after gamma irradiation with even less than half of the 18-Gy threshold dose needed to produce changes in cortical activities (24, 227). Hippocampal spike discharges were first identified in cats (226) and later confirmed in rabbits (227). This spiking developed soon after irradiation (2–4 Gy x-rays) when no other clinical signs of neurological damage or radiation sickness were present.

The apparent radiosensitivity of the hippocampus and its importance in critical functions, such as learning, memory, and motor performance, have led others to investigate the electrophysiology of this brain area. The firing of hippocampal neurons was found to be altered by exposure to 4 Gy of 60Co gamma radiation in rabbits (40). In guinea pigs exposed to 5 or 10 Gy x-rays, significant changes in hippocampal neuronal function were observed to be time, dose, and dose-rate dependent (523). Higher doses (40–65 Gy x-rays) decreased the ability of hippocampal neurons to generate an action potential (524). In addition, in vitro experiments suggested that spontaneous discharges of hippocampal pacemaker-like neurons were induced by X- and gamma rays at a dose of only 0.08 Gy (522). If confirmed, these data suggest that hippocampal electrophysiology may be the most sensitive measure of functional brain changes after irradiation.

Alterations in the thresholds and patterns for audiogenic and electroconvulsive seizures have been produced by exposing animals to ionizing radiations. Such effects are interpreted as reflecting gross changes in CNS reactivity. Early work with dogs showed that spontaneous seizures sometimes occurred following very large doses of radiation (399). Later experiments confirmed that seizures can be induced by whole-body or head-only exposures to 30–250 Gy in a variety of species. For example, rats were exposed to 5 Gy of X-radiation and the electroconvulsive shock (ECS) threshold was determined for 180

days after irradiation (560). ECS thresholds were reduced in irradiated rats over the entire test period. Later studies (561) reported that considerably lower doses (perhaps as little as 0.01 Gy) also reduced the thresholds for ECS seizures and audiogenic seizures (457, 532).

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Unlike the CNS, peripheral nerves are quite resistant to the functional alterations produced by ionizing radiation. Most data indicate that peripheral nerves do not show any changes in electrophysiology with x-ray exposures below 100 Gy (577). After higher doses, the action-potential amplitude and the conduction velocity temporarily increase but then gradually decrease (32–35, 577). Also, alpha and beta particles are more destructive to peripheral nerves than gamma or x-rays, and usually cause a monophasic depression of function without the initial enhancement of activity (222, 229, 717). Perhaps the lowest dose of ionizing radiation ever found to produce an alteration in the function of peripheral nerves was reported in a study in which T-shaped preparations of isolated frog sciatic nerves were produced when the nerves were partially divided longitudinally (364). Electrical stimulation was applied to the intact stem of the T, and electrical recordings were made from the ends of the two branches. A small segment of one of the branches was irradiated with 0.04–0.06 Gy of alpha particles, producing a definite decrease in action-potential amplitude and an increase in chronaxy. These results were remarkable because of the much higher doses required to affect these peripheral nerve functions in most other studies.

Paralysis of the hind limbs of animals can result from localized irradiation of the spinal cord. Rabbits developed this paralysis at 4–33 weeks after exposure of the upper thoracic region to 30–110 Gy of X-radiation at 2.5 Gy/day (586). The minimum single exposure found to produce paralysis at 5 months was 20 Gy (587). As in other model systems, the interval between irradiation and the appearance of neurological symptoms decreased as dose increased. For example, 50 Gy of x-rays to the monkey midthoracic spinal cord produced immediate paraplegia, whereas 40 Gy was effective only after a latent period of about 5.5 months (164).

Radiation effects on the electrophysiology of the synapse were first studied using the cat spinal reflex (394, 578–581). These studies showed that excitatory synaptic transmission was significantly increased by x-ray exposures of 4–6 Gy. Synaptic transmission at the upper cervical ganglion of the cat was also facilitated 15–20 min after exposure to 8 Gy of x-rays (481). Both monosynaptic and polysynaptic spinal reflexes were significantly augmented immediately after exposure to 5 Gy of X-radiation. Interestingly, significant augmentation of monosynaptic excitatory postsynaptic potentials (EPSPs) was found immediately after exposure to 6–12 Gy of x-rays, whereas inhibitory postsynaptic potentials (IPSPs) recorded from the same cell were not significantly affected by a 12-Gy exposure (578, 581). Similarly, polysynaptic EPSPs were significantly augmented as the dose increased, whereas the polysynaptic IPSPs were little influenced even by an exposure of 158 Gy. At higher doses (50–200 Gy), ionizing radiation may damage both synaptic and postsynaptic functioning, probably through different molecular mechanisms (649). These radiogenic changes in synaptic transmission may be important factors underlying the complicated functional changes that occur in the CNS following radiation exposures.

Neurochemistry

Ion flow across the neuronal semipermeable membrane is one of the most important mechanisms of postirradiation nervous transmission to be studied. In particular, the flow of sodium ions is believed to be involved in the control of neuronal excitability (99) and apparently can be disrupted after either a very high or very low dose of radiation. A study using the radioactive isotope 24Na compared the sodium intake across the membrane of the squid giant axon before and after exposure to x-rays (563). A significant increase in sodium intake was found to occur during the initial hyperactive period induced by a dose of 500 Gy. These observations were confirmed, although a simultaneous decrease in the rate of sodium extrusion also occurred in a study of frog sciatic nerves that had been irradiated with 1500–2000 Gy of alpha particles (222). As was described earlier, peripheral nerves may be less radiosensitive than CNS neurons and perhaps differ in their radiation response. In a study that used a different technique, the artificially stimulated uptake of sodium into brain synaptosomes was significantly reduced by an ionizing radiation exposure (high-energy electrons or gamma radiation) of 0.1–1000.0 Gy (485, 709).

The brain has been described as a radiosensitive biochemical system (194); in fact, many significant changes in brain neurochemistry have been observed after irradiation. An early study revealed that 1–2 days after an exposure to 3 Gy of X-radiation, neurosecretory granules in the hypophysial-hypothalamic system showed a transient increase in number over the controls (634). A leaking of brain monoamines from the neuronal terminals of rats irradiated with 40 Gy of x-rays was also observed (153). These changes in neuronal structure may correlate with radiogenic alterations of neurotransmitter systems. Normal catecholamine functioning appears to be damaged following exposure to intermediate or high doses of ionizing radiation. After 100 Gy 60Co gamma radiation, a transient disruption in dopamine functioning (similar in some ways to dopamine-receptor blockade) was demonstrated (303). This

radiogenic change in dopaminergic systems is further supported by the finding that a 30-Gy 60Co radiation exposure increased the ability of haloperidol (a dopamine-receptor-blocking drug) to produce cataleptic behavior (328). Relatively low doses of 56Fe (0.1–1.0 Gy) also caused a profound reduction

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in K+-stimulated dopamine release from perfused striatal slices of rat brain (326, 327). This decrement lasted as long as 180 days postexposure. Radiation-induced effects on dopamine have been correlated in time with behavioral deficits. However, other neuromodulators (such as prostaglandins) also seemed to influence dopaminergic systems to help produce some radiation-induced behavioral changes (328). A transient reduction in the norepinephrine content of a monkey hypothalamus was observed on the day of exposure to 6.6 Gy of gamma radiation. Levels of this neurotransmitter returned to normal 3 days later (367). Similar effects were reported elsewhere (673), but another study found no change in noradrenaline after 8.5 Gy of x-rays (323). An increase in the catecholamine enzyme monoamine oxidase (MAO) was reported within 4 min of exposure and lasted for at least 3 h (98).

A variety of functions involving the neurotransmitter acetylcholine (ACH) are significantly altered by exposure to ionizing radiation. ACH synthesis rapidly increased in the hypothalamus of the rat after as little as 0.02 Gy of beta radiation, but it was inhibited at only slightly higher radiation doses (194). A dose of 4 Gy of 60Co gamma radiation produced a long-term increase in the rate of ACH synthesis in dogs (165). Also, high-affinity choline uptake (a correlate of ACH turnover and release) slowly increased to 24% above control levels 15 min after irradiation with 100 Gy (303). Choline uptake returned to normal by 30 min after exposure. Massive doses of gamma or x-rays (up to 600 Gy) were required to alter brain acetylcholinesterase activity (570), whereas much smaller doses depressed plasma acetylcholinesterase by 30% (397).

Exposure to large doses of ionizing radiation resulted in postirradiation hypotension in monkeys (82, 133, 279), with arterial blood pressure decreasing to less than 50% of normal (184). Postirradiation hypotension also produced a decrease in cerebral blood flow immediately after a single dose of either 25 or 100 Gy of 60Co gamma radiation (106, 107, 128, 130). This hypotension may be responsible for the early transient incapacitation (ETI; see later description) observed after a supralethal dose of ionizing radiation (82, 108, 658). A study with untrained monkeys, whose postirradiation blood pressures were maintained by norepinephrine or other pressor drugs, showed that as long as arterial pressure was above a critical level, the monkeys remained attentive and alert (456). However, in a follow-up study on monkeys trained to perform a task, norepinephrine maintained blood pressure but did not consistently improve performance during the first 30 min after irradiation (660). Other authors observed no close association between blood pressure and behavioral changes (424). Further contrary evidence was obtained from experiments with the spontaneously hypertensive rat (SHR), in which exposure to ionizing radiation reduced the blood pressure of most rats to near-normal levels. However, the irradiated SHRs still showed a significant behavioral deficit after exposure to 100 Gy of high-energy electrons (453). Finally, a significant association was found between the degree of hypotension and the frequency of early performance deficits (EPDs) (82). Still, half the monkeys with a 50% drop in blood pressure did not show behavioral decrements. Thus, even though the relationship between decreased blood pressure and impaired performance is intriguing, simple changes in blood pressure may not be sufficient to explain transient behavioral changes.

The massive release of histamine observed after exposure to a large dose of ionizing radiation was proposed as a mediator of radiogenic hypotension and EPDs (183). Histamine was found to be a very active biogenic amine and putative neurotransmitter located in neurons and mast cells throughout the body, especially around blood vessels (180). Attempts to alter the development of behavioral deficits by treating animals with antihistamines before exposure have been encouraging (73, 182, 184). Monkeys pretreated with chlorpheniramine (H1-receptor blocker) performed better and survived longer after irradiation than did controls (184). Similar benefits were observed in irradiated rats (442). Further, the use of diphenhydramine (a histamine H1-receptor antagonist) inhibited radiation-induced cardiovascular dysfunction (11). Because these antagonists produced only partial relief from radiation effects, it appeared that the histamine hypothesis explained just a portion of the behavioral and physiological deficits observed after radiation exposure (94).

When most animal species were exposed to a sufficiently large dose of ionizing radiation, they exhibited lethargy, hypokinesia, and deficits in performance (109, 344, 452). Because these behaviors seemed similar to those observed after a large dose of morphine, a role was proposed for endogenous opioids (endorphins) in the production of radiation-induced behavioral changes (171, 314). Endogenous morphine-like substances were thought to be released as a reaction to some (5, 254, 283), but not all (413), stressful situations. Like a sufficiently large injection of morphine, endogenous opioids produced lethargy, somnolence, and reduction in behavioral responsiveness (335, 413). Cross-tolerance between endorphins and morphine was demonstrated for a variety of behavioral and physiological measures (81, 632). Because of the similarity of radiation- and opiate-induced symptoms, it is not surprising that

endorphins are involved in some aspects of radiogenic behavioral change. Ionizing radiation produced dose-dependent analgesia in mice, and this radiogenic analgesia was reversed by the opiate antagonist naloxone (642). In another experiment, morphine-induced analgesia of the rat was significantly enhanced 24 h after neutron (but not

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gamma) irradiation, suggesting some combined delayed effects of endogenous and exogenous analgesics that may be radiation specific (89). Ionizing radiation exposure also attenuated the naloxone-precipitated abstinence syndrome in morphine-dependent rats (152).

Further supporting the hypothesis that endorphins are involved in radiation-induced behavioral change, C57Bl/6J mice exhibited a stereotypic locomotor hyperactivity similar to that observed after morphine injection, after receiving 10–15 Gy of 60Co gamma radiation (451). This radiogenic behavior was reversed by administering naloxone or by pre-exposing the mice to chronically stressful situations, a procedure that produces endorphin tolerance (447). In addition, opiate-experienced mice reduced the self-administration of morphine after irradiation, suggesting that the internal production of an endorphin reduced the requirement for an exogenous opioid compound (450). Biochemical assays also revealed changes in mouse brain beta-endorphin after exposure to ionizing radiation (449). Rats and monkeys had enhanced blood levels of beta-endorphin after irradiation (10, 158), and morphinetolerant rats showed less performance decrement after irradiation than nontolerant subjects (448). Further, naloxone (1 mg/kg) given immediately before exposure to 100 Gy of high-energy electrons significantly attenuated the early behavioral deficits observed in rats (10). Conversely, rats either underwent no change or were made more sensitive to radiation effects after chronic treatment with naloxone on a schedule that increased the number of endorphin receptors (479). However, the manipulation of opioid systems did not produce total control over postirradiation performance deficits. Thus, these data do not suggest an exclusive role for endorphins in radiogenic behavioral change.

BEHAVIORAL EFFECTS OF IONIZING RADIATION

Behavioral and Neurophysiological Eff effects of Prenatal or Neonatal Radiation Exposure

The developing nervous system is significantly more radiosensitive than the adult nervous system, because ionizing radiation, like other teratogenic agents, is apt to affect embryonic cells with high proliferative and metabolic activity (for reviews, see refs. 347, 645). Thus, prenatal exposure to ionizing radiation can cause either organogenic malformations (abnormal closure of the neural tube) or histogenic abnormalities (abnormal proliferation or migration of neurons) (331, 542). Because postnatal neurogenesis may be protracted (256), radiosensitivity of selected brain areas (e.g., the dentate gyrus) may well extend into the neonatal period and even young adulthood in some species. For example, 4 Gy of 60Co gamma radiation caused a significant reduction in synaptic contacts made by hippocampal neurons from 7-day-old rats (272). Early postnatal exposure to 137Cs (5–25 Gy) produced a dose-dependent reduction in myelin synthesis (316).

Other cells important to the functioning of the CNS (e.g., glia) develop early in gestation and continue to divide in the mature organism. Glia guide neuronal migration in the fetal brain and subserve neuronal activity in the adult. The present limit for detection of morphological changes in glia was determined to be as low as 0.2 Gy x-rays (549).

Corresponding behavioral alteration may also be observed after relatively low doses of ionizing radiation if exposure occurs prenatally or soon after birth when many cells are actively dividing or migrating (104). In fact, behavioral indicators were shown to be more sensitive indicators of radiogenic damage than were morphological assessments of brain development (331, 513). Schull and Otake (590) estimated that survivors exposed in utero to the atomic bombings in Hiroshima and Nagasaki exhibited a diminution in intelligence score of 21–27 points/Gy. The highest risk of severe mental retardation occurred during the 8th-15th week of gestation when radiation exposure coincided with the most rapid period of proliferation of neuronal elements and the migration of immature neurons to the cerebral cortex (514, 589).

The behavioral results of prenatal irradiation are discussed in several in-depth reviews (76, 344, 347). Most of the work focuses on motor performance. Prenatal irradiation can cause significant alterations in gait. For example, D'Amato and Hicks (157) observed a hopping locomotion in rats exposed to 1.5 Gy on gestation days 14 and 15. After some initial difficulty, these animals learned to traverse horizontal ladders by adapting their hopping gait to navigate the rungs. Norton and Kimler (498) also found that rats exposed to 1 Gy from 137Cs source on gestation day 15 showed deficits in muscular endurance as measured by their ability to sustain their own weight by hanging from a rod. This behavioral deficit (and others) were correlated with reduced thickness of the cerebral cortex. Fractionation of the radiation dose resulted in less damage to the developing rat cerebral cortex, as measured by postnatal growth, behavioral tests, and morphological assessment (348, 675). Motor deficits were also observed in rats exposed to a low dose (0.6 Gy) on gestation day 16 (73). These animals had difficulty obtaining a reward if they were required to make motor responses in rapid succession (pressing a lever four times in 2 s).

Locomotor hyperactivity was reported after prenatal or perinatal radiation exposure (445). In particular, locomotor activity was enhanced in mice irradiated with 1 Gy 137Cs on gestation day 14 (461), and in rats exposed

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to 1.25 Gy x-rays on gestation days 14 or 15 (500) as well as 2 Gy x-rays on gestation day 17 (321). Mice exposed to 1.0 Gy 137Cs on day 14 of gestation exhibited higher levels of open-field activity at 19–20 months, but not at 6–7 or 12–13 months of age. Thus, it was concluded that later behavioral changes of prenatally irradiated animals may depend on the age of testing (460).

Other behaviors were also affected by prenatal irradiation. Male mice irradiated with 2 Gy 137Cs on gestation day 14 and tested at 100–135 days of age exhibited increased aggressiveness compared to controls (459). When rats were exposed to 2 Gy X-irradiation on gestation day 17 and tested as adults, they showed enhanced performance on an active avoidance task (requiring movement in a shuttle box) (321, 633), whereas passive avoidance (requiring a freezing response) was impaired (633). Rats exposed to 1.5 Gy 60Co gamma radiation on gestation day 15 exhibited a hyperresponse and delayed habituation on an acoustic startle test (462). Mice receiving 0.1–0.5 Gy x-rays (609) or 0.5 Gy mixed neutron/gamma radiation (175) on gestation day 18 and tested in adulthood showed impairment on a spatial memory task (609). Administration of *d*-amphetamine 10 min before testing as adults, however, alleviated neonatally induced X-irradiation-related deficits in short-term memory of rats (286).

Radiogenic deficits were also observed when animals (previously irradiated in utero) performed tasks with substantial cognitive components (79). In 90-day-old squirrel monkeys exposed to gamma radiation (0.5 or 1 Gy) on gestation days 89–90, the correct responses in visual orientation, discrimination, and reversal learning tasks were significantly lower than that of controls. Decrements in reversal learning persisted undiminished in the irradiated subjects at 2 years of age.

Cognitive dysfunction is a common sequela of cranial radiation therapy (16), particularly in young children treated for acute lymphoblastic leukemia (1, 13, 287, 672). A dose of 24 Gy or more of radiation to the CNS of children under 5 years of age resulted in neurocognitive deficits that, it was reported, may not become apparent until 2–5 years after treatment (478). Young children are most vulnerable due to toxicity to the developing brain. Mullenix and her colleagues, at this writing, are using a neonatal rat model to investigate the use of drugs to mitigate the effects of cranial radiation-induced behavioral deficits (483, 484).

Significant changes in neurotransmitter levels can be measured in brains of rats exposed prenatally (e.g., 0.95 Gy on day 10, 12, or 15 of pregnancy). Marked changes in serotonin and serotonin receptors were found in several brain structures (e.g., hippocampus) and dopamine increased significantly in striatum. There were also significant increases in glutamate, glutamine, and γ-aminobutyric acid (GABA) in cortex, hippocampus, striatum, and thalamus (170). Interestingly, these radiogenic changes in concentrations of neurotransmitters did not correspond with changes in receptor binding (648).

Administration of the N-methyl-D-aspartate (NMDA) receptor antagonist, dizocilpine (MK-801), a glutamate blocker, before neonatal X-irradiation produced a dose-dependent behavioral protection in adult rats with radiation-induced hippocampal damage (446). In a related study, dizocilpine administered 20 min after neonatal 60Co irradiation significantly reduced neuronal damage in rats 6 h after exposure (7).

Behavioral Effects of Adult Radiation Exposure

Naturalistic Behaviors

Naturalistic behaviors (normal parts of an animal's response repertoire) may be altered by radiation exposure. In particular, spontaneous locomotor activity is of interest because this behavior is an important component of many other responses. Jones et al. (324) reported an immediate depression in rat volitional activity-wheel performance following an acute, whole-body dose of 2–7 Gy x-rays. Exposure of rats to 10 Gy high-energy electrons (200) or mice to 10 Gy 60Co gamma radiation (408) resulted in reductions in both horizontal and vertical activity within 1 h of exposure. Radiation-induced hypoactivity has also been reported for a variety of species (for review, see ref. 444). A biphasic locomotor response to radiation exposure (initial decrease, followed by partial recovery and then secondary hypoactivity) was reported for mice (371), and is consistent with the phasic postirradiation clinical symptomatology observed in humans (219).

Exposure to ionizing radiation also produces a dose-dependant reduction in food and water consumption, as well as nausea and vomiting (emesis) in a variety of species (217, 218, 344, 350, 422, 650). Taste-aversion learning (an association between a distinctive taste and radiation-induced malaise) appears to be an especially sensitive indicator of radiation's effects on consummatory behaviors (539, 541). The ED50 for the formation of a taste aversion following 56Fe exposure was only 0.21 Gy (540). Diltiazem, a calcium channel blocker, prevented the onset of radiation-induced taste aversions in rats (482).

Another indicator of gastrointestinal malaise, vomiting, is more likely observed after irradiation with

neutrons than gamma rays (185). Increasing doses of radiation up to 10 Gy (neutron: gamma=0.4), corresponded with the enhanced likelihood of vomiting in the monkey (454). Above 10 Gy, however, the number of monkeys that vomited decreased with increasing dose. The ED50 was approximately 4.5 Gy. Eighty percent

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of monkeys exposed to mixed neutron/gamma radiation (6 Gy), with a high neutron: gamma ratio of 0.85, vomited in about 45 min (415).

The relationship between vomiting and performance decrement was shown to be complex (721), with irradiated animals rarely vomiting during early behavioral incapacitations (see later discussions). For example, Franz (210), found no relationship between vomiting and early performance deficits in monkeys performing in a physical activity wheel and exposed to less than 50 Gy of mixed neutron/gamma radiation. Animals that were not incapacitated but received the same dose as the incapacitated animals vomited as expected (721, 723). Although these data are revealing, the relationship between radiation-induced vomiting and behavioral deficits remains to be fully elucidated. Serotonin receptor blocking agents were shown to be effective against radiation-induced vomiting (51, 186, 211, 415), and to have relatively few behavioral side effects (53, 54, 68).

Social behaviors have not received much attention from radiobiologists. Miyachi and colleagues reported a paradoxical effect when both sexual (468) and aggressive (466, 469) behavior of mice were reduced following exposure to doses of 0.05–0.15 Gy x-rays but not after higher doses of 0.25–0.35 Gy. Maier and Landauer (407, 408) found that aggressive behaviors were surprisingly robust after 10 Gy (electron or 60Co gamma radiation) and persisted until radiogenic moribund behavior was evident.

Motor Performance

Several studies revealed chronic deterioration of motor performance after doses of radiation at or below the LD50. For example, Stapleton and Curtis (616) reported a long-term (42-week) progressive deterioration of forced wheel running behavior in mice exposed to a LD50 dose of neutron radiation. Kimeldorf et al. (346) also found significant reductions in motor capacity of rats swimming daily to exhaustion before and after exposure to 3–10 Gy of x-rays. Rats performing a task where they had to press a bar 20 times rapidly to avoid footshock showed significant performance decrements following 7.5 Gy 60Co gamma radiation. This decrement persisted for the first 4 weeks after exposure (434). Performance of a physically demanding motor task can alter survival after irradiation. Kimeldorf and Jones (345) reported that swimming to exhaustion before and after X-irradiation significantly reduced rat performance and lowered the LD50 by about 2 Gy. Bogo and colleagues (67, 71) observed a similar phenomenon in rats performing a strenuous, shock-motivated motor task after irradiation.

Learning, Memory, and Cognition

A number of studies suggest learning can be altered by ionizing radiation exposure. Meyerson (438) conditioned rabbits to associate a light and tone stimulus with the respiratory reflex of apnea (cessation of breathing) produced by inhalation of ammonia vapor. A 15-Gy exposure to 60Co gamma radiation produced an absent, or considerably reduced, conditioned apnea response to the light/tone. In contrast, the unconditioned apnea (normal response to ammonia inhalation) was enhanced after irradiation, suggesting the performance capacity of the animal was intact. Another investigator (204) found reduced maze-learning behavior after up to 10 Gy of x-rays. Urmer and Brown (662) also reported a temporary reduction in the ability of rats to reorganize previously learned material after 4 Gy 60Co gamma radiation.

These data support the notion of radiation's effects on some components of learning, and they are consistent with other results (90) suggesting that radiogenic disruptions in behavior may not merely reflect defects in nonassociative factors. Although the predominant work in this field concludes there are postirradiation learning deficits, improved or unaltered learning capacity after irradiation was observed under certain circumstances (for review see ref. 444).

Radiation exposure also may disrupt memory. For example, Wheeler and Hardy (697) reported a significant retrograde amnesia in rats after low doses of electron irradiation. Human memory may be impaired by radiation exposure as well. A few cases of acute retrograde amnesia were reported by people who survived the bombing of Hiroshima (320). Five years after the attack, deficits in memory and intellectual capacity were noted in individuals experiencing radiation sickness (138). Although there may be alternative explanations for these amnesias (e.g., psychological trauma), the data seem consistent with Soviet literature, which reported memory deficits in patients undergoing therapeutic irradiations (319).

Exposure to ionizing radiation is known to alter performance on behavioral tasks requiring nondemanding physical movements and the involvement of functional cognitive processes, such as timing, decision making, or concept formation. For example, Cynomolgus monkeys tested 2–3.5 months after a 20-Gy head-only exposure to X- or gamma rays showed a deficit on a series of discrimination problems (551). Cranial irradiation of rats with single doses of 20 or 25 Gy of x-rays produced delayed impairment of spatial learning and working memory (291). Chimpanzees exhibited a chronic inability to

perform an oddity discrimination task after a whole-body 60Co radiation of 4 Gy (551). Highly-trained complex behavior in laboratory rats can be disrupted with

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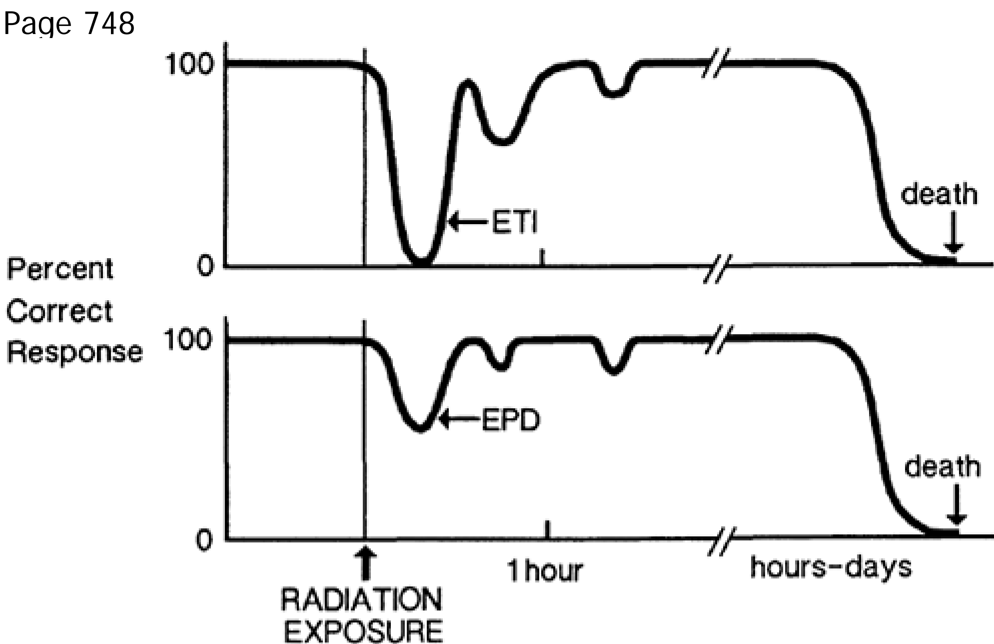

FIG. 15.6. Idealized performance time-courses for acute radiation-induced behavioral decrement. As shown, soon after a sufficiently large dose of radiation, several animal species exhibit an early transient incapacitation (ETI; *upper panel*) or an early performance decrement (EPD; *lower panel*). Subsequent smaller transient deficits may occur approximately 45 min and 4 h later. From References 210, 595, 723.

sublethal levels (4.5–6.75 Gy) of 60Co gamma radiation (433–435, 705, 706). Typically, radiation-induced disruptions in rates and patterns of responding became evident within 1 day of exposure, with a duration of 24–96 h, before returning to pre-exposure levels (435).

Early, Transient Performance Deficits

Transient performance deficits were observed in animals and humans after a large, rapidly delivered dose of ionizing radiation. This response was termed early transient incapacitation (ETI) (590). An idealized, individual ETI profile is shown in the upper part of Figure 15.6. As shown, 5–10 min after radiation exposure, performance rapidly fell to near zero, followed by partial or total recovery 10–15 min later. Delayed ETIs also occurred about 45 min and 4 h after irradiation.

A less severe variant of ETI is early performance decrement (EPD), in which performance is significantly degraded rather than totally suppressed. ETI and EPD were presumed to occur only after supralethal radiation doses in which, following behavioral recovery, death occurred in hours or days. However, some data suggest lower doses may also produce these effects (70).

Early transient performance decrements are modulated by several factors. For instance, the radiation dose required to disrupt behavior is directly related to the requirements of the task being performed. Demanding/complex tasks and tasks that require rapid responding are most disrupted after radiation exposure (for review, see ref. 444). Radiation dose and dose rate can also influence behavioral deficits. When 10 Gy 60Co gamma radiation was given at dose rates from 0.3–1.8/min, it produced 7–81% ETI, respectively (82).

The type of radiation can also differentially influence early transient behavioral deficits. The median effective doses required to disrupt rat performance on an accelerating rotating rod were 61 Gy for 18.6 MeV electrons, 81 Gy for 18.1 MVp Bremsstrahlung, 89 Gy for 1.25 MeV gamma photons, and 98 Gy for 1.67 MeV neutrons. Thus, in contrast to typical lethality data, electrons are significantly more effective than neutrons in producing motor deficits (69).

Several human accidents have involved very large doses of ionizing radiations sufficient to produce behavioral incapacitation. One of these exposures occurred in the early days of fissionable material production at Los Alamos Scientific Laboratory (United States) and resulted in the fatal radiation injury of a worker known as Mr. K. The accident victim received a rapid total body dose of 45 Gy and an estimated upper abdominal dose of 120 Gy of mixed neutrons and gamma radiations (606). During the event, Mr. K either fell or was knocked to the floor. For a short period, he was apparently dazed as he turned his plutonium-mixing apparatus off and then back on again. He was able to run to another room but soon became ataxic and disoriented. He could not stand unaided, was incapacitated, and drifted in and out of consciousness for more than a half hour before he was rushed to a local hospital. Later, Mr.

K regained consciousness and coherence. From 2–30 h after the accident he showed significant behavioral recovery, at some points experiencing euphoria, although his clinical signs were grave. The few hours before his death (35 h postirradiation) were

characterized by irritability, uncooperativeness, mania, and eventually coma. This case is consistent with the animal literature suggesting that a supralethal dose of radiation can produce early transient performance deficits. The physiological and behavioral symptoms associated with acute radiation effects in humans following exposure to doses of 0.5–30 Gy were summarized by Anno and his colleagues (17). The Chernobyl (Ukraine) nuclear reactor accident in 1986 also produced behavioral deficits in personnel attempting to perform duties in high-radiation environments. A firefighter who fought the blaze of the burning reactor core suffered performance deficits and eventually had to withdraw because of radiation exposure (L.Telyatnikov, personal communication, 1987). Another individual exposed to an estimated 2.0–3.5 Gy radiation during the accident was reported to have permanent headaches and vision impairment (64). These human accidents add to the animal literature suggesting sublethal doses of radiation can also induce performance decrements.

Protection Against Radiogenic Behavioral Disruption

Few studies have attempted to normalize behavioral changes observed immediately (up to 24 h) after irradiation. However, as described earlier, antihistamines (i.e., chlorpheniramine) (183, 442) and opiate antagonists (e.g., naloxone) may offer behavioral radioprotection under certain circumstances (446). Other data suggest that estrogens, already known for their ability to reduce the lethal effects of ionizing radiation (642), can reduce the intensity and duration of radiation-induced early transient behavioral deficits in castrated rats trained to perform an avoidance task (441). Currently, no definitive data identify agents capable of completely restoring normal behavior after irradiation-induced changes.

Separate experiments evaluated behavioral toxicity of radioprotectants used for their ability to reduce the lethal effects of irradiation (for reviews, see ref. 87, 411, 695). Most of these radioprotectants act as free-radical scavengers. Early studies administered *n*-decylaminoethanethiosulfuric acid, WR-1607 (10 mg/kg, IV) to monkeys and reported some protection against ETI (598, 659). WR-1607 also extended the life of the subjects beyond that of controls. Today, the leading radioprotective drug is amifostine, formerly known as WR-2721 (635). Although amifostine is available for clinical use in conjunction with radiotherapy (72, 636, 683), the drug's side effects (emesis, hypotension) limit its use (694). Unfortunately, in all species and tasks studied, WR-2721 alone at the best radioprotective doses was behaviorally toxic (that is, it disrupted trained behavior or it reduced locomotor activity), and it potentiated rather than attenuated radiation-induced performance decrements (372, 373, 376, 423). In general, the behavioral toxicity of each of a variety of radioprotective compounds increases in parallel with the radioprotective properties (374, 375).

Psychological Factors of Radiation Exposure

Compared to what we know about the physiological changes brought about by ionizing radiation exposure, we know little about the psychological changes that may also be exhibited. The information we do have is derived from the Japanese atomic bomb experience, human radiation accidents, clinical radiation exposures, and selected animal studies. The data from animal studies reflects behavioral changes resulting from direct effects of radiation on nervous system functioning. The human data reflect additional social, cognitive, and cultural factors that modulate human emotional and psychological phenomena (443).

The animal data have shown, for example, that we can expect motivational changes after sufficiently large doses of ionizing radiation. Rats that initially exerted similar amounts of work to receive rewarding brain stimulation of several different brain nuclei, following exposure to 100 Gy high-energy electrons, stopped working for stimulation of some nuclei (e.g., septum) but continued working for stimulation of the lateral hypothalamus (452). Although these irradiated subjects maintained the capacity to perform barpressing, there was selective modification of their motives such that they chose to exert work to obtain a selected subset of the incentives that were previously all rewarding.

In addition to the physiologically mediated changes in psychological variables that might be revealed by animal studies, human perceptions, interactions, and expectations can combine to produce distinct changes in emotional responses after radiation exposure (136). For example, the nuclear reactor accident in 1979 at Three Mile Island (United States) produced virtually no radiation exposure above background levels. Still, the perceived radiation hazard and the public's "nuclear phobia" evoked long-term emotional, behavioral, and physiological signs of stress (41, 135). The particular fear associated with potential radiation exposure from nuclear power plants seems to be heightened by the fact that ionizing radiation presents an invisible, unfamiliar, man-made (and therefore "unnatural") hazard (572). Interestingly, fear and dread of radiation is lessened when the radiation source is natural and the individual may encounter it in a familiar setting (e.g., his/her own basement), as is the case with radon (693).

Psychological symptoms that have been reported following radiation accidents have been quite dramatic. Fear, anxiety, stress, depression, neurasthenia, and hypochondria were reported as part of the clinical course of persons exposed to radiation during the Chernobyl nuclear power plant accident (150, 151, 244, 336, 651, 676). Deficits in memory, attention, and sensorimotor activities were also observed in these patients (116). Many individuals, and children in particular, reported symptoms of fatigue, pallor, inattention, abdominal pain, and headache as a result of the Chernobyl accident. Ukrainian doctors have labeled this syndrome "vegetative dystonia" (412, 625).

In one of the most highly contaminated areas of Belarus, the results of a large health survey found that the Chernobyl accident caused long-standing loss of health-related quality of life, psychological well-being, and changes in illness behavior (276). Even immigrants from the former Soviet Union were found to have various stress-related disorders many years after leaving the Chernobyl-contaminated areas (151). As might be expected, one of the highest risk groups for these effects were the Chernobyl liquidators, the large number of people who were involved in cleaning up after the accident (676). The clinical significance of this well-documented psychological stress due to the Chernobyl accident, however, is still unknown (240, 277, 677).

The reaction of the public, and even the medical establishment, to radiation accident victims is often characterized as fearful (167). Following the radiation accident in Goiânia, Brazil, in 1987, the radiation exposure victims were subjected to chronic stress (137) and intense ostracism (167). Everyone fled from them, including doctors and nurses who were afraid they would become contaminated themselves (167). These fears are not totally unrealistic. Although the intrinsic radiation produced by a single patient may not be life threatening under these circumstances (139), care of many of these individuals may produce some risk to medical staff.

Summary

Radiation doses below the LD50 (whole body) do not produce permanent sensory changes; however, transient alterations were reported in several modalities at doses from 1–5 Gy. High radiation doses can cause more permanent sensory and perceptual impairments. Radiogenic damage to mature brain morphology may occur after an exposure of less than 15 Gy and is an accepted finding at higher doses. The developing CNS is significantly more sensitive than the mature nervous system to radiation-induced changes in a variety of histological, morphological, and behavioral parameters. Likewise, indicators of brain functioning (e.g., electrophysiology, neurochemistry) are more radiosensitive than are neuroanatomical end points.

Under many circumstances, exposure to ionizing radiations can significantly impede performance. This conclusion is supported by extensive research on experimental animals and limited human experiences. At low to intermediate doses of radiation (up to 10 Gy), performance deficits may be slow in developing and relatively long-lasting. After high doses, the behavioral effects are often rapid (within minutes) and usually abate before the debilitation of chronic radiation sickness begins. These rapid effects can also occur at intermediate doses under certain circumstances.

Not all task performance is equally radiosensitive. Tasks with complex, demanding requirements are more easily disrupted than simple ones. The exception may be found in certain naturalistic behaviors (e.g., eating and drinking behavior) that are also quite radiosensitive. Postirradiation deficits in memory, cognitive ability, and motor performance have been reported. Radiation parameters such as dose, dose rate, and radiation quality can all influence the degree of performance decrement observed.

Many of the pharmacological compounds that protect animals from the lethal effects of ionizing radiation also have severe behavioral effects. In addition to the well-studied physiological and behavioral effects of ionizing radiations, psychological changes (e.g., motivational deficits, anxiety, depression) may also accompany radiation exposure.

CONCLUSION

To assess the average exposure of residents of the United States to ionizing radiation, the National Council on Radiation Protection and Measurements obtained the collective effective dose equivalent from each of six main radiation source categories (492). The collective effective dose equivalent is calculated by multiplying the average per capita effective dose equivalent by the estimated number of people exposed (34). The average effective dose equivalent was then calculated by dividing the collective effective dose equivalent by the total U.S. population. The dose equivalent accounts for differences in relative biological effectiveness by multiplying the absorbed dose by the quality factor while the effective dose equivalent relates the dose equivalent to risk.

As seen in Table 15.2, natural radiation sources contribute 82% of the total average annual effective dose equivalent of 3.6 mSv. By far the largest contribution (55%) is made by radon and its decay

products. Radon in domestic water supplies is also the chief contributor to radiation exposure from consumer products (34).

Although much is written about radiation exposure from nuclear power production and nuclear weapons testing fallout, their contributions are negligible compared to the importance of environmental radon, the largest source of human exposure to ionizing radiation.

Unfortunately, the exception to the rule occurred on April 26, 1986, when the graphite-moderated reactor of Unit No. 4 of the Chernobyl nuclear power station of the former Soviet Union exploded, distributing a large amount of a variety of radionuclids throughout the northern hemisphere in what has been estimated to be the single most costly industrial accident in history (20, 122, 196, 214, 310, 355, 596, 661). One result of the accident is that the contaminated areas of Belarus, Ukraine, and Russia have become a living laboratory of the consequences of radioactive contamination. The ecotoxicological effects of the Chernobyl accident have provided unprecedented observations of radionuclides in the environment and the human health effects are only now becoming evident with additional reports appearing each year. Many of these late reports have been included in this chapter because they are changing what is known about the toxicity of ionizing radiation.

QUESTIONS

1. What processes are involved in the amplification of a single exposure of ionizing radiation to a final end point of biological expression (injury, cancer, or death)?
2. The most important sensitizer of biological tissues to ionizing radiation is________________.
3. Describe how radiation is thought to affect the cell cycle. In which phase of the cell cycle are cells more sensitive to ionizing radiation? In which phase are they more sensitive?
4. Why is a 20 cGy dose of neutron radiation able to result in cataract formation, but not a 20 cGy dose X-radiation? What is the difference between a high LET radiation and a low LET radiation? How are RBE and LET related?
5. The "Law of Bergonie and Tribondeau" put forth in 1906 describes basically why some cells are more sensitive to ionizing radiation than other cells. Why are bone marrow cells and cancer cells sensitive to ionizing radiation?
6. What is the difference between *ionization* and *excitation*? What is a thiol, and how does it interact with a free radical?
7. What is the estimated lethal dose of ionizing radiation for humans? What are the lethality syndromes induced by ionizing radiation? Why can an organism tolerate a larger dose if it is protracted or fractionated, whereas a smaller, acute exposure dose can be lethal?
8. What is the role of apoptosis in response to radiation injury?
9. What have we learned about the effects of human populations living in the relatively highly contaminated areas around the Chernobyl reactor?
10. What is the reality of the actual incidence of birth defects from human exposure to environmental (not medical) radiation?
11. Discuss the behavioral alterations that can occur as a result of prenatal irradiation.
12. What is radiation-induced "early transient incapacitation"?
13. Explain the relatively high incidence of thyroid cancer in Belarus, Ukraine, and Russia in the 1990s.
14. How would a diet high in Brazil nuts contribute to a high body-burden of radionuclides and where in the body would they be concentrated?
15. Explain the difference in the risk of consuming clams, oysters, or scallops from an area contaminated with 60Co and from one contaminated with 90Sr.
16. Explain why denudation of the gastrointestinal mucosa may not be seen with death occurring from the neurovascular syndrome associated with the acute radiation syndrome.
17. Why does the conceptus exhibit the lowest irradiation LD50 of any stage of an organism's life?
18. How does irradiation depress both the specific and nonspecific immune responses?

REFERENCES

1. Abayomi, O.K. (1996): Pathogenesis of irradiation-induced cognitive dysfunction. *Acta. Oncol.*, 35:659–663.
2. Abbas, B., Boyle, F.C., Wilson, D.J., et al. (1990): Radiation induced changes in the blood capillaries of rat duodenal villi: A corrosion cast, light and transmission electron microscopical study. *J. Submicrosc. Cytol. Pathol. (BOLOGNA)*, 22:63–70.
3. Abbas, B., Hume, S.P., McCullough, J.S., et al. (1990): Early morphological changes in blood capillaries of mouse duodenal villi induced by X-irradiation. *J. Submicrosc. Cytol. Pathol. (BOLOGNA)* 22:609–614.
4. Abdullin, G.Z. (1962): Study of comparative radiosensitivity of different parts of brain in terms of altered function. *Atomic Energy Commission TR-5141.* OTS/Department of Commerce, Washington, DC.

5. Akil, H., Madden, J., Patrick, R.L. III, and Barchas, J.D. (1976): Stress-induced increase in endogenous opioid peptides: Concurrent analgesia and its reversal by naloxone. In: *Opiates and Endogenous Opiate Peptides*, edited by H.W.Kosterlitz, p. 63. Elsevier North-Holland, Amsterdam.
6. Akiyama, M. (1995) Late effects of radiation on the human immune system: An overview of immune response among the atomic-bomb survivors. *Int. J. Radiat. Biol.*, 68:497–508.

7. Alaoui, F., Pratt, J., Trocherie, S., et al. (1995): Acute effects of irradiation on the rat brain: Protection by glutamate blockade. *Eur. J. Pharmacol.*, 276:55–60.
8. Alman, R.W., Rosenberg, M., and Fazekas, J.F. (1952): Effects of histamine on cerebral hemodynamics and metabolism. *A.M.A. Arch. Neurol Psychiat.*, 67:354–356.
9. Al-sarraf, M., LeBlanc, M., Giri, P.G.S., et al. (1998): Chemoradiation therapy versus radiation therapy in patients with advanced nasopharyngeal cancer: Phase III randomized Intergroup study 0099. *J. C. O.*, 16:1310–1317.
10. Alter, W., Mickley, G.A., Catravas, G., et al. (1980): Role of histamine and beta-endorphin in radiation-induced hypotension and acute performance decrement in the rat. In: *Proceedings of the 51st Annual Meeting of the Aerospace Medical Association*, pp. 225–226. Anaheim, CA; 7–11 May 1980.
11. Alter, W.A., Catravas, G.N., Hawkins, R.N., and Lake, C.R. (1984): Effect of ionizing radiation of physiological function in the anesthetized rat. *Radiat. Res.*, 99:394–409.
12. Altman, K.I., Gerber, G.B., and Okada, S. (1970): *Radiation Biochemistry.* Academic Press, New York.
13. Anderson, V., Godber, T., Smibert, E., and Ekert, H. (1997): Neurobehavioural sequelae following cranial irradiation and chemotherapy in children: An analysis of risk factors. *Pediatr. Rehabil.*, 1:63–76.
14. Andrew, F.D., and Lytz, P.S. (1981): Biochemical disturbances associated with developmental toxicity. In: *Developmental Toxicology*, edited by C.Kimmel and J.Buelke-Sam, pp. 145–165. Raven Press, New York.
15. Andrushchak, L.I., Gol'Dshmid, B.Y., Nikitchenko, V.V., et al. (1993): Morphological and ultrastructural changes in small intestine of rats under long-term constant action of low doses of ionizing radiation (in Russian). *Tsitologiya I Genetika*, 27:13–19.
16. Andrykowski, M.A., Altmaier, E.M., Barnett, R.L., et al. (1990): Cognitive dysfunction in adult survivors of allogeneic marrow transplantation: Relationship to dose of total body irradiation. *Bone Marrow Transplant*, 6:269–276.
17. Anno, G.H., Baum, S.J., Withers, H.R., and Young, R.W. (1989): Symptomatology of acute radiation effects in humans after exposure to doses of 0.5–30=Gy. *Health Phys.*, 5:821–838.
18. Anscher, M.S., Kong, F.-M., and Jirtle, R.L. (1998): The relevance of transforming growth factor *B*1 in pulmonary injury after radiation therapy. *Lung Cancer*, 19:109–120.
19. Antonellia, A., Silvano, G., Bianchi, F., et al. (1995): Risk of thyroid nodules in subjects occupationally exposed to radiation: A cross-sectional study. *Occup. Environ. Med.*, 52:500–504.
20. Aoyama, M., Hirose, K., Inoue, H., et al. (1989): 30 years records of the radioactive fallout in Japan. *J. Radiat. Res. (Tokyo)* 30:11.
21. Apanasenko, Z.I. (1967): Combined effect of double exposure to vibration and chronic irradiation on the functional state of vestibular apparatus. In: *NASA Technical Translation, F-413*, pp. 212–228. National Aeronautic and Space Administration, Washington, DC.
22. Archambeau, J.O. (1987): Relative radiation sensitivity of the integumentary system: Dose response of the epidermal, microvascular, and dermal populations. In: *Advances in Radiation Biology, Vol. 12, Relative Radiation Sensitivities of Human Organ Systems*, edited by J.T.Lett and K.I.Altman, pp. 147–203. Academic Press, San Diego.
23. Arena, V. (1971): *Ionizing Radiation and Life.* Mosby, St. Louis.
24. Arnold, A., Bailey, P., and Harvey, R.A. (1954): Intolerance of primate brain stem and hypothalamus to conventional high energy radiations. *Neurology*, 4:575–585.
25. Arnold, M., and Kummermehr, J. (1988): Radiation induced damage to the regenerative capacity of surgically traumatized rat femur after single doses of x-rays. In: Terrestrial Space Radiation and its Biological Effects, edited by P.D.McCormack, C.E.Swenberg, and H.Bücker, pp. 475–486. Plenum Press, New York.
26. Arsenian, M.A. (1991): Cardiovascular sequelae of therapeutic thoracic radiation. *Prog. Cardiovasc. Dis.*, 33:299–311.
27. Asfandiyarova, N.S., Romadin, A.E., Kolcheva, N.G., et al. (1998): Immunity system in residents of territories contaminated with radionuclides after the Chernobyl accident (in Russian). *Terapevticheskii Arkhiv.*, 70:55–59.
28. Astakhova, L.N., Anspaugh, L.R., Beebe, G.W., et al. (1998): Chernobyl-related thyroid cancer in children of Belarus: A case-control study. *Radiat. Res.*, 150:349–356.
29. Astor, M.B., Anderson, M.E., and Meister, A. (1988): Relationship between intracellular GSH levels and hypoxic cell radiosensitivity. *Pharmac. Ther.*, 39:115–121.
30. Auerbach, C. (1958): Radiomimetic substances. *Radiat. Res.*, 9:33–47.

31. Babadzhanova, Sh. A., and Busakov, B.S. (1997): Radiation as a risk factor for nerve system diseases. *Uzbekiston Tibbiet Zhurnali*, 5–7:41–43.
32. Bachofer, C.S. (1957): Enhancement of activity of nerves by x-rays. *Science*, 125:1140–1141.
33. Bachofer, C.S., and Gautereaux, M.E. (1959): X-ray effects on single nerve fibers. *J. Gen. Physiol.*, 42:723–735.
34. Bachofer, C.S., and Gautereaux, M.E. (1960): Bioelectric activity of mammalian nerves during X-irradiation. *Radiat. Res.*, 12:575–586.
35. Bachofer, C.S., and Gautereaux, M.E. (1960): Bioelectric response in situ of mammalian nerves exposed to x-rays. *Am. J. Physiol.*, 198:715–717.
36. Baetcke, K.P., Sparrow, A.H., Nauman, C.H., and Schwemmer, S.S. (1967): The relationship of DNA content to nuclear and chromosome volumes and to radiosensitivity (LD50). *Proc. Natl. Acad. Sci. U.S.A.*, 58:533–540.
37. Baisakhatov, R., and Khanson, K.P. (1971): Comparison of the content of adenylic nucleotides and the activity of the process of oxidative phosphorylation in the rat thymus after total X-irradiation. *Radiobiologiya,* 11:155–159.
38. Baker, D.G., and Krochak, R.J. (1989): The response of the microvascular system to radiation: A review. *Cancer Invest.*, 7:287–294.
39. Barcellos-Hoff, M.H. (1993): Radiation-induced transforming growth factor *B* and subsequent extracellular matrix reorganization in the murine mammary gland. *Cancer Res.*, 53:3880–3886.
40. Bassant, M.H., and Court, L. (1978): Effects of whole-body irradiation on the activity of rabbit hippocampal neurons. *Radiat. Res.*, 75:593–606.
41. Baum, A., Gatchel, R.J., and Schaeffer, M.A. (1983): Emotional, behavioral, and physiological effects of chronic stress at Three Mile Island. *J. Consult. Clin. Psychol.*, 51:565–572.
42. Baum, S.J., Anno, G.H., Young, R.W., and Withers, H.R. (1984): Nuclear weapon effect research at PSR-1983: Vol. 10, Symptomatology of acute radiation effects in humans after exposure to doses of 75 to 4500 Rads (cGy) free-in-air. *DNA TR-85–50.* Defense Nuclear Agency, Washington, DC.
43. Bayraktar, M., Gedik, O., Akalin, S., et al. (1990): The effect of radioactive iodine treatment on thyroid C cells. *Clin. Endocrinol., 33:625–630.*
44. Beard, C.M., Heath, H., 3d, O'Fallon, W.M., et al. (1989): Therapeutic radiation and hyperparathyroidism. A case-control study in Rochester, Minn. *Arch. Intern. Med.*, 149:1887–1890.
45. Becciolini, A. (1987): Relative radiosensitivities of the small and large intestine. In: *Advances in Radiation Biology. Vol. 12, Relative*

Radiation Sensitivities of Human Organ Systems, edited by J.T. Lett and K.I.Altman, pp. 83–128. Academic Press, San Diego.
46. Bedford, J.S., and Mitchell, J.B. (1973): Dose rate effects in synchronous mammalian cells in culture. *Radiat. Res.*, 54:316–327.
47. Beetz, A., Messer, G., Oppel, T., et al. (1997): Induction of interleukin 6 by ionizing radiation in a human epithelial cell line: Control by corticosteroids. *Int. J. Radiat. Biol.*, 72:33–43.
48. BEIR III. (1980): The effects on populations of exposure to low levels of ionizing radiation. Report of the Committee on the Biological Effects of Ionizing Radiations, National Research Council. National Academy Press, Washington, DC.
49. BEIR IV. (1988): Health risks of radon and other internally deposited alpha-emitters. Report of the Committee on the Biological Effects of Ionizing Radiations, National Research Council. National Academy Press, Washington, DC.
50. BEIR V. (1990): Health effects of exposure to low levels of ionizing radiation. Report of the Committee on the Biological Effects of Ionizing Radiations, National Research Council. National Academy Press, Washington, DC.
51. Belkacemi, Y., Ozsahin, M., Pene, F., et al. (1996): Total body irradiation prior to bone marrow transplantation: Efficacy and safety of granisetron in the prophylaxis and control of radiationinduced emesis. *Int. J. Radiat. Oncol. Biol. Phys.*, 36:77–82.
52. Bengtsson, G. (1991): Introduction: Present knowledge on the effects of radioactive contamination on pregnancy outcome. *Biomed. Pharmacother.*, 45:221–223.
53. Benline, T.A., and French, J. (1997): Anti-emetic drug effects on cognitive and psychomotor performance: granisetron vs. ondansetron. *Aviat. Space Environ. Med.*, 68:504–511.
54. Benline, T.A., French, J., and Poole, E. (1997): Anti-emetic drug effects on pilot performance: Granisetron vs. ondansetron. *Aviat. Space Environ. Med.*, 68:998–1005.
55. Berg, N.O., and Lindgren, M. (1958): Time dose relationship and morphology of delayed radiation lesions of the brain of the rabbit. *Acta Radiol.*, 167:1–118.
56. Berger, N.A., Sims, J.L., Catino, D.M., and Berger, S.J. (1983): Poly(ADP-ribose)polymerase mediates the suicide response to massive DNA damage: Studies in normal and DNA-repair defective cells. In: *ADP-Ribosylation, DNA Repair and Cancer*, edited by M.Miwa, O.Hayaisha, S.Shall, M.Smulson, and T. Sugimura, pp. 219–226. Japan Sci Soc Press, Tokyo.
57. Bergonie, J., and Triblondeau, L. (1906): De quelques resultats de la radiotherapie et essai de fixation d'une technique rationannelle. *Comptes rendus des seances de l'acadenie des sciences*, 143:983–985. English translation by Fletcher G.H. (1959): Interpretation of some results of radiotherapy and an attempt at determining a logical technique of treatment. *Radiat. Res.*, 11:587–588.
58. Bermudez, J., Boyle, E.A., Miner, W.D., and Sanger, G.J. (1988): The anti-emetic potential of the 5-hydroxytryptamine3 receptor antagonist BRL 43694. *Br. J. Cancer*, 58:644–650.
59. Biaglow, J.E., Varnes, M.E., Clark, E.P., and Epp, E.R. (1987): Role of glutathione and other thiols in cellular response to radiation and drugs. In: *Radiation Research. Proceedings of the 8th International Congress of Radiation Research, Vol 2, Edinburgh, July 1987*, edited by E.M.Fielden, J.F.Fowler, J. H.Hendry, and D.Scott, pp. 677–682. Taylor and Francis, New York.
60. Biard, D.S.F., Saintigny, Y., Maratrat, M., et al. (1997): Enhanced expression of the Kin17 protein immediately after low doses of ionizing radiation. *Radiat. Res.*, 147:442–450.
61. Bigbee, W.L., Jensen, R.H., Veideaum, T., et al. (1996): Glycophorin A biodosimetry in Chernobyl cleanup workers from the Baltic countries. *B. M. J.*, 312:1078–1079.
62. Bigbee, W.L., Jensen, R.H., Veidebaum, T., et al. (1997): Biodosimetry of Chernobyl cleanup workers from Estonia and Latvia using the glycophorin A in vivo somatic cell mutation assay. *Radiat. Res.*, 147:215–224.
63. Billen, D. (1987): Free radical scavenging and the expression of potentially lethal damage in X-irradiated repair deficient *Escherichia coli. Radiat. Res.*, 111:354–360.
64. Birioukov, A., Meurer, M., Peter, R.U., et al. (1993): Male reproductive system in patients exposed to ionizing irradiation in the Chernobyl accident. *Arch. Androl.*, 30:99–104.
65. Blank, K.R., Rudolz, M.S., Kao, G.D., et al. (1997): The molecular regulation of apoptosis and implications for radiation oncology. *Int. J. Radiat. Biol.*, 71:455–466.
66. Bleuer, J.P., Averkin, Y.I., and Abelin, T. (1997): Chernobyl-related thyroid cancer: What evidence for role of short-lived iodines? *Environ. Health Perspect.*, 105:1483–1486.
67. Bogo, V. (1988): Radiation: Behavioral implications in space. *Toxicology*, 49:299–307.
68. Bogo, V., Boward, C., Fiala, N., et al. (1989): Zacopride: A nonbehaviorally toxic radiation antiemetic.

In: *Proceedings of the 60th Annual Meeting of the Aerospace Medical Association*, A31. Washington, DC; 12–15 May, 1989.
69. Bogo, V., Dennison, B.A., and Mulvihill, M. (1989): Motor performance, radiation and mortality in rats. In: *Proceedings of the 37th Annual Meeting of the Radiation Research Society*, 1:139. Seattle, WA; 18–23 March 1989.
70. Bogo, V., Franz, C.F., and Young, R.W. (1987): Effects of radiation on monkey visual discrimination performance. In: *Proceedings of the 8th International Congress of Radiation Research, Edinburgh, July 1987*, edited by E.M.Fielden, J.F.Fowler, J. H., Hendry, and D.Scott, p. 259. International Association for Radiation Research, Edinburgh.
71. Bogo, V., Zeman, G.H., and Dooley, M. (1989): Radiation quality and rat motor performance. *Radiat. Res.*, 118:341–352.
72. Bohuslavizki, K.H., Brenner, W., Klutmann, S., et al. (1998): Radioprotection of salivary glands by amifostine in high-dose radioiodine therapy. *J. Nucl. Med.*, 39:1237–1242.
73. Bornhausen, M. (1986): Analysis of behavioral changes induced by prenatal irradiation. In: *Radiation Risks to the Developing Nervous System*, edited by H.Kriegel, W.Schmahl, G.B.Gerber, and F.-E. Stieve, pp. 283–293. Gustav Fischer Verlag, Stuttgart.
74. Bossola, M., Merrick, H.W., Eltaki, A., et al. (1990): Rat liver tolerance for partial resection and intraoperative radiation therapy: Regeneration is radiation dose dependent. *J. Surg. Oncol.*, 45:196–200.
75. Breiter, N., Trott, K.R., and Sassy, T. (1989): Effect of X-irradiation on the stomach of the rat. *Int. J. Radiat., Oncol. Biol. Phys.*, 17:779–784.
76. Brent, R.L. (1984): The effects of ionizing radiation, microwaves, and ultrasound on the developing embryo: Clinical interpretations and applications of the data. *Curr. Prob. Pediatr.*, 14:1–87.
77. Brent, R.L., Beckman, D.A., and Jensh, R.P. (1987): Relative radiosensitivity of fetal tissues. In: *Advances in Radiation Biology, Vol. 12, Relative Radiation Sensitivities of Human Organ Systems*, edited by J.T.Lett, and K.I.Altman, pp. 239–256. Academic Press, San Diego.
78. Breuer, R., Tochner, Z., Conner, M.W., et al. (1992): Superoxide dismutase inhibits radiation-induced lung injury in hamsters. *Lung*, 170:19–29.
79. Brizzee, K.R., and Ordy, J.M. (1986): Effects of prenatal ionizing radiation on neural function and behavior. In: *Radiation Risks to the Developing Nervous System*, edited by H.Kriegel, W.Schmahl, G.B.Gerber, and F.-E.Stieve, pp. 255–282. Gustav Fischer Verlag, Stuttgart.

80. Brown, J.M., and Kovacs, M.S. (1993): Visualization of nonreciprocal chromosome exchanges in irradiated human fibroblasts by fluorescence in situ hybridization. *Radiat. Res.*, 136:71–96.
81. Brown, R.G., and Segal, D.S. (1980): Alterations in beta-endorphin-induced locomotor hyperactivity in morphine tolerant rats. *Neuropharmacology*, 19:619–621.
82. Bruner, A. (1977): Immediate dose-rate effects of 60Co on performance and blood pressure in monkeys. *Radiat. Res.*, 70:378–390.
83. Bruni, J.E., Persaud, T.V., Froese, G., et al. (1994): Effects of in utero exposure to low dose ionizing radiation on development in the rat. *Histol. Hisiopathol.*, 9:27–33.
84. Bruni, J.E., Persaud, T.V., Huang, W., et al. (1993): Postnatal development of the rat CNS following in utero exposure to a low dose of ionizing radiation. *Exp. Toxicol. Pathol.*, 45:223–231.
85. Bucky, G., Blank, F., and Distelheim, I.H. (1950): Influence of genz rays on histamine-induced manifestations. *Arch. Derm. Syph.*, 62:319–322.
86. Buell, M.G., and Harding, R.K. (1989): Proinflammatory effects of local abdominal irradiation on rat gastrointestinal tract. *Dig. Dis. Sci.*, 34:390–399.
87. Bump, E., and Malaker, K. (1998): *Radiprotectors: Chemical, Biological, and Clinical Perspectives.* CRC Press, Washington, DC.
88. Burger, A., Loeffler, H., Bamburg, M., and Rodemann, H.P. (1998): Molecular and cellular basis of radiation fibrosis. *Int. J. Radiat. Biol.*, 73:401–408.
89. Burghardt, W.F., and Hunt, W.A. (1984): The interactive effects of morphine and ionizing radiation on the latency of tail withdrawal from warm water in the rat. In: *Proceedings of the 9th Symposium on Psychology in the Department of Defense*, edited by G. E.Lee, T.E.Ulrich, pp. 73–76. USAFA Technical Report No. 84–2. U.S. Air Force Academy, Colorado Springs, CO.
90. Burt, D.H., and Ingersoll, E.H. (1965): Behavioral and neuropathological changes in the rat following X-irradiation of the frontal brain. *J. Comp. Physiol. Psychol.*, 59:90–93.
91. Byelorussia and Chernobyl. (1991): The delegation of the Byelorussian SSR at the 45th session of the UN General Assembly: A review, pp. 6–52. Minsk Belarus Publishers, Minsk, Belarus.
92. Calvo, W. (1993): Experimental radiation damage of the central nervous system. *Recent Results Cancer Res.*, 130:175–188.
93. Carlson, R.G., Mayfield, W.R., Normann, S., and Alexander, J. A. (1991): Radiation-associated valvular disease. *Chest*, 99:538–545.
94. Carpenter, D.O. (1979): Early transient incapacitation: A review with considerations of underlying mechanisms. *AFRRI Scientific Report, SR 79–1.* Armed Forces Radiobiology Research Institute, Bethesda, MD.
95. Carter, S., Auer, K.L., Reardon, D.B., et al. (1998): Inhibition of mitogen activated protein (MAP) kinase cascade protentiates cell killing by low dose ionizing radiation in A431 human squamous carcinoma cells. *Oncogene*, 16:2787–2796.
96. Cassaret, G.W. (1980): *Radiation Histopathology.* CRC Press, Boca Raton, FL.
97. Castillo, L.A., Craft, A.W., Kernahan, J., et al. (1990): Gonadal function after 12-Gy testicular irradiation in childhood acute lymphoblastic leukaemia. *Med. Pediatr. Oncol.*, 18:185–189.
98. Catravas, G.N., and McHale, C.G. (1973): Activity changes of brain enzymes in rats exposed to different qualities of ionizing radiation. *AFRRI Scientific Report, SR 73–19.* Armed Forces Radiobiology Research Institute, Bethesda, MD.
99. Catterall, W.A. (1984): The molecular basis of neuronal excitability. *Science*, 223:653–661.
100. Caveness, W.F. (1977): Pathology of radiation damage to the normal brain of the monkey. *Natl. Cancer. Inst. Monogr.*, 46:57–76.
101. Caveness, W.F. (1980): Experimental observations: Delayed necrosis in normal monkey brain. In: *Radiation Damage to the Nervous System*, edited by H.A.Gilbert and A.R.Kagen, pp. 1–38. Raven Press, New York.
102. Cerveny, T.J., and Cockerham, L.G. (1986): Medical management of internal radionuclide contamination. *Med. Bull. U.S. Army Eur.*, 43:24–27.
103. Cerveny, T.J., MacVittie, T.J., and Young, R.W. (1989): Acute radiation syndrome in humans. In: *Medical Consequences of Nuclear Warfare*, edited by R.I.Walker and T.J.Cerveny, pp. 15–36. TMM Publications, Falls Church, VA.
104. Chaillan, F.A., Devigne, C, Diabira, D., et al. (1997): Neonatal gamma-ray irradiation impairs learning and memory of an olfactory associative task in adult rats. *Eur. J. Neurosci.*, 9:884–894.
105. Champlin, R. (1988): Treatment for victims of nuclear accidents: The role of bone marrow transplantation. *Radiat. Res.*, 113:205–210.

106. Chapman, P.H., and Young, R.J. (1968): Effect of cobalt-60 gamma irradiation on blood pressure and cerebral blood flow in the Macaca mulatta. *Radiat. Res.*, 35:78–85.
107. Chapman, P.H., and Young, R.J. (1968): Effect of head versus trunk fission-spectrum radiation on learned behavior in the monkey. *USAF SAM Technical Report, TR 68–80*. Brooks Air Force Base: School of Aerospace Medicine. San Antonio, TX.
108. Chapman, P.H., and Young, R.J. (1968): Effect of high energy X-irradiation of the head on cerebral blood flow and blood pressure in the Macaca mulatta. *Aerosp. Med.*, 3:1316–1321.
109. Chaput, R.L., and Wise, D. (1970): Miniature pig incapacitation and performance decrement after mixed gamma-neutron irradiation. *Aerosp. Med.*, 41:290–293.
110. Charlton, D.E., Nikjoo, H., and Humm, J.L. (1989): Calculation of initial yields of single- and double-strand breaks in cell nuclei from electrons, protons and alpha particles. *Int. J. Radiat. Biol.*, 56:1–19.
111. Chaterjee, J., De, K., Basu, S.K., and Das, A.K. (1994): Low-level x-ray exposures on rat skin: Hyperkeratinization and concomitant changes in biometal concentration. *Biol. Trace Element Res.*, 46:203–210.
112. Chen, A.Y., Okunieff, P., Pommier, Y., and Mitchell, J.B. (1997): Mammalian DNA topoisomerase I mediates the enhancement of radiation cytotoxicity by camptothecin derivatives. *Cancer Res.*, 57:1529–1536.
113. Cherkasova, L.S., and Mironova, T.M. (1976): Effects of ionizing radiation on enzymes of carbohydrate metabolism. *Radiobiologiya*, 16:657–664.
114. Chesser, R.K., Sugg, D.W., Lomakin, M.D., et al. 134,137Cesium, 90Strontium and partial dose rate estimates in small mammals at Chernobyl. *J. Environ. Toxicol. Chem.*, 2000; 19:305–312.
115. Chieng, P.U., Huang, T.S., Chang, C.C., et al. (1991): Reduced hypothalamic blood flow after radiation treatment of nasopharyngeal cancer: SPECT studies in 34 patients. *A.J.N.R. Am. J. Neuroradiol.*, 12:661–665.
116. Chinkina, O.V. (1991): Psychological characteristics of patients exposed to accidental irradiation at the Chernobyl atomic-power station. In: *The Medical Basis for Radiation-Accident Preparedness III: The Psychological Perspective*, edited by R.C.Ricks, M.E.Berger, and F.M.O'Hara, pp. 93–103. Elsevier, New York.
117. Chiu, S.-M., Xue, L.-Y., Friedman, L.R., and Olenik, N.L. (1992): Chromatin compaction and the efficiency of formation of DNA-protein cross-links in γ-irradiated mammalian cells. *Radiat. Res.*, 129:184–191.
118. Chmura, S.J., Nodzenski, E., Beckett, M.A., et al. (1997): Loss of ceramide production confers resistance to radiation-induced apoptosis. *Radiat. Res.*, 57:1270–1275.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_754.html[2010/10/28 18:47:31]

119. Christensen, H.D., Flesher, A.M., and Haley, T.J. (1969): Changes in brain self-stimulation rates after exposure to X-irradiation. *J. Pharm. Sci.*, 58:128–129.
120. Cilliers, G.D., Harper, I.S., and Lochner, A. (1989): Radiation-induced changes in the ultrastructure and mechanical function of the rat heart. *Radiother. Oncol.*, 16:311–326.
121. Clarke, R.H. (1987): Dose distributions in western Europe following Chernobyl. In: *Radiation and Health. The Biological Effects of Low-Level Exposure to Ionizing Radiation*, edited by R.Jones, and R.Southwood, pp. 251–264. John Wiley & Sons, New York.
122. Clarke, R.H. (1989): Current radiation risk estimates and implications for the health consequences of Windscale, TMI and Chernobyl accidents. In: *Medical Response to Effects of Ionizing Radiation*, edited by W.A.Crosbie, and J.H., Gittus, pp. 103–118. Elsevier Science, New York.
123. Cockerham, L.G., Arroyo, C.M., and Hampton, J.D. (1988): Effects of 4-hydroxypyrazolo (3,4-d) pyrimidine (Allopurinol) on postradiation cerebral blood flow: Implications of free radical involvement. *Free Radic. Biol. Med.*, 4:279–284.
124. Cockerham, L.G., Cerveny, T.J., and Hampton, J.D. (1986): Postradiation regional cerebral blood flow in primates. *Aviat. Space Environ. Med.*, 57:578–582.
125. Cockerham, L.G., Doyle, T.F., Donlon, M.A., and Gossett-Hagerman, C.J. (1985): Antihistamines block radiation-induced increased intestinal blood flow in canines. *Fundam. Appl. Toxicol.*, 5:597–604.
126. Cockerham, L.G., Doyle, T.F., Donlon, M.A., and Helgeson. E. A. (1984): Canine postradiation histamine levels and subsequent response to Compound 48/80. *Aviat. Space Environ. Med.*, 55:1041–1045.
127. Cockerham, L.G., Doyle, T.F., Paulter, E.L., and Hampton, J.D. (1986): Disodium cromoglycate, a mast-cell stabilizer, alters postradiation regional cerebral blood flow in primates. *J. Toxicol. Environ. Health*, 18:91–101.
128. Cockerham, L.G., and Forcino, C.D. (1995): Effect of antihistamines, disodium cromoglycate (DSCG) or methysergide on post-irradiation cerebral blood flow and mean systemic arterial blood pressure in primates after 25 Gy, whole-body, gamma irradiation. *J. Radiat. Res. (Tokyo)*, 36:77–90.
129. Cockerham, L.G., Forcino, T.C., Pellmar, T.C, and Smart, S.W. (1987): Effect of methysergide on postirradiation hypotension and cerebral ischemia. In: *Proceedings of the Cerebral Hypoxia and Stroke Symposium*. Budapest, Hungary, August 22–24.
130. Cockerham, L.G., Hampton, J.D., and Doyle, T.F. (1986): Dose dependent radiation-induced hypotension in the canine. *Life Sci.*, 39:1543–1547.
131. Cockerham, L.G., and Hawkins, R.N. (1987): Radiation injury and the splanchnic circulation. In: *Pathophysiology of the Splanchnic Circulation*, Vol. II, edited by P.R.Kvietys, J.A. Barrowman, and D.N.Granger, pp. 55–66. CRC Press, Boca Raton, FL.
132. Cockerham, L.G., Pautler, E.L., Carraway, R.E., et al. (1988): Effect of disodium cromoglycate (DSCG) and antihistamines on postirradiation cerebral blood flow and plasma levels of histamine and neurotensin. *Fundam. Appl. Toxicol.*, 10:233–242.
133. Cockerham, L.G., and Prell, G.D. (1989): Prenatal radiation risk to the brain. *Neurotoxicology*, 10:467–474.
134. Cockerham, L.G., Prell, G.D., and Cerveny, T.J., et al. (1991): Effects of aminoguanidine on pre- and postirradiation regional cerebral blood flow and systemic blood pressure in the primate. *Agents Actions*, 32:237–244.
135. Collins, D.L. (1991): Stress at Three Mile Island: Altered perceptions, behaviors, and neuroendocrine measures. In: *The Medical Basis for Radiation-Accident Preparedness III: The Psychological Perspective*, edited by R.C.Ricks, M.E.Berger, and F.M.O'Hara, pp. 71–79. Elsevier, New York.
136. Collins, D.L. (1992): Behavioral differences of irradiated persons associated with the Kyshtym, Chelyabinsk, and Chernobyl nuclear accidents. *Mil. Med.*, 157:548–552.
137. Collins, D.L., and de Carvalho, A.B. (1993): Chronic stress from the Goiania 137Cs radiation accident. *Behav. Med.*, 18:149–157.
138. Committee for the Compilation of Materials on Damage Caused by the Atomic Bombs in Hiroshima and Nagasaki. (1981): Psychological trends among A-bomb victims. In: *Hiroshima and Nagasaki: Physical, Mental and Social Effects of the Atomic Bombings*, translated by E.Ishikawa, and D.Swain, pp. 485–500. Basic Books, New York.
139. Conklin, J.J., and Walker, R.I. (1987): Diagnosis, triage, and treatment of casualties. In: *Medical Radiobiology*, J.J.Conklin, and R.I.Walker, pp. 231–240. Academic Press, Orlando, FL.
140. Cooper, J.S. (1997): Classic and acquired immunodeficiency syndrome (AIDS)-related Kaposi's

sarcoma. In: *Principles and Practice of Radiation Oncology*, 3rd ed, edited C.A.Perez, and L.W.Brady, pp. 745–762. Lippincott-Raven Publishers, Philadelphia.
141. Cox, A.B., Lee, A.C., and Lett, J.T. (1988): Delayed effects of proton irradiation in the lens and integument: A primate model. In: *Terrestrial Space Radiation and its Biological Effects*, edited P.D.McCormack, C.E.Swenberg, and H.Bücker, pp. 415–422. Plenum Press, New York.
142. Cristaldi, M., D'Arcangelo, E.D., and Leradi, L.A., et al. (1990): 137Cs determination and mutagenicity tests in wild Mus musculus domesticus before and after the Chernobyl accident. *Environ. Pollut.*, 64:1–9.
143. Cristaldi, M., Leradi, L.A., Mascanzoni, D., and Mattei, T. (1991): Environmental impact of the Chernobyl accident: Mutagenesis in bank voles from Sweden. *Int. J. Radiat. Biol.*, 59:31–40.
144. Croft, J.R. (1989): The Goiânia accident. In: *Medical Response to Effects of Ionizing Radiation*, edited by W.A.Crosbie and J.H. Gittus, pp. 83–101. Elsevier Science, New York.
145. Crompton, M.R., and Layton, D.D. (1961): Delayed radionecrosis of the brain following therapeutic X-irradiation of the pituitary. *Brain*, 84:85–101.
146. Cross, F.T., Palmer, R.F., Busch, R.H., et al. (1981): Development of lesions in Syrian Golden hamsters following exposure to radon daughters and uranium ore dust. *Health Phys.*, 41:135–153.
147. Cross, F.T., Palmer, R.F., Filipy, R.E., et al. (1982): Carcinogenic effects of radon daughters, uranium ore dust and cigarette smoke in beagle dogs. *Health Phys.*, 42:33–52.
148. Currey, J.D., Foreman, J., Laketic, I., et al. (1997): Effects of ionizing radiation on the mechanical properties of human bone. *J. Orthopaedic Res.*, 15:111–117.
149. Cust, M.P., Whitehead, M.I., Powles, R., Hunter, M., and Milliken, S. (1989): Consequences and treatment of ovarian failure after total body irradiation for leukaemia. *B. M. J.*, 299:1494–1497.
150. Cwikel, J. (1997): Comments on the psychosocial aspects of the International Conference on Radiation and Health. *Environ. Health Perspect.*, 105:1607–1608.
151. Cwikel, J., Abdelgani, A., Goldsmith, J.R., et al. (1997): Two-year follow up study of stress-related disorders among immigrants to Israel from the Chernobyl area. *Environ. Health Perspect.*, 105:1545–1550.
152. Dafny, N., and Pellis, N.R. (1986): Evidence that opiate addiction is in part an immune response: Immune system destruction by irradiation altered opiate withdrawal. *Neuropharmacology*, 25:815–818.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_755.html[2010/10/28 18:47:31]

153. Dahlstrom, A., Haggendal, J., and Rosengren, B. (1973): The effect of Roentgen irradiation on monoamine containing neurons of the rat brain. *Acta Radiol. Ther. Phys. Biol.*, 12:191–200.
154. Dallas, C.E. (1993): Aftermath of the Chernobyl Nuclear Disaster: Pharmaceutical needs in the Republic of Belarus. *Am. J. Pharm. Ed.*, 57:182–185.
155. Dallas, C.E., Jagoe, C.H., Fisher, S.K., et al. (1995): Evaluation of genotoxicity in wild organisms due to the Chernobyl Nuclear Disaster. *Ecol. Ind. Regions*, 1:44–54.
156. Dallas, C.E., Lingenfelser, S.F., Lingenfelser, J.T., et al. (1998): Flow cytometric analysis of leukocyte and erthrocyte DNA in fish from Chernobyl-contaminated ponds in the Ukraine. *Ecotoxicology*, 7:211–219.
157. D'Amato, C.J., and Hicks, S.P. (1980): Development of the motor system: Effects of radiation on developing corticospinal neurons and locomotor function. *Exp. Neurol.*, 70:1–23.
158. Danquechin-Dorval, E., Mueller, G.P., Eng, R.R., et al. (1985): Effect of ionizing radiation on gastric secretion and gastric motility in monkeys. *Gastroenterology*, 89:374–380.
159. Danylash, M.M., Voshchepynets, H.A., Urban, V.I., and Fekiishgazi, S.B. (1996): Immune status parameters and renal function in subjects with prior ionizing radiation exposure (in Ukranian). *Likars'Ka Sprava*, 1–2:18–20.
160. Darby, S.C., and Weiss, H.A. (1995): Human studies in radiation leukaemogenesis. In: *Radiation Toxicology: Bone Marrow and Leukaemia*, edited by J.H.Hendry, and B.I.Lord, pp. 335–353. Taylor & Francis, Washington.
161. Dare, A., Hachisu, R., Yamaguchi, A., et al. (1997): Effects of ionizing radiation on proliferation and differentiation of osteoblast-like cells. *J. Dental Res.*, 76:658–664.
162. d'Avella, D., Cicciarello, R., Albiero, F., et al. (1992): Quantitative study of blood-brain barrier permeability changes after experimental whole-brain radiation. *Neurosurgery*, 30:30–34.
163. d'Avella, D., Cicciarello, R, Angileri, F.F., et al. (1998): Radiation-induced blood-brain barrier changes: Pathophysiological mechanisms and clinical implications. *Acta Neurochir. Suppl. (Wien)*, 71:282–284.
164. Davidoff, L.M., Dyke, C.G., Elsberg, C.A., and Tarlov, I.M. (1938): The effect of radiation applied directly to brain and spinal cord. I. Experimental investigations on Macaca rhesus monkeys. *Radiology*, 31:451–463.
165. Davydov, B.I. (1961): Acetylcholine metabolism on the thalamic region of the brain of dogs after acute radiation sickness. *Radiobiologiia*, 1:550–554.
166. Day, R., Gorin, M.B., and Eller, A.W. (1995): Prevalence of lens changes in Ukrainian children residing around Chernobyl. *Health Phys.*, 68:632–642.
167. de Carvalho, A.B. (1991): The psychological effects of the Goiania radiological accident on the emergency responders. In: *The Medical Basis for Radiation-Accident Preparedness III: The Psychological Perspective*, edited by R.C.Ricks, M.E.Berger, and F.M. O'Hara, pp. 132–141. Elsevier, New York.
168. Deininger, M.W.N., Bose, S., Gora-Tybor, J., et al. (1998): Selective induction of leukemia-associated fusion genes by high-dose ionizing radiation. *Cancer Res.*, 58:421–425.
169. Deng, C., Zhang, P., Harper, J.W., et al. (1995): Mice lacking p21(CIP1/WAF1) undergo normal development, but are defective in G1 checkpoint control. Cell, 82:675–684.
170. Deroo, J., Gerber, G.B., and Maes, J. (1986): In: *Radiation Risks to the Developing Nervous System*, edited by H.Kriegel, W. Schmahl, G.B.Gerber, and F.-E.Stieve, pp. 211–219. Gustav Fischer Verlag, Stuttgart.
171. DeRyck, M., Schallert, T., and Teitelbaum, P. (1980): Morphine versus haloperidol catalepsy in the rat: A behavioral analysis of postural support mechanisms. *Brain Res.*, 201:143–172.
172. Deshmukh, B.D., and Suryawanshi, S.A. (1989): Effects of gamma irradiation on histomorphology of some endocrine glands of the rain quail, Coturnix coromandelica (Gmelin). *Ind. J. Exp. Biol.*, 27:780–784.
173. de Toledo, S.M., Azzam, E.I., Gasmann, Mk., and Mitchell, R.E. (1995): Use of semiquantitative transcription polymerase chain reaction to study gene expression in normal human skin fibroblasts following low dose-rate irradiation. *Int. J. Radiat. Biol.*, 67:135–143.
174. Dewey, W.C., Ling, C.C., and Meyn, R.E. (1995): Radiation-induced apoptosis: Revelance to radiotherapy. *Int. J. Radiat. Oncol. Biol. Phys.*, 33:781–796.
175. Di Cicco, D., Antal, S., and Ammassari-Teule, M. (1991): Prenatal exposure to gamma/neutron irradiation: Sensorimotor alterations and paradoxical effects on learning. *Teratology*, 43:61–70.
176. Donlon, M.A., and Walden, T.L., Jr. (1988): The release of biologic mediators in response to acute radiation injury. *Comments Toxicol.*, 2:205–216.

177. Dopico, A.M., and Zieher, L.M. (1993): Neurochemical characterization of the alterations in the noradrenertic afferents to the cerebellum of adult rats exposed to X-irradiation at birth. *J. Neurochem.*, 61:481–489.
178. Dorr, R.T. (1998): Radioprotectants: Pharmacology and clinical applications of amifostine. *Semin. Radiat. Oncol.*, 4(Suppl 1):10–13.
179. Dörr, W., and Schultz-Hector, S. (1992): Early changes in mouse urinary bladder function following fractionated X irradiation. *Radiat. Res.*, 131:35–42.
180. Douglas, W.W. (1985): Histamine and 5-hydroxytryptamine (serotonin) and their antagonists. In: *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, edited by A.G. Gilman, L.S.Goodman, T.W.Rall, and F.Murad, pp. 605–615. Macmillan, New York.
181. Doull, J. (1967): Pharmacological responses in irradiated animals. *Radiat. Res.*, 30:334–341.
182. Doyle, T.F., Curran, C.R., and Turns, J.E. (1974): The prevention of radiation-induced early transient incapacitation of monkeys by an antihistamine. *Proc. Soc. Exp. Biol. Med.*, 145:1018–1024.
183. Doyle, T.F., and Strike, T.A. (1977): Radiation-released histamine in the rhesus monkey as modified by mast cell depletion and antihistamine. *Experientia*, 33:1047–1049.
184. Doyle, T.F., Turns, J.E., and Strike, T.A. (1971): Effect of antihistamine on early transient incapacitation of monkeys subjected to 4000 rads of mixed gamma-neutron radiation. *Aerosp. Med.*, 42:400–403.
185. Dubois, A. (1988): Effect of ionizing radiation on the gastrointestinal tract. *Comments Toxicol.*, 2:233–242.
186. Dubois, A., Fiala, N., Boward, C.A., and Bogo, V. (1988): Prevention and treatment of the gastric symptoms of radiation sickness. *Radiat. Res.*, 115:595–604.
187. Dubrova, Y.E., Nesterov, V.N., Krouchinsky, N.G., et al. (1996): Human minisatellite mutation rate after the Chernobyl accident. *Nature*, 380:683–686.
188. Dubrova, Y.E., Nesterov, V.N., Krouchinsky, N.G., et al. (1997): Further evidence for elevated human minisatelllite mutation rate in Belarus eight years after the Chernobyl accident. *Mutat. Res. Fundam. Mol. Mech. Mutagenesis*, 38:267–278.
189. Dulic, V., Kaufmann, W.K., Wilson, S.J., et al. (1994): p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblast during radiation-induced G1 arrest. *Cell*, 76:1013–1023.
190. Dygalo, N.N., Sakharov, D.G., and Shishkina, G.T. (1997): Corticosterone and testosterone in the blood of adult rats: The effects of low doses and the times of the action of ionizing radiation

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during intrauterine development (in Russian). *Radiat. Biol. Radioecol.*, 37:377–381.
191. Dynes, J.B., and Smedal, M.J. (1960): Radiation myelitis. *Am. J. Roentgenol. Radium Ther. Nuc. Med.*, 83:78–87.
192. Edwards, A.A. (1997): The use of choromosomal aberrations in human lymphocytes for biological dosimetry. *Radiat. Res.*, 148:S39–S44.
193. Edwards, J.C., Cramp, W.C., Chapman, D., and Yatvin, M.B. (1984): The effects of ionizing radiation on biomembrane structure and function. *Prog. Biophys. Mol. Biol.*, 43:71–93.
194. Egana, E. (1962): Some effects of ionizing radiations on the metabolism of the central nervous system. *Int. J. Neurol.*, 3:631–647.
195. Ehrhart, E.J., Segarini, P., Tsang, M.L.-S., et al. (1997): Latent transforming growth factor B1 activation in situ: Quantitative and functional evidence after low-dose gamma-irradiation. *F. A. S. E. B. J.*, 11:991–1002.
196. Eisenbud, M. (1987): *Environmental Radioactivity From Natural, Industrial, and Military sources*, 3rd Academic Press, New York.
197. Elliott, T.B., Brook, I., and Stiefel, S.M. (1990): Quantitative study of wound infection in irradiated mice. *Int. J. Radiat. Biol.*, 58:341–350.
198. Faure, E., Cavard, C., Zider, A., et al. (1995): X-irradiation-induced transcription from HIV type 1 long term repeat. *AIDS Res. Hum. Retroviruses*, 11:41–43.
199. Feldman, J.E. (1989): Ovarian failure and cancer treatment: Incidence and interventions for premenopausal women. *Oncol. Nurs. Forum*, 16:651–657.
200. Ferguson, J.L., Kandasamy, S.B., Harris, A.H., et al. (1996): Indomethacin attenuation of radiation-induced hyperthermia does not modify radiation-induced motor hypoactivity. *J. Radiat. Res. (Tokyo)*, 37:209–215.
201. Fetter, S.A., and Tsipis, K. (1981): Catastrophic releases of radioactivity. *Sci. Am.*, 244:41–47.
202. Feyerabend, T., Kapp, B., Richter, E., et al. (1990): Incidence of hypothyroidism after irradiation of the neck with special reference to lymphoma patients. A retrospective and prospective analysis. *Acta Oncol.*, 29:597–602.
203. Fici, G.J., Althaus, J.S., and von Voigtlander, P.F. (1997): Effects of lazaroids and a peroxynitrite scavenger in a cell model of peroxynitrite toxicity. *Free Radic. Biol. Med.*, 22:223–228.
204. Fields, P.E. (1957): The effect of whole-body X-irradiation upon activity drum, straight runway, and maze performances of white rats. *J. Comp. Physiol. Psychol.*, 50:386–391.
205. Fischbein, A., Zabludovsky, N., Eltes, F., et al. (1997): Ultramorphological sperm characteristics in the risk assessment of health effects after radiation exposure among salvage workers in Chernobyl. *Environ. Health Perspect.*, 105:1445–1450.
206. Flam, F. (1992): Quasars: Ablaze with gamma rays. *Science*, 256:311.
207. Fornace, A.J. (1992): Mammalian genes induced by radiation: Activation of genes associated with growth control. *Annu. Rev. Genet.*, 26:507–526.
208. Francois, A., Aigueperse, J., Gourmelon, P., et al. (1998): Exposure to ionizing radiation modifies neurally-evoked electrolyte transport and some inflammatory responses in rat colon in vitro. *Int. J. Radiat. Biol.*, 73:93–101.
209. Franke, T.F., and Lewis, C.C. (1997): A bad kinase makes good. *Nature*, 390:116–117.
210. Franz, C.G. (1985): Effects of mixed neutron-gamma total body irradiation on physical activity performance of rhesus monkeys. *Radiat. Res.*, 101:434–441.
211. Freud, A., Canfi, A., Sod-Moriah, U.A., and Chayoth, R. (1990): Neonatal low-dose gamma irradiation-induced impaired fertility in mature rats. *Isr. J. Med. Sci.*, 26:611–615.
212. Freud, A., and Sod-Moriah, U.A. (1990): Progesterone and estradiol plasma levels in neonatally irradiated cycling rats. *Endocr. Res.*, 16:221–229.
213. Fritzell, J.A., Narayanan, L., Baker, S.M., et al. (1997): Role of DNA mismatch repair in the cytotoxicity of ionizing radiation. *Cancer Res.*, 57:5143–5147.
214. Fry, F.A. (1987): Doses from environmental radioactivity. In: *Radiation and Health. The Biological Effects of Low-Level Exposure to Ionizing Radiation*, edited by R.Jones, and R. Southwood, pp. 9–17. John Wiley & Sons, New York.
215. Fujiwara, S., Sposto, R., Ezaki, H., et al. (1992): Hyperparathyroidism among atomic bomb survivors in Hiroshima. *Radiat. Res.*, 130:372–378.
216. Fukuda, T., Setoguchi, M., Inaba, K., et al. (1991): The antiemetic profile of Y-25130, a new selective 5-HT3 receptor antagonist. *Eur. J. Pharmacol.*, 196:299–305.
217. Furchtgott, E. (1963): Behavioral effects of ionizing radiations: 1955–61. *Psychol. Bull.*, 60:157–

199.
218. Furchtgott, E. (1971): Behavioral effects of ionizing radiations. In: *Pharmacology and Biophysical Agents and Behavior*, edited by E. Furchgott, pp. 1–64. Academic Press, New York.
219. Furchtgott, E. (1975): Ionizing radiations and the nervous system. In: *Biology of Brain Dysfunction*, Vol. 3, edited by G.E.Galli, pp. 343–379. Plenum Press, New York.
220. Fushiki, S. (1997): Pathogenesis of the neuronal migration disorder, with special reference to the animal model of prenatal exposure to low-dose ionizing radiation (in Japanese). *No To Hattatsu*, 29:102–107.
221. Fushiki, S., Hyodo-Taguchi, Y., Kinoshita, C., et al. (1997): Shortand long-term effects of low-dose prenatal X-irradiation in mouse cerebral cortex, with special reference to neuronal migration. *Acta Neuropathol. (Berlin)*, 93:443–449.
222. Gaffey, C.T. (1962): Bioelectric effects of high energy irradiation on nerve. In: *Response of the Nervous System to Ionizing Radiation*, edited by T.J.Haley, and R.S.Snider, pp. 277–296. Academic Press, New York.
223. Gaichenko, V.A., Kryzhanovsky, V.I., and Stovbchaty, V.N. (1994): Post-accident state of the Chernobyl Nuclear Power Plant Alienated Zone faunal complexes. *Radiat. Biol. Ecol.*, Special Issue:27–32.
224. Gal, D., Strickland, D.M., Lifshitz, S., et al. (1984): Effect of radiation on prostaglandin production by human bowel in vitro. *Int. J. Radiat. Oncol. Biol. Phys.*, 10:653–657.
225. Gallegos, A., Berggren, M., Gasdaska, J.R., and Powis, G. (1997): Mechanisms of the regulation of thioredoxin reductase activity in cancer cells by the chemopreventive agent selenium. *Cancer Res.*, 57:4965–4970.
226. Gangloff, H. (1962): Acute effects of X-irradiation on brain electrical activity in cats and rabbits. In: *Effects of Ionizing Radiation on the Nervous System; Proceedings*, pp. 123–138. International Atomic Energy Agency, Vienna. Vienna, Austria; 5–9 June 1961.
227. Gangloff, H., and Haley, T.J. (1960): Effects of X-irradiation on spontaneous and evoked brain electrical activity in cats. *Radiat. Res.*, 12:694–704.
228. Ganong, W.F. (1989): *Review of Medical Physiology*, 14th ed. Appleton and Lange, San Mateo, CA.
229. Gasteiger, E.L., and Campbell, B. (1962): Alteration of mammalian nerve compound action potentials by beta irradiation. In: *Response of the Nervous System to Ionizing Radiation*, edited by T.J.Haley and R.S.Snider, pp. 597–605. Academic Press, New York.
230. Geist, B.J., Lauk, S., Bornhausen, M., and Trott, K.R. (1990): Physiologic consequences of local heart irradiation in rats. *Int. J. Radiat. Oncol. Biol. Phys.*, 18:1107–1113.

231. Geist, B.J., and Trott, K.R. (1992): Radiographic and function changes after partial lung irradiation in the rat. *Strahlenther. Onkol.*, 168:168–173.
232. Gembicki, M., Stozharov, A.N., Arinchin, A.N., et al. (1997): Iodine deficiency in Belarusian children as a possible factor stimulating the irradiation of the thyroid gland during the Chernobyl Catastrophe. *Environ. Health Perspect.*, 105:1487–1490.
233. George, A.M., and Cramp, W.A. (1987): The effects of ionizing radiation on structure and function of DNA. *Prog. Biophys. Mol. Biol.*, 50:121–169.
234. Geraci, J.P., Mariano, M.S., and Jackson, K.L. (1991): Hepatic radiation injury in the rat. *Radiat. Res.*, 125:65–72.
235. Geraci, J.P., Mariano, M.S., and Jackson, K.L. (1992): Radiation hepatology of the rat: Microvascular fibrosis and enhancement of liver dysfunction by diet and drugs. *Radiat. Res.*, 129:322–332.
236. Gershey, E.L., Klein, R.C., Party, E., and Wilkerson, A. (1990): *Low-Level Radioactive Waste. From Cradle to Grave*. Van Nostrand Reinhold, New York.
237. Gersterner, H.B. (1956): Effect of high-intensity X-irradiation on the A group fibers of the frog sciatic nerve. *Am. J. Physiol.*, 184:333–337.
238. Giambarresi, L., and Jacobs, A.J. (1987): Radioprotectants. In: *Military radiobiology*, edited by J.J.Conklin and R.I.Walker, pp. 265–301. Academic Press, San Diego, CA.
239. Gibson, D.P., DeGowin, R.L., and Knapp, S.A. (1982): Effect of X irradiation on release of prostaglandin E from marrow stromal cells in culture. *Radiat. Res.*, 89:537–545.
240. Giel, R. (1991): The psychosocial aftermath of two major disasters in the Soviet Union. *J. Traumatic. Stress*, 4:381–393.
241. Gillette, S.L., Gillette, E.L., Powers, B.E., et al. (1989): Ureteral injury following experimental intraoperative radiation. *Int. J. Radiat. Oncol. Biol. Phys.*, 17:791–798.
242. Gillette, S.M., Powers, B.E., Orton, E.C., and Gillette, E.L. (1991): Early radiation response of the canine heart and lung. *Radiat. Res.*, 125:34–40.
243. Gilmore, S.A., Sims, T.J., Davies, D.L., et al. (1997): Microglial development is altered in immature spinal cord by exposure to radiation. *Int. J. Dev. Neurosci.*, 15:1–14.
244. Ginzburg, H.M. (1993): The psychological consequences of the Chernobyl accident—findings from the International Atomic Energy Agency Study. *Public. Health Rep.*, 108:184–192.
245. Girinsky, T., Baume, D., Socie, G., et al. (1991): Blood cell kinetics after a 385 cGy total body irradiation given to a CML patient for bone marrow transplantation. *Bone Marrow Transplant.*, 7: 317–320.
246. Giwercman, A., von der Maase, H., Berthelsen, J.G., et al. (1991): Localized irradiation of testes with carcinoma in situ: Effects on Leydig cell function and eradication of malignant germ cells in 20 patients. *J. Clin. Endocrinol. Metab.*, 73:596–603.
247. Gluzman, D.F., Moutet, A., Simmonet, M.-L., et al. (1994): Oncohematological aspects of ionizing radiation exposure on human embryo and fetus (in Russian). *Eksperimental'Naya Onkologiya*, 16:279–287.
248. Gobbel, G.T., Seilhan, T.M., and Fike, J.R. (1992): Cerebrovascular response after interstitial irradiation. *Radiat. Res.*, 130:236–240.
249. Goldman, M. (1997): The Russian radiation legacy: Its integrated impact and lessons. *Environ. Health Perspect.*, 105:1385–1392.
250. Goncharova, R.I., and Ryabokon, N.I. (1995): Dynamics of cytogenetic injuries in natural populations of bank vole in the republic of Belarus. *Radiat. Protect. Dosim.*, 62:37–40.
251. Gonzales, S. (1982): Host rocks for radioactive-waste disposal. *Am. Sci.*, 70:191–200.
252. Goud, S.N. (1995): Effect of irradiation of lymphocyte proliferation and differentiation: Potential of IL-6 in augmenting antibody responses in cultures of murine spleen cells. *Int. J. Radiat. Biol.*, 67:461–468.
253. Graham, M.M., Evans, M.L., Dahlen, D.D., et al. (1990): Pharmacological alteration of the lung vascular response to radiation. *Int. J. Radiat. Oncol. Biol. Phys.*, 19:329–339.
254. Grevert, D., and Goldstein, A. (1977): Some effects of naloxone on behavior in the mouse. *Psychopharmacology (Berlin)*, 53:111–113.
255. Grosch, D.S., and Hopwood, L.E. (1979): *Biological Effects of Radiation*. Academic Press, New York.
256. Gueneau, G., Baille, V., Dubos, M., and Court, L. (1986): Protracted postnatal neurogenesis and radiosensitivity in the rabbit's dentate gyrus. In: *Radiation Risks to the Developing Nervous System*, edited by H.Kriegel, W.Schmahl, G.B.Gerber, and F.-E.Stieve, pp. 133–140. Gustav Fischer Verlag,

Stuttgart.
257. Guinee, V.F., Guido, J.J., Pfalzgraf, K.A., et al. (1985): The incidence of herpes zoster in patients with Hodgkin's Disease. *Cancer*, 56:642–648.
258. Gunter-Smith, P.J. (1987): Effect of ionizing radiation on gastrointestinal physiology. In: *Military Radiobiology*, edited by J.J. Conklin and R.Walker, pp. 135–151. Academic Press, San Diego.
259. Gunter-Smith, P.J. (1989): Gamma radiation affects active electrolyte transport by rabbit ileum. II. Correlation of alanine and theophylline response with morphology. *Radiat. Res.*, 117:419–432.
260. Gupta, M.L., and Umadevi, P. (1990): Response of reptilian liver to external gamma irradiation. *Radiobiol. Radiother. (Berlin)*, 31:285–288.
261. Gupta, M.L., and Umadevi, P. (1990): Response of piscine liver to external gamma irradiation. *Radiobiol. Radiother. (Berlin)*, 31:289–292.
262. Guyton, A.C. (1986): *Textbook of Medical Physiology*, 7th ed. W. B.Saunders, Philadelphia.
263. Hagen, U. (1989): Biochemical aspects of radiation biology. *Experientia*, 45:7–12.
264. Haimovitz-Friedmann, A., Kolesnick, R.N., and Fuks, Z. (1996): Modulation of the apoptotic response: Potential for therapeutic applications in radiation oncology. *Semin. Radiat. Oncol.*, 6:273–283.
265. Hakem, R., Hakem, A., Duncan, G.S., et al. (1998): Differential requirement for caspace 9 in apoptotic pathway in vivo. *Cell*, 94:339–352.
266. Haley, T.J. (1962): Changes induced in brain activity by low doses of X-irradiation. In: *Effects of Ionizing Radiation on the Nervous System; Proceedings*, pp. 171–185. International Atomic Energy Agency, Vienna. Vienna, Austria; 5–9 June 1961.
267. Hall, E.C., and Cox, J.D. (1989): Physical and biologic basis of radiation therapy. In: *Radiation Oncology. Rationale, Technique, Results*, 6th ed., edited by M.T.Moss, and J.D.Cox, pp. 1–57. C.V.Mosby, St. Louis.
268. Hall, E.J. (1984): *Radiation and Life*, 2nd ed. Pergammon Press, New York.
269. Hall, E.J. (1994): *Radiobiology for the Radiologist*, 4th ed. J.B. Lippincott, New York.
270. Hallahan, D.E. (1996): Radiation-medicated gene expression in the pathogenesis of the clinical radiation response. *Semin. Radiat. Oncol.*, 6:250–267.
271. Halpern, J., Kishel, S.P., Park, J., et al. (1984): Radiation-induced brain edema in primates, studies with sequential brain CAT scanning and histopathology. *Res. Commun. Chem. Pathol. Pharmacol.*, 45:463–470.
272. Hamdorf, G., Shahar, A., Cervos-Navarro, J., et al. (1992): Irradiation neurotoxicity assessed in organotypic cultures of rat hippocampus, *Neurotoxicology*, 13:165–170.

273. Hancock, S.L., Cox, R.S., McDougall, I.R. (1991): Thyroid diseases after treatment of Hodgkin's disease. *N. Engl. J. Med.*, 325:599–605.
274. Hansen, P.V., Trykker, H., Svennekjaer, I.L., and Hvolby, J. (1990): Long-term recovery of spermatogenesis after radiotherapy in patients with testicular cancer. *Radiother. Oncol.*, 18:117–125.
275. Harley, N.H., Cross, F.T., and Stuart, B.O. (1984): Evaluation of occupational and environmental exposures to radon and radon daughters in the United States. *NCRP Report No. 78.* National Council on Radiation Protection and Measurement, Washington, DC.
276. Havenaar, J., Rumyantzeva, G., Kasyanenko, A., et al. (1997): Health effects of the Chernobyl disaster: Illness or illness behavior? A comparative general health survey in two former Soviet regions. *Environ. Health Perspect.*, 105:1533–1538.
277. Havenaar, J.M., van den Brink, W., Kasyanenko, A.P., et al. (1995): Mental health problems in the Gomel Region (Belarus). An analysis of risk factors in an area affected by the Chernobyl disaster. *Psychol. Med.*, 26:845–855.
278. Hawkins, R.N., Alter, W.A., Jr, Doyle, T.F., and Catravas, G.N. (1983): Radiation-induced cardiovascular dysfunction in the rhesus monkey. *Radiat. Res.*, 94:654.
279. Hawkins, R.N., and Cockerham, L.G. (1987): Postirradiation cardiovascular dysfunction. In: *Military Radiobiology*, edited by J.J.Conklin and R.Walker, pp. 153–163, Academic Press, San Diego.
280. Hawkins, R.N., and Forcino, C.D. (1988): Effects of radiation on cardiovascular function. *Comments Toxicol.*, 2:243–252.
281. Haymaker, W. (1962): Morphological changes in the nervous system following exposure to ionizing radiation. In: *Effects of Ionizing Radiation on the Nervous System; Proceedings*, pp. 309–358. International Atomic Energy Agency, Vienna. Vienna, Austria; 5–9 June 1961.
282. Henderson, E.E., Tudor, G., and Yang, J.Y. (1992): Inactivation of the human immunodeficiency virus type 1 (HIV-1) by ultraviolet and X irradiation. *Radiat. Res.*, 131:169–176.
283. Herman, B.H., and Panksepp, I. (1978): Effects of morphine and naloxone on separation distress and approach attachment: Evidence for opiate mediation of social effect. *Pharmacol. Biochem. Behav.*, 9:213–220.
284. Hewitt, M., Cornish, J., Pamphilon, D., and Oakhill, A. (1991): Effective emetic control during conditioning of children for bone marrow transplantation using ondansetron, a 5-HT3 antagonist. *Bone Marrow Transplant.*, 7:431–433.
285. Hienz, R.D. (1992): Effects of ionizing radiation on auditory and visual thresholds. *DNA-TR-91–47.* Defense Nuclear Agency, Alexandria, VA.
286. Highfield, D.A., Hu, D., and Amsel, A. (1998): Alleviation of x-irradiation-based deficit in memory-based learning by D-amphetamine: Suggestions for attention deficit-hyperactivity disorder. *Proc. Natl Acad. Sci. U.S.A.*, 95:5785–5788.
287. Hill, J.M., Kornblith, A.B., Jones, D., et al. (1998): A comparative study of the long term psychosocial functioning of childhood acute lymphoblastic leukemia survivors treated by intrathecal methotrexate with or without cranial radiation. *Cancer*, 82:208–218.
288. Hirata, Y., Matsukado, Y., Mihara, Y., and Kochi, M. (1985): Occlusion of the internal carotid artery after radiation therapy for the chiasmal lesion. *Acta Neurochir. (Wien)*, 74:141–147.
289. Hoar, R.M., and Monie, I.W. (1981): Comparative development of specific organ systems. In: *Developmental Toxicology*, edited by C.A.Kimmel and J.Buelke-Sam, pp. 13–33. Raven Press, New York.
290. Hobbs, C.H., and McClellan, R.O. (1986): Toxic effects of radiation and radioactive materials. In: *Casarett and Doull's Toxicology, 3rd ed.*, edited by C.D.Klaassen, M.O.Amdur, and J.Doull, pp. 669–750. Macmillan, New York.
291. Hodges, H., Katzung, N., Sowinski, P., et al. (1998): Late behavioural and neuropathological effects of local brain irradiation in the rat. *Behav. Brain Res.*, 91:99–114.
292. Hoekstra, H.J., Mehta, D.M., Oosterhuis, J.W., et al. (1990): The short- and long-term effect of single high-dose intra-operative electron beam irradiation of retroperitoneal structures—an experimental study in dogs. *Eur. J. Surg. Oncol.*, 16:240–247.
293. Hofer, M., Viklicka, S., Tkadlecek, L., and Karpfel, Z. (1989): Haemopoiesis in murine bone marrow and spleen after fractionated irradiation and repeated bone marrow transplantation. II. Granulopoiesis. *Folia Biol. (Praha)*, 35:418–428.
294. Hohwieler, M.L., Lo, T.C., Silverman, M.L., and Freiberg, S.R. (1986): Brain necrosis after radiotherapy for primary intracerebral tumor. *Neurosurgery*, 18:67–74.
295. Hole, E.D., Nelson, W.H., Sagstuen, E., and Close, D.M. (1992): Free radical formation in single crystals of 2′-deoxyguanosine 5′-monophosphate tetrahydrate disodium salts: An EPR/ENDOR study.

Radiat. Res., 129:119–138.
296. Hollahan, E.V., Jr. (1987): Cellular radiation biology. In: *Military Radiobiology*, edited by J.J.Conklin, and R.I.Walker, pp. 87–110. Academic Press, Orlando.
297. Hollinden, G.E., and Pellmar, T.C. (1989): Attenuation of synaptic transmission in hippocampal slices following whole animal exposure to ionizing radiation. *Soc. Neurosci. Abstr.*, 15:134.
298. Holloman, K., Dallas, C.E., Jagoe, C.H., et al. (1998): Interspecies differences in oxidative stress response and radiocesium uptake in rodents inhabiting areas highly contaminated by the Chernobyl nuclear disaster. *J. Environ. Toxicol. Chem. 2000;* (In Press).
299. Hopewell, J.W. (1979): Late radiation damage to the central nervous system: A radiobiological interpretation. *Neuropathol. Appl. Neurobiol.*, 5:329–343.
300. Hopewell, J.W., and Wright, E.A. (1970): The nature of latent cerebral irradiation damage and its modification by hypertension. *Br. J. Radiol.*, 43:161–167.
301. Hopewell, J.W., and Wright, E.A. (1975): The effects of dose and field size on late radiation damage to the rat spinal cord. *Int. J. Radiat. Biol.*, 28:325–333.
302. Huang, T.S., Chen, S.T., Lui, L.T., et al. (1990): Early effects of cranial irradiation on hypothalamic pituitary function. *Taiwan I Hsueh Hui Tsa Chih*, 89:541–547.
303. Hunt, W.A., Dalton, T.K., and Darden, J.H. (1979): Transient alterations in neurotransmitter activity in the caudate nucleus of rat brain after a high dose of ionizing radiation. *Radiat. Res.*, 80:556–562.
304. Hunter, A.E., Prentice, H.G., Pothecary, K., et al. (1991): Granisetron, a selective 5-HT3 receptor antagonist, for the prevention of radiation induced emesis during total body irradiation. *Bone Marrow Transplant.*, 7:439–441.
305. Hwang, A., and Muschel, R.J. (1998): Radiation and the G2 phase of the cell cycle. *Radiat. Res.*, 150(Suppl):S52–S59.
306. Hyer, M., and Nielsen, O.S. (1992): Influence of dose on regeneration of murine hematopoietic stem cells after total body irradiation and 5-fluorouracil. *Oncology*, 49:166–172.
307. Ibrahim, M.Z.M., Haymaker, W., Miquel, J., and Riopelle, A.J. (1967): Effects of radiation on the hypothalamus in monkeys. *Archiv für Psychiatrie und Zeitschrift f.d. ges. Neurologie*, 210:1–15.
308. Il′enko, A.I., and Krapivko, T.P. (1994): Radioresistance of populations of bank voles Clethrionomys glareolus in radionuclide-contaminated areas. *Doklady Biological Sciences*, 336:262–266.

309. Iliakis, G., Wang, Y., Pantelias, G.E., and Metzger, L. (1992): Mechanism of radiation sensitization by halogenated pyrimidines: Effect of BrdU on repair of DNA breaks, interphase chromatin breaks, and potentially lethal damage in plateau-phase CHO cells. *Radiat. Res.*, 129:202–211.
310. Imanaka, T., Seo, T., and Koide, H. (1988): Radioactivity release from the Chernobyl-4 accident and its cancer consequences. *J. Radiat. Res. (Tokyo)*, 29:80.
311. Inano, H., Suzuki, K., Ishii-Ohba, H., et al. (1989): Steroid hormone production in testis, ovary, and adrenal gland of immature rats irradiated in utero with 60Co. *Radiat. Res.*, 117:293–303.
312. Innes, J.R., and Carsten, A. (1961): Demyelination or malacic myelopathy. *Arch. Neurol.*, 4:190–199.
313. Ito, M., Patronas, N.J., Di Chiro, G., et al. (1986): Effect of moderate level X-radiation to brain on cerebral glucose utilization. *J. Comput. Assist. Tomogr.*, 10:584–588.
314. Iverson, S.D., and Iverson, L.L. (1981): *Behavioral Pharmacology*, Oxford University Press, New York.
315. Izrael, Yu. A., Petrov, V.A., Avdjushin, S.I., et al. (1987): Radioactive pollution of the natural environment in the zone of the accident of the Chernobyl Atomic Power Plant (in Russian). *Meterologiya Hydrologiya*, 2:5–18.
316. Jacobs, A.J., Maniscalco, W.M., Parkhurst, A.B., and Finkelstein, J.N. (1986): In vivo and in vitro demonstration of reduced myelin synthesis following early postnatal exposure to ionizing radiation. *Radiat. Res.*, 105:97–104.
317. Jaenke, R.S., and Angleton, G.M. (1990): Perinatal radiation-induced renal damage in the beagle. *Radiat. Res.*, 122:58–65.
318. Jagoe, C.H., Chesser, R.K., Smith, M.H., et al. (1998): Radiocesium, mercury and lead in fish, and sediment radiocesium in waters near Chernobyl, Ukraine. *Ecotoxicology*, 7:202–210.
319. Jammet, H., Mathe, G., Pendic, B., et al. (1959): Study of six cases of accidental whole-body irradiation. *Rev. Fr. Etud. Clin, Biol.*, 4:210–225.
320. Janis, I.L. (1951): *Air War and Emotional Stress.* McGraw-Hill, New York.
321. Jensh, R.P., Eisenman, L.M., and Brent, R.L. (1995): Postnatal neurophysiologic effects of prenatal X-irradiation. *Int. J. Radiat. Biol.*, 67:217–227.
322. Jirtle, R.L., Anscher, M.S., and Alati, T. (1990): Radiation sensitivity of the liver. In: *Advances in Radiation Biology, Vol. 14, Relative Radiation Sensitivities of Human Organ Systems, Part II.* edited by K.I.Altman and J.T.Lett, pp. 269–311. Academic Press, San Diego.
323. Johnsson, J.E., Owman, C.H., and Sjoberg, N.O. (1970): Tissue content of noradrenaline and 5-hydroxytryptamine in the rat after ionizing radiation. *Int. J. Radiat. Biol.*, 18:311–316.
324. Jones, D.C., Kimeldorf, D.J., Rubadeau, D.O., et al. (1954): Effects of X-irradiation on performance of volitional activity by the adult male rat. *Am. J. Physiol.*, 177:243–250.
325. Jones, G.D.D., Ward, J.F., Limoli, C.L., et al. (1995): Mechanisms of radiosensitization in iododeoxyuridine-substituted cells. *Int. J, Radiat. Biol.*, 67:647–653.
326. Joseph, J.A., Erat, S., Rabin, B.M. (1998): CNS effects of heavy particle irradiation in space: Behavioral implications. *Adv. Space Res.*, 22:209–216.
327. Joseph, J.A., Hunt, W.A., Rabin, B.M., and Dalton, T.K. (1992): Possible "accelerated striatal aging" induced by 56Fe heavy particle irradiation: Implications for manned space flight. *Radiat. Res.*, 130:88–93.
328. Joseph, J.A., Kandasamy, S.B., Hunt, W.A., et al. (1988): Radiation-induced increases in sensitivity of cataleptic behavior to haloperidol: Possible involvements of prostaglandins. *Pharmacol. Biochem. Behav.*, 29:335–341.
329. Jung, M., and Dritschilo, A. (1996): Signal transduction and cellular responses to ionizing radiation. *Semin. Radiat. Oncol.*, 6:268–272.
330. Kader, H.A., and Rostom, A.Y. (1991): Follicle stimulating hormone levels as a predictor of recovery of spermatogenesis following cancer therapy. *Clin. Oncol., (R. Coll. Radiol.)*, 3:37–40.
331. Kameyama, Y., and Hoshino, K. (1986): Sensitive phases of CNS development. In: *Radiation Risks to the Developing Nervous System*, edited by H.Kriegel, W.Schmahl, G.B.Gerber, and F.-E. Stieve, pp. 75–92. Gustav Fischer Verlag, Stuttgart.
332. Kandasamy, S.B., Hunt, W.A., and Mickley, A.G. (1988): Implications of prostaglandins and histamine H1 and H2 receptors in radiation-induced temperature responses of rats. *Radiat. Res.*, 114:42–53.
333. Kaplan, M.I., and Morgan, W.F. (1998): The nucleus is the target for radiation-induced chromosomal instability. *Radiat. Res.*, 150:382–390.

334. Karaoglou, A., Desmet, G., Kelly, G.N., and Menzel, H.G. (1995): The radiological consequences of the Chernobyl accident. In: *Proceedings of the First International Conference*, Minsk, Belarus. Office for Official Publications of the European Communities, Luxembourg. May 1995.
335. Katz, R.J., Carroll, B.J., and Baldright, G. (1978): Behavioral activation by enkephalins in mice. *Pharmacol. Biochem. Behav.*, 8:493–496.
336. Kaul, A., Landfermann, H., and Thieme, M. (1996): One decade after Chernobyl: Summing up the consequences. *Health Phys.*, 71:634–640.
337. Kekcheyev, K. (1941): Changes in the threshold of achromatic vision of man by the action of ultrashort, ultraviolet and x-ray waves. *Probl. Fisiol. Optics*, 1:77–79.
338. Kemper, T.L., O'Neill, R., and Caveness, W.F. (1977): Effects of single dose supervoltage whole brain radiation in Macaca mulatta. *J. Neuropathol. Exp. Neurol.*, 36:916–940.
339. Kent, C.R.H., Eady, J.J., Ross, G.M., and Steel, G.G. (1995): The comet moment as a measure of DNA damage in the comet assay. *Int. J. Radiat. Biol.*, 67:655–660.
340. Kerr, J.F.R., Wyllie, A.H., and Currie, A.R. (1972): Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer*, 26:239–256.
341. Keyse, S.M. (1998): Protein phosphatases and the regulation of MAP kinase activity. *Cell Dev. Biol.*, 9:143–152.
342. Khodarev, N.N., Sokolova, I.A., and Vaughan, A.T.M. (1998): Mechanisms of induction of apoptotic DNA fragmentation. *Int. J. Radiat. Biol.*, 73:455–467.
343. Kim, J.H., Mandell, L.R., and Leeper, R. (1990): Radiation effects on the thyroid gland. In: *Advances in Radiation Biology, Vol. 14, Relative Radiation Sensitivities of Human Organ Systems, Part II*, edited by K.I.Altman and J.T.Lett, pp. 119–156. Academic Press, San Diego.
344. Kimeldorf, D.J., and Hunt, E.L. (1965): *Ionizing Radiation: Neural Function and Behavior*, Academic Press, New York.
345. Kimeldorf, D.J., and Jones, D.C. (1951): The relationship of radiation dose to lethality among exercised animals exposed to Roentgen rays. *Am. J. Physiol.*, 167:626–632.
346. Kimeldorf, D.J., Jones, D.C., and Castanera, T.J. (1953): Effect of X-irradiation upon the performance of daily exhaustive exercise by the rat. *Am. J. Physiol.*, 174:331–335.
347. Kimler, B.F. (1998): Prenatal irradiation: A major concern for the developing brain. *Int. J. Radiat. Biol.*, 73:423–434.
348. Kimler, B.F., Vidal-Pergola, G.M., Peterson, S.L., et al. (1994): Effect of in utero radiation dose fractionation on rat postnatal

development, behavior and brain structure: 3-hour interval. *Neurotoxicology*, 15:183–189.
349. King, G.L. (1988): Characterization of radiation-induced emesis in the ferret. *Radiat. Res.*, 114:599–612.
350. King, G.L., and Landauer, M.R. (1990): Effects of Zacopride and BMY25801 (Batanopride) on radiation-induced emesis and locomotor behavior in the ferret. *J. Pharmacol. Exp. Ther.*, 253:1026–1033.
351. Kinsella, T.J. (1989): Effects of radiation therapy and chemotherapy on testicular function. *Prog. Clin. Biol. Res.*, 302:157–177.
352. Klatzo, I., Suzuki, R., Orzi, F., et al. (1989): Pathomechanisms of ischemic brain edema. In: *Recent Progress in the Study and Therapy of Brain Edema*, edited by K.G.Co and A.Raathmann, pp. 1–17. Plenum Press, New York.
353. Kligerman, M.M., Liu, T., Scheffler, B., et al. (1992): Interim analysis of a randomized trial of radiotherapy of rectal cancer with/without WR-2721. *Int. J. Radiat. Oncol. Biol. Phys.*, 22:799–802.
354. Klouwen, H.M., and Betel, I. (1963): Radiosensitivity of nuclear ATP synthesis. *Int. J. Radiat. Biol.*, 6:441–461.
355. Koga, T., Morishima, H., Niwa, T., and Kawai, H. (1991): Tritium precipitation in European cities and in Osaka, Japan owing to the Chernobyl nuclear accident. *J. Radiat. Res. (Tokyo)*, 32:267–276.
356. Kondakova, N.V., Lisakovskii, S.V., Sakharova, V.V., et al. (1994): Effect of ionizing radiation on human muscle tissue (in Russian). *Voprosy Meditsinskoi Khimii*, 40:46–50.
357. Konings, A.W.T. (1987): Role of membrance lipid composition in radiation-induced death of mammalian cells. In: *Prostaglandin and Lipid Metabolism in Radiation Injury*, edited by T.L.Walden, Jr, and H.N.Hughes, pp. 29–43. Plenum Press, New York.
358. Konoplyannikov, A.G. (1997): Molecular and cellular mechanisms of late radiation damages. *Radiatsionnaya Biologiya Radioekologiya*, 37:621–628.
359. Koshurnikova, N., Buldakov, L., Bysogolov, G., et al. (1994): Mortality from malignancies of the hematopietic and lympathic tissue among personnel of the first nuclear plant in the USSR. *Sci. Total Environ.*, 142:19–23.
360. Koshurnikova, N.A., Bysogolov, G.D., Bolotnikova, M.G., et al. (1996): Mortality among personnel who worked at the MAYAK complex in the first years of its operation. *Health Phys.*, 71:90–93.
361. Kossenko, M.M. (1996): Cancer mortality among Techa river residents and their offspring. *Health Phys.*, 71:77–82.
362. Krabbe, A.A., and Olesen, J. (1982): Effect of histamine on regional cerebral blood flow in man. *Cephalalgia*, 2:15–18.
363. Krapivko, T.P., and Il′enko, A.I. (1988): First features of radioadaptation in a population of red-backed voles (Clethrionomys glareolus) in a radiation biogeocenosis. *Doklady Akademii Nauk SSR*, 302:1272–1274.
364. Krobel, W., and Kroem, G. (1959): Die Wirkung geringer strahlungsdosen auf die Signalerzeugungs und Fortleitungs eigenshaftens-eigenschaften in Froschnerven. *Atomkernergie*, 4:280–286.
365. Kryshev, I.I. (1992): *Radioecological Consequences of the Chernobyl Accident*. Nuclear Society International, Moscow.
366. Kuettle, M.R., Thraves, P.J., Jung, M., et al. (1996): Radiation-induced neoplastic transformation in human prostate epithelial cells. *Cancer Res.*, 56:5–10.
367. Kulinski, V.I., and Semenov, L.F. (1965): Content of catecholamines in the tissues of macaques during the early periods after total gamma irradiation. *Radiobiologiia*, 5:494–500.
368. Kumar, K.S., Vaishnav, Y.N., and Weiss, J.F. (1989): Radioprotection by antioxidant enzymes and enzyme mimetics. *Pharma. Ther.*, 39:301–309.
369. Kundurovic, Z., Scepovic, M., Causevic, A., and Mornjakovic, Z. (1991): Histochemical aspects and fine structural characteristics of thyreocytes in pinealectomized and melatonin treated rats prior to irradiation. *Acta Med. Croatica*, 45:347–355.
370. Lam, K.S., Tse, V.K., Wang, C., et al. (1991): Effects of cranial irradiation on hypothalamic-pituitary function—a 5-year longitudinal study in patients with nasopharyngeal carcinoma. *Q.J. Med.*, 78:165–176.
371. Landauer, M.R., Davis, H.D., Dominitz, J.A., and Weiss, J.F. (1987): Effects of acute gamma radiation exposure on locomotor activity of Swiss-Webster mice. *Toxicologist*, 7:253.
372. Landauer, M.R., Davis, H.D., Dominitz, J.A., and Weiss, J.F. (1987): Dose and time relationships of the radioprotector WR-2721 on locomotor activity in mice. *Pharmacol. Biochem. Behav.*, 27:573–576.
373. Landauer, M.R., Davis, H.D., Dominitz, J.A., and Weiss, J.F., (1988): Long-term effects of

radioprotector WR-2721 on locomotor activity and body weight of mice following exposure to ionizing radiation. *Toxicology*, 49:315–323.
374. Landauer, M.R., Davis, H.D., Kumar, K.S., and Weiss, J.F. (1992): Behavioral toxicity of selected radioprotectors. *Adv. Space Res.*, 12:273–283.
375. Landauer, M.R., McChesney, D.G., and Ledney, G.D. (1997): Synthetic trehalose dicorynomycolate (S-TDCM): Behavioral effects and radioprotection. *J. Radiat. Res. (Tokyo)*, 38:45–54.
376. Landauer, M.R., Walden, T.L., and Davis, H.D., (1990): Behavioral effects of radioprotective agents in mice: Combination of WR-2721 and 16,16-dimethyl prostaglandin E2. In: *Frontiers in Radiation Biology*, edited by E.Riklis, pp. 199–207. VCH Publishers, New York.
377. Langlois, R.G., Ariyama, M., Kusunoki, Y., et al. (1993): Analysis of somatic cell mutations at the glycophorin A locus in atomic bomb survivors: A comparative study of assay methods. *Radiat. Res.*, 136:111–117.
378. Lauk, S., Bohm, M., Feiler, G., et al. (1989): Increased number of cardiac adrenergic receptors following local heart irradiation. *Radiat. Res.*, 119:157–165.
379. Law, M.P., and Ahier, R.G. (1989): Vascular and epithelial damage in the lung of the mouse after X rays or neutrons. *Radiat. Res.*, 117:128–144.
380. Le, X.C., Xing, J.Z., Lee, J., et al. (1998): Inducible repair of thymine glycol detected by an ultrasensitive assay for DNA damage. *Science*, 280:1066–1069.
381. Leadon, S.A. (1996): Repair of DNA damage produced by ionizing radiation: A minireview. *Semin. Radiat. Oncol.*, 6:295–305.
382. Lebedinsky, A.V., Grigoryev, U.G., and Demirchoglyan, G.G. (1958): On the biological effect of small doses of ionizing radiation. In: *Proceedings of Second United Nations International Conference on Peaceful Uses of Atomic Energy*, pp. 17–28. Geneva, Switzerland; 1982.
383. Lenoir, A. (1944): Adaptation and rontgenbestrahlung. *Radiol. Clin., (Basel)*, 13:264–276.
384. Liebster, J., and Kopoldova, J.K. (1964): The radiation chemistry of amino acids. *Adv. Radiat. Biol.*, 1:157–226.
385. Lindgren, M. (1958): On tolerance of brain tissue and sensitivity of brain tumors to irradiation. *Acta Radiol.*, 170:5–75.
386. Lingenfelser, S.K., Dallas, C.E., Jagoe, C.H., et al. (1997): Variation in blood cell DNA content in *Carassius carrassius* from ponds near Chernobyl, Ukraine. *Ecotoxicology*, 6:187–203.
387. Little, J.B., McGrandy, R.B., and Kennedy, A.R. (1978): Interactions between polonium-210, alpha-radiation, benzo(a)pyrene, and 0.9% NaCl solution instillations in the induction of experimental lung cancer. *Cancer Res.*, 38:1929–1935.
388. Littley, M.D., Shalet, S.M., and Beardwell, C.G. (1990): Radiation and hypothalamic-pituitary function. *Baillieres Clin. Endocrinol. Metab.*, 4:147–175.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_761.html[2010/10/28 18:47:35]

389. Littley, M.D., Shalet, S.M., and Beardwell, C.G. (1991): Radiation and the hypothalamic-pituitary axis. In: *Radiation Injury to the Nervous System*, edited by P.H.Gutin, S.A.Leibel, and G.E.Sheline, pp. 303–324. Raven Press, New York.
390. Littley, M.D., Shalet, S.M., Morgenstern, G.R., and Deakin, D. P. (1991): Endocrine and reproductive dysfunction following fractionated total body irradiation in adults. *Q. J. Med.*, 78:265–274.
391. Livingston, G.K., Jensen, R.H., Silberstein, E.B., et al. (1997): Radiobiological evaluation of immigrants from the vicinity of Chernobyl. *Int. J. Radiat. Biol.*, 72:703–713.
392. Lo, E.H., Frankel, K.A., Steinberg, G.K., et al. (1992): High-dose single-fraction brain irradiation: MRI, cerebral blood flow, electrophysiological, and histological studies. *Int. J. Radiat. Oncol. Biol. Phys.*, 22:47–55.
393. Lomat, L., Galburt, G., Quastel, M.R., et al. (1997): Incidence of childhood disease in Belarus associated with the Chernobyl accident. *Environ. Health Perspect.*, 105:1529–1532.
394. Lott, J.R. (1962): Changes in ventral root potentials during X-irradiation of the spinal cord in the cat. In: *Effects of Ionizing Radiation on the Nervous System; Proceedings*, pp. 85–92. International Atomic Energy Agency, Vienna. Vienna, Austria; 5–9 June 1961.
395. Lucas, J.N. (1997): Dose reconstruction for individuals exposed to ionizing radiation using chromosome painting. *Radiat. Res.*, 148:S33–S38.
396. Lundbeck, F., Uls, N., and Overgaard, J. (1989): Cystometric evaluation of early and late irradiation damage to the mouse urinary bladder. *Radiother. Oncol.*, 15:383–392.
397. Lundin, J., Clemedson, C.J., and Nelson, A. (1957): Early effects of whole-body irradiation on cholinesterase activity in guinea pig's blood with special regard to radiation sickness. *Acta Radiol.*, 48:52–64.
398. Luxin, W., Yongru, Z., Zufan, T., et al. (1990): Epidemiological investigation of radiological effects in high background radiation areas of Yangjiang, China. *J. Radiat. Res. (Tokyo)*, 31:119–136.
399. Lyman, R.S., Kupalov, R.S., and Scholz, W. (1933): Effects of roentgen rays on the central nervous system. Results of large doses on the brains of adult dogs. *A.M.A. Arch. Neurol. Psychiat.*, 29:56–87.
400. Macklis, R.M., Bellerive, M.R., and Humm, J.L. (1990): The radiotoxicology of Radithor. Analysis of an early case of iatrogenic poisoning by a radioactive patent medicine. *J. A. M. A.*, 264:619–621.
401. MacNaughton, W.K., Aurora, A.R., Bhamra, J., et al. (1998): Expression, activity and cellular localization of inducible nitric oxide synthase in rat ileum and colon post-irradiation. *Int. J. Radiat. Biol.*, 74:255–264.
402. MacNaughton, W.K., and Prud'Homme-LaLonde, L. (1995): Exposure to ionizing radiation alters vasoreactivity in rat jejunum ex vivo. *Can. J. Physiol. Pharmacol.*, 73:699–705.
403. MacNaughton, W.K., Leach, K.E., Prud'Homme-LaLonde, L., and Harding, R.K. (1997): Exposure to ionizing radiation increases responsiveness to neural secretory stimuli in the ferret jejunum in vitro. *Int. J. Radiat. Biol.*, 72:219–226.
404. MacNaughton, W.K., Leach, K.E., Prud'Homme-LaLonde, L., et al. (1994): Ionizing radiation reduces neurally evoked electrolyte transport in rat ileum through a mast cell-dependent mechanism. *Gastroenterology*, 106:324–335.
405. MacVittie, T.J., Monroy, R.L., Patchen, M.L., and Souza, L.M. (1990): Therapeutic use of recombinant human G-CSF (rhG-CSF) in a canine model of sublethal and lethal whole-body irradiation. *Int. J. Radiat. Biol.*, 57:723–736.
406. MacVittie, T.J., Monroy, R.L., Vigneulle, R.M., et al. (1991): The relative biological effectiveness of mixed fission-neutron-γ radiation on the hematopoietic syndrome in the canine: Effect of therapy on survival. *Radiat. Res.*, 128:S29-S36.
407. Maier, D.M., and Landauer, M.R. (1989): Effects of acute sublethal gamma radiation exposure on aggressive behavior in male mice: A dose-response study. *Aviat. Space Environ. Med.*, 60:774–778.
408. Maier, D.M., and Landauer, M.R. (1990): Onset of behavioral effects in mice exposed to 10 Gy 60Co radiation. *Aviat. Space Environ. Med.*, 61:893–898.
409. Maier, D.M., Landauer, M.R., Davis, H.D., and Walden, T.L. (1989): Effect of electron radiation on aggressive behavior, activity, and hemopoiesis in mice. *J. Radiat. Res. (Tokyo)*, 30:255–265.
410. Maisin, J.R. (1988): Acute radiation syndromes in man. In: *Terrestrial Space Radiation and Its Biological Effects*, edited by P.D.McCormack, C.E.Swenberg, and H.Bücker, pp. 445–463. Plenum Press, New York.
411. Maisin, J.R. (1998): Chemical radioprotection: Past, present, and future prospects. *Int. J. Radiat. Biol.*, 73:443–450.
412. Malysheva, O.A., and Shirinskii, V.S. (1998): Seasonal changes of secondary immunodeficiency in

patients with vascular dystonia (in Russian). *Klin. Med. (Mosk.)*, 76:34–36.
413. Margules, D.L. (1979): Beta-endorphin and endoloxone: Hormones of the autonomic nervous system for the conservation of expenditure of bodily resources and energy in anticipation of famine or feast. *Neurosci. Biobehav. Rev.*, 3:155–162.
414. Marks, J.E., and Wong, J. (1985): The risk of cerebral radionecrosis in relation to dose, time and fractionation. *Prog. Exp. Tumor Res.*, 29:210–218.
415. Martin, C., Roman, V., Agay, D., and Fatome, M. (1998): Antiemetic effect of ondansetron and granisetron after exposure to mixed neutron and gamma irradiation. *Radiat. Res.*, 149:631–636.
416. Martin, S., Stratford, M.R.L., Watfa, R.R., et al. (1992): Collagen metabolism in the murine colon following X irradiation. *Radiat. Res.*, 130:38–47.
417. Martin, S., Vojnovic, B., Murray, J.C. (1991): Determination of x-ray-induced damage to the murine colon using tissue compliance measurements. *Int. J. Radiat. Biol.*, 59:503–515.
418. Maruyama, Y., and Feola, J.M. (1987): Relative radiosensitivities of the thymus, spleen, and lymphohemopoietic systems. In: *Advances in Radiation Biology, vol. 14, Relative Radiation Sensitivities of Human Organ Systems, Part II*, edited by K.I. Altman and J.T.Lett, pp. 1–82. Academic Press, San Diego.
419. Mastaglia, F.L., McDonald, W.I., Watson, J.V., and Yogendran, K. (1976): Effects of X-irradiation on the spinal cord: An experimental study of the morphological changes in central nerve fibers. *Brain*, 99:101–122.
420. Mattsson, A., Ruden, B.-I., Hall, P., et al. (1993): Radiation-induced breast cancer: Long-term follow-up of radiation therapy for benign breast disease. *J. Natl. Cancer Inst.*, 85:1679–1685.
421. Mattsson, A., Ruden, B.-I., Palmgren, J., et al. (1995): Dose- and time-response for breast cancer risk after radiation therapy for benign breast disease. *Br. J. Cancer*, 72:1054–1061.
422. Mattsson, J.L., and Yochmowitz, M.G. (1980): Radiation-induced emesis in monkeys. *Radiat. Res.*, 82:191–199.
423. McDonough, J.H., Mele, P.C., and Franz, C.G. (1992): Comparison of behavioral and radioprotective effects of WR-2721 and WR-36–89. *Pharmacol. Biochem. Behav.*, 42:233–243.
424. McFarland, W.L., and Levin, S.G. (1974): Electroencephalographic responses of 2500 rads of whole-body gamma-neutron radiation in the monkey Macaca mulatta. *Radiat. Res.*, 58:60–73.
425. McKay, M.J., Bull, C.A., Houghton, C.R., and Langlands, A.O. (1990): Persisting cyclical uterine bleeding in patients treated with

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_762.html[2010/10/28 18:47:36]

radical radiation therapy and hormonal replacement for carcinoma of the cervix. *Int. J. Radiat. Oncol. Biol. Phys.*, 18:921–925.
426. McMahon, T., and Vahora, S. (1986): Radiation damage to the brain. *Neuropsychiatr. Aspects*, 8:437–441.
427. Mechanick, J.I., Hochberg, F.H, and LaRocque, A. (1986): Hypothalamic dysfunction following whole brain irradiation. *J. Neurosurg.*, 65:490–494.
428. Medvedev, Z.A. (1979): *Nuclear Disaster in the Urals.* W.W. Norton, New York.
429. Medvedev, Z.A. (1990): *The Legacy of Chernobyl*, p. 187. W.W. Norton, New York.
430. Medvedev, Z.A. (1994): Chernobyl: Eight years after. *TREE*, 9:369–371.
431. Mefferd, J.M., Donaldson, S.S., and Link, M.P. (1989): Pediatric Hodgkin's disease: Pulmonary, cardiac, and thyroid function following combined modality therapy. *Int. J. Radiat. Oncol. Biol. Phys.*, 16:679–685.
432. Meistrich, M.L., and van Beek, M.E.A.B. (1990): Radiation sensitivity of the human testis. In: *Advances in Radiation Biology, Vol. 14, Relative Radiation Sensitivities of Human Organ Systems, Part II*, edited by K.I.Altman and J.T.Lett, pp. 227–268. Academic Press, San Diego.
433. Mele, P.C., Franz, C.G., and Harrison, J.R. (1988): Effects of sublethal doses of ionizing radiation on schedule-controlled performance in rats. *Pharmacol. Biochem. Behav.*, 30:1007–1014.
434. Mele, P.C., Franz, C.G., and Harrison, J.R. (1990): Effects of ionizing radiation on fixed-ratio escape performance in rats. *Neurotoxicol. Teratol.*, 12:367–373.
435. Mele, P.C., and McDonough, J.H. (1995): Gamma radiation-induced disruption in schedule-controlled performance in rats. *Neurotoxicology*, 16:497–510.
436. Melkonyan, H.S., Ushakova, T.E., and Umansky, S.R. (1995): Hsp 70 gene expression in mouse lung cells upon chronic gamma-irradiation. *Int. J. Radiat. Biol.*, 68:277–280.
437. Mettler, F.A., Jr., and Moseley, R.D., Jr. (1985): *Medical Effects of Ionizing Radiation.* Grune and Stratton, New York.
438. Meyerson, F.G. (1962): Effect of damaging doses of gamma-radiation on unconditioned and conditioned respiratory reflexes. In: *Works of the Institute of Higher Nervous Activity, Pathophysiological Series*, vol. 4, pp. 25–41. Izvestia Akademi USSR, Moscow, 1958; Israel Program Scientific Translation.
439. Michailov, M.C., Neu, E., Tempel, K., et al. (1991): Influence of X-irradiation on the motor activity of rat urinary bladder in vitro and in vivo. *Strahlenther. Onkol.*, 167:311–318.
440. Michel, C. (1989): Radiation embryology. *Experientia*, 45:69–77.
441. Mickley, G.A. (1980): Behavioral and physiological changes produced by a supralethal dose of ionizing radiation: Evidence for hormone-influenced sex differences in the rat. *Radiat. Res.*, 81:48–75.
442. Mickley, G.A. (1981): Antihistamine provides sex-specific radiation protection. *Aviat. Space Environ. Med.*, 52:247–250.
443. Mickley, G.A. (1991): Can animals serve as useful models for research on the psychological effects of radiation exposure? In: *The Medical Basis for Radiation-Accident Preparedness III: The Psychological Perspective*, edited by R.C.Ricks, M.E. Berger, and F.M.O'Hara, pp. 25–38. Elsevier, New York.
444. Mickley, G.A., Bogo, V., and West, B. (1989): Behavioral and neurophysiological changes with exposure to ionizing radiation. In: *Textbook of Military Medicine*, edited by R.Zajtchuck, D. P.Jenkins, R.F.Bellamy, V.M.Ingram, R.I.Walker, and T. J.Cerveny, pp. 105–151. U.S. Army, Washington, DC.
445. Mickley, G.A., Ferguson, J.L., Mulvihill, M.A., and Nemeth, T. J. (1989): Progressive behavioral changes during the maturation of rats with early radiation-induced hypoplasia of fascia dentata granule cells. *Neurotoxicol. Teratol.*, 11:385–393.
446. Mickley, G.A., Ferguson, J.L., and Nemeth, T.J. (1992): Serial injections of MK 801 (Dizocilpine) in neonatal rats reduce behavioral deficits associated with x-ray-induced hippocampal granule cell hypoplasia. *Pharmacol. Biochem. Behav.*, 43:785–793.
447. Mickley, G.A., Sessions, G.R., Bogo, V., and Chantry, K.H. (1983): Evidence for endorphin-mediated cross-tolerance between chronic stress and the behavioral effects of ionizing radiation. *Life Sci.*, 33:749–754.
448. Mickley, G.A., Stevens, K.E., Burrows, J.M., et al. (1983): Morphine tolerance offers protection from radiogenic performance decrements. *Radiat. Res.*, 93:381–387.
449. Mickley, G.A., Stevens, K.E., Moore, G.H., et al. (1983): Ionizing radiation alters beta-endorphin-like immunoreactivity in brain but not blood. *Pharmacol. Biochem. Behav.*, 19:979–983.
450. Mickley, G.A., Stevens, K.E., White, G.A., and Gibbs, G.L. (1983): Changes in morphine self-administration after exposure to ionizing radiation: Evidence for the involvement of endorphins. *Life Sci.*, 33:711–718.

451. Mickley, G.A., Stevens, K.E., White, G.A., and Gibbs, G.L. (1983): Endogenous opiates mediate radiogenic behavioral change. *Science*, 220:1185–1187.
452. Mickley, G.A., and Teitelbaum, H. (1978): Persistence of lateral hypothalamic-mediated behaviors after a supralethal dose of ionizing radiation. *Aviat. Space Environ. Med.*, 49:863–873.
453. Mickley, G.A., Teitelbaum, H., Parker, G.A., et al. (1982): Radiogenic changes in the behavior and physiology of the spontaneously hypertensive rat: Evidence for a dissociation between acute hypotension and incapacitation. *Aviat. Space Environ. Med.*, 53:633–638.
454. Middleton, G.R., and Young, R.W. (1975): Emesis in monkeys following exposure to ionizing radiation. *Aviat. Space Environ. Med.*, 46:170–172.
455. Miki, T., Fukiu, Y., Hisano, S., et al. (1996): Histogenetic abnormalities of the hippocampus in prenatally gamma-irradiated rats. *Teratology*, 54:15A.
456. Miletich, D.J., and Strike, T.A. (1970): Alteration of postirradiation hypotension and incapacitation in the monkey by administration of vasopressor drugs. *AFRRI Scientific Report, SR70–1*. Armed Forces Radiobiology Research Institute, Bethesda, MD.
457. Miller, D.S. (1962): Effects of low level radiation on audiogenic convulsive seizures in mice. In: *Response of the Nervous System to Ionizing Radiation*, edited by T.J.Haley and R.S.Snider, pp. 513–531. Academic Press, New York.
458. Miller, R.W. (1990): Effects of prenatal exposure to ionizing radiation. *Health Phys.*, 59:57–61.
459. Minamisawa, T., Hirokaga, K., Sasaki, S., and Noda, Y. (1992): Effects of fetal exposure to gamma rays on aggressive behavior in adult male mice. *J. Radiat. Res. (Tokyo)*, 33:243–249.
460. Minamisawa, T., and Hirokaga, K. (1995): Long term effects of prenatal exposure to low level gamma rays on spontaneous circadian motor activity of male mice. *J. Radiat. Res. (Tokyo)*, 36:179–184.
461. Minamisawa, T., and Hirokaga, K. (1995): Long-term effects of prenatal exposure to low levels of gamma rays on open-field activity in male mice. *Radiat. Res.*, 144:237–240.
462. Mintz, M., Yovel, G., Gigi, A., and Myslobodsky, M.S. (1998): Dissociation between startle and prepulse inhibition in rats exposed to gamma radiation at day 15 of embryogeny. *Brain Res. Bull.*, 45:289–296.
463. Mitchell, J.B., and Russo, A. (1987): The role of glutathione in radiation and drug induced cytotoxicity. *Br. J. Cancer*, 55:96–104.
464. Mitchell, J.B., Wink, D.A., DeGraff, W., et al. (1993): Hypoxic mammalian cell radiosensitization by nitric oxide. *Cancer Res.*, 53:5845–5848.

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465. Miyachi, Y. (1997): Analgesia induced by repeated exposure to low dose x-rays in mice, and involvement of the accessory olfactory system in modulation of the radiation effects. *Brain Res. Bull*, 44:177–182.
466. Miyachi, Y., Kasai, H., Ohyama, H., and Yamada, T. (1994): Changes of aggressive behavior and brain serotonin turnover after very low-dose X-irradiation of mice. *Neurosci. Lett.*, 175:92–94.
467. Miyachi, Y., Koizumi, T., and Yamada, T. (1994): Immediate arousal response and adaptation to low-dose x-rays in mouse and its disappearance by olfactory bulbectomy and nitric oxide inhibitor. *Neurosci. Lett.*, 177:32–34.
468. Miyachi, Y., and Yamada, T. (1994): Low-dose x-ray-induced depression of sexual behavior in mice. *Behav. Brain Res.*, 65:113–115.
469. Miyachi, Y., and Yamada, T. (1996): Head-portion exposure to low-level x-rays reduces isolation-induced aggression of mouse, and involvement of the olfactory caraosine in modulation of the radiation effects. *Behav. Brain Res.*, 81:135–140.
470. Mole, R.H. (1986): Problems related to prenatal exposure of the nervous systems: History and perspective. In: *Radiation Risks to the Developing Nervous System*, edited by H.Kriegel, W. Schmahl, G.B.Gerber, and F.-E.Stieve, pp. 1–20. Gustav Fischer, Stuttgart, Germany.
471. Mole, R.H. (1990): Severe mental retardation after large prenatal exposures to bomb radiation. Reduction in oxygen transport to fetal brain: A possible abscopal mechanism. *Int. J. Radiat. Biol.*, 58:705–711.
472. Mole, R.H. (1992): Expectation of malformations after irradiation of the developing human in utero: The experimental basis for predictions. In: *Advances in Radiation Biology, Vol. 15, Relative Radiation Sensitivities of Human Organ Systems, part III*, edited by K.I.Altman and J.L.Lett, pp. 217–301. Academic Press, San Diego.
473. Molostovov, G.S., and Shavrova, E.N. (1997): Immunophenotyping of peripheral blood lymphocytes in children and adolescents with Hashimoto's thyroiditis (in Russian). *Vyestsi Akademii Navuk Byelarusi Syeryya Biyalahichnykh Navuk*, 1:93–100.
474. Monnier, M., and Krupp, P. (1962): Action of gamma radiation on electrical brain activity. In: *Response of the Nervous System to Ionizing Radiation*, edited by T.J.Haley, and R.S.Snider, pp. 607–617. Academic Press, New York.
475. Monroy, R.L. (1987): Radiation effects on the lymphohematopoietic system: A compromise in immune competency. In: *Military Radiobiology*, edited by J.J.Conklin and R.I.Walker, pp. 113–134. Academic Press, San Diego.
476. Monson, R.R., and MacMahon, B. (1984): Prenatal x-ray exposure and cancer in children. In: *Radiation Carcinogenesis: Epidemiology and Biological Significance*, edited by J.D.Boice, Jr, and J.F.Faumeni, Jr, pp. 97–105. Raven Press, New York.
477. Mooibroek, J., Trieling, W.B., and Konings, W.T. (1982): Comparison of the radiosensitivity of unsaturated fatty acids, structured as micelles or liposomes, under different experimental conditions. *Int. J. Radiat. Biol.*, 42:601–609.
478. Moore, I.M., Kramer, J.H., Wara, W., et al. (1991): Cognitive function in children with leukemia. Effect of radiation dose and time since irradiation. *Cancer*, 68:1913–1917.
479. Morse, D.E., and Mickley, G.A. (1988): Interaction of the endogenous opioid system and radiation in the suppression of appetite behavior. *Soc. Neurosci. Abstr.*, 14:1106.
480. Moulder, J.E., Fish, B.L., Holcenberg, J.S., and Sun, G.X. (1990): Hepatic function and drug pharmacokinetics after total body irradiation plus bone marrow transplant. *Int. J. Radiat. Oncol. Biol. Phys.*, 19:1389–1396.
481. Mtskhvetadze, A.V., and Kucherenko, T.M. (1968): Direct and indirect effect of irradiation on the transmission of the stimulus in the upper neck sympathetic ganglion of cats. *Radiobiologiia*, 8:624–627.
482. Mukherjee, S.K., Goel, H.C., Pant, K., and Jain, V. (1997): Prevention of radiation induced taste aversion in rats. *Ind. J. Exp. Biol.*, 35:232–235.
483. Mullenix, P.J., Kernan, W.J., Schunior, A., et al. (1994): Interactions of steroid, methotrexate, and radiation determine neurotoxicity in an animal model to study therapy for childhood leukemia. *Pediatr. Res.*, 35:171–178.
484. Mullenix, P.J. (1998): Radiation protection in the developing central nervous system: Investigation of a biological approach. In: *Radiprotectors: Chemical, Biological, and Clinical Perspectives*, edited by E.Bump, pp. 349–371. CRC Press LLC, Washington, DC.
485. Mullin, M.J., Hunt, W.A., and Harris, R.A. (1986): Ionizing radiation alters the properties of sodium channels in rat brain synaptosomes. *J. Neurochem.*, 47:489–495.

486. Munro, T.R. (1970): The relative radiosensitivity of the nucleus and cytoplasm of Chinese hamster fibroblasts. *Radiat. Res.*, 42:451–470.
487. Muscel, R.J., Zhang, H.B., and McKenna, W.G. (1993): Differential effect of ionizing radiation on the expression of cyclin A and cyclin B in Hela cells. *Cancer Res.*, 53:1128–1135.
488. Myers, J.H., Blackwell, L.H., and Overman, R.R. (1972): Early functional hemodynamic impairment in baboons after 1000 R or less of gamma radiation as revealed by hemorrhagic stress. *Radiat. Res.*, 52:564–578.
489. Nagata, S., and Golstein, P. (1995): The fas death factor. *Science*, 267:1449–1456.
490. Nam, S.Y., Kim, J.H., Cho, C.K., et al. (1997): Enhancement of radiation-induced hepatic microsomal epoxide hydrolase gene expression by oltipraz in rats. *Radiat. Res.*, 147:613–620.
491. Nandchahal, K. (1990): Mitotic figures and pyknotic nuclei and necrotic cells in the mouse jejunum during injury and repair after whole-body gamma irradiation. *Radiobiol. Radiother. (Berlin)*, 31:333–336.
492. National Council on Radiation Protection and Measurements (NCRP). (1987): Ionizing radiation exposures of the population of the United States. *NCRP Report No. 93.* National Council on Radiation Protection and Measurements, Washington, DC.
493. National Council on Radiation Protection and Measurements (NCRP). (1990): The relative biological effectiveness of radiations of different quality. *NCRP Report 104.* National Council on Radiation Protection and Measurements, Bethesda, MD.
494. Neta, R., and Okunieff, P. (1996): Cytokine-induced radiation protection and sensitization. *Semin. Radiat. Oncol.*, 6:306–320
495. Nicklas, J.A., O'Neill, J.P., and Albertini, R.J. (1986): Use of T-cell receptor gene probes to quantify the in vivo hprt mutations in human T-lymphocytes. *Mutat. Res.*, 173:67–72.
496. Nikiforov, Y.E., Nikiforova, M., and Fagin, A. (1998): Radiation-induced post-Chernobyl pediatric thyroid carcinomas. *Oncogene,* 17:1983–1988.
497. Nokta, M., Belli, J., and Pollard, R. (1992): X-irradiation enhances in vitro human immunodeficiency virus replication. Correlation with cellular levels of cAMP. *Proc. Soc. Exp. Biol. Med.*, 200:402–408.
498. Norton, S., and Kimler, B.F. (1988): Comparison of functional and morphological deficits in the rat after gestational exposure to ionizing radiation. *Neurotoxicol. Teratol.*, 10:363–371.
499. Norton, S., Kimler, B.F., and Mullenix, P.J. (1991): Progressive behavioral changes in rats after exposure to low levels of ionizing radiation in utero. *Neurotoxicol. Teratol.*, 13:181–188.

500. Norton, S., Mullenix, P., and Culver, B. (1976): Comparison of the structure of hyperactive behavior in rats after brain damage from X-irradiation, carbon monoxide and pallidal lesions. *Brain Res.*, 116:49–67.
501. Nozue, M., and Ogata, T. (1989): Correlation among lung damage after radiation, amount of lipid peroxides, and antioxidant enzyme activities. *Exp. Mol. Pathol.*, 50:239–252.
502. Obe, G., Johannes, I., Johannes, C., et al. (1997): Chromosomal aberrations in blood lymphocytes of astronauts after long-term space flights. *Int. J. Radiat. Biol.*, 72:727–734.
503. O'Connel, J.F.A., and Brunschwig, A. (1937): Observations on the Roentgen treatment of intracranial gliomata with special reference to the effects of irradiation upon the surrounding brain. *Brain*, 60:230–258.
504. Odum, E.P. (1971): *Fundamentals of Ecology*, 3rd ed. W.B. Saunders, Philadelphia.
505. Ogilvy-Stuart, A.L., Shalet, S.M., and Gattamaneni, H.R. (1991): Thyroid function after treatment of brain tumors in children. *J. Pediatr.*, 119:733–737.
506. Okada, S., Okeda, R., Matsushita, S., and Kawano, A. (1998): Histopathological and morphometric study of the late effects of heavy-ion irradiation on the spinal cord of the rat. *Radiat. Res.*, 150:304–315.
507. Oleinick, N.L., and Rustad, M. (1976): Interrelationships between ionizing radiation, protein synthesis, and the physiological expressions of radiation damage. *Adv. Radiat. Biol.*, 6:107–160.
508. Olinski, R., Nackerdien, Z., and Dizdaroglu, M. (1992): DNA-protein cross-linking between thymine and tyrosine in chromatin of gamma-irradiated or H2O2-treated cultured human cells. *Arch. Biochem. Biophys.*, 297:139–143.
509. Olschowka, J.A., Kyrkanides, S., Harvey, B.K., et al. (1997): ICAM-1 induction in the mouse CNS following irradiation. *Brain Behav. Immunol.*, 11:273–285.
510. Onoda, J.M., Piechocki, M.P., and Honn, K.V. (1992): Radiation-induced increase in expression of the alpha IIb beta 3 integrin in melanoma cells: Effects on metastatic potential. *Radiat. Res.*, 130:281–288.
511. Ord, M.G., and Stocken, L.A. (1968): Variations in the phosphate content and thiol/disulfide ratio of histones during the cell cycle. *Biochem. J.* 107:403–410.
512. Ortin, T.T., Shostak, C.A., and Donaldson, S.S. (1990): Gonadal status and reproductive function following treatment for Hodgkin's disease in childhood: The Stanford experience. *Int. J. Radiat. Oncol. Biol. Phys.*, 19:873–880.
513. Otake, M., and Schull, W.J. (1984): In utero exposure to A-bomb radiation and mental retardation; a reassessment. *Br. J. Radiol.*, 57:409–414.
514. Otake, M., and Schull, W.J. (1998): Radiation-related brain damage and growth retardation among prenatally exposed atomic bomb survivors. *Int. J. Radiat. Biol.*, 74:159–171.
515. Overgaard, J., and Matsui, M. (1990): Effect of radiation on glucose absorption in the mouse jejunum in vivo. *Radiother. Oncol.*, 18:71–77.
516. Pallis, C.A., Louis, S., and Morgan, R.L. (1961): Brain myelopathy. *Brain*, 84:460–479.
517. Parshkov, E.M., Chebotareva, I.V., Sokolov, V.A., and Dallas, C.E. (1998): Additional thyroid dose factor from transportation sources in Russia following the Chernobyl disaster. *Environ. Health Perspect.*, 105:1491–1496.
518. Patchen, M.L., MacVittie, T.J., Solberg, B.D., and Souza, L.M. (1990): Therapeutic administration of recombinant human granulocyte colony-stimulating factor accelerates hemopoietic regeneration and enhances survival in a murine model of radiation-induced myelosuppression. *Int. J. Cell Cloning*, 8:107–122.
519. Patchen, M.L., MacVittie, T.J., Solberg, B.D., and Souza, L.M. (1990): Survival enhancement and hemopoietic regeneration following radiation exposure: Therapeutic approach using glucan and granulocyte colony-stimulating factor. *Exp. Hematol.*, 18:1042–1048.
520. Patchen, M.L., MacVittie, T.J., and Souza, L.M. (1992): Postirradiation treatment with granulocyte colony-stimulating factor and preirradiation WR-2721 administration synergize to enhance hemopoietic reconstitution and increase survival. *Int. J. Radiat. Oncol. Biol. Phys.*, 22:773–779.
521. Patchen, M.L., MacVittie, T.J., Williams, J.L., et al. (1991): Administration of interleukin-6 stimulates multilineage hematopoiesis and accelerates recovery from radiation-induced hematopoietic depression. *Blood*, 77:472–480.
522. Peimer, S.I., Dudkin, A.O., and Swerdlov, A.G. (1986): Response of hippocampal pacemaker-like neurons to low doses of ionizing radiation. *Int. J. Radiat. Biol.*, 49:597–600.
523. Pellmar, T.C., and Lepinski, D.L. (1993): Gamma radiation (5–10 Gy) impairs neuronal function in

the guinea pig hippocampus. *Radiat. Res.,* 136:255–261.
524. Pellmar, T.C., Schauer, D.A., and Zeman, G.H. (1990): Timeand dose-dependent changes in neuronal activity produced by X radiation in brain slices. *Radiat. Res.*, 122:209–214.
525. Peter, R.U., Braun-Falco, O., Birioukov, A., et al. (1994): Chronic cutaneous damage after accidental exposure to ionizing radiation: The Chernobyl experience. *J. Am. Acad. Dermatol.,* 30(5 Part 1):719–723.
526. Peterson, L.M., Evens, M.L., Graham, M.M., et al. (1992): Vascular response to radiation injury in the rat lung. *Radiat. Res.*, 129:139–148.
527. Petridou, E., Trichopoulos, D., Dessypris, N., et al. (1996): Infant leukaemia after in utero exposure to radiation from Chernobyl. *Nature,* 382:352–353.
528. Philpott, D.E., Sapp, W., Miquel, J., et al. (1985): The effect of high energy (HZE) particle radiation (40 Ar) on aging parameters of mouse hippocampus and retina. In: *Scanning Electron Microspy,* III, edited by A.M.F.O'Hare, pp. 1177–1182. SEM, Inc, Chicago, IL.
529. Pineau, C., Velez de la Calle, J.F., Pinon-Lataillade, G., and Jegou, B. (1989): Assessment of testicular function after acute and chronic irradiation: Further evidence for an influence of late spermatids on Sertoli cell function in the adult rat. *Endocrinology,* 124:2720–2728.
530. Pinon-Lataillade, G., Viguier-Martinez, M.C., Touzalin, A.M., et al. (1991): Effect of an acute exposure of rat testes to gamma rays on germ cells and on Sertoli and Leydig cell functions. *Reprod. Nutr. Dev.* 31:617–629.
531. Pollack, A., and Zagars, G.K. (1998): Androgen ablation in addition to radiation therapy for prostate cancer. Is there a true benefit? *Semin. Radiat. Oncol.,* 8:95–106.
532. Pollack, M., and Timiras, P.S. (1964): X-ray dose and electroconvulsive responses in adult rats. *Radiat. Res.,* 21:111–119.
533. Pourquier, H., Baker, J.R., Giaux, G., and Benirschke, K. (1958): Localized roentgen-ray beam irradiation of the hypophysohypothalamic region of the guinea pig with a 2 million volt van de Graaf generator. *Am. J. Roentgenol. Radium Ther. Nucl. Med.,* 80:840–850.
534. Powers, D.A., Kress, T.S., and Jankowski, M.W. (1987): The Chernobyl source term. *Nuclear Safety*, 28:10.
535. Prasad, A.V., Mohan, N., Chandrasekar, B., and Meltz, M. (1995): Induction of transcription of "immediate early genes" by low-dose ionizing radiation. *Radiat. Res.,* 143:263–272.
536. Priestman, T., Challoner, T., Butcher, M., and Priestman, S. (1987): Control of radiation induced emesis with GR38032F (GR). *Proc. Am. Soc. Clin. Oncol.,* 7:281.

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537. Quastel, M.R., Goldsmith, J.R., Cwikel, J., et al. (1997): Commentary: Lessons learned from the study of immigrants to Israel from areas of Russia, Belarus, and Ukraine contaminated by the Chernobyl accident. *Environ. Health Perspect.*, 105:1523–1528.
538. Quastel, M.R., Goldsmith, J.R., Mirkin, L., et al. (1997): Thyroid-stimulating hormone levels in children from Chernobyl. *Environ. Health Perspect.*, 105:1497–1498.
539. Rabin, B.M. (1996): Free radicals and taste aversion learning in the rat: Nitric oxide, radiation and dopamine. *Prog. Neuropsychopharmacol. Biol. Psychiatry*, 20:691–707.
540. Rabin, B.M., Hunt, W.A., Joseph, J.A., et al. (1991): Relationship between linear energy transfer and behavioral toxicity in rats following exposure to protons and heavy particles. *Radiat. Res.*, 128:216–221.
541. Rabin, B.M., Joseph, J.A., and Erat, S. (1998): Effects of exposure to different types of radiation on behaviors mediated by peripheral or central systems. *Adv. Space Res.*, 22:217–225.
542. Rakic, P. (1986): Normal and abnormal neuronal migration during brain development. In: *Radiation Risks to the Developing Nervous System*, edited by H.Kriegel, W.Schmahl, G.B.Gerber, and F.-E. Stieve, pp. 35–44. Gustav Fischer Verlag, Stuttgart.
543. Raleigh, J.A. (1987): Radiation peroxidation in model membranes. In: *Prostaglandin and Lipid Metabolism in Radiation Injury*, edited by T.L.Walden, Jr. and H.N.Hughes, pp. 1–27. Plenum Press, New York.
544. Randolph, M.L., and Brewen, J.G. (1980): Estimation of whole-body doses by means of chromosome aberrations observed in survivors of the Hiroshima A-bomb. *Radiat. Res.*, 82:393–407.
545. Reinhold, H.S., Fajardo, L.F., and Hopewell, J.W. (1990): The vascular system. In: *Advances in Radiation Biology, Vol. 14, Relative Radiation Sensitivities of Human Organ Systems, Part II*, edited by K.I.Altman and J.T.Lett, pp. 177–226. Academic Press, San Diego.
546. Reiter, R., Tang, L., Garcia, J.J., and Munoz-Hoyos, A. (1997): Pharmacological actions of melatonin in oxygen radical pathophysiology. *Life Sci.*, 60:2255–2271.
547. Reyners, H., Gianfelici de Reyners, E., and Maisin, J.R. (1982): The beta-astrocyte: A newly recognized radiosensitive glial cell type in the cerebral cortex. *J. Neurocytol.*, 11:967–983.
548. Reyners, H., Gianfelici de Reyners, E., and Maisin, J.R. (1986): Early cell regeneration processes after split-dose X-irradiation of the cerebral cortex of the rat. *Br. J, Cancer Suppl VII*, 53:218–220.
549. Reyners, H., Gianfelici de Reyners, E., and Maisin, J.R. (1986): The role of the glia in late damage after prenatal irradiation. In: *Radiation Risks to the Developing Nervous System*, edited by H.Kriegel, W.Schmahl, G.B.Gerber, and F.-E.Stieve, pp. 117–131. Gustav Fischer Verlag, Stuttgart.
550. Riches, A.C. (1995): Experimental radiation leukaemogenesis. In: *Radiation Toxicology: Bone Marrow and Leukaemia*, edited by J.H.Hendry and B.I.Lord, pp. 311–334. Taylor & Francis, Washington, DC.
551. Riopelle, A.J. (1962): Some behavioral effects of ionizing radiation on primates. In: *Response of the Nervous System to Ionizing Radiation*, edited by T.J.Haley, and R.S.Snider, pp. 719–728. Academic Press, New York.
552. Robbins, M.E., Bywaters, T., Rezvani, M, et al. (1991): Residual radiation-induced damage to the kidney of the pig as assayed by retreatment. *Int. J. Radiat. Biol.*, 60:917–928.
553. Robbins, M.E., Campling, D., Rezvani, M., et al. (1989): Nephropathy in the mature pig after the irradiation of a single kidney: A comparison with the immature pig. *Int. J. Radiat. Oncol. Biol. Phys.*, 16:1519–1528.
554. Robbins, M.E., Campling, D., Rezvani, M., et al. (1989): Radiation nephropathy in mature pigs following the irradiation of both kidneys. *Int. J. Radiat. Biol.*, 56:83–98.
555. Robertson, J.B. (1989): Toxicology of ionizing radiation. In: *A Guide to General Toxicology*, 2nd ed., edited by J.K.Marquis, pp. 141–156. S.Karger AG, New York.
556. Rodvall, Y., Pershagen, G., Hrubec, Z., et al. (1990): Prenatal x-ray exposure and childhood cancer in Swedish twins. *Int. J. Cancer*, 46:362–365.
557. Roizin, L., Akai, K., Carsten, A., et al. (1976): Post-x-ray myelinopathy (pathogenic mechanisms). In: *International Symposium on the Aetiology and Pathogenesis of Demyelinating Diseases*, edited by T.Yonawa, pp. 29–57. Japan Press Co, Heiho-Sha, Japan.
558. Romanova, L.K., and Zhorova, E.S. (1994): The effect of irradiation at small doses on human embryos and fetuses (in Russian). *Ontogenez*, 25:55–65.
559. Ron, E., Lubin, J.H., Shore, R.E., et al. (1995): Thyroid cancer after exposure to external radiation: A pooled analysis of seven studies. *Radiat. Res.*, 141:259–277.
560. Rosenthal, F., and Timiras, P.S. (1961): Changes in brain excitability after whole-body X-irradiation

in the rat. *Radiat. Res.*, 18:648–657.
561. Rosenthal, F., and Timiras, P.S. (1961): Threshold and pattern of electroshock seizures after 250 R whole-body X-irradiation in rats. *Proc. Soc. Exp. Biol. Med.*, 208:267–270.
562. Ross, J.A.T., Levitt, S.R., Holst, E.A., and Clemente, C.D. (1954): Neurological and electroencephalographic effects of X irradiation of the head in monkeys. *A.M.A. Arch. Neurol. Psychiatry*, 71:238–249.
563. Rothenberg, M.A. (1950): Studies on permeability in relation to nerve function. II. Ionic movements across axonal membranes. *Biochim. Biophys. Acta*, 4:96–114.
564. Rowland, R.E., and Lucas, H.F., Jr. (1984): Radium-dial workers. In: *Radiation Carcinogenesis: Epidemiology and Biological Significance*, edited by J.D.Boice, Jr., and J.F.Faumenir, Jr., pp. 231–240. Raven Press, New York.
565. Rubin, P., Johnston, C.J., Williams, J.P., et al. (1995): A perpetual cascade of cytokines postirradiation leads to pulmonary fibrosis. *Int. J. Radiat. Biol. Phys.*, 33:99–110.
566. Rudoltz, M.S., Kao, G., Blank, K.R., et al. (1996): Molecular biology of the cell cycle: Potential for therapeutic applications in radiation oncology. *Semin. Radiat. Oncol.*, 6:284–294.
567. Russel, D.S., Wilson, C.W., and Tansley, K. (1949): Experimental radionecrosis in the brains of rabbits. *J. Neurol. Neurosurg. Psychiatry*, 12:187–195.
568. Russo, I.H., and Russo, J. (1996): Mammary gland neoplasia in long-term rodent studies. *Environ, Health Perspect.*, 105:938–967.
569. Rytomaa, T. (1996): Ten years after Chernobyl. *Ann. Med.*, 28:83–87.
570. Sabine, J.C. (1956): Inactivation of cholinesterases by gamma radiation. *Am. J. Physiol.*, 187:280–282.
571. Sadekova, S., Lehnert, S., and Chow, T.Y. (1997): Induction of PBP74/mortalin/Grp75, a member of the hsp 70 family, by low doses of ionizing radiation: A possible role in induced radioresistance. *Int. J. Radiat. Biol.*, 72:653–660.
572. Saenger, E.L., and Hinnefeld, J. (1991): Perception of radiation injury versus radiogenic effect. In: *The Medical Basis for Radiation-Accident Preparedness*, III: *The Psychological perspective*, edited by R.C.Ricks, M.E.Berger, and F.M.O'hara, pp. 39–50. Elsevier, New York.
573. Sakuma, S., Saya, H., Ijichi, A., and Tofilon, P. (1995): Radiation induction of the receptor tyrosine kinase gene Ptk-3 in normal rat astrocytes. *Radiat. Res.*, 143:1–7.

page_767

574. Samaan, N.A. (1990): Hypothalamic-pituitary failure after radiotherapy for tumors of the head and neck. In: *Advances in Radiation Biology, Vol. 14, Relative Radiation Sensitivities of Human Organ Systems, Part II*, edited by K.I.Altman and J.T.Lett, pp. 111–117. Academic Press, San Diego.
575. Sanders, C.L. (1986): *Toxicological Aspects of Energy Production*. Battelle Press, Columbus, OH, and Richland, WA.
576. Sarri, Y., Conill, C., Verger, E., et al. (1991): Effects of single dose irradiation on pancreatic beta-cell function. *Radiother. Oncol.*, 22:143–144.
577. Sato, M. (1978): Electrophysiological studies on radiation-induced changes in the adult nervous system. In: *Advances in Radiation Biology, Vol. 7, Relative Radiation Sensitivities of Human Organ Systems*, edited by J.T.Lett and H.Adler, pp. 181–221. Academic Press, New York.
578. Sato, M., and Austin, G. (1964): Acute radiation effects on mammalian synaptic activities. In: *Response of the Nervous System to Ionizing Radiation*, edited by T.J.Haley and R.S.Snider, pp. 279–289. Little, Brown and Co, Boston.
579. Sato, M., Austin, G.M., and Stahl, W. (1962): The effects of ionizing radiation on spinal cord neurons. In: *Response of the Nervous System to Ionizing Radiation*, edited by T.J.Haley and R. Snider, pp. 561–671. Academic Press, New York.
580. Sato, M., Austin, G.M., and Stahl, W. (1962): Delayed radiation effects on neuronal activity in the spinal cord of the cat. In: *Effects of Ionizing Radiation on the Nervous System; Proceedings of the Symposium on the Effects of Ionizing Radiation on the Nervous System*. pp. 93–110. International Atomic Energy Agency, Vienna. Vienna, Austria; 5–9 June 1961.
581. Sato, M., Stahl, W., and Austin, G.M. (1963): Acute radiation effects on synaptic activity in the mammalian spinal cord. *Radiat. Res.*, 18:307–320.
582. Scheid, W., Weber, J., Petrenko, S., and Traut, H. (1992): Chromosome aberrations in human lymphocytes apparently induced by Chernobyl fallout. *Health Phys.*, 64:531–534.
583. Schmidt-Ullrich, R.K., Mikkelsen, R.B., Dent, P., et al. (1997): Radiation-induced proliferation of the human A431 squamous carcinoma cells is dependent on EGRF tyrosine phosphorylation. *Oncogene*, 15:1191–1197.
584. Schmidt-Ullrich, R.K., Valerie, K.C., Chan, W., and McWilliams, D. (1994): Altered expression of epidermal growth factor receptor and estrogen receptor in MCF-7 cells after single and repeated radiation exposure. *Int. J. Radiat. Oncol. Biol. Phys.*, 29:813–819.
585. Schmitt, G., and Zamboglou, N. (1990): Radiation effects on bone and cartilage. In: *Advances in Radiation Biology, Vol. 14, Relative Radiation Sensitivities of Human Organ Systems, Part II*, edited by K.I.Altman and J.T.Lett, pp. 157–176. Academic Press, San Diego.
586. Scholz, W., Ducho, E.G., and Breit, A. (1959): Experimentelle Roentgenspatschaden am ruchenmark des erwachsenen kaninchens. Ein weiterer beitrag zur wirkungsweise ionisierender strahlen auf das zentralnervose gewebe. *Psychiat. Neurol. Japan*, 61:417–442.
587. Scholz, W., Schlote, W., and Hirschberger, W. (1962): Morphological effect of repeated low dosage and single high dosage of X-irradiation to the central nervous system. In: *Response of the Nervous System to Ionizing Radiation*, edited by T.J.Haley and R.S.Snider, pp. 211–232, Academic Press, New York.
588. Schrag, S.D., and Dixson, R.L. (1985): Occupational exposures associated with male reproductive dysfunction. *Ann. Rev. Pharmacol. Toxicol.*, 25:567–592.
589. Schull, W.J. (1995): *Effects of Atomic Radiation: A Half Century of Studies From Hiroshima and Nagasaki*. Wiley-Liss, New York.
590. Schull, W.J., and Otake, M. (1986): Neurological deficit among the survivors exposed in utero to the atomic bombing of Hiroshima and Nagasaki: A reassessment and new directions. In: *Radiation Risks to the Developing Nervous System*, edited by H.Kriegel, W.Schmahl, G.B.Gerber, and F.-E.Stieve, pp. 399–419. Gustav Fischer Verlag, Stuttgart.
591. Schultz-Hector, S., Böhm, M., Blöchel, A., et al. (1992): Radiation-induced heart disease: Morphology, changes in catecholamine synthesis and content, β-adrenoceptor density, and hemodynamic function in an experimental model. *Radiat. Res.*, 129:281–289.
592. Schwartz, G.N., Neta, R., Vigneulle, R.M., et al. (1988): Recovery of hematopoietic colony-forming cells in irradiated mice pretreated with interleukin 1 (IL-1). *Exp. Hematol.*, 16:752–757.
593. Scott, B.R., and Dillehay, L.E. (1990): A model for hematopoietic death in man from irradiation of bone marrow during radioimmunotherapy. *Br. J. Radiol.*, 63:862–870.
594. Segerstahl, B. (1991): The Costs. In: *Chernobyl: A Policy Response Study*, edited by B.Segerstahl, p. 59. Springer-Verlag, New York.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_767.html[2010/10/28 18:47:39]

595. Seigneur, L.J., and Brennan, J.T. (1966): Incapacitation in the monkey (Macaca Mulatta) following exposure to a pulse of reactor radiation. *AFRRI Scientific Report SR 66–2*. Armed Forces Radiobiology Research Institute, Bethesda, MD.
596. Severa, J., and Bár, J. (1991): *Hand Book of Radioactive Contamination and Decontamination*. Elsevier Science, New York.
597. Shalet, S.M., Tsatsoulis, A., Whitehead, E., and Read, G. (1989): Vulnerability of the human Leydig cell to radiation damage is dependent upon age. *J. Endocrinol.*, 120:161–165.
598. Sharp, J.C., Kelly, D.D., and Brady, J.V. (1986): The radio-attenuating effects of n-decylaminoethanethiosulfuric acid in the rhesus monkey. In: *Use of Nonhuman Primates in Drug Evaluation*, edited by H.Vagtborg, pp. 338–346. Southwest Foundation for Research and Education, San Antonio, TX.
599. Sharplin, J., and Franko, A.J. (1989): A quantitative histological study of strain-dependent differences in the effects of irradiation on mouse lung during the intermediate and late phases. *Radiat. Res.*, 119:15–31.
600. Shaw, E.G., Gunderson, L.L., Martin, J.K., et al. (1990): Peripheral nerve and ureteral tolerance to intraoperative radiation therapy: Clinical and dose-response analysis. *Radiother. Oncol.*, 18:247–255.
601. Sheline, G.E., Wara, W.M., and Smith, V. (1980): Therapeutic irradiation and brain injury. *Int. J. Radiat. Oncol. Biol. Phys.*, 6:1215–1228.
602. Shenkin, H.A. (1951): Effects of various drugs upon cerebral circulation and metabolism in man. *J. Appl. Physiol.*, 3:465–471.
603. Shevchenko, V.A., Pomerantseva, M.D., Ramaiya, L.K., et al. (1992): Genetic disorders in mice exposed to radiation in the vicinity of the Chernobyl nuclear power station. *Sci. Tot. Environ.*, 112:45–56.
604. Shigematzu, I. (1991): *The International Chernobyl Project. An Overview. Assessment of Radiological Consequences and Evaluation of Protective Measures*. Report by an International Advisory Committee. International Atomic Energy Agency, Vienna.
605. Shimada, Y., Yasukawa-Barnes, J., Kim, R.Y., et al. (1994): Age and radiation sensitivity of rat mammary clonogenic cells. *Radiat. Res.*, 137:118–123.
606. Shipman, T.L., Lushbaugh, C.C., Peterson, D.F., et al. (1961): Acute radiation death resulting from an accidental nuclear critical excursion. *J. Occup. Med.*, 3:146–192.
607. Shubik, V.M., Zaitseva, M.B., and Kositskaya, L.S. (1996): Role of immune deviations in some diseases observed in areas contaminated with radionuclides after the accident in Chernobyl NPP (in Russian). *Radiatsionnaya Biologiya Radioekologiya*, 36:332–337.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_767.html[2010/10/28 18:47:39]

page_768

608. Sich, A.R. (1994): Cheraobyl accident management actions: Implications for source term estimates. *Nuclear Safety*, 35:1–24.
609. Sienkiewicz, Z.J., Haylock, R.G., and Saunders, R.D. (1994): Prenatal irradiation and spatial memory in mice: Investigation of dose-response relationship. *Int. J. Radiat. Biol.*, 65:611–618.
610. Sklar, C.A., Kim, Th., and Ramsay, N.K.C. (1982): Thyroid dysfunction among long-term survivors of bone marrow transplantation. *Am. J. Med.*, 73:668–694.
611. Sklar, C.A., Robison, L.L., Nesbit, M.E., et al. (1990): Effects of radiation on testicular function in long-term survivors of childhood acute lymphoblastic leukemia: A report from the Children Cancer Study Group. *J. Clin. Oncol.*, 8:1981–1987.
612. Sloan, S.R., Newconb, E.W., and Pellicer, A. (1990): Neutron radiation can activate K-ras via a point mutation in codon 146 and induce a different spectrum of ras mutations than does gamma irradiation. *Mol. Cell. Biol.*, 10:405–408.
613. Sokolov, V.E., Rjabov, I.N., Ryabtsev, I.A., et al. (1993): Ecological and genetic consequences of the Chernobyl atomic power plant accident. *Vegetatio*, 109:91–99.
614. Southwood, R. (1987): Opening remarks. In: *Radiation and Health. The Biological Effects of Low-Level Exposure to Ionizing Radiation*, edited R.Jones and R.Southwood, pp. 3–6. John Wiley & Sons, New York.
615. Spotheim-Maurizot, M., Gardier, F., Sabattier, R., and Charlier, M. (1992): Metal ions protect DNA against strand blockage induced by fast neutrons. *Int. J. Radiat. Biol.*, 62:659–666.
616. Stapleton, G.E., and Curtis, H.J. (1946): The effects of fast neutrons on the ability of mice to take forced exercise. *US Atomic Energy Report No. 9. MDDC-696.* Oak Ridge National Laboratory, Oak Ridge, TN.
617. Stather, J.W., Dionian, J., Brown, J., et al. (1987): Assessing risks of childhood leukaemia in Seascale. In: *Radiation and Health. The Biological Effects of Low-Level Exposure to Ionizing Radiation*, edited by R.Jones and R.Southwood, pp. 65–80. John Wiley & Sons, New York.
618. Stephan, G., and Oestreicher, U. (1989): An increased frequency of structural chromosome aberrations in persons present in the vicinity of Chernobyl during and after the reactor accident: Is this effect caused by radiation exposure? *Mut. Res.*, 223:7–12.
619. Stevenson, M.A., Pollock, S.S., Coleman, N.C., and Calderwood, S.K. (1994): X-irradiation, phorbolesters, and H2O2 stimulate mitogen activated protein kinase activity in NIH-3T3 cells through the formation of reactive oxygen intermediates. *Cancer Res.*, 54:12–15.
620. Stewart, F.A., Lundbeck, F., Oussoren, Y., and Luts, A. (1991): Acute and late radiation damage in mouse bladder: A comparison of urination frequency and cystometry. *Int. J. Radiat. Oncol. Biol. Phys.*, 21:1211–1219.
621. Stewart, F.A., Luts, A., and Lebesque, J.V. (1989): The lack of long-term recovery and reirradiation tolerance in the mouse kidney. *Int. J. Radiat. Biol.*, 56:449–462.
622. Stewart, F.A., Oussoren, Y., and Luts, A. (1990): Long-term recovery and reirradiation tolerance of mouse bladder. *Int. J. Radiat. Oncol. Biol. Phys.*, 18:1399–1406.
623. Stewart, F.A., Soranson, J.A., Alpen, E.L., et al. (1984): Radiation-induced renal damage: The effects of hyperfractionation. *Radiat. Res.*, 98:407–420.
624. Stieve, F.-E. (1986): Experiences with accidents and consequences for treatment. *Br. J. Radiol.*, 59(Suppl 19): 18–22.
625. Stiehm, E.R. (1992): The psychologic fallout from Chernobyl. *Am. J. Dis. Child.*, 146:761–762.
626. Stryker, J.A., Robins, D.B., and Velkley, D.E. (1990): Relative radiosensitivity of the urinary bladder in cancer therapy. In: *Advances in Radiation Biology, Vol. 14, Relative Radiation Sensitivities of Human Organ Systems, Part H,* edited by K.I. Altman and J.T.Lett, pp. 1–21. Academic Press, San Diego.
627. Stuart, B.O., Palmer, R.F., Filipy, R.E., et al. (1977): Respiratory tract carcinogenesis in large and small experimental animals following daily inhalation of radon daughters and uranium ore dust. In: *Proceedings of the IVth Congress of the International Radiation Protection Association.* pp. 104–117. Foutenay, Aux Roses, France. IRPA.
628. Sugg, D.W., Bickham, J.W., Brooks, J.A., et al. (1996): DNA damage and radiocesium in channel catfish from Chernobyl. *Environ. Toxicol. Chem.*, 15:1057–1063.
629. Suzuki, K., Takahashi, M., Ishii-Ohba, H., et al. (1990): Steroidogenesis in the testes and the adrenals of adult male rats after gamma-irradiation in utero at late pregnancy. *J. Steroid. Biochem.*, 35:301–305.
630. Suzuki, R., Yamaguchi, T., Kirno, T., et al. (1983): The effects of 5-minute ischemia in mongolian gerbils. I. Blood-brain barrier, cerebral blood flow, and local cerebral glucose utilization changes. *Acta*

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_768.html[2010/10/28 18:47:40]

Neuropathol. (Berlin), 60:207–216.
631. Switzer, R.C., Bogo, V., and Mickley, G.A. (1991): High energy electron and proton irradiation of rat brain induces degeneration detectable with the cupric-silver stain. *Soc. Neurosci. Abstr.*, 17:1460.
632. Szekely, J.E., Ronai, A.Z., Duna-Kovacs, Z., et al. (1977): Cross tolerance between morphine and beta-endorphin in vivo. *Life Sci.*, 20:1259–1264.
633. Tamaki, Y., and Inouye, M. (1988): Go/No-go discriminated avoidance learning in prenatally X-irradiated rats. *Neurotoxicol. Teratol.*, 10:35–38.
634. Tanimura, H. (1957): Changes of the neurosecretory granules in hypothalamo-hypophysical system of rats by irradiating their heads with x-rays. *Acta Anat. Nippon*, 32:529–533.
635. Tannehill, S.P., and Mehta, M.P. (1996): Amifostine and radiation therapy: Past, present, and future. *Semin. Oncol.*, 23:69–77.
636. Tannehill, S.P., Mehta, M.P., Larson, M., et al. (1997): Effect of amifostine on toxicities associated with sequential chemotherapy and radiation therapy for unresectable non-small-cell lung cancer: Results of a phase II trial. *J. Clin. Oncol.*, 15:2850–2857.
637. Tateno, H., and Mikamo, K. (1989): Effects of neonatal ovarian X-irradiation in the Chinese hamster. I. Correlation between the age of irradiation and the fertility span. *J. Radiat. Res. (Tokyo)*, 30:185–190.
638. Taylor, G.M. (1995): Genetic effects of ionising radiation with respect to leukaemia. In: *Radiation Toxicology: Bone Marrow and Leukaemia*, edited by J.H.Hendry and B.Lord, pp. 275–310. Taylor & Francis, Washington, DC.
639. Taylor, J.A., Watson, M.A., Devereux, T.R., et al. (1994): p53 mutation hotspot in radon-associated lung cancer. *Lancet*, 343:86–87.
640. Teicher, B.A., Holden, S.A., Al-Achi, A., and Herman, T.S. (1990): Classification of antineoplastic treatments by their differential toxicity toward putative oxygenated and hypoxic tumor subpopulations in vivo in the FSaIIC murine fibrosarcoma. *Cancer Res.*, 50:3339–3344.
641. Teicher, B.A., Lazo, J.S., and Sartorelli, A.C. (1981): Classification of antineoplastic agents by their selective toxicities toward oxygenated and hypoxic tumor cells. *Cancer Res.*, 41:73–81.
642. Teskey, G.C., and Kavaliers, M. (1984): Ionizing radiation induces opioid-mediated analgesia in male mice. *Life Sci.*, 35:1547–1552.
643. Thacker, J., and Stretch, A. (1985): Responses of four x-ray-sensitive CHO cell mutants to different radiations and to irradiation conditions promoting cellular recovery. *Mut. Res.*, 146:99–108.

644. Thomas, D.B., Rosenblatt, K., Jimenez, L.M., et al. (1994): Ionizing radiation and breast cancer in men (United States). *Cancer Causes & Control*, 5:9–14.
645. Thorne, M.C., ed. (1986): Developmental effects of irradiation on the brain of the embryo and fetus. *ICRP Publication 49.* Pergamon Press, Oxford.
646. Tillman, B.F., Loyd, J.E., Malcolm, A.W., et al. (1989): Unilateral radiation pneumonitis in sheep: Physiological changes and bronchoalveolar lavage. *J. Appl. Physiol.*, 66:1273–1279.
647. Timmermans, R., and Gerber, G.B. (1984): The effect of X-irradiation on cardiac β-adrenergic receptors following local heart irradiation. *Radiat. Res.*, 100:510–518.
648. Timmermans, R., Maes, J., Deroo, J., and Gerber, G.B. (1986): Serotonin receptors, vascular functions and lipids in rat brain after prenatal irradiation. In: *Radiation Risks to the Developing Nervous System*, edited by H.Kriegel, W.Schmahl, G.B.Gerber, and F.-E. Stieve, pp. 221–230. Gustav Fischer Verlag, Stuttgart.
649. Tolliver, J.M., and Pellmar, T.C. (1987): Ionizing radiation alters neuronal excitability in hippocampal slices of the guinea pig. *Radiat. Res.*, 112:555–563.
650. Torii, Y., Shikita, M., Saito, H., and Matsuki, N. (1993): X-irradiation-induced emesis in Suncus murinus. *J. Radiat. Res. (Tokyo)*, 34:164–170.
651. Torubarov, F.S. (1991): Psychological consequences of the Chernobyl accident from the radiation neurology point of view. In: *The Medical Basis for Radiation-Accident Preparedness. III: The Psychological Perspective*, edited by R.C.Ricks, M.E. Berger, and F.M.O'Hara, pp. 81–91. Elsevier, New York.
652. Trabalka, J.R., Eyman, L.D., and Auerbach, S.I. (1980): Analysis of the 1957–1958 Soviet Nuclear disaster. *Science*, 209:345–353.
653. Trakhtenbrot, L., Kelman, Z., Rotter, V., and Haaran-Ghera, N. (1990): Chromosomal mapping of the murine c-abl proto-oncogene by in situ hybridization. *Leukemia*, 4:136–137.
654. Travis, E.L. (1987): Relative radiosensitivity of the human lung. In: *Advances in Radiation Biology, Vol. 12, Relative Radiation Sensitivities of Human Organ Systems*, edited by J.T.Lett, pp. 205–238. Academic Press, San Diego.
655. Trnovec, T., Kallay, Z., and Bezek, S. (1990): Effects of ionizing radiation on the blood brain barrier permeability to pharmacologically active substances. *Int. J. Radiat. Oncol. Biol. Phys.*, 19:1581–1587.
656. Trnovec, T., Volenec, K., Bezek, S., et al. (1991): The effect of high energy electron irradiation on blood-brain barrier permeability to haloperidol and stobadin in rats. *Radiat. Environ. Biophys.*, 30:277–287.
657. Trotti, A. (1998): Toxicity antagonists in head and neck cancer. *Semin. Radiat. Oncol.*, 8:282–291.
658. Turbyfill, C.L., Roudon, R.M., and Kieffer, V.A. (1972): Behavior and physiology of the monkey (Macaca mulatta) following 2500 rads of pulse mixed gamma-neutron radiation. *Aerosp. Med.*, 7:41–45.
659. Turbyfill, C.L., Roudon, R.M., Young, R.W., and Kieffer, V.A. (1972): Alteration of radiation effects by 2-(n-decylamino) ethanethiolsulfuric acid (WR-1607) in the monkey. *AFRRI Scientific Report SR72–3.* Armed Forces Radiobiology Research Institute, Bethesda, MD.
660. Turns, J.E., Doyle, T.F., and Curran, C.R. (1971): Norepinephrine effects on early post-irradiation performance decrement in the monkey. *AFRRI Scientific Report SR71–16*. Armed Forces Radiobiology Research Institute, Bethesda, MD.
661. Uchiyama, M., Nakamura, Y., Kobayashi, S., et al. (1989): Radiocesium body burden of Japanese who returned from European countries following the Chernobyl accident. *J. Radiat. Res.*, 30:51.
662. Urmer, A.H., and Brown, W.L. (1960): The effect of gamma radiation on the reorganization of a complex maze habit. *J. Gen. Psychol.*, 97:67–76.
663. U.S. Environmental Protection Agency (USEPA). (1986): *Citizen's Guide to Radon.* U.S. Environmental Protection Agency, Washington, DC.
664. USSR State Committee on the Utilization of Atomic Energy. (1986): *The Accident at Chernoyl Nuclear Power Plant and its Consequences.* Information compiled for the IAEA Experts' Meeting, 24–9 August, 1986, Vienna. Working Document for the Post-Accident Review Meeting. Draft. Part I: General Material. Part II: Annexes 1–7, August 1986 (hereafter referred to as The Accident…Soviet IAEA Report). Part I was subsequently published in Russian in *Atomnaya Energiya*, 61(5), Moscow.
665. Utley, J.F. (1987): Relative radiosensitivities of the oral cavity, larynx, pharynx, and esophagus. In: *Advances in Radiation Biology, Vol. 12, Relative Radiation Sensitivities of Human Organ Systems*, edited by J.T.Lett and K.I.Altman, pp. 129–146. Academic Press, San Diego.
666. Vahakangas, K.H., Samet, J.M., Metcalf, R.A., et al. (1992): Mutations of p53 and *ras* genes in radon-associated lung cancer from uranium miners. *Lancet*, 339:576–580.

667. Valk, P.E., and Dillon, W.P. (1991): Radiation injury of the brain. *Am. J. Neuroradiol.*, 12:45–62.
668. van Bekkum, D.W., Jongeiper, H.J., Nieuwerkerk, H.T.M., and Cohenm, J.A. (1954): The oxidative phosphorylation by mitochondria isolated from the spleen of rats after total body exposure to x-rays. *Br. J. Radiol.*, 27:127–130.
669. van der Kogel, A.J. (1986): Radiation-induced damage in the central nervous system: An interpretation of target cell responses. *Br. J. Cancer Suppl VII*, 53:207–217.
670. van der Meer, Y., Huiskamp, R., Davids, J.A.G., et al. (1992): The sensitivity of quiescent and proliferating mouse spermatogonial stem cells to X irradiation. *Radiat. Res.*, 130:289–295.
671. van der Meer, Y., Huiskamp, R., Davids, J.A.G., et al. (1992): The sensitivity to X rays of mouse spermatogonia that are committed to differentiate and of differentiating spermatogonia. *Radiat. Res.*, 130:296–302.
672. Van Dongen-Melman, J.E., De Groot, A., Van Dongen, J.J., et al. (1997): Cranial irradiation is the major cause of learning problems in children treated for leukemia and lymphoma: A comparative study. *Leukemia*, 11:1197–1200.
673. Varagic, V., Stepanovic, S., Svecenski, N., and Hajdukovic, S. (1967): The effect of X-irradiation on the amount of catecholamines in heart atria and hypothalamus of the rabbit and in brain and heart of the rat. *Int. J. Radiat. Biol.*, 12:113–119.
674. Verger, P. (1997): Down syndrome and ionizing radiation. *Health Phys.*, 73:882–893.
675. Vidal-Pergola, G.M., Kimler, B.F., and Norton, S. (1993): Effect of in utero irradiation on the postnatal development, behavior, and brain structure of rats: Dose fractionation with a 6-h interval. *Radiat. Res.*, 134:369–374.
676. Viel, J.F., Curbakova, E., Dzerve, B., et al. (1997): Risk factors for long-term mental and psychosomatic distress in Latvian Chernobyl liquidators. *Environ. Health Perspect.*, 105:1539–1544.
677. Viinamäki, H., Kumpusalo, E., Myllykangas, M., et al. (1995): The Chernobyl accident and mental well being—a population study. *Acta Psychiat. Scand.*, 91:396–401.
678. Vijayalaxmi, Reiter, R.J., Sewerynek, E., et al. (1995): Marked reduction of radiation-induced micronuclei in human blood lymphocytes pretreated with melatonin. *Radiat. Res.*, 143:102–106.

679. Voevodskaya, N.A., and Vanin, A.F. (1992): Gamma-irradiation potentiates L-arginine-dependent nitric oxide formation in mice. *Biochem. Biophys. Res. Commun.*, 186:1423–1428.
680. von Sonntag, C. (1987): *The Chemical Basis of Radiation Biology*. Taylor and Francis, New York.
681. von Sonntag, C. (1991): The chemistry of free-radical-mediated DNA damage. *Basic Life Sci.*, 58:287–317.
682. Vyatleva, O.A., Katargina, T.A., Puchinskaya, L.M., and Yurkin, M.M. (1997): Electrophysiological characterization of the functional state of the brain in mental disturbances in workers involved in the clean-up following the Chernobyl atomic energy station accident. *Neurosci. Behav. Physiol.*, 27:166–172.
683. Wagner, W., Prott, F., and Schonekas, K. (1998): Amifostine: A radioprotector in locally advanced head and neck tumors. *Oncol. Rep.*, 5:1255–1257.
684. Wakeford, R. (1995): The risk of childhood cancer from intrauterine and preconceptional exposure to ionizing radiation. *Environ. Health Perspect.*, 103:1018–1025.
685. Walden, T.L., Jr. (1989): Long-term and low level effects of ionizing radiation. In: *Medical Consequences of Nuclear Warfare*, edited by R.I.Walker and T.J.Cerveny, pp. 171–126. TMM Publications, Falls Church, VA.
686. Walden, T.L., Jr., and Farzaneh, N.K. (1990): *Biochemistry of Ionizing Radiation*, Raven Press, New York.
687. Walden, T.L., Jr., and Farzaneh, N.K. (1991): Biochemical response of normal tissues to ionizing radiation. In: *Radiation Injury to the Nervous System*, edited by P.H.Gutin, S.A.Leibel, and G.E.Sheline, pp. 17–36. Raven Press, New York.
688. Walden, T.L., Jr., Farzaneh, N.K., and Richards, L. (1989): Lipoxygenase products in radiation injury and protection. *New Trends Lipid Mediators*, 3:154–160.
689. Walker, D.I., and Eisen, V. (1979): Effect of ionizing radiation on 15-hydroxy prostaglandin dehydrogenase (PGDH) activity in tissue. *Int. J. Radiat. Biol.*, 36:399–407.
690. Wallace, W.H., Shalet, S.M., Crowne, E.C., et al. (1989): Ovarian failure following abdominal irradiation in childhood: Natural history and prognosis. *Clin. Oncol. (R. Coll. Radiol.)*, 1:75–79.
691. Wallace, W.H., Shalet, S.M., Hendry, J.H., et al. (1989): Ovarian failure following abdominal irradiation in childhood: The radiosensitivity of the human oocyte. *Br. J. Radiol.*, 62:995–998.
692. Ward, J.F. (1990): The yield of DNA double-strand breaks produced intracellularly by ionizing radiation: A review. *Int. J. Radiat. Biol.*, 57:1141–1150.
693. Weinstein, N.D. (1991): Public response to home radon exposure. In: *The Medical Basis for Radiation-Accident Preparedness. III: The Psychological Perspective*, edited by R.C.Ricks, M.E. Berger, and F.M.O;'Hara, pp. 173–178. Elsevier, New York.
694. Weiss, J.F. (1997): Pharmacologic approaches to protection against radiation-induced lethality and other damage. *Environ. Health Perspect.*, 105(Suppl 6): 1473–1478.
695. Weiss, J.F., Kumar, K.S., Walden, T.L., et al. (1990): Advances in radioprotection through the use of combined agent regimens. *Int. J. Radiat. Biol.*, 57:709–722.
696. Weshler, Z., Ligumsky, M., Brufman, G., et al. (1987): Functional and morphological alterations following isolated rat stomach irradiation. A model for estimation of radiation injury. *In Vivo*, 1:357–361.
697. Wheeler. T.G., and Hardy, K.A. (1985): Retrograde amnesia produced by electron beam exposure: Causal parameters and duration of memory loss. *Radiat. Res.*, 101:74–80.
698. Wheeler, T.G., and Tilton, B.M. (1983): Duration of memory loss due to electron beam exposure. *USAF SAM Technical Report TR 83–33*. USAF School of Aerospace Medicine, Brooks AFB, TX.
699. Whicker, F.W. (1989): Impact on plant and animal populations. In: *Health Impacts of Large Releases of Radionuclides. Ciba Foun dation Symposium No. 203*, pp. 74–93. The Ciba Foundation, London.
700. Whicker, F.W., Pinder, J.E. III, Bowling, J.W., et al. (1990): Distribution of long-lived radionuclides in an abandoned reactor cooling reservoir. *Ecol. Monogr.*, 60:471–496.
701. Whicker, F.W., and Schultz, V. (1982): *Radioecology: Nuclear Energy and the Environment*, Vols. I and II. CRC Press, Boca Raton, FL.
702. Wientroub, S., Weiss, J.F., Catravas, G.N., and Reddi, A.H. (1990): Influence of whole body irradiation and local shielding on matrix-induced endochondral bone differentiation. *Calcif. Tissue Int.*, 46:38–45.
703. Williams, D. (1994): Chernobyl, eight years on. *Nature*, 371:556.
704. Winkler, H. (1957): Untersuchungen uber die Wirkung von Roentgensstrahlen auf die bluthirschranke mit hilfe von P32 Zbl allg. *Pathol. Anat.*, 97:301–307.
705. Winsauer, P.J., Bixler, M.A., and Mele, P.C. (1995): Differential effects of ionizing radiation on the

acquisition and performance of response sequences in rats. *Neurotoxicology*, 16:257–269.
706. Winsauer, P.J., and Mele, P.C. (1993): Effects of sublethal doses of ionizing radiation on repeated acquisition in rats. *Pharmacol. Biochem. Behav.*, 44:809–814.
707. Wishkerman, Y.V., Quastal, M.R., Douvdevani, A., and Goldsmith, J.R. (1997): Somatic mutations at the glycophorin A (GPA) locus measured in red cells of Chernobyl liquidators who immigrated to Israel. *Environ. Health Perspect.*, 105(Suppl 6):1451–1454.
708. Withers, R.H. (1975): The four R's of radiotherapy. *Adv. Radiat. Biol.*, 5:241–271.
709. Wixon, H.N., and Hunt, W.A. (1983): Ionizing radiation decreases veratridine stimulated uptake of sodium in rat brain synaptosomes. *Science*, 220:1073–1074.
710. Wolfle, G., Bleyer, H., Muller, D., and Klinger, W. (1991): The influence of the radiation syndrome on cytochrome P450-dependent monooxygenation in rat liver. *Exp. Pathol.*, 43:89–95.
711. Wondergem, J., van der Laarse, A., van Ravels, F.J., et al. (1991): In vitro assessment of cardiac performance after irradiation using an isolated working rat heart preparation. *Int. J. Radiat. Biol.*, 59:1053–1068.
712. World Health Organization. (1995): *Health Consequences of the Chernobyl Accident. Results of the IPHECA Pilot Projects and Related National Programmes.* World Health Organization, Geneva.
713. Wright, E.G. (1995): The pathogenesis of leukaemia. In: *Radiation Toxicology: Bone Marrow and Leukaemia*, edited by J.H.Hendry and B.I.Lord, pp. 245–274. Taylor & Francis, Washington, DC.
714. Yaes, R.J. (1992): Radiation damage to the kidney. In: *Advances in Radiation Biology, Vol. 15, Relative Radiation Sensitivities of Human Organ Systems, Part III*, edited by K.I.Altman and J. T.Lett, pp. 1–35. Academic Press, San Diego.
715. Yamada, T., and Ohyama, H. (1988): Radiation-induced interphase cell death of rat thymocytes is internally programmed (apoptosis). *Int. J. Radiat. Biol.*, 53:65–75.
716. Yamamoto, M., Ueno, K., Igarashi, Y., et al. (1990): Determination of low-level Ra-226 in human bone by α-spectrometry. *J. Radiat. Res.*, 31:85.
717. Yamashita, H., and Miyasaka, T. (1952): Effects of beta rays upon a single nerve fiber. *Proc. Soc. Exp. Biol. Med.*, 80:375–377.
718. Yamashita, S., Namba, H., and Nagataki, S. (1993): Thyroid and radiation (in Japanese). *Folia Endocrinol. Jpn.*, 69:1035–1043.
719. Yeung, T.K., Lauk, S., Simmonds, R.H., et al. (1989): Morphological and functional changes in the rat heart after X irradiation: Strain differences. *Radiat. Res.*, 119:489–499.

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720. Yoshii, Y., Maki, Y., Tsunemoto, H., et al. (1981): The effect of total-head irradiation C3H/He of X irradiation of the head in monkeys. *Radiat. Res.*, 86:152–170.
721. Young, R.W. (1986): Mechanisms and treatment of radiation-induced nausea and vomiting. In: *Nausea and Vomiting: Mechanisms and Treatment*, edited by C.J.Davis, G.V. Lake-Bakaar, and G.V.Grahame-Smith, pp. 94–109. Springer-Verlag, New York.
722. Young, R.W. (1987): Acute radiation syndrome. In: *Military Radiobiology*, edited by J.J.Conklin and R.I.Walker, pp. 165–190. Academic Press, New York.
723. Young, R.W., and Myers, P.H. (1986): The human response to nuclear radiation. *Med. Bull.*, 43:20–23.
724. Yuhas, J.M. (1970): Biological factors affecting the radioprotective efficiency of S-2-(3-aminopropylamino)ethylphosphorothioic acid (WR-2721): LD 50/30 doses. *Radiat. Res.*, 44:621–628.
725. Yuhas, J.M. (1980): Active versus passive absorption kinetics as the basis for selective normal tissue protection by S-2-(3-aminopropylamino) ethylphosphorothioic acid. *Cancer Res.*, 40:1519–1524.
726. Zaman, M.S., Lancaster, F.E., and Hupp, E.W. (1997): Physical and motor development in male and female rat offspring prenatally exposed to gamma radiation. *J. Environ. Sci. Health B*, 32:313–325.
727. Zamora, M.L., Tracy, B.L., Zielinski, D., et al. (1998): Chronic ingestion of uranium in drinking water: A study of kidney bioeffects in humans. *Toxicol. Sci.*, 43:68–77.
728. Zaridze, D.G. (1997): Epidemiology of leukemias in children (in Russian). *Arkhiv Patologii*, 59:65–70.
729. Zeman, W. (1963): Disturbances of nuclei acid metabolism preceding delayed radionecrosis of nervous tissue. *Proc. Natl. Acad. Sci. U.S.A.*, 50:626–630.
730. Zhavoronkova, L.A., Kholodova, N.B., Zubovskii, G.A., et al. (1995): Electroencephalographic correlates of neurological disturbances at remote periods of the effect of ionizing radiation (sequelae of the Chernobyl' NPP accident). *Neurosci. Behav. Physiol.*, 25:142–149.
731. Ziboh, V.A., Mallia, C., Mohart, E., and Taylor, L. (1982): Induced biosynthesis of cutaneous prostaglandins by ionizing radiation. *Proc. Soc. Exp. Biol. Med.*, 169:386–391.
732. Zmudzka, B.Z., and Beer, J.Z. (1990): Activation of human immunodeficiency virus by ultraviolet radiation. *Photochem. Photobiol.*, 52:1153–1162.

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Chapter **16**

The Use of Laboratory Animals in Toxicologic Research

William J.White

Principles and Methods of Toxicology,
Fourth Edition, edited by A.Wallace Hayes.
Taylor & Francis, Philadelphia © 2001.

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This chapter provides an overview of laboratory animal science and medicine as it pertains to the use of laboratory animals in toxicologic research. The purpose of this chapter is to familiarize the toxicologist with a number of important issues involving the use of laboratory animals. It is not possible to explore all subjects that pertain to this topic nor to comprehensively review the subjects in this chapter. The reader is urged to seek more detail in the references provided and to consult specialists in the field for more information.

Veterinarians with specialized training in laboratory animal medicine have played important supporting roles in institutions conducting toxicologic research. Much of the information used by them, as well as by other laboratory animal professionals and toxicologists, has been developed as a direct result of studies initiated in the basic sciences and in toxicology. By their very nature, toxicologic studies have required

an understanding of the biological characteristics and needs of laboratory animals as well as an understanding of those variables that impact the performance of laboratory animals in research studies. Our understanding of laboratory animals is far from complete and is further complicated by the fact that there is a wide variety of species from which the toxicologist may choose. Species such as mice and rats are extensively used with vast amounts of background data available and whose biological characteristics have been well explored. Less commonly used species, such as guinea pigs, hamsters, and gerbils, have proportionally less known about them and much less published background data available. Recent efforts by a number of organizations to collect, analyze, and publish background

data on a continuing basis in a variety of disciplines is helping to extend the knowledge of certain laboratory animal species (48, 191).

Like any field, the quality of published information can vary widely. Moreover, a lot of dogma and unsubstantiated opinion still remain in the literature. A number of recommended practices still cannot be well substantiated in the peer-reviewed literature. In designing experimental protocols, the toxicologist should familiarize himself with the important variables associated with the animals that will be used in the study and put in place measures to control or to account for these variables.

In today's society the use of animals for research purposes has become a subject of much public discussion. Over the last century, economically developed countries in which most biomedical research takes place have moved further and further away from extensive public involvement with agricultural use of animals and replaced that exposure with zoos, family pets, and stylized animal characters in the media. This has caused society to reexamine their relationship with animals both in agriculture and in research. This has resulted in the development of laws, regulations, and guidelines that address responsible animal use. As with any controversial subject, there are those who find the measures taken to be insufficient and who advocate even further change through dialogue, protest, or terrorism. In many cases, radical views and actions are based on misinformation and a basic distrust of large organizations and governments. There are, however, a large number of individuals concerned with animal welfare who are open-minded and seek to strike an appropriate balance in their use in biomedical research. They, like the members of the research community, are focused on responsible animal usage, including, where appropriate, refinement, reduction, and replacement (the three Rs) of laboratory animals (278).

Responsible animal usage has stimulated interest in in vitro alternatives, computer-simulated models, and computer structure-activity analyses to screen for appropriate drug candidates. These efforts have had some limited success in the initial stages of the drug discovery process. They have not yet led to suitable in vitro replacements for most animal usage in toxicologic studies, especially product registration studies, nor do they appear likely to in the near future. This failure to develop complete substitutes for intact living organisms used in research is likely due to the complex interactions that exist on organ, cellular, and subcellular levels. A suitable replacement for animals will need to reliably predict biological phenomena, including being at least as good and consistent a model for risk assessment in humans as animals. Such systems will need to be extensively validated and accepted by regulatory bodies as suitable substitutes.

The use of animals in toxicologic research cannot be taken for granted. With their use comes responsibilities not only to adherence to institutional, governmental, and scientific principles, policies, laws, regulations, and guidelines, but also an ethical and moral responsibility for the lives of the animals used in research or product manufacture. Each researcher is also responsible for the quality of the care that they receive, the appropriateness of their use, and the minimization or relief of pain.

REGULATIONS, LAWS, POLICIES, AND GUIDELINES

The use of animals in research, as well as the assurance that provisions for appropriate animal welfare and care have been made, is controlled by a number of mechanisms. These can be subdivided into two general categories: (1) guidelines and recommendations and (2) laws and regulations. In addition, policies can be developed for either of these two categories. For example, guidelines or recommendations that are not regulated by law can be included in an overall policy that governs institutional activities or eligibility for receiving funding. Moreover, policies may also be created and used as accepted interpretations of regulations developed in response to laws.

Laws and regulations require mandatory compliance. Failure to meet the requirements imposed by laws or regulations usually is attended by legal actions that may culminate in fines, revocation of the ability to conduct animal-related activities, or imprisonment. In the case of laboratory animals in the United States, laws and regulations are administered by the U.S. Department of Agriculture (USDA). The regulatory body is charged with conducting regular inspections and registering all facilities using laboratory animals. Within the USDA, the Animal Plant and Health Inspection Service (APHIS) is charged with making such inspections. Within APHIS, the Regulatory Enforcement and Animal Care (REAC) unit is responsible for enforcing regulations developed in response to legislation governing the use of laboratory animals in research.

Guidelines and recommendations usually are developed by independent groups with expertise in one or more aspects of laboratory animal science and medicine. Compliance with guidelines or recommendations is voluntary; however, failure to do so may be accompanied by undesirable consequences, such as denial of funding by government institutions, inability to have data accepted for

publication, or inability to use data in submissions filed in response to mandated regulatory processes (81, 95). Compliance with such guidelines or recommendations may need to be assured through filing of legally binding statements, submission of regular reports to

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agencies tracking such activities, or by participation in a voluntary accreditation program, such as the one conducted by the Association for Assessment and Accreditation of Laboratory Animal Care-International (AAALAC), who uses a combination of regular reports and periodic site visits to evaluate programs, facilities, and animal care.

INTERNATIONAL ASSURANCE AND REGULATION OF LABORATORY ANIMAL CARE AND USE

The existence and complexity of laws, regulations, guidelines, and recommendations governing the care and use of laboratory animals varies significantly between countries. In general, nonindustrial countries commonly do not have laws governing the use of animals in research, teaching, or product production. Some international guidelines or recommendations may be followed, but only to the extent that they impact the suitability of work or products for registration in other countries.

Countries within the European Economic Union use certain minimal standards developed through the council of Europe and subsequently ratified by member states. These standards are used as the basis for individual country laws and regulations that meet or exceed these standards. Variation between countries can exist. Within an individual country, standards may be different for different aspects of animal production and use. These laws and standards may apply not only to research use of animals, but also to transportation of animals, as well as their exhibition and sale for other purposes. A comprehensive review of all of these items, including regulatory oversight and reporting requirements, are beyond the scope of this text. There are, however, a few important considerations that should be mentioned.

Protocol review at either a regional or national level is a common component of the regulations governing animal care and use in European countries. Protocols must fulfill certain guidelines and provide a detailed description of the proposed study as well as supporting rationale. There can be a significant time lag from the submission of a protocol until a decision is made by reviewers.

Licensing of researchers to perform specific procedures is often a component of the regulatory process. Guidelines for credentials and training are specified and licensing is often done on an individual procedure basis.

Evaluation of programs and unannounced inspection of facilities is often done through a government agency. Activities can be suspended based on findings of inspections, and licensure can be revoked. Other penalties can be imposed, depending on the country.

Unlike the United States, all species used in research conducted in Europe are covered by guidelines and regulations. Differences can exist between countries in terms of acceptable care and use practices, and there should be no assumption that all of these standards are similar to ones in the United States or elsewhere. The level of detail and the emphasis on various topics can differ substantially from United States standards.

The Federated European Laboratory Animal Science Associations (FELASA) is a European consortium of laboratory animal science associations. FELASA has developed a number of recommendations for health monitoring, accreditation of animal diagnostic laboratories, and other topics that can impact the quality of laboratory animals used in toxicologic research. Adherence to these recommendations is voluntary, and in some cases the recommendations may differ from accepted practices in other parts of the world.

In Canada, the Canadian Council on Laboratory Animal Care has produced guidelines for the care and use of laboratory animals. Inspection of research facilities is conducted on a voluntary basis by this organization (46, 47). In Japan, efforts are underway to develop voluntary guidelines similar to those in the United States, Canada, and Europe. Laws governing the use of laboratory animals in research are not present.

UNITED STATES LAWS, REGULATIONS, GUIDELINES, RECOMMENDATIONS, AND POLICIES

The Animal Welfare Act

Regulation of laboratory animals used in research is governed by the Laboratory Animal Welfare Act of 1966, Public Law (P.L. 89–544) (308). The USDA administers the Animal Welfare Act. The act establishes legal requirements for research facilities to provide certain minimum standards for the care of animals in research. In 1970, the Laboratory Animal Welfare Act was amended (P.L. 91–579), at which time its name was changed to the Animal Welfare Act because the term *animal* was changed to include all warm-blooded animals except those used for food or fiber (308). The 1970 Act required registration of research facilities as well as annual reporting requirements. Animals used for other purposes, such as exhibition or as pets, were also extended coverage.

The Act was again amended in 1976 (P.L. 94–279) in order to include more rigorous standards for transportation of animals (308). The Act was last amended in 1985 (P.L. 99–198) to include requirements for exercising dogs, the psychological well being of nonhuman primates, the consideration

of alternative procedures to ones causing pain or distress, training of personnel, and the establishment of an institutional animal care and use committee of specified composition (308).

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In response to the Act and its amendments, a series of regulations and policies were developed. Significant additions to the regulations were added in, August of 1989, April of 1990, and February of 1991 that refined definitions and terms: set standards for humane handling, care, treatment, and transportation; set space requirements for primary enclosures for guinea pigs, rabbits, and hamsters; set standards for humane handling, care, treatment, and transportation of dogs, cats, and nonhuman primates (188, 280). The regulations promulgated by the USDA in response to the Animal Welfare Act are published in the Code of Federal Regulations (CFR, Title 9, Chapter A, Parts I, II, and III) (71). Currently, the Animal Welfare Act covers dogs, cats, nonhuman primates, hamsters, guinea pigs, rabbits, marine mammals, and any other domestic animal, as well as those animals normally found in the wild, used in research, testing, exhibition, experimentation, or kept as pets. The term *animal,* as defined in the Animal Welfare Act, has excluded rats, mice, and birds, as well as horses and other farm animals used or intended for use as food or fiber or for the use in improving animal nutrition, breeding, or production. Farm animals, including horses, are covered under the Animal Welfare Act if they are used for nonagricultural research or exhibition, which includes biomedical research (167).

Extension of the USDA's regulation of laboratory animals to include rats, mice, and birds is viewed by some as being inevitable. This exclusion was still in place as of 2000 and reflects a balancing of public and private resources with the perceived benefits to regulating these excluded species. The effects on toxicologic research in the United States of extension of regulation to these species is hard to predict, as these species represent the bulk of all animals used in such research, and hence the limited degree of experience with the present regulatory process may not be predictive of the consequences of extended coverage. Given the USDA's broad mandate for regulation, which includes transportation, the cost to the government and feasibility of providing the appropriate level of regulation of this activity may be difficult to accurately estimate.

The Guide for the Care and Use of Laboratory Animals

In the United States, the primary set of guidelines for the care and use of laboratory animals is *The Guide for the Care and Use of Laboratory Animals (The Guide)* which was developed by the National Academy of Sciences (243). *The Guide* is used as the primary reference for voluntary assurance and accrediting bodies, such as National Institutes of Health's Office for Laboratory Animal Welfare (OLAW) and AAALAC. This document was first prepared and published in 1963 and has been revised many times since. The last revision (1996) made substantial changes in the overall approach to laboratory animal care and use.

The Guide cannot be appropriately used without an institutional animal care and use committee (222, 243). The institutional animal care and use committee (IACUC) is appointed by the chief executive officer of the institution and is advisory to him. The committee, in order to comply with both the requirements of *The Guide* and USDA regulations, should include a doctor of veterinary medicine who is certified or has training or experience in laboratory animal science and medicine or in the use of the species used in research at the institution. The committee should also have at least one practicing scientist experienced in research involving animals as well as one non-scientist who may or may not be employed by the institution. The committee should also include at least one public member to represent community interest with respect to care and use of laboratory animals. The public member should not be affiliated with the institution, should not be an immediate family member of anyone affiliated with the institution, and should not be involved in the use of laboratory animals.

The IACUC is responsible for the evaluation and oversight of the institution's animal care and use program and all related issues set forth in *The Guide*. The IACUC must inspect all animal facilities used by the institution's research program as well as carry out a programmatic review of the research program every 6 months. The committee subsequently analyzes the findings of their inspection and review and prepares a written report for the responsible institutional official that details their findings and any recommendations for action. The institutional official, in turn, must address any major deficiencies detailed in the report by providing a reasonable plan for corrective action and a timetable. The committee must maintain written records of their meetings and all decisions taken. Meetings should be held as frequently as necessary to accomplish their designated tasks, but it should meet at a minimum of twice a year. A mechanism for documenting minority views must also be provided.

An essential component of the IACUC's activity is the review and approval of animal use protocols prior to the initiation of research or other animal-related activities. Protocols submitted for committee review should be complete with respect to their description of the animal care and use components. To prepare a protocol that covers all of the areas and issues posed both by the USDA regulations and *The Guide* recommendations, the IACUC often requires the researcher to place the protocol in a standard format

that may take the form of a questionnaire. Topics such as surgery, anesthesia, provision of analgesics during painful procedures, determination of acceptable alternatives to painful procedures, methods of euthanasia, criteria for

ending an animal's participation in a study or procedure, and assurance of appropriate housing and care practices are some of the many issues that are considered in the review of any protocol.

The Guide charges the users of research animals with the responsibility of achieving specific outcomes with respect to the care and use of animals, but by using a performance-based approach, provides latitude as to how these outcomes are achieved (222). Performance standards define an outcome in detail and provide criteria for assessing that outcome. They do not restrict the method by which this outcome is achieved. This is in contrast to engineering standards that do not provide for interpretation and modification of prescribed methods or procedures in the event that acceptable alternative methods are available or usual circumstances occur. The IACUC is charged with developing additional performance standards to evaluate alternative methods to achieve specified outcomes and is given the latitude to modify recommendations set forth in *The Guide* based on performance data generated by researchers to establish the adequacy of alternative methods.

Public Health Service Policy

The administration and coordination of the Public Health Services' (PHS) policy on Humane Care and Use of Laboratory Animals (The Policy) is administered by the OLAW (264). This policy requires institutions to establish and maintain proper measures to assure the appropriate care and use of all animals involved in research, research training, and biological testing activities conducted or supported by the PHS. The policy also endorses a set of principles developed by the Interagency Research Animal Committee (IRAC) that covers the use and care of vertebrate animals used in testing, research, and training (140).

The Policy applies to all research supported by PHS and places a heavy emphasis on training of all personnel involved in the care and use of animals in a research setting. The Policy requires the biannual preparation and evaluation of reports by the IACUC on the institution's programs and facilities that involve animal activities. The Policy requires the filing of an assurance statement with OLAW binding the institution to The Policy as well as to the use of *The Guide* as a basis for developing and implementing institutional programs for activities involving animals. OLAW also requires the filing of annual reports by the institution. Achieving and maintaining such assurance is a prerequisite for consideration for funding of activities supported by the PHS, and, by extension, may be adopted by agencies as an essential criteria for consideration for funding.

THE ASSOCIATION FOR ASSESSMENT AND ACCREDITATION OF LABORATORY ANIMAL CARE-INTERNATIONAL

The Association for Assessment and Accreditation of Laboratory Animal Care-International is a voluntary organization that was founded in 1965. AAALAC is a United States-based nonprofit corporation with national and international representation from scientific and educational organizations and whose goal is to promote responsible and high-quality animal care and use by research institutions through the use of a peer-review process. The organization assists institutions in developing and improving programs and facilities for animal care and use.

The accreditation process is designed to assess the institution's conformance with appropriate locally applicable guidelines, recommendations, laws, regulations, and policies, as well as evaluate the program's ability to ensure that animals' health and well-being is safeguarded by institutional processes and operational procedures. In the United States, AAALAC uses *The Guide* as its primary standard, outside of the United States, applicable regulations, laws, and other standards are used that are relevant to that country and the institution's activities.

The accreditation process is confidential. It is initiated by the submission of a comprehensive application that is designed to provide a detailed description of the institution's animal programs and facilities. An initial site visit is made by two or more individuals representing AAALAC, one of whom may be an ad hoc consultant with special expertise in programmatic areas that are relevant to the institution applying for accreditation. Following the initial site visit, a written report is filed along with recommendations. This is then considered by AAALAC's Council on Accreditation to determine if the facility should be granted accreditation. Facilities that have obtained accreditation are revisited every 3 years to determine eligibility to maintain continued accreditation. Annual reports of activities that include programmatic changes and animal usage must be filed.

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DESIGN AND CONSTRUCTION OF ANIMAL HOUSING FACILITIES

The design of animal facilities for biomedical research has evolved over time, as have housing methods for research animals. Key design concepts that have survived the test of time relate to practical matters, such as waste handling, sanitation, investigator access, disease control, and the size of the animal species being used. There is no universally accepted design for animal facilities, nor is it likely that any one given design for animal holding rooms will be appropriate for all species of animals that could be used in toxicologic research. Some reviews on the subject of animal facilities design have been prepared, as have design process considerations for specialized support facilities for research animals (9, 17, 45, 61, 64, 79, 113, 128, 130, 139, 174, 189, 222, 223, 258, 281, 297, 345).

The process of animal facility design requires, first and foremost, a clear documentation of the intended type and extent of use of the facility based on past and present toxicologic research programs, as well as a reasonable expectation of the growth of these programs over a period of 5–10 years (345). In considering the intended facility use, the numbers and types of animals by species must be determined. While studies involving small rodent species often consume the bulk of toxicologic research that use animals, other larger species are frequently used and may have to be provided for within a facility. As an alternative, facilities may be designed to accommodate only a limited range of species, with housing and usage of other species being outsourced to other facilities that are more well suited to their maintenance.

Once the level of usage has been determined, specialized requirements—such as the use of biohazardous material requiring biocontainment facilities, a surgical program requiring facilities for aseptic procedures, specialized radiographic or imaging facilities, diet preparation facilities, specialized equipment or procedures facilities, diagnostic laboratory or necropsy facilities, or other specialized spaces requiring specific types of equipment or housing for animals used in toxicologic research—need to be identified, as well as the level of activity that will occur in such facilities. Because these types of facilities often represent a significant cost to construct and operate, as well as consume considerable amounts of floor space, it is important to carefully assess the realistic level of activity projected for such facilities.

The animal care program itself must also be carefully described, and details of the program should be well worked out before the design process is started. The reason for this is that the location of various components of the animal facility will depend greatly on the methodology and equipment to be used in the animal care program. For example, caging and racking within an ani mal facility must move to and from cage washing facilities. The amount of labor involved in performing these tasks is directly related to the distance of various components of the animal housing facility from the cage washing facilities, as is the opportunity for transmission of adventitious microorganisms potentially infecting one group of animals spreading to others through the cage sanitation process. The frequency of cage changing, as well as the location in which cages are changed and the equipment required for such cage changing, will also influence the amount of storage space and operational space within individual animals rooms and storage space within the cage washing facility.

The type of caging and the level of bioexclusion of microorganisms to be achieved for certain groups of animals maintained within the animal research facility will also dictate many aspects of the design (344). Facilities housing large numbers of rodents in microisolation cages or in flexible or semirigid isolators will require different designs and operational practices, as compared to facilities that use conventional opencage housing.

Animal holding rooms can be classified into two general types. The first *rodent housing,* is characterized by smaller rooms with few fixtures whose design is orientated toward removal of caging from the room for sanitation and perhaps sterilization (204, 222, 223, 344). Such rooms usually have a small sink for hand washing and, if microisolation caging is used, will often be outfitted with a laminar flow hood designed for cage changing using aseptic technique. When microisolation caging is used, such rooms commonly have only a single access door to minimize traffic through the room. Such rooms generally do not contain floor drains or specialized equipment for spraying water under pressure during cleaning of the room or equipment.

The second type of animal holding room is designed to handle larger caging or runs/pens to house animals the size of rabbits or larger (222). Such rooms are equipped with floor drains or some form of trough-drain system to allow in-the-room cleaning of cage components or, in the case of rabbits, the frequent spillage of liquids onto the floor (222). While some caging may be removed from the room and taken to the cage washing area for more complete sanitation, certain components of daily cleaning are carried out within the room itself.

With the exception of some aspects of cage washing, most routine animal care activities are conducted manually, making the animal facility labor intensive. Moreover, the use of the animal facility by researchers also involves regular and often complex manual manipulations of the animals for study purposes. For this reason, distances between support facilities and the animal holding areas need to be minimized and provisions often need to be made within the design for strategic location of procedural rooms and, where appropriate,

critical laboratory facilities necessary to conduct determinations that cannot be adequately conducted at great distances from the animal facility. The final design of an animal facility needs to be analyzed for efficient traffic flow of both personnel and equipment. It is important that both users of the animal facility, as well as the animal care staff, analyze the design with respect to day-to-day tasks that need to be accomplished and the distances that need to be traversed by both people and equipment to accomplish these tasks.

Over time, two common arrangements of rooms within animal facilities have been developed (129). These have evolved from considerations of material and personnel flow patterns and from the use of specialized equipment and practices to control the spread of adventitious organisms and contaminants within animal facilities (288). The most common facility is one with a single corridor design. Often, these are a result of renovation of existing space originally designed for laboratory use in which a single corridor that provides both entrance and exit from individual animal rooms was necessitated by the available space and location or, in some cases, by the need to isolate specialized animal facilities, such as microbiological barriers, in which shower and clothing change for people and decontamination of supplies are required. Such barriered animal facilities have become popular in support of some forms of toxicologic research, especially those programs using transgenic or immunologically deficient animals (344).

The other common animal facility arrangement is the dual-corridor system orientated toward cage-washing facilities. In such a system, one or more corridors (i.e., supply or clean) that exit the clean side of the cage wash connect with one of two entrance doors to each animal room. The second door in each room is used to remove caging and other material for return through a second corridor (i.e., return or dirty) to the cage wash by a separate entrance in which soiled caging is collected for subsequent sanitation. This design addresses logical flow of personnel and equipment through animal rooms so as not to have crossing of equipment within common corridors. It is unlikely that such a design provides any realistic measure of disease control by preventing cross contamination, given the many other opportunities for movement of microorganisms through a facility (344). Commonly, there is an air pressure gradient maintained across the two-corridor system such that the supply corridor (clean) is at the highest pressure, with the next highest air pressure being maintained in the animal room and the lowest pressure in the return (dirty) corridor (222, 223).

Because toxicologic research programs are dynamic and requirements can change dramatically over time, most animal facility designs try to maintain some degree of flexibility (113). Animal rooms designed for large or small animals usually are designed with a minimum of fixed equipment or specialized mechanical systems. This allows easy conversion from use of one species to another within the categories of large or small animal holding areas. Because most caging and racking is more efficiently located along the perimeter of the room, rectangular designs for animal holding rooms are common, as they maximize wall space while minimizing the relatively unused center of the room space. It is the usual practice to keep the center of the room clear in order to allow the orderly movement of equipment, supplies, and personnel through the room and for the conduct of animal care and research functions. For this reason, common designs, whether on a one- or two-corridor system, usually have rooms with widths ranging from 12–14 feet and lengths of 18–20 feet.

Room sizes are also often dictated by building support structures which, in the case of multiple story buildings, result in support columns that must be maintained throughout the structure. The spacing of these columns is done at fixed intervals, and hence room dimensions must conform to these constraints in order to prevent columns being placed within the room itself rather than in the walls.

Animal rooms, as well as support areas, are often grouped by function in order to minimize the distance traveled between related functions and to minimize the runs of piping and other mechanical services between areas that have higher use of mechanical services (e.g., cage washing, operating rooms, and holding rooms containing dogs rooms, etc.).

In considering the design of animal research facilities, provisions must be made for future expansion (113). Certain items, such as cage washing facilities, specialized laboratories, and surgical facilities, are expensive components of new construction, and during the initial construction of animal facilities may be able to be designed with sufficient capacity to support larger animal populations through logical expansion of the existing building. In developing a design for an animal facility, planners often include provisions for future expansion that must be considered in the animal facility design so that architectural and mechanical barriers are not put in place.

An overriding concern in the construction of animal facilities is the need to frequently clean and disinfect surfaces within the facility. In some cases, specialized components of the animal facility, such as

biocontainment facilities or bioexclusion facilities, must also have the capability of being totally disinfected or sterilized using aggressive agents that would be unsuitable for use in the rest of the animal facility. Many of the construction guidelines presented below are directed at making such tasks and routine cleaning/sanitization easier to accomplish on a regular basis, as well as preventing

the deterioration of the physical facilities by conducting such practices. By their very nature, these guidelines are quite general and are based on common practices and equipment.

Floors

Opinion varies greatly on the best type of flooring to use for animal facilities. Each type of flooring has its advantages and disadvantages, and it is likely that no perfect flooring exists for animal facilities. Those materials showing the greatest chemical resistance often have difficulties withstanding impact with sharp or heavy objects, whereas those showing the greatest resistance to mechanical damage may have difficulty in resisting the action of chemicals and urine. To withstand repeated sanitation and spillage of urine and water, floors must be moisture resistant and nonabsorbent (222). They should be resistant to the action of hot water, cleaning, agents, disinfectants, urine, and other biological materials. They should be relatively smooth for ease of cleaning, although they may need to have textured surfaces, especially in high moisture areas, where injury to personnel could occur from slipping or to animals, such as hoof stock kept in pens that are regularly cleaned with water.

Floors need to be impact resistant and capable of supporting equipment, cage racks, and other stored items without becoming cracked, pitted, or gouged. To facilitate cleaning, there should be a minimum number of joints in which debris can become entrapped. Materials that have proved particularly satisfactory in many applications are epoxy aggregates, sealed concrete, and other hardened synthetic-based aggregates. Correct surface preparation of subflooring and experienced installation of the flooring itself is essential to its satisfactory performance.

Drains

Drains in animal facilities are a source of contention, but in some areas they are a necessity. Facilities housing large animals require drains. Floors in such areas should be gently sloped toward the drain, and in the case of pens and runs, a dedicated trough for draining should be considered. In such applications, drainage should be designed to allow rapid removal of water and the drying of surfaces to minimize elevation of humidity. Where waste is to be flushed down drains along with water, larger-diameter drains (4 inches or greater) should be considered (222). To facilitate this process, rim-flush drains that sweep waste collected from trough drainage systems down into drain pipes through coarse basket strainers fitted in the drains minimize maintenance considerations. It is critical in the sanitation program for animal facilities that drain traps be kept filled with liquid and that drains that are not in use for long periods of time be capped and sealed to prevent backflow of sewage, gases, and other contaminants into the animal holding areas.

Floor drains are not essential in all animals rooms. Rooms containing rodents can be sanitized better by using alternative cleaning methods, such as wet vacuuming or mopping with cleaning compounds and disinfectants.

Walls and Ceilings

Walls within animal facilities need to be frequently sanitized and therefore should be moisture resistant and nonabsorbent. They also should be free of cracks or holes, such as those created by unsealed utility penetrations. Junctions of the walls with the ceiling and floors, as well as to themselves, should be adequately caulked or otherwise sealed to prevent build-up of debris and harborage for insects. The construction of the walls can take a variety of forms, with masonry being the most durable; however, other wall construction materials may be more appropriate in certain situations. The surface coating of walls should be resistant to the chemical actions of disinfectants and cleaning agents and should have significant resistance to abrasion and impact. Common wall materials acceptable in other applications, such as painted gypsum board, may not prove satisfactory in some animal facility applications given their low impact resistance and significant damage when exposed to moisture. Some of the difficulties associated with certain wall construction materials can be overcome by protecting the wall surfaces through the use of strategically placed guard rails or bull-nosed curbing on the wall to floor junction in order to minimize the impact of equipment on the walls (222).

Like walls, ceilings should be smooth and moisture resistant. Their surfaces should be capable of withstanding detergents and disinfectants and be free of imperfect junctions that would allow the build-up of debris and harborage of insects. Generally, suspended ceilings are undesirable in animal facilities unless they are constructed in such a way as to be readily sanitizable and free of imperfect junctions or penetrations. Gypsum board that has been appropriately sealed and finished with a durable coating or concrete that has been smoothed and sealed or painted are the most appropriate and long-lasting choices for ceilings.

Doors to Animal Rooms

Doors to animal rooms should be constructed of and/or coated with materials that resist corrosion and

are amenable to disinfection. They should be appropri

ately sealed/gasketted and fitted with door sweeps to ensure adequate sealing with the floor and the door frames. This is critical in preventing the incursion of pests and the uncontrolled movement of air and particulates beneath and around the door. Doors should be self-closing and should open into animal rooms to prevent injury to personnel or damage to equipment moving in hallways. For safety reasons, viewing windows often are appropriate, but the ability to cover the viewing windows in certain instances where close control of lighting is required must be considered.

Doors should be equipped with recessed or shielded handles (to prevent damage from equipment) and equipped with metal or plastic strike plates to prevent equipment damage to the surfaces. Generally, doors that are 42 in ×84 in accommodate most animal equipment; however, larger doors may be required in certain portions of the animal facilities. Consideration of room level security is important in order to limit access to animals on study or in areas in which bioexclusion or biocontainment is required. This is best accomplished with mechanical or electrical locks; however, it is important that doors be designed to be opened from the inside without a key for emergency egress purposes.

Corridors

Corridors within the animal facility are the principal means of access to animal rooms and support areas. For this reason, they should be wide enough to accommodate the movement of equipment and personnel and be amenable to cleaning and disinfection. Generally, a width of 6–8 feet is sufficient for most purposes (45, 130, 222). All of the considerations reviewed for floors, walls, and ceilings generally apply to corridors; however, suspended ceilings are more commonly used in corridors due to the need to access mechanical fixtures, such as electrical conduits and plumbing. It is good practice to ensure that access to water lines, drain pipes, and other utilities supporting animal rooms is available through panels or chases in the corridor outside of the animal rooms. Wall-mounted equipment in the corridors, such as, telephones, fire extinguishers, fire alarms, and record-keeping stations, should be recessed or installed high enough to prevent damage from movement of equipment and personnel through the corridors.

Support Areas

In addition to the basic animal rooms, animal facilities must incorporate certain facilities and equipment to support the basic day-to-day investigational and animal care needs, including locker rooms often coupled with shower facilities and restrooms, break areas, cage washing facilities, feed and bedding storage, waste handling and storage areas, supply rooms, general storage areas, administrative offices, and procedural rooms for use by researchers. Although a comprehensive treatment of these areas will not be addressed in this chapter, information regarding their essential features and construction can be found elsewhere (222). A few essential points, however, bear emphasizing.

In designing animal facilities, it is a common error to undersize these facilities in comparison to the intended animal populations or types of research to be conducted. This is especially true of support areas that experience cyclic levels of activity during their normal function. For example, locker rooms where clothing change occurs are intensively used at the beginning and end of work days and, depending on the type of facility, may also experience significant activity at lunch time or during designated break periods. Commonly, the size of these areas are designed to only allow a few individuals to use them at a single time, requiring adaptation of schedules and difficulty in access to the facility during certain times throughout the day.

Such areas must be large enough to accommodate reasonable numbers of employees and to provide sufficient locker space for clothing and storage of uniforms and other supplies. If mandatory showering is required, as is the case in some barriered facilities, sufficient capacity for showers or other clothing change functions must be provided. From a programmatic standpoint, the necessity of certain types of clothing change or the use of certain items of disposable clothing, such as face masks or shoe covers, must be carefully considered. The more complex the clothing change, the more complex the support area for it needs to be. Similarly, if reusable garments are used, adequate storage space and provisions for laundering/disinfection must be made. If clothing changes are to be made at the level of the animal holding room, such changing must be provided for in the design of the facility and must be logical if it is to be routinely followed. Human use support areas, such as lockers rooms and break areas or administrative offices, should be separated from animal areas. They are often located at the periphery of the animal facility and are used as control points for entrance into the animal facility.

The location of support areas is dictated by the location of utilities and certain architectural features in the animal facility. For example, cage-washing facilities are usually located at the end of corridors servicing groups of animal rooms. These facilities usually are located near storage areas for bedding, feed, and building utility runs in order to minimize distances traveled and construction costs. Waste-

handling facilities are commonly located near cage-washing facilities, and such facilities usually are located within a convenient distance of loading docks or other exterior entrances.

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Cage-washing facilities are designed to allow the use of chemical detergents, and disinfectants, and large amounts of hot water. Regular cleaning of room surfaces is an essential part of their operation, and the facility must be designed to allow this to be easily done. Large amounts of equipment are moved in and out of these facilities and they must be big enough to accommodate such equipment both in process and for short periods of storage. Substantial quantities of heat and moist air are generated within the cage-washing facility, and Heating, Ventillation, and Air Conditioning (HVAC) systems must be designed to accommodate these loads.
Often the locations of support areas are selected to decrease the amount of unwanted noise and traffic passing by animal rooms (222). Noise-producing areas, such as, mechanical rooms, waste processing areas, and cage washers, are often grouped together and separated from animal holding areas by noise traps, such as air locks or sound-proofing surfaces. Commonly, species of animals such as dogs, nonhuman primates, and swine that produce loud vocalizations as part of their normal behavior are also separated from other holding areas that contain more noise-sensitive animals, such as rodents, by using similar building concepts. Housing for these noise-producing species is commonly located near support areas that produce noise, although the two functions usually are separated by air locks, doors, or other means.
Areas used for storage of feed are kept at lower environmental temperatures than other areas to inhibit decomposition of liable ingredients and the development of immature forms of insect pests that may contaminate the packaging or the feed.

Specialized Components of Animal Facilities

Within animal facilities there is often a need for specialized structures and equipment to support toxicologic research. Such facilities include, but are not limited to, barrier facilities for maintenance of animals in defined microbiological states, bio-containment facilities for housing and manipulation of animals exposed to biohazardous materials, surgical facilities, imaging facilities, necropsy facilities, and facilities for conducting diagnostic tests on animals.

Barrier Facilities/Barrier Rooms

Barrier facilities or individual barrier rooms may be used in toxicologic research for either bioexclusion or biocontainment purposes (344). Most commonly, they are used for bioexclusion. In this role, the room or series of rooms (facility) is separated from other components of the animal facility using a variety of construction methodologies in a manner to prevent undesirable microorganisms from entering the room whether carried on personnel, equipment, or materials. Barrier rooms are composed of durable materials that are impervious to liquids and free of unsealed penetrations. Personnel entry is usually through a lock system requiring progressive decontamination of personnel by shower and/or clothing change. Air to the room is independently supplied and HEPA (high efficiency particulate air) filtered. Water supplied to the area is independently decontaminated or sterilized, and provisions are available to provide sterilized feed, bedding, and other supplies for use in the room. Waste is removed using processes designed to prevent the incursion of unwanted microorganisms during the removal process.
Most commonly, multiple methods of introducing supplies and equipment are built into the barrier room. Generally, a through-the-wall autoclave is provided with sufficient capacity to handle reasonably large loads of materials. For maintenance purposes, the autoclave should be able to be serviced from the outside of the barrier. It is essential that the autoclave be calibrated and validated based on material types and load configurations. This calibration and validation should be done on a regular basis, usually yearly. The use of cumulative heat-sensitive or biological indicators placed at one or two points in a load to be autoclaved is not sufficient to confirm adequate disinfection or sterilization.
In addition to autoclaves for processing large volumes of materials that are unaffected or marginally affected by heat, alternative means of disinfection need to be provided for introduction of heat-sensitive materials into the barrier room. This is accomplished by using a double-door chamber into which materials that have been immersed in disinfectants can be placed and additional disinfectants sprayed into the port before it is closed and the materials held for an appropriate contact time. Once this chemical disinfectant process has taken place, materials can be removed from the chamber using the inside door for subsequent use in the barrier. As with autoclaving procedures, the use of chemical disinfectants must be calibrated so that adequate application procedures, concentrations and contact times are maintained. Materials processed by this method must be wrapped in coverings that can withstand the application of the disinfectants, and the materials inside of the packaging must be already disinfected by alternative means. For example, vacuum-packed containers of paper products that have been previously gamma irradiated could be introduced using this spray-port method.
Introduction of personnel into barrier rooms poses the greatest risk to the microbiological integrity of the

barrier room. Because it is impossible to adequately disinfect the external surfaces of people, it is necessary to place a barrier between them and the animals maintained within the room. This is accomplished in an entry lock system by an orderly removal of clothing and a re-gowning in

attire that covers the body surfaces using materials that are known to be free of organisms of concern (344).

The entry lock system usually is composed of four compartments. The first compartment, often referred to as an insect lock or air pressure lock, is used for static pressure control between other areas of the animal facility and the lock system. This first lock also serves to inhibit the passage of insects into the other more critical areas of the lock system. It is also used in some instances for storage of external clothing, such as jackets or for depositing of shoes worn elsewhere in the animal facility. The next component of the lock system is an undress lock, where all clothing is removed and placed in lockers or other suitable receptacles. Presumably, most, if not all, of the contamination that might be associated with personnel resides on such clothing.

At this point in the lock system there are two possible configurations. The first is termed the *wet entry system* and involves personnel taking a water shower. Depending on the rigors of the barrier facility, an electromechanical system may be placed in this portion of the lock system to assure that a wet shower is actually taken. This consists of interlocking doors with timers requiring a wait period within the shower area and an electrical interface with the water supply to the showers such that the release timers are only activated if water has run for a certain period of time. In the end, even such rigorous measures do not necessarily guarantee that the shower taken is adequate or consistent (344).

Although some decontamination of external surfaces of a person's body may occur to varying degrees with a shower, the principal rationale for the use of the water shower is to ensure that street clothing has been removed. There are a number of disadvantages associated with a shower in a lock entry system, including the time constraints associated with the entry procedure and the considerable requirements for expendable supplies used in the showering procedure. Following the shower, a separate room is provided for drying off and putting on a sterile/disinfected uniform, including components such as a cap, mask, gloves, and dedicated footwear. Once these procedures have been conducted, entrance into the barrier room can be made. Leaving the barrier room, the employee reverses the process; however, often the showering step is omitted.

Because the wet entry system is quite cumbersome and limits the number of employees that can enter the barrier facility at any given time, a new methodology, the *dry entry system,* has gained increasing favor in many research settings. This latter method involves the elimination of the wet shower and a direct change in a separate room from the de-gowning portion of the lock into clean, disinfected clothing as occurs in the last step of the wet entry system. Once clothed in appropriate attire, the employee enters an air shower that blows high-velocity HEPA-filtered air across all surfaces, removing particulates that may contain any contaminants imparted to the external surfaces of the gown during the changing process. Doors to the air shower are electrically interlocked, requiring that a full cycle be completed before the employee can enter the barrier room. Upon exiting the barrier room, all aspects of the process, including the air shower, are again used by the employee in reverse order.

In addition to time savings, the air shower also provides a very positive interlock between the barrier room and the lock system, preventing escape of any animals that have left their cages and any potential incursion of insects or other pests into the barrier room through the lock system. The air shower also greatly decreases the amount of particulates that are brought out of the barrier room serving as a safeguard for the general animal facility should a contamination occur within the barrier (344).

As a general rule, cage washing and other sanitation procedures are done within the barrier facilities rather than bringing caging materials out of the barrier. This is because each transport of materials across the barrier entails some degree of risk of introduction of unwanted microorganisms. Movement of caging from the barrier through common animal facility hallways and cage-washing facilities also poses a risk of contamination that may or may not be eliminated by the disinfection processes used to re-enter such materials into the barrier. Similarly, rigidly operated barrier rooms generally do not provide for personnel break areas or other administrative support facilities within the barrier. Such areas tend to encourage removal of protective clothing and inappropriate activities given the microbiological status of the barrier.

Barrier rooms are expensive to operate and are best used to house large numbers of animals or to house large animals under defined microbiological conditions. Due to their large size and the large amount of materials that must be passed across the barrier to maintain it, failure of the barrier, as measured by changes in the microbiological status of the animals maintained within the barrier, are common. Once an unwanted microorganism gains entrance, all animals within the barrier room are at risk of becoming contaminated unless some form of secondary containment/bioexclusion system (e.g., microisolation cages) is also used within the barrier (344). This practice is seldom done, given the high

cost of such secondary bioexclusion practices.

Biohazard Containment Facilities

Toxicologic research often requires the use of biohazardous materials in conjunction with animals. Such materials, whether they be toxic, carcinogenic, radioactive, or infectious, must be contained within a very

Table 16.1 Comparison of conventional animal care strategies with strategies for containing biohazards

	Recommended Animal Care Practices Strategies *a*	Biocontainment Strategies *b*	Rationale
Air flow	High	Low	A
Cage cleaning	Frequent	Infrequent	A
Bedding change	Frequent	Infrequent	A
Equipment/supplies	Reusable	Disposable	B
Animal handling	Frequent	Infrequent	C

a Practices or approaches commonly used to provide a clean, healthful environment for animals.
b Practices or approaches designed to minimize the release of hazardous materials from animals or their environment.
A—The more potentially contaminated material generated, the more that has to be decontaminated and the greater chance for error; B—processing materials for reuse while assuming proper decontamination is costly, logistically difficult, and has the potential for error; C—the more handling, the greater risk for personnel injury and contamination.

defined space and handled appropriately so as not to contaminate other animals or personnel (287, 288). It is a popular misconception that barrier facilities or certain other bioexclusion systems, such as microisolation cages, provide, in normal operation, the necessary level of biocontainment to meet most research needs. In general, good biocontainment practices are at odds with recommended animal husbandry and bioexclusion techniques (Table 16.1).

The type of biocontainment facilities required when using biohazardous materials in animals depends on the nature of the hazard and the level of risk posed by it. In the case of infectious materials, the Centers for Disease Control and Prevention (CDC) has published classification systems for microorganisms and have described biological containment facilities of four increasingly secure types designated BL I through BL IV (49, 50). These safety practices and equipment used in conjunction with these increasing levels of hazards are designed to prevent inadvertent transmission or release of contaminants from the work area. These guidelines set standards for reducing aerosol generation, hazards associated with sharp objects, the use of protective clothing, and the operational standards for hoods and other devices used to manipulate infected materials, including animals.

Biocontainment facilities are resource intensive, and their complexity increases with the size of the animals that must be contained at the various biosafety levels. Laboratory animals the size of rabbits or larger require considerable housing space and generate large amounts of waste material that is potentially contaminated. Normal operations of cleaning and disinfection, as well as removal of biological materials, including animal carcasses, may require complex systems, making such facilities expensive to construct and to operate. For this reason, those institutions conducting limited work in larger species involving biohazardous materials may choose to subcontract these tasks to more specialized institutions in which the volume of such work is sufficient to justify the cost of constructing and maintaining these facilities.

Most institutions, however, use cage-level biocontainment practices for studies involving rodents and other small mammals. Studies up through biosafety level III can be conducted in small, dedicated facilities using these systems, along with appropriate safety practices.

Surgical Facilities

The use of surgically altered animals is common in toxicologic research. Traditionally, the complexity of surgical procedures performed and the nature of the surgical facilities required was directly proportional to the body size of the animals being used. Small laboratory animals, such as rats and mice, were often used in studies in which relatively simple procedures involving organ removal was required. The facilities to support these operative procedures were not complex in nature, and often a portion of a multipurpose area could be used on a temporary basis for conducting them. Larger, more complex procedures were usually reserved for animals the size of dogs or domestic farm animals in which the operative field was much larger, and techniques used in human surgery, as well as the necessary support equipment, such as specialized gas anesthesia machines, bypass equipment, and specialized imaging equipment used in human or traditional veterinary practice, could be employed. Such procedures require a much larger, dedicated surgical facility.

A few guidelines for the design and construction of surgical facilities for use in biomedical research have been published (222, 345). In general, such facilities should have certain basic components, including the following:

an operating room in which the surgical procedure is conducted, a postoperative care area separated from the rest of the surgical facility in which animals can recover from the surgical procedure, an animal preparation area in which the animal can be anesthetized and other preoperative procedures conducted, an instrument and supply preparation area in which materials can be assembled, cleaned, and sterilized, and a surgeon preparation area in which personnel can decontaminate their hands and then become suitably attired for the surgical procedure. A number of support facilities may also be associated with the surgical facility, including a diagnostic laboratory, imaging facilities, and specialized procedure rooms to study instrumented animals.

Existing guidelines for surgical facilities endeavor to separate the various functions to be performed by physical barriers, distance, or by separating the functions in time (222). Surfaces and construction materials used in surgical suites are designed to be easily cleaned and disinfected (222). Air provided to surgical facilities should be highly filtered to remove microorganisms and should be appropriately conditioned in order to minimize temperature loss or gain during the surgical procedure. Such facilities are utility intensive and often require access to medical gases and emergency power.

Over the last decade there has been a trend toward miniaturization, and complex surgical procedures in support of toxicologic research can now be performed on small laboratory rodents. The increasing commercial availability of vascular catheterized rodents, coupled with numerous types of telemetry devices for continuous recording of a variety of parameters from nonrestrained animals, has provided new possibilities in drug discovery research. If sufficient numbers of such procedures are to be done, a dedicated surgical facility for rodents may be appropriate. In general, many of the same components used in traditional large-animal surgical facilities are present in such a dedicated rodent surgical facility but may not be separated by physical barriers, such as walls.

Due to the small size of the operative field in small laboratory animals, the use of sterile instrument tip procedures can be coupled with chemical disinfectants and table-top dry heat sterilizers to allow large numbers of rodents to be instrumented aseptically during a single surgical session (38, 68, 69). To facilitate these procedures, horizontal laminar-flow hoods may be used to minimize the chance of cross-contamination between animals during surgery and to reduce bacteriologic contamination of the operative site.

Unlike traditional operative procedures on larger animals, most rodent surgery is done in a sitting position and may require the use of magnification and focused, point-source illumination. For this reason, provision needs to be made for adequate counter space and electrical power. Because multiple groups of animals may be used in the surgical facility over a short period of time, preoperative and postoperative housing is often done in cage-level bioexclusion systems to prevent the chance of cross-contamination between projects when animals are used from either different sources of supply or from different locations employing different housing methods.

It is important to remember that the size of the animal or the species of the animal does not diminish the need to maintain appropriate aseptic procedures during the course of the surgical manipulation (20, 32), however, due to the small size of rodents and the relatively small operative field, the risk of postoperative infection and the need for maintaining large areas of a facility in an aseptic fashion is greatly reduced (68, 69).

CAGING AND HOUSING SYSTEMS

The environment of the laboratory animals used in toxicologic research is classified by levels of enclosure. A cage, pen, or stall is the immediate limit of an animal's environment in a research facility and is designated as its *primary* enclosure. The room or space in which the primary enclosure is located is termed the *secondary enclosure* (222).

The environmental conditions in the secondary enclosure influence, but do not control, the environment in the primary enclosure (26, 354, 355). The animal's primary enclosure needs to allow the animal to remain clean and dry, securely contain the animal so that it cannot escape and cannot injure itself while residing in the enclosure, and provide adequate ventilation in order to allow the animal a sufficient supply of fresh air that is appropriately conditioned to meet its needs. The primary enclosure should also provide for normal physiologic and behavioral requirements of the animals, including reproduction, movement, urination, and defecation. It should allow for investigator access and observation of the animal with minimal disturbance. In addition to these criteria, primary enclosures may also be used to maintain the animal and its environment in a specified microbiological status. This bioexclusion function requires a heavy reliance on aseptic techniques and personnel training, as well as significant support facilities, including autoclaves and laminar flow work stations (222, 344).

Primary Enclosures

Microisolation Cages

A microisolation cage consists of a plastic cage bottom with stainless steel wire top that is covered by a fitted plastic lid containing a filter. The filter usually is comprised of a polyester material that can be fabricated to

different thickness and average pore sizes. In principle, by the lid remaining on the cage, all ventilation occurs through the filter, which excludes particulates that may contain infectious organisms. As long as all manipulations are done in a laminar flow work station and all materials are adequately disinfected/sterilized, unwanted organisms should be excluded from the cage environment (72). For this to occur, all manipulations must be done using aseptic technique. Such systems can be effective in maintaining a particular microbiological status of animals, but manipulation of the animals may be cumbersome for researchers and requires that all research equipment and materials undergo rigorous and frequent decontamination/sterilization to ensure the microbiological status of the animals.

Microisolation caging does provide some disadvantages in terms of the environmental conditions surrounding the animals. Because static microisolation caging does not allow for significant air exchange through the filter, the concentration of water vapor, gases, and heat may build up to levels well above that in the secondary enclosure (161, 298). Another difficulty may be related to the adequacy of the fit of the filtered top to the cage itself. Studies have shown that in some designs of microisolator caging, ventilation occurs under the lip of the cage, allowing unfiltered air to migrate across cage surfaces and other areas into the sterilized environment of the microisolation cage (202, 203). This makes practices such as the exterior disinfection of the cage before manipulation in the change station an important consideration. In addition, when manipulating such caging in a laminar flow change station, it is important to minimize the amount of materials in the change station, as laminar flow air upon striking objects will eddy for a distance of at least three times the diameter of the object struck, causing unwanted movement of air between materials in the station, resulting in airborne cross-contamination (349).

Ventilated Microisolation Caging

To address some of the difficulties associated with poor ventilation of static microisolation caging, specialized racks designed to hold the microisolation caging have been developed. These racks contain a source of HEPA-filtered air that is discharged either through a nozzle directly into the cage or through small openings above the filter cage top such that the air moves directly into the cage. Ventilated racks usually have a mechanism for removing air from the cage, often through a port located directly above the filter top in another area of microisolation cage. This exhaust air is either HEPA filtered and discharged into the room or directly dis charged into the room exhaust system. These racks usually are equipped with individual blowers to pass air through the HEPA filters and generate the necessary static pressure. Like static microisolation caging, ventilated microisolators must be handled and managed in the same manner as static microisolation caging using aseptic technique, sterilized materials, and laminar flow change stations (344).

By supplying relatively large volumes of conditioned fresh air to each cage, heat, moisture, and gas contaminant build-up is minimized. Bedding materials often stay drier than in unventilated cages, improving the animal's environment and allowing longer intervals between bedding changes (222). In the case of the ventilated microisolation cage, it is important to remember that the cage itself is maintained under positive pressure with respect to the secondary enclosure. As such, if hazardous or unwanted microorganisms are present within any given cage, the possibility exists of leakage of contaminants to surfaces outside of the cage through leakage in individual filter tops. For this reason, ventilated microisolation cages must be used with caution in research involving the use of hazardous materials (344).

Conventional Caging

By far the most commonly used type of caging in toxicologic research is conventional open caging. In the case of rodents, this is of two types. The first is solid bottom plastic or metal caging with contact bedding. The cage body is covered with a lid that often contains an integrated feeder for holding feed pellets and a space for a water bottle. As an alternative, water may be provided by automatic watering systems, in which case a small hole is found in one end of the cage, through which a small water valve is introduced.

The most common type of cage is constructed of plastic with a solid bottom. These can be opaque or transparent. Transparent materials have the advantage of allowing easy visualization of the animals, whereas opaque materials provide a more sheltered environment for the animals by decreasing the amount of light striking them. The type of plastic materials used in the construction of the caging is important, as procedures such as frequent autoclaving and washing with high-temperature water and chemical compounds that are designed to disinfect can rapidly deteriorate some forms of plastic (58, 177). In addition, animal urine, which contains significant quantities of minerals and proteinaceous material, can cling tenaciously to plastic, requiring the use of mineral acids or other compounds to

remove them (222). Over time, all plastic caging will deteriorate and need to be replaced. Cracks or other deficits in the plastic make

sanitation difficult and may affect the structural integrity of the caging, requiring replacement.
A second type of caging is referred to under the general classification of *suspended caging*. Such caging usually is constructed of metal, most commonly stainless steel. The floors usually are of wire mesh, containing either two or four wires per inch. Some types of suspended caging have punched metal floors that have been electropolished or otherwise smoothed to prevent injury to feet. Punched metal flooring is commonly used for housing rabbits, as it provides greater support for their feet. As an alternative, some suspended caging is constructed of heavy duty plastics. Floors may also be constructed of the same plastic using the same principle as the punched metal flooring. Suspended cages for larger animals, such as dogs, cats, or swine, commonly use coated wire or coated, metal punched flooring to minimize the chance of damage to the animal's feet.
The mesh size of any suspended cage flooring is important. It must be sufficiently wide to allow the passage of feces while still providing sufficient support to the feet to prevent pressure injuries. In theory, suspended caging should allow feces and urine to pass onto catch pans containing sheet or loose bedding or, in some cases, onto catch pans that can be flushed into trough drain systems. Because most animals, including rodents, are coprophagic, if feces pass through the flooring, the animals will have limited access to it. Many species may consume feces as it is being defecated and before it can drop through the cage floor. Coprophagy is critical for normal health of some species, such as rabbits.
Larger suspended caging usually is cleaned in place, whereas caging for rodents and other small mammals usually is taken to a wash area for cleaning. Watering can be provided to suspended caging by means of automatic watering devices, water bottles, or, in the case of some large animals, water bowls. Feed usually is dispensed either in feeders that hang on the cages or within it or, for some larger animals, by use of feed bowls. Caging for some larger animals, such as nonhuman primates, cats, and dogs, often contain a resting board or perch that is suspended off of the floor. This allows the animals to express normal behaviors and provides them an elevated place on which to rest.
Neither solid-bottom nor suspended conventional caging is designed to prevent the airborne or fomite transmission of adventitious microorganisms. Control of such organisms when these cages are used is at the secondary enclosure level.
Pens and Runs
Domestic farm animals and dogs often are maintained in either runs or pens. A pen is a large indoor enclosure whose floor usually is the floor of the secondary enclosure. In the case of those animals that do not exhibit climbing behavior, indoor pens may be constructed such that the top of the pen is open. Most commonly, however, pens form a complete enclosure. Pens usually have an incorporated drainage system either by the use of a slanted floor underneath a raised pen floor that allows liquids to drain toward the back of the pen into a trough drain system or through the incorporation of a trough drain system in the floor of the pen itself. Pens are cleaned using water under pressure and detergent/disinfectants. Pens may also be bedded with loose bedding, which is removed and replaced on a regular basis. Whenever in-place cleaning of pens is done, it is important that the design of the pens be such that floor drains are large enough to accommodate fecal material and other items, such as bedding that may be flushed down the drainage system.
In theory, pens provide large animals the ability to move about normally and to acquire some exercise (131). Studies have shown, however, that such exercise is rarely spontaneous and usually is associated with the presence of humans (42, 43, 134). For this reason, components of some legislation, as well as certain guidelines, require regular periods of exercise for certain penned animals (308). Special provisions must be made to allow such exercise to occur.
The term *run* is often reserved for pens that have both an indoor and outdoor component. In such cases, the indoor component of the run often has a smaller floor area than the outdoor component. The indoor component usually is in a conditioned space or has other provisions for the thermal comfort of the animal, such as heated flooring. Access to the outdoor portion of the run may be limited through the use of animal-operated doors or doors that must be opened by animal facility personnel. Feeding and provision of water to animals housed in runs usually are done in the indoor portion of the run. Cleaning is done using water under pressure accompanied by detergents or disinfectants. Care must be taken during the process of cleaning such enclosures so as not to unduly stress or cause the animals to become wet during the process (222).
Housing of animals in pens or runs requires that consideration be given to handling and training of animals housed in such enclosures (256). Toxicologic research often requires repeated sampling or other manipulations. Removing animals from such enclosures, as well as protection of implanted sampling

ports or catheters while animals are in the enclosures, must be given adequate attention. Many problems can be overcome by conditioning periods during which the animals are handled and trained to respond in certain ways.

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SECONDARY ENCLOSURES

The construction of typical animal holding rooms has been previously discussed. A number of specialized secondary enclosures other than the conventional animal holding rooms have been developed for the purpose of minimizing transmission of adventitious microorganisms between groups of animals. In contrast to individual primary enclosure bioexclusion systems, these secondary enclosure systems provide some increased flexibility in animal handling and some decreased reliance on maintaining strict technical practices. They also pose an additional advantage of allowing the use of existing caging systems.

Cubicles

A cubicle is a small room partitioned off within another larger room and is used to decrease the chance of airborne cross-contamination between groups of animals housed in adjacent cubicles. The air supplied to the cubicle may come from a separate air supply within the cubicle or from the room in which the cubicle is located. In the latter instance, a negative pressure is created within the cubicle by an exhaust system, usually located in the ceiling of the cubicle, which pulls air into the cubicle through a space under the doors to the cubicle in a fashion similar to a fume hood. The cubicle is entered by opening the doors, at which point all directional control of air movement is lost (347). Unlike cage-level containment systems, cubicles only address airborne cross-contamination between cubicles, not fomite transmission, as might occur on dust particles or by manipulation by investigators or by animal care personnel. Due to the low face velocity associated with the air movement underneath the door to the cubicle, absolute prevention of airborne cross-contamination cannot be assured (344, 347).

Cubicles have been shown to be useful in some applications where separation of species is required in limited space, and they apparently can limit the spread of certain microorganisms under the right conditions (171). They are probably best used in conjunction with cage-level bioexclusion systems if reliable prevention of microbiological contamination is required.

Ventilated Cabinets

Ventilated cabinets consist of a cabinet with one or more doors. The cabinet has a small fan and HEPA-filter that extracts air from the room and releases it into the cabinet. Air is exhausted either between the doors to the cabinet or through an exhaust duct into the room. The exhaust air may or may not be filtered (185). Like cubicles, when the cabinet door is opened, caging within the cabinets no longer receives the benefit of the HEPA-filtered air supply. Airborne cross-contamination between cages within the system is not controlled unless a cage-level bioexclusion system is also used. Fomite transmission is not prevented by ventilated cabinets. Their use in toxicologic studies is limited.

Mass Air Displacement and Laminar Air Flow Rooms

These secondary enclosure bioexclusion systems are designed to decrease the airborne transfer of microorganisms between groups of animals. Both systems use a HEPA filter and blower to supply air to either diffusers or a plenum located in the ceiling of an animal room. In the case of mass air displacement rooms, large volumes of appropriately filtered air is released through a series of diffusers located in the ceiling in an attempt to wash any airborne contaminants to the floor and out through exhaust ducts located at floor level at several locations in the room (186, 194). By contrast, laminar air flow rooms pressurize a space above the ceiling (plenum) and discharge air out through the thousands of tiny holes in the ceiling, creating laminar movement of air (19). In theory, particles released from individual cages will be caught up in the laminar air stream and drop to the floor (65). In practice, once the laminar air flow strikes caging or other objects within the room, it eddies for distances of three or more times the diameter of any object that it strikes, causing mixing and producing an effect similar to mass air displacement (349).

Both of these room types do not effectively address airborne cross-contamination between adjacent cages and do not address fomite or other means of transmission of microorganisms. By themselves they are not a complete bioexclusion system (344). Depending on the design of the ventilation equipment, these rooms can be quite noisy and may be prone to excessive heat build up. Prior to the advent of cage-level bioexclusion systems, mass air displacement and laminar flow rooms were a common feature in toxicological research facilities using animals. They are less commonly used today.

ILLUMINATION

Light is an important environmental factor that can affect biological and behavioral processes in animals and the conduct of routine animal care duties. Numerous studies have documented effects of light on the morphology, physiology, and behavior of various animals (33, 82, 233, 316, 338). Not all of these effects have been adequately explored in all species or in sufficient detail

to predict the magnitude and the extent of impact on various toxicologic studies.

There are three factors that characterize lighting: (1) lighting spectrum (wavelength), (2) light intensity as measured in foot candles (English) or lux (metric), and (3) photoperiodicity (light/dark cycle) (22, 300). In addition, factors such as the light history of the animal, animal pigmentation, time of light exposure during the circadian cycle, species, sex, age, body temperature, hormonal status, and stock or strain of the animal can alter the influence of specific characteristics of the light on an animal (33, 76, 240, 285, 294, 331).

The one characteristic of light that has the most profound effect on animals is photoperiodicity. It regulates circadian (one day in length) and ultradian (greater than one day) biological rhythms in animals. These, in turn, can alter a variety of basic processes. Photoperiodicity is a critical regulator of reproduction and behavior (118, 196, 268, 339), including a behavioral influence on processes such as feed consumption and resulting body weight gain, nutrient intake, and hormone secretion (34, 54, 316). It appears that in most species, a period of 10–14 hours of light is required to maintain normal biological rhythms (200). Some species such as rats and mice are more tolerant of light cycle length than are species such as hamsters, which require at least 14 hours of daylight for normal reproductive function (54, 99). Continuous daylight, which can occur with malfunctioning light timers or inadvertent overriding of light timing devices, can have serious consequences for some toxicologic studies (28). For this reason, it is important to regularly assess the function of light timing devices and to build in safeguards to any light timer overriding mechanism to ensure that a consistent photoperiodicity is maintained (222).

A consistent photoperiodicity with adequate dark cycles is also essential to allow for the regular daily renewal of rods and cones in the eye, especially in nocturnal and crepuscular species (60). Without adequate dark periods, retinal degeneration cannot occur and can magnify the effects of retinal degeneration associated with high light intensity (236). Every effort should be made to minimize disruption of the dark cycle of the photoperiod. When procedures need to be conducted during the dark cycle, infrared illumination, low-intensity red lighting, and the use of point source low-intensity lighting, such as hand-held flashlights, may allow the necessary procedures to be performed without significant disruption. Alternatively, light cycles can be adjusted to fit work required (reverse light cycles), or the necessary procedures may need to be rescheduled into the light cycle.

Consistency in the photoperiod is important, and for this reason the use of windows in animal rooms needs to be carefully considered. Exterior windows allow seasonal ambient lighting conditions to alter photoperiodicity. This may not be important in certain species, such as nonhuman primates, dogs, and some agricultural or other large mammals, and may be considered a form of enrichment. In small rodents in which reproduction may be a study parameter, this lack of control may be an important variable (222).

Other significant concerns posed by exterior windows may be difficulties in temperature regulation within the animal holding room because of heat loss or gain through the windows and difficulties in providing adequate security for exterior windows (222).

Light intensity is another important characteristic of light that can dramatically affect certain laboratory animals. Many commonly used laboratory animals, including rats and mice, are nocturnal. Moreover, many of these species have been developed on albino backgrounds, thereby lacking pigment in their eyes, skin, and other tissues that provide some protection from the effects of light intensity. The most damaging effect appears to be phototoxic retinal atrophy, which occurs in albino rats and mice (175, 172, 271).

Because light intensity is affected by the type, location, and number of lighting fixtures in a room, considerable variation can occur between toxicologic research facilities. The age of the light-producing fixture (i.e., bulbs/tubes) is also important, as the output intensity will vary with length of use. It is unclear whether the effects reported for light intensity are related to a single wavelength/group of wavelengths or occurs equally over the whole spectrum. Light intensity is also affected by caging type and location as well as the reflective ability of other room surfaces (234, 339). Studies have shown that the position of mice in cages at various levels on the caging rack can influence the incidence of retinal atrophy, with an incidence of affected animals as high as 30.2% being recorded for animals housed in upper cages, as compared to 0.7% incidence for animals housed in lower cages (28, 115). For this reason, randomization of caging, as well as rotation of cages, is an important consideration if such effects are to be equally distributed between test and control groups (116).

When specifying light intensity, it is important that a uniform point of measure be used. In recent studies, as well as recommendations by various organizations, a measuring point of 1 m off of a floor in

the center of the animal holding room has been used and is considered to be the standard point of measure for intensity (22, 59, 222). This is used only to set an overall room intensity level for the purposes of comparing animal rooms and does not reflect the actual intensity experienced by the animals.

The light exposure history of individual animals can alter their sensitivity to phototoxicity. At least one study suggests that light intensities that are 130 to 270 lux

above the intensity under which the animal was raised may be the point at which retinal damage may begin to occur (294). Young albino and pigmented mice appear to have the ability to reverse some of the retinal damage associated with elevated light intensities (331, 341). Current recommendations suggest that a light level of 325 lux (30-foot candles) at 1.0 m (3.4 feet) above the floor is sufficient for routine sanitation while still avoiding significant retinal degeneration in albino animals (22). Other recommendations exist and are based on cage-level lighting intensities but are difficult to assess and administer (201).

The spectral distribution of light as it relates to adverse effects on laboratory animals has not been extensively studied. No artificial light has exactly the same spectrum as sunlight. Some fluorescent bulbs have a much wider spectrum with more output in the ultraviolet and infrared ranges than others. This can be partially compensated for by mixing standard fluorescent fixtures produced by different manufacturers in the same lighting fixture. Because different fluorescent compounds may be used by different manufacturers, this procedure can produce greater spectral diversity than bulbs from a single manufacturer. Overall, there is no definitive evidence to suggest that a wide spectral diversity in artificial lighting provides any direct enhancement of animal health or well being.

Some species of new-world primates are capable of synthesizing certain forms of vitamin D from dietary constituents and sunlight. Because it is common practice to provide enriched diets for these species that contain the appropriate forms of already converted vitamin D, access to full-spectrum artificial sunlight would not appear to be necessary.

A number of practical issues should be considered with respect to lighting in animal facilities. Many of these are covered in recommendations produced by the Illuminating Engineering Society of North American (IESNA) Handbook, which provides practical information on selection and installation of lighting (153, 154). It is generally recommended that light bulbs or fixtures have protective covers to ensure the safety of both the animals and animal care staff. Cleaning operations and the use of water in animal husbandry procedures can pose hazards from breakage and electrical shock.

NOISE

There is little doubt that extreme levels of acoustic energy (80–120 decibels of sound pressure) can, under the right circumstances, produce auditory and extra-auditory changes in laboratory animals (18, 92, 107, 108, 224, 249, 250, 251, 358). The assessment of sound-related interaction and injury in laboratory animals is complex, as the potential effects of noise on ani mals not only must take into consideration the intensity of the sound but also the frequency pattern of sound presentation, including rate of onset, duration, and vibration effects (12, 59, 241, 252, 255). The hearing range, noise-exposure history, and susceptibility to adverse effects of sound based on species, age, and strain further complicate any analyses (37, 145, 197, 199, 266, 284, 328). Many diverse effects have been reported in the literature and could pose unwanted variation in any toxicologic study. There are no comparative damage risk criteria for each of the common laboratory animal species, making it difficult to know what sound presentation is harmful (22).

In general, noise-producing animals and animal care activities should be separated from species and activities that do not generate noise (103, 222, 252, 283). This can be done by careful consideration of facility design and operational practices (11, 245). Physical barriers such as doors, air locks, and sound-absorbing materials can be useful in minimizing the effect of sound (245). Excessive and unpredictable noise patterns can be minimized through personnel training and the use of alternative practices and equipment in routine animal care duties.

VENTILATION

The purpose of ventilating an animal holding room is to dilute gaseous and particulate contaminants, supply adequate oxygen, remove thermal loads caused by animal respiration, lights, and equipment, adjust the moisture content of room air, and, where appropriate, create static-pressure differentials between adjoining areas (58, 222). It is, unfortunately, a common mistake to place heavy reliance on a ventilation system as the primary mechanism to prevent the movement of undesirable microbiological organisms from one location within a room or a facility to another. Other factors, including, movement of personnel between areas, movement of equipment, and transfer of animals within a facility, can quickly overcome any benefits associated with well-controlled ventilation. In theory, high ventilation rates should dilute out particulate and gaseous contaminants including undesirable microorganisms and allergens (104, 301, 307). Because, it is difficult to control the movement of air within secondary enclosures, viable particulates can easily escape any containment, provided strictly on the basis of ventilation.

As a general rule, air supplied to any secondary or primary enclosure should be free of contaminants

and be properly conditioned. HEPA filtration is recommended to provide some assurance that supplied air is free of particulates that could potentially harbor infectious organisms (189). HEPA filters are given efficiency ratings that can exceed 99% for particulate

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exclusion within certain particle size ranges (5). Average pore diameters of HEPA filters are designed to exclude particulates the size of bacteria or larger but may not be small enough to exclude individual viral particles. Because viruses and other organisms tend to travel on particulates due to their high electrostatic charge, HEPA filters are effective in excluding them. Unfortunately, large volumes of air are processed by HEPA filters, and becasue their efficiency rating is not 100%, a certain amount of infectious particles will pass HEPA filters over time. Given the dilution in the air stream, this small amount of passage may not pose a significant threat in most situations. Moreover, because incoming air is seldom challenged with high concentrations of infectious particles, this deficiency usually is not a problem.

If HEPA filters become wet or excessively laden with particulates, bacteria associated with such particles may grow through the HEPA filters and be discharged on the clean side (77). Clogging of HEPA filters usually is monitored by measuring the static pressure across the HEPA filter. Increases in static pressure indicate that the HEPA filter is becoming laden with particulates and at some predetermined static pressure reading, should be changed.

Correct installation of HEPA filters is critical. If they are incorrectly mounted, untreated air can pass by the HEPA filters, producing the same effect as if they contained a large hole. For this reason, HEPA filters should be tested in place using particulate generation equipment (e.g., DOP testing) (77). It is important that such testing be done on a regular basis and should be thoroughly done when the HEPA filter is first mounted. It is critical that such testing be done by individuals experienced in the intricacies of the testing procedure, as inappropriate testing can fail to reveal difficulties in the mounting or construction of the HEPA filter. HEPA filters should be protected by pre-filters designed to catch the majority of particulates of large to intermediate size. This usually is accomplished by a series of pre-filters of different efficiencies. Such pre-filters should be changed regularly.

Air exhausted from animal holding rooms may or may not be filtered prior to discharge to the environment. In the case of toxicologic research involving hazardous agents, exhaust air usually is filtered using filters that are capable of excluding the material in question from the air stream. If the nature and level of hazard is low enough, dilution with large volumes of air by discharge into combined exhaust or into the environment directly may be sufficient.

Facility design guidelines emphasize the need for regulating air-pressure differentials either within animal holding facilities as a whole or within specialized support facilities, such as surgical, procedural, service, housing, or quarantine areas. In general, areas maintained under negative pressure with respect to adjacent areas are designed to prevent the escape in the air stream of unwanted materials from the area maintained under negative pressure (99, 287, 288). This would be most appropriate, for example, in an animal holding area being used for quarantining of animals. Conversely, maintenance of an animal holding area under positive pressure with respect to the surrounding areas would be undertaken under those circumstances where animals within the positive pressure area are free of certain microorganisms and whose exposure to particulates from other adjacent areas might increase their risk of contamination. Such positive pressure housing might be used in areas maintaining specific pathogen-free animals or used for surgery. While maintenance of static pressure differentials may pose some advantage with respect to contamination control, it should not be relied on for containment of chemical or infectious agents that could be transferred between areas. Most air handling systems have neither the capacity nor the necessary control mechanisms to maintain pressure differentials when doors, passthroughs, or other structures are opened even for brief periods (222).

For years the ventilation rate for animal holding rooms and primary enclosures has been an important focus of many recommendations for research animal facilities. The ventilation rate or air exchange rate in the context of animal facilities refers to the number of times the total volume of air in the room is exhausted and resupplied with appropriately conditioned air per unit of time. This often is expressed in air exchanges per hour. Rules of thumb have been developed based on experience and limited data. These guidelines have suggested the air exchange rates of 10–15 changes per hour are generally satisfactory for animal facilities (45, 222). Unfortunately, they do not take into account the range of possible heat loads, the type of bedding or frequency of cage cleaning; the species and number and size of animals housed; differences in room dimensions; or the efficiency of air distribution linking the secondary and primary enclosures (192, 193, 198, 222, 355). Given these variables, either under- or overventilation can occur, with resulting problems in heat and odor accumulation (58).

Instead of using general guidelines, such as 10–15 air changes per hour, more recent recommendations suggest that the heat generated by animals be calculated using an average total heat gain formula as developed by the American Society of Heating, Refrigerating and Air Conditioning Engineering (6). Using

this, the minimum ventilation required can be calculated from the total cooling load necessary to control heat generated by the animals and other heat sources. This, in turn, will allow determination of ventilation rates.

Additional ventilation capacity may be added as a margin of safety and to address any unusual odor generation (222).

The adequacy with which air is distributed within a room is an important consideration (193). If it is inadequately distributed, heat and water vapor build-up may occur unevenly. The coupling of secondary enclosure to the primary enclosure environments may also vary within the room (354). The use of computational fluid dynamic modeling has revealed that supply and exhaust diffuser placement, as well as type, can have a profound effect on air distribution (136, 137, 269). This has important implications not only for the environment of the animals with the resulting effect on studies, but also for researchers and technicians working in the animal room.

The concept of drafts as they relate to human comfort has been explored (192, 193). Unfortunately, little work has been done with animals to demonstrate whether or not there are biological consequences or if there is discomfort associated with the detection of movement of air. In the case of small laboratory rodents and rabbits, caging designs, and cage placements are unlikely to allow significant air movement within these enclosures to affect the animals contained therein. The one exception may be individually ventilated cages where high air exchange rates can be produced. Even so, there is no evidence to suggest that harmful effects occur. Similarly, larger species kept in pens, runs, or open cages have not been reported to have any ill effects associated with the detection of air movement.

Current guidelines allow the use of recycled air under certain conditions (222). In general, no less than 50% fresh air should be mixed with recycled air for reuse in animal holding areas. Recycled air must be appropriately conditioned and mixed with sufficient fresh air to control temperature, and humidity and other appropriate conditioning should be applied to remove particulates. Mixing recycled air from different animal holding areas is discouraged (222). Recycling of animal room air or the use of appropriately conditioned air from human use areas for animal rooms can provide energy cost savings and be an adjunct to other energy recovery efforts.

Ventilation should not be used as a substitute for good husbandry practices. Adjusting cage densities and bedding, or cage cleaning frequencies may prove a better solution to odor generation problems than trying to increase the capacity of air handling systems. The conditions within animal holding rooms should be regularly monitored to ensure that the ventilation system is working correctly. Plans should be made for action in the event of conditions that may affect the operation of the air handling system such as power failures, failure of mechanical parts, or failure of air filters.

TEMPERATURE AND HUMIDITY

The maintenance of body temperature within certain critical ranges is essential for animals if they are to maintain normal metabolic and physiologic processes (162, 263, 337). Thermal regulation has been extensively studied in some species (110, 111). Clinically observable effects can occur in unadapted animals that have been exposed to temperatures above 85°F (29.4°C) or below 40°F (4.4°C) that are denied access to shelter or other protective mechanisms (110, 162, 265, 343). Depending on the extent and length of time that the animals are exposed to temperatures outside of these ranges, the effects produced can vary from changes in reproductive ability to loss of life. In general, animals are quite adaptable to temperature changes and use behavioral, morphologic, and physiologic mechanisms to maintain body temperature (106, 110, 111, 121, 246, 343). Unfortunately, such adaptation takes time and can affect the animal's performance in toxicologic studies.

For the most common laboratory animals, a dry bulb temperature range from 61°F (16°C) to 84°F (29°C) has been suggested as acceptable (222). Within this range, depending on species, clinical effects are unlikely to occur, and experience has shown that there is little likelihood of toxicologic interaction. Of the common laboratory animals, rabbits are generally kept in a somewhat cooler environment because of their dense hair coat. The recommended temperature range for rabbits is 61°F (16°C) to 72°F (22°C) (222). Close control of temperatures within this recommended range may reduce some variation in certain studies. Unfortunately, very close control of temperature is not always possible within animal facilities, and some fluctuation during the course of any 24-hour period is to be expected. It has also been demonstrated that temperature will stratify within an animal room, especially one containing many cages. Temperature fluctuations of 2°F to 6°F within any given animal room as measured from the floor to the ceiling are not uncommon (26, 27). This is another reason that rotation of cages within any given animal room may be valuable. Variables such as cage construction, population density, the use of filter tops, and animal activity can also cause variations in temperature and humidity between the cage, the room, and between cages (4, 27, 234, 295, 298, 354).

Some means of regularly assessing the temperature within individual animal holding rooms should be used. Most commonly, temperature sensors placed in the return air ducts or mounted at a height of

approximately 5 feet off the floor near the door are used to monitor room temperature.
In addition to temperature, humidity may add additional stress to animals and can affect an animal's ability to efficiently lose heat. Many animals, such as rats,

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mice, dogs, and certain domestic farm animals, cannot sweat and as such cannot take advantage of evaporative cooling (26, 337). These animals must use insensible heat loss through the respiratory track or alterations in blood supply to heat radiating structures, such as the tail, soles of the feet, and tips of the ears to dissipate excess heat or to protect against excessive heat loss (110, 111). Some animals, such as dogs, can increase the frequency of their respiration (panting) to further lose heat. If the humidity in the environment is high, evaporative heat loss through insensible means is decreased and internal temperatures build. At very low humidities there is significant, insensible water loss that must be compensated for in order to maintain adequate fluid balance. Within recommended temperature ranges, however, the effects of relative humidity appear to be minimal. There is some human epidemiological evidence relating low relative humidity to increased susceptibility to certain respiratory diseases (16). Unfortunately, convincing evidence relating humidity to respiratory disease in animals is not available.

In rodents, low relative humidity has been suggested to cause ringtail (an annular notching of the tail that can lead to necrosis) in young rats and mice. Studies conducted on this condition indicate that it occurs at a high environmental temperature (81°F) with a low relative humidity (less than 30°) (93, 235, 314). While possible, these conditions are unlikely to occur under most laboratory animal housing conditions. Moreover, in those studies that have been reported, a variety of other factors, including diet and caging type, can effectively eliminate the condition (93, 235). When the condition is seen, there generally are very few animals affected and often there is no correlation to relative humidity.

The concept of thermal neutrality as a desirable state in which to maintain animals on study has been explored (35, 336). In theory, a temperature or temperature range exists for any given animal species in which oxygen consumption is minimal and no energy is expended to heat or cool the animal in order to maintain a constant body temperature. Ambient temperatures above the range result in increases in metabolism, physiologic alterations, and behavioral changes that favor heat loss. Conversely, temperatures below the range result in adjustments in the same systems designed to produce or conserve heat. Ambient temperatures outside of the thermal neutral zone can result in observable changes, such as alternations in feed consumption, activity, reproduction, and growth. When coupled with relative humidity, climatograms can be constructed in which it is presumed that animals are most comfortable (336). In practice, such optimal conditions are hard to determine for various species of animals, and there is evidence that climatograms may vary significantly with age, reproduction, and other factors (336).

BEDDING

Although many toxicologic studies are conducted on animals housed on suspended wire flooring with no contact bedding, there is increased use of contact bedding for many studies. This is due principally to the increase in bioexclusion housing that uses cage level containment. Even in the case of animals housed on suspended wire flooring, bedding often is placed below the flooring to absorb the moisture from urine and feces. Bedding can influence experimental data and animal well being (41, 239, 253, 254, 312).

Bedding is constructed of natural products, most commonly wood, paper, or corncobs. No bedding is ideal for all occasions and all species, but must be chosen based upon its characteristics. There have been a number of descriptions of the desirable characteristics of different types of bedding, as well as methods for evaluating various bedding types based upon these characteristics (27, 149, 169, 261, 309, 335). Of all the characteristics of bedding, absorbency would seem to be the most important. It is the purpose of bedding to absorb moisture to keep the environment dry and thereby minimize the growth of microorganisms that would otherwise flourish on the organic material and moisture within the cage. Moreover, by diluting feces and urine with bedding, the animals within the cage have less contact with excreta and hence have less chance of being soiled by it.

Wood products, although probably the most common form of bedding, are not the most absorbent, with their absorbency being directly related to their total surface area. The finer the wood product bedding, the more surface area and hence the more moisture it can retain. Paper product bedding, unlike wood product, tends to absorb large amounts of water directly into the material, which cause it to swell. The absorbency of paper product bedding can be much greater than wood product bedding. Unfortunately, some forms of paper product bedding lose their structural integrity when wet, making them difficult to manipulate, which can be a problem if used under suspended catch pans. To address this, some types of paper product bedding have a thin plastic backing, which makes them easier to remove from catch pans.

Another important factor with respect to bedding is its potential for introducing contaminants. Wood product bedding is obtained as a waste product from the milling of lumber. Wood chip bedding is

composed of the small wood chips produced by sawing lumber with coarse saws. Prior to processing, it may contain a range of chip sizes, including very coarse and very fine materials, such as saw dust. Wood from which bedding is produced may come from a variety of locations with different histories of exposure to compounds and microorganisms. Raw wood chips are processed by first screening out large debris and then heating them to a temperature of 140°F

to 150°F in an air convection furnace for approximately 20 minutes. This dries the product, which reduces bacterial contamination and the potential for growth, and also drives off volatile oils. The wood chip bedding is then run through a series of screens to develop products of varying sizes. After being screened, it is placed in bags and made available for animal housing.

Wood shavings are the product of planing kiln-dried lumber. Lumber is dried in large ovens maintained at a temperature of 140°F for periods of up to 14 days. The lumber is then run through shaping planes that produce shavings. These shavings are then bagged and used for bedding.

Both hardwood and softwood bedding are available as processed products. Storage conditions for these products are critical to preventing contamination. Certain softwoods, such as cedar, are no longer used for bedding for laboratory animals, with their use being relegated primarily to bedding for pets. Studies using softwood bedding have shown that volatile oils in them have the ability to induce liver microsomal enzymes, which can alter drug metabolism (56, 86, 320, 321, 322, 324, 334) and increase the incidence of neoplasia (146, 323). Softwood bedding that is kiln dried and hardwood bedding are both much less likely to produce hepatic microsomal effects, as essential oils associated with these inductive effects are driven off in the drying process. Hardwood beddings have not been shown to induce liver microsomal enzymes but may have other effects (309). If autoclaving or other heat treatments are applied to the bedding after receipt, the chance of essential oils producing any inductive effect are even further reduced (70, 260). In general, any such induction takes 3 or more days to occur and is rapidly lost when animals are removed from bedding materials, such as unprocessed cedar.

Paper bedding can come in a variety of shapes and sizes. Flat cardboard-like sheets, as well as plastic-backed absorbent pads, are commonly used in bedding trays under suspended wire cages. Paper chips and shredded paper products that may be bleached or unbleached provide a highly absorbent, contaminant-free contact bedding. Recently, compressed pads of paper that are cut to the size of individual cages have been introduced. These materials can come presterilized and are inserted into the cage at changing. When placed in the cages bedded with this material, animals scratch up the paper product into a loose bedding that is highly absorbent. This activity by the animals is considered by some to be a form of psychological enrichment. Similarly, the ability to burrow in contact bedding is also considered to be beneficial to some rodents.

Corn cob bedding is produced from coarsely ground corn cobs that have been heat treated. Their absorbency is similar to wood product bedding. The bedding itself is of irregular shape, is not easily compressed, and retains its structure when wet. There has been little indication that this bedding can cause significant research interactions; however, it is not widely used (259).

Larger animals, such as swine, dogs, cats, and other species, may also benefit from the use of contact bedding. In general, the use of such bedding is designed primarily to aid in cleaning of the cages, however, in the case of swine, it may assist in providing for their natural routing behavior.

A number of problems have been associated with varying types of bedding. Nude mice and SKH-1 mice that do not have eyelashes have a tendency to collect debris under their eyelids. This effect is less noticeable with screened wood product bedding; however, paper product bedding appears to release many more fines that are capable of lodging under the eyelids and causing swelling, and in the presence of the right microorganism, produce abscesses. Corn cob bedding seems to be similar to wood product bedding in this regard.

The ingestion of bedding can be problematic for newborn animals. If the particles are too small or do not easily pass the GI tract, newborn animals can ingest the bedding and develop fatal gastrointestinal tract obstructions.

It is important to periodically assess bedding materials for the presence of toxic and carcinogenic materials. Whereas some of these materials may be driven off during the processing of the bedding, others may be more stable.

Bedding, especially contact bedding, needs to be changed frequently based on a variety of factors, including the wetness of the bedding, the amount of feces present, the number of animals in the cage, and a variety of other factors. In the case of large animals or group-housed animals, bedding change often is required several times a week. Smaller animals or individuallyhoused animals may require less frequent bedding change. Failure to change the bedding and replace it with new bedding can result not only in an unhealthful environment, but can also increase the concentration of materials excreted in the feces and urine within the animal's environment, making ingestion or inhalation much more likely. There is also evidence that a soiled environment may also affect liver metabolism, which could interfere with toxicologic research (322).

WATER

Access to a sufficient quantity of clean, potable water is essential for normal hydration of the animal and for the maintenance of normal physiologic and metabolic processes. Water consumption and drinking patterns of laboratory animals vary with the species (311). Water supplied to toxicologic laboratories may be either from municipal sources or from privately owned wells. In either

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case, the potential exists for contaminants to be introduced into animal holding facilities or for significant variation to occur in water quality over time.

To address this problem, animal facility drinking water, as well as water used for other husbandry tasks, is treated in order to adjust chemical and biologic characteristics. Water treatments may include one or more of the following: filtration, ion exchange, ultraviolet (UV) disinfection, halogenation (chlorination or iodination), reverse osmosis, or ozonization. At the local or room level, water may be acidified using mineral acids. Acidification usually is reserved for treatment of water used in water bottles although it is occasionally used in automatic drinking water systems.

Water filtration can be done using either depth or membrane filters. Depth filters are used for coarse filtration and involve passage of water through a coarse medium, such as sand. Finer filtration, down to 0.1 μm, can be obtained using a series of membrane filters often constructed out of paper or synthetic materials. These filters are only reliable for removing nonviable particulates, as many bacteria that collect on such filters can grow through them. If membrane filters are to be used for bacterial filtration, some regular means of disinfecting the filters must be employed. Some chemical components in water, such as suspended gases and certain organic compounds, can be removed by charcoal filters. These filters consist of canisters of activated charcoal that have varying degrees of retentivity for different compounds. These filters must be changed regularly and suffer from similar problems with respect to microbiological growth as membrane filters. Ion exchange resins can be used to remove inorganic compounds but offer no biological treatment.

Often, reverse osmosis is applied to animal water supplies. This treatment involves placing water under pressure on one side of a membrane that is designed to exclude all but a few types of molecules and collecting the water that passes through the membrane in an area of lower pressure on the opposite side of the membrane. To be effective in animal facilities, the capacity of the reverse osmosis equipment must be large enough to accommodate the necessary water usage. This entails either many such units or very large units. Water produced by reverse osmosis is devoid of all minerals and hence is chemically very aggressive to surfaces. Over time, water treated by reserve osmosis can recapture metal from stainless steel surfaces and other materials. Because the reverse osmosis process depends on an intact membrane, absolute bacteriologic or viral exclusion is only possible if there are no deficits in the membrane that might allow untreated water to pass through. Although water produced by reverse osmosis, in theory, should be free of microorganisms, in practice this is not always the case.

UV disinfection is a common and rapid means of killing a variety of microorganisms that might be found in water supplies. Because the process involves the exposure of water to UV light, the water must be reasonably free of minerals so that deposits do not form inside the exposure chambers. Moreover, light intensities of UV-producing fixtures decrease over time, and hence the effectiveness of the process. For this reason, regular measurements of the irradiance level of the light source, as well as regular replacement of the light source, must be done.

Halogenation, most commonly chlorination, is a relatively simple but effective means of disinfecting water supplies (133). Chlorine is effective against a wide range of microorganisms (21). Depending on the chlorine species being measured, ranges of effective concentrations have been established that will not adversely affect animals but still accomplished disinfection. It is important to realize that a certain minimum contact time is required for the chlorine or any chemical disinfectant to be effective. For this reason, chlorination usually is done centrally with the treated water being held in a contact tank for a period of several minutes to several hours in order to ensure adequate disinfection.

Depending on the form of chlorine being used, the chlorine reacts in variable fashion with organic materials present in the water, causing the generation of halogenated organic compounds (225). The importance and the likelihood of such occurrence is difficult to assess. It should be noted, however, that chlorine-based compounds are the most commonly used disinfectants for water and for husbandry practices in animal facilities, as well as for the treatment of drinking water for humans. The use of chlorine to disinfect drinking water has an important advantage over filtration and reverse osmosis methods in that it provides residual disinfection within watering systems. This prevents build up of bacteria in the watering systems as the result of back flow into water bottles or automatic watering devices of saliva and feed debris during drinking.

Ozonization is another means of assuring microbiological decontamination of water supplies and the destruction of dissolved organic compounds in the water. Such systems use an ozone generator to produce ozone gas and bubble it into a water contact tank. Contact periods of less than 90 seconds are required in order to adequately sterilize water. The resulting water is highly aggressive to pipes and surfaces, requiring that the ozone be broken down into less aggressive oxygen-containing compounds.

This is done either by exposure to UV light or by the addition of chlorine-containing compounds (sometimes both). Water that has been ozonated is devoid of any residual disinfection capacity and hence must be treated with halogen-containing compounds (e.g.,

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chlorine) in order to have residual disinfection capabilities.

Acidification of drinking water was first used to control the growth of pseudomonas in the drinking water in water bottles used for mice that had been irradiated (94, 184, 187). Water of a low pH (acidic) is bacteriostatic to certain vegetative forms of some bacteria. To be most effective, acidification should be at pH 2.3 or lower (127). Usually this is done with mineral acids; however, such acids have little buffering capacity, and hence the addition of saliva or other alkaline containing compounds may rapidly increase the pH of the drinking water.

The use of acidification, as well as other forms of water treatment, are not without potential research effects (91, 119). Any water treatment should be considered an experimental variable and should be well-understood by the toxicologist (58, 127). Usually it is necessary to group together a number of different water treatments in order to provide a water system that is capable of not only adjusting the chemical composition of the water, but also to ensure that it is free from harmful microorganisms.

FEED

Feed is considered by some to be one of the principal uncontrolled variables in toxicology today. Many textbooks and articles, as well as guidelines, have been written on nutrition for laboratory animals (209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 220, 221, 230, 226). There are numerous examples of how diet composition or administration can affect the outcome of toxicological research either directly or indirectly (30, 57, 144, 227, 232, 237, 292, 340). There also are examples of individual nutrients either in excess or deficiency producing a wide range of metabolic, morphologic, or physiologic conditions that have influenced the outcome of studies (8, 44, 229, 231, 330). It is not the purpose of this review to cover all of these. The reader is encouraged to pursue such information from available texts, reports, and journal articles, as well as seek the advice of those skilled in laboratory animal nutrition for specific recommendations. Subcommittees of the National Research Council's Committee on Animal Nutrition have published reviews of the nutrient requirements of laboratory animals that can be used as a guide in assessing the adequacy of specific formulations (209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 220, 221).

Feed provided to laboratory animals should be palatable, nutritionally complete, and free of contaminants unless the lowering or removal of a nutrient or the addition of other compounds is required by the study design. The feed should be provided in sufficient quantity and be of the necessary quality to assure normal growth and provide for the overall health of the animal (46, 222). Increased demands of reproduction and lactation may require slightly different feed formulations. Many commercially available diets are calorie dense, protein rich, and often are fed ad libitum. Such practices encourage feed wastage and may adversely affect some toxicology studies (159).

The physical and taste characteristics of laboratory animal feed can be important variables in study design. Some common laboratory animal species, such as nonhuman primates, guinea pigs, and rabbits, have well-developed taste and dietary consistency preferences. Guinea pigs, for example, do not easily accept changes in the texture of the diet or in its taste. These animals may need to be habituated to changes in diet by gradual introduction of the new diet during the course of feeding a previously accepted diet. This can prove challenging when artificial diets need to be substituted for natural ingredient diets.

Laboratory animal feeds can be classified as natural ingredient diets, semipurified (or purified) diets, or chemically defined diets (273). These classifications are based on the ingredients used to construct the feed. Natural ingredient diets are composed of unprocessed or crudely processed materials, such as cereal grains, milk products, and animal product meals, such as fishmeal or meatmeal. Semi-purified diets (also referred to as synthetic, semi-synthetic, and purified) are constructed of processed ingredients that are refined so as to be uniform in content. Examples of such ingredients include casein, dextran, soy protein, hemicellulose, and fats, such as corn oil. Chemically defined diets are composed of chemically pure compounds, including specific amino acids, fatty acids, inorganic salts, vitamins, and sugars, such as glucose and fructose (351).

Natural ingredient diets can be pelletized (extruded), expanded (baked), or processed as a meal (powdered). They can also be suspended in agar or other types of hydrocolloids, providing both feed and a source of water. Semi-purified and chemically defined diets are provided in either a powdered or liquid form. Experimental compounds can be incorporated in any of these physical forms, but as a rule are usually fed as a gel or in liquid or powdered form.

The exact formulation of specific feeds may or may not be available to the toxicologist. Open-formula diets are diets that are manufactured to published specifications based on quantities of specific ingredients (165, 166). The quantitative and often qualitative ingredient compositions of open-formula

diets are readily available from the manufacturer. Open-formula diets usually are made from natural ingredients, and as such may easily suffer from variability due to differences in source of ingredient supply, harvest times, and storage conditions (234). Detailed ingredient analysis prior to manufacture in both open- and closed-formula diets is seldom done, and when

analysis is conducted, it usually is done for only a few key constituents. For this reason, open-formula diets can still exhibit significant variability between suppliers and within any given supplier.
Closed-formula diets are those manufactured by feed companies who hold the exact composition of the diet in terms of the quantities of specific ingredients as proprietary information. They will provide a list of the ingredients used and usually will provide a calculated analysis. Closed-formula diets are often less costly than open-formula diets, as the manufacturer can adjust the formula periodically, based on the availability and costs of various ingredients. It must be remembered that like open-formula diets, closed-formula diets may have variable nutrient levels. The calculated analysis on such products is not the same as a laboratory analysis of an individual batch of closed-formula diet.
Recently, the concept of constant nutrition or variable-ingredient diets has been introduced. This is a natural extension of closed-formula diets in that the manufacturer is allowed to select the type and quantity of various natural product ingredients to be used in a diet formulation based on availability and cost. The maximum percentage that any given ingredient can constitute in the diet is limited by published specifications; however, the final product must have analyzed nutrient levels that fall within prescribed ranges. Generally, constant nutrition diets are coupled with regular analysis by the manufacturer of the product for an extensive list of nutrients in order to assure that the product provides the specified levels of such nutrients as set forth in the diet specifications. Given the inherent variability with both open- and closed-formula diets, as well as the lack of regular laboratory analysis for specific nutrients in such diets, constant nutrition diets provide a means for standardizing diets between locations as well as suppliers.
Certain basic analyses are required for interstate shipment of dietary ingredients in the United States. Analyses for up to five constituents, including protein, are made, and certain minimal levels based on the guaranteed analysis stated on the feed container must be met (17). It is important to realize that the actual level of these nutrients is not guaranteed, only that the level is not less than that listed. Similarly, diets can be certified with respect to levels of certain toxic and carcinogenic compounds. This process involves holding in a secured location a batch of feed from which samples are taken for analysis of 15 or more toxic or carcinogenic compounds. The analysis for that particular lot of feed is used to certify the diet as containing specific levels of these compounds which may interfere with some toxicologic research (80, 100, 227, 230, 296, 350). Certification of feed does not imply a laboratory analysis for specific nutrients, only that the material does not contain more than a stated level of certain compounds as determined by the analyses. Hence, a certified diet could be deficient in nutrients and yet still be certified (57, 117, 228, 267, 352, 353).
For convenience in feeding and to minimize wastage, diets often are pelletized (97). During this process, the diets are briefly exposed to temperatures exceeding 60°C. The process of pelletizing diets is one of compression and extrusion. The amount of time that the pellet is exposed to elevated temperatures is very brief, and hence there is no assurance that vegetative or nonnegative forms of bacteria or other microorganisms are consistently killed.
Expanded diets undergo a process of prolonged mixing and heating with added liquid in a manner similar to mixing a cake batter. This material is mixed with air and expanded through a die into pellets that are dried with heated air. This process forms a much harder and durable product. The process also exposes the mixture to temperatures in excess of 100°C for periods of 15 minutes or more. This causes some reduction in microorganisms; however, the product itself is not sterile. Contamination can occur in both pelletized and expanded diets after processing. Expanded diets have a lower specific gravity, and hence a lower caloric density than pelletized or powered diets of equal volume. Hence, to obtain the same caloric intake, animals will consume greater volumes of an expanded diet.
A number of applications in toxicological research require the decontamination or sterilization of feed (204). If true reductions in bacteria and viruses are to be achieved in feed products, feed can be treated by application of steam under pressure (autoclaving), dry heat, or irradiation. Heat applied in these processes can destroy certain labile ingredients within the diets (62, 96, 242). Irradiation produces very little elevation in temperature and hence is less likely to require additional fortification if sterilization is contemplated (66, 356).
Any process used to sterilize feed must be calibrated and carefully controlled. Minor items, such as variation in stacking patterns of bags of feed within an autoclave or the magnitude and number of vacuum pulses, can yield significant differences in the total reduction of microorganisms during the autoclaving process (222). Equipment used for such feed treatment must be regularly calibrated. The use of indictor tapes or other sterilization indicators in a single spot does not assure the adequacy of the process. Moreover, cycles designed for pelletized feed seldom are effective in processing powdered feed

or other forms of diet.

When irradiation is used, it is important to specify dosages based on the minimum dosage received throughout the entire load or container. Once an item has been treated, it should be labeled as to the date of treatment so that judgments can be made as to the appro

priate time in which the product should be used. Feed should be sealed in durable and preferably water-resistant packaging prior to irradiation.

With or without heat or radiation treatment, certain liable components within diets will degrade over time (102, 242). Diet manufacturers place milling dates on containers of diet using either sequential dating codes or Julian dating. As a general rule, natural ingredient, dry laboratory-animal diets that have been stored properly can be used for 180 days after manufacture (222). Some diets that contain supplemental vitamin C or other very labile ingredients should be used in approximately 3 months from milling unless special stabilized forms of these especially labile nutrients are added to extend the shelf life (222). Supplemental vitamins and other labile ingredients can be provided separately through the use of drinking water supplements, sprayed on dietary additives, or in the case of nonhuman primates and certain other species, by the feeding of fruits and vegetables.

Feeding can also be used as part of a psychological enrichment program for certain species of animals. In the case of nonhuman primates, specialized feeders, the use of a variety of different types of food items, and the presentation of feed in a way that foraging is required can all be used to stimulate these animals and reinforce natural behaviors. For the purposes of toxicologic research, maintaining constancy in a diet is important. Varying the diet with a wide selection of unbalanced foods, as might occur through nonstructured supplementation of diets, can cause health-related problems (195). It is also important to minimize abrupt changes in diet because in many species these can lead to digestive and metabolic disturbances.

The storage of feed and other dietary ingredients should be done in areas that are regularly cleaned and enclosed to prevent entry of pests. In general, feed should be stored off of the floor and care should be taken to prevent exposure of these items to temperatures above 70°F for prolonged periods of time (102, 228, 272). Containers used to hold loose feed should be covered and constructed of materials that prevent the entrance of vermin and other pests.

The presentation of feed to animals is another important variable. With few exceptions, feed should be administered to animals in feeders that allow easy access to the feed while minimizing the possibility of contamination with feces or urine. Feeders should also minimize wastage, which is particularly important with powdered diets. The size and number of openings in feeders, as well as the location of the feeder, can affect the animals' consumption of feed. Some designs of feeders can actually restrict an animal's access to the feed, resulting in caloric restriction.

Dogs, cats, nonhuman primates, and certain other species are commonly "meal" fed such that feed is pro vided one to three times a day in limited quantities (178). Rodents are more commonly fed *ad libitum*. Feed consumption can be influenced by the light cycle, with some species preferring to consume more feed in the dark part of the light cycle than others. Rodents consume feed both in the light and dark portions of the light cycle, with more feed being consumed during the dark part of the cycle than the light. Poultry consume feed primarily during the light cycle, as do many other nonrodent species. Hamsters characteristically horde feed, and for that reason will tend to remove feed from feeders in order to form feed piles on the floor. To minimize injury associated with this behavior, hamsters are commonly fed off of the floor when pelletized diets are used (122). The ready availability of a water source is also important in feeding behavior for many animals. Many species eat and drink at the same time.

Of all the variables associated with feed, perhaps that which has the greatest impact on toxicologic studies, other than outright nutritional deficiency, is overfeeding (159). Animals will consume feed until an internal caloric limit is reached. The imposition of mild to moderate caloric restriction in all species, including rodents, can increase longevity, postpone the development of spontaneously occurring neoplasms, decrease the incidence of background lesions in the kidneys, cardiovascular, and endocrine systems, and cause other changes that in aggregate can produce a more consistent model for toxicologic research (3, 23, 24, 52, 53, 183, 319). To adequately interpret data from long-term studies on animals using caloric restriction, other primary studies, including range-finding studies for dosages, need to be conducted in calorically restricted animals.

Caloric restriction or limited feeding is not a new concept (225, 226, 227, 274, 302, 303, 304, 305, 306, 317). The effects produced by this procedure have been recognized for decades (63, 150, 182). A number of publications have reviewed various segments of this relatively large body of literature (123, 124, 156, 157, 158, 357). The phenomena appears to be closely correlated with total calories and not with specific nutrient concentrations, such as protein levels (170). The technique appears to be most easily applied to those studies in which compounds are administered by gavage; however, there is little reason to believe that it could not be applied to studies in which the compound was administered by

other routes. Errors associated with administering compounds incorporated into the diet and administered under a caloric restriction regimen may not pose any greater error than those associated with similar administration using *ad lib* feeding, which assumes a consistent intake on a daily basis and a consistent level of wastage.

The usefulness of caloric restriction in group-housed animals, however, still needs to be explored (55). It has been assumed that any dominance hierarchy estab

lished within a group would lead to significant differences in diet consumption between members of the group with, perhaps, the introduction of unwanted variation, however, any such dominance hierarchy that already exists within the group will still result in variation even when feed is available *ad libitum*. This problem could be further magnified if one or more members of the group died during the course of a study. The interaction of group size, sex, and caloric restriction still needs additional investigation.

The decision of the toxicologist to use caloric restriction as a means of decreasing the variability encountered in product registration studies using rodents must be based on a careful examination of the literature and a dialogue with regulatory authorities. Caloric restriction in rodents is used by a growing number of pharmaceutical companies and has gained acceptance by regulatory bodies, provided that adequate control data are provided.

HEALTH AND HEALTH MONITORING

Animals used in toxicologic research come from several different categories of sources. A limited number of animals are occasionally obtained from either wild populations or open colonies in which there is little attempt to control the introduction of adventitious organisms. Such conventional or random source animals have the potential for significant interindividual variability with respect to health status.

A second-source classification are closed colonies of animals in which the introduction of new animals is minimized or eliminated and certain control measures are put in place either by means of vaccination, antimicrobial therapy, or anthelminthic treatment to control and, in some cases, eliminate a limited number of microorganisms. Interindividual variability in health profiles may still exist; however, the maintenance of these control measures at the toxicology laboratory can provide some assurance that certain organisms have been eliminated.

The last source classification of animals are animals from colonies that have originated through procedures designed to totally eliminate unwanted microorganisms and parasites, usually caesarean section or embryo transfer. These animals are maintained within bioexclusion systems, such as barrier production facilities, that are designed to prevent contamination with species-specific pathogens and, in the case of some bioexclusion systems, even common opportunistic organisms that could pose unwanted research interference (315).

The availability of any species in any one of these source categories may be limited or even nonexistent. Larger domestic animals, such as dogs, cats, and farm animals, and nondomestic animals, such as nonhuman primates, generally are not available in a highly defined microbiological status. Health programs designed to control or improve the health status of these animals will not eliminate the possibility of species-specific or opportunistic disease that may interfere with toxicologic research using these animals.

Health monitoring programs for nonbarriermaintained or random source animals depend heavily upon a comprehensive initial quarantine and screening process that is supplemented by individual and/or colony health data available from the supplier (84, 85). Some organisms that are seldom found in barrier-reared animals are not uncommon in many research facilities (147). Routine vaccination and anthelmintic therapy, as well as the application of antimicrobial agents, may all be used to control or eliminate undesirable organisms. The toxicologist must be aware that during the course of a study, individual animals may develop clinical illness or may exhibit individual variation as a result of underlining infectious processes. The frequency of such occurrences cannot be predicted, and allowances must be made for this in the study design when using such animals.

To minimize the impact of health issues on toxicologic research, each institution must develop an institutional philosophy with respect to the impact of certain organisms in their research animals. Not all organisms have a significant impact on research, and those that do may not interfere with certain types of research. Simplistically, organisms can be divided in terms of their relative importance and the amount of peer-reviewed information available to support their impact on research (Figure 16.1).

A number of reviews of microorganisms affecting laboratory animals have been assembled (29, 205, 206, 120, 247). Unfortunately there is no universally accepted list of organisms that should be excluded from all animals under all circumstances. Many adventitious organisms do not cause clinical disease. Instead they cause subclinical infections that do not produce histologic changes associated with their presence. In some cases they may have only limited or subtle research effects that are self-limiting and cease after protective antibodies are formed (344). In a few instances more significant effects occur only when the organism is first introduced into a naïve population (epizootic phase of infection). After the organism becomes established and protective antibodies are present either through maternal transfer or the development of a controlled infection, clinical signs or research effects may disappear. Often, agents that are commonly recommended to be screened for have been selected on a historical

basis rather than through an analysis of their potential to cause effects or the prevalence of the organism.

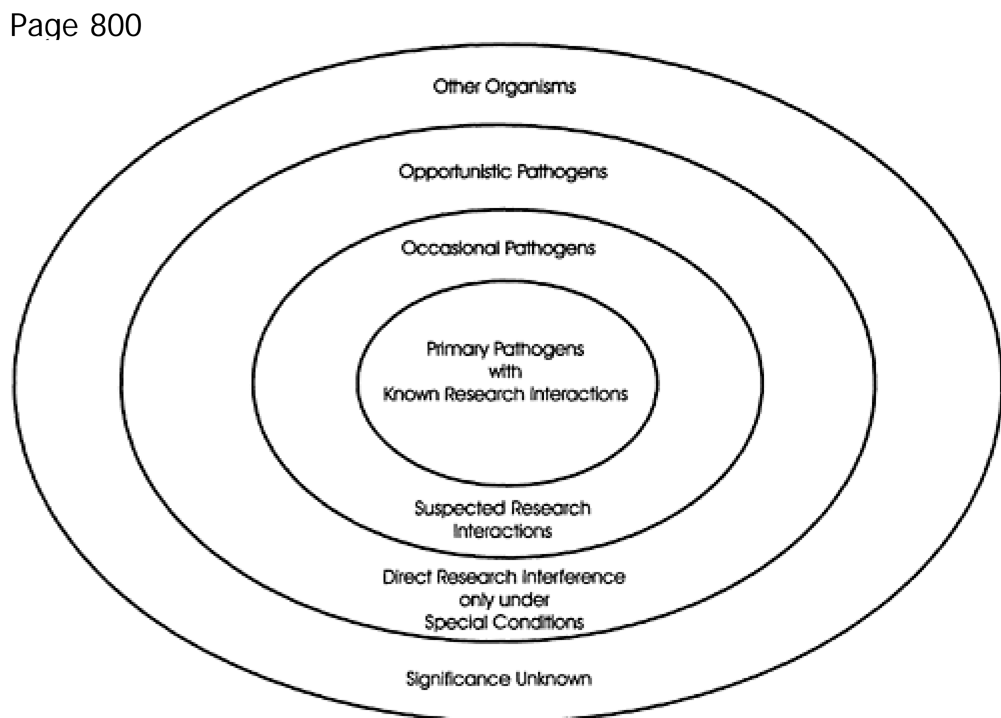

FIG. 16.1. Classification of organisms by their significance and impact on research.

Suppliers of barrier-produced animals have developed exclusionary lists of organisms that define the status of the animals that they produce. These exclusionary lists set forth microorganisms that are to be screened for on a regular basis and whose presence is deemed to be a cause for the supplier to eliminate that colony of animals from production. In some cases, an additional group of opportunistic microorganisms may be screened for by suppliers to document the presence of these organisms in the event that they might interfere with certain types of research. These opportunistic organisms may have little potential to cause research interaction and are not a cause to eliminate colonies. These organisms may be common human or environmental commensals that can only be excluded by very specialized bioexclusion housing techniques that may be impractical in the toxicologic research environment.

Animals free of one or more specified microorganisms are termed *specific pathogen free* (SPF) (206). There is no universal definition of SPF with respect to what organisms are to be excluded from each common laboratory species. Proprietary terms or abbreviations may be used to designate a particular supplier's or research institution's definition of SPF.

In developing an institutional health standard for laboratory animals to be used in toxicologic research, the advice of a competent veterinary professional with experience in laboratory animal medicine science should be sought. Decisions on which organisms to exclude and to test for will need to be based on the nature of the research being conducted, species being utilized, a realistic assessment of the facilities and equipment available for maintaining animals, and a thorough review of peer-reviewed literature with respect to the disease-causing potential, host range, and research interactions posed by specific microorganisms. Table 16.2 lists a number of organisms that may infect common laboratory animal species. This table includes species-specific organisms and some common environmental and human commensals whose exclusion might require extraordinary efforts although their presence may have little research impact; however, such opportunistic organisms may be considered for inclusion in the screening program if their presence would need to be documented in order to help

Table 16.2 Organisms capable of infecting laboratory rodentsa

	Mice	Rats	Guinea Pigs	Hamsters
Sendai virus	X	X	Xb	X
Pneumonia virus of mice	X	X	X	X
Mouse hepatitis virus	X			
Minute virus of mice	X			
GD-VIIc	X			
Reo-3 virus	X	X	X	X
Epizootic diarrhea of infant mice	X			
Lymphocytic chroiomeningitis virus	X	X	X	X
Polyoma virus	X			
Mouse cytomegalovirus	X			
Ectromelia virus	X			
Mouse parvovirus	X			
K virus	X			
Mouse thymic virus	X			
Hanta viruses	X	X		
Sialodacryoadenitis virus		X		
Rat coronavirus		X		
Rat parvovirus		X		
Kilham rat virus		X		
H-1 virus		X		
Mouse adenovirus	X	X		
Guinea pig adenovirus			X	
Cilia associated respiratory bacillus	X	X		
Bordetella bronchiseptica	X	X	X	X
Citrobacter Freundii 4248	X			
Corynebacterium kutscheri	X	X	X	X
Salmonella spp.	X	X	X	X
Mycoplasma pulmonis	X	X	X	X
Mycoplasma spp.		X		
Streptobacillus moniliformis	X	X		
Helicobacter hepaticus	X	X		
Helicobacter bilis	X	X		
Helicobacter spp.	X	X	X	X
Klebsiella oxytoca	X	X	X	X
Klebsiella pneumoniae	X	X	X	X
Pasteurella pneumotropica	X	X	X	X
Pasteurella multocida	X	X	X	X
Pasteurella spp.	X	X	X	X
Pseudomonas aeruginosa	X	X	X	X
Pseudomonas spp.	X	X	X	X
Staphylococcus aureus	X	X	X	X
Streptococcus pneumoniae	X	X	X	X
β Hemolytic Streptococcus spp.	X	X	X	X
Streptococcus spp.	X	X	X	X
Streptococcus zooepidemicus			X	
Encephalitozooan cuniculi	X	X	X	X

a Infection does not imply disease or interference with research results.
b Also referred to as Theiler's Murine Encephalorayelitis Virus
c Probably is not really infected with this virus; can acquire other Parainfluenza viruses that cross-react serologically.

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explain unexpected variation in certain specialized studies (84). It is unrealistic for most toxicologic research facilities, however, to set as a goal the exclusion of all the organisms on this list, as the likelihood of long-term success, unless extraordinary measures are used, is quite low (344).

No matter what institutional exclusionary list is decided upon, each organism on the list should have documentation in the peer-reviewed literature to support its inclusion. Moreover, in developing an exclusionary list, consideration should be given to the formulation of a plan to address the potential discovery of positive findings in research populations within the institution. Development of an exclusionary list presumes that some method is available to limit the spread of the organism if it gains entrance into the facility and that some course of action, such as elimination of infected animals or the imposition of some form of control measures, such as drug therapy, will be used to eliminate it (344). Because the toxicologist will be impacted by the health and health monitoring program at the institution, it is important that he/she participate in the formulation of that program.

There are a number of microorganisms that can be carried by animals that have health implications for man. Zoonotic organisms that can be transmitted from animals to man are often included in the screening and control processes that are part of the health program for laboratory animals. Many of these organisms, such as herpes B virus (nonhuman primates), *Salmonella, Shigella,* Hantaan virus, and Lymphocytic Choriomeningitis virus, may be included in screening programs not because of their possible research effects, but because of their ability to cause disease in people who work with the animals (101, 138, 289).

Health monitoring programs require consideration of sample size and frequency; the type, availability, sensitivity, and specificity of assays for various microorganisms; biological characteristics of the microorganisms; sampling procedures and preparation; and the interpretation of results. Discussions of these subjects are beyond the scope of this review but are available elsewhere (10, 75, 84, 85, 176, 293, 325, 342). In light of all of these considerations, as well as the limitations of the health monitoring techniques currently available, it is imperative that no single set of results be used to make a determination of the health status of any group of animals used in toxicologic research. Any positive findings should be confirmed with alternative tests or additional samples. False-positive and false-negative results do, in fact, occur, and drastic actions taken without confirmatory data can cause irreparable damage to ongoing studies (342).

Special problems are imposed when immunocompromised animals are used in pharmacological research. The very property that makes these animals unique (i.e., defects in their immune system) makes them susceptible to many organisms that would not be of concern to animals with an intact immune system. Manipulation of these animals requires the use of aseptic technique and rigorous attention to detail to assure that they are not exposed to even common opportunistic organisms that could set up life-threatening infections or cause research interactions. Health monitoring programs for these animals often require the use of immunocompetent sentinel animals that are housed either on soiled bedding obtained from the immunocompromised animals or in direct contact with the animals themselves (73, 310, 342). Such animals must be obtained from suppliers in a microbiologically defined status that limits the microorganisms to which the animals are exposed to a small number of commensals that have no disease-causing capacity or research implications. Maintenance of these animals requires very special bioexclusion housing practices and modification of research techniques.

The collection of background data can be incorporated into the health monitoring program. In addition to documenting the presence or absence of various microorganisms, samples may also be collected for histopathology, clinical chemistry, and hematology using representative animals obtained from the same populations as the animals participating in the study.

In some instances it may be necessary to eliminate one or more microorganisms from a group of animals in order to make it acceptable for introduction into a toxicologic research facility if alternatives to the use of these animals are not available. This process is referred to as rederivation and can be accomplished either by caesarean section with cross-fostering of young onto lactating females of the correct microbiological status or by hand rearing the young using the appropriate milk substitute. As an alternative, embryos collected from donor females can be transferred, after washing, to recipient mothers of the appropriate health status. Both of these techniques require considerable technical skill and extensive health monitoring after the procedures to assure that the appropriate health status of the rederived animals has been obtained. Each technique has advantages and disadvantages. Both of these procedures are expensive and time consuming, especially if they are to be applied to outbred animals that require relatively large founding populations.

In the case of a few viral agents, cessation of breeding and the elimination of naive individuals for a

period of 6 weeks or more, coupled with environmental disinfection, has been shown to eliminate certain viruses from infected populations (342). With some bacteria and parasites, medications can be used but usually are only practical when applied to small numbers of animals, as such therapies are seldom 100% effective.

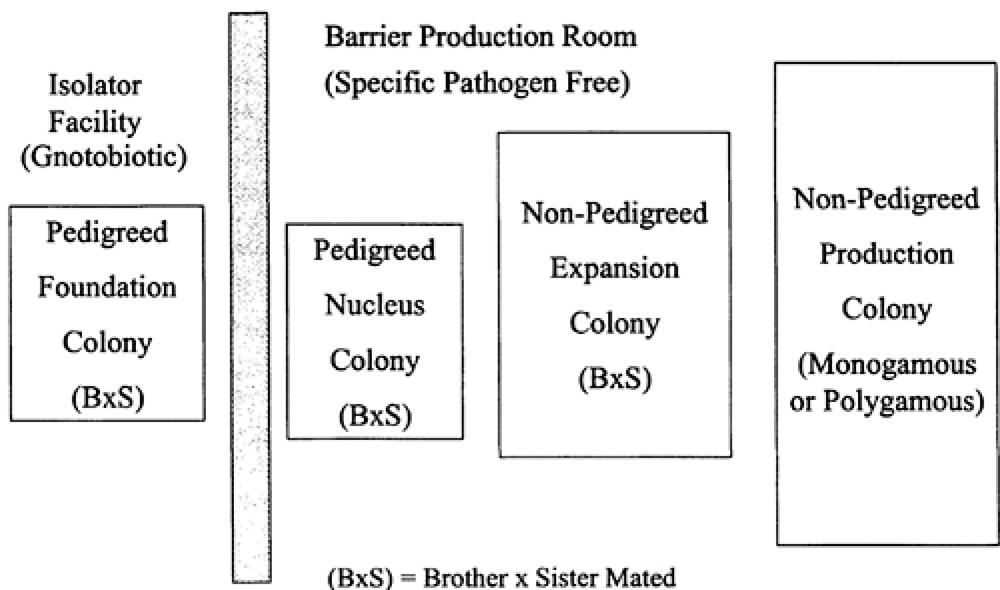

FIG. 16.2. Schematic representation of the segments of an inbred production colony.

GENETICS AND GENETIC MONITORING

Laboratory animals need to be appropriately identified in terms of nomenclature and genetics if studies are to be confirmed either at the same institution or at other institutions. Laboratory animals may be referred to by their common names as well as their scientific names for the purposes of description. In the case of laboratory rodents, specific rules of nomenclature exist for stock and strain designations that cover the genetic classification of the animal and identify its origins and its methods of derivation (87, 90, 142, 143, 207, 208). Listing of various stocks and strains, as well as other genetic classifications of animals, are periodically published (88,89). The correct designation of the animals used should be included in any reports or publications as well as other information regarding the source of supply.

The majority of rodents used in toxicologic research fall into three general genetic classifications: outbred/ random bred, inbred, and F1 hybrid. Transgenic animals can be produced on any of these backgrounds and represent a rapidly growing segment of rodents used in toxicologic research.

Inbred animals are produced as the result of 20 generations or more of brother-sister mating and possess more than 98% genetic homozygosity. They are produced by most breeders by means of a colony structure that allows breeding of large numbers of these animals that are, at most, separated from a brother-sister mating by no more than one generation (Figure 16.2). A gnotobiotic foundation colony is maintained in isolators or other bioexclusion systems and is periodically used to replace the pedigreed nucleus colony of animals within the production facility. The nucleus colony animals are brother-sister mated with pedigrees being maintained. The progeny from these matings form nonpedigreed brother-sister matings in an expansion colony that pro duces breeders for either a pair-mated or polygamous-mated production colony, the offspring of which are used in research.

Inbred animals are useful in toxicologic research in which genetic homogeneity is required. Inbred strains may differ significantly in their response to various compounds due to variations in their genotype and phenotype. They are particularly useful when studying pharmacological mechanisms. Conclusions drawn using a single inbred strain should be verified using other inbred strains or outbred stocks if the findings are to be applied to more genetically diverse populations, such as domestic animals or man.

F1 hybrids are produced from the mating of two inbred strains. Offspring are genetically identical at all alleles at

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which the parental strains differ. F1 hybrids are not self-perpetuating in that breeding F1 hybrids to each other to yield F2 generation allows the various genes that were heterozygous in the F1 alleles to segregate. Commercial production of F1 hybrids requires maintenance of two inbred colonies. Use of F1 hybrids in toxicologic research is limited; however, they do exhibit greater genetic diversity than inbred strains.

Outbred or random-bred animals posses a high degree of individual genetic diversity. The term *random bred* refers to a system of mating by which there is no conscious selection for specific traits or kinships. Unfortunately, random mating assumes that a population is of infinite size and that all reproductively fit members of the population participate in the mating program. It also assumes that no conscious or unconscious selection process issue is used when new matings are set up. Even under the most careful circumstances, these assumptions cannot be fulfilled. Random mating programs, especially those that start with small numbers of individuals, will rapidly result in an increasing level of inbreeding or loss of heterozygosity. Because random-bred or outbred animals are presumed to be useful in toxicology and because of their great degree of individual diversity, loss of this diversity through random mating diminishes their usefulness.

Outbred animals are produced using a purposeful system of mating that seeks to retain the heterozygosity within a group of animals. A number of rotational systems for outbreeding have been devised, as have systems based on the coefficient of inbreeding of pedigreed groups of breeders (114, 163, 257). The latter system provides the greatest assurance of continued genetic diversity, provided that the colony is started with a sufficiently large number of genetically unrelated individuals.

Because established colonies of outbred animals undergo independent genetic assortment of polymorphic alleles and will independently fix certain nondeleterious mutations, different colonies of outbred animals separated by time as well as by geographic location will undergo genetic divergence even if they were started from the same colony of animals. To minimize this genetic divergence, breed stock can be traded between colonies of the same stock within the same supplier's organization. Systems for linking colonies in this fashion have been described. These rely on well-established population genetic techniques (125).

The genetic authenticity of inbreds and F1 hybrids can be monitored by assaying a variety of polymorphic alleles located on multiple chromosomes (126, 238). If mismatings occur, variations in this allelic profile will be found indicating a genetic contamination. Any polymorphic marker can be used, including biochemical, immunologic, and DNA assays.

The authenticity of outbred stocks or of individual outbred animals cannot be determined. Populations of outbred animals can be compared using markers similar to those used for inbreds and F1 hybrids, but large enough groups from a population must be sampled to characterize the allele frequency within the population. Using this allele frequency data, population genetic calculations can be made that will allow the similarity of different populations to be compared (48, 125).

In the case of transgenic animals, the presence of a transgene can be determined using PCR or other DNA analysis techniques in order to verify its presence and zygosity. This information can be used to adjust the breeding program or to authenticate a transgenic animal. Finding the presence of a particular transgene, however, does not determine if it has any functional significance.

SANITATION

Sanitation refers to the cleaning processes required for the maintenance of environmental conditions that are conducive to health. Sanitation of the animal's primary and secondary enclosure includes bedding change and cage cleaning and disinfection. The act of cleaning, either by manual or mechanical methods, removes excessive amounts of dirt and debris from surfaces while disinfection reduces or eliminates unacceptable concentrations of microorganisms that can interfere with toxicologic studies (222). The frequency with which these processes of cleaning and disinfection are done, as well as the methods used, depends on a number of factors, including species, age, and size of the animals; the type, construction, and size of the enclosure; the nature and quantity of the feces, urine, or debris soiling the environment; the temperature and relative humidity; the rate at which the surfaces of the enclosure becomes soiled; and the normal behavioral and physiological characteristics of the animals housed in the enclosure (222, 322).

To facilitate cleaning, the surfaces of both the secondary and primary enclosures should be impervious to liquids and smooth to allow easy application of cleaning materials and disinfectants. The surfaces should also be capable of withstanding scrubbing and should be free of cracks or other deficits that may inhibit the cleaning process (177). The use of agents designed to mask animal odors should not be used in animal facilities designed to support toxicologic research. Such agents expose the animals to chemical

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compounds that may interact with metabolic or physiologic processes involved in drug uptake and metabolism. Such agents simply obscure poor sanitation practices or inadequate ventilation. Conversely, characteristic animal odors released from the decomposition of urine or feces cannot be used as the sole criteria for the adequacy of sanitation practices or ventilation. Some studies suggest a relationship between

certain decomposition products of urine and feces (principally ammonia) and the potentiation of the effects of certain disease-causing organisms (36, 104, 179).

The sanitation of cages and associated equipment, such as watering devices and feed containers, can be done either manually or with mechanical equipment. The frequency of cleaning is dictated by cage type and the husbandry program. The use of wire-bottom or perforated-bottom cages, the use of regularly changed contact or noncontact bedding, and regular flushing of catch pans suspended under certain types of caging, coupled with the number, size, and type of animals in the cage, will influence how often the cage will need to be washed and sanitized. Hamsters, guinea pigs, and rabbits produce urine that contains high concentrations of minerals and proteins that can adhere to caging equipment. Regular cleaning will minimize the build up of such materials on the caging; however, pretreatment with acid solutions prior to cleaning may be required in order to remove such deposits from cages housing these species of animals (223).

Disinfection of caging can be accomplished, with hot water or chemicals applied either singly or in combination. The conditions under which these disinfection regimens are used must be adjusted so that they adequately kill vegetative forms of common bacteria and other organisms to be controlled by the sanitation program. It must be remembered that cage-washing practices, including the use of disinfectants in the cage-washing regimen, will not necessarily produce sterilized equipment nor guard against contamination after the disinfectants have been rinsed off the surfaces. The process of disinfection with chemicals must be carefully controlled in terms of preparation of the agent, method of application, conditions of application, properties of the agent, and contact time (13, 31, 180).

The use of hot water to disinfect surfaces must also be done in a controlled fashion. It is the combination of the temperature of the water, coupled with the length of time that the surfaces are exposed to a given water temperature (cumulative heat factor), that must be controlled. The same total exposure in terms of time and temperature can be obtained by washing at lower water temperatures for longer periods of time (327). Water temperatures ranging from a 140°F to 180°F have been used effectively in mechanical cage washers for disinfection with hot water alone. If detergents and/or disinfectants are combined with the hot water washing process, it is important that these compounds be thoroughly rinsed from all surfaces, as residual materials may interfere with some types of studies. If manual washing of cages is undertaken, as might be necessary in some specialized housing facilities, special care must be given to standardize the washing process and to assure that personnel are provided with the necessary personal protection equipment to minimize their exposure to hot water or chemical agents (222).

Although conventional methods for washing cages may be satisfactory for most toxicologic studies, the use of specialized bioexclusion systems, such as microisolation caging, may require a greater level of assurance of disinfection. In such cases, wrapping caging after cleaning in steam-permeable materials and autoclaving may be required to achieve the necessary level of decontamination.

Runs and pens may be sanitized by using high-pressure water coupled with detergents and/or disinfectants. Some organisms, such as gastrointestinal parasites, may not be eliminated by such treatments and may require periodic application of chemical disinfectants for prolonged periods of time. In all cases, thorough rinsing of the enclosures is critical so that carry over of materials that might adversely affect the animals or research does not occur.

Secondary enclosures, such as animal holding rooms, hallways, and support areas, should also undergo regular cleaning and disinfection. These areas can easily become contaminated with infectious organisms that can reinfect clean and disinfected caging. Contamination of secondary enclosures can also allow the spread of organisms from one area of a facility to another. To break the cycle, it is necessary to regularly clean the floors, walls, and other surfaces in the room using agents similar to those used in the cage-washing process.

The choice of disinfectants to use in an animal facility is difficult, may not be limited to a single agent, and will rely on a variety of factors. Discussion of disinfection principles is beyond the scope of this text, but useful information that will aid in selecting agents can be found in a number of references (13, 31, 180). For general purpose disinfection, agents should be active both by direct contact and in the vapor phase. The agent should have a broad spectrum of activity, including the ability to kill spores and nonenveloped viruses. Halogen-based compounds, most commonly those containing chlorine, appear to have the widest range of activity and are commonly selected. Care must be taken when using such agents, as regular use of any aggressive disinfectant can also damage surfaces.

PEST CONTROL

Animal facilities by their very nature generate large amounts of waste and use significant amounts of

feed and bedding. Their complex design and extensive mechanical systems provide excellent harborages for rodent and insect pests. Pests can serve as vectors for the introduction into research animal colonies of unwanted parasites and pathogenic microorganisms. Inappropriate

application of materials designed to kill pests can pose a risk to toxicologic research either through direct toxic effects on the animals or by interaction on a molecular level with experimental protocols (98, 155, 329).

Pest control is best conducted by professionals who can design an integrated pest management system that puts in place control methods and monitoring systems to track the effectiveness of the program. The use of potentially toxic chemicals should be avoided whenever possible in favor of nonchemical control measures (73, 105, 109, 164, 223). Application of pesticides should only be conducted with the knowledge of the animal care and research staff and done in compliance with federal, state, or local regulations (222).

WASTE DISPOSAL

Animal facilities generate large amounts of waste in either solid or liquid form. These wastes can either be nonhazardous or hazardous in nature. Depending on the country, wastes from animal facilities may be regulated and may have to be tracked and disposed of in a prescribed manner (244). In the United States, the Medical Waste Tracking Act (MWTA) of 1988 charged the EPA with tracking certain potentially hazardous wastes. Additional state and local regulations may also be imposed, causing the complexity of research waste management to vary from state to state (282). Although these regulations were designed to limit exposures to biohazardous wastes, all animal wastes may be subject to specific tracking and disposal procedures, depending on the interpretation of these regulations. For this reason, a local safety committee with expertise in these regulations should oversee the disposal process and ensure that all personnel handling waste are properly trained.

Some forms of waste may require special handling and may be incinerated on site. The operation of an on-site incinerator is heavily regulated in most countries and is not available at all research facilities. More commonly, contract waste handlers are used to dispose of research/medical waste using methods that comply with existing regulations. Waste water from cage washing, room cleaning, and flushing of pens and runs is flushed into drains to be subsequently treated either on site using local sewage treatment facilities or sent to municipal treatment systems.

Bedding constitutes the largest waste component in animal facilities. This is seldom treated on site and usually is removed either as bagged waste or in large containers for off-site disposal. Final disposal of noninfectious waste bedding is often by composting, land application, or landfills. Noninfectious animal carcasses and carcasses exposed to infectious or other hazardous materials are commonly refrigerated or frozen in plastic bags for sub sequent incineration (222). Recently, cost-effective chemical digestion methods for carcasses and other solid biological wastes have become available, allowing on-site treatment of such items with minimal environmental impact. Wherever possible, waste that is known to contain infectious or heat-liable hazardous materials should be treated by autoclaving or other destructive methods prior to storage for final disposal. Care should be taken to ensure that biological materials with a high liquid content, such as carcasses, are placed in sturdy leak-proof containers that can be sealed to minimize the potential spread of contamination (222).

Areas in which wastes are stored should be adequately ventilated and appropriately labeled. Individual waste containers should also be labeled as to the contents and any hazardous materials that they might contain. In the case of biological materials that are being collected for disposal, refrigerated or freezer storage may be required; in such instances a properly labeled, dedicated refrigerator or freezer should be available.

ANESTHESIA, ANALGESIA, AND SURGERY

Some toxicologic research protocols involve the surgical modification of animals or may subject animals to procedures that are likely to cause pain. Such procedures have been an important focus of the animal use oversight processes in most countries that have legislation regulating the use of animals in research. The researcher bears ultimate responsibility for the use of the appropriate procedures and the administration of the necessary drugs in such circumstances to assure the welfare of animals. Many references are available to assist the toxicologist in developing an appropriate surgical, anesthetic, or analgesic regimen (39, 68, 69, 152, 168). Assistance in this process should be sought from veterinarians experienced in laboratory animal medicine. The conduct of surgery in a research setting usually is a team effort requiring advanced planning, training to acquire the necessary skills, the appropriate facilities and equipment, and regular evaluation of outcomes and objective oversight of the entire surgical process (39, 40, 69).

Because both infection or lack of appropriate materials to successfully complete the surgery can result in the failure of the surgical procedure with the subsequent loss of animals, it is important that all aspects of the surgical procedure, from the selection of the animal through its preparation, anesthesia, surgical

manipulation, and postoperative recovery, be carefully planned in detail and all necessary supplies, equipment, and assistance be provided (14, 15, 222, 291). It is well established that animals are no less susceptible to infection than humans (20, 32, 68, 332, 333); For this reason, any materials or

equipment used in the operative procedure must be appropriately disinfected or sterilized and techniques be used to maintain the critical components of surgical supplies and equipment in aseptic condition throughout the surgical procedure (25, 151, 270, 279, 290).

The complexity of this task varies with the size and number of animals that must undergo a surgical procedure. Large animals often require a much larger surgical field from which hair is removed and the skin is scrubbed, disinfected, and covered by sterile drapes (132, 348). Often the incision site is quite large and the procedures undertaken may be more complex and require more instrumentation than those conducted on small laboratory rodents (69). Personnel must be provided with appropriate operating attire that is decontaminated or sterile. The surgeon's hands and arms should be disinfected by scrubbing with an antibacterial soap and water, and the necessary clothing and gloves should be packaged in a manner that will allow them to be put on by the surgeon in a location and manner so they do not become contaminated. The extent of surgical clothing required will vary with species of animals being used and will be proportional to the size of the operative site and incision (51, 248, 348). In the case of surgery involving rodents, the use of sterile gloves, a face mask, and a clean garment that covers the upper torso including the arms may be all that is required, whereas surgery on larger animals, such as rabbits, dogs, or nonhuman primates, may require much more extensive coverage of the surgeon with disinfected/sterile attire (38, 67, 69).

It is critical to review in detail each component of the procedure to determine if assistance is needed either from a person who is in surgical attire and capable of manipulating instruments or tissues in the operative field or by personnel who are not in full surgical attire and can handle materials that have not been disinfected or sterilized, conduct anesthesia, or aseptically dispense surgical supplies.

To be qualified to conduct the surgical procedure, the toxicologist must undergo training. Most commonly, this is done using a combination of techniques, including assisting in similar surgical procedures, observing others performing the surgical procedure, practicing on cadavers, or in the case of basic surgical technique, using training aids to acquire the necessary skills to conduct the surgery (1). The surgeon should also be prepared to deal with possible complications, including anesthesia difficulties, hemorrhage, difficulties in wound closure, and other similar, unwanted occurrences that could be associated with the procedure.

The postoperative recovery of the animal should be carefully planned and the clinical parameters that would signal need for additional intervention, including supportive therapy, which might consist of supplemental heat, administration of fluids, use of parental drugs (antibiotics, analgesics), nutritional support, or resuscitative procedures, prepared for. The criteria for a successful surgical outcome should be determined, as should the criteria for terminating a process when the outcome is unsuccessful. The toxicologist should have planned for and be trained in appropriate methods of euthanasia if and when it is required (7, 160, 181).

When new procedures are being developed, veterinary assistance and oversight may be necessary to further refine techniques and minimize failures. If animals die unexpectedly or unfavorable outcomes occur, the process should be analyzed and steps taken to improve it. Often critical to this is a comprehensive diagnostic examination of the animals that have undergone the procedure, either at the end of the study or when unexpected deaths or failure of the procedure occurs. Postmortem examinations, including histopathology and, where appropriate, other diagnostic measures, are essential to this process.

Surgical procedures can be classified as either recovery or nonrecovery and further subdivided as major or minor (222). Each requires respect for the animals being used and provision of adequate anesthesia. There are many complex issues in selecting the appropriate anesthetic regimen for any surgical procedure. Anesthetic techniques, equipment, and drugs are constantly being refined. With few exceptions, most techniques that can be applied to man can be applied to animals. Most anesthetic regimens used in toxicologic research, however, tend to be relatively simple and effective. Commonly, only one or two drugs are administered, and agents are favored that are easy to administer and whose use is well characterized in the species selected.

Because most toxicologic research involves small rodent species, agents that can be given intraperitoneally or by inhalation are easiest to administer and are commonly used. Selecting a few anesthetic regimens and being skilled in their use in a particular species often proves to be the most successful approach for the toxicologist. Switching between new anesthetic regimens without adequate training can lead to undesirable outcomes. Conversely, if an anesthetic regimen does not work well in a particular species or for a specific procedure, the toxicologist should not be reticent to seek an alternative technique that is more successful.

If inhalational agents are used, care must be taken to minimize human exposure, especially to women of childbearing age. Simple systems have been devised to scavenge waste anesthetic gases, and devices exist to monitor personnel exposure. Many anesthetic agents and analgesics are categorized as controlled substances that require secure storage, record keeping, and licensure.

When administering anesthesia, it is necessary to be cognizant of the signs and reflexes used to gauge the depth of anesthesia and what dosages will be used to re-dose the animals should additional anesthesia be required.

Similarly, a number of important factors can influence the course of anesthesia, including the state of hydration of the animal, decreases in the animal's core temperature due to loss of heat into its surroundings, the route and site of administration of the agent, and variability in the depth of anesthesia associated with failure to fast the animal. Techniques have been described to address all of these issues as well as others (168, 346).

Once an animal has recovered from anesthesia, there may be instances in the postoperative period where analgesics may be required. Similarly, in some studies, pain may result from nonsurgical procedures or conditions that may need to be relieved for the study to continue. The diagnosis of pain in animals is difficult and does not rely on any single clinical sign (135, 299). Analgesics have not been well studied in laboratory animals. It is clear that animals can and do experience pain and that analgesics can provide relief from pain, as evidenced by alterations in parameters such as weight gain, feed consumption, and activity measurements (219). Determination of the presence or absence of pain in small laboratory animals is particularly difficult, as is the assessment of the effectiveness of various analgesics administered prior to or following the onset of pain. Because the information available for choosing analgesics is limited and in some cases relatively subjective, the toxicologist should seek to assess the effectiveness of any drug or regimen selected through measurement of clinical parameters that are affected by pain.

ACQUISITION, QUARANTINING, AND CONDITIONING ANIMALS

The choice of a particular species for use in toxicologic research may be based on past work using the species, evaluation of metabolic, physiologic, or morphologic characteristics of a given species, the incidence of the development of certain spontaneous lesions, conditions, or an assessment of other biologic parameters, including lifespan, reproductive cycles, and behavioral characteristics that would make them suitable for a particular type of toxicological study. Laws and guidelines in many countries require consultation of literature or other sources of information to verify the appropriateness of a particular species to assure unnecessary duplication of work and to determine if alternatives to painful procedures exists. Other factors, such as the availability of a particular species and its conservation status, may also impact the decision.

When ordering animals, the only truly verifiable specifications are weight and sex. With the exception of nonhuman primates, dogs, and cats, there are very few instances in which a complete clinical or historical record is kept on individual animals. In some cases, a vaccination history and records of preshipment conditioning programs, including dosing with anthelminthic and antimicrobial treatment, may also be available for cats, dogs, and nonhuman primates, as well as limited colony health history, as previously discussed.

Commercially bred rodents and rabbits maintained under defined microbiological conditions by large commercial breeders are easily obtainable, but care must be taken when ordering these animals. Most commercial breeders produce rodents and rabbits in large numbers, often using polygamous or harem mating systems. Stock animals are maintained after weaning either in weight groups or in age groups by week of birth. Animals are seldom held by day of birth unless specific arrangements are made to set aside groups of animals for this purpose.

When ordering, animals usually are specified by weight or age but seldom both. With outbred animals and, to a lesser extent, with inbred and F1 hybrid animals, the weight range at any given age can be relatively broad and significantly overlap weight ranges for other age groups. Hence, it is possible to have animals of two different ages have the same weight. These overlaps can span several weeks of growth. Suppliers construct growth charts that can be used to estimate age based on weight within specific weight ranges. These are useful when a specific weight range of animals is required for a particular study, but some assurance still needs to be given that this represents a certain age range. If both an exact age and a specific weight range are selected, only a small portion of an outbred population of animals (and to a lesser extent, inbred and F1 hybrid animals) will be represented, resulting in unconscious selection for certain traits associated with animals within the population that fall within this specific age/weight range. Such overselection can adversely skew study populations and can lead to circumstances where findings cannot be repeated. For these reasons, it is best to specify animals by either age or weight.

When selecting animals for a study, it is important that the toxicologist carefully determine the size of the study group to be used. If very small groups of outbred animals are used, it is possible to select, purely through sampling error, a nonrepresentative group of animals from a much larger population (125). Attempts to repeat the findings of the study with another small group or even with larger groups may yield different results purely due to inappropriate sampling. It is also a reason why care must be

taken to adequately randomize the assignment of animals to both test and control groups. Populations of animals are dynamic and constantly changing their profile of expressed phenotypes. This occurs to a much smaller degree with inbred animals and F1 hybrids but can occur, as previously described, through the process of fixation and assortment of natural mutations. The problem is magnified in outbred col

onies due to random genetic drift (125). Hence, the toxicologist should expect that historical controls will vary over time in an unpredictable fashion and that such variation will occur regardless if the same colony is used for a source of animals for subsequent studies or if alternative colonies are selected. It is also important to appreciate that while different suppliers may produce strains or stocks of similar designations, perhaps derived from a common source, the longer the time interval from the point of stocking until the present, the more the groups of animals have likely drifted apart genetically and the more likely they are to have different phenotypic expressions, either through continual genetic reassortment and fixation or through the development of new allelic polymorphisms. Switching sources of supply of animals can cause changes in historical controls as well as in standardized assays. These changes usually are not dramatic but can be a source of concern for those assuming that there is some consistency between different populations of animals.

Animals are transported from suppliers to toxicology laboratories in shipping containers that are designed to meet national and certain international standards (141). Depending on the country, various regulations may be applicable to the control of animals in transport. Animals containing infectious agents or whose genetic material has been altered, and animals that have been exposed to hazardous materials of a noninfectious nature, must be shipped in conformance to regulations that can differ significantly between countries. Commercial animal suppliers are experienced in shipping animals in conformance with these requirements; however, if the toxicologist intends to ship animals between institutions, it is important to work with experienced brokers or other shipping agents who can assist in making the necessary arrangements and acquire the necessary shipping containers to legally conduct this process.

For the most part, animals are shipped in new, disposable containers that often are filtered to prevent the incursion of microorganisms. Containers are sometimes sterilized or disinfected, as may be the bedding, feed, and water used in shipping, depending on the supplier and the animals being transported. Most transportation either entirely or in part is done by truck. Most large laboratory animal suppliers maintain independent trucking routes and dedicated vehicles that are disinfected between shipments that transport animals. Some animal suppliers have only limited trucking capabilities and rely on air shipments. All suppliers ship some portion of their animals by air to supply customers not served by truck routes or to accommodate special conditions or orders that cannot be handled by truck. Unless very special shipping containers are used, shipment of animals by air allows the possibility of excessive stress and contamination during shipment (190, 222, 223). Moreover, the supplier does not have control of the shipment while it is being handled by the air carrier, and hence the control of environmental conditions may vary depending on the circumstances.

It is generally good practice to process animals immediately upon receipt at the research institution. Disinfection of the outside of the containers with solutions of general-purpose disinfectants is often a prudent step, especially if the animals have been shipped by air. Animals should be removed from their containers and examined upon arrival to confirm their clinical condition and to verify order specifications. They should be placed in appropriate caging that has been labeled to identify the animals, and they should be given access to feed and water as soon as possible after arrival. Most toxicologic research facilities provide a period of stabilization and/or quarantine for newly arrived animals. During quarantine periods, animals are observed for clinical signs of disease, samples are taken for health assessment, and in some cases, vaccinations, treatment with antimicrobials, or treatment with anthelminthics may also be undertaken (222). Other forms of diagnostic testing may also be conducted during the quarantine period. The length of quarantine can vary considerably, depending on the institutional exclusionary list of organisms for a particular species and health monitoring program.

A stabilization period differs from quarantine in that a stabilization period is designed to allow the animals time to recover from the stress of transportation, become rehydrated, and to gain back weight that may have been lost during transportation (2, 74, 78, 148, 326). This period of acclimation also allows the animal to become accustomed to using the water and feed sources and to adapt to any changes in diet (112, 173, 262, 313). In the case of group-housed animals, it allows the establishment of both social hierarchies and other behavioral adaptations. Stabilization and quarantine should be done concurrently. Some institutions do not have a quarantine period in which health monitoring is conducted, but rather, health monitoring information from the animal supplier is used to determine the fitness of the animals for incorporation into the research program. A stabilization period, however, may be instituted for the group to allow the necessary acclimation to occur prior to use. Typical stabilization periods range from 3 to 7 days for most species and are based upon some limited work to suggest that periods of 48 or 72 hours are necessary to overcome the stress of transportation (173, 286, 318).

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QUESTIONS

1. What housing systems can be used to minimize the risk of introduction and spread to animals of unwanted microorganisms that could alter toxicologic research?
2. What microorganisms that infect animals have the ability to alter toxicologic research results?
3. If outbred (non inbred) rodents are used in toxicologic research or product registration studies, what factors associated with their breeding methods and source colonies, as well as ordering specifications, can cause variation in research results?
4. What are the differences between closed formula, open formula, and constant nutrition natural ingredient diets used to feed research animals?
5. What laws, regulations, and guidelines affect the use of laboratory animals in toxicological and product registration research?
6. What constituents of the research animals' environ ment can cause variation in toxicologic research results?

REFERENCES

1. Academy of Surgical Research (ASR) (1989): Guidelines for training in surgical research in animals. *J. Invest. Surg.*, 2:263–268.
2. Aguila, H.N., Pakes, S.P., Lai, W.C., and Lu, Y.S. (1988): The effect of transportation stress on splenic natural killer cell activity in C57BL/6J mice. *Lab. Anim. Sci.*, 38:148–151.
3. Albanes, D. (1987): Total calories, body weight, and tumor incidence in mice. *Cancer Res.*, 47:1987–1992.
4. Allander, C., and Abel, E. (1973): Some aspects of the differences of air conditions inside a cage for small laboratory animals and its surroundings. *Z. Versuchstierkd, Bd.*, 15:20–34.
5. American Society of Heating, Refrigeration, and Air Conditioning Engineers, Inc. (ASHRAE) (1992): Air cleaners for particulate contaminants. In: *1992 ASHRAE Handbook: HVAC Systems and Equipment, I-P edition.* ASHRAE, Atlanta.
6. American Society of Heating, Refrigeration, and Air Conditioning Engineers, Inc. (ASHRAE) (1993): Environmental control of animals and plants. In: *1993 ASHRAE Handbook: Fundamentals, I-P edition.* ASHRAE, Atlanta.
7. American Veterinary Medical Association (AVMA) (1993): 1993 Report of the AVMA Panel on Euthanasia. *J. Am. Vet. Med. Assoc..* 202:229–249.
8. Ames, B.N., Shigenaga, M.K., and Hagen, T.M. (1993): Review: Oxidants, antioxidants, and the degenerative disease of aging. *Proc. Natl. Acad. Sci. USA,* 90:7915–7922.
9. Animal Welfare Institute (1979): *Comfortable Quarters for Laboratory Animals.* Animal Welfare Institute, Washington, DC.
10. Anonymous (1976): Long-term holding of laboratory rodents. *ILAR News.* 19:L1–L25.
11. Anthony, A. (1962): Criteria for acoustics in animal housing. *Lab. Anim. Care,* 13:340–347.
12. Armario, A., Castellanos, J.M., and Balasch, J. (1985): Chronic noise stress and insulin secretion in male rats. *Physiol. Behav.*, 34:359–361.
13. Ascenzi, J.M. (1996): *Handbook of Disinfectants and Antiseptics.* Marcel Dekker, New York.
14. Association of Operating Room Nurses (AORN) (1982): Recommended practices for traffic patterns in the surgical suite. *Assoc. Oper. Room Nurs. J.*, 15:750–758.
15. Ayliffe, G.A.J. (1991): Role of the environment of the operating suit in surgical wound infection. *Rev. Infect. Dis.*, 13(suppl 10):S800–S804.
16. Baetjer, A.M. (1968): Role of environment temperature and humidity in susceptibility to disease. *Arch. Environ. Health.*, 16:565–570.
17. Barker, H.J., Lindsey, J.R., and Weisbroth, S.H. (1979): Housing to control research variables. In: *The Laboratory Rat, Volume 1: Biology and Diseases*, edited by H.J.Baker, J.R.Lindsey, and S.H.Weisbroth, pp. 169–192. Academic Press, Orlando.
18. Barrett, A.M., and Stockham, M.A. (1963): The effect of housing conditions and simple experimental procedures upon the corticosterone level in the plasma of rats. *J. Endocrinol.*, 26:97–105.
19. Beall, J.R., Torning, F.E., and Runkle, R.S. (1971): A laminar flow system for animal maintenance. *Lab. Anim. Sci.*, 21:206–212.
20. Beamer, T.C. (1972): Pathological changes associated with ovarian transplantation. In: *The 44th Annual Report of the Jackson Laboratory,* p. 104. Jackson Laboratory, Bar Harbor, Maine.
21. Beck, R.W. (1963): The control of *Pseudomonas aeruginosa* in mouse breeding colony by the use of chlorine in the drinking water. *Lab. Anim. Care,* 13:41–45.
22. Bellhorn, R.W. (1980): Lighting in the animal environment. *Lab. Anim. Sci.*, 30:440–450.

23. Berg, B.N., and Simms, H.S. (1960): Nutrition and longevity in the rat. II: Longevity and onset of disease with different levels of food intake. *J. Nutr.*, 71:255–263.
24. Berg, B.N., and Simms, H.S. (1961): Nutrition and longevity in the rat. III: Food restriction beyond 800 days. *J. Nutr.*, 74:23–32.
25. Berg, J. (1993): Sterilization. In: *Textbook of Small Animal Surgery, 2nd edition*, edited by D.Slatter, pp. 124–129. W.B. Saunders, Philadelphia.
26. Besch, E.L. (1975): Animal cage-room dry-bulk and dew-point temperatures differential. *ASHRAE Trans.*, 88:549–557.
27. Besch, E.L. (1980): Environmental quality within animal facilities. *Lab. Anim. Sci.*, 30:385–406.
28. Besch, E.L. (1990): Environmental variables and animal needs. In: *The Experimental Animal in Biomedical Research, Volume 1: A Survey of Scientific and Ethical Issues for Investigators*, edited by B.E.Rollin and M.L.Kesel, pp. 113–131. CRC Press, Boca Raton, FL.
29. Bhatt, P.N., Jacoby, R.O., Morse, H.C., III, and New, A.E. (1986): *Viral and Mycoplasmal Infections of Laboratory Rodents: Effects on Biomedical Research*. Academic Press, Orlando.
30. Birt, D.F., and Conrad, R.D. (1981): Weight gain, reproduction, and survival of Syrian hamsters fed five natural ingredients diets. *Lab. Anim. Sci.*, 31:149–155.
31. Block, S.S. (1991): *Disinfection, Sterilization and Preservation, 4th edition*, Lea & Febiger, Philadelphia.
32. Bradfield, J.F., Schachtman, T.R., McLaughlin, R.M., and Steffen, E.K. (1992): Behavioral and physiological effects of inapparent would infection in rats. *Lab. Anim. Sci.*, 42:572–578.
33. Brainard, G.C. (1989): Illumination of laboratory animal quarters: Participation of light irradiance and wavelength in the regulation of the neuroendocrine system. In: *Science and Animals: Addressing Contemporary Issues*, pp. 69–74. Scientists Center for Animal Welfare, Greenbelt, Maryland.
34. Brainard, G.C., Vaughan, M.K., and Reiter, R.J. (1986): Effect of light irradiance and wavelength on the Syrian hamster reproductive system. *Endocrinology*, 119:648–654.
35. Brewer, N.R. (1964): Estimating heat produced by laboratory animals: New data on animal heat and vapor transmission account

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_810.html[2010/10/28 18:48:05]

for activity and other factors to provide a more reliable basis for conditioning design calculations. *Heating Piping Air Cond.*, 36:139–141.
36. Broderson, J.R., Lindsey, J.R., and Crawford, J.E. (1976): The role of environmental ammonia in respiratory mycoplasmosis of rats. *Am. J. Pathol.*, 85:115–130.
37. Brown, A.M., and Pye, J.D. (1975): Auditory sensitivity at high frequencies in mammals. *Adv. Comp. Physiol. Biochem.*, 6:1–73.
38. Brown, M.J. (1994): Aseptic surgery for rodents. In: *Rodents and Rabbits: Current Research Issues*, edited by S.M.Niemi, J.S. Venable, and H.N.Guttman, pp. 67–72. Scientists Center for Animal Welfare, Bethesda, Maryland.
39. Brown, M.J., Pearson, P.T., and Tomson, F.N. (1993): Guidelines for animal surgery in research and teaching. *Am. J. Vet. Res.*, 54:1544–1559.
40. Brown, M.J., and Schofield, J.C. (1994): Perioperative care. In: *Essentials for Animal Research: A Primer for Research Personnel*, edited by B.T.Bennett, M.J.Brown, and J.C.Schofield, pp. 79–88. National Agricultural Library, Washington, DC.
41. Burkhart, C.A., and Robinson, J.L. (1978): High rat pup mortality attributed to the use of cedar-wood shavings as bedding. *Lab. Anim.*, 12:221–222.
42. Campbell, S.A. (1990): Effects of exercise programs on serum biochemical stress indicators in purpose-bred beagle dogs. In: *Canine Research Environment*, edited by J.A.Mench and L.Krulisch, pp. 77–82. Scientists Center for Animal Welfare, Bethesda, MD.
43. Campbell, S.A., Hughes, H.C., Griffin, H.E., Landi, M.S., and Mallon, F.M. (1988): Some effects of limited exercise on purpose-bred beagle dogs. *Am. J. Vet. Res.*, 49:1298–1301.
44. Campbell, T.C., and Hayes, J.R. (1974): Role of nutrition in the drug-metabolizing enzyme system. *Pharmacol. Rev.*, 26:171–197.
45. Canadian Council on Animal Care (1980): *Guide and Care and Use of Experimental Animals, Vol. 1.* Canadian Council on Animal Care, Ottawa, Canada.
46. Canadian Council on Animal Care (1984): *Guide and Care and Use of Experimental Animals, Vol. 2.* Canadian Council on Animal Care, Ottawa, Canada.
47. Canadian Federation of Humane Societies (1990): *Guidelines for Community Members of Animal Care Committees.* Experimental Animals Committee, Canadian Federation of Humane Societies, Nepean, Canada.
48. CD(SD)IGS Study Group (1998): Biological reference data on CD(SD)IGS rats—1998. Best Printing Co., Yokohama, Japan.
49. Centers for Disease Control and Prevention (CDC) and National Institutes of Health (NIH) (1993): *Biosafety in Microbiological and Biomedical Laboratories, 3rd edition.* HHS Publication No. (CDC) 93–8395, U.S. Government Printing Office, Washington, DC.
50. Centers for Disease Control and Prevention (CDC) and National Institutes of Health (NIH) (1995): *Primary Containment for Biohazards: Selection, installation and use of Biological Safety Cabinets.* U.S. Government Printing Office, Washington, DC.
51. Chamberlain, G.V., and Houang, E. (1984): Trial of the use of masks in gynecological operating theatre. *Ann. R. Coll. Surg.*, 66:432–433.
52. Cheney, K.E., Liu, R.K., Smith, G.S., Leung, R.E., Mickey, M. R., and Walford, R.L. (1980): Survival and Disease patterns in C57BL/6J mice subjected to undernutrition. *Exp. Gerontol.*, 15:237–258.
53. Cheney, K.E., Liu, R.K., Smith, G.S., Meredith, P.J., Mickey, M.R., and Walford, R.L. (1983): The effect of dietary restriction of varying duration on survival, tumor patterns, immune function, and body temperature in B10C3F1 female mice. J. *Gerontrol.*, 38:420–430.
54. Cherry, J.A. (1987): The effect of photoperiod on development of sexual behavior and fertility in golden hamsters. *Physiol. Behav.*, 39:521–526.
55. Chvedoff, M., Clarke, M.R., Irisarri, E., Faccini, J.M., and Monro, A.M. (1980): Effects of housing conditions on food intake, body weight and spontaneous lesions in mice: A review of the literature and results of a 18-month study. *Food Cosmetics Toxicol.*, 18:517–522.
56. Cinti, D.K., Lemelin, M.E., and Christian, J. (1976): Induction of liver microsomal mixed-function oxidases by volatile hydrocarbons. *Biochem. Pharmacol.*, 25:100–103.
57. Clapp, M.J.L. (1980): The effect of diet on some parameters measured in toxicological studies in the rat. *Lab. Anim.*, 14:253–261.
58. Clough, G. (1976): The immediate environment of the laboratory animal. In: *Control of the Animal House Environment*, edited by T.McSheehy, pp. 77–94. Trevor Laboratory Animals, London.
59. Clough, G. (1982): Environmental effects on animals used in biomedical research. *Biol. Rev.*,

57:487–523.
60. Clough, G. (1987): The animal: Design, equipment and environmental control. In: *The UFA W Handbook on the Care and Management of Laboratory Animals, 6th edition*, edited by T.B. Poole, pp. 108–143. Longman Scientific & Technical, London.
61. Clough, G., and Gamble, M.R. (1976): *Laboratory Animal Houses: A Guide to the Design and Planning of Animal Facilities*, LAC Manual Series No. 4, Medical Research Council Laboratory Animals Council Laboratory Animals Centre, Abbey Press, Abingdon, Oxon.
62. Collins, T.F.X., Hinton, D.M., Welsh, J.J., and Black, T.N. (1992): Evaluation of heat sterilization of commercial rat diet for use in FDA toxicological studies. *Toxicol. Ind. Health.*, 8:9–20.
63. Conybeare, G. (1979): Effect of quality and quantity of diet on survival of tumour incidence in outbred Swiss mice. *Food Cosmetics Toxicol.*, 18:65–75.
64. Cooper, E.C. (1989): Design considerations for research animal facilities. *Lab. Anim.*, 18:23–26.
65. Coriell, L.L., and McGarrity, G.J. (1973): Biomedical applications of laminar airflow. In: *Germ-free Research Biological Effect of Gnotobiotic Environments*, edited by J.B.Henegham, p. 43. Academic Press, New York.
66. Cover, C.E., and Belcher, L.A. (1992): Effect of an irradiated rodent diet on growth and food consumption: A comparative study. *Contemp. Top. Lab. Anim. Sci.*, 31:13–17.
67. Cunliffe-Beamer, T.L. (1983): Biomethodology and surgical techniques. In: *The Mouse in Biomedical Research, Vol. III: Normative Biology, Immunology and Husbandry*, edited by H. L.Foster, J.D.Small, and J.G.Fox, pp. 419–420. Academic Press, New York.
68. Cunliffe-Beamer, T.L. (1990): Surgical techniques. In: *Guidelines for the Well-Being of Rodents in Research*, edited by H.N. Guttman, pp. 80–85. Scientists Center for Animal Welfare, Bethesda, Maryland.
69. Cunliffe-Beamer, T.L. (1993): Applying principles of aseptic surgery to rodents. *AWIC Newsl.*, 4:3–6.
70. Cunliffe-Beamer, T.L., Freeman, L.C., and Myers, D.D. (1981): Barbiturate sleeptime in mice exposed to autoclaved or unautoclaved wood beddings. *Lab. Anim. Sci.*, 31:672–675.
71. Department of Agriculture (1987): Animal and plant health inspection service: 9 CFR Parts 1 and 2; animal welfare; proposed rules. *Fed. Reg.*, 52:10292–10322.
72. Dillehay, D.L., Lehner, N.D.M., and Huerkamp, M.J. (1990): The effectiveness of a microisolator cage system and sentinel mice for controlling and detecting MHV and Sendai virus infections. *Lab. Anim. Sci.*, 40:367–370.

73. Donahue, W.A., VanGundy, D.N., Satterfield, W.C., and Coghlan, L.G. (1989): Solving a tough problem. *Pest Control*, August:46–50.
74. Drozdowicz, C.K., Bowman, T.A., Webb, M.L., and Lang, C.M. (1990): Effect of in-house transport on murine plasma corticosterone concentration and blood lymphocyte populations. *Am. J. Vet. Res.*, 51:1841–1846.
75. Dubin, S., and Zietz, S. (1991): Sample size for animal health surveillance. *Lab. Anim.*, 20:29–33.
76. Duncan, T.E., and O'Steen, W.K. (1985): The diurnal susceptibility of rat retinal photoreceptors to light-induced damage. *Exp. Eye Res.*, 41:497–507.
77. Dyment, J. (1976): Air filtration. In: *Control of the Animal Housing Environment*, edited by T.McSheehy, pp. 209–246. Laboratory Animals, London.
78. Dymsza, H.A., Miller, S.A., Maloney, J.F., and Foster, H.L. (1963): Equilibration of the laboratory rat following exposure to shipping stresses. *Lab. Anim. Care*, 13:60–65.
79. Eaton, P. (1987): Hygiene in the animal house. In: *The UFAW Handbook on the Care and Management of Laboratory Animals, 6th edition*, edited by T.B.Poole, pp. 144–148. Longman Scientific & Technical, London.
80. Edwards, G.S., Fox, J.G., Policastro, P., Goff, U., Wolf, M.H., and Fine, D.H. (1979): Volatile nitrosamine contamination of laboratory animal diets. *Cancer Res.*, 39:1857–1858.
81. Environmental Protection Agency (1978): Proposed guidelines for registering pesticides in the U.S. Hazard evaluation: Humans and domestic animals. *Fed. Reg.*, 43:37336–37403.
82. Erkert, H.G., and Grober, J. (1986): Direct modulation of activity and body temperature of owl monkeys *(Aotus lemurinus griseimembra)* by low light intensities. *Folia Primatol.*, 47:171–188.
83. Everett, R. (1984): Factors affecting spontaneous tumor incidence rates in mice: A literature review. *CRC Crit. Rev. Toxicol.*, 13:235–251.
84. Federation of European Laboratory Animal Science Associations (1994): Recommendations for the health monitoring of mouse, rat, hamster, guinea pig and rabbit breeding colonies. *Lab. Anim.*, 28:1–12.
85. Federation of European Laboratory Animal Science Associations, Supplement on Health Monitoring (1999): Health monitoring of non-human primate colonies. *Lab. Anim.*, 33(suppl. 1) S1:3-S1:18.
86. Ferguson, H.C. (1966): Effect of red cedar chip bedding on hexobarbital and pentobarbital sleep time. *J. Pharm. Sci.*, 55:1142–1143.
87. Festing, M., and Staats, J. (1973): Standardized nomenclature for inbred strains of rats: Fourth listing. *Transplantation*, 16:221–245.
88. Festing, M.F.W. (1993): *International Index of Laboratory Animals, 6th edition*. M.F.W.Festing, Leicester, United Kingdom (available from M.F.W.Festing, PO Box 301, Leicester LE1 7RE, UK).
89. Festing, M.F.W., and Greenhouse, D.D. (1992): Abbreviated list of inbred strains of rats. *Rat News Letter*, 26:10–22.
90. Festing, M.F.W., Kondo, K., Loosli, R., Poiley, S.M., and Spiegel, A. (1972): International standardized nomenclature for outbred stocks of laboratory animals. *ICLA Bull.*, 30:4–17.
91. Fidler, I.J. (1977): Depression of macrophages in mice drinking hyperchlorinated water. *Nature*, 270:735–736.
92. Fletcher, J.L., (1976): Influence of noise on animals. In: *Control of Animal House Environment—Laboratory Animal Handbooks, Vol. 7*, edited by T.McSheehy, pp. 51–62. Trevor Laboratory Animals, London.
93. Flynn, R.J. (1959): Studies of the etiology of ringtail of rats. *Proc. Anim. Care Panel*, 9:155–160.
94. Flynn, R.J. (1963): *Pseudomonas aeruginosa* infection and radiobiological research at Argonne National Laboratory: Effects, diagnosis, epizootiology, control. *Lab. Anim. Care*, 13:25–35.
95. Food and Drug Administration (1978): Nonclinical laboratory studies, good laboratory practice recommendations. *Fed. Reg.*, 43:59986–60025.
96. Ford, D.J. (1977): Effect of autoclaving and physical structure of diet on their utilization by mice. *Lab. Amin.*, 11:235–239.
97. Ford, D.J. (1977): Influence of diet pellet hardness and particle size on food utilization by mice, rats and hamsters. *Lab. Anim.*, 11:241–246.
98. Fouts, J.R. (1970): Some effects of insecticides on hepatic microsomal enzymes in various animal species. *Rev. Can. Biol.*, 29:377–389.
99. Fox, J.G. (1986): Interrelationships of disease and environmental variables in laboratory animals. In: *Safety Evaluation of Drugs and Chemicals*, edited by W.E.Lloyd, pp. 91–114. Hemisphere Publishing, Washington, DC.
100. Fox, J.G., Aldrich, F.D., and Boylen, G.W., Jr. (1976): Lead in animal foods. J. *Toxicol. Environ.*

Health, 1:461–467.
101. Fox, J.G., Newcomer, C.E., and Rozmiarek, H. (1984): Selected zoonoses and other health hazards. In: *Laboratory Animal Medicine*, edited by J.G.Fox, B.J.Cohen, and F.M.Loew, pp. 614–648. Academic Press, New York.
102. Fullerton, F.R., Greenman, D.L., and Kendall, D.C. (1982): Effects of storage conditions on nutritional qualities of semipurified (AIN-76) and natural ingredient (NIH-07) diets. *J. Nutr.*, 112:567–473.
103. Gamble, M.R. (1979): Fire alarms and oestrus in rats. *Lab. Anim.*, 10:93–104.
104. Gamble, M.R., and Clough, G. (1976): Ammonia build-up in animal boxes and its effect on a rat tracheal epithelium. *Lab. Anim.*, 10:161–163.
105. Garg, R.C., and Donahue, W.A. (1989): Pharmacologic profile of methoprene, an insect growth regulator, in cattle, dogs and cats. *J. Am. Vet. Med. Assoc.*, 194:410–112.
106. Garrard, G., Harrison, G.A., and Weiner, J.S. (1974): Reproduction and survival of mice at 23°C. J. *Reprod. Fertil.*, 37:287–298.
107. Gerber, W.F., and Anderson, T.A. (1967): Cardiac hypertrophy due to chance audiogenic stress in the rat and rabbit. *Comp. Biochem. Physiol.*, 21:237.
108. Gerber, W.F., Anderson, T.A., and Van Dyne, B. (1966): Physiologic responses of the albino rat to chronic noise stress. *Arch. Environ. Health*, 12:751–754.
109. Gibson, S.V., Besch-Williford, C., Raisbeck, M.F., Wagner, J.E., and McLaughlin, R.M. (1987): Organophosphate toxicity in rats associated with contaminated bedding. *Lab. Anim.*, 37:789–791.
110. Gordon, C.J. (1990): Thermal biology of the laboratory rat. *Physiol. Behav.*, 47:963–991.
111. Gordon, C.J. (1993): *Temperature Regulation in Laboratory Animals.* Cambridge University Press, New York.
112. Grant, L., Hopkinson, P., Jennings, G., and Jenner, F.A. (1971): Period of adjustment of rats used for experimental studies. *Nature*, 232:135.
113. Graves, R.G., (1990): Animal facilities: Planning for flexibility. *Lab. Anim.*, 19:29–50.
114. Green, E.L. (1981): Breeding systems. In: *The Mouse in Biomedical Research, Vol. I: History, Genetics and Wild Mice*, edited by H.L.Foster, J.D.Small, and J.G.Fox, pp. 91–104. Academic Press, New York.
115. Greenman, D.L., Bryant, P., Kodell, R.L., and Sheldon, W. (1982): Influence of cage shelf level on retinal atrophy in mice. *Lab. Anim. Sci.*, 32:353–356.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_812.html[2010/10/28 18:48:06]

116. Greenman, D.L., Kodell, R.L., and Sheldon, W.G. (1981): Association between cage shelf level and spontaneous and induced neoplasms in mice. *J. Natl. Cancer Inst.*, 73:107–113.
117. Greenman, D.L., Oller, W.L., Littlefield, N.A., and Nelson, C.J. (1980): Commercial laboratory animal diets: Toxicant and nutrient variability. *J. Toxicol. Environ. Health*, 6:235–246.
118. Halberg, F., Halberg, E., Barnum, C.P., and Bittner, J.J. (1959): Physiologic 24-hour periodicity in human beings and mice, the lighting regimen and daily routine. In: *Photoperiodism and Related Phenomena in Plants and Animals: Proceedings of a Conference on Photoperiodism*, edited by R.G.Withrow, pp. 803–879, Publ. No. 55. American Association for Advancement of Science, Washington, DC.
119. Hall, J.E., White, W.J., and Lang, C.M. (1980): Acidification of drinking water: Its effects on selected biologic phenomena in male mice. *Lab. Anim. Sci.*, 30:643–651.
120. Hamm, T.E. (1986): *Complications of Viral and Mycoplasmal Infections in Rodents to Toxicology Research and Testing.* Hemisphere Publishing, Washington, DC.
121. Hardy, J.D. (1961): Physiology of temperature regulation. *Physiol. Rev.*, 41:521–606.
122. Harkness, J.E., Wagner, J.E., Kusewitt, D.F., and Frisk, C.S. (1977): Weight loss and imparied reproduction in the hamster attributable to an unsuitable feeding apparatus. *Lab. Anim. Sci.*, 27:117–118.
123. Hart, R.W., Keenan, K., Turturro, A., Abdo, K.M., Leakey, J., and Lyn-Cook, B. (1995): Symposium overview: Caloric restriction and toxicity. *Fund. Apply. Toxicol.*, 25:184–195.
124. Hart, R.W., Leakey, J., Duffy, P.H., Feuers, R.J., and Turturro, A. (1996): The effects of dietary restriction on drug testing and toxicity. *Exp. Toxic. Pathol.*, 48:24–35.
125. Hartl, D.L. (1988): *A Primer of Population Genetics, 2nd edition.* Sinauer Associates, Sunderland, Massachusetts.
126. Hedrich, H.J., and Adams, M. (1990): *Genetic Monitoring of Inbred Strains of Rats: A Manual on Colony Management, Basic Monitoring Techniques, and Genetic Variants of the Laboratory Rat.* Gustav Fischer Verlag, Stuttgart.
127. Hermann, L.M, White, W.J., and Lang, C.M. (1982): Prolonged exposure to acid, chlorine, or tetracycline in the drinking water: Effects on delayed-type hypersensitivity, hemagglutination titers and reticuloendothelial clearance rates in mice. *Lab. Anim. Sci.*, 32:603–608.
128. Hessler, J.R., (1991): Facilities to support research. In: *Handbook of Facilities Planning: Volume 2—Laboratory Animal Facilities*, edited by T.Ruys, pp. 34–54. Van Nostrand Reinhold, New York.
129. Hessler, J.R. (1991): Single versus dual-corridor systems: Advantages, disadvantages, limitations, and alternatives for effective contamination control. In: *Handbook of Facilities Planning: Volume 2—Laboratory Animal Facilities*, edited by T.Ruys, pp. 59–66. Van Nostrand Reinhold, New York.
130. Hessler, J.R., and Moreland, A.F. (1984): Design and management of animal facilities. In: *Laboratory Animal Medicine*, edited by J.G.Fox, B.J.Cohen, and F.M.Loew, pp. 505–526. Academic Press, Orlando.
131. Hite, M., Hanson, H.M., Bohidar, N.R., Conti, P.A., and Mattis, P.A. (1977): Effect of cage size on patterns of activity and health of beagle dogs. *Lab. Anim. Sci.*, 27:60–64.
132. Hofmann, L.S. (1979): Preoperative and operative patient management. In: *Small Animal Surgery: An Atlas of Operative Technique*, edited by W.E.Wingfield and C.A.Rawlings, pp. 14–23. W.B.Saunders, Philadelphia.
133. Homberger, F.R., Pataki, Z., and Thomann, P.E. (1993): Control of *Pseudomonas aeruginosa* infection in mice by chlorine treatment of drinking water. *Lab. Anim. Sci.*, 43:635–637.
134. Hughes, H.C, Compbell, S., and Kenney, C. (1989): The effects of cage size and pair housing on exercise of beagle dogs. *Lab. Anim. Sci.*, 39:302–305.
135. Hughes, H.C., and Lang, C.M. (1983): Control of pain in dogs and cats. In: *Animal Pain: Perception and Alleviation*, edited by R.L. Kitchell and H.H.Erickson, pp. 207–216. American Physiological Society, Bethesda, Maryland.
136. Hughes, H.C., and Reynolds, S. (1995): The use of computational fluid dynamics for modeling air flow design in a kennel facility. *Contemp. Topics*, 34:49–53.
137. Hughes, H.C., Reynolds, S., and Rodriguez, R. (1996): Designing animal rooms to optimize air flow using computational fluid dynamics. *Pharm. Eng.*, Vol. 16(2):46–65.
138. Hugh-Jones, M.E., Hubbert, W.T., and Hagstad, H.V. (1995): *Zoonoses: Recognition, Control and Prevention.* Iowa State University Press, Ames, Iowa.
139. Institute of Laboratory Animal Resources (1978): *Laboratory Animal Housing*, proceedings of a symposium held at Hunt Valley, MD, September 22–23, 1976. National Academy of Sciences,

Washington, DC.
140. Interagency Research Animal Committee (IRAC) (1985): U.S. Government Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training. *Federal Register*, May 20, 1985, Office of Science and Technology Policy, Washington, DC.
141. International Air Transport Association (IATA) (1995): *IA TA Live Animal Regulations.* International Air Transport Association (IATA), Montreal, Quebec. (Available from IATA, 2000 Peel Street, Montreal, Quebec H3A 2R4, Canada.)
142. International Committee on Standardized Genetic Nomenclature for Mice (1994): Rules for nomenclature of inbred strains. *Mouse Genome*, 92:xxviii–xxxii.
143. International Committee on Standardized Genetic Nomenclature for Mice (1994): Rules and guidelines for gene nomenclature. *Mouse Genome*, 92:viii–xxiii.
144. International Life Sciences Institute (1995): *Dietary Restriction: Implications for the Design and Interpretation of Toxicity and Carcinogenicity Studies*, edited by R.W.Hart, D.A.Neumann, and R.T.Robertson. ILSI Press, Washington, DC.
145. Iturrian, W.B. (1971): Effect of noise in the animal house on experimental seizures and growth of weanling mice. In: *Defining the Laboratory Animal,* Proceedings of the IVth International Symposium on Laboratory Animals, pp. 332–352. National Academy of Sciences, Washington, DC.
146. Jacobs, B.B., and Dieter, D.K. (1978): Spontaneous hepatomas in mice inbred from Ha: ICR swiss stock: Effects of sex, cedar shavings in bedding, and immunization with fetal liver or hepatoma cells. *J. Natl. Cancer Inst.*, 61:1531–1534.
147. Jacoby, R.O., and Lindsey, J.R. (1997): Health care for research animals is essential and affordable. *FASEB J.*, 11:609–614.
148. Jelinek, V. (1971): The influence of the condition of the laboratory animals employed on the experimental results. In: *Defining the Laboratory Animal*, pp. 110–120. National Academy of Sciences, Washington, DC.
149. Jones, D.M. (1977): The occurrence of dieldrin in sawdust used as bedding material. *Lab. Anim.*, 11:137.
150. Jose, D.G., and Good, R.A. (1973): Quantitative effects of nutritional protein and caloric deficiency on immune responses to tumors in mice. *Cancer Res.*, 33:807–812.
151. Kagan, K.G. (1992): Care and sterilization of surgical equipment. *Vet. Tech.*, 13:65–70.
152. Kagan, K.G., (1992): Aseptic technique. *Vet. Tech.*, 13:205–210.
153. Kaufman, J.E. (1984): *IES Lighting Handbook Reference Volume.* Illuminating Engineering Society, New York.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_813.html[2010/10/28 18:48:07]

154. Kaufman, J.E. (1987): *IES Lighting Handbook Application Volume.* Illuminating Engineering Society, New York.
155. Keast, D., and Coales, M.F. (1967): Lymphocytopenia induced in a strain of laboratory mice by agents commonly used in treatment of ectoparasites. *Aust. J. Exp. Biol. Med. Sci.*, 45:645–650.
156. Keenan, K.P., Ballam, G.C., Dixit, R., Soper, K.A., Laroque, P., Mattson, B.A., Adams, S.P., and Coleman, J.B. (1997): The effects of diet, overfeeding and moderate dietary restriction on Sprague-Dawley rat survival, disease and toxicology. J. *Nutr.*, 127:851S-856S.
157. Keenan, K.P., Laroque, P., Ballam, G.C., Soper, K.A., Dixit, R., Mattson, B.A., Adams, S.P., and Coleman, J.B. (1996): The effects of diet, *ad libitum* overfeeding, and moderate dietary restriction on the rodent bioassay: The uncontrolled variable in safety assessment. *Toxicol. Pathol.*, 24:757–768.
158. Keenan, K.P., Laroque, P., and Dixit, R. (1998): Need for dietary control by caloric restriction in rodent toxicology and carcinogenicity studies. J. *Toxicol. Envior. Health* (Part B), 1:135–148.
159. Keenan, K.P., Smith, P.F., and Soper, K.A. (1994): Effect of dietary (caloric) restriction on aging, survival, pathobiology and toxicology. In: *Pathobiology of the Aging Rat, Vol. 2,* edited by W.Notter, D.L.Dungworth, and C.C.Capen, pp. 609–628. International Life Sciences Institute. Washington, DC.
160. Keller, G.L. (1982): Physical euthanasia methods. *Lab. Anim.*, 11(4): 20–26.
161. Keller, L.S.F., White, W.J., Snider, M.T., and Lang, C.M. (1989): An evaluation of intra-cage ventilation in three animal caging systems. *Lab. Anim. Sci.*, 39:237–242.
162. Keplinger, M.L., Lanier, G.E., and Deichmann, W.B. (1959): Effects of environmental temperature on the acute toxicity of a number of compounds in rats. *Toxicol. Appl. Pharmacol.*, 1:156–161.
163. Kimura, M., and Crow, J.F. (1963): On maximum avoidance of inbreeding. *Genet. Res.*, 4:399–415.
164. King, J.E., and Bennett, G.W. (1989): Comparative activity of fenoxycarb and hydroprene in sterilizing the German cockroach (Dictyoptera: Blattellidae). J. *Econ. Entomol.*, 82:833–838.
165. Knapka, J.J. (1983): Nurtition. In: *The Mouse in Biomedical Research, Vol. III,* edited by H.L.Foster, J.D.Small, and J. G.Fox, pp. 51–67. Academic Press, New York.
166. Knapka, J.J., Smith, K.P., and Judge, F.J. (1974): Effect of open and closed formula rations on the performance of three strains of laboratory mice. *Lab. Anim. Sci.*, 24:480–487.
167. Knauff, D.R. (1987): Revised laboratory animal policy, *Lab. Anim.*, 16:11.
168. Kohn, D.F., Wixson, S.K., White, W.J., and Benson, G.J. (1997): *Anesthesia and Analgesia In Laboratory Animals.* Academic Press, New York.
169. Kraft, L.M. (1980): The manufacture, shipping and receiving, and quality control of rodent bedding materials. *Lab. Anim. Sci.*, 30:366–376.
170. Krichevsky, D., Weber, M.M., and Klurfeld, D.M. (1984): Dietary fat versus caloric content in initiation and promotion of 7.12-dimethylbenz(a)anthracene induced mammary tumorigenesis in rats. *Cancer Res.*, 44:3174–3177.
171. Kuntz, M.J. (1989): Cubicles—Rational approach to specialized laboratory animal housing. *Anim. Technol.*, 40:203–209.
172. Kupp, R.P., Jr., Pinto, C.A., Rubin, L.F., and Griffin, H.E. (1989): Effects of ambient lighting on the eyes of rats. *Lab. Anim.*, 18:32–35,37.
173. Landi, M.S., Kreider, J.W., Lang, C.M., and Bullock, L.P. (1982): Effects of shipping on the immune function in mice. *Am. J. Vet. Res.*, 43:1654–1657.
174. Lang, C.M. (1983): Design and management of research facilities for mice. In: *The Mouse in Biomedical Research, Vol. III,* edited by H.L.Foster, J.D.Small, and J.G.Fox, pp. 37–50. Academic Press, New York.
175. Lanum, J. (1979): The damaging effects of light on the retina: Empirical findings, theoretical and practical implications. *Surv. Ophthalmol.*, 22:221–249.
176. LaRegina, M.C, and Lonigro, J. (1988): Serologic screening for murine pathogens: Basic concepts and guidelines. *Lab. Anim.*, 17:40–47.
177. LeBlanc, D.A., and Danforth, D.D. (1992): Substrate compatibility of animal cage wash products. *Contemp. Top. Lab. Anim. Sci.*, 31:13–16.
178. Leveille, G.A., and Hanson, R.W. (1966): Adaptive changes in enzyme activity and metabolic pathways in adipose tissue from meal-fed rats. J. *Lipid Res.*, 7(1):7–46.
179. Lindsey, J.R., and Conner, M.W. (1978): Influences of cage sanitation frequency on intracage ammonia (NH3) concentration and progression of murine respiratory mycoplasmosis in the rat. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg.*, 241:215–216.
180. Linton, A.H., Hugo, W.B., and Russell, A.D. (1987): *Disinfection in Veterinary and Farm Animal Practice.* Blackwell Scientific Publications, Oxford.

181. Lumb, W.V., and Moreland, A.F. (1982): Chemical methods for euthanasia. *Lab. Anim.*, 11:29–35.
182. Maeda, H., Gleiser, C.A., Masoro, E.J., Murata, I., McMahan, C.A., and Yu, B.P. (1985): Nutritional influences on aging of Fischer 344 rats. II: Pathology. J. *Gerontol.*, 40:671–688.
183. Masoro, E.J., (1992): Aging and proliferative homeostasis: modulation by food restriction in rodents. *Lab. Anim. Sci.*, 42:132–137.
184. McDougall, P.T., Wolf, N.S., Stenback, W.A., and Trentin, J.J. (1967): Control of *Pseudomonas aeruginosa* in an experimental mouse colony. *Lab. Anim. Care*, 17:204–214.
185. McGarrity, G.J., and Coriell, L.L. (1973): Mass airflow cabinet for control of airborne infection of laboratory rodents. *Appl. Micro.*, 26:167–172.
186. McGarrity, G.J., and Coriell, L.L. (1976): Maintenance of axenic mice in open cages in mass air flow. *Lab. Anim. Sci.*, 26:746–750.
187. McPherson, C.W. (1963): Reaction of *Pseudomonas aeruginosa* and coliform bacteria in mouse drinking water following treatment with hydrochloric acid or chlorine. *Lab. Anim. Care*, 13:737–744.
188. McPherson, C.W. (1984): Laws, regulations, and policies affecting the use of laboratory animals. In: *Laboratory Animal Medicine*, edited by J.G.Fox, B.J.Cohen, and F.M.Loew, pp. 19–30. Academic Press, Orlando.
189. Megna, V.A. (1984): Engineering needs and trends of a toxicology laboratory. *Concepts Toxicol.*, 1:118–137.
190. Meskin, L.H., and Shapiro, B.L. (1971): Teratogenic effect of air shipment on A/Jax mice. J. *Dent. Res.*, 50:169.
191. Middle Atlantic Reproduction and Teratology Association (MARTA) and Midwest Teratology Association (MTA) (1996): *Historical Control Data (1992–1994) for Developmental and Reproductive Toxicity Studies Using the CRL: CD®(SD)BR Rat.* Charles River Laboratories, Wilmington, Massachusetts.
192. Miller, P.L., and Nash, R.T. (1971): A further analysis of room air distribution performance. *ASHRAE Trans.*, 77:205–215.
193. Miller, P.L., and Nash, R.T. (1979): Analysis, evaluation and comparison of room air distribution performance: A summary. *ASHRAE Trans.*, 78:235–242.
194. Miller, P.L., and Nevins, R.G. (1969): Room air distribution with an air distribution ceiling: Part II. *ASHRAE Trans.*, 75:118–131.
195. Moore, B.J. (1987): The California diet: An inappropriate tool for studies of thermogenesis. J. *Nutr.*, 117:227–231.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_814.html[2010/10/28 18:48:08]

196. Mulder, J.B. (1971): Animal behavior and electromagnetic energy waves. *Lab. Anim. Sci.*, 21:389–393.
197. Mulligan, S.R., et al. (1993): Sound levels in rooms housing laboratory animals: An uncontrolled daily variable. *Physiology & Behav.*, 53:1067–1076.
198. Murakami, H. (1971): Differences between internal and external environments of the mouse cage. *Lab. Anim. Sci.*, 21:680–684.
199. Murata, M., and Takigawa, H. (1989): Teratogenic effects of noise in mice. *J. Sound Vibration*, 132:11–18.
200. Nair, V., and Casper, R. (1969): The influence of light on daily rhythm in hepatic drug metabolizing enzymes in rat. *Life Sci.*, 8(Part I):1291–1298.
201. National Aeronautics and Space Administration (NASA) (1988): Summary of conclusions reached in workshop and recommendations for lighting animal housing modules used in microgravity related projects. In: *Lighting Requirements in Microgravity: Rodents and Nonhuman Primates: NASA Technical Memorandum 101077*, edited by D.C.Holley, C.M.Winget, and H.A.Leon, pp. 5–8. Ames Research Center, Moffett Field, California.
202. National Institutes of Health Office of the Director, Division of Engineering Services, F.Memarzadeh Principal Investigator (1998): *Ventilation Design Handbook on Animal Research Facilities Using Static Microisolator, Vol. I.* National Institutes of Health, Bethesda, Maryland.
203. National Institutes of Health Office of the Director, Division of Engineering Services, F.Memarzadeh Principal Investigator (1998): *Ventilation Design Handbook on Animal Research Facilities Using Static Microisolator, Vol. II.* National Institutes of Health, Bethesda, Maryland.
204. National Research Council (1989): *Immunodeficient Rodents: A Guide to Their Immunobiology, Husbandry, and Use.* National Academy Press, Washington, DC.
205. National Research Council (1991): *Companion Guide to Infectious Diseases of Mice and Rats.* National Academy Press, Washington, DC.
206. National Research Council (1991): *Infectious Diseases of Mice and Rats.* National Academy Press, Washington, DC.
207. National Research Council (NRC), Institute of Laboratory Animal Resources, Committee on Rat Nomenclature (1992): Definition, nomenclature, and conservation of rats strains. *ILAR News*, 34:S1–S26.
208. National Research Council (NRC), Institute of Laboratory Animal Resources, Committee on Transgenic Nomenclature (1992): Standardized nomenclature for transgenic animals. *ILAR News*, 34:45–52.
209. National Research Council (NRC) (1977): *Nutrient Requirements of Rabbits: A Report of the Committee on Animal Nutrition.* National Academy Press, Washington, DC.
210. National Research Council (NRC) (1978): *Nutrient Requirements of Nonhuman Primates: A Report of the Committee on Animal Nutrition.* National Academy Press, Washington, DC.
211. National Research Council (NRC) (1981): *Nutrient Requirements of Cold Water Fishes: A Report of the Committee on Animal Nutrition.* National Academy Press, Washington, DC.
212. National Research Council (NRC) (1981): *Nutrient Requirements of Goats: A Report of the Committee on Animal Nutrition.* National Academy Press, Washington, DC.
213. National Research Council (NRC) (1982): *Nutrient Requirements of Mink and Foxes: A Report of the Committee on Animal Nutrition.* National Academy Press, Washington, DC.
214. National Research Council (NRC) (1983): *Nutrient Requirements of Warm Water Fishes and Shellfishes: A Report of the Committee on Animal Nutrition.* National Academy Press, Washington, DC.
215. National Research Council (NRC) (1985): *Nutrient Requirements of Dogs: A Report of the Committee on Animal Nutrition.* National Academy Press, Washington, DC.
216. National Research Council (NRC) (1985): *Nutrient Requirements of Sheep: A Report of the Committee on Animal Nutrition.* National Academy Press, Washington, DC.
217. National Research Council (NRC) (1986): *Nutrient Requirements of Cats: A Report of the Committee on Animal Nutrition.* National Academy Press, Washington, DC.
218. National Research Council (NRC) (1988): *Nutrient Requirements of Swine: A Report of the Committee on Animal Nutrition.* National Academy Press, Washington, DC.
219. National Research Council (NRC) (1992): *Recognition and Alleviation of Pain and Distress in Laboratory Animals: A Report of the Institute of Laboratory Animals Resources Committee on Pain and Distress in Laboratory Animals.* National Academy Press, Washington, DC.
220. National Research Council (NRC) (1994): *Nutrient Requirements of Poultry: A Report of the*

Committee on Animal Nutrition. National Academy Press, Washington, DC.
221. National Research Council (NRC) (1995): *Nutrient Requirements of Laboratory Animals: A Report of the Committee on Animal Nutrition.* National Academy Press, Washington, DC.
222. National Research Council, Commission on Life Sciences, Institute of Laboratory Animal Resources (1996): *Guide for the Care and Use of Laboratory Animals.* National Academy Press, Washington, DC.
223. National Research Council, Commission of Life Sciences, Institute of Laboratory Animals Resources, Committee on Rodents (1996): *Laboratory Animal Management—Rodents.* National Academy Press, Washington, DC.
224. Nayfield, K.C., and Besch. E.L. (1981): Comparative responses of rabbits and rats to elevated noise. *Lab. Anim. Sci.,* 31:386–390.
225. Newall, G.W. (1980): The quality, treatment and monitoring of water for laboratory rodents. *Lab. Anim. Sci.,* 30:377–384.
226. Newberne, P.M. (1975): Diet: The neglected experimental variable. *Lab. Anim.,* 4:20–24.
227. Newberne, P.M. (1975): Influence on pharmacological experiments of chemicals and other factors in diets of laboratory animals. *Fed. Proc.,* 34:209–218.
228. Newberae, P.M., and Fox, J.G. (1980): Nutritional adequacy and quality control of rodent diets. *Lab. Anim. Sci.,* 30:352–365.
229. Newberne, P.M., and McConnell, R.G. (1979): Nutrition of the Syrian golden hamster. *Prog. Exp. Tumor Res.,* 24:127–138.
230. Newberne, P.M., and McConnell, R.G. (1980): Dietary nutrients and contaminants in laboratory animal experimentation. *J. Environ. Pathol. Toxicol.,* 4:105–122.
231. Newberae, P.M., and Rogers, A.E., (1973): Rat colon carcinomas associated with aflatoxin in marginal vitamin A. J. *Natl. Cancer Inst.,* 50:439–448.
232. Newberne, P.M., Rogers, A.E., and Wogan, G.N. (1968): Hepatorenal lesions in rats fed a low lipotrope diet and exposed to aflatoxin. J. *Nutr.,* 94:331–343.
233. Newbold, J.A., Chapin, L.T., Zinn, S.A., and Tucker, H.A. (1991): Effects of photoperiod on mammary development and concentration of hormones in serum of pregnant dairy heifers. J. *Dairy Sci.,* 74:100–108.
234. Newton, W.M. (1978): Environmental impact on laboratory animals. *Adv. Vet. Sci. Comp. Med.,* 22:1–28.
235. Njaa, L.R., Utne, F., and Braekkan, O.R. (1957): Effect of relative humidity on rat breeding and ringtail. *Nature,* 180:290–291.
236. Noell, W.K., and Albrecht, R. (1971): Irreversible effects of visible light on the retina: Role of vitamin A. *Science,* 172:76.

237. Nolen, G.A., and Alexander, J.C. (1966): Effects of diet and type of nesting material on the reproduction and lactation of the rat. *Lab. Anim. Care*, 16:327–336.
238. Nomura, T., Esaki, K., and Tomita, T. (1984): *ICLAS Manual for Genetic Monitoring of Inbred Mice.* University of Tokyo Press, Tokyo.
239. Noris, M.L., and Adams, C.E. (1976): Incidence of pup mortality in the rat with particular reference to nesting material, maternal age and parity. *Lab. Anim.*, 10:165–169.
240. O'Steen, W.K. (1980): Hormonal influences in retinal photodamage. In: *The Effects of Constant Light on Visual Processes*, edited by T.P.Williams and B.N.Baker, pp. 29–49. Plenum Press, New York.
241. Ogle, C.W., and Lockett, M.F. (1968): The urinary changes induced in rats by high pitched sound (20 kcyc/sec). J. *Endocrinol.*, 42:253–260.
242. Oller, W.L., Greenman, D.L., and Suber, R. (1985): Quality changes in animal feed resulting from extended storage. *Lab. Anim. Sci.*, 35:646–650.
243. Orlans, F.B., Simmonds, R.C., and Dodds, W.J. (1987): Consensus recommendations on effective institutional animal care and use committees. *Lab. Anim. Sci.*, 37(special issue):11–13.
244. Party, E., and Wilkerson, A. (1991): Implications of new medical waste regulations on laboratory animal research. *Lab. Anim.*, 20(8):28–36.
245. Pekrul, D. (1991): Noise control. In: *Handbook of Facilities Planning: Volume 2—Laboratory Animal Facilities*, edited by T.Ruys, pp. 166–173. Van Nostrand Reinhold, New York.
246. Pennycuik, P.R. (1967): A comparison of the effects of a range of high environmental temperatures and of two different periods of acclimatization on the reproductive performances on male and female mice. *Aust. J. Exp. Biol. Med. Sci.*, 45:527–532.
247. Percy, D.H., and Barthold, S.W. (1993): *Pathology of Laboratory Rodents and Rabbits.* Iowa State University Press, Ames Iowa.
248. Pereira, L.J., Lee, G.M., and Wade, K.J. (1990): The effect of surgical handwashing routines on the microbial counts of operating room nurses. *Am. J. Inf. Control*, 18:354–364.
249. Peterson, E.A. (1980): Noise and laboratory animals. *Lab. Anim. Sci.*, 30:422–436.
250. Peterson, E.A., Augenstein, J.S., Tanis, D.C., and Augenstein, D. G. (1981): Noise raises blood pressure without impairing auditory sensitivity. *Science*, 211:1450–1452.
251. Pfaff, J. (1974): Noise as an environmental problem in the animal house. *Lab. Anim.*, 8:347–354.
252. Pfaff, J., and Stecker, M. (1976): Loudness levels and frequency content of noise in the animal house. *Lab. Anim.*, 10:111–117.
253. Pick, J.R., and Little, J.M. (1965): Effect of type of bedding material on thresholds of pentylenetetrazol convulsions in mice. *Lab. Anim. Care*, 15:29–33.
254. Plank, S.J., and Irwin, R. (1966): Infertility of guinea pigs on sawdust bedding. *Lab. Anim. Care*, 16:9–11.
255. Poche, L.B., Jr., Stockwell, C.W., and Ades, H.W. (1969): Cochlear hair cell damage in guinea-pigs after exposure to impulse noise. J. *Acoust. Soc. Am.*, 46:947–951.
256. Podberscek, A.L., Blackshaw, J.K., and Beattie, A.W. (1991): The effects of repeated handling by familiar and unfamiliar people on rabbits in individual cages and group pens. *Appl. Anim. Behav. Sci.*, 28:365–373.
257. Poiley, S.M. (1960): A systematic method of breeder rotation for non-inbred laboratory animal colonies. *Proc. Anim. Care Panel*, 10:159–166.
258. Poiley, S.M. (1974): Housing requirements-general consideration. In: *Handbook of Laboratory Animal Science, Vol. 1*, edited by E.C.Melby, Jr., and H.H.Altman. CRC Press, Cleveland.
259. Port, C.D., and Kaltenbach, J.P. (1969): The effect of corncob bedding on reproductivity and leucine incorporation in mice. *Lab. Anim. Care*, 19:46–49.
260. Porter, G., and Lane-Petter, W. (1965): The provision of sterile bedding and nesting materials with their effects on breeding mice. J. *Anim. Technol. Assoc.*, 16:5–8.
261. Potgieter, F.J., and Wilke, P.I. (1991): Laboratory animal bedding: A review of specifications and requirements. *J.S. Afr. Vet. Assoc.*, 62:143–146.
262. Prasad, S., Gatmaitan, B.R., and O'Connell, R.C. (1978): Effect of a conditioning method on general safety test in guinea pigs. *Lab. Anim. Sci.*, 28:591–593.
263. Prychodko, H. (1958): Effect of aggregation of laboratory mice *(Mus Cusculus)* on food intake at different temperatures. *Ecology*, 39:500.
264. Public Health Service (PHS) (1996): Public Health Service Policy on Human Care and Use of Laboratory Animals. U.S. Department of Health and Human Services, (PL 99–158, Health Research Extension Act 1985), Washington, DC.

265. Pucak, G.J., Lee, C.S., and Zaino, A.S. (1977): Effects of prolonged high temperature on testicular development and fertility in the male rat. *Lab. Anim. Sci.*, 27:76–77.
266. Ralls, K. (1967): Auditory sensitivity in mice: *Peromyscus and Mus musculus. Anim. Behav*., 15:123–128.
267. Rao, G.N., and Knapka, J.J. (1987): Contaminant and nutrient concentrations of natural ingredient rat and mouse diet used in chemical toxicology studies. *Fundam. Appl. Toxlcol*, 9:329–338.
268. Reiter, R.J. (1973): Comparative effects of continual fighting and pinealectomy on the eyes, the Harderian glands and reproduction in pigmented and albino rats. *Comp. Biochem. Physiol.*, 44:503–509.
269. Reynolds, S.D., and Hughes, H.C. (1994): Design and optimization of air flow patterns. *Lab. Anim.*, 23:46–49.
270. Ritter, M.A., and Marmion, P. (1987): The exogenous sources and controls of microorganisms in the operating room. *Orthopaedic Nursing*, 7:23–28.
271. Robison, W.G., Jr., and Kuwabara, T. (1976): Light-induced alterations of retinal pigment epithelium in black, albino, and beige mice. *Exp. Eye Res.*, 22:549–557.
272. Rogers, A.E. (1985): Factors influencing the results of animal experiments in toxicology. In: *Basic Toxicology: Fundamentals, Target Organs, and Risk Assessment*, edited by F.C.Lu, pp. 254–267. Hemisphere Publishing, Washington, DC.
273. Rose, R.J. (1990): Practical aspects of formulating research diets. *Lab. Anim.*, 19:47–49.
274. Ross, M.H., and Bras, G. (1971): Lasting influences of early caloric restriction on prevalence of neoplasma in the rat. J. *Natl. Cancer Inst.*, 47:1095–113.
275. Ross, M.H., and Bras, G. (1973): Influence of protein under- and over-nutrition on spontaneous tumor prevalence in the rat. *J. Nutr.*, 103:944–963.
276. Ross, N.H., Bras, G., and Ragbeer, N.S. (1970): Influence of protein and caloric intake upon spontaneous tumor incidence of the anterier pituitary gland and the rat. *J. Nutr.*, 100:177–189.
277. Ross, N.H., Lustbader, E.D., and Bras, G. (1983): Body weight, dietary practices, and tumor susceptibility in the rat. J. *Natl. Cancer Inst.*, 71:1041–1046.
278. Russell, W.M.S., and Burch, R.L. (1959): The principles of human experimental techniques. Methuen & Co., London (reprinted as a special edition in 1992 by the University Federation for Animal Welfare).
279. Rutala, W.A. (1990): APIC guideline for selection and use of disinfectants. *Am. J. Inf. Control*, 18:99–117.
280. Ruys, T. (1991): Codes, regulations and standards: Appendix E: Comments on the federal animal welfare regulations dealing with

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dogs, cats and nonhuman primates (9 CFR Part 3. Subpart A. Feb. 15, 1991). In: *Handbook of Facilities Planning, Volume 2: Laboratory Animal Facilities,* edited by T.Ruys, pp. 398–405. Van Nostrand Reinhold, New York.
281. Ruys, T. (1991): The effect of animal species and types of the design of animal facilities. In: *Handbook of Facilities Planning, Volume 2: Laboratory Animal Facilities,* edited by T.Ruys, pp. 55–59. Van Nostrand Reinhold, New York.
282. Ruys, T. (1991): Waste. In: *Handbook of Facilities Planning, Volume 2: Laboratory Animal Facilities,* edited by T.Ruys, pp. 241–244. Van Nostrand Reinhold, New York.
283. Sales, G.D. (1991): The effect of 22 kHz calls and artificial 38 kHz signals on activity in rats. *Behav. Processes,* 24:83–93.
284. Sales, G.D., Wilson, K.J., and Spencer, K.E.V. (1988): Environmental ultrasound in laboratories and animal houses: A possible cause for concern in the welfare and use of laboratory animals. *Lab. Animals,* 22:369–375.
285. Saltarelli, D.G., and Coppola, C.P. (1979): Influenced of visible light on organ weights of mice. *Lab. Anim. Sci.,* 29:319–322.
286. Sanhouri, A.A., Jones, R.S., and Dobson, H. (1989): The effects of different types of transportation on plasma cortisol and testosterone concentrations in male goats. *Br. Vet. J.,* 145:446–450.
287. Sansone, E.G., and Losikoff, A.M. (1979): Potential contamination from feeding test chemicals in carcinogen bioassay research: Evaluation of single- and double-corridor animal housing facilities. *Toxicol. Appl. Pharmacol.,* 50:115–121.
288. Sansone, E.G., Losikoff, A.M., and Pendleton, R.A. (1977): Potential hazards from feeding test chemicals in carcinogen bioassay research. *Toxicol. Appl. Pharmacol.,* 39:435–450.
289. Schnurrenberger, P.R., and Hubbert, P.R. (1981): *An Outline of Zoonoses.* Iowa State University Press, Ames, Iowa.
290. Schofield, J.C. (1994): Principles of aseptic technique. In: *Essentials for Animal Research: A Primer for Research Personnel,* edited by B.T.Bennett, M.J.Brown, and J.C.Schofield, pp. 59–77. National Agricultural Library, Washington, DC.
291. Schonholtz, G.J. (1976): Maintenance of aseptic barriers in the conventional operating room. J. *Bone and Joint Surg.,* 58-A:439–445.
292. Schroeder, H.A., Balassa, J.J., and Vinton, W.H., Jr. (1965): Chromium, cadmium and lead in rats: Effects on life span, tumors and tissue levels. *J. Nutr.,* 86:51–66.
293. Selwyn, M.R., and Shek, W.R. (1994): Sample sizes and frequency of testing for health monitoring in barrier rooms and isolators. *Cont. Topics,* 33:56–60.
294. Semple-Rowland, S.L., and Dawson, W.W. (1987): Retinal cyclic light damage threshold for albino rats. *Lab. Anim. Sci.,* 37:289–298
295. Serrano, L.J. (1971): Carbon dioxide and ammonia in mouse cages: Effect of cage covers, population and activity. *Lab. Anim. Sci.,* 21:75–85.
296. Silverman, J., and Adams, J.D. (1983): N-nitrosamines in laboratory animal feed and bedding. *Lab. Anim. Sci.,* 33:161–164.
297. Simmonds, R.C. (1991): Characteristics of laboratory animal facilities. In: *Handbook of Facilities Planning, Volume 2: Laboratory Animal Facilities,* edited by T.Ruys, pp. 1–33. Van Nostrand Reinhold, New York.
298. Simmons, M.L., Robie, D.M., Jones, J.B., and Serrano, L.J. (1968): Effect of a filter cover on temperature and humidity in a mouse cage. *Lab. Anim.,* 2:113–120.
299. Soma, L.R. (1987): Assessment of animal pain in experimental animals. *Lab. Anim. Sci.,* 37:71–74.
300. Stoskopf, M.K. (1983): The physiological effects of psychological stress. *Zoo Biology,* 2:179–190.
301. Swanson, M.C., Campbell, A.R., O'Hollaren, M.T., and Reed, C. E. (1990): Rate of ventilation, air filtration, and allergen product rate in determining concentrations of rat allergies in the air of animal quarters. *Am. Rev. Respir. Dis.,* 141:1578–1581.
302. Tannebaum, A. (1942): The genesis and growth of tumors. II: Effect of caloric restriction per se. *Cancer Res.,* 2:460–467.
303. Tannenbaum, A., (1942): The genesis and growth of tumors. III: Effects of a high-fat diet. *Cancer Res.,* 2:468–475.
304. Tannenbaum, A. (1945): The dependence of tumor formation on the degree of caloric restriction. *Cancer Res.,* 5:609–615.
305. Tannenbaum, A. (1945): The dependence of tumor formation on the composition of the calorie-restricted diet as well as on the degree of restriction. *Cancer Res.,* 5:616–625.

306. Tannebaum, A. (1959): Nutrition and cancer. In: *The Physiopathology of Cancer 2nd edition*, edited by F.Homberger, pp. 517–562. Hoeber-Harper, New York.
307. Teelman, K., and Weihe, W.H. (1974): Microorganism counts and distribution patterns in air-conditioned animal laboratories. *Lab. Anim.*, 8:109.
308. The Animal Welfare Act of 1966. PL 89–544; as amended by Animal Welfare Act of 1970. PL 91–579; by the 1976 Amendments to the Animal Welfare Act. PL 94–297; and by the 1985 Food Security Act. PL 99–198.
309. Thigpen, J.E., Lebetkin, E.H., Dawes, M.L., Clark, J.L., Langely, C.L., Amyx, H.L., and Crawford, D. (1989): A standard procedure for measuring rodent bedding particle size and dust content. *Lab. Anim. Sci.*, 39:60–62.
310. Thigpen, J.E., Lebetkin, E.H., Dawes, M.L., et al. (1989): The use of dirty bedding for detection of murine pathogens in sentinel mice. *Lab. Anim. Sci.*, 39:324–327.
311. Thompson, R. (1971): The water consumption and drinking habits of a few species and strains of laboratory animals. *J. Inst. Anim. Technol.*, 22:29–36.
312. Torronen, R., Pelkonen, K., and Karenlampi, S. (1989): Enzyme-inducing and cytotoxic effects of wood-based materials used as bedding for laboratory animals: Comparison by a cell culture study. *Life Sci.*, 45:559–565.
313. Toth, L.A., and January, B. (1990): Physiological stabilization of rabbits after shipping. *Lab. Anim. Sci.*, 40:384–387.
314. Totton, M. (1958): Ringtail in new-born Norway rats: A study of the effect of environmental temperature and humidity on incidence. J. *Hyg.*, 56:190–196.
315. Trexler, P.C. (1987): Animals of defined microbiological status: Animal production and breeding methods. In: *The UFA W Handbook on the Care and Management of Laboratory Animals, 6th edition*, edited by T.B.Poole, pp. 85–98. Longman Scientific and Technical, London.
316. Tucker, H.A., Petitclere, D., and Zinn, S.A. (1984): The influence of photoperiod on body weight gain, body composition, nutrient intake and hormone secretion. *J. Anim. Sci.*, 59:1610–1620.
317. Tucker, M.J. (1979): The effect of long-term food restriction on tumours in rodents. *Int. J. Cancer*, 23:803–807.
318. Tuli, J.S., Smith, J.A., and Morton, D.B. (1995): Stress measurements in mice after transportation. *Lab. Anim.*, 29:132–138.
319. Turnbull, G.J., Lee, P.N., and Roe, F.J.C. (1985): Relationship of body-weight gain to longevity and to risk of development of nephropathy and neoplasia in Sprague-Dawley rats. *Food Chem. Toxicol.*, 23:355–361.
320. Vesell, E.S. (1967): Induction of drug-metabolizing enzymes in liver microsomes of mice and rats by softwood bedding. *Science*, 157:1057–1058.
321. Vesell, E.S., Lang, C.M., White, W.J., Passananti, G.T., Hill, R. N., Clemens, T.L., Liu, D.K., and Johnson, W.D. (1976): Environmental and genetic factors affecting the response of laboratory animals to drugs. *Fed. Proc.*, 35:1125–1132.

322. Vesell, E.S., Lang, C.M., White, W.J., Passananti, G.T., and Tripp, S.L. (1973): Hepatic drug metabolism in rats: Impairment in a dirty environment. *Science*, 179:896–897.
323. Vlahakis, G. (1977): Possible carcinogenic effects of cedar shavings in bedding of C3H-AvyfB mice. J. *Natl. Cancer Inst.*, 58:149–150.
324. Wade, A.E., Holl, J.E., Hilliard, C.C., Molton, E., and Greene, F. E. (1968): Alteration of drug metabolism in rats and mice by an environment of cedarwood. *Pharmacology*, 1:317–328.
325. Waggie, K., Kagiyama, N., Allen, A.M., and Nomura, T. (1994): *Manual of Microbiologic Monitoring of Laboratory Animals, 2nd edition.* NIH Pub. No. 94–2498, U.S. Department of Health and Human Services, Washington, DC.
326. Wallace, M.E. (1976): Effect of stress due to deprivation and transport in different genotypes of house mouse. *Lab. Anim.*, 10:335–347.
327. Wardrip, C.L., Artwohl, J.E., and Bennett, B.T. (1994): A review of the role of temperature versus time in effective cage sanitation program. *Contemp. Topics*, 33:66–68.
328. Warfield, D. (1973): The study of hearing in animals. In: *Methods of Animal Experimentation, IV*, edited by W.Gay, pp. 43–143. Academic Press, London.
329. Wassermann, M., Wassermann, D., Gershon, Z., and Zellermayer, L. (1969): Effects of organochlorine insecticides on body defense systems. *Ann. N.Y. Acad. Sci.*, 160:393–401.
330. Wattenberg, L.W. (1975): Effects of dietary constituents on the metabolism of chemical carcinogens. *Cancer Res.*, 35:3326–3331.
331. Wax, T.M. (1977): Effects of age, strain, and illumination intensity on activity and self-selection of light-dark schedules in mice. *J. Comp. Physiol. Psychol.*, 91:51–62.
332. Waynforth, H.B. (1980): *Experimental and Surgical Technique in the Rat.* Academic Press, London.
333. Waynforth, H.B. (1987): Standards of surgery for experimental animals. In: *Laboratory Animals: An Introduction for New Experimenters*, edited by A.A.Tuffery, pp. 311–312. Wiley-Interscience, Chichester.
334. Weichbrod, R.H., Cisar, C.F., Miller, J.G., Simmonds, R.C., Alvares, A.P., and Ueng, T.H. (1988): Effects of cage beddings on microsomal oxidative enzymes in rat liver. *Lab. Anim. Sci.*, 38:296–298.
335. Weichbrod, R.H., Hall, J.E., Simmonds, R.C., and Cisar, C.F. (1986): Selecting bedding material. *Lab. Anim.*, 15(6):25–29.
336. Weihe, W.H. (1965): Temperature and humidity climatograms for rats and mice. *Lab. Anim. Care*, 15:18–28.
337. Weihe, W.H. (1973): The effect of temperature on the action of drugs. *Annu. Rev. Pharmacol.*, 13:409–425.
338. Weihe, W.H. (1976): The effect of light on animals. In: *Control of the Animal House Environment: Laboratory Animal Handbooks, Vol. 7*, edited by T.McSheehy, pp. 63–76. Trevor Laboratory Animals, London.
339. Weihe, W.H., Schidlow, J., and Strittmatter, J. (1969): The effect of light intensity on the breeding and development of rats and golden hamsters. *Int. J. Biometerol.*, 13:69–79.
340. Weindruch, R., and Walford, R.L. (1988): *The Retardation of Aging and Disease by Dietary Restriction.* Charles C.Thomas, Springfield, Illinois.
341. Weis, I., Stotzer, H., and Seitz, R. (1974): Age- and light-dependent changes in the rat eye. *Vichows Arch. A Pathol. Anat. Histopathol.*, 362:145–156.
342. Wesibroth, S.H., Peters, R., Riley, L.K., and Shek, W. (1998): Microbiological assessment of laboratory rats and mice. *ILAR Jour.*, 39:272–290.
343. White, W.J. (1990): The effect of cage space and environmental factors. In: *Guidelines for the Well-being of Rodents in Research*, edited by H.N.Guttman, pp. 29–45. Scientists Center for Animal Welfare, Bethesda, Maryland.
344. White, W.J., Anderson, L.C., Geistfeld, J., and Martin, D.C. (1998): Current strategies for controlling/eliminating opportunistic microorganisms. *ILAR Jour.*, 39:291–305.
345. White, W.J., and Blum, J.R. (1997): Design of surgical suites and postsurgical care units. In: *Anesthesia and Analgesia in Laboratory Animals*, edited by D.F.Kohn, S.K.Wixson, W.J.White, and G. J.Benson, pp. 149–163. Academic Press, New York.
346. White, W.J., and Field, K.J. (1987): Anesthesia and surgery of laboratory animals. *Vet. Clin. North Am.*, 17:989–1017.
347. White, W.J., Hughes, H.C., Singh, S.B., and Lang, C.M. (1983): Evaluation of a cubicle containment system in preventing gaseous and particulate airborne cross-contamination. *Lab. Anim. Sci.*, 33:571–576.
348. Whyte, W. (1988): The role of clothing and drapes in the operating room. J. *Hosp. Inf.*, 11(Suppl

C): 2–17.
349. Whyte, W., and Shaw, B.H. (1974): The effect of obstructions and thermals in laminar-flow systems. *Journal of Hygiene*, 72:415–423.
350. Williams, G.M. (1984): The significance of environmental chemicals as modifying factors in toxicity studies. In: *Concepts in Toxicology, Volume 1: Toxicology Laboratory Design and Management for the 80's and Beyond*, edited by A.S.Tegeris, pp. 14–19. S.Kargar AG, Basel.
351. Wise, A. (1982): Interaction of diet and toxicity: The future role of purified diet in toxicological research. *Arch. Toxicol.*, 50:287–299.
352. Wise, A., and Gilburt, D.J. (1980): The variability of dietary fiber in laboratory animal diets and its relevance to the control of experimental conditions. *Cosmet. Toxicol.*, 18:643–648.
353. Wise A., and Gilbert, D.J. (1981): Variation of minerals and trace elements in laboratory animal diets. *Lab. Anim.*, 15:299–303.
354. Woods, J.E. (1975): Influence of room air distribution on animal cage environments. *ASHRAE Trans.*, 81:559–571.
355. Woods, J.E., Nevins, R.G., and Besch, E.L. (1975): Experimental envaluation of heat and moisture in metal dog cage environments. *Lab. Anim. Sci.*, 25:425–433.
356. Wostman, B.S. (1975): Nutrition and metabolism of the germfree mammal. *World Rev. Nutr. Diet*, 22:40–92.
357. Yu, B.P. (1990): Food restriction research: Past and present status. *Rev. Biol. Res. Aging*, 4:349–371.
358. Zondek, B., and Tamari, I. (1964): Effect of audiogenic stimulation on genital function and reproduction. III: Infertility induced by auditory stimuli prior to mating. *Acta Endocrinol.*, 45(Suppl. 90):227–234.

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Chapter **17**

Genetic Toxicology

David Brusick

Principles and Methods of Toxicology,
Fourth Edition, edited by A.Wallace Hayes.
Taylor & Francis, Philadelphia © 2001.

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Genetic toxicology, as a subspecialty of toxicology, involves the identification and analysis of agents with toxicity directed toward the hereditary components of living organisms. A large proportion of human disease is either directly or indirectly associated with genetic dysfunction. Many agents are able to produce genetic damage at high exposure concentrations following the induction of acute, nonspecific cytotoxic effects across a wide range of cellular processes, however, the ultimate objectives of genetic toxicologists are to detect and access genetic hazard from agents that are highly specific for nucleic acids and are capable of producing genetic damage at subtoxic concentrations. Such agents are classified as *genotoxic*.

The term *genotoxic* is a general descriptor and is used to distinguish chemicals that have an intrinsic affinity for DNA from those that do not. Genotoxic substances have several common chemical or physical properties facilitating their interaction with nucleic acids (i.e., electrophilicity). A report of the International Commission for Protection Against Environmental Mutagens and Carcinogens (ICPEMC) provides a more detailed definition of genotoxic and emphasizes that categorization of a chemical as genotoxic is not a priori indication of a health hazard (46).

Mutagens comprise a subset of genotoxicants and are characterized by their ability to induce specific classes of stable changes in (a) the nucleotide sequence of genes, (b) chromosome structure, or (c) chromosome number. Changes in nucleotide sequence are described as point mutations, and chromosomal damage is referred to as clastogenicity. These two major classes of genetic damage are responsible for a large proportion of the array of human genetic diseases and contribute significantly to congenital malformations.

Because the original goal of genetic toxicology was to protect the human gene pool from increases in

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mutational load, the discipline of genetic toxicology initially focused on transmissable damage. Test methods employed during this early period were primarily in vivo and focused on measuring damage to mammalian germ cells. Later, reports from several independent groups of investigators were published showing a correlative relation between mammalian carcinogens and mutagens (3, 14, 18, 60, 93).

Table 17.1 Basic biochemical characteristics of all double-standard DNA

DNA consists of two purines (guanine, adenine) and two pyrimidines (thymine and cytosine).

A nucleotide pair consists of one purine and one pyrimidine (adenine/thymine (AT) or guanine/cytosine (GC)).

Nucleotide pairs are connected to a double helix molecule by sugar-phosphate backbone linkages and hydrogen bonding.

The AT base pair is held by two hydrogen bonds and the GC is held by three hydrogen bonds (see Figure 17.2).

The distance between each base pair in a molecule is 3.4 Å, producing 10 nucleotide pairs per turn of the DNA helix.

The number of adenine molecules must equal the number of thymine molecules in a DNA molecule. The same relation exists for guanine and cytosine molecules. The ratio at AT to GC base pairs, however, may vary in DNA from species to species.

The two strands of the double helix are complementary and antiparallel with respect to the polarity of the two sugar-phosphate backbones, one strand being 3′-5′ and the other 5′-3′ with respect to the terminal OH group on the ribose sugar.

DNA replicates by a semiconservative method in which the two strands separate, and each is used as a template for the synthesis of a new complementary strand.

The rate of DNA nucleotide polymerization during replication is approximately 600 nucleotides per second. The helix must unwind to form templates at a rate of 3600 rpm to accommodate this replication rate.

The DNA content of cells is variable (1.8×109 daltons for *Escherichia coli* to 1.9×1011 daltons for human cells).

Thus, genetic toxicology has evolved to play a dual role in safety evaluation programs. One role is the implementation of testing and risk assessment methods to determine the impact of genotoxic agents found in the environment on the integrity of the human gene pool. The second role is the application of genetic test methods to the detection and mechanistic understanding of carcinogenic processes.

BASIC GENETIC CONCEPTS

Gene Structure

The hereditary informational molecules of all living systems, with the exception of some viruses that use RNA, are composed of DNA, and those organisms which store their hereditary information in RNA go through a DNA intermediate during replication. Some common characteristic features of DNA molecules are listed in Table 17.1. The mechanisms of information storage and gene expression are similar for all organisms with DNA composition.

The simplest complete functional unit in a DNA molecule is termed a *gene*. Most of the early knowledge concerning structure and operation of genes was acquired from studies with bacteria or bacteriophages. During the past 10 years, development in molecular biology of mammalian cells has resulted in equivalent information in this cell type. The differences between the genes of prokaryotic (bacteria) and eukaryotic (plant and animal cells) organisms center primarily on the number, location of the respective chromosome, and mechanisms of gene regulation (Table 17.2). In prokaryotic cells there is a single *chromosome* with little or no differentiation along the DNA molecule so far as function is concerned. Eukaryotic cells, on the other hand, have DNA with nonfunctional, repeated sequences of some genes; these cells also have regions of noncoding DNA, called *introns*, inserted between coding sequences called *exons*. The exact function of repeated DNA sequences and intron regions is not known.

The nucleotide composition and the mechanisms by which information encoded in a gene is transformed into gene products is universal. Universality was confirmed by recombinant DNA genetic engineering studies in which genes continue to function properly after having been transplanted from human cells to bacterial cells or from bacterial cells to plant cells (15, 53).

In eukaryotic cells, the process of gene expression follows the pattern shown in Figure 17.1. Enzymes located in the nucleus of the cell excise intron regions and splice the coding sequence back together. The resulting mRNA is then transported to ribosomes outside the nucleus for translation. Intron regions are not present in prokaryotic cells, and the gene is read in one sequence. DNA repair processes are also influenced by transcription activity. Important to a full appreciation of the functions of genes coding for structural proteins and enzymes is the identification of regulatory genes that control gene expression.

Somatic and Germ Cell Characteristics

From a genetic perspective, multicellular eukaryotic organisms are composed of two cell types: somatic cells and germ cells. Somatic cells constitute the major portion

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Table 17.2 Characteristics of DNA in prokaryotic and eukaryotic cell types

Prokaryotic Cells	Eukaryotic Cells
Primarily haploid	Primarily diploid
DNA uncomplexed	DNA complexed with proteins forming chromosomes
DNA nonlocalized in the cell cytoplasm	DNA localized primarily within the nucleus of the cell
No morphologic stages in DNA replication	DNA replication described by mitotic cycle consisting of specific cytologic stages
DNA often found as a closed circle	DNA found in linear chromosomes
Replication not associated with cellular organelles	Replication and separation of chromosome associated with cellular organelles called centrioles
All genes encoded in the DNA are functional	Repetitive, nonfunctional gene sequences are common
Spacer sequences have not been identified	Noncoding spacer sequences identified as introns occur along the DNA molecule

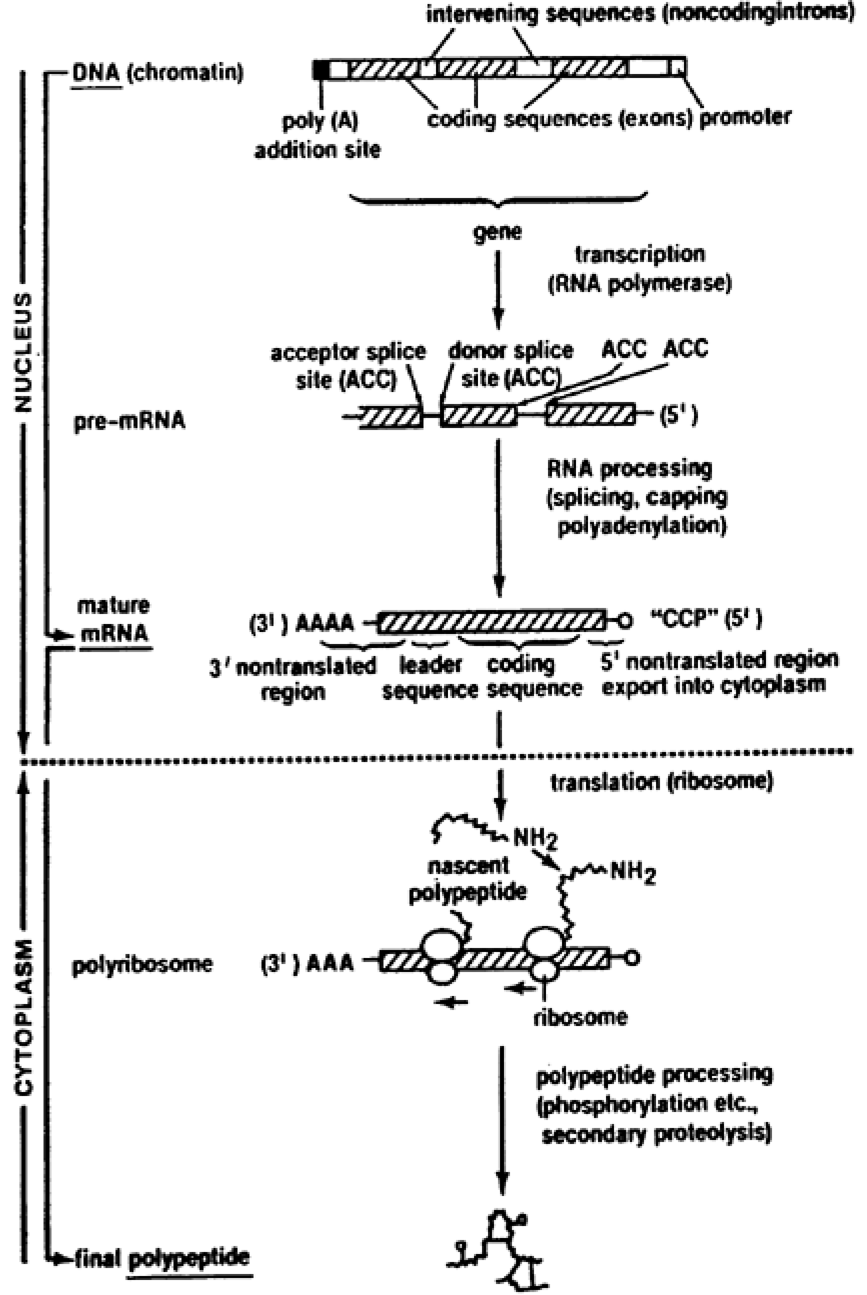


FIG. 17.1 Transcription and translocation of mammalian genes.

of the mammalian organism. The *genomes* of most somatic cells are *diploid* (two complete sets of chromosomes), and genetic alterations in somatic cells are not transmissible to subsequent generations. Virtually all in vitro mammalian cell assays used in genetic toxicology employ somatic cell types. Germ cells (sperm and eggs) are a special cell population in multicellular organisms. Their function is to form the next generation. Mutations carried in these cells produce a broad array of heritable genetic diseases, congenital malformations, and other disorders. Germ cells are derived from diploid stem cells in gonadal tissues following meiosis and carry a *haploid* set of chromosomes. *Mutations* carried in germ cells are classified as recessive or dominant, dependent on their expression in the diploid state. A large proportion of human genetic diseases are associated with recessive mutant genes that are expressed only when two mutant alleles (one contributed by each parent) are present in the homozygous condition. Recessive mutations are maintained at a constant level in the gene pool in the heterozygous state and are carried in that configuration by individuals who appear phenotypically normal. The inability to identify heterozygous from normal individuals is one of the primary reasons that the human genetic burden has not been reduced. Table 17.3 summarizes the spectra of mutations in humans for germ cell and somatic cell effects.

DNA ALTERATIONS RESULTING IN GENOTOXIC EFFECTS IN CELLS

DNA synthesis and replication are not flawless processes, and in rare instances, genetic alterations occur

Table 17.3 Mutational spectrum in humansa

Mutation Type	Examples of Inherited Effects	Examples of Somatic Effects
Single base changes	Sickle cell disease, Phenylketonuria	Epithelial cancers, activation of *ras* oncogenes
Small deletions and/or translocations	Haemophilias, Duchenne muscular dystrophy	Lymphomas, leukemias, enhanced activation of *myc, abl* oncogenes
Whole chromosome losses or gains	Down's syndrome, Turner's syndrome	Loss of tumor suppressor genes, retinoblastoma, Wilms' tumor, breast cancer

a From Reference 35.

spontaneously during normal cell division. In addition, endogenous oxidative metabolism produces reactive species capable of damaging DNA. The occurrence of intrinsic damage follows a predictable rate per gene and forms the basis of "background" or "spontaneous" mutation frequencies. In addition to errors in replication or repair, the origin of spontaneous mutations may arise from unavoidable environmental exposures (e.g., background radiation, diet).

Classification Scheme for Genotoxic Effects

DNA damage may be classified into several broad categories based on the nature (presumed mechanism) of the DNA change. The following is one type of such classification:

A. DNA disruption damage involves the breakage of and/or interchange of DNA segments between chromosomal structures. This type of damage may be visible through cytologic analysis of condensed chromosomes. Although genotoxicants such as alkylating agents induce DNA disruption damage, a characteristic of DNA disruption damage is that it may also be caused by secondary mechanisms (e.g., processes that result in cell stress such as high temperature) that do not target nucleic acids specifically (43, 16).

B. DNA microlesions are nonvisible alterations occurring at the nucleotide level. Nucleotide damage generally produces point mutations through base-pair substitution or insertion/deletion or it may induce recombination between sister chromatids. Microlesions generally are induced by agents that specifically target nucleic acids (e.g., electrophilic agents).

In addition, some genotoxic effects may be induced by other mechanisms that do not fall readily into either of the two mechanisms defined previously. Examples of such genotoxic damage are aneuploidy/polyploidy that may be caused by damage to chromosome movement proteins and morphological cell transformation resulting from altered gene expression patterns.

Processes Leading to Mutation

The expected DNA base pairings are shown in Figure 17.2: adenine and thymine form two hydrogen bonds, and guanine and cytosine form three. Hydrogen bonds are weak electrostatic forces involving oxygen and nitrogen atoms at specific sites on the purine and pyrimidine molecules. If electrophilic chemical species covalently bind to portions of the DNA bases involved in the formation of hydrogen bonds (Figure 17.3), these covalently bound species *(adducts)* can produce electron shifts from the H-bonding sites to areas within the molecules, giving opportunities for short-lived mispaired bases (e.g., A:C

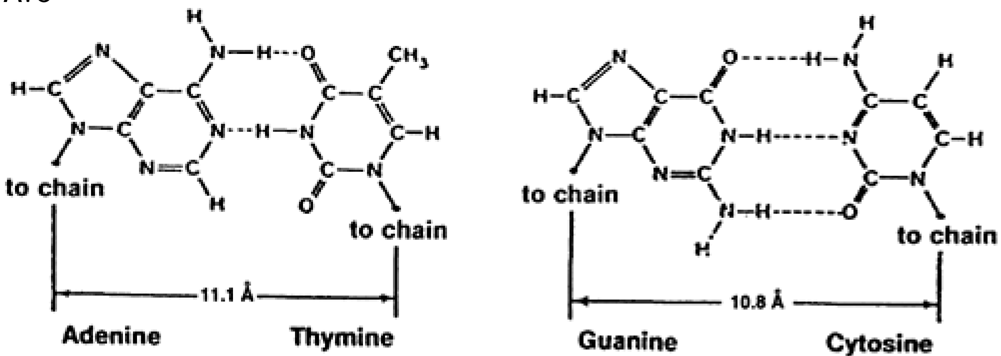


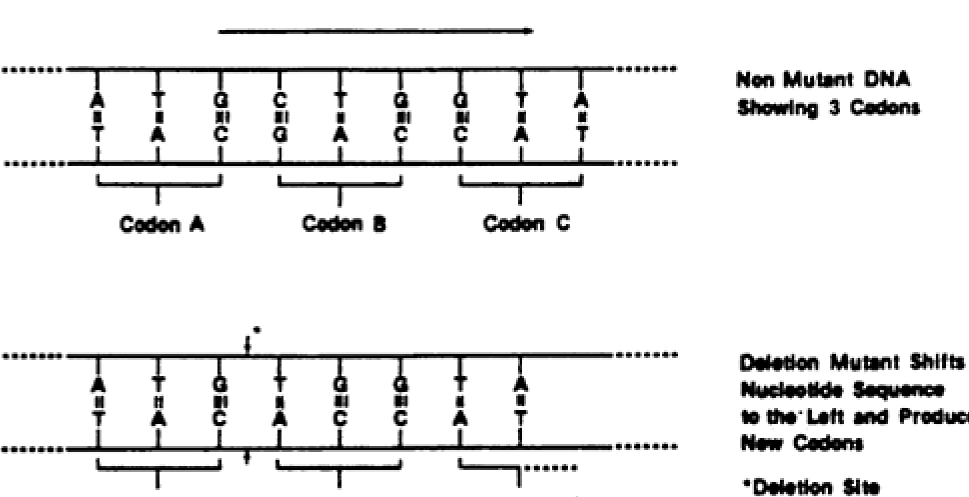
FIG. 17.2. Hydrogen bonding of nucleotides normally found in DNA.

FIG. 17.3. Two examples of DNA chemical adducts. (Top) an aromatic amine adduct to guanine. (Bottom) a benzo[a]pyrene metabolite adduct to guanine.

or G:T). If this mispairing occurs before or during a DNA replication cycle, the result may be the substitution of an incorrect base pair for the original pair. The cycle of DNA replication to fix base-pair substitution mutations is called the "expression period" in in vitro mutation assays.

Base-pair addition-deletion mutations, also called *frameshift mutations*, result from the addition or deletion of one or a few nucleotide pairs from the nucleotide sequence in an exon or gene. Because the codon sequence reads in one direction and is nonpunctuated, the loss or gain of a single base pair changes the reading frame of the gene—hence frameshift mutation. This type of mutagenic mechanism is illustrated in Figure 17.4. Both frameshift and *basepair substitution* gene mutations result in alterations in translation of mRNA into the proper sequence of amino acids in the gene products (See Figure 17.1) and produce a mutant cell or organism. Mutations or DNA methylation in regulatory genes are capable of modulating the production of functional gene products (44).

DNA is visible microscopically as a chromosome. Figure 17.5 illustrates the generalized anatomy of a chromosome. The chromosome illustrated is typical of a metaphase structure and the anatomy shows distinctive bands as a result of giemsa staining. Mammalian chromosomes have a constriction known as the *centromere*. To either side of the centromere are the chromosome arms that terminate in structures called *telomeres*. The dark G+ bands represent highly condensed DNA and are areas of little or no gene expression (transcription). The light G− bands represent active genomic areas where gene expression (transcription) is occurring. The telomeric regions at the ends of the chromosome struc



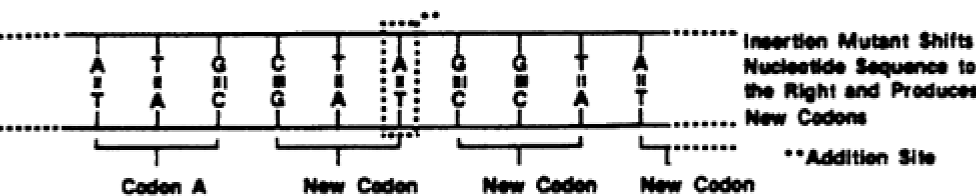


FIG. 17.4. The base pair changes involved with frameshift mutations. An insertion or deletion of a base pair results in the shift of the gene or exon reading frame.

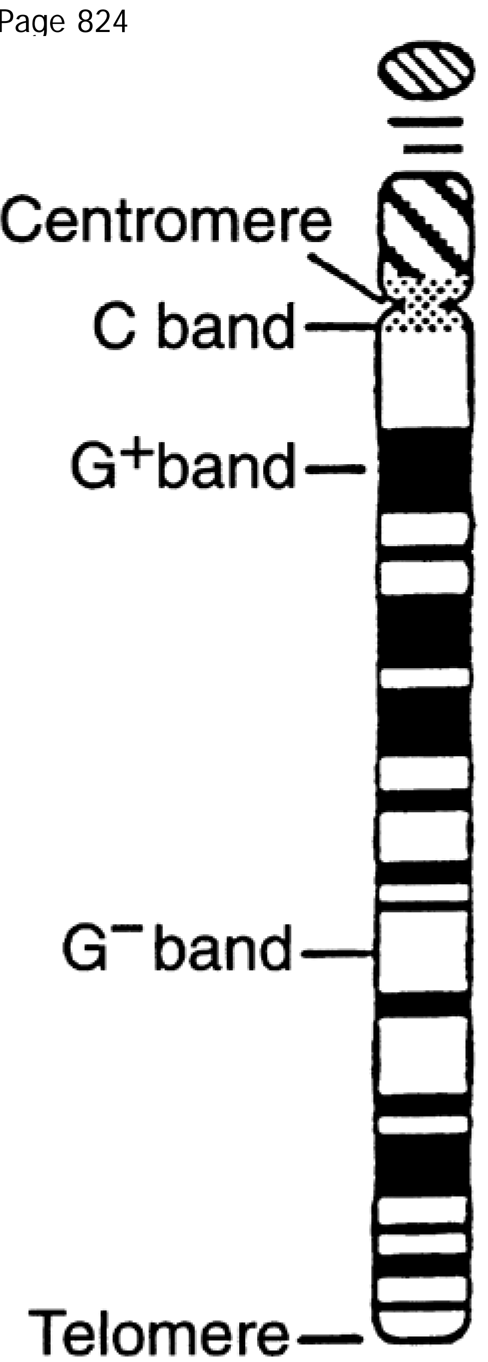


FIG. 17.5. The anatomy of a typical chromosome to show active (unstained) and inactive (stained) regions.

tures are important for chromosome stability. Telomeres consist of repeat units of a small number of nucleotides that stabilize the linear DNA molecule. At each cell division, chromosomes lose small amounts of telomeric DNA. The ability of the cell to replace the lost DNA with telomerase determines whether the cell continues to proliferate or, if the DNA loss reaches a critical level, stops dividing (28). Microscopically visible DNA disruption damage is subdivided into changes in chromosome number (gain or loss of single chromosomes or sets of chromosomes) and changes in chromosome structure (breaks, deletions, rearrangements). Each type of chromosome change has a characteristic morphometric description so that a reasonably high degree of uniformity can be maintained when scoring them microscopically. Variations in chromosome number can result from incomplete dissociation of single or entire sets of chromosomes at metaphase, resulting in aneuploidy or polyploidy (34). A wide range of in vitro and in vivo genetic test methods are designed to assess chromosome breakage and numerical changes. Chromosome breakage can be induced by several mechanisms (e.g., disruption of DNA synthesis or DNA cross-linking) that result in double-strand breaks. In addition to the tests that

specifically measure nucleotide substitutions and chromosome alterations, a third group of tests to measure other mechanisms of genotoxicity has been employed in screening. Included in this group were tests for DNA binding and repair, DNA strand breakage, sister chromatid exchange (SCE), and mitotic recombination. These methods were categorized as tests for measuring primary DNA damage. Few testing guidelines specify primary DNA damage tests any longer, as these tests have been found to lack adequate specificity and sensitivity. In some instances they may be useful biomarkers of exposure to genotoxicants.

A significant amount of research has focused on the induction and measurement of DNA adducts. The initial hope of this technology was to use adduct formation as a method to demonstrate exposure and calculate cancer risk. To date, that hope has not been fulfilled. Adduct formation remains a sensitive method to demonstrate exposure; however, we still do not know enough about adduct processing and repair to use this information to define genetic or cancer risk except for a small number of agents. Binding sites on DNA vary with the compound and are influenced by complex factors that are not yet understood. What is known is that organs/tissues in mammals have different capacities for repairing adducts and that some adducts, such as the O-alkyl adducts, are repaired inefficiently and probably produce most of the mutagenic damage responsible for tumor cell initiation.

Repair of DNA Damage

The fidelity and integrity of genetic information in organisms are maintained by several types of enzymatic DNA repair. The characteristic of self repair is unique to DNA and illustrates how important the integrity of this molecule is to the survival of an organism. Table 17.4 summarizes the major classes of DNA repair that exist in virtually all higher organisms. DNA repair kinetics are variable, but, in general, highest rates of DNA repair are associated with gene transcription processes (i.e., transcription-coupled-repair (TCR)).

DNA damage is induced by environmental agents and normal cellular proceses of DNA replication or oxidative stress. Some estimates of endogenous damage and repair capacities for human cells are given in Table 17.5. As indicated, endogenous cellular repair capacities are more than adequate to compensate for background DNA

Table 17.4 Classification and properties of DNA repair processes

Class	Properties
Base excision repair	Elimination of single nucleotide through cleavage of the glycosyl bond connecting the altered base to the deoxyribose sugar, resulting in an abasic site in the DNA followed by resynthesis using the opposite strand as template.
Nucleotide excision repair	Removal of bulky DNA adducts from DNA. Process involves 20 proteins that remove up to 100 nucleotides associated with damaged region. Repair synthesis using the opposite strand as template fills in repair patch.
Mismatch repair	A "second chance" repair system that corrects mismatch base pairs post-DNA replication. This process catches lesions missed by nucleotide excision repair and base excision repair (BER) processes.
Recombinational repair	This process acts on double-strand DNA breaks and/or DNA crosslinks, where both DNA strands are damaged.

damage; however, massive or frequent exposures to exogenous genotoxic agents may saturate the DNA repair capacity leading to human genetic disease, aging, and cancer (83).

The most important feature of most repair processes is the ability to enzymatically remove and replace damaged segments of DNA with higher fidelity. If a DNA lesion can be repaired prior to mutation fixation, the net effect of the DNA damage to an organism may be nil. This is especially true following intermittent, low-level exposures to genotoxicants, where repair enzymes are not fully saturated by excessive numbers of damaged DNA sites. DNA adducts are not all recognized or repaired equally by excision repair. The size and effect that an adduct has on the conformation of DNA determines how readily it is detected by the repair enzymes and is excised. As a consequence of this situation, it is not advisable to use the number of adducts per cell as a predictor of damage or genetic hazard unless definitive information is available about the elimination kinetics for the specific adducts (99).

Test systems measuring some parameter of the DNA repair process have been used as genotoxicity screens for detection of primary DNA damage (80, 103). Normal organisms are capable of some type of DNA repair

Table 17.5 Estimates of human endogenous DNA damage and repair processes[a]

Type of Damage	Estimated Occurrences of Damage per Hour per Cell[b]	Maximal Repair Rate, Base Pairs per Hour per Cell
Depurination	1000	–[c]
Depyrimidination	55	–[c]
Cytosine Deamination	15	–[c]
Single-stranded breaks	5000	2×10^5
N7-methylguanine	3500	Not reported
O6-methylguanine	130	10^4
Oxidation products	120	10^5

[a] Modified from data in Reference 69.
[b] Might be higher or lower by a factor of 2.
[c] Not reported, but the rates are at least 10^4, based on concentration of repair activities in cell extracts.

activity following chemical insult; thus, stimulation or induction of repair activity following chemical treatment at sublethal concentrations is a good indicator that the target organism has experienced DNA-directed toxicity. These tests are generally identified as measures of unscheduled DNA synthesis (UDS).

Studies of DNA repair kinetics indicate that once permutational lesions have been induced in the DNA, both error-prone and error-free repair processes are activated. Error-prone repair processes generate nucleotide mismatches (i.e., A:C or G:T) that produce mutations de novo. Error-free repair replaces the damaged DNA site with a correct nucleotide sequence. The fidelity of repair depends on the degree to which the two different processes are involved. Factors that determine whether error-prone or error-free pathways predominate include (a) the target species, (b) cell type involved, (c) chemical mutagen, and (d) the specific DNA lesion induced. Some data suggest that the error-free repair pathways predominate at low exposure levels, and error-prone pathways come into play only following saturation of the error-free enzymes.

The basic processes of Nucleotide Excision Repair (NER) and Base Excision Repair (BER), the primary repair mechanisms for chemical damage, are shown in Figure 17.6. The enzyme complex responsible for NER of bulky adducts requires more than 20 proteins and consists of several steps (63). One step is an

endonuclease activity that cleaves the DNA at the site of the damage; an exonuclease cuts out the damaged region, including nucleotides to either side. The correct bases are replaced by a DNA polymerase using an editing function to ensure that the correct bases are incorporated into the repair patch. DNA ligase seals the repair patch. Occasionally, even in error-free repair, incorrect bases are incorporated

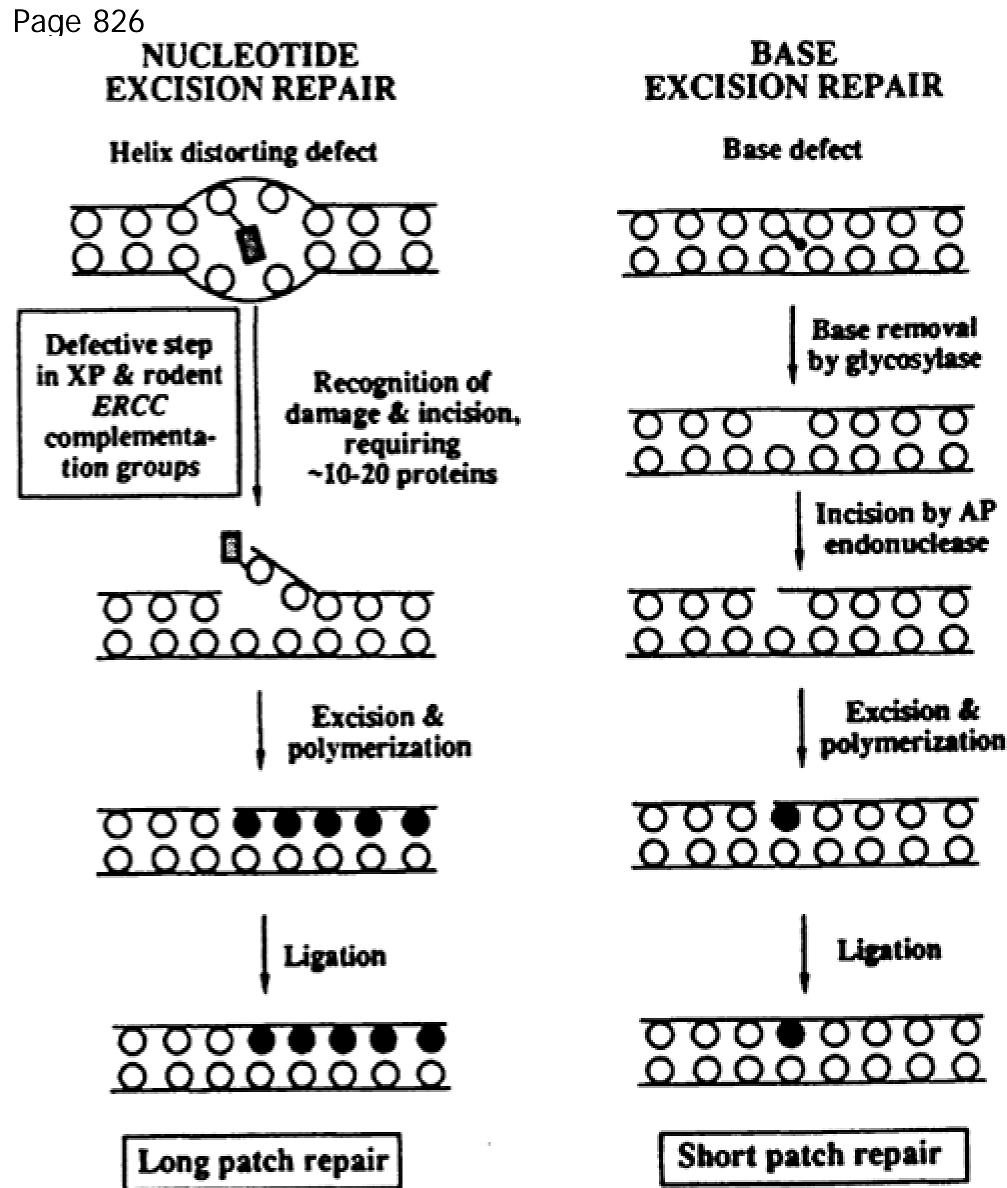


FIG. 17.6. Long and short patch excision repair processes.

by the polymerase, resulting in mismatched bases that do not properly hydrogen bond. In BER, a number of different glycosylases catalyze release of the inappropriate base, followed by replacement and ligation (38).

Mismatch repair (MR) enzymes recognize nonhydrogen bonding base pairs. A short segment of the DNA duplex is excised and filled by the repair polymerase. This is a second-chance repair process that occurs after BER and NER and improves the accuracy of those processes. Finally, recombinational repair acts on double-strand breaks and interstrand cross links, resulting in damage to both strands of the DNA. Collectively, these processes maintain integrity of DNA against endogenous and exogenous sources of damage.

Repair of mutational damage has been shown to be inducible by low-level exposures to DNA-damaging agents (79, 105). Inducibility of repair processes above constitutional levels will increase the magnitude of exposure required to exceed the intrinsic capacity producing a “threshold” for mutation and produce a protective effort for subsequent exposures. Theoretical assumptions and data from studies of repair support the belief that at background or low-exposure levels, an error-free removal of alkyl groups from DNA can be virtually 100% effective. Thus, one observes survival shoulders and nonlinear kinetics for mutation induction in repair-proficient cells and the loss of apparent “no effect” regions in repair-deficient cells.

Because of the influence that species- and cell-specific genetic background have on repair capacity, an appreciation of the variability of DNA repair in somatic and germ cells of humans as well as the animal

models is essential in assessing genetic risk (36, 71). Several general characteristics of mammalian DNA repair are summarized in Table 17.6.

RELATIONSHIP OF GENOTOXIC DAMAGE TO TOXICITY

The proposed sequence of events from DNA interaction to the expression of human toxicity is represented

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Table 17.6 DNA repair characteristics

DNA repair characteristics
DNA repair is tissue- and species-specific (e.g., human capacity approximately 10-fold greater than mouse)
DNA repair is increased in genes involved in transcription (e.g., chromosome loops)
DNA adducts are repaired with different efficiency (e.g., bulky >small)
Effects of DNA repair are tied closely to cell stringency and apoptosis (e.g., cell lines with low cycling stringency have higher error rates)

GENOTOXIC AGENT
↓ ACTIVATION
DNA ADDUCT
↓ REPAIR ↗
PREMUTATIONAL LESION
↓ REPAIR ↗ FIXATION
MUTATION
↓
HUMAN DISEASE

FIG. 17.7. The hypothetical process from exposure to genotoxin to the induction of genetic disease in humans. Repair processes can eliminate the formation of mutations that produce disease.

in Figure 17.7. The nature of the toxicity or disease is dependent on which cells or tissues and genes are altered by the mutation.

Because of the fundamental role genes play in all aspects of organisms, the mutational basis for many human disorders and anomalies is well documented (61). The genetic disease burden in humans is estimated to be approximately 5% (Table 17.7), and its effects contribute significantly to the health care costs of most developed countries. The origin of the mutations in the gene pool is unknown, but Table 17.8 provides several probable sources. Animal experiments offer convincing evidence that environmental agents are capable of inducing permanent, transmissible mutations in either somatic or germ cells and support the assumption that humans are susceptible to genetic risk factors in the environment (76, 77). Other types of toxic phenomena appear to be determined or influenced by somatic cell genotoxicity. Among these endpoints are the following:

1. Oncogenesis-inherited and induced forms (12, 54).
2. Teratogenesis (49).
3. Sterility or semisterility (23).
4. Atherosclerosis (47).
5. Aging (20).

Tests for DNA binding and repair, point mutation, and clastogenicity have been used to monitor human populations, putatively exposed to genotoxic agents, for evidence of somatic cell genotoxicity (27). In many studies there have been strong associations between somatic cell toxicity and the production of primary DNA damage 127–138. PAH exposure, DNA adducts, and tumor incidences are highly associated in human populations, but DNA adducts are not general predictors of carcinogenesis (57); however, the consistency between cancer and other types of DNA damage, such as chromosome aberrations, SCEs, or micronuclei in humans exposed to carcinogens, is not sufficient for routine human monitoring (11).

Carcinogens and Mutagens

During the early 1970s, the introduction of a *Salmonella* assay detecting reverse mutation, combined with an in vitro metabolic activation system, appeared to offer a rapid, inexpensive solution to the identification of chemical carcinogens (4, 58). The Ames test was the forerunner for an array of submammalian and mammalian cell assays proposed as rapid screens for carcinogens (73, 74). The rationale for the use of these methods consisted of several investigations demonstrating that properties associated with the transformed (malignant) cell phenotype were encoded in the DNA. During the late 1970s, studies were published showing that DNA isolated from transformed mouse cells could be

purified of other contaminants, cut into discrete fragments by bacterial restriction enzymes, transfected into "normal" cells resulting in the conversion of "normal" cells into a transformed phenotype (101). Investigators conducting these experiments ultimately identified a series of genes in the restriction fragments (proto-oncogens) responsible for transforming the cells (12, 75). Proto-oncogens are highly conserved genes found in most eukaryotic organisms, and some, such as the *ras, myc,* and *neu* genes, are known to be activated by base-pair substitution mutations or chromosome aberrations (34). Additional loci have been discovered that code for tumor suppressor molecules. The suppressor molecules, which prevent cell proliferation, become inactive following mutation in the genes coding for them (45). *Oncogenes* and *tumor suppressor* have been studied in humans and in rodents used for experimental carcinogenesis studies (7). It has been established that some strains of animals carry activated oncogenes in their germ line and are predisposed, therefore, to specific tumor types following exposures to promoting agents alone (55). Apoptosis is an important cellular process that functions

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Table 17.7 Examples of genetic disorders in humansa

Category of Genetic Alteration	Estimated Frequency/103 Population	Typical Examplesb
Chromosome abnormalities	6.86	• Down's syndrome (trisomy) • Klinefelter's syndrome (XXY) • Turner's syndrome (XO) • Cri du chat (deletion of chromosome) • Numerous other trisomies (XYY)
Dominant mutations	1.85–2.64	• Familial polyposis (AD) • Neurofibromatosis (AD) • Huntington chorea (AD) • Crouzon's disease • Craniofacial dystosis (AD) • Retinoblastoma (AD) • Anitidia (AD) • Chrondrodystrophy (AD)
Recessive mutations	2.23–2.54 0.78–1.99	• Xeroderma pigmentosum (AR) • Duchenne's muscular dystrophy (XR) • Hemophilia (XR) • Lesch-Nyhan disease (XR) • Sickle cell disease (AR) • Galactosemia (AR) • Phenylketonuria (AR) • Diabetes mellitus (AR) • Fanconi's syndrome (AR) • • Albinism (AR) • Cystic fibrosis (AR)
Polygenic (complex inheritance)	26.00–32.00	• Cleft lip • Anencephaly • Spina bifida • Clubfoot • Idiopathic epilepsy • Congenital heart defects

a From Reference 109.
b AD—autosomal dominant; AR—autosomal recessive; XR—X-linked recessive.

in the immature and adult animals. In adult animals it is responsible for elimination of potentially preneoplastic cells by nuclear degredation (97). The p53 tumor suppressor gene is a major apoptosis-inducing protein. Elimination or reduction in the expression of p53 by mutation is believed to be a critical step in the process of neoplasia.

Support for genotoxicity as a means of carcinogen detection was derived from studies correlating the results of specific genotoxicity tests (or batteries of tests) on chemicals with tumor responses, for the same chemicals, in long-term tests using mice or rats. The animal cancer classifications were usually consensus responses derived from multiple tests employing several rodent species or strains. During the early 1970s, concordance between rodent carcinogenicity and results reported for chemicals in the Ames test ranged from 90% to 95% (89); however, by 1984 the concordance on a wider base of data had dropped to just over 60%. There are several reasons for this reduction in concordance (9); however, the factor that had the most influence was the selection of chemicals

Table 17.8 Origin of mutations in the human gene pool

- The majority are inherited
- A small number arise from spontaneous events that occur during normal DNA replication and repair
- Unavoidable environmental exposures (radiation, products of combustion, mycotoxin, pesticides, manufacturing emissions, etc.)
- Therapeutic treatments that are directly mutagenic (e.g. radiation)
- Successful treatment of individuals with lethal genetic diseases, thereby elevating the gene frequency in the reproducing population

used in the comparisons (17). Reports in the early to mid-1970s showing high concordance employed groups of chemicals highly biased toward electrophilc carcinogens (72). Reports with lower concordance employed groups of chemicals (a) not selected for electrophilicity and (b) with a greater proportion of noncarcinogens. A good comparison of the two approaches is summarized in Table 17.9, which compares the results of the EPA Gene-Tox analysis of concordance and the National Institute of Environmental Health Sciences (NIEHS) National Toxicology Program (NTP) analysis of concordance (52). The EPA Gene-Tox analysis, which demonstrated high positive correlations between mutagens and carcinogens, used published data heavily skewed toward positive responses in both the cancer and genotoxicity tests (Table 17.10). Consequently, it is impossible to exclude chance as an explanation for the high concordance found in the EPA Gene-Tox assessment.

Another outcome of the cancer concordance analyses has been the use of genotoxicity tests to help interpret the mechanism of rodent carcinogens. Carcinogens with positive effects in genetic tests are considered to act through direct genotoxic effects on DNA in contrast to carcinogens with predominantly negative results in genetic tests. Thus, a classification scheme of genotoxic and nongenotoxic carcinogens has been proposed by some individuals as a method to interpret the mechanisms of tumor initiation and aid in the selection of the most appropriate data extrapolation model for cancer risk assessment (84). A genotoxic mechanism implies a no-threshold mechanism, and risk data would be analyzed with more conservative methods than would data from nongenotoxic carcinogens. While conceptually this scheme appears reasonable, a better understanding of carcinogenic processes is necessary to support its general appreciation to risk assessment. With the correlation between carcinogens and mutagens averaging about 70%, the appropriate integration of genetic toxicology results into toxicological assessments is less than certain.

The use of genetic tests as part of an assessment for carcinogenic potential may be justified by mechanistic considerations alone and may not have to be supported by high correllations. New mutation models, such as the in vivo *transgenic* models developed for measuring

Table 17.9 A comparison of the sensitivities and specificities of several short-term tests used in carcinogen screening trialsa

	Sensitivityb (%)		Specificityb (%)	
Assay	Gene Toxc	NTPd	Gene Toxc	NTPd
Ames/*Salmonella*	175/23 (78)	20/44 (45) 66/119 (54)	29/47 (62)	25/29 (86) 51/73 (70)
Mouse lymphoma	45/54 (87)	31/44 (70)	0/5 (0)	13/29 (45)
CHO/HGPRT	40/41 (98)	–	1/1 (100)	–
V79/HGPRT	84/104 (81)	–	3/3 (100)	–
Drosophila SLRL	77/106 (73)	4/18 (22)	9/16 (60)	9/9 (100)
In vitro cytogenetics	40/54 (74)	24/44 (55)	2/6 (33)	20/29 (69)
In vivo cytogenetics	8/9 (89)	9/15 (60)	0/0 (—)	11/12 (92)
In vitro SCE	100/101 (99)	31/44 (70)	0/10 (0)	13/29 (45)
In vivo SCE	21/21 (100)	10/15 (67)	0/0 (—)	5/12 (42)
UDS in hepatocytes	19/22	6/30 (20)	0/0 (—)	13/14 (93)

a From Reference 53
b Sensitivity—proportion of positive results for carcinogens; specificity—proportion of negative results for noncarcinogens.
c Combined results for sufficient and limited evidence carcinogens and noncarcinogens.
d Assumes equivocal evidence compounds are noncarcinogens.

Table 17.10 Relative distribution of positive and negative agents among several types of bioassays reviewed under the EPA Gene-Tox program

	Chemicals with Either Sufficient Negative or Positive Responses	Positive Agents (%)	Negative Agents (%)
Rodent cancer tests	413	351 (85)	62 (15)
Ames test	1262	820 (65)	442 (35)
Mouse lymphoma test	138	114 (83)	24 (17)
In vitro cytogenetics	116	81 (70)	35 (30)
Micronucleus test	63	60 (95)	3 (5)
Drosophila (SLRL)	345	238 (69)	107 (31)

mutation, may offer a much more relevant assessment of carcinogenicity than in vitro assays (78). Two transgenic models for mutation detection employing transgenes from the bacteria *lac* operon have been developed and partially validated (64, 85). Other transgenic mouse models with altered tumor suppressor genes (p53 heterozygote) or activated proto-oncogenes (TG:AC) are currently under validation in short-term bioassays for chemical carcinogens. The p53 model is used for genotoxic agents as the mode of action for enhanced tumorigenesis is inactivation of the normal p53 allele by chemical-induced mutation, deletion, or rearrangement (98).

EFFECTS OF MUTAGENS ON THE HUMAN GENE POOL

Induction of damage to the germ-line DNA of plant and animal species has the potential to create serious adverse consequences for the health and survival of those organisms. In humans, genetic damage is a cause of hereditary diseases, cancer, congenital anomalies, and even reduced life expectancy (94).

The genes needed to produce the current human population were all acquired from the previous generation's *gene pool*. The gene pool is the sum total of genes, at a given point in time in the reproductively active population of a species, available for transmission to the next generation. Deleterious genes are present in the gene pool at a set frequency, as evidenced by predictable rates of recurring genetic diseases in the human species. The origin of this genetic burden (genetic load) is not known, but it is imperative that the current caretakers of the gene pool use all precautions to transmit it to the next generation in no worse shape than it was received.

Genetic disease in humans appears to be produced by the same types of mutations identified in animal models:

Table 17.11 Examples of human diseases and conditions caused by mutations in germs cellsa

Genetic Disease or Condition	Estimated No. of Cases in the United States
Dyslexia	15,000,000
Manic depression	2,000,000
Schizophrenia	1,500,000
Juvenile diabetes	1,000,000
Adult polycystic kidney disease	500,000
Familial Alzheimer's disease	250,000
Multiple sclerosis	250,000
AAT deficiency (emphysema)	120,000
Myotonic muscular dystrophy	100,000
Fragile X chromosome syndrome	100,000
Sickle cell anemia	65,000
Duchenne muscular dystrophy	32,000
Cystic fibrosis	30,000
Huntington's chorea	25,000
Hemophilia	20,000
Phenylketonuria	16,000
Retinoblastoma (childhood eye cancer)	10,000

a From Reference 96

(a) chromosome abnormalities resulting in stable changes in chromosome number or structure, (b) dominant gene mutations in which only a single mutant allele (of the normal gene pair) is required to produce the disease, (c) recessive gene mutations in which both alleles of the pair must be mutant for

expression of the trait, or (d) polygenic mutations in which the mutant trait is determined by the interaction of several genes.

Table 17.11 provides examples of human genetic diseases as well as the frequency of these disorders in the United States population. The examples given in the table represent only a small portion of the total human diseases and defects known to be of genetic origin (61). It is estimated that the human genome consists of approximately 140,000 genes controlling all aspects of an organism's biology and behavior. Of the total number of genes, we currently know the function for about 1500 to 2000 genes (30). The current human genome project plans to complete sequencing the entire human genome by 2001; however, it will be many more years before all gene functions are mapped to a physical structure.

The degrees of impact to the gene pool from the separate categories of germ cell alterations (listed in Table 17.7) are not equivalent; for example, most of the chromosome abnormalities, other than balanced translocations, result in cell lethality, and if induced in either the ova or sperm, generally produce dominant lethal effects not transmitted to the next generation.

Dominant viable mutations will be expressed in the first generation after induction and may contribute only moderately to the genetic burden. The impact of these mutations on the gene pool may be limited because the affected individuals are aware that they are carrying the mutant form of the gene and that there will be a 50% probability of transmitting the trait to their children if married to an unaffected (normal) individual. Thus, depending on the severity of the effect, the parents can decide, prior to reproducing, if the risk associated with transmission is acceptable.

Recessive mutations are not expressed unless both alleles of the gene in a diploid organism are defective or if the mutation is on the X chromosome in male offspring. Therefore, two normal-appearing heterozygous carriers for an autosomal mutant allele could produce offspring (25% incidence) exhibiting a recessive disease. Consequently, increases in new recessive mutations pose the most serious threat to the gene pool because they would tend to accumulate, over time, in the gene pool as expressed heterozygotes. Due to this latent period, the ultimate expression of a new recessive mutation in the population will have no apparent association with the environmental exposure that induced it. This situation severely limits the opportunity to use human epidemiological studies for proof of human genetic risk.

Intrinsic Differences Affecting Susceptibility to Genotoxic Effects

Unlike the genetic uniformity intrinsic in the animals used as experimental models in toxicology testing, humans exhibit a broad range of variability in their capacity to process and repair DNA damage (30, 41, 63). Studies on DNA repair capacity in mice also indicate a substantial decrease in global DNA repair capacity compared with humans (40).

As described previously, DNA repair in organisms, including humans, is controlled by a complex genetic system of structural and regulatory genes involving almost 50 different proteins. Human *polymorphisms* exist for many of these genes. Individual DNA repair capacity may range as low as 65% of the normal average level (63), placing such individuals at a different risk to mutagens than most of the population. Additional information about repair genes comes from studies of human genetic diseases, such as xeroderma pigmentosum (XP). Diseases such as XP are caused by mutations in NER genes. Individuals with XP lack one of the enzymes in the repair process, and affected individuals, with as little as l%-2% of the normal repair capacity, usually experience high levels of intrinsic DNA damage (63). Cancer susceptibility among XP cases is believed to be a consequence of the genetic damage and can be as much as 1000 times greater than non-XP individuals. Other human syndromes associated with reduced repair capacity (e.g., ataxiatelangiectasia, Bloom Syndrome, and Fanconi anemia) are inherited traits that also exhibit increased cancer risk (64). Thus, it is unlikely that genetic damage induced in genetically homogenous animal models with uniform repair will be easily extrapolated to human populations because factors such as DNA repair exhibit such broad response ranges. It is also clear from this illustration that quantitative estimates of hazardous or safe exposures to genotoxic chemicals based on results from animal data are likely to have large margins of error.

Pharmacogenetics

In addition to DNA repair polymorphisms, other allele variations confer important differences that will influence the impact of genotoxic agents. Some of these differences affect metabolic conversion kinetics. Individuals genetically predisposed to be either a fast or slow metabolizer of specific chemicals may be at higher risk from the parent compound or metabolite. Determination of an individual's genotype can be made with only a small sample of their DNA (15).

Identification of single gene polymorphisms (one nucleotide change), which influence the susceptibility of an individual to the pharmacologic/toxic effects of therapeutic agents, has developed into a major field of medical genetics (107). Institutions have assembled alleles from hundreds of polymorphic genes, and large human populations can be screened for single gene polymorphisms with automated high through-put screening systems employing techniques such as DNA binding and transcription activation. Integration of such information into a genetic profile may eventually be used to direct an individual's lifestyle optimization. Studies in humans exposed to carcinogenic hydrocarbons documented a genotypic influence on the level of adducts. Polymorphism in GSTMI 2 (gluthathione transferase), CYP1A1, and CYP2D6 alleles all affected the formation of DNA adducts found in white blood cells of exposed individuals (21).

Life Style and Exposure to Genotoxicants

There are numerous examples of heritable cancer, where acquisition of specific genes confer a high risk of cancer (e.g., retinoblastoma, colon cancer, breast cancer). These alleles may contribute to as much as 5% of the total cancer incidence, the other 95% is a product of life style. It is well documented that occupation and life style can influence genetic hazard (48, 86).

Table 17.12 Foods and mutagenic activity in the Ames assay

Coffee
Tea
Broiled beef and pork
Broiled fish
Pickled vegetables (Japanese)
Flavanoids in many edible plants
Mushrooms (Agaricus bisporus)
Salted fish (Chinese)
Caramelized sugars (glucose/fructose)
Pyrolysates of onion and garlic
Aflatoxin and other mycotoxins (food contamination)
Safrole

See References 2, 66, 67, and 68 for general discussion.

Tobacco smoke contains a broad range of mutagenic agents detectable in human lymphocytes and urine (51). Consumption of alcoholic beverages has been associated with genetic alterations in humans (69). Diet is also an important source of mutagens. The average human consumes about 10 tons (dry weight) of food by the age of 50 (92), and the list in Table 17.12 illustrates that many food items normally consumed by humans contain substances found to be mutagenic in microbial assays. Many of these agents are also mutagenic when tested in mammalian cell systems.

Other ubiquitous materials that have been reported to produce positive effects in mutagenicity tests include cosmetic ingredients, drugs, food additives, and pesticide residues (1, 5, 6, 87). At present, it is not possible to assess, in quantitative terms, the relevance, if any, of these agents to the mutational or cancer risk in humans.

In addition to tobacco smoke, alcoholic beverages, and consumption of preformed mutagens in or on food products, other life style factors that may be important are less subject to individual control. Exposure to ultraviolet light and ionizing radiation are common. Ambient air contains carbonaceous particles coated with agents producing mutagenic responses in a range of assays (57). The level of particle loading is location dependent, and exposures encountered in urban areas or in certain occupations (e.g., coke oven workers) are considerably higher than exposures in more rural areas.

Ames (2) hypothesized that endogenously formed mutagens and intermediates found in various plants are significant contributors to genotoxic risk. He reported the formation of numerous mutagens resulting from lipid peroxidation of fatty acids. The agents include aldehydes, peroxides, and other free radicals. Considerable efforts are currently under way to evaluate the relative contributions of dietary, environmental, and endogenously formed mutagens to the human disease burden, as well as the modulating influence of antimutagenic agents (e.g., antioxidants) on mutation expressions and cancer (48, 91).

Methods for Assessing Genetic Hazard and Risk

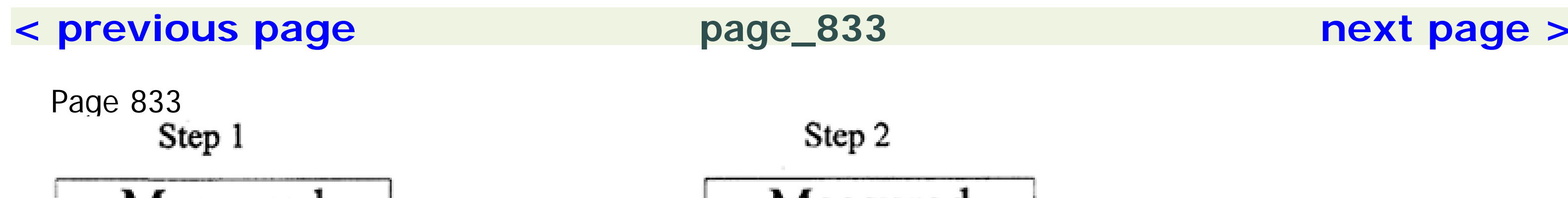
Observations of "new" dominant mutations in human populations and experimental induction of mutations in the germlines of mice indirectly document the fact that mammalian species, including humans, are susceptible to induced mutation. The ability to demonstrate genetic toxicity to human populations through epidemiological methods has been limited by

1. the small number of instances in which sufficient induced sentinel mutations were induced that could be detected in an epidemiology study;
2. the small number of genetic diseases or marker genes identified with specific genetic diseases;
3. the difficulty in identifying reproductively active populations exposed to biologically significant levels of mutagenic agents.

In 1992, the United Nations Environment Program (UNEP) reviewed the status of and methods available for genetic risk assessment (95). The report outlined the situations in which genetic risk assessment might be appropriate and the criteria for selection of risk assessment strategies. Risk information can be developed using qualitative methods resulting in the assignment of a classification, such as "human mutagen" and "probable human mutagen," based on a set of test results criteria. This classification method has been proposed for environmental health uses due to the absence of sufficient in vivo germ cell data to perform quantitative assessments.

When information is limited or when only a general characterization of risk is required, a qualitative assessment can be made. The results are expressed as a ranking for classification based on the test results from a predetermined scheme in which exposure and intrinsic bioactivity are known (Figure

17.8). In most cases, this type of classification is adequate for making risk management decisions (90). When essential, quantitative risk analysis for genetic damage can be performed using dose response results from animal models such as the mouse-specific locus test or the mouse heritable translocation assay. From these data, the population incidence of mutation can be calculated for anticipated exposure levels and expressed as the probability of new disease occurrence in the population. The same problems that confound the production of cancer risk estimates from animal studies affect the ability to generate accurate genetic risks (i.e.,



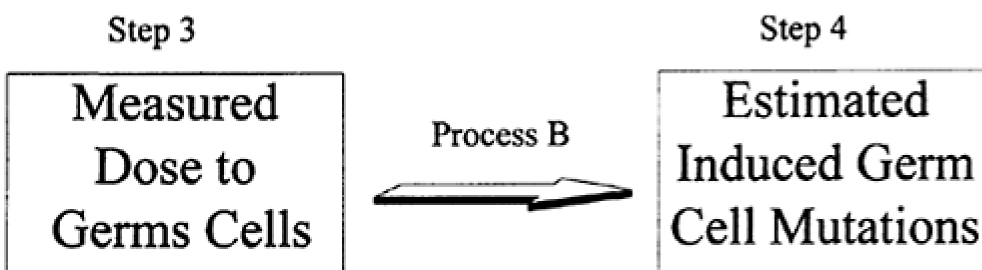


FIG. 17.8. Diagrammatical representation of the parallelogram method of estimating germ cell risk using somatic results and target site dosimetry. This method has been used with rodent and human data to develop probable mutation risks.

Table 17.13 Risk estimates for somatic and germ cell mutations for selected mammalian mutagensa

		Dose (mg/kg) required to double the spontaneous background		
		Germ Cell		
Compound	Stage	Gene	Chromosomal	Somatic Cell
Cyclophosphamide	Pg	8	69	nt
	g	320	nt	
Methylmethane sulfate	Pg	2	4	7
	g	17	nt	
Procarbazine	Pg	43	33	11
	g	110	nt	
Mitomycin C	Pg	nt	4	0.3

g=gonial stages; pg=postgonial stages; nt=not tested.
a From Reference 23

understanding the dose response kinetics, species differences in susceptibility, and factors that influence the phenotypic expression of new mutations). A few quantitative genetic risk assessments have been made (30) using data from mouse models for mutation and clastogenesis (Table 17.13). The major deficiencies in current risk assessment models are the lack of accurate methods to extrapolate animal mutation data to estimates of anticipated new disease incidences in exposed human populations. Mutagens are not as site specific (i.e., organ or tissue) as carcinogens.

INTERPRETING THE RESULTS OF GENETIC RISK ASSESSMENTS

Assessing human germ cell risk to mutagenic substances represents a formidable task, and no chemicals have been proven to induce new mutations in offspring of exposed individuals. Animal mutagens have been detected in rodent germ cells, and quantitative estimates of induced mutation rates per gene locus or the dose required to double a specific mutation rate have to be calculated from results of the in vivo-specific locus or

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heritable translocation assays (37). These estimates may be of limited value in calculating human risk or in setting safe exposure levels because they are based only on male gametes and, in the case of specific locus assay, generally on premeiotic stem cells (spermatogonia). The data do not reflect the risk to later cell stages in spermatogenesis or in female germ cells. Estimates of mutation in postmeiotic sperm and from female gametes will become available; but even so, other important biological variables would interfere with reliable risk estimates and extrapolation across species boundaries. Factors such as differences in endocrine profiles, gene structure, mutation specificity, DNA repair, mutation expression, and disease homology between rodents and humans will make extrapolation tenuous. In addition, postzygotic repair of damaged sperm has been demonstrated in mice and is a factor that may affect genetic risk.

Exposure assessment is a critical component of risk assessment. Exposure may occur by different routes and duration; however, most in vivo mutagenicity studies used in hazard assessment are dosed acutely by oral or intraperitoneal routes. Information derived from the physical/chemical properties of the agent, its concentration in environmental matrices, and exposure modeling are important factors that must be included in the development of quantitative genetic risk assessments. Table 17.14 outlines the variable factors essential for an accurate genetic risk assessment.

GENETIC TOXICOLOGY TESTING STRATEGIES AND DATA EVALUATION

Genetic toxicology assessments seldom consist of a single assay. Due to the multiplicity of mechanisms involved in mutation induction, most evaluations are made using a battery of several tests (102). Test batteries may consist of screening tests (in vitro), hazard assessment tests (in vivo), or both. It is important at the outset of testing to carefully define the objectives desired in a testing program. Screening to prioritize agents for further testing will require different types of tests than would be used to quantify somatic cell hazard to humans.

After conducting the tests, the more complex task is that of interpreting the results generated from the test battery. A genotoxic compound may be defined as

"an agent that produces a positive response in a bioassay measuring any genetic endpoint (e.g., mutation, unscheduled DNA synthesis (UDS) or chromosome breakage)."

Table 17.14 A list of required information for genetic risk analysisa

Endogenous DNA Repair: Capacities and mechanisms for repairing damaged DNA differ among organisms.

Metabolic Specificity: Many agents are not mutagenic themselves but require conversion to a chemical form that can react with DNA and cause mutation. This conversion is called metabolic activation and is accomplished by enzymes which vary in specificity and amount among (a) species (b) individual organisms within a species, and (c) different tissues in an organism.

Background Mutation Rate: All organisms and genes have an inherent background rate of mutations. Genetic factors that alter normal error rates in repair will alter this among individuals.

Age: Somatic mutations may accumulate during the lifetime of an organism. An older organism may thus be more vulnerable to disease, due to a greater body burden of background and induced mutations, than a younger organism.

Diet: Deficiencies in the levels of some vitamins, such as folate, may increase susceptibility to chromosomal mutations. Many foods contain both mutagens and antimutagens. Consumption of large quantities of mutagenic foods may account for the occurrence of certain types of cancer.

Economic and Sound Factors: Poor diet, inadequate health care, prevalence of infectious diseases, and excess exposure to known environmental mutagens, such as cigarette smoke and sunlight, could interact to increase susceptibility to mutagens.

Duration of Exposure: Duration of exposure to a mutagenic substance may affect the resulting genetic risk, depending on the form of the dose-response curve and the specificity of the agent for particular stages of germ-cell development.

Germ Cell Specificity: Acute exposure to chemicals that induce mutation in late stages of germ-cell growth will result in a transitory genetic risk, confined to conceptions resulting from the gametes exposed during the sensitive stage. Acute exposure to chemicals that induce mutation in early stages of germ-cell growth will result in a permanent genetic risk.

a From Reference 96

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Although this definition considers virtually all forms of damage to DNA to classify an agent as genotoxic, genotoxicity should not be interpreted, a priori, as an indication of hazard/risk. The label genotoxic is only a convenient method of classifying chemicals, according to their DNA reactivity, into genotoxic or nongenotoxic subgroups. Additional experimental information beyond this initial classification is necessary to resolve concerns of genetic hazard to somatic or germ cells.

Test results are interpreted on both an individual test basis (i.e., positive or negative in a specific assay) and on a test battery basis. Most decisions made for regulatory purposes are based on the response profile from a battery of tests specified by regulations or guidelines. The international scientific community has proposed a number of genetic test batteries for the evaluation of new chemicals, pesticides, food additives, and pharmaceutical products.

CONSIDERATIONS FOR ASSAY SELECTION

Regulatory Guidelines

Standard study designs for the routine genetic toxicology assays have been published by the EPA (32), Organization for Economic Co-operation and Development (OECD) (70), Canadian Health and Welfare (22), and European Economic Community (EEC) (29). Table 17.15 summarizes many of the recommended testing schemes for nonpharmaceuticals. Most test batteries include, at a minimum, (a) the Ames test, (b) a test for in vitro cytogenetic analysis, and usually (c) an in vivo test for cytogenetic damage. Other tests may be included to expand the profile on the agent if there were positive results or if human exposure was anticipated to be very high. It is also possible to add tests that may be particularly informative for special chemical classes. The EPA (33) has proposed guidelines for the interpretation of data, including in vivo tests. These guidelines provide a general framework for qualitative evaluation of results using a weight-of-evidence approach but do not define criteria to class agents as nonmutagenic.

A standard core battery of tests for pharmaceuticals has been developed under the ICH (officially, the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use). The ICH process resulted in two guidelines: (1) guidance and recommendations for the conduct of genetic tests and (2) establishment of a standard genotoxicity test battery. This battery consists of the Ames test modified by the addition of an *Escherichia coli* tester strain, the mouse micronucleus test, and either the Mouse Lymphoma test (with colony sizing) or an in vitro test for chromosome aberrations (Table 17.16). Adoption of the battery has been completed by the European Union, Japanese *MHW* (Ministry of Health & Welfare), and the U.S. Food and Drug Administration.

Development of a standard core battery of the type recommended by ICH has raised a number of issues regarding which genotoxic endpoints are relevant to hazard assessment, and which tests should be conducted. The two tests agreeable to all participants in the harmonization process were the Ames test for gene

Table 17.15 Testing requirements and guidelines other than for pharmaceuticals

AGENCY	AMES	IN VITRO	IN VIVO	OTHER
U.S. EPA FIFRA	+		+	Mouse lymphoma[a]
U.S. FDA				
Devices/Implants[b]	+	+		Mouse lymphoma[a]/UDS
Drug or Food Additive	+	+		
EUROPEAN COMMUNITY				
Industrial Chemicals	+	+		
Food Additive	+	+	+	Mouse lymphoma
JAPAN				
Industrial Chemicals	+	+		

a The mouse lymphoma must be conducted with colony sizing. Alternatively, the mouse lymphoma or CHO/HGPRT assay plus in vitro cytogenetics may be used.

b Usually conducted on extracts.

In most instances, where the mouse lymphoma is designated, the CHO cell HGPRT assay may be substituted.

Protocol designs for the tests in Table 17.16 follow the most recent OECD guidelines except for the mouse lymphoma assay, which should follow the ICH design.

Table 17.16 ICH test battery

Study	OECD Guideline Design	Testing Expectations
Bacteria reverse mutation assay	471/472	*Salmonella* and *Escherichia* tester strains Maximum concentration of 5 mg/plate or 5 μl/plate Equivocal response requires further testing Negative response confirmed on a case-by-case basis
In vitro chromosome aberration test	473	Maximum concentration of 5 mg/ml or 10 mM Test into the insoluble range Toxicity should exceed LC50 One harvest time at 1.5×normal cell cycle length If negative/-S9, do continuous treatment for 1.5 cycles Record polyploidy and endo replication when seen Equivocal responses require further testing Negative response/-S9 need to be confirmed on a case-by-case basis
Mouse lymphoma assay	476	Maximum concentration of 5 mg/ml or 10 mM Test into the insoluble range Toxicity should achieve 80%-90% Duplicate treatments or eight concentrations Sizing of mutant colonies required Equivocal responses require further testing Negative results must be confirmed on a case-by-case basis
Rodent micronucleus assay (mouse or rat)	474	Maximum dose of 2 g/kg Single sex is sufficient, if toxicity is similar in both May do acute or multiple dosing regimen Three dose levels For acute dosing, harvest high dose at 48 h; the rest at 24 h Must have 5 scorable animals/group Score 2000 PCE/animal Equivocal results should be clarified by further testing

mutation and the in vivo micronucleus for chromosome breakage. The use of in vitro tests for chromosome analysis and gene mutation were more controversial. The use of the mouse lymphoma assay was accepted, in part, because it could serve as both an in vitro measure of gene mutation and chromosome aberrations, and because induction of mutation at the target gene (thymidine kinase) can be induced by either base-pair substitution mutation at the mutant site on the normal chromosome or by deletions of the allele through chromosome breakage. The former mechanism results in large colony mutants and the latter mechanism produces small mutant colonies. Consequently, use of the mouse lymphoma assay for ICH purposes requires colony sizing. Chromosome analysis using cultured cell lines or human lymphocytes is an acceptable alternate to the mouse lymphoma assay.

NEW TECHNOLOGY AND ITS PLACE IN GENETIC TESTING

Limitations of Current Testing Strategies

By 1980, over 150 different tests for genotoxicity had contributed data to scientific journals. Many of the tests were redundant (i.e., measured same endpoint in a different organism or cell type) but each method had a champion who attempted to define the unique value of the particular technique or target organism. The following decade saw extensive efforts to validate and evaluate the best test or set of tests for the purposes of detecting relevant genotoxicant (carcinogens and/or germ cell mutagens). In the process, several valuable lessons were learned:

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1. Many test methods are very sensitive and respond positively to rodent carcinogens; however, the tests do not discriminate agents based on DNA reactivity and they also respond to agents that are not carcinogenic or mutagenic. Examples of such tests are (a) in vitro SCE measures and (b) alkaline elution and other measures of single- and double-strand breakage. The use of these tests in genetic toxicology evaluations has dropped dramatically.
2. Several test methods worked extremely well in the laboratory of the inventor but did not reliably transfer to other testing laboratories. Some of the most difficult methods to transfer from lab to lab are the in vitro morphological cell transformation assays. Currently, only the Syrian hamster embryo (SHE) assays are used as a short-term test for carcinogenicity, and extensive standardization is required to acheive interlaboratory agreement on scoring colonies.
3. The array of mechanisms detected by the available tests may not include mechanisms that are relevant to genetic hazard in mammals. Examples of such deficiencies are tests that reliably measure aneuploidy, induction of tandom repeat errors, transposons, or effects from DNA methylation.
4. Commonly used tests, such as in vitro cytogenetic analysis and the Mouse Lymphoma assay, are susceptible to false-positive responses generated by (a) nonphysiological treatment conditions; (b) chemical-induced cytoxicity, and (c) reactive oxidation species generated by S9 mix chemistry (19).

Several actions were taken in an attempt to resolve the issues listed. The most dramatic was a reduction in test methods requested by regulatory agencies. Genetic toxicology testing schemes were simplified for the purposes of regulatory toxicology safety testing. Most agencies now expect to see the results from a limited test battery consisting of tests for gene mutation and chromosome aberrations before asking for further tests. The ICH core battery of tests is an example of this approach, and this battery is expected, over time, to become the international standard for most testing requirements. Other tests, such as in vitro cell transformation, DNA breakage and repair, mitotic recombination, and DNA adduct formation, are now used in research or as supplemental methods to support or explain findings from core battery assessments. Finally, treatment conditions and confirmatory testing were spelled out in more detail and have been structured to minimize false-positive responses.

Weight-of-evidence methods have been developed to evaluate complex response patterns from test batteries. Some of the methods, such as the Mutagenic Activity Pro files created by ICPEMC, have been validated with large data sets and encoded into computer software (62).

The technological deficiencies indicated in the prior list have not been resolved. The tests included in the ICH battery do not provide a full coverage of mechanisms known to operating in higher mammals and can potentially fail to detect agents that may be relevant to elements of the human genome. In addition, there are no routine methods capable of detecting gene mutation in vivo. This is a serious deficiency when attempting to confirm or extrapolate the results from the Ames test to organisms with eukaryotic DNA and chromosome structure.

Use of alternative methods for carcinogenicity testing, such as the p53 and TG:AC transgenic mice, may tend to reduce reliance on genotoxicity tests because these tests detect tumor endpoints and are both faster and more information rich than conventional rodent cancer bioassays, however, there will continue to be a need to assess the hazard to somatic and germ cell DNA from exposure to new products.

Proposed New Methods for Genetic Testing

Table 17.17 lists several technologies that have become widely available during the past 5 years and appear to be directly applicable to gaps identified in the existing methods used in genetic toxicology. Specific tests have been developed as a consequence of the technology. These new methods are currently being evaluated and validated as either replacements or additions to the current test batteries. Their primary advantage is that they allow more testing to be conducted in vivo using DNA from virtually any tissue.

Transgenic Models for in vivo Mutation Analysis

Transgenic animals for mutation detection were developed in 1990 using shuttle vector technology (40, 86). The two models that are commercially available today are summarized in Table 17.18. A third model that is based on the *lac Z* gene integrated in plasmid DNA was developed by Boerringter and co-workers (13) and is in commercial development. In all commercial models, a gene from the lactose operon of *E. coli* was combined with DNA sequences from lambda phage to form a recoverable shuttle vector (Figure 17.9). The transgenic animals were generated following micro-injection of the vectors into fertilized mouse ova that were subsequently replaced back into the uteri of psuedo-pregnant dams. Founder animals with integrated shuttle vectors were recovered and used to develop stable homozygous strains of mice (and rats) from which the target *lac* genes can be recovered and analyzed for mutation

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Table 17.17 Technologies leading to the development of new genetic testing methods

Method	Properties	In Vivo Test Developed
Polymerase chain reaction (PCR)	Specific gene amplification which permits isolation, amplification, and analysis of specific endogenous mammalian genes	Gene mutation at the HGPRT gene in mice and rats
Shuttle vectors	Engineer-specific DNA sequences into a rescuable vector that can integrate into the host organism's genomic DNA	Transgenic mice and rat models for gene mutation
DNA gel electrophoresis	The use of various types of gels to separate DNA on the basis of size or base composition	COMET assay for DNA strand breakage; gene mutation using single strand conformated polymorphism
Flourescent immunochemical straining combined with in situ hybridization	The use of fluorescent stains attached to specific nucleotide sequences to identify gene locations	Highly specific chromosome mapping and tests to detect small delection and rearrangements

Table 17.18 Characteristics of commercially available transgenic model systems

MUTAMOUSE
80 copies of the *gt10lac* Z shuttle vector
40-merconcameters (head-to-tail) chromosome 3
lac Z target DNA is 3126 base pairs in length
Nontranscribed target gene
Positive selection system for mutant identification
BIG BLUE MOUSE
80 copies of *lac* I reported gene per diploid genome
lac I target DNA is 1080 base pairs in length
Nontranscribed target gene
Visual selection of mutants
Rat model also available

in vivo exposure. This technology offers an ideal model for in vivo mutation detection and molecular analysis (Figure 17.10).

The recoverable shuttle vector containing the *lac* gene can be isolated from virtually any cell type in the treated animal (Table 17.19). Validation studies with transgenic models have demonstrated that they generally are reliable. For example, studies of known rodent carcinogens have demonstrated tissue-specific mutation associated with the target organs associated with induced tumors (98). Both transgenic models show very close con cordance with the Ames test for known genotoxic carcinogens (>90%), and may, therefore, serve as a confirmatory test for the Ames or other in vitro gene mutation screens.

All of the currently available models employ either *lac I* or *lac Z* target genes. The genes are not transcribed but appear to respond in a similar fashion to endogenous genes (96). The primary difference is associated with the ability of the transgenes to detect large deletions. Approximately 1%–1.5% of the spontaneous mutants from Big Blue or MutaMouse models are deletions of 50 base pairs or larger (98, personal communication); however, the *lac Z* plasmid model developed by Borringter and co-workers (13) is able to detect large deletions, and approximately 50% of the spontaneous mutations are due to deletions of 50 base pairs or larger. Recent development of positive selection methods for target genes has made the transgenic models faster and much less expensive to conduct.

Transgenic models for mutation have been used to show that many organ-specific carcinogens show a parallel organ specificity for mutation induction (35). These models are excellent models for the study of mutagenic specificity, as both *lac I* and *lac Z* genes can be sequenced. They may also prove to be good models to investigate the mechanisms of antimutagens and tumor promotors. Transgene sequences can be easily recovered from germinal tissue, making these models potentially ideal for genetic risk assessments. Validation studies for heritable risk analysis have not been conducted with transgenic models.

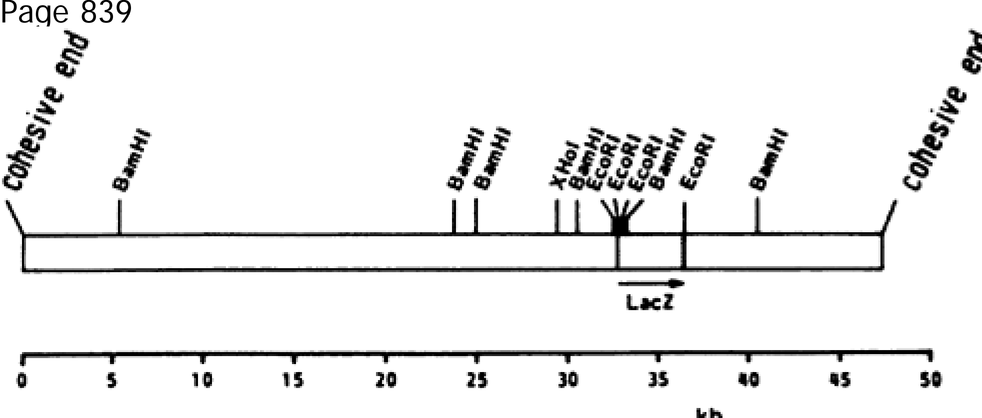

FIG. 17.9. The shuttle vector used to produce the MutaMouse transgenic model for mutation detection. The vector consists of the *lac Z* gene from *E. coli* with Lambda phage genes on either end. The vector is rescuable from mammalian DNA with lambda packaging extracts.

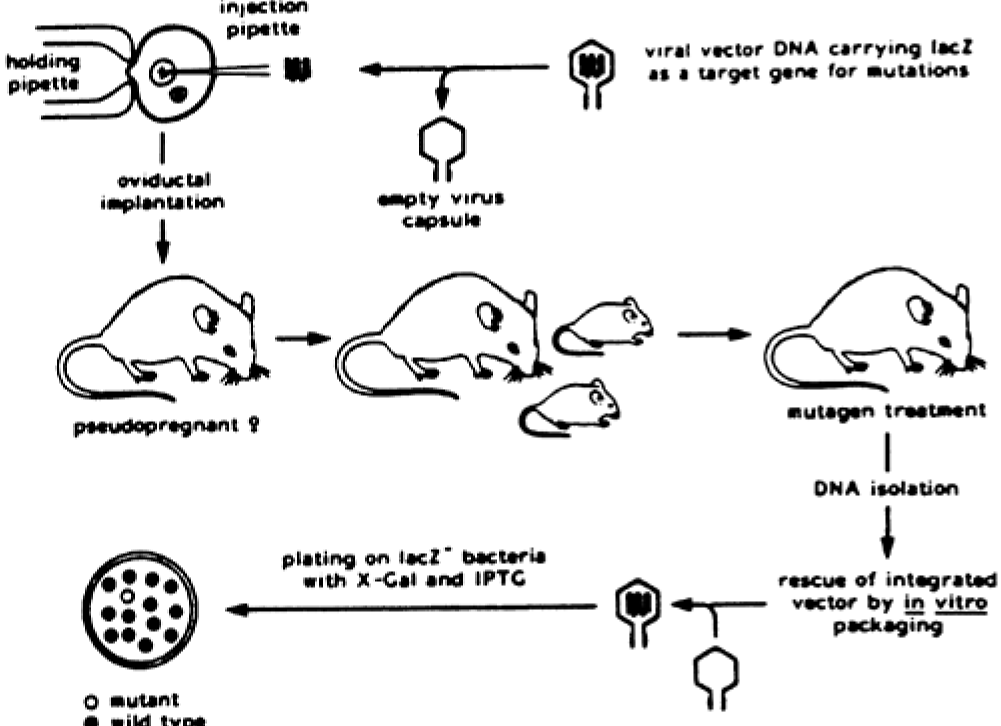

FIG. 17.10. The steps involved in the use of the MutaMouse transgenic mutation model. Exposure can be by any desired route. DNA can be recovered and analyzed from any tissue. The mutation detection part of the process is an in vitro technique.

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Table 17.19 Chemicals tested with MutaMouse

Chemicals	Skin	Bone marrow	Liver	Lung	Testes	Other
ENU		+	+	+	+	Brain
Chlorambucil		+	+		+	
Procarbazine		+				
Cyclophosphamide		+				
Acrylamide		+				
Benzene		Inc.	Inc.			
MNNG	+					
DMBA	+	Inc.				
Acetic acid	Inc.					
TPA	−					
Diethyl nitrosamine		−	+			
Ethylmethane sulfonate		+				
Mitomycin C		+	Inc.			
1,3-Butadiene		−	−	+		

+—mutagenic effects reported;−—no mutagenic effect using conventional study design; Inc.—mutagenic effects reported but inconclusive based on small sample sizes.

Transgenic models have not been introduced into testing schemes but are under intense review by regulatory agencies worldwide. It is likely that one or more of these models will be used as (a) a method to confirm in vitro gene mutation screening in vivo, or (b) replacements for many of the current in vitro gene mutation models.

Gene Amplification Approaches to Mutation Detection

Development of polymerase chain reaction (PCR) has resulted in the ability to select specific DNA sequences and create millions of copies of them rapidly and inexpensively. Although PCR technology has had its greatest impact in other areas of biotechnology, it has been used to develop some new methods of mutation analysis (Table 17.17). Two such models appear promising as methods to detect mutation in vivo, and are compared to the transgenic model. The advantages of these methods over transgenic models are that (a) the target DNA sequences are endogenous in orgin rather than transplanted from other organisms, (b) the technology can be applied to any mammalian system from mouse to primate, and (c) the types of damage detected are relatively unrestricted to specific mechanisms (although DNA deletions are not detected). The first method involves the isolation of splenic T cells from treated rodents and subjecting these cells to 6-thioguanine resistant cells, amplification of the DNA by PCR, and classification of the mutations by sequence analysis. This technique works very well, but the mutation target is lim ited to the spleen. A second method, called single-strand conformational polymorphism, allows mutations to be detected in a larger selection of tissues. In this method, DNA from specific sites (e.g., targeted exons of the gene of interest) can be amplified by PCR. The amplified DNA is then subjected to denaturation and placed on nondenaturing polyacrylamide gel electrophoresis. DNA damage as small as single base changes will alter the mobility of the amplified sequences separating mutant DNA from nonmutant DNA. The shifts will be visible on stained gels. The mutant DNA bands can be removed and sequenced to classify the DNA changes. This method has been used to study organ-specific mutations in various regions of the p53 tumor suppressor gene.

Single Cell Gel Electrophoresis (COMET) Assay

This technique is dependent on the analysis of DNA integrity in single cells. The advantages of this technology are that the source of the cells is almost unrestricted. The basis of the test is that if single- or double-strand breaks are induced in DNA in situ, when cells are isolated, treated to denature nucleic acids, and placed in an electric field, DNA will migrate from the negative to the positive charge at a rate related to the length of the DNA. Fragmented DNA pieces migrate rapidly and, when stained, form a tail on the cells similar to that of a comet (Figure 17.11). The COMET assay is a very versatile technique that can be used in vitro or in vivo (88).

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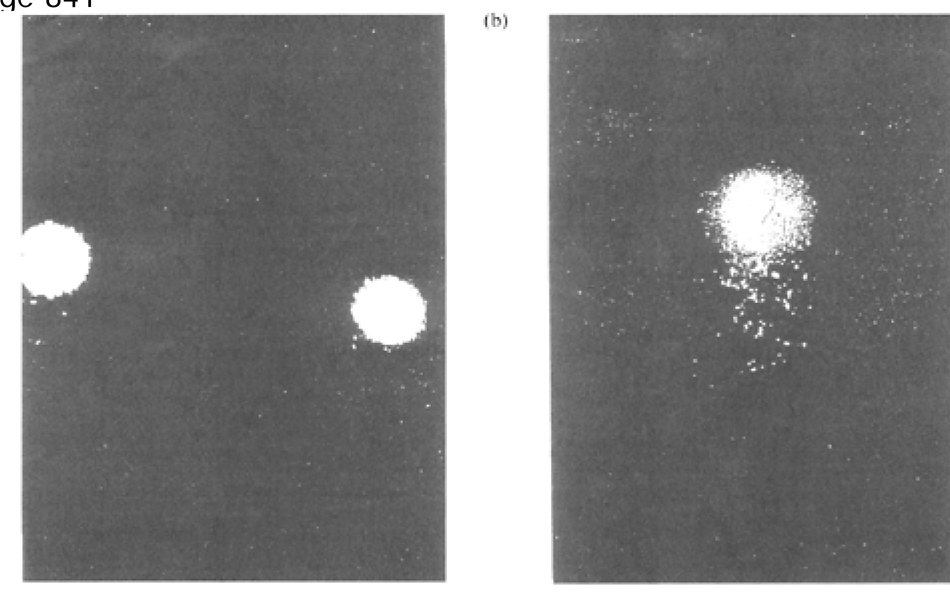

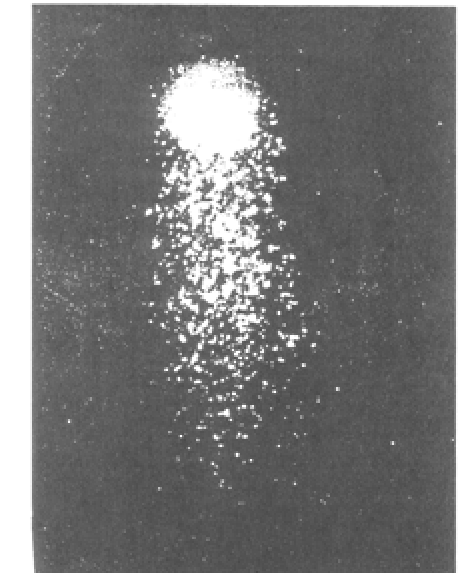

FIG. 17.11. Development of a "tail" in the COMET assay. A normal nucleus (a), followed by some leakage of broken DNA (b) forming ultimately a "tail" of DNA (c). The length of the tail is indicative of the amount of DNA damage.

The COMET assay has been used to screen for genotoxicity under the in vivo exposure conditions. The types of exposures have ranged from radio frequency radiation and agricultural and industrial chemicals to pharmaceutical products. Because the analysis can be conducted with small cell populations, studies of tissue-specific genotoxicity have been published (81). The method may also be applied directly to human populations. Wojewodzka and co-workers (104) studied a group of men and women with chronic low level exposure to radiation and reported that cells from blood cultures showed significant increases in DNA breaks compared to matched controls.

The use of this method increased dramatically from 1994 through 1996, but has slowed somewhat due to the technical problems associated with the sensitivity of the method to stress-induced positive effects occurring during the isolation and fixation steps of the methods. Interpretation of the results from this test might be compromised by cytotoxic effects from normal processes, such as programmed cell death (apoptosis). Extreme care must be taken during these steps of the procedure to eliminate false-positive effects. Some of the limitations and advantages of the COMET assay are listed in Table 17.20.

The introduction of one or more of these in vivo methods or others that are currently in development

Table 17.20 Potential advantages and limitations of the COMET assaya

Advantages

Studies can be conducted in vivo in almost any tissue

The endpoint is developed rapidly and seen visually as increased tail length

The endpoint is nonspecific and should respond to any agent causing DNA breakage

Measurement of the endpoint is sensitive and quantitative

Limitations

Cytotoxicity and induction of programmed cell death (apoptosis) can lead to false responses

The test is sensitive to physiological changes in vivo and stress alone may induce increased tail lengths

The endpoint is an indicator of reparable damage and may not be useful in quantifying risk or impact on germ cell DNA

a Either the mouse lymphoma assay or in vitro cytogenetics is required

Precipitates—if no toxicity is seen, the highest dose may use the lowest precipitating concentration. If toxicity is evident at precipitating concentrations, the high dose should be based on toxicity.

Exposure confirmation in vivo—demonstration of bone marrow exposure is essential for acceptability of negative results.

Mouse lymphoma repeat tests—if the results of the initial trial are negative, the treatment time in a repeat test (−S9) should be increased to 24 h.

would significantly improve the relevance of test batteries such as the ICH core battery. In vivo data from the mouse micronucleus and, for example, a transgenic mutation assay conducted using the anticipated route of human exposure, coupled with toxicokinetic data from the site of cell harvest, could provide a realistic set of results upon which quantitative hazard/risk decisions might be developed.

SELECTED STUDY DESIGNS AND GENETIC TESTING

This section contains a set of study designs that will meet the ICH guideliness for the United States, EC, and Japan. Although not fully described in every protocol, it is recommended that each test article be examined for several chemical, physical, and biological parameters. Included in this preliminary assessment is an examination of the test article's solubility, volatility, and light sensitivity, as well as its stability in the recommended solvent. A preliminary toxicity study is performed to establish a maximum concentration or dose level. Careful selection of concentrations is important. Because of the potential for artifacts produced by excessive ionic levels or low pH levels (19), these parameters should be monitored during the exposure period of in vitro procedures. The protocols all contain specific criteria related to interpretation of toxicity, but it is often possible to save time and materials by knowing the approximate concentration range needed to achieve the desired toxicity.

This group of protocols does not represent the entire repertoire of methods available to genetic toxicologists. It provides, however, the essential test's needed for routine screening for most new compounds under development for use in commerce.

Tests Measuring Gene Mutation

Protocol 1: Salmonella-Eschericia colil mammalian-microsome Reverse Mutation Assay

The objective of the bacteria/microsome assay is to evaluate the test article for mutagenic activity in a bacterial reverse mutation system with and without a mammalian S9 activation component. The assay design is based on OECD Guideline 471–472 (1997 version).

Materials. The strains of *Salmonella typhimurium* and *E. coli* that are used routinely in the plate assay are described in the following table:

Table 17.21 Strains used in the plate assay

Tester Strains	*Target Group*	*Additional Mutations*		
Salmonella				
TA1535	*his* G46	*rfa*	*uvr B*	–
TA1537	*his* C3076	*rfa*	*uvr B*	–
TA98	*his* D3052	*rfa*	*uvr B*	+R
TA100	*his* G46	*rfa*	*uvr B*	+R
Escherichia				
WP2	*trp*		*uvr A*	–

An S9 homogenate is used as the activation system. The 9000×g supernatant fluid is prepared from SpragueDawley adult male rat liver induced by Aroclor 1254 (5). The components of the S9 mix are described in (4).

Test procedures. An appropriate number of tubes of molten overlay agar (at least three per control or concentration level) are prepared. An equivalent number of Vogel Bonner plates also are prepared and properly labeled.

The test article is weighed or measured and diluted in a solvent to set up the stock solutions. The test article and other components are prepared fresh and added to the overlay. The contents then are mixed and poured on the surfaces of the Vogel Bonner plates. For activation studies, 0.5 ml of the S9 mix is added to each overlay tube. For highly reactive chemicals that might have short half-lifes in aqueous environments, a liquid preincubation method has been developed (107). The entire test consists of nonactivation and activation (+S9 mix) test conditions, each with appropriate negative and positive controls.

The plates (once the overlay has solidified) are incubated at 37°C for 36 to 72 h and scored for numbers of revertants per plate. Colonies are selected at random for verification of histidine independence by growth on minimal agar medium.

For the standard plate test, at least five dose levels of the test article, dissolved in a suitable solvent, are added to the test system. Triplicate plates per dose per strain are used in all standard assays. The test concentrations should be based on preliminary toxicity dose selection tests conducted both with and without S9 mix at 10 concentrations ×1 plate each. When no toxicity is observed, concentrations of up to 50 *µ*l or 5000 to 10,000 *µ*g per plate should be employed.

The chemicals that may be used as positive control agents are given in Table 17.22.

Assay acceptance criteria. Several methods for statistical analysis of the Ames test are suggested in the EPA Gene-Tox work group report for *S. typhimurium* mutation assay (50). Plate test data consist of direct revertant colony counts obtained from a set of selective agar plates seeded with populations of mutant cells suspended in a semisolid overlay. Because the test article and the cells are incubated in the overlay for 36 to 72 h, and because a few cell divisions occur during the incubation period, the test is semiquantitative in nature. Although these features of the assay reduce the quantitation of results, they provide certain advantages not contained in a quantitative suspension test:

1. The small number of cell divisions permits potential mutagens to act on replicating DNA, which is often more sensitive than nonreplicating DNA.
2. The combined incubation of the test article and the cells in the overlay permits constant exposure of the indicator cells for 36 to 72 h.

Surviving populations. Plate test procedures do not permit exact quantitation of the number of cells surviving chemical treatment. At low concentrations of the test article, the surviving populations on the treatment plates are essentially the same as that on the negative control plate. At high concentrations, the surviving population usually is reduced by some unknown fraction. This protocol normally employs several doses ranging over two or three log concentrations; the highest of these doses is selected to show toxicity as determined by subjective criteria, such as background clearing or a reduction in

Table 17.22 Positive control agents for the standard plate test

Assay	*Chemical*	*Solvent*	*Concentration per plate (µg)*	*Responding* Salmonella *strains*
Nonactivation	Sodium azide	Water	1	TA-1535, TA-100
	2-Nitrofluorene	DMSO	10	TA-1538, TA-98
	9-Aminoacridine	Ethanol	50	TA-1537
	4-Nitroquinoline	Oxide	1	*E. coli*
Activation	2-Anthramine	DMSO	2.5	All strains of *Salmonella*
	2-Anthramine	DMSO	25	*E. coli*

the number of spontaneous colonies on treated plates compared with the solvent control.

Dose-response phenomena. The demonstration of a dose-related increase in mutant counts is an important criterion for establishing mutagenicity. A factor that might modify dose-response results for a mutagen is the selection of doses that are too low (usually mutagenicity and toxicity are related). If the highest dose is far lower than a toxic concentration, no increase may be observed over the dose range selected. Conversely, if the lowest dose employed is highly cytotoxic, the test article may kill any mutants that are induced, and thus does not appear to be mutagenic. Occasionally, high levels of toxicity produce microcolonies (not mutants), which can be confused for revertants by inexperienced investigators.

Control tests. Positive and negative control assays are conducted with each experiment and consist of direct-acting mutagens for nonactivation assays and mutagens that require metabolic biotransformation in activation assays. Negative controls consist of the test article solvent in the overlay agar together with the other essential components. The negative control plates for each strain give a reference point to which the test data can be compared. The positive control assay is conducted to demonstrate that the test systems are functional with known mutagens.

Replication. OECD guidelines require independent repeat of all tests for confirmation of both negative and equivocal responses.

Assay Evaluation acceptance criteria. Because the procedures used to evaluate the mutagenicity of the test article are semiquantitative, the criteria used to determine positive effects are inherently subjective and based primarily on a historical database. Most data sets are evaluated using the following criteria:

1. Strains TA-1535 and TA-1537. If the solvent control value is within the typical range for the laboratory, a test article that produces a positive dose response over three concentrations, with the highest increase equal to three times the solvent control value, is considered mutagenic.
2. *Strains TA-98, TA-100, and WP2uvrA.* If the solvent control value is within the normal range for the laboratory, a test article that produces a positive dose response over three concentrations, with the highest increase equal to twice the solvent control value, is considered mutagenic. Occasionally, a doubling is not necessary for TA-100 if a clear dose-related pattern is observed over several concentrations.
3. *Pattern.* Because TA-1535 and TA-100 are derived from the parental strain (G-46), and because TA-1538 and TA-98 are derived from the same par ental strain (D3052), to some extent there is a built-in redundancy in the microbial assay. In general, the two strains of a set respond to the same mutagen, and such a pattern is sought. Generally, if a strain responds to a mutagen in nonactivation tests, it does so in activation tests.

The preceding criteria are not absolute, and other extenuating factors may enter into a final evaluation decision, however, these criteria can be applied to most situations and are presented to aid individuals not familiar with this procedure. It must be emphasized that modifications of the procedure involving preincubation conditions or source of S9 mix are necessary for evaluation of specific chemicals or classes of chemicals. Attached to reports should be results of analytical chemical analyses for stability and dose-verification. Laboratory historical control ranges should also be included.

Protocol 2: Forward Mutation at the TK Locus in L5178Y Mouse Lymphoma Cells

This method is developed from OECD protocol 473 (1997 version). The objective of the mouse lymphoma assay is to evaluate a test article for its ability to induce forward mutation in the L5178Y TK +/− mouse lymphoma cell line, as assessed by colony growth in the presence of 5-trifluorothymidine (TFT).

Thymidine kinase (TK) is a cellular enzyme that allows cells to salvage thymidine from the surrounding medium for use in DNA synthesis. If a thymidine analog such as TFT is included in the growth medium, the analog is phosphorylated via the TK pathway and incorporated into DNA, eventually resulting in cellular death. Cells that are heterozygous at the TK locus (TK+/−) may undergo a single step forward mutation to the TK−/− genotype, in which little or no TK activity remains. Such mutants are as viable as the heterozygotes in normal medium because DNA synthesis proceeds by *de novo* synthetic pathways that do not involve thymidine as an intermediate. The basis for selection of the TK−/− mutants is the lack of any ability to use toxic analogs of thymidine, which enable only TK−/− mutants to grow in the presence of TFT. Cells that grow to form colonies in the presence of TFT are therefore assumed to have mutated to the TK−/− genotype either spontaneously or by the action of a test article.

Materials.

Indicator cells. The mouse lymphoma cell line used in this assay, L5178Y TK+/−, is derived from the Fischer L5178Y line. Stocks are maintained in liquid nitrogen and laboratory cultures are periodically

checked for the absence of *Mycoplasma* contamination and karyotype stability. To reduce the negative control frequency (spontaneous frequency) of TK−/− mutants to

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as low a level as possible, cell cultures are exposed to conditions that select against the TK−/− phenotype and then returned to normal growth medium for 3 days or more before use.

Media. The cells are maintained in RPMI 1640 or Fischer's mouse leukemia medium supplemented with L-glutamine, sodium pyruvate, and horse serum (10% by volume). Cloning medium consists of the preceding growth medium with the addition of agar to achieve a semisolid state. Selection medium is cloning medium containing 3 μg/ml of TFT (25).

Control articles. A negative control consisting in assay procedures performed on cells exposed to solvent in the medium is assayed as the solvent-negative control article to determine any effects on survival or mutation caused by the solvent alone. For test articles assayed with activation, the solvent-negative control articles include the activation mixture.

Reference substances for use as positive control articles should be based on the historical data from the laboratory performing the assay. The positive control articles listed below are chosen because of the large database available and because both chemicals detect both small and large colonies (108).

Methyl methanesulfonate (MMS) can be used at concentrations of 10.0 to 15 nl/ml as a positive control for nonactivation studies. Ethylmethane sulfonate is highly mutagenic via alkylation of cellular DNA and may be used at 0.25 to 0.50 μl/ml as a positive control article for nonactivation studies. 3-Methylcholanthrene requires metabolic activation by microsomal enzymes to become mutagenic and may be used at 1.0 to 4.0 μg/ml as a positive control article for assays performed with activation.

Sample forms. All types of test articles can be evaluated in the mouse lymphoma assay. Solid articles can be dissolved in water, if possible, or in dimethylsulfoxide (DMSO), ethanol, or acetone unless another solvent is requested. Liquids can be tested by direct addition to the test system at predetermined concentrations or following dilution in a suitable solvent.

Experimental design.

Dose selection. The solubility of the test article in water or an organic solvent is determined first, and then a wide range of test article concentrations are tested for cytotoxicity, starting with a maximum applied dose of 1 to 5 mg/ml for a solid test article or 1 to 5 μl/ml for a liquid test article and using twofold dilution steps. After an exposure time of 4 h, the cells are washed and a viable cell count is obtained the next day. Relative cytotoxicities expressed as the reduction in growth compared to the growth of negative control cells are used to select 7 to 10 doses that cover the range from 0–50% to 80%-90% reduction in 24-h growth. These selected doses subsequently are applied to cell cultures prepared for mutagenicity testing, but only five or six of the doses may be carried through the mutant selection process. This procedure compensates for daily variations in cellular cytotoxicity and ensures the choice of five or six doses spaced from little or no survival to no apparent effect on cell growth.

Mutagenicity testing. The procedure used for the nonactivation assay is based on that which has been reported (1, 24, 25). Cultures exposed to the test article for 4 h at the preselected doses are washed and placed in growth medium for 2 to 3 days to allow recovery, growth, and expression of the induced TK−/− phenotype. Cell counts are determined daily and appropriate dilutions made to allow optimal growth rates.

At the end of the expression period 3×104 cells for each selected dose are seeded in soft agar plates with selection medium, and resistant (mutant) colonies are counted after 10 to 14 days incubation. To determine the number of cells capable of forming colonies, a portion of the cell suspension is also cloned in normal medium (nonselective). The ratio of resistant colonies to total viable cell number is the mutant frequency.

The activation assay can be run concurrently with the nonactivation assay. The only difference is the addition of the S9 fraction of rat liver homogenate and necessary co-factors (CORE) during the 4-h treatment period. CORE consists of NADP (sodium salt) and isocitric acid. The final concentrations of the activation system components in the cell suspension should be 2.4 mg NADP (sodium salt)/ml, 4.5 mg isocitric acid/ml, and S9/ml. The optimal concentration of S9 (usually 10 to 50 μl S9/ml) should be determined in a pretest with control and MCA-treated cells.

Preparation of 9000g supernatant fluid (S9). Sprague- Dawley or Fischer 344 male rats are normally used as the source of hepatic microsomes; the S9 can be obtained commercially. Briefly, induction with Aroclor 1254 or another agent is performed by injection 5 days prior to killing. After decapitation and bleeding, the liver is immediately dissected from the animal using aseptic technique and placed in ice-cold buffer at pH 7.4. When an adequate number of livers are obtained, the collection is washed twice with fresh buffer and completely homogenized. The homogenate is centrifuged for 10 min at 9000g in a refrigerated centrifuge, and the supernatant fluid (S9) from this centrifuged sample is retained and frozen at −80°C until used in the activation system.

Test materials identified as mutagenic should have representative control and mutagenic selection plates recounted to permit mutant colony sizing. Selection plates are recounted using the Artek colony counter at size settings between 0.0 to 4.0 in increments of 0.2.

If the initial trial is determined to be negative or equivocal, a report test of the nonactivation portion of the assay is conducted with a 24-h exposure period. It is suggested that the activation portion be repeated using a different dose range.

Assay acceptance criteria. An assay is considered acceptable for evaluation of test results only if all of the criteria in the following list are satisfied. The activation and nonactivation portions of the mutation assays usually are performed concurrently, but each portion is in fact an independent assay with its own positive and negative controls. The activation or nonactivation assays are repeated independently to satisfy general acceptance and evaluation criteria:

1. The average absolute cloning efficiency of the negative controls (average of the solvent and untreated controls) is 100%±30%. Assay variables can lead to artificially low cloning efficiencies in the range of 50%–70% and still yield internally consistent and valid results. Assays with cloning efficiencies in this range are conditionally acceptable and dependent on the scientific judgment of the investigator. All assays below 50% cloning efficiency are unacceptable.
2. The average negative control suspension growth factor is not less than about eight. The optimal value is 25, which corresponds to fivefold increases in cell number for each of the 2 days following treatment of the experimental cultures.
3. The background mutant frequency (average of the solvent and untreated negative controls) is calculated separately for concurrent activation and nonactivation assays, even though the same population of cells is used for each assay. For both conditions, the normal range of background frequencies for assays performed with different cell stocks is 20 to 120×10^{-6}. Assays with backgrounds outside this range are not necessarily invalid and should be considered in the context of the historical data in the laboratory performing the assay.
4. A positive control is included with each assay to provide confidence in the procedures used to detect mutagenic activity. The normal range of mutant frequencies induced by MMS (nonactivation assay) is 200×10^{-6} to 800×10^{-6}; for MCA 4.0 μg/ml (activation assay), the normal range is 200×10^{-6} to 1000×10^{-6}. An assay is considered acceptable in the absence of a positive control (loss due to contamination or technical error) only if the test article clearly shows mutagenic activity as described in the evaluation criteria.
5. For test articles with little or no mutagenic activity, an assay must include applied concentrations that reduce the relative growth to between 10% and 20% of the average solvent control or reach the maximum applied concentrations given in the evaluation criteria. This requirement is waived if a dose increment of about 1.5 or less causes excessive toxicity.
6. An experimental treatment that results in fewer than 3.0×10^{-6} cells by the end of the 2-day growth period is not cloned for mutant analysis.
7. An experimental mutant frequency is considered acceptable for evaluation only if the relative cloning efficiency is 10% or greater and the total number of viable clones exceeds 20.
8. Mutant frequencies are derived normally from sets of three dishes for both the mutant and the viable colony count. To allow for contamination losses, an acceptable mutant frequency can be calculated from a minimum of two dishes per set.
9. The mutant frequencies for six treated cultures are normally determined in each assay. A required number of various concentrations cannot be explicitly stated, although a minimum of four analyzed cultures is considered necessary under the most favorable test conditions to accept a single assay for evaluation of the test article.

Assay evaluation criteria. The minimum condition considered necessary to demonstrate mutagenesis for any given treatment is a mutant frequency that is at least two times the concurrent background frequency. *Background frequency* is defined as the average mutant frequency of the solvent controls. The minimum increase is based on extensive experience that indicates that the calculated minimum increase is often a repeatable result.

The observation of a mutant frequency that meets the minimum criterion for a single treated culture within a range of assayed concentrations is not sufficient evidence to evaluate a test article as a mutagen. The following test results must be obtained to reach this conclusion for either activation or nonactivation conditions:

1. A dose-related or toxicity-related increase in mutant frequency is observed. It is desirable to obtain this relation for at least three doses, but it depends on the concentration steps chosen for the assay and the toxicity at which mutagenic activity appears.
2. An increase in mutant frequency may be followed by only small or no further increases at higher concentrations or toxicities; however, a decrease in mutant frequency to values below the minimum

criterion is not acceptable in a single assay to classify the test article as a mutagen. If the mutagenic activity at lower concentrations or toxicities is large, a repeat assay is performed to confirm the mutagenic activity.

3. If an increase of at least four times the background mutant frequency is observed for a single dose near

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the highest testable toxicity, as defined in the Assay Acceptance Criteria section, the test article is considered mutagenic. Smaller increases at a single dose near the highest testable toxicity require confirmation by a repeat assay.

4. For some test articles the correlation between toxicity and applied concentration is poor. The proposition of the applied article that effectively interacts with the cells to cause genetic alterations is not always repeatable or under control. Conversely, measurable changes in the frequency of induced mutants may occur with concentration changes that cause only small changes in observable toxicity. Therefore, either parameter—applied concentration or toxicity (percent relative growth)—can be used to establish if the mutagenic activity is related to an increase in effective treatment. A negative correlation with dose is acceptable only if a positive correlation with toxicity exists. An apparent increase in mutagenic activity as a function of decreasing toxicity is not acceptable evidence for mutagenicity.

A test article is evaluated as nonmutagenic in a single assay only if the minimum increase in mutant frequency is not observed for a range of applied concentrations that extends to toxicity causing 10%–20% relative suspension growth. If the test article is relatively nontoxic, the maximum applied concentrations are normally 5 mg/ml (or 5 μl/ml) unless limited by solubility. If a repeat assay does not confirm an earlier, minimal response as discussed previously the test article is evaluated as nonmutagenic in this assay system.

Tests Detecting Chromosome-Breaking (Clastogenic) Agents

Protocol 4: Chromosome Aberrations in Chinese Hamster Ovary Cells

This design meets the OECD Guideline 473 (1997 version). The objective of the Chinese hamster in vitro assay is to evaluate the ability of a test article to induce chromosome aberrations in Chinese hamster ovary (CHO) cells.

Indicator cells. Cells to be used in this assay can be obtained from the American Type Culture Collection Repository No. CCL61 (Rockville, MD). The original cells were obtained from an ovarian biopsy of a Chinese hamster. It is a permanent cell line with an average cycle time of 10–12 h.

The CHO cells for this assay are grown in Ham's F12 medium supplemented with 10% fetal calf serum. The cells are split back to 3×10^5 per 75 cm^2 plastic flask and fed 24 h prior to treatment with 10 ml of fresh medium.

Control articles. The solvent for the test article is used as the solvent vehicle for the control article. Ethylmethane sulfonate, a known mutagen and chromosome-breaking (clastogenic) agent, may be dissolved in the culture medium and used as a positive control article for the nonactivation studies at a final concentration of 0.5 μl/ml.

Experimental design. Toxicity and dose determination. The solubility, toxicity, and doses for the test article are determined prior to screening. The effect of each test article on the survival of the indicator cell is determined by exposing the cells to a wide range of article concentrations in complete growth medium. Toxicity is measured as the loss in growth potential of the cells induced by a 4-hour expression period in growth medium. Doses are selected from the range of concentrations by bracketing the highest dose that shows no loss in growth potential with at least one higher and three lower doses. Otherwise, a half-log series of doses are employed, with the highest dose being perhaps limited by solubility, but in any case not to exceed 5 mg/ml. The doses cover at least four orders of magnitude, and all doses that yield sufficient numbers of scorable metaphase cells are considered in the analysis. Alternatively, inhibition of cell cycling (BrdU staining, discussed later) can be used to set dose levels.

Cell treatment. For the nonactivation assay, approximately 10^6 cells are treated with the test article at predetermined doses for 3 h at 37°C in growth medium. The exposure period is terminated by washing the cells twice with saline. Four replicate cultures per dose are employed in this assay; all receive fresh medium after washing, and 5-bromo-2′-deoxyuridine (BrdU) is added to two of the four replicates (10 μM final concentration). Incubation is continued in the dark for 20 h. Colcemid is added for the last 3 h of incubation (2×10^{-7} M final concentration), and metaphase cells are collected by mitotic shake-off. These cells are swollen with 0.075 M KCl hypotonic solution, washed three times in fixative (methanol/acetic acid, 3:1), dropped onto slides, and air dried.

For the activation assay, the test article is tested in the presence of an S9 rat liver activation system. This assay differs from the nonactivation assay in that the S9 reaction mixture is added to the growth medium, together with the test article, for 3 h. The S9 mix is the same as that used in the mouse lymphoma assay. The exposure period is terminated by washing the cells twice with saline. From this point, they are treated as described for the nonactivation assay.

If the 3-h exposure studies are equivocal or negative, a repeat nonactivation study employing a 17.5-h exposure will be conducted to confim the negative results. It is

suggested that the activiation test also be repeated using a different dose range.

Staining and scoring of slides. Slides are stained with 10% Giemsa at pH 6.8 for subsequent scoring of chromosome aberration frequencies. About 50 to 100 cells are scored from each of two replicate cultures per dose.

Standard forms are used to score and record gaps, breaks, fragments, and reunion figures, as well as numerical aberrations such as polypoid cells. The complete list of aberrations to be scored are as follows:

Chromatid gap
Chromosome break
Chromosome gap
Chromosome break
Chromatid deletion
Fragment
Acentric fragment
Translocation
Pulverized chromosomes
Pulverized chromosomes
Pulverized cells
Complex rearrangement
Ring chromosome
Dicentric chromosome
Minute chromosome
More than 10 aberrations
Triradial
Quadradial
Polypoid
Hyperdiploid

For control of bias, all slides are coded for scoring.

Assay evaluation criteria. A number of general guidelines have been established to aid in determining the meaning of CHO chromosome aberrations. Basically, an attempt is made to establish if a test article or its metabolites can interact with chromosomes to produce gross lesions or changes in chromosome numbers and if these changes are of a type that can survive more than one mitotic cycle of the cell. All aberration figures detected by this assay result from breaks in the chromatin that either fail to repair or repair in atypical combinations.

It is anticipated that many of the cells bearing breaks or reunion figures would be eliminated (i.e., fail to divide again) after their first mitotic division and, as a corollary, that those cells that survive the first division would primarily bear balanced lesions. The detection of these lesions, and hence a complete risk evaluation usually must rely on additional testing. In general, a cell bearing configurations such as small deletions or reciprocal translocations may be perpetuated and therefore constitutes a greater risk to an individual than one with large deletions or complex rearrangements.

Data are summarized in tabular form and evaluated. Gaps are not counted as significant aberrations unless they are present at a much higher than usual frequency. Open breaks are considered indicators of genetic damage, as are configurations resulting from the repair of breaks. The latter includes, for example, translocations, multiradials, rings, and multicenters. Reunion figures such as these are weighed higher than breaks and may lead to stable configurations.

The number of aberrations per cell are also considered significant. Cells with more than one aberration indicate more genetic damage than those containing evidence of single events. Frequently, one is unable to locate sufficient suitable metaphase spreads. Possible causes appear to be related to cytoxic effects, which alter the duration of the cell cycle, kill the cell, or cause clumping of the chromosomes. Additional information can be gained from the mitotic index, which appears to reflect cytotoxic effects, as well as from the frequencies of M1, M2, and M3 cells.

Comparison with a concurrent negative control that shows an unusually low frequency of aberrations can suggest undue statistical significance. Therefore, treatment data are considered against historical control data. In either event, the type of aberration, its frequency, and its correlation to dose trends within a given time period are all considered when evaluating a test article as being mutagenically positive or negative.

Statistical analysis employs a two-tailed t-test. This test can be performed on the number of breaks per

chromosome in treated and control samples. Dose regression analysis also is useful.

Protocol 5: Rodent Bone Marrow Micronucleus Assay

This assay design is based on OECD Guideline 474 (1997 version). The objective of the mouse micronucleus assay is to evaluate a test article for clastogenic activity in polychromatic erythrocyte (PCE) stem cells in treated mice. The micronucleus test can serve as a rapid screen for clastogenic agents and test articles that interfere with normal mitotic cell division (42, 82). Micronuclei are believed to be formed from chromosomes or chromosome fragments left behind during anaphase and scored during interphase because they persist. Thus, the time involved in searching for metaphase spreads in treated cell populations is eliminated. Test articles affecting spindle fiber function or formation, as well as clastogenic agents, can be detected through micronucleus induction.

Materials (This design will describe a study in mice). Adult male and/or female mice, strain CD-1, from a randomly bred closed colony may be used as well as other strains or species. A healthy, random-bred strain

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is selected to maximize genetic heterogeneity and at the same time ensure access to a common source. Animals usually are 8–10 weeks old at the time of dosing.

Trimethylene melamine (TEM) at 1.0 mg/kg may be used as the positive control article and is administered via a split-dose intraperitoneal injection. The negative control consists of the solvent or vehicle used for the test article and is administered by the same route as, and concurrently with, the test article in volumes equal to the maximum amount administered to the experimental animals.

Experimental design.

Animal husbandry. Animals are group-housed 7 mice per cage. A commercial diet and water are available *ad libitum* unless contraindicated by the particular experimental design.

Five animals per dose level are uniquely identified and assigned to study groups at random. Prior to study initiation, animals are weighed to calculate dose levels. The volume of test article administered per animal is established using a mean weight unless there is significant variation among individuals; in this case, individual calculations are made.

Sanitary cages and bedding are used. Personnel handling animals or working within the animal facilities are required to wear suitable protective garments. When appropriate, individuals with respiratory or other overt infections are excluded from the animal facilities.

Dose selection. If acute toxicity information (e.g., LD50) is available, it can be used to determine dose levels. If it is not available, dose levels can be determined using five groups of six animals each in a toxicity study. For nontoxic agents, a limit dose of 2000 mg/kg is employed. For toxic agents, the high dose is selected based on some evidence of toxicity (e.g., clinical signs, death, depression in bone marrow cell maturation). Two lower doses at 1/2 and 1/4 of the high dose will be selected. An attempt is made in mutagenesis studies, as well as other toxicology work, to evaluate the extremes of dosage as well as values close to the use level.

Dosing schedule and route of administration. The test article administered may be in one of two groups: (1) animals may be treated once or (2) two or more daily doses are given 24 h apart. The single-dose administration is the most common. In the event that test article characteristics preclude oral gavage, intraperitoneal injection may be employed. These routes of administration are the ones most commonly used for this test procedure.

Extraction of bone marrow. For the single-dose regimen, sampling times will be at 24 and 48 hours after compound administration. Animals are killed with CO2 and the adhering soft tissue and epiphyses of both femurs removed. The marrow is aspirated from the bone and transferred to centrifuge tubes containing 5 ml fetal calf serum (one tube for each animal).

Preparation of slides. After centrifugation to pellet the tissue, the supernatant fluid is drawn off and portions of the pellet spread on slides and air-dried. The slides are then stained in May-Gruenwald solution and Giemsa, followed by clearing in distilled water.

Screening the slides. One thousand PCEs per animal are scored. The frequency of micronucleated cells is expressed as percent micronucleated cells based on the total PCEs present in the scored optic field. The frequencies of other bone marrow cell types are recorded for analysis of cytotoxic effects (reduced production of specific blood cell types).

Evaluation criteria. In tests performed for this evaluation, only PCEs are scored for micronuclei. Mature erythrocytes and other cells in the field are recorded but not scored. Loss of nucleated cells is an indication of cytotoxicity. The dose levels are established to ensure that a nontoxic level of the test article is scored. Dose-response data are not necessary to define a test article as active. Responses considered active are assumed to reflect clastogenic and related activities of test articles. Agents that break chromosomes and induce nondisjunction, as well as other events that produce structural or numerical changes in chromosomes, can produce micronuclei.

The data generated in this study may be analyzed by a two-tailed *t*-test. Individual animal results are used as data points in the analysis. The set of micronuclei frequencies among the controls are compared to the set for each treatment level. Male and female animal data are combined unless there appears to be a sex difference, in which case the data are analyzed separately. Increases above the negative control frequency that are significant at $p<0.01$ are considered indicative of an active agent.

For control of bias, all slides are coded prior to scoring and scored blind.

QUESTIONS

1. Would transcription-coupled DNA repair (TCR) be more active in an exon or an intron?
2. Which type of gene mutation would be corrected by mismatch repair: a basepair substitution or a frameshift mutation?
3. Which part of a chromosome is believed to influence cell mortality: the centromere, the telomere, or

the centriole?
4. Which short-term test shows the best overall concordance with animal carcinogenicity: SCEs, the Ames test, or the mouse micronucleus test?

5. The p53 gene is a member of which class of genes: an oncogene, a dominant gene, or a tumor suppressor gene?
6. A disruption in the nucleotide sequence of which of the following is likely to result in a mutation: a codon, a gene, or a chromosome?
7. Which of the following technologies was critical to the development of the transgenic mouse models for mutation: shuttle vector, PCR, or gel electrophoresis?
8. Chemically-induced heritable mutations have not been documented in which of the following species: fruit fly, mouse, or human?

REFERENCES

1. Amacher, D.E., Paillet, S.C., Turner, G.N., Ray, V.A., and Salsburg, D.S.: Point mutations at the thymidine kinase locus in L5178Y mouse lymphoma cells II. Test validation and interpretation. *Mutation Research* 72:447–474.
2. Ames, B.N. (1984): Dietary carcinogens and anti-carcinogens. *Clin. Toxicol.*, A 22:291–301.
3. Ames, B.N. (1979): Indentifying environmental chemicals causing mutations and cancer. *Science*, 204:587–593.
4. Ames, B.N., Durston, W.E., Yamasaki, E., and Lee, F.D. (1973): Carcinogens are mutagens: A simple test system combining liver homogenates for activation and bacteria for detection. *Proc. Natl Acad. Sci. USA*, 70:2281.
5. Ames, B.N., Kammen, H.L., and Yamasaki, E. (1975): Hair dyes are mutagenic: Identification of a variety of mutagenic ingredients. *Proc. Natl. Acad. Sci. USA*, 72:2423–2427.
6. Ames, B.N., McCann, J., and Yamasaki, E. (1975): Methods for detecting carcinogens and mutagens with the *Salmonella* mammalian microscome mutagenicity test. *Mutation Res.*, 31:347–364.
7. Anderson, M.W., Maronpot, R.R., and Reynolds, S.H. (1988): Role of oncogenes in chemical carcinogenesis: Extrapolation from rodents to humans. In: *Methods for Detecting DNA Damaging Agents in Humans*, edited by H.Bartsch, K.Hemminki, and I. K.O'Neill, pp. 477–485. IARC Scientific Publications No. 89, Lyon.
8. Ashby, J., de Serres, F.J., Draper, M., Ishidate, M., Margolin, B., Matter, B., and Shelby, M.D. (1985): *Short-Term Tests for Carcinogens: Results of the IPCS Study.* Elsevier, Amsterdam.
9. Ashby, J., and Purchase, I.F.H. (1988): Reflections on the declining ability of the *Salmonella* assay to detect rodent carcinogens as positive. *Mutation Res.*, 205:51–58.
10. Ashby, J., and Richardson, C.R. (1985): Tabulation and assessment of 113 human surveillance cytogenetics studies conducted between 1965 and 1984. *Mutation Res.*, 154:111–133.
11. Au, W.W., Cajas-Salazar, N., and Salama, S. (1998): Factors contributing to discrepancies in population monitoring studies. *Mutation Res.*, 400:467–478.
12. Barbacid. M. (1987): Mutagens, oncogenes and cancer. In: *Oncogenes and Growth Factors*, edited by R.A.Bradshaw and S.Prentis, pp. 90–99. Elsevier, Amsterdam.
13. Boerringter, M., Dolle, M., Martus, H., Gossen, J., and Vijg, J. (1995): Plasmid-based transgenic mouse model for studying in vivo mutations. *Nature*. 377:657–659.
14. Bridges, B.A. (1976): Short-term screening tests for carcinogens. *Nature*, 261:195–200.
15. Brousseau, R., Scarpulla, R., Sung, W., Hsing, H.M., Narang, S. A., and Ulu, R. (1982): Synthesis of a human insulin gene. V: Enzymatic assembly, cloning and characterization of the human proinsulin DNA. *Gene*, 17:279–289.
16. Brusick, D., Albertini, R., McRee, D., Peterson, D., Williams, G., Hanawalt, P., and Preston, J. (1998): Genotoxicity of Radiofrequency Radiation. Environmental and Molecular Mutagenesis. 32:1–16.
17. Brusick, D. (1983): Mutagenicity and carcinogenicity correlations between bacteria and rodents. In: *Cellular Systems for Toxicity Testing 407*, reprinted from Annals of the New York Academy of Sciences, pp. 164–176.
18. Brusick, D.J. (1978): The role of short-term testing in carcinogen detection. *Chemosphere*, 5:403–417.
19. Brusick, D.J. (1987): Implications of treatment-condition-induced genotoxicity for chemical screening and data interpretation. *Mutation Res.*, 189:1–6.
20. Burnet, F.M. (1974): *Intrinsic Mutagenesis: A Genetic Approach to Aging.* Medical & Technical Publishing, Lancaster, England.
21. Butkiewicz, D., Grzybowska, E., Hemminki, K., Ovrebo, S., Haugen, A., Motykiewicz, G., and Chorazy, M. (1998): Modulation of DNA adduct levels in human mononuclear white blood cells and granulocytes by CYP1A1, CYP2D6 and GSTM1 genetic polymorphisms. *Mutation Res.*, 415:97–108.
22. Canadian Health and Welfare (1986): Guidelines on the use of mutagenicity tests in the toxicological

evaluation of chemicals. *Environ. Molec. Mutagen.*, 11:261–304.
23. Cacheiro, N.L.A., Russell, L.B., and Swartout, M.S. (1974): Translocations, the predominant cause of total sterility in sons of mice treated with mutagens. *Genetics*, 75:73–91.
24. Clive, D., Caspery, W., Kirby, P.E., Krehl, R., Moore, M., Mayo, J., and Oberly, T.J. (1987): Guide for performing the mouse lymphoma assay for mammalian cell mutagenicity. *Mutation Res.*, 189:143–156.
25. Clive, D., Johnson, K.O., Spector, J.F.S., Batson, A.G., and Brown, M.M.M. (1979): Validation and characterization of the L5178Y TK +/− mouse lymphoma mutagen assay system. *Mutation Res.*, 59:61–108.
26. Counter, C. (1996): The roles of telomeres and telomerase in cell life span. *Mutation Res.*, 366:45–63.
27. Perera., F., Dickey, C., Santella, R., O'Neill, J., Albertini, R., Ottman, R., Tsai, W., Mooney, L., Savela, K., and Hemminki, K. (1994): Carcinogen—DNA adducts and gene mutation in foundry workers with low-level exposure to polycyclic aromatic hydrocarbons. *Carcinogenesis*, 15:2905–2910.
28. deLange, T. (1994): Activation of telomerase in a human tumor. *Proc. Natl. Acad. Sci. USA*, 91:2882–2885.
29. EEC (European Economic Community) Official Journal of the European Communities, 6th Amendment to Directive 67/548/EEC, Annex VII, 15.10.79, and Annex V, EEC Directive 79–831, part B, Toxicological Methods of Annex VIII, Draft, July 1983.
30. Ehling, U.H. (1988): Quantification of the genetic risk environmental mutagens. *Risk Analysis*, 8:45–56.
31. Ehling, U.H., Averby, D., Cerutti, P.A., Friedman, J., Greim, H., Kolbye, A.C., Jr., and Mendelsohn, M.L. (1983): Review of the evidence for the presence or absence of thresholds in the induction of genetic effects by genotoxic chemicals. *Mutation Res.*, 123:281–341.
32. EPA (Environmental Protection Agency) Office of Pesticides and Toxic Substances (1982): *Health Effects Test Guidelines*. EPA Publication 560/682–001. National Technical Information Service, Springfield, Virginia.
33. EPA (1986): EPA guidelines for mutagenicity risk assessment. *Fed. Register*, 51:34006–34012.

34. Evans, H.J.: Cytogenetics: Overview. In: *Mutation and the Environment*, Part B, edited by M.L.Mendelsohn, and R.J. Albertini, pp. 301–323. Wiley-Liss, Inc., New York.
35. Fletcher, K., Tinwell, H., and Ashby, J. (1998): Mutagenicity of the human bladder carcinogen 4-aminobiphenyl to the bladder of MutaMouse transgenic mice. *Mutation Res.*, 400:245–250.
36. Generoso, W.M., Cain, K.I., and Banby, A.J. (1983): Some factors affecting the mutagenic response of mouse germ cells to chemicals. In: *Utilization of Mammalian Specific Locus Studies in Hazard Evaluation and Estimation of Genetic Risk*, edited by F.J.de Serres, and W.Sheridan, pp. 227–239. Plenum Press, New York.
37. Generoso, W.M, Cain, K.T., Huff, S.W., and Gosslee, D.G. (1978): Heritable translocation test in mice. In: *Chemical Mutagens: Principles and Methods for Their Detection*, *Vol. 5*, edited by A.Hollandaer and E.I.de Serres. Plenum Press, New York.
38. Glassner, B.J., Posnick, M., and Samson, L.D. (1998): The influence of DNA glycosylases on spontaneous mutation. *Mutation Res.*, 400:33–44.
39. Gossen, J., deleeuw, W., Tan, C., Zwarthoff, E., Berends, F., Lohman, P., Knock, D., and Viig, J. (1989): Efficient rescue of integrated shuttle vectors from transgenic mice: A model for studying mutations in vivo. *Proc. Nat. Acad. Sci. USA,* 86:7971–7975.
40. Hanawalt, P. (1998): Genomic instability: Environmental invasion and the enemies within. *Mutation Res.*, 400:117–125.
41. Harris, C.C. (1998): Interindividual variation among humans in carcinogen metabolism, DNA adduct formation and DNA repair. *Carcinogenesis*, 10:1563–1566.
42. Heddle, J. (1973): A rapid in vitro test for chromosomal damage. *Mutation Res.*, 18:187–190.
43. Hilliard, C., Armstrong, M., Bradt, C., Hill, R., Greenwood, S., and Galloway, S. (1998): Chromosome aberrations in vitro related to cytotoxicity of nonmutagenic chemicals and metabolic poisons. *Environmental and Molecular Mutagenesis*, 31:316–326.
44. Holliday, R., and Ho, T. (1988): Gene silencing in mammalian cells by uptake of 5-methyl deoxycytidine 5′ phospate. *Somatic Cell Mol. Genet.*, 17:537–542.
45. Horowitz, J., Yandell, D., and Park, S. (1989): Point mutational inactivation of the retinoblastoma antioncogene. *Science*, 243:937–940.
46. ICPEMC (1988): Testing for mutagens and carcinogens: The role of short-term genotoxicity assays. *Mutation Res.*, 205:3–12.
47. ICPEMC (1990): The possible involvement of somatic mutations in the development of atheriosclerotic plaques. *Mutation Res.*, 239:143–148.
48. Inger-Lise, H. (1990): Occupational and lifestyle factors and chromosomal aberrations of spontaneous abortions. In: *Mutation and the Environment*, Part B, pp. 467–475. Wiley-Liss, Inc., New York.
49. Kalter, H. (1977): Correlation between teratogenic and mutagenic effects of chemicals in mammals. In: *Chemical Mutagens: Principles and Methods for Their Detection*, *Vol. 6*, edited by A.Hollandaer. Plenum Press, New York.
50. Kier, L.D., Brusick, D.J., Auletta, A.E., Von Halle, E.S., Brown, M.M., Simmon, V.F., Dunkel, V., McCann, J., Mortelmans, K., Prival, M., Rao, T.K., and Ray, V. (1986): The *Salmonella typhimurium/mammalian* microsomal assay: A report of the U.S. EPA Gene-Tox Program. *Mutation Res.*, 168:69–240.
51. Kier, L.D., Yamasaki, E., and Ames, B.N. (1974): Detection of mutagenic activity in cigarette smoke condensates. *Proc. Natl. Acad. Sci. USA*, 71:4159–4163.
52. Kier, L.D. (1988): Comments and perspectives on the EPA workshop on the relationship between short-term test information and carcinogenicity. *Environ. Molec. Mutagen.*, 11:147–157.
53. Kleinhofs, A., and Behki, R. (1977): Prospects for plant genome modification by nonconventional methods. *Annu. Rev. Genet.*, 11:79–101.
54. Knudsen, A.G., Jr. (1973): Mutation and human cancer. *Adv. Cancer Res.*, 17:317–352.
55. Kopelovich, L., Bias, N.E., and Helson, L. (1979): Tumor promotor alone induces neoplastic transformation of fibroblasts from humans genetically predisposed to cancer. *Nature*, 282:619–621.
56. Kriek, E., Rojas, M., Alexandrov, K., and Bartsch, H. (1998): Polycyclic aromatic hydrocarbon-DNA adducts in humans: Relevance as biomarkers for exposure and cancer risk. *Mutation Res.*. 400:215–231.
57. Lewtas, J. (1986): A quantitative cancer risk assessment methodology using short-term genetic bioassays: The comparative potency method. In: *Risk and Reason: Risk Assessment in Relation to Environmental Mutagens and Carcinogens*, edited by P.Oftedal and A.Bragger, pp. 107–120. Alan R.Liss, New York.

58. Malling, H.V. (1971): Dimehthylnitrosamine: Formation of mutagenic compounds by interation with mouse liver microsomes. *Mutation Res.*, 13:425–429.
59. Maron, D.M., and Ames, B.N. (1983): Revised methods for the *Salmonella* mutagenicity test. *Mutation Res.*, 113:173–215.
60. McCann, J., Choi, E., Yamasaki, E.. and Ames B.N. (1975): Detection of carcinogens as mutagens in the *Salmonella/* microsome test: Assay of 300 chemicals. *Proc. Natl. Acad. Sci. USA*, 72:5135–5139.
61. McKusick, V.A. (1978): *Mendelian Inheritance in Man: Catalogs of Autosomal Dominant, Autosomal Recessive and X-linked Phenotypes, 5th ed.* Johns Hopkins University Press, Baltimore.
62. Mendelsohn, M.L., Moore, D.H., II, and Lohman. P.H.M. (1992): A method for comparing and combining short-term genotoxicity test data: Results and interpretation. *Mutation Res.*, 266:43–60.
63. Mohrenweiser, H.W., and Jones, I.M. (1998): Variation in DNA repair is a factor in cancer susceptibility: A paradigm for the promises and perils of individual and population risk estimation. *Mutation Res.*, 400:15–24.
64. Myhr, B. (1991): Validation studies with MutaMouse: A transgenic mouse model for detection mutations in vivo. *Environ. Molec. Mutagen.*, 18:308–315.
65. Nagao, M., Sugimura, T., and Matsushima, T. (1978): Environmental mutagens and carcinogens. *Annu. Rev. Genet.*, 12:117–159.
66. Nagao, M., Takahashi, Y., Yamanaka, H., and Sugimura, T. (1979): Mutagens in coffee and tea. *Mutation Res.*, 68:101–106.
67. Nagao, M., Yahagi, T., Kawachi, T., Seino, Y., Honda, M., Matsukura, N., Sugimura, T., Wakabayashi, K., Tsuji, K., and Kosuge, T. (1977): Mutagens in foods. and especially pyrolysis products of protein. In: *Progress in Genetic Toxicology*, edited by D.Scott, B.A.Bridges, and F.H.Sobels, pp. 259–264. Elsevier/North-Holland, New York.
68. NAS (1989): Biological significance of DNA adducts and protein adducts. In: *Drinking Water and Health, Volume 9*, pp. 6–37. National Academy Press, Washington, D.C.
69. Obe, G., and Anderson, D. (1987): Genetic effects of ethanol. *Mutation Res.*, 186:177–200.
70. OECD (Organization for Economic Co-operation and Development) (1981, revised May 1983): *Guidelines for Testing of Chemicals.*
71. Pedersen, R.A., and Brandriff, B. (1980): Radiation- and drug-induced DNA repair in mammalian occytes and embryos. In: *DNA Repair and Mutagenesis in Eukaryocytes*, edited by W.M.Generoso, M.D.Shelby, and F.J.de Serres, pp. 389–410. Plenum Press, New York.

72. Pet-Edwards, J., Chankong, V., Rosenkranz, H.S., and Haimes, Y.Y. (1985): Applications of the carcinogenicity prediction and battery selection (CPBS) method to the Gene-Tox data base. *Mutation Res.*, 153:187–200.
73. Pienta, R.J., Kuschner, L.M., and Russell, L.S. (1984): The use of short-term tests and limited bioassays in carcinogenicity testing. *Regul. Toxicol. Pharmacol.*, 4:249–260.
74. Ray, V.A. (1979): Application of microbial and mammalian cells to the assessment of mutagenicity. *Pharmacol. Rev.*, 30:537–546.
75. Reedy, E.P., Reynolds, R.K., Santos, E., and Barbacid, M. (1982): A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. *Nature*, 300:145–152.
76. Russell, L.B., Aaron, C.S., de Serres, F., Generoso, W.M., Kannan, K.L., Shelby, M., Springer, J., and Voytele, P. (1984): Evaluation of mutagenicity assays for purposes of genetic risk assessment. *Mutation Res.*, 134:143–157.
77. Russell, L.B., Selby, P.B., van Halle, E., Sheridan, W., and Valcovic, L. (1981): The mouse specific locus test with agents other than radiation: Interpretation of data and recommendations for future work. *Mutation Res.*, 86:329–354.
78. Saffer, J.D. (1992): Transgenic mice in biomedical research. *Lab. Animal* 21:30–38.
79. Samson, L., and Schwartz, J.L. (1980): Evidence for an adaptive DNA repair pathway in CHO and human skin fibroblast cell lines. *Nature*, 287:861–863.
80. San, R.H.C., and Stich, H.F. (1975): DNA repair synthesis of cultured human cells as a rapid bioassay for chemical carcinogens. *Int. J. Cancer*, 16:284–291.
81. Sasaki, Y., Saga, A., Yoshida, K., Su, Y.Q., Ohta, T., Matsusaka, N., and Tsuda, S. (1998): Colon-specific genotoxicity of heterocyclic amines detected by the modified alkaline single cell gell electrophoresis assay of multiple mouse organs. *Mutation Res.*, 414: 9–14.
82. Schmid, W. (1975): The micronucleus test. *Mutation Res.*, 31:9–15.
83. Setlow, R.B. (1978): Repair deficient human disorders and cancer. *Nature*, 271:713–717.
84. Shelby, M.D. (1988): The genetic toxicity of human carcinogens and its implications. *Mutation Res.*, 204:3–15.
85. Short, J.M., Kohler, S.W., Provost, G.S., Ferik, A., and Kretz, P. L. (1990): The use of lambda phage shuttle vectors in transgenic mice for development of a short-term mutagenicity assay. In: *Mutation and the Environment*, Part A, edited by M.Mendelsohn, and R.Albertini, pp. 355–367. Wiley-Liss, Inc., New York.
86. Simic, M.C., and Bergtold, D.S. (1991): Dietary modulation of DNA damage in human. *Mutation Res.*, 250:17–24.
87. Simmon, V.F., Mitchell, A.D., and Jorgenson, T.A.: Evaluation of selected pesticides as chemical mutagens, in vivo and in vivo studies. EPA-600/1–77–028. National Technical Information Center, Springfield, Virginia.
88. Singh, N.P., McCoy, M.T., Tice, R.R., and Schneider, E.L. (1988): A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell. Res.*, 175:184–191.
89. Slater, E.E., Anderson, M.D., and Rosenkranz, H.S. (1971): Rapid detection of mutagens and carcinogens. *Cancer Res.*, 31:970–973.
90. Sobels, F.H. (1982): Extrapolation from experimental test systems for evaluation of genetic risks in man. In: *Progress in Mutation Research, Vol 3*, edited by K.C.Bora, et al., pp. 323–327. Elsevier, Amsterdam.
91. Stich, H.F. (1991): The beneficial and hazardous effects of simple phenolic compounds. *Mutation Res.*, 259:307–324.
92. Sugimura, T. (1978): Let's be scientific about the problem of mutagens in cooked food. *Mutation Res.*, 55:149–152.
93. Sugimura, T., Sato, S., Nagao, M., Yahagi, T., Matsushima, T., Seino, Y., Takeurchi, M., and Kawachi, T. (1976): Overlapping of carcinogens and mutagens. In: *Fundamentals in Cancer Prevention*, edited by P.N.Magee, T.Matsushima, T.Sugimura, and S.Takayama, pp. 191–215. University of Tokyo Press, Tokyo, and University Park Press, Baltimore.
94. Turturro, A., and Hart, R.W. (1984): DNA repair mechanisms in aging. In: *Comparative Pathology of Major Age-Related Diseases*, edited by D.G.Scarpelli and G.Migaki, p. 1946. Mark R.Liss, New York.
95. UNEP (1992): *Assessing the Risk of Genetic Damage*, edited by D. J.Brusick, W.B.Gopalon, E.Hesletine, J.W.Huismans, and P.H. M.Lohman. Lewis Press, Boca Raton.
96. van Delft, J., Bergmans, A., van Dam, F., Tates, A., Howard, L., Winton, D., and Baan, R. (1998):

Gene-mutation assays in lacZ transgenic mice: Comparison of lacZ with endogenous genes in splenocytes and small intestinal epithelium. *Mutation Res.*, 415:85–96.
97. Venkatachalam, S., and Donehower, L.A. (1998): Murine tumor suppressor models. *Mutation Res.*, 400:391–407.
98. Vijg, J., and van Steeg, H. (1998): Transgenic assays for mutations and cancer: current status and future perspectives.
99. Vrieling, H., van Zeeland, A., and Mullenders, L.H.F. (1998): Transcription coupled repair and its impact on mutagenesis. *Mutation Res.*, 400:135–142.
100. Waters, M., and Auletta, A. (1981): The Gene-Tox program. *J. Chem. Inf. Comput. Sci.*, 21:35–38.
101. Weinberg, R.A. (1981): Use of transfection to analyze genetic information and malignant transformation. *Biochem. Biophys. Acta*, 651:25–35.
102. Weisburger, J.H., and Williams, G.M. (1981): The decision point approach for systematic carcinogen testing. *Food Cosmet. Toxicol.*, 19:561–566.
103. Williams, G.M. (1977): The detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell cultures. *Cancer Res.*, 37:1845–1851.
104. Wojewodzka, M., Kruszewsk, M., Iwanenko, T., Collins, A., Szumiel, I. (1998): Application of the comet assay for monitoring DNA damage in workers exposed to chronic low-dose irradiation I: Strand breakage. *Mutation Res.*, 416:21–35.
105. Wolff, S., Afzal, V., and Olivieri, G. (1990): Inducible repair of cytogenetic damage to human lymphocytes: Adaption to low-level exposures to DNA-damaging agents. In: *Mutation and the Environment*, Part B, pp. 397–405. Wiley-Liss, Inc., New York.
106. Woychik, R.P., Klebig, M.L., Justice, M.J., Magnuson, T.R., and Avrer, E.D. (1998): Functional genomics in the post-genome era. *Mutation Res.*, 400:3–14.
107. Yahagi, T., Nagao, M., Seino, Y., Matsushima, T., Sugimura, T., and Oleada, M. (1977): Mutagenicities of N-nitrosamines on *Salmonella*. *Mutation Res.*, 48:121–130.
108. Young, R., Oveisistork, F., Harrington-Brock, K., Schalkowsky, S., Moore, M., and Myhr, B. (1991): Quantitative size analysis of L5178Y TK +/− mutant colonies in soft agar: An interlaboratory comparison. *Environ. Molec. Mutagenenesis*, 17(Suppl. 19):79.
109. Department of Health and Social Security (1979): *Committee on Mutagenicity, Committee on Mutagenicity of Chemicals in Food, Consumer Products, and the Environment. A consultative document on guidelines for the testing of chemicals for mutagenicity*. Department of Health and Social Security, Great Britain.

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Chapter **18**

Acute Toxicity and Eye Irritancy

Louis C.DiPasquale and A.Wallace Hayes

Principles and Methods of Toxicology, Fourth Edition, edited by A.Wallace Hayes.
Taylor & Francis, Philadelphia © 2001.

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The methods and principles of evaluating two categories of hazards, acute systemic toxicity and eye irritation, both resulting from a single or very short-term exposure, are described in this chapter. In

recent years, economics and concerns over animal welfare have raised many issues in animal testing. Alternate methods for acute toxicity and eye irritation are being developed and, in some cases, accepted by regulatory agencies for hazard assessment purposes. This chapter describes the classical methods for determining acute systemic toxicity and eye irritation, and the more modern methods requiring fewer animals are also discussed. In addition, criteria have been put in place for the validation and regulatory acceptance of alternative tests and these will be reviewed.

PRINCIPLES OF ACUTE TOXICOLOGY

Acute toxicity testing began nearly a century ago when physicians and pharmacologists were concerned with potent poisons and drugs. In 1927, Trevan (355) introduced the concept of a median lethal dose (LD50) for the standardization of digitalis extracts, insulin, and diphtheria toxin. He recognized that the precision of the value was dependent on many factors such as seasonal variation and the number of animals used in a test. High-precision LD50 values can only be established with a large number of animals.

The list of extraneous factors that affect the precision of the LD50 has increased since Trevan's work and

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now includes, among other factors, sex, species, strain, age, diet, nutritional status, general health conditions, animal husbandry, experimental procedures, route of administration, stress, dosage formulation (vehicle), and intra- and inter-laboratory variations. In spite of the many variables affecting the LD50 determination, many government agencies still regard the LD50 as the sole measurement of the acute toxicity of all materials, though a change in this attitude has emerged.

It is important to obtain a sound measurement of the killing power of highly toxic substances because a small difference in exposure can distinguish a safe from a lethal situation. However, a precise LD50 is not necessary for less toxic materials such as pesticides and consumer household products. For these substances, an approximate measurement of the killing ability is sufficient and still desirable, since overexposure to these products is possible and lethal cases are not uncommon. There are errors inherent in the determination of LD50 values, some of which cannot be controlled by the experimenter, and it is therefore not scientifically sound to obtain a precise LD50 on low to moderate toxic substances. Many methods have been developed over the years to calculate LD50 values and to evaluate the acute toxicity of chemicals with a small number of animals. These methods are discussed later in this chapter. Many scientists have advocated changes in the emphasis of acute toxicity testing. To date, there is a general consensus among toxicologists in academia, industry, and government that a change in the emphasis of acute toxicity testing is needed (24, 122, 132, 208, 227, 262, 321, 339). The value of a precise LD50, except for highly toxic substances, is being de-emphasized and the focus is now on obtaining as much information as possible on the toxic manifestation and mechanism with the smallest number of animals. Undoubtedly, such information will be for physicians more useful than the LD50 in treating overexposure.

Even though the emphasis of acute toxicity testing is changing, the principles of dose-response and development of signs of toxicity remain the basis of the science of toxicology. This section refreshes the knowledge of the experienced and introduces the novice to these general concepts.

Definition of Acute Toxicity

Toxicity is defined as the harmful effect of a chemical or a drug on a living organism. Various expert groups have defined acute and subchronic toxicities. The Organization for Economic Cooperation and Development (OECD) defines acute toxicity as "the adverse effects occurring within a short time of (oral) administration of a single dose of a substance or multiple doses given within 24 hours" (275). In terms of human exposure, this definition of acute toxicity refers to life-threatening crises such as accidental catastrophes, overdoses, and suicide attempts.

Dose-Response Relationship

Toxicologists often obtain two types of data, quantal and graded. The quantal response is called the "all-or-none" response; it either happens or it does not happen. On the other hand, the graded response can be determined quantitatively and it is continuous. Mortality and incidences of pharmacotoxic signs are examples of quantal data, whereas enzyme activity, protein concentration, body weight, feed consumption, and electrolyte concentration are quantitative parameters. However, many apparently quantal responses are quantitative. If technical measurements permit, they may be graded. For example, the severity of a pharmacotoxic sign can be graded if detection methods are available.

At the molecular level, the graded dose-response relationship often can be explained by the receptor, a relatively old concept but still a valid one. Let S be a particular substance that produces a specific response by interacting with a target protein molecule, the receptor R, in the body to form a substance-receptor complex, SR. Assuming the reaction is reversible and there is only one binding site on every target receptor molecule, this process can be described by the following expression:

$$\mathrm{S} + \mathrm{R} \underset{K_2}{\overset{K_1}{\rightleftarrows}} \mathrm{SR}$$

and the mass equation for this reversible process is

$$\frac{K_2}{K_1} = k_d = \frac{[\mathrm{S}][\mathrm{R}]}{[\mathrm{SR}]} \quad (1)$$

where [S] [R], and [SR] are the concentrations of the substance, the receptor, and the substance-receptor complex at any particular time, respectively, and *Kd* is the dissociation constant of the process. Let [R]O be the initial concentration of the receptor, which is usually very small and constant in number when compared with the concentration of the substance. Then

[R]0=[R]=[SR]

thus

$$[R]=[R]_0-[SR]$$

Substituting the above into the mass equation [(Eq. (1)]

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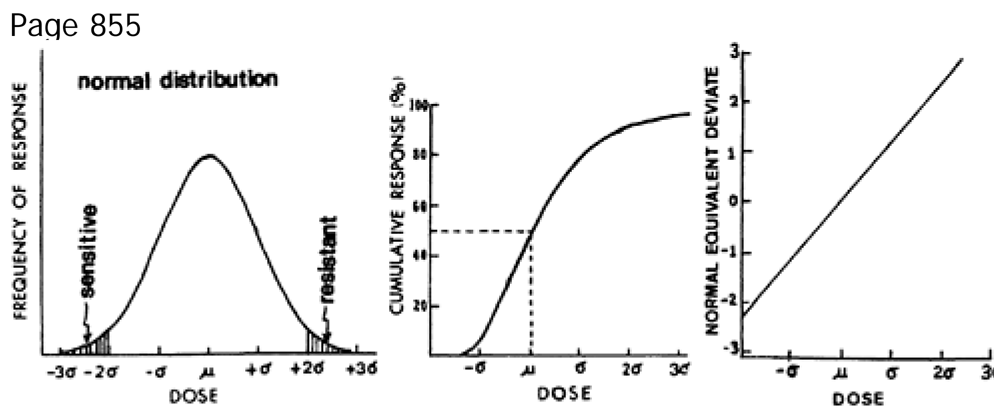

FIG. 18.1. Normal distribution of dose-response relationships: frequency of response, cumulative response, and cumulative response in terms of normal equivalent deviate.

and rearranging:

$$[SR]K_d = [S]([R]_0 - [SR])$$

or

$$[SR](K_d + [S]) = [R]_0[S]$$

which can be rearranged to

$$\frac{[SR]}{[R]_0} = \frac{[S]}{k_d + [S]} \quad (2)$$

SR/[R]O is the fraction of receptor that has reacted with the substance to form the substance-receptor complex. If we assume that the response *(E)* resulting from the interaction of the substance with the receptor is dependent on the fraction of total receptor concentration that has reacted with the substance, then

$$E = \frac{[S]}{k_d + [S]} \quad (3)$$

Equation (3) is a hyperbolic function; therefore, the response *(E)* is related to the concentration of the substance in a hyperbolic function relationship. If the concentration of the substance at the receptor site is dependent on the dose, then the response is dependent on the dose administered. This phenomenon is perhaps the simplest version of the receptor kinetic concept relating the dose of the chemical to a biological response. The kinetics of the receptor-substrate interaction may be more complicated, and different dose-response relationships could be drawn based on these complicated kinetics. Readers who are interested in different receptor-substance kinetics are referred to Ferdinard (135).

The quantal dose-response relationship often is difficult to conceptualize based on the receptor theory. However, quantal response also can be viewed as a graded response if the whole population is considered as an individual. This relationship can best be explained in terms of a probability distribution. For a particular response, members of a population, for example, all the rats in the world, respond differently to a particular stimulus such as exposure to a chemical. Some rats will be highly sensitive and others will be very resistant. If these different responses are distributed normally within the population (i.e., with most members of the population being neither extremely sensitive nor resistant), the well-known bell-shaped population distribution curve results. If the probability of dose-response is expressed in terms of cumulative response, a sigmoidal curve can be obtained, as shown in Figure 18.1. However, most biological response distributions are not exactly normal and tend to be skewed to the higher dose, that is, extreme resistors have a larger *range of dose* to response than the extremely sensitive portion of the population. In general, a logarithmic dose transformation can normalize the distribution (i.e., convert the skewed distribution to a normal distribution) (Figure 18.2). After this logarithmic dose transformation, if the probability of the log dose-response is expressed cumulatively, the sigmoidal response curve is obtained (Figure 18.2). How is this lognormal transformation related to a regular dose-response curve? Is there justification or basis for a log dose transformation? To answer these questions, let us again look at Eq. (3). This equation can be arranged to

$$E = \frac{[S]}{k_2/k_1 + [S]}$$

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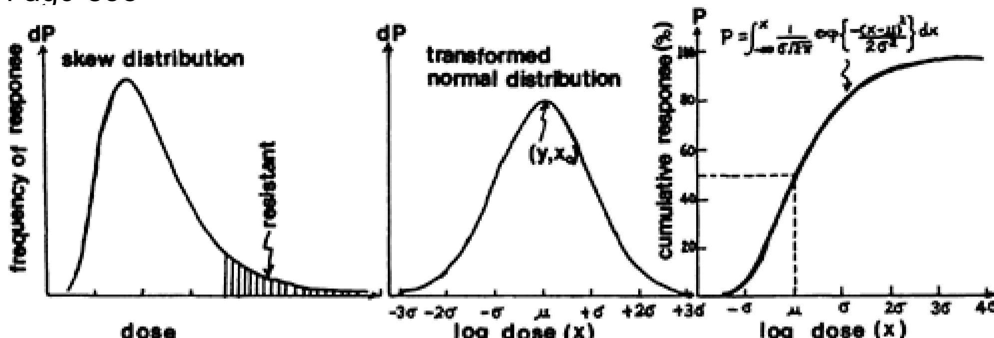

FIG. 18.2. Skew of dose-response can be normalized by log-dose transformation.

which can be rearranged to

$$E = \frac{k_1[S]}{k_2 + k_1[S]} \tag{4}$$

Over a certain concentration range, Eq. (4) will produce a curve very similar to the logarithmic function $E=K1 \log (k2 [S]+1)$ (77). Therefore, there may be justification for the log transformation besides simply a mathematical convenience.

Because a sigmoidal curve is more difficult to analyze than a straight line, many experts feel that further transformation of the log dose-response hyperbolic function is necessary to obtain a *straight-line* function curve. Perhaps the most widely used transformation is the normal equivalent deviate (NED) or the similar probit transformation (34, 78, 82, 95, 105, 138, 242). This technique involves the log dose transformation and the transformation of the cumulative response probability to the NED or probit. After both the probability and the dose are transformed, their transformed values are directly related to each other. A brief derivation of the straight-line direct function relationship between the log-dose and NED or probit is presented later in this chapter.

LD50and Its Determination

Definition

The LD50 in its simplest form is the dose of a compound that causes 50% mortality in a population. A more precise definition has been provided by the OECD panel of experts as the "statistically derived single dose of a substance that can be expected to cause death in 50% of the animals" (275). In other words, an LD50 of a compound is not a constant, even though it has been treated that way by many; rather, it is a statistical term designed to describe the lethal response of a compound in a particular population under a discrete set of experimental conditions.

The significance of LD50 values has been examined by many scientists (24, 122, 132, 208, 227, 262, 321, 339) who have arrived at similar conclusions: the LD50 is an imprecise value, it is not a biological constant and should be de-emphasized for most materials. They agree, furthermore, that approximate LD50 values often are sufficient for all practical purposes and that more emphasis should be placed on signs of toxicity, target organs, and other factors.

The numeric value of the LD50 has been used to classify and compare toxicity among chemicals. The extent of involvement of the LD50 in safety evaluation has almost reached a level of abuse. Although determining the LD50 under a set of experimental conditions can provide valuable information about the toxicity of a compound, the numeric LD50 per se is not equivalent to acute toxicity. One must always remember that lethality is only one of many reference points in defining acute toxicity. The slope (response/dose) of the dose-response curve, the time to death, pharmacotoxic signs, and pathological findings are all vital or even more critical than the LD50 in the evaluation of acute toxicity. Therefore, defining acute toxicity based only on the numeric value of an LD50 is inappropriate.

As pointed out previously, lethality is a quantal response, and the probability of a cumulative response is related to dose in a hyperbolic (sigmoidal) function. The cumulative probability of response is directly related to the standard deviates of a log dose population (Figure 18.1). Therefore, the slope of the log dose-response curve will indicate the relationship between the range of dose and the lethal response. This relationship is more important in risk assessment than the numeric value of the LD50 because more insight is available about the intrinsic toxic characteristics of a compound.

Sometimes the slope can give a clue to the mechanism of toxicity. For example, a steep slope may indicate rapid onset of action or faster absorption. A large margin of safety is predicted when a compound has a flat slope, that is, only a small increase in response with a large increase in dose. With the slope, it often is possible to extrapolate the response to a low dose (e.g., LD0, LD1) or even to a no-observed-effect level. It is especially important to know the slope when comparing a set of compounds. Two compounds may have identical LD50 values but different slopes and thus have quite different toxicological characteristics depending on the range of doses. Parallel dose-response curves may indicate a similar mechanism of toxicity, kinetic pattern, and probably similar prognosis. Neither the LD50 nor the slope can reveal absolutely a specific mechanism, but with pharmacokinetic and other biochemical studies elucidation of the mechanism of toxicity may be possible.

*Determination of LD***50**

Many methods are available for the determination of the LD50. They can be grouped into two categories, the *normal population assumption* and *the normal population assumption-free* methods. The former usually can be analyzed by graphic procedures.

The normal population assumption-free methods are represented by Thompson's moving average interpolation (353, 372) and the "up-and-down" method (51–54, 77, 98). The former method is widely accepted, and convenient tables (105, 372) are available for estimation of the value of the LD50 with confidence limits when either 0 or 100% mortality incidences are observed. However, there are some restrictions on the use of this method, that is, four doses at equal log dose intervals and the number of animals per dose level must be equal. The up-and-down or the pyramid method is designed to estimate the LD50 with a small number of samples. It has a relative cost advantage because fewer animals are needed, but the test may be time-consuming and require excessive test material. Because of the advantage of using only a few animals, this method is popular when the test has to be conducted in large animals such as cows or sheep or expensive animals such as monkeys. A study comparing LD50 values obtained by the up-and-down method and other methods revealed an excellent agreement (52). There are apparently two shortcomings for this method—it is not adequate for estimating the incidence of delayed deaths, and a dose-response of mortality or signs of toxicity cannot easily be obtained. However, Weil (373) has adapted the up-and-down method to calculate the slope of acute toxicity response.

The normal population assumption method is represented by the probit analysis approach, which can be performed either by graphic means (138) or by mathematical calculation (139). Because the probit analysis is used widely in evaluating acute toxicity data, the priniciples are discussed briefly. This method involves the transformation of both the cumulative response probability and the dose.

When the dose is transformed into a log dose (x), the frequency of response versus log doses follows a normal distribution (Figure 18.2), which can be expressed mathematically as

$$dP = \frac{1}{\sigma\sqrt{2\pi}} \exp\left(\frac{-(x-u)^2}{2\sigma^2}\right) \tag{5}$$

where σ^2 and u are the variance and the mean of the population, respectively, and P is the probability corresponding to each value of x (Figure 18.2). The LD50 is defined as the log dose that can produce 50% mortality in a population (i.e., P=0.5 or 50% cumulative response). Let x be the log LD50; then P=0.5 will correspond to the area under the log normal distribution curve from $-\infty$ to x_0; or P=0.5 will correspond to the integration of Eq. (5) from $-\infty$ to x_0: That is,

$$P = 0.5 = \int_{-\infty}^{X_0} \frac{1}{\sigma\sqrt{2\pi}} \exp\left(\frac{-(x-u)^2}{2\sigma^2}\right) dx \tag{6}$$

The solution of Eq. (6) is $x=u$, the true mean or the median of the log normal distribution. One way to solve this equation is by a graphic method. The integration of Eq. (5) from $x=-\infty$ to $+\infty$ can be represented graphically by a sigmoidal curve as illustrated in Figure 18.2. Analysis of the sigmoidal curve is more difficult than a straight line. One way to transform the sigmoidal curve to a straight line is by NED analysis or, similarly, by probit analysis. For a detailed description of this analysis, the reader should consult Finney's text (138). A brief derivation of the straight-line function between log dose and the transformed probability of response is described below.

Probability *(P)* is normally expressed in terms of percentage or with values between 0 and 1; but Gaddum (152) has proposed to measure the probability of response on a transformed scale called the normal equivalent deviate (NED), or the standard deviation of a normal distribution, which can be

described mathematically by Eq. (5). In a particular case, the normal distribution of response with mean equal to 0 and the standard deviation equal to 1, Eq. (5) can be written as

$$dP = \frac{1}{\sqrt{2\pi}} \exp\left(\frac{-x^2}{2}\right) dx$$

Similarly, if this distribution of response is plotted on the

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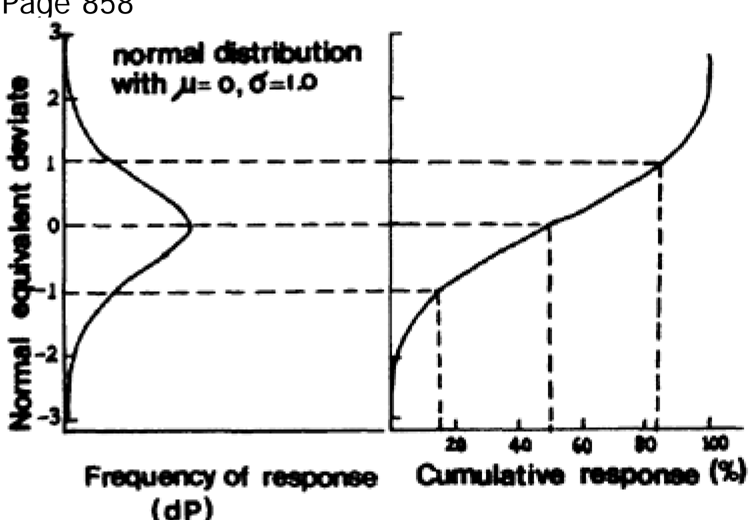

FIG. 18.3. Probability of response can be expressed in terms of percentage of the population or the NED of a normal distribution with mean=0 and standard deviation=1.

y axis (Figure 18.3), then

$$dP=\frac{1}{\sqrt{2\pi}}\exp\left(\frac{-y^2}{2}\right)dx \quad (7)$$

The probability in such a case is defined by a value on the *y* axis of Figure 18.3, that is, the integration of Eq. (7) from −∞ to *y:*

$$P=\frac{1}{\sqrt{2\pi}}\int_{-\infty}^{y}\exp\frac{-y}{2}dy \quad (8)$$

In other words, for each value of *y* (from −∞ to +∞) expressed in terms of the standard deviation of a normal distribution with the mean equal to 0 and the standard deviation equal to 1, there is a corresponding value of probability *(P)* expressed in terms of percentage or having a value ranging from 0 to 1. Thus, equivalent values on the *y* axis can be used to define the value of *P* or vice versa; *y* and *P* define each other. This relationship is illustrated in Figure 18.3.

The particular probability of response to a particular log dose value *x,* as described in Eq. (6), will be

$$P=\int_{-y}^{-x}\frac{1}{\sigma\sqrt{2\pi}}\exp\left(\frac{-(x-u)^2}{2\sigma^2}\right)dx \quad (9)$$

where *u* and *σ* are the mean and standard deviation of the log dose, respectively.

If *P* is expressed by a value of *y* on the *y* axis (standard deviations), then

$$P=\frac{1}{\sqrt{2\pi}}\int_{-\infty}^{y}\exp\left(\frac{-y^2}{2}\right)dy$$

$$=\int_{-\infty}^{x}\frac{1}{\sigma\sqrt{2\pi}}\exp\left(\frac{-(x-u)^2}{2\sigma^2}\right)dx$$

The solution of this equation is $x=u+\sigma y$ or

$$y=\frac{(x-u)}{\sigma}-\frac{1}{\sigma}x-\frac{u}{\sigma} \quad (10)$$

Therefore, the probability when expressed in terms of *y* (the NED scale) is related linearly to *x,* the log dose. If *x* is plotted against the corresponding *y,* a straight line with slope=1/*σ* will be obtained. To further facilitate calculation, Bliss (33) suggested a slightly different NED unit called the probit, such that the new *y* value is equal to $[(x-u)/\sigma]+5$. This procedure eliminates the negative values of NED when *P* has a value of less than 50%. Therefore, the probit is equal to the NED plus 5. The linear relationship between probits and log dose is similar to the relationship between NED and log dose. Thus, when *y*=5, from Eq. (10),

$$5 = \frac{(x-u)}{\sigma} + 5$$

and $x=\mu$ (i.e., the median log dose which has a probability of response of 50%).

Logistic Transformation

Waud (371) suggested a logistic approach to calculate the LD50. Thus

$$P = \frac{D^E}{(D^E + K^E)}$$

where P is the probability of response, D is the dose, E and K are scale and location parameters, respectively, and K corresponds to the LD50. With the procedure of iteration, K and E can be estimated with a range of confidence. The derivation of this equation is beyond the scope of this chapter, and interested readers are referred to the original article by Waud (371).

Nonlethal Parameters

Although the LD50 and the slope of the dose-response curve can provide valuable information on the toxicity of a compound, the LD50 *is not equivalent to toxicity*. Chemicals can induce damage to the physiological, biochemical, immunological, neurological, or anatomical systems. Depending on the severity and the extent of the disturbance of the normal biological functions, the animal may survive the toxic response but some irreversible damage may occur. These nonlethal, adverse effects are as undesirable as lethality and certainly should be taken into consideration in the risk assessment of a chemical.

The major problem in the analysis of nonlethal responses is that the data often are not quantal. For example, dermal toxicity ranges from slight to severe.

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These polychotomous data may be handled by RIDIT analysis, which was designed to analyze quantal responses with more than two outcomes (2, 50, 176).
Although toxic effects may contribute to lethality, any attempt to correlate a particular nonlethal response to mortality may be irrational (340) unless that response is the only one responsible for the eventual death of the animal. Identification of the response or responses related to mortality is not often a straightforward matter. Nonlethal responses that affect the general well-being of an animal should be considered in the risk assessment of a compound. If nonlethal responses can be viewed as true quantal data, the *median effective dose* (ED50) and the corresponding dose-response curve may apply. The ED50, which often is used in the standardization of a biologically active compound such as a drug, has a meaning similar to the LD50 except that it is designated to examine nonlethal parameters such as pharmacological responses and other nonlethal adverse effects. The ED50 is defined as a statistically derived single dose of a substance that can be expected to cause a particular effect to occur in 50% of the animal population. The therapeutic index (TI), defined by the ratio of LD50/ED50 or LD1/ED99, has been applied to establish the safety margin of some biologically active drugs. The higher the index, the greater the margin of safety with the drug, that is, a large difference exists between the amount of compound predicted to kill 50% of the animals and the amount of compound predicted to elicit a particular response in 50% of the animals. The TI gives an even greater estimate of safety when the LD1 is compared with the ED99.

Reversibility of Nonlethal Parameters

In general, reversible responses are those that diminish with elimination of the chemical from the body. A true reversible response will cause no residual effects when the chemical is completely eliminated from the body. Such responses are commonly seen in drugs used at therapeutic dose levels. As the amount of drug in the body increases, the magnitude of the effect also increases. If it is truly reversible, the effect will wear off when the drug is completely eliminated.
The reversibility of a particular response is dependent on the organ or system involved, the intrinsic toxicity of the chemical, the length of exposure, the total amount of the chemical in the body at a specific time, and the age and general health of the animal. If the amount of chemical in the body is high enough, the intensity of the response may overwhelm a particular organ. Effects indicated through hormonal imbalance such as thyroid effects generally are reversible unless the threshold is surpassed. Damage in rapidly regenerating organs such as the liver is usually more likely to be reversible than damage in nonregenerating tissues such as nerves. A good example is the delayed onset of neuropathy caused by many organophosphate insecticides. The chemical may be completely eliminated from the body before the effect manifests itself. Animals with renal or liver diseases are often more susceptible to damage (reversible or irreversible) by a chemical insult because of their decreased ability to eliminate the chemical. Exposure to a chemical at an early age may induce irreversible damage more easily than at an older age because of the limited development of the kidneys and/or functional capacity of other organs such as the liver.
In risk assessment, it is important to know whether a toxic effect is reversible. Irreversible effects seen in animals obviously are weighted more heavily in reaching a conclusion on the toxicity and hazard a chemical may pose for humans.

ACUTE TOXICITY TESTING

The objectives of acute toxicity testing are to define the intrinsic toxicity of the chemical, predict hazard to nontarget species or toxicity to target species, determine the most susceptible species, identify target organs, provide information for risk assessment of acute exposure to the chemical, provide information for the design and selection of dose levels for prolonged studies, and, most important and practical of all, provide valuable information for clinicians to predict, diagnose, and prescribe treatment for acute overexposure (poisoning) to chemicals. Acute studies often are called the “first line of defense” in the absence of data from long-term studies. These data help industrial, government, and academic institutions formulate safety measures for their researchers and for limited segments of their worker population during the early stage of the development of a chemical. From a regulatory standpoint, acute toxicity data are essential in the classification, labeling, and transportation of a chemical. From an academic standpoint, a carefully designed acute toxicity study can often provide important clues on the mechanism of toxicity and the structure-activity relationship for a particular class of chemicals.
Many acute toxicity studies have been conducted solely for the purpose of determining the LD50 of a chemical. However, the reader is reminded that acute toxicity is not equivalent to the LD50, and that the LD50 is not an absolute biological constant to be equated, as many investigators have, with such chemical constants such as pH, pKa, melting point, and solubility. The LD50 is only one of many indices

used in defining acute toxicity. A well-designed acute toxicity study should include consideration of the dose-response relationship of both lethal

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and nonlethal parameters, as discussed above. Sometimes biochemical measurements in an acute test can aid in elucidating the mechanism of toxic actions. Histopathology of organs may be helpful in determining the cause of death and identifying the target organs.

Types of Acute Testing

Because acute toxicity data may provide the first line of defense, a battery of tests under different conditions and exposure routes should be considered. In general, these tests should include oral, dermal, and inhalation toxicities, and skin and eye irritation studies. Other tests such as acute pre-neonatal and neonatal exposure, dermal contact sensitization, and phototoxicity should be considered. Depending on sound scientific factors, which may vary from one chemical to another, the number and kind of acute tests needed to establish the initial toxicity database may not be the same. For example, when inhalation exposure is not expected to occur because of the physical properties of the chemical, inhalation testing may not be needed. Such a case is not uncommon if respirable particles cannot be generated, even under the most favorable laboratory conditions. Nonetheless, for most purposes, oral, dermal, skin, and eye tests should be considered in the initial acute investigation. These four tests are often sufficient for regulatory purposes, labeling, and classification of a chemical, although increasing concerns also are placed on inhalation and skin sensitization studies. This chapter is concerned only with acute oral and dermal toxicity and eye irritation.

Acute Oral Toxicity

Principles

The test substance, undiluted or diluted with the appropriate solvent or suspending vehicle, is given to several groups of animals by gavage with a feeding needle or by gastric intubation. A vehicle control group is included if needed, but generally this group is not necessary if the toxicity of the vehicle is known. Clinical signs, morbidity, and mortality are observed at specific intervals. Animals that die or become extremely moribund during the study are subjected to necropsies. Animals that survive the test period are killed and necropsied at the end of the observation period. Tissues may be saved for histopathological examination to facilitate the understanding of the acute toxicity of the compound. To increase the reproducibility of the study, all experimental conditions and procedures should be standardized, and the study should be conducted according to generally recognized good laboratory practices (GLP) outlined by the U.S. Environmental Protection Agency (EPA) and the OECD (120, 121, 281).

Animal Species

The responses elicited by a compound often vary greatly among species. Ideally, toxicity tests should be conducted with an animal that will elicit compound-related toxic responses similar to those that occur in humans, that is, an animal that metabolizes the compound identically to humans and that has the same susceptible organ system(s). Under such conditions, the animal data may be extrapolated to humans. Unfortunately, finding such an ideal animal is a difficult if not impossible task.

A less ideal but more manageable approach is to conduct acute toxicity studies in a variety of animal species under the assumption that if the toxicity of a compound is consistent in all the species tested, then a greater chance exists that such a response may also occur in humans. Even though the response in different species is not consistent, it generally is considered better to err on the safe side with the risk assessment being based on the most sensitive species unless there is justification that such responses are less likely to occur in humans, for example, because of dissimilarity in metabolism between the less sensitive animal species and humans. Although these are logical assumptions and generally quite reliable, the danger of underestimating or overestimating the response in humans still exists. Therefore, there is no absolute criterion for selecting a particular animal species. However, priority should be given to species with metabolism or other physiological and biochemical parameters similar to humans. Animal species also should be selected on the basis of convenience, economic factors, and the existing database for the animal. Rats, mice, rabbits, and guinea pigs are most commonly chosen for acute toxicity studies.

Other Animal Variations

Acute toxicity, even within a particular species, can vary with health conditions; age; sex; genetic makeup; body weight; differences in absorption, distribution, metabolism, and excretion of the compound; and the influence of hormones (101). A conscientious investigator should be aware of the possible interaction of chemical treatment with these parameters. For example, immature animals may lack an effective drug-metabolizing enzyme system; this may contribute to higher toxicity of the compound in an immature animal if an enzyme is responsible for detoxification of the compound, or to decreased toxicity if an enzyme is responsible for activation of the compound. Obesity may affect the

distribution and storage of a compound, especially when it is highly

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lipophilic. Sex hormones may be the target, or sex hormones may modify a particular toxic response which then may account for different toxic responses between sexes. Liver and renal diseases associated with old age may contribute to higher toxicity. Variations in genetic makeup among different strains may alter metabolism or other parameters, which may affect the toxicity of a particular compound. It is therefore important to document all data on animals: age, sex, body weight, strain, general health condition, and source. In general, healthy, young adults should be used. For example, if a rat is the test animal, then young adults weighing between 150 and 250 g should be used. The weight variation among animals should not exceed ±20% of the mean body weight.

Animal Numbers and Sex

The precision of the acute test is dependent to a large extent on the number of animals employed per dose level. Historically, 10 rats (5 male and 5 female) have been recommended in most regulatory guidelines (109, 124, 125, 275, 337), although more recently modified protocols are acceptable using as few as three animals per dose level (278, 279). The degree of precision needed and, in turn, the number of animals per dose group needed depend on the purpose of the study. In screening tests or tests designed to define the range of toxicity, fewer animals per dose level or fewer numbers of dose levels may be considered. In rare situations where a fairly precise LD50 is needed, the number of dose levels (at least three dose levels) and animals per dose group may need to be increased. The number of animals and dose levels should be based on sound scientific judgement.

In 1986, the OECD updating committee recommended that acute toxicity studies be conducted first in the male and at one dose level in females. If a marked sex difference appears when one dose level is used, then the test should be cross-checked. This approach reduces the number of animals used in acute toxicity testing. The more recent guideline (279), which is even more effective in reducing the number of animals used, specifies that in the absence of information suggesting that males are more sensitive, only females should be used. Literature surveys have shown that when there are sensitivity differences between the sexes, females, in general, are more sensitive (225).

Animal Housing and Environment

Studies should be conducted in a controlled environment. The temperature (22±3°C), relative humidity (30–70%), light and dark cycle (12 h dark, 12 h light), diet, and quality of drinking water should be standardized and maintained continuously. Animals may be caged in groups by sex or caged individually, depending on the species and size of the animals and on the needs of the particular study. Rodents can be caged in groups, usually not more than three per cage, but larger animals such as rabbits and dogs should be housed individually.

Dose levels. In general, the dose levels should be sufficient in number to allow a clear demonstration of a dose-response relationship and to permit an acceptable determination of the LD50, if required. Three dose levels generally are sufficient. The selected dose levels should bracket the expected LD50 value with at least one dose level higher than the expected LD50 but not causing 100% mortality, and one dose level below the expected LD50 value but not causing 0% mortality, when the probit analysis method is applied to estimate the LD50. However, with a method such as the moving average under some specific conditions (at least four dose levels with equal logarithmic intervals between each dose level, and with equal numbers of animals in each dose group), the LD50 can be estimated even with 0% mortality at the lower dose levels and 100% mortality at the two higher dose levels.

In any event, three or more dose levels with a wide range of toxicity responses are recommended if no other toxicity data are available. A pilot study may be needed for the selection of dose levels. Fewer animals per dose level and wider logarithmic intervals between dose levels usually are selected for the pilot study to ensure bracketing of the expected LD50 value. For example, if one desires an equal log interval of 0.6 between dose levels, and if the lowest dose is 1 mg/kg (log dose=0), the next log doses would be 0+0.6, 0.6+0.6, 1.2+0.6, and so on, corresponding to dose levels of antilogs 0.6, 1.2, 1.8 (i.e., the dose levels are: 1, 4, 16, 64, etc., in a geometric progression ratio of 4).

Dosages. If necessary, the test substance should be dissolved or suspended in a suitable vehicle, preferably in water, saline, or an aqueous suspension such as 0.5% methyl cellulose in water. If a test substance cannot be dissolved or suspended in an aqueous medium to form a homogeneous dosage preparation, corn oil or another solvent can be used. If the toxicity of the vehicle is not known, a vehicle control group should be included in the test. The animals in the vehicle control group should receive the same volume of vehicle given to animals in the highest dose group.

The test substance can be administered to animals at a constant concentration across all dose levels (i.e., varying the dose volume) or at a constant dose volume (i.e., varying the dose concentration). However, the investigator should be aware that the toxicity observed by administration in a constant

concentration may be different from that observed when given in a constant dose volume. For instance, when a large volume of corn

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oil is given orally, the gastrointestinal motility is increased, causing diarrhea and decreasing the time for absorption of the test substance in the gastrointestinal tract. This situation arises when a highly lipid-soluble chemical is tested. A large fraction of such a material may quickly pass through the gastrointestinal tract and remain unabsorbed. Local irritation of a test substance generally decreases when the material is diluted. If the objective of the study is to establish systemic toxicity, the test substance should be administered in a constant volume (diluted concentration) to minimize gastrointestinal irritation that may in turn affect the absorption of the test substance. On the other hand, the test substance should be administered undiluted to assess the irritation potential of the test substance. The choice of constant concentration versus constant dose volume should be based on sound scientific judgment. The OECD guideline suggests a constant dose volume approach (275). The maximum dose volume in rodents should not exceed 10 ml/kg body weight for non-aqueous vehicles or 20 ml/kg body weight for aqueous solution or suspension. In any event, for scientific and humane reasons the dose volume should be as small as possible.

Observation. As has been discussed previously, the emphasis in acute toxicity studies is on the determination of the dose-response and the onset of toxic signs. The observation period should be flexible depending on the purpose of the study. This period should be based on the onset of signs, the nature of the toxicity, time to death, and the rate of recovery. For most highly toxic substances, the onset of toxic signs and the time to death may be very short, and prolonged observation may not be necessary. The slope of the dose-response curve for such test substances is usually very steep, and the treated animals either die or survive within a very short time. For other substances, the onset of signs and the time to death may be delayed for a few days to a few weeks or longer. Obviously, a longer observation period is needed to detect these delayed acute effects. The observation period also should be long enough for the determination of reversibility or the recovery of an adverse effect. Under specific circumstances the observation period might be longer, but it normally does not exceed 14 days.

Test limit. All chemicals can produce toxicity under some experimental conditions; for instance, if a sufficiently large dose is given. It is therefore scientifically misleading and inhumane to conduct acute toxicity studies at unreasonably high dose levels for the sake of demonstrating lethality and/or toxicity, which may be irrelevant to the compound itself. For example, an extremely high dose of a practically nontoxic compound can cause gastrointestinal blockage, which, in turn, can result in gastrointestinal tract dysfunction. Toxicity in this case is not related to the intrinsic characteristic of the test substance, because it is a direct result of the physical blockage caused by the biologically inert substance. There must be a point, however, at which an investigator can conclude that a test substance is practically nontoxic or nonlethal after an acute exposure. The traditional test limit for acute oral toxicity was considered to be 5.0 g/kg body weight. If no mortality was observed at this dose level, a higher dose level generally was not necessary. The more recently accepted protocols for acute toxicity (277–279) have a test limit of 2.0g/kg-body weight.

Testing methods and procedures. The purpose of this section is to describe practical experimentation in detail, but it is not the authors' intention to list all technical procedures. A manual is available that describes technical procedures such as handling and dosing animals (286). Since the Good Laboratory Practices Act has been implemented in both the United States and most OECD countries, all experimental procedures should be documented and the studies conducted by trained personnel. For more details on GLP guidelines, the reader may consult several EPA, OECD, and Japanese publications (120, 121, 281, 337). The Japanese guidelines contain some unique requirements that are not duplicated by other countries.

Grouping, randomization, and preparation of animals. Animals not previously treated with test substances in other studies should be identified individually by coded marks, metal ear tags, or tattoos. The animals then should be quarantined for at least a week prior to dosing to acclimatize them to the conditions of the animal room. The animals should be fasted prior to administration of the test substance if the route of administration is oral. The purpose of fasting the animal is to eliminate feed in the gastrointestinal tract, which may complicate absorption of the test substance. Rats usually are fasted overnight. Because mice have a higher metabolic rate, withholding feed for 3–4 h may be adequate. Over-fasting small animals with a high metabolic rate may induce undesirable effects.

The animals should be randomly assigned to dose groups. Randomization, needed not only in acute toxicity studies but also in subchronic and chronic studies, ensures a homogeneous population and can minimize errors due to sampling bias. All animals with body weights and health conditions out of the normal range should be eliminated prior to the randomization procedure. With computer-generated random digit numbers or with tables of random digit numbers such as the one below, animals can be

randomly assigned to groups by cage number or by individually assigned animal number.

Random numbers

	00–04	05–09	10–14	15–19	20–24
00	01826	72696	67261	13748	57834
01	70371	12890	90395	45245	71282
02	46616	84522	17249	78172	14197
	25–29	30–34	35–39	40–44	45–49
00	27748	47492	43428	85524	19311
01	15960	02749	86763	80564	02631
02	84272	53226	96719	83462	05628

For example, by using a random number table, 30 rats from a total of 60 rats are assigned to 3 dose groups of 10 rats each. Assume that 12 rats will be excluded from the study because of overweight, underweight, or other health problems; this leaves 48 rats, from which 30 would be chosen. One method of randomization is to arbitrarily number the 48 rats from 1 to 48. The random number table gives the following series of random numbers (row 00): 01826, 72696, 67261, 13748, 57834, 27748, 47492, and 43428. Because there are only 48 rats, the numbers should be two-digit numbers. The first group of rats would be rat numbers 01, *82, 67,* 26, *96, 67, 26,* 11, 37, *48, 57, 83,* 42, *77, 48,* 47, *49,* 24, 34, and 28. The italicized numbers should be discarded because they are either repeated numbers or greater than 48. The second group of rats would be chosen similarly. The same procedure is repeated until 30 rats have been assigned to the 3 dose groups.

When the number of animals to be grouped is small, a large number of random numbers may be required to complete the grouping. Another way to use the random number table under such circumstances is by cycling. For example, 6 rabbits are assigned to 2 groups of 3 rabbits each from a total of 10 rabbits. From the second row of the same random digit number table, the first 3 two-digit numbers are 70, 73, and 11. Dividing these numbers by the total number of rabbits (i.e., 10), the remainders of these three numbers are 0, 3, and 1. If the remainder is zero, the denominator (in this case 10) will remain. Therefore, the 3 rabbits in the first group would be rabbit numbers 10, 3, and 1. Again, if the remainder numbers are repeated, they would be discarded. Also, if the number is smaller than the total number of animals, the number would be used and no division is needed. For the second group, the next series of numbers from the table are 28, 90, 90, 39, 54, 52, 45, 71, 28, 21, 59, 60, 02, 74, 98, and 67, and the corresponding numbers are 8, 0, 0, 9, 4, 2, 5, 1, 8, 1, 9, 10, 2, 4, 8, and 7. Therefore, the 3 rabbits in the second dose group would be rabbit numbers 8, 9, and 4. If a third group is needed, the 3 rabbits in this group would be rabbit numbers 2, 5, and 7. In general, the following rules apply when using the cycling method:

1. When the number from the random number table is less than the total number of animals to be chosen from, no division is needed.
2. When the number is greater than the total number of animals, the random number is divided by the total number of animals, and the remainder is used.
3. When the remainder is zero, the denominator is used.
4. No numbers or remainders should be allowed to repeat: any repeated numbers should be discarded.

The entry (the starting point) in the random number table should *not* be consistent. Thus, the entry should be made at a different point each time the random number table is used (one can start along the rows or the columns).

Calculation and Preparation of Doses

Doses in general are based on the body weight of the animal (expressed as weight of the test substance per kilogram of body weight of the animal), although for larger animals, the surface area may be more appropriate. The weight (or dose) of the test substance often is expressed in milligrams or grams of active ingredient if the test substance is not 100% pure. Ideally, only 100% pure sample should be tested; however, impurity-free samples are difficult to obtain. Although some toxicologists strongly advocate the use of pure samples in toxicity testing, others see the appropriateness of using technical samples, formulations, or crude products. It is acceptable to study the test substance in its pure form or in its technical or product form; however, the toxicity of impurities should be examined separately if the investigator feels that the impurities may contribute significantly to the toxicity of the test substance.

Selection of doses often is based on a pilot study, on existing toxicity data in other species, or on data obtained with a similar analog of the test substance. In an example based on the traditional LD50 determination, if 0 of 3 and 3 of 3 rats died at dose levels of 500 and 1000 mg/kg, respectively, in the pilot study, the expected LD50 numeric value would be between these two dose levels. If the investigator decides to select five doses that will bracket the expected LD50, the logical approach would

be to set the LD50 at 750 mg/kg for the definitive study with the following dose levels: 520, 625, 750, 900, and 1080 mg/kg. The dose levels progress by a value of the antilog 0.08 (i.e., multiplied or divided by 1.2 of the assumed LD50). Although more modern methods

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may require fewer dose levels, the following procedure for calculation and preparation of doses is similar.

If the test substance contains only 75% active ingredient and the investigator chooses a constant dose volume of 10 ml/kg body weight across all dose levels, it will be more convenient to prepare a stock solution so that when 10 ml/kg of this stock solution is given to the animal, the dose will be 1080 mg/kg (active ingredient). The concentration of this stock solution would be

$(1080 \text{ mg}/10 \text{ ml}) \div 0.75 = 144$ mg of test substance/ml.

Aliquots of the test substance for other dose levels can be prepared by dilution of the stock solution. For example, the solution concentration for the 900 mg/kg dose is

$(900 \text{ mg}/10 \text{ ml}) \div 0.75 = 120$ mg of test substance/ml.

This solution can be prepared by diluting the stock solution 1.2 times, that is, for each ml of the 120 mg/ml solution to be prepared,

$$\frac{120 \text{ mg/ml} \times 1 \text{ ml}}{144 \text{ mg/ml}} = 0.833 \text{ ml of the stock solution should be diluted to a final volume of 1 ml with the vehicle.}$$

The vehicle should be one with limited or no toxicity. In preparing dosage solutions or suspensions, the vehicle of choice is water; other choices include an aqueous suspending vehicle such as 0.5% (w/v) methyl cellulose (Methocel) in water; corn oil; or other solvents such as aqueous ethanol, propylene glycol, or diluted DMSO in 5.0% $NaHCO_3$ solution. Magnetic stirring bars, micromills, or homogenizers can be used in preparing suspensions. Sometimes a small amount of a surfactant such as Tween 80, Span 20, or Span 60 is helpful in obtaining a homogeneous suspension. If surfactants are used, one must be aware of the potential effect of these surfactants on the absorption of the test substance.

On many occasions, it is desirable to prepare the dosage in an aqueous medium because of the undesirable effect of corn oil or other vehicles on the animals. In this case, one may try to prepare the dosage in a simple, watersuspensible, uniform formulation even though the test material is not soluble or cannot be suspended in aqueous medium. For example, a formulation can be prepared by dissolving a specific amount of the test material in an adequate amount of acetone, followed by mixing the acetone solution with a known amount of small particle size and biologically inert dispensing agent such as HiSil, and evaporating to dryness under a hood. In the resulting formulation, the test material is uniformly coated on the small particles. Then, a dosage in a uniform suspension can be prepared from the formulated test substance. This procedure has been used successfully in many studies.

Administration of the Dose

The test substance can be administered as a solution or suspension as long as it is homogeneous. The solution or suspension is gavaged to the animal with a suitable stomach tube or feeding needle attached to a syringe. For most acute oral toxicity studies with a test limit of 2.0 g/kg, the dose can be administered with one treatment. If unusual circumstances require a total dose that is too large to be administered at a single time, it should be divided into equal doses with 3 or 4 h between each administration. Feed should be withheld until the last dose, which should be within 24 h of the first dose.

Observations

Clinical examination, observation, and mortality checks should be made shortly after dosing, at frequent intervals over the next 4 h, and at least once daily thereafter. The intervals and frequency of observation should be flexible enough to determine the onset of signs, onset of recovery, and the time to death. The mortality checks should be frequent enough to minimize unnecessary loss of animals due to autolysis or cannibalism. The cage side observation should include any changes in the skin, fur, eyes, mucous membranes, circulatory system, autonomic and central nervous systems, somatomotor activities, behavior, and so on. Any pharmacotoxic signs such as tremor, convulsions, salivation, diarrhea, lethargy, sleepiness, morbidity, fasciculation, mydriasis, miosis, droppings, discharges, or hypotonia should be recorded. The most common pharmacotoxic signs are listed in Tables 18.1–18.3.

Individual body weights should be determined just prior to dosing, once weekly, and at death or at termination. The body weights of animals found dead generally are not as useful as the body weights of live animals. Necropsies should be performed on animals that are moribund, found dead, and killed at the conclusion of the study. All changes in the size, color, or texture of any organ should be recorded. Any gross change observed at necropsy should be described according to the size, color, and position of the lesion. Definitive pathological diagnostic terms should be avoided. Even though a complete

microscopic examination of tissues and organs is ideal and would be helpful in defining acute toxicity, economic and time factors may preclude such a study. If the investigator feels that microscopic examination of a lesion is essential, tissues from the lesion should be preserved in an appropriate fixative such as 10% buffered formalin.

Table 18.1 Common signs and observations in acute toxicity tests

Clinical observation	Observed signs	Organs, tissues, or systems most likely to be involved
I. Respiratory blockage in the nostril, changes in rate and depth of breathing, changes in color of body surface	A. Dyspnea: difficult or labored breathing, essentially gasping for air, respiration rate usually slow	
	1. Abdominal breathing: breathing by diaphragm, greater deflection of abdomen upon inspiration	CNS respiratory center, paralysis of costal muscles, cholinergic
	2. Gasping: deep labored inspiration, accompanied by a wheezing sound	CNS respiratory center, pulmonary edema, secretion accumulation in airways, increased cholinergic
	B. Apnea: a transient cessation of breathing following a forced respiration	CNS respiratory center, pulmonary-cardiac insufficiency
	C. Cyanosis: bluish appearance of tail, mouth, foot pads	Pulmonary-cardiac insufficiency, pulmonary edema
	D. Tachypnea: quick and usually shallow respiration	Stimulation of respiratory center, pulmonarycardiac insufficiency
	E. Nostril discharges: red or colorless	Pulmonary edema, hemorrhage
II. Motor activities: changes in frequency and nature of movements	A. Decrease or increase in spontaneous motor activities, curiosity, preening, or locomotions	Somatomotor, CNS
	B. Somnolence: animal appears drowsy, but can be aroused by prodding and resumes normal activities	CNS sleep center
	C. Loss of righting reflex, loss of reflex to maintain normal upright posture when placed on the back	CNS, sensory, neuromuscular
	D. Anesthesia: loss of righting reflex and pain response (animal will not respond to tail and toe pinch)	CNS, sensory
	E. Catalepsy: animal tends to remain in any position in which it is placed	CNS, sensory, neuromuscular, autonomic
	F. Ataxia: Inability to control and coordinate movement while animal is walking with no spasticity, epraxia, paresis, or rigidity	CNS, sensory, autonomic
	G. Unusual locomotion: Spastic, toe walking, pedaling, hopping, and low body posture	CNS, sensory, neuromuscular
	H. Prostration: immobile and rests on belly	CNS, sensory, neuromuscular
	I. Tremors: involving trembling and quivering of the limbs or entire body	Neuromuscular, CNS
	J. Fasciculation: involving movements of muscles, seen on the back, shoulders, hind limbs, and digits of the paws	Neuromuscular, CNS, autonomic
III. Convulsion (seizure): marked involuntary contraction or seizures of contraction of	A. Clonic convulsion: convulsive alternating contraction and relaxation of muscles	CNS, respiratory failure, neuromuscular, autonomic

voluntary muscle	B. Tonic convulsion: persistent contraction of muscles, attended by rigid extension of hind limbs	CNS, respiratory failure, neuromuscular, autonomic
	C. Tonic-clonic convulsion: both types may appear consecutively	CNS, respiratory failure, neuromuscular, autonomic
	D. Asphyxial convulsion: usually of clonic type but accompanied by gasping and cyanosis	CNS, respiratory failure, neuromuscular, autonomic
	E. Opisthotonos: tetanic spasm in which the back is arched and the head is pulled toward the dorsal position	CNS, respiratory failure, neuromuscular, autonomic

Clinical observation		Observed signs	Organs, tissues, or systems most likely to be involved
IV.	Reflexes	A. Corneal eyelid closure: touching of the cornea causes eyelids to close	Sensory, neuromuscular
		B. Primal: twitch of external ear elicited by light stroking of inside surface of ear	Sensory, neuromuscular
		C. Righting: ability of animal to recover when placed dorsal side down	CNS, sensory, neuromuscular
		D. Myotact: ability of animal to retract its hind limb when limb is pulled down over the edge of a surface	Sensory, neuromuscular
		E. Light (pupillary): constriction of pupil in presence of light	Sensory, neuromuscular, autonomic
		F. Startle reflex: response to external stimuli such as touch, noise	Sensory, neuromuscular
V.	Ocular signs	A. Lacrimation: excessive tearing, clear or colored	Autonomic
		B. Miosis: constriction of pupil regardless of the presence or absence of light	Autonomic
		C. Mydriasis: dilation of pupils regardless of the presence or absence of light	Autonomic
		D. Exophthalmos: abnormal protrusion of eye in orbit	Autonomic
		E. Ptosis: dropping of upper eyelids, not reversed by prodding animal	Autonomic
		F. Chromodacryorrhea: red lacrimation	Autonomic, hemorrhage, infection
		G. Relaxation of nictitating membrane	Autonomic
		H. Corneal opacity, iritis, conjunctivitis	Irritation of the eye
VI.	Cardiovascular signs	A. Bradycardia: decreased heart rate	Autonomic, pulmonary-cardiac insufficiency
		B. Tachycardia: increased heart rate	Autonomic, pulmonary-cardiac insufficiency
		C. Vasodilation: redness of skin, tail, tongue, ear, foot pad, conjunctivae, sac, and warm body	Autonomic, CNS, increased cardiac output, hot environment
		D. Vasoconstriction: blanching or whitening of skin, cold body	Autonomic, CNS, decreased cardiac output, cold environment
		E. Arrhythmia: abnormal cardiac rhythm	CNS, autonomic, pulmonary-cardiac insufficiency, myocardiac infraction
VII.	Salivation	A. Excessive secretion of saliva: hair around mouth becomes wet	Autonomic
VIII.	Piloerection	A. Contraction of erectile tissue of hair follicles resulting in rough hair	Autonomic
IX.	Analgesia	A. Decrease in reaction to induce pain (e.g., hot plate)	Sensory, CNS
X.	Muscle tone	A. Hypotonia: generalized decrease in muscle tone	Autonomic
		B. Hypertonia: generalized increase in muscle tension	Autonomic
XI.	Gastrointestinal signs: Droppings (feces)	A. Solid, dried, and scant	Autonomic, constipation, GI motility
		B. Loss of fluid, watery stool	Autonomic, diarrhea, GI motility
	Emesis	A. Vomiting and retching	Sensory, CNS, autonomic (in rat, emesis absent)
	Diuresis	A. Red urine	Damage in kidney
		B. Involuntary urination	Autonomic sensory

XII. Skin	A. Edema: swelling of tissue filled with fluid	Irritation, renal failure, tissue damage, long-term immobility
	B. Erythema: redness of skin	Irritation, inflammation, sensitization

Table 18.2 Autonomic signs

Sympathomimetic	Piloerection
	Partial mydriasis
Sympathetic block	Ptosis
	Diagnostic if associated with sedation
Parasympathomimetic	Salivation (examined by holding blotting paper)
	Miosis
	Diarrhea
	Chromodacryorrhea in rats
Parasympathomimetic block	Mydriasis (maximal)
	Excessive dryness of mouth (detect with blotting paper)

Table 18.3 Toxic signs of acetylcholinesterase inhibition

Muscarinic effects[a]	Nicotinic effects[b]	CNS effects[c]
Bronchoconstriction	Muscular twitching	Giddiness
Increased bronchoconstriction		Anxiety
	Fasciculation	Insomnia
Nausea and vomiting (absent in rats)	Cramping	Nightmares
	Muscular weakness	Headache
Diarrhea		Apathy
Bradycardia		Depression
Hypotension		Drowsiness
Miosis		Confusion
Urinary incontinence		Ataxia
		Coma
		Depressed reflex
		Seizure
		Respiratory depression

a Blocked by atropine.
b Not blocked by atropine.
c Atropine might block early signs.

Other Nonroutine Determinations

The cause of death in acute poisoning generally involves the nervous, cardiovascular, or respiratory systems. Effects on other organs such as the liver or kidney sometimes are masked by lethality or cannot be detected without special determinations. Clinical laboratory studies (hematology, clinical chemistry, or specific functional tests) may be needed to identify these adverse effects. Because these laboratory tests are costly, they are only justifiable when there is evidence indicating that a particular laboratory test would be helpful in identifying the target organ or mechanism of toxic action. For example, if the test substance has a chemical structure that belongs to a class of hepatotoxins, clinical chemistry measurements may be conducted to verify its hepatotoxicity.

Histopathology can be very valuable in identifying a target organ. Like clinical laboratory tests, histopathological examination of organs is expensive. However, the cost should not be the limiting factor in deciding whether histopathological examination is needed; rather the limiting factor should be whether there is evidence that a particular organ is involved.

Data Processing

All signs of toxicity, onset of signs, time to recovery, time to death, mortality, and necropsy findings can be summarized in tabular form. If a defined LD50 is required, it may be determined by an acceptable method (226, 353, 373). Although most laboratories are equipped with calculators or computer programs to facilitate the estimation of LD50 values, fiducial limits, and the slopes of the dose-response curve, the reader is advised to review the manual estimation procedure for two widely adopted methods, the probit analysis and the moving average method. The graphic procedure of the probit analysis is rapid and is sufficient for most purposes. But for a more precise estimate of the LD50, mathematical calculation may be necessary, and the reader is referred to the maximum likelihood estimation described in Finney's text (138).

*Graphic estimation of LD*50 *by probit analysis.* The basic linear equation for the probit analysis as described in the previous section is

$$y = 5 + \frac{1}{\sigma}(x - u)$$

where y is the probit, (σ is the standard deviation of a log normal distribution with mean u, and x is the log dose. This equation is linear with respect to y and x often can be expressed as a linear equation, for example, $y=\alpha+\beta x$, where $\beta=1/\sigma=$ slope, and $\alpha=5-(u/\sigma)$. When $y=5$, $(x-u)/\sigma=0$; thus $x=\mu$ (the median log dose). Further, y is related to P (the probability of response which has a value of 0 to 1 by the following equation:

$$P = \frac{1}{\sqrt{2\pi}} \int_{-x}^{y} \exp\left(\frac{-y}{2}\right) dy$$

The reader should bear in mind that both the u and x are in log dose scale.

The following steps should be taken for graphic estimation of LD50 by probit analysis.

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1. Convert response probabilities to probit units by a probit transformation table (96, pp. 54–55).
2. Convert all doses into log dose units (e.g., log10 dose=*x*). (Steps 1 and 2 may be eliminated if probit-log graphic paper is available.)
3. Using the probit as the abscissa and log10 dose as the ordinate, plot the response probit units against the log10 dose.
4. Draw a straight line such that the vertical deviations of points (the probits) at each *x* value are as small as possible. Extreme probits, for example, those outside the range of probit 7 and 1, carry little weight in the fitting of the probit-log dose-response line and thus should be excluded.
5. From the regression of the probit-log dose line, extrapolate the log dose corresponding to probit units of 5, which also correspond to the P=0.5. Thus, this extrapolated dose should be the median lethal *log dose,* and the LD50 value would be the *antilog* of this log dose value.
6. Calculate the slope of the probit-log dose line. This slope, $\beta=1/\sigma$, is defined as the number of increases in probit units for a unit increase in log dose. The slope defined by Litchfield and Wilcoxon (226) is equal to

$$\frac{1}{2}\left(\frac{LD_{84}}{LD_{50}}+\frac{LD_{50}}{LD_{16}}\right)=\sigma$$

This slope is different but related to the slope described here, thus, the larger the slope value, the steeper the probit-log dose response. The opposite is true in the Litchfield and Wilcoxon definition.
7. A *x*2 test should be conducted to determine if the fitted line is adequate. A small value of *x*2 statistic (within the limits of random variation) may indicate satisfactory agreement between the theoretically expected line and the fitted line. A significantly large *x*2 statistic may indicate either that the animals do not respond independently or that the fitted line (probit-log dose) does not adequately describe the dose-response relationship of the test substance. If the latter is true, forms of the dose-response curve other than the probit-log dose linearity may exist, and further transformation may be needed (138). If the former is the case, then precision of the line is reduced.
8. Determination of precision is by weighting the coefficient. The standard deviation of a binomial distribution is, $\sqrt{PQ/n}$, where *P* and *Q* are the mean probabilities, *P* equals (1–0, and *n* is the number of test subjects. Thus, the variance is *PQ/n,* the square of the standard deviation. It is obvious that the variance (i.e., the spread of a distribution) is inversely related to *n*. This relationship means that the larger the number of test subjects, the smaller the variance and the better the precision. The reciprocal of the variance is invariance, which measures the weight, *nW*. Here, *W* (weighting coefficient)=*Z2/PQ,* where $Z=(1/\sqrt{2\pi})$ exp(−*y*2/2) dand is related to the normal frequency function corresponding to the NED. A table of weighting coefficients (see 96, p. 53) corresponding to probits *(y)* is available (139). The standard error for the log LD50 is given by

$$\sigma/\sqrt{\Sigma nW}$$

if the estimated log LD50 does not greatly differ from the true mean log LD50, because this estimation does not take into consideration the error in the estimation of σ for the probit-log dose-response line. A better equation for the estimation of the variance of the estimated log LD50 is given by

$$V(m)=\sigma^2\left(\frac{1}{\Sigma nW}+\frac{(m-\bar{x})^2}{\Sigma nW(x-\bar{x})^2}\right)$$

where *V(m)* is the variance of LD50, $\bar{x}$ is the weighted mean log dose, *m* is the median log dose, *x* is the log dose, and $1/\sigma=1/1\Sigma nW\ (x-\bar{x})$. If the χ2 is large, indicating that the test subjects do not respond inde- pendently to the dose, the estimation of variance of log LD50 may not apply, and adjustment due to the sampling variation of the slope (1/σ) of the probit-log dose line may have to be made (138). For a quick estimation of the LD50 this adjustment may be dropped, and the standard error would be the square root of the variance, i.e., $\sqrt{V(m)}$. One must remember that the dose is expressed in log dose; therefore, the estimation of the standard error (SE) for the LD50 in the original dose unit (e.g., mg/kg) is impossible. However, an approximation is given:

$$SE(LD_{50})=(10^m)\cdot([\log_e(10)\cdot(S_m)]$$

where *Sm* (which equals $\sigma/\sqrt{\Sigma nW}$ or $\sqrt{V(m)}$) is the estimated standard error for the median log dose

m (i.e., m=log LD50 or 10^m=LD50).
A more rapid approximation of the standard error of log LD50 was given by Litchfield and Wilcoxon (226) as

$$S_m = \frac{S}{N'/2}$$

where S is the difference between two log doses of expected effects (as indicated by the probiting dose

line) that differ by one unit of probit and *N′* is the total number of animals between the log dose limits, corresponding to the expected probit 4.0–6.0 (i.e., the 16% and 80% responses).

9. *Fiducial limits.* The concept of fiducial limit is similar to the confidence limit. The value of the two may be the same, but they are not always identical. The fiducial probability *F*(e.g., *95%*) can be defined as the situation when the true value of a parameter lies between the calculated upper and lower limits, which would not be contradicted by a significance test at the 1/2(1–*F*) probability level. These higher and lower limits are called the fiducial limits. For rapid analysis, the fiducial limits at the *F*=95% level can be estimated by log LD50±1.96 *(Sm)*. A more detailed estimation can be obtained by the maximum likelihood estimation (138).

Another simple approximation of the fiducial limits is given by Litchfield and Wilcoxon (226) as LD50/*f*LD50 or LD50×*f*LD50 for the lower and upper limit, respectively, where LD50 is defined as the LD50 factor equal to *(s)* $^{(2.77/\sqrt{N'})}$. Here *s* is the slope, which is defined as

$$\frac{1}{2}\left(\frac{\mathrm{LD}_{54}}{\mathrm{LD}_{50}}+\frac{\mathrm{LD}_{50}}{\mathrm{LD}_{16}}\right)=\frac{1}{2}(3.55+3.55)=3.55$$

in this example, and *N′* is the total number of animals used between response probabilities 16% and 84% (i.e. probit 4 and *6,* equal to 30 in this example). Then *f*LD50 equals 1.896. Therefore, the lower fiducial limit is equal to 8.91/1.896=4.70, and the upper fiducial limit is equal to 8.96×1.896=16.90.

For all practical purposes, the graphic method should be sufficient to estimate the LD50. Nonetheless, because computer programs are available to handle the mathematics, the more detailed maximum likelihood estimation (98, 138) is used in many laboratories. Indeed the up-and-down procedure (52–54, 279) (See Alternative Methods for Oral LD50 Test), which is one of the more modern methods of estimating the LD50, is based on the maximum likelihood method. Calculations can be performed using either SAS (316) or BMDP (99) computer program packages which are available to many toxicology laboratories. Other examples of programming for the estimation of the LD50 with a small computer have been reported (223, 311).

Acute Dermal Toxicity

Dermal exposure is an important route of exposure. The objective of conducting an acute dermal toxicity study is the same as an acute oral toxicity study. Such testing may provide information on the adverse effects resulting from a dermal application of a single dose of a test substance. The acute dermal test also provides the initial toxicity data for regulatory purposes, labeling, classification, transportation, and subsequent subchronic and chronic dermal toxicity studies. Comparison of acute toxicity by the oral and dermal routes may provide evidence of the relative penetration of a test material.

Although the general experimental design and principles of acute dermal toxicity testing are similar to those of acute oral testing, there are differences. These differences include selection of the animal species, number of animals per dose level, preparation of animals, dosage, and administration of the test substance. Only differences in the acute dermal test are described in this section.

Animals

The same concern about animal factors raised in connection with acute oral toxicity testing, such as species, age, health conditions, body weight, sex, and housing environment, can affect the outcome of an acute dermal test. The three most commonly used animal species are young, healthy adult rabbits (2–3 kg), rats (200–300 g), and guinea pigs (350–450 g). Other species can be used. The animals should be housed individually in a controlled environment. Quarantine, acclimatization, and randomization are as described for acute oral studies. The back of the animal or a band around the trunk should be clipped free of hair. When clipping the hair, care must be taken not to abrade the skin. If abraded skin is called for, a needle may be used, but care must be taken not to damage the dermis. Increasingly, investigators have come to question the value of conducting tests on abraded skin, and many consider such tests to be irrelevant. To date, almost all testing guidelines call for conducting the dermal test only on intact skin (109, 124, 125, 275, 337). Fasting the animals overnight is not necessary for the dermal test. Generally, five animals per dose level per sex is sufficient to allow for an acceptable estimation of the dermal LD50. Smaller numbers of animals can be used.

Doses

Dose selection is similar to the acute oral test. Higher doses do not need to be tested when a test substance at 2000 mg/kg has not produced test substance-related mortality.

While a control group generally is not needed, a vehicle control group should be included in the study if the toxicity of the vehicle is not known. Its influence on dermal penetration of the test substance should

be fully established prior to the study.

Preparation of Dosage and Dosing Procedure

The test substance should be applied uniformly to approximately 10% of the body surface of the animal (e.g., 4 cm×5 cm for rats, 12 cm×14 cm for rabbits, 7 cm×10 cm for guinea pigs). This area may vary. For example, the area of application for highly toxic substances may be small because a smaller volume is applied. Liquid test substances generally are applied undiluted. If the test substance is a solid, it should be pulverized, weighed, placed on a plastic sheet or porous gauze dressing, moistened to a paste with normal saline (one part test substance for one part saline) or with the appropriate solvent, and spread evenly on the closely clipped skin to ensure uniform contact with the skin. Grinding of the solid test substances may not be needed under some conditions. For example, when a granular formulation is tested, it may be more relevant to test the substance in its formulation state than to destroy the formulation by grinding.

Because rabbits are the most widely used animal for acute dermal testing, the dosing procedure for the rabbit is detailed, especially for liquid substances. Dermal application of the test substance ranges from occlusive to semiocclusive to unocclusive.

The choice of the application method depends on what the most likely exposure pattern is in humans. Skin irritation is usually the worst after occlusive exposure, followed by semiocclusive and unocclusive exposure. Skin irritation may not only cause stress to the animal but also may increase dermal penetration. For unocclusive application, the application site remains uncovered but the volume of liquid test substance that can be applied to the skin may be limited depending on the volatility of the liquid. Immobilizing the animal or using a device such as a collar is needed to prevent ingestion through licking the application site. For occlusive or semiocclusive application, the application site is covered with an impervious material such as a plastic sheet, or with a porous gauze dressing as described in the following paragraph. The volume that can be applied with the occlusive or semiocclusive patch generally is larger than that of the unocclusive method.

Dosing procedures for liquid test substances. Rabbits are clipped free of hair with an electric animal hair clipper. The rabbit may have to be restrained by tightening the hind legs to a secured post and holding the nape of the neck during clipping. The area of skin to be clipped should be based on the need of the experiment and generally involves the entire band around the trunk between the flank and the shoulders if the dose exceeds 5.0 mg/kg. If abraded skin is to be tested (generally not required), abrasions to the stratum corneum may be made with a hypodermic needle (201/2 G) 2–3 cm apart longitudinally over the appli cation site. A plastic cuff in a cylindrical shape (approximately 12–15 in. long and 10 in. in diameter) open at both ends can be used. The cuff is put on the trunk of the rabbits, covering the application site. With the help of another investigator, the plastic cuff is folded around the trunk and secured at the thorax and flank of the rabbit with surgical adhesive tape. Care should be exercised so that the cuff is sufficiently secured but not too tight to affect breathing. Using a long feeding needle, the correct amount of the liquid test substance is drawn into a syringe of appropriate size. The needle then is placed under the cuff and half of the dose is delivered evenly on each side of the vertebral column. After withdrawal of the needle, the test substance is evenly distributed over the application site by gently rubbing the top of the plastic cuff. A piece of cloth of appropriate size is then wrapped around the plastic cuff and taped in place to absorb any test substance that may spill off the cuff. After dosing, the investigator should observe the animal for a moment to see if breathing is affected, prior to putting the animal back into the cage. In the semiocclusive method, a porous gauze dressing replaces the plastic cuff. In unocclusive exposure, the test substance is applied uniformly over the skin: care must be taken to minimize run off from the skin, especially for aqueous dosing solutions. Applying the test substance in small amounts at a time may help.

Dosing procedure for solid test substances. If the test substance is a solid, it should be ground with a mortar and pestle unless there is justification not to pulverize it. The correct dose of the ground solid is weighed, placed in the center of a plastic sheet of appropriate size, and moistened with sufficient normal saline or another appropriate vehicle. If a vehicle other than saline or water is used, the effect of the vehicle on the skin penetration of the test substance should be considered, and its toxicity should be known. The type of vehicle selected should be based on the expected mode of exposure of the test substance and should be mixed into a paste. The paste then is spread evenly around the center of the plastic sheet. With one person holding the rabbit by grasping it at the back, another person moistens its belly and its back with paper towels soaked with saline. Then the rabbit is placed with its belly on the test substance paste on the plastic sheet, and another investigator wraps the sheet around the trunk of the rabbit. The plastic cuff is secured in place with surgical tape at the thorax and the flank. A piece of cloth of appropriate size then is wrapped around the plastic cuff and secured in place in the same

manner. In the semiocclusive method, a porous gauze dressing replaces the plastic sheet.

Dosing procedures for rats and guinea pigs. Similar dosing procedures can be applied to rats and guinea pigs. Liquid samples should be placed on the back instead of the belly or on the lateral trunk. If unocclusive

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exposure is called for in rats, the test substance should be applied to the skin as near to the head as possible to prevent ingestion by preening of the application site. A plastic collar may be used to further limit access to the treatment site. Generally, the plastic collar produces more stress in the rat, as indicated by chromodacryorrhea (red stain around the eye), than in the rabbit. To minimize stress in rats, small collars can be handmade from light cardboard. The collar is lined with cut rubber tubing around the neck area and stapled in place. The cardboard collar is lighter and easier to place on small animals. It can readily be replaced if needed (the collar placed on the neck usually will last about three days), and it is more economical than the commercially available plastic collars.

Exposure Period and Removal of Cuff

Almost all testing guidelines (109, 124, 125, 275, 337) call for 24-h continous exposure. After the 24-h exposure, the cuff is removed and the application site is gently wiped with a paper towel soaked with saline, water, or any appropriate solvent to remove residual test substance remaining on the application site.

Observation Period

As in the acute oral toxicity test, the observation period and intervals should be flexible enough to establish onset of signs, time to death, and time to recovery, but should be frequent enough such that the loss of animals due to autolysis and cannibalizing is minimal. In addition, skin irritation should be assessed according to a scoring system such as the one described by Draize et al. (102).

Data Processing

Data should be analyzed and handled as in the acute oral toxicity test. Because the exposure period in a dermal toxicity study is longer than in a skin irritancy test, the skin irritation resulting from the 24-h exposure may not be relevant for assessing the skin irritancy potential of the test substance but may be considered as the worst case if the occlusive or semiocclusive method is used.

Test Limit

If no test substance-related mortality is observed at 2000 mg/kg, testing at higher doses may not be necessary because additional test substance may only be applied on top of the test substance layer already present. This layering may form a physical barrier to prevent further absorption of the test substance from the application site.

ALTERNATIVE METHODS FOR ORAL LD50TEST

Animal testing has been widely debated. Many regard the issue as no more than animal protectionism and solely

Table 18.4 Summary of current recommendations on acute oral toxicity testing

Provisions	EEC and OECD	U.S. EPA	U.S. CPSC	U.S. FDA	U.S. DOT
Use of LD50	Discourages	Same as OECD	Strongly discourages	Does not require	Same as OECD
Limit test (2g/kg Dose)	Recommends	Same as OECD*a*	Same as OECD	Refers to	Recommends
Defined test (if limit>2g/kg)	Test guideline 401 Test guideline 420 Test guideline 423 Test guideline 425	Same as OECD	NS	NS	NS
Endpoint evaluations	Onset, nature, reversibility of effects, gross necropsy, histopathology	Same as OECD	NS	NS	NS
Alternatives	NS	Structurally related activities (SAR)	Existing animal data Prior human experience Expert opinion	Accepts alternatives	Existing animal data Prior human experience

NS, not specified.

a Under current policy and regulations for pesticide products, precautionary statements may still be required unless there are data to indicate the LD50 is greater than 5g/kg.

based on humanitarian reasons. Aside from valid scientific concerns on the usefulness of classic LD50 values (e.g., uncertainty in species extrapolation, seldom needed for potent drug standardization), there are broader issues on animal testing, some political in nature and others economically based. The cost of animal testing has been increasing at a skyrocketing rate over the last decades, and even without animal rights activism, the scientific community will need less costly alternatives to cope with the increasing demand for safety evaluation of a vast number of existing and new chemicals.

The approach to animal testing today is being altered by the so called Three Rs: reduction, refinement, and replacement. Reduction of the number of animals used in testing and refinement of existing testing methods to minimize pain and suffering of animals represent the short-term objective. Replacement of animal testing with non-animal-based methods, e.g., in vitro ("in glass," i.e., test tube) methods, is the ultimate goal. However, genuine, validated, and regulatory accepted nonanimal alternative methods to replace whole animal acute toxicity testing are still more of a goal than a reality, even though the concept has been widely accepted by scientists from industry, professional societies, and certain regulatory bodies (10, 240, 338, 339).

As far as the oral LD50 test is concerned, a number of these classical, precise tests, using large number of animals, have been conducted for the purpose of labeling and classification of chemicals. Recently, there have been many positive developments in the reduction and refinement of acute oral toxicity tests. For example, the limit test (151), the British Society of Toxicology method (49), the up-and-down method (52–54), the fixed dose procedure (359), and the acute toxic class method (309), can achieve reduction in the number of animals. These methods, in general, have been endorsed by the scientific and regulatory communities and animal advocates alike (10, 240, 338, 339), and some have been adopted as regulatory guidelines for the testing of chemicals (277–279). However, the classification and labeling schemes of many regulatory bodies around the world still use the LD50 value, and it may be some time before the classical, more precise test, using large numbers of animals, is eliminated. Perhaps it is time for regulatory bodies to redefine the scheme for classification and labeling of chemicals on the basis of reduced and refined acute toxicity testing results. An international study using the fixed dose procedure has shown that this is possible (359).

Modified LD50Tests

The aim of these tests is to obtain adequate information on toxic signs, approximate LD50 values, and, in some,

Table 18.5 Approximate lethal dose (ALD) versus conventional rat LD50 (g/kg)

	Conventional		Approximate	
Chemical	n	LD50	n	ALD
Tetraethyllead	36	20	5	26
Methomyl	53	40	5	26
Hexachlorophene	46	165	11	90
Adiponitrite	65	301	7	300
Caffeine	40	483	8	450
N-Butylhexamethylene amine	35	536	7	1000
Hexamethylene diamine	92	1127	5	1500
Bromobenzene	35	3591	8	3400
Carbon tetrachloride	105	10,054	5	7500

Adapted from Reference 208.

the slope of the dose-response. Many studies have shown that adequate acute toxicity and lethality information can be obtained by using fewer animals than in the classical LD50 studies. DePass (93) and Lipnick et al. (225) have reviewed several modified LD50 tests. Although the main endpoint remains lethality, these tests generally fulfill the goal of reducing the number and suffering of animals and in some cases provide adequate information for hazard classification and labeling. The key alternative tests are described below.

Approximate Lethal Dose Method

This method involves sequential dosing until the lowest lethal dose is obtained. Initially, an arbitrary dose is given to an animal. If the animal survives, a second animal is given 1.5 times the initial dose, and, sequentially, several animals are given increasing doses in the same manner until a lethal dose is achieved. This dose is the approximate lethal dose (ALD). In general, only 6–10 animals are required to achieve the ALD. Comparison of classical LD50 values and the ALD indicates that the ALD can be used to closely predict the LD50 (Table 18.5).

The Up-and-Down Method

Animals are dosed one at a time, starting at an estimated LD50 dose. If the first animal survives, the next one receives a higher dose. If the first animal dies, the next one receives a lower dose. The spacing of doses generally is adjusted by a factor of 1.3 up or down depending on the outcome of the previous animal. Comparison of classical LD50 values to the up-and-down-derived LD50 shows close agreement (Table 18.6). This test has been adopted by the OECD as an alternative to the more traditional methods of LD50 determination (279). The

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Table 18.6 Up-and-down method versus conventional rat Oral LD50 (g/kg)

	Conventional		Up-and-down	
Chemical no.	n	LD50	n	LD50
1	50	0.273	6	0.388
2	40	0.344	9	0.421
3	40	3.490	8	4.120
4	40	3.520	6	4.020
5	40	4.040	6	3.520
6	40	5.560	6	5.700
7	40	9.280	6	8.770
8	20	>10.00	3	>10.10
9	50	10.11	7	11.09
10	10	>20.00	8	22.40

Adapted from Reference 54.

OECD guideline also contains the provision for a limit test. The upper-limit dose is 2000 mg/kg (5000 mg/kg). If the first animal survives the upper-limit dose, then the second animal receives the same dose. If three animals survive the limit dose, then three animals of the other sex are tested at the limit dose. If all survive, the test is terminated.

The British Society of Toxicology (BST) Protocol

This protocol starts with three doses (5, 50, and 500 mg/kg) with five animals per sex per group, to minimize pain and suffering observed typically at the higher doses. The three initial doses generally will produce toxicity but no mortality. Depending on the response from the initial doses, subsequent doses can be increased or decreased by a factor of 10. Based on survival and toxic signs at a given dose, substances are placed into one of four categories. At least one study showed that classification of chemicals based on the BST protocol and the standard OECD protocol produced good agreement, but only use 74% of the animals and produce 87% less mortality than the OECD protocol (360).

The Fixed Dose Procedure

This modification of the BST protocol has been described by Van den Heuvel et al. (359). Basically, this procedure calls for testing at a dose selected from a series of pre-set doses (5, 50, 500, and 2000 mg/kg) for discriminating classification of the test substance into toxicity categories. The selected dose (discriminating dose) should be nonlethal, nonpainful, nonstressful, but toxicityevident. It could be selected by using available information, or by conducting a "sighting study" using three or four animals. The focus of the test should not be limited to mortality (found dead or killed for humane reasons), but should include other toxicity endpoints such as time course of signs of toxicity and necropsy findings. These data and the "discriminating dose" should provide adequate data for hazard assessment, comparative reference, and labeling classification (Table 18.7).

A multinational validation study in 33 laboratories with 20 materials using the fixed dose approach produced consistent results on the time course of signs of toxicity which was adequate for acute toxicity risk assessment and acute toxicity classification based on the European Economic Community (EEC) criteria. Fewer animals were used and less stress occurred (359). This test has been adopted by the OECD as an alternative acute oral toxicity method (277).

The Acute Toxic Class Method

This method has been described by Roll et al. (309) and is based on the assumption that using a minimum number of animals in a stepwise procedure will provide enough information on the acute toxicity of a substance to allow classification according to the most commonly used classification schemes. Three animals of one sex are used for each step; either sex can be used. The initial dose is selected from one of three fixed levels, 25, 200, or 2000 mg/kg body weight, and should be chosen to produce some mortality. If existing information suggests that mortality is unlikely at the 2000 mg/kg dose, then a limit test at that level may be conducted with three animals of each sex. If deaths occur, further testing at the lower dose levels may be necessary. This method was evaluated in national and international validation studies (319, 320), and has been adopted by the OECD as an alternative acute oral toxicity method (278).

Current Regulatory Status of Acute Oral Toxicity Testing

The current regulatory status of acute oral toxicity testing is in a state of flux. Although some form of acute toxicity information is required, most international regulatory agency guidelines are being revised to accept alternative methods based on the principles of reduction and refinement. A new regulatory philosophy is emerging which discourages the use of the classical LD50 test except when specifically

justified for reasons of scientific necessity. Indeed, the OECD has agreed to phase out its Guideline 401 —Acute Oral Toxicity, which requires an LD50 with 95% confidence interval, dose-mortality curve and slope, and to replace it with the more recently approved Acute Oral Toxicity Guidelines (277–279). Various regulatory agency recommendations on acute oral toxicity testing are compared in Table 18.4. For specific information on regulatory agency requirements,

Table 18.7 Investigation of acute oral toxicity using the fixed-dose method of interpretation of results

Fixed dose	Results	Interpretation
5 mg/kg[a]	Less than 100% survival[b]	Compounds that may be very toxic if swallowed
	100% survival but evident toxicity	Compounds that may be toxic if swallowed
	100% survival; no evident toxicity	Retested at 50 mg/kg if not already tested at that level
50 mg/kg	Less than 100% survival[b]	Compounds that may be toxic or very toxic if swallowed; retested at 5 mg/kg if not already tested at that level
	100% survival but evident toxicity	Compounds that may be harmful if swallowed
	100% survival; no evident toxicity	Retested at 500 mg/kg if not already tested at that level
500 mg/kg	Less than 100%, survival[b]	Compounds that may be toxic or harmful if swallowed; retested at 50 mg/kg if not already tested at that level
	100% survival but evident toxicity	Compounds that do not present a significant acute toxic risk if swallowed
	100% survival; no evident toxicity	Retested at 2000 mg/kg if not already tested at that level
2000 mg/kg[c]	Less than 100% survival[b]	Compounds that may be harmful if swallowed; retested at 500 mg/kg if not already tested at that level
	100% survival with or without evident toxicity	Compounds that do not present a significant acute toxic risk if swallowed

Adapted from Reference 359.

a Where a dose of 5 mg/kg produces significant mortality, or where a sighting study suggests that mortality will result at that dose level, the substance should be investigated at a lower dose level. The level chosen should be one that is likely to produce evident toxicity but no mortality.

b Includes compound-related mortality and humane kills but not accidental deaths.

c Testing mortality at this dose level is carried out primarily for risk assessment purposes. However, where no evident toxicity is seen at 500 mg/kg its results are relevant to classification if there is greater than 50% mortality (including humane kills).

the reader is encouraged to check the most recent regulatory guidelines that apply.

Refining the Acute Oral Toxicity Test

Humane Endpoints

Refinement of the acute oral toxicity test to minimize pain and suffering of animals, is an area that is also receiving considerable attention. Current OECD Test Guidelines require that animals which are moribund or in obvious pain and distress should be humanely killed. An ad hoc working group has been established to develop an OECD guidance document to provide a more humane perspective on when to euthanize animals used in toxicity studies. The final document will give specific criteria to determine when an animal is in moribund condition or experiencing significant pain and distress. Although the guidance document has not been finalized, the working group has agreed that a harmonized approach to defining humane endpoints in acute toxicity testing is essential. For additional information, the reader is referred to the accumulating literature on the subject of humane endpoints (217, 252–256, 319, 359).

Quantitative Structure-Activity Relationship (QSAR) Analysis

Analysis of the structure-activity relationships within a class of chemicals can yield valuable information and may reduce the number of bioassays conducted. QSAR analysis is particularly useful during the discovery stage for selection of chemicals for further development. QSAR also can be used for prioritization of chemicals for various actions related to health and safety and environmental assessment. The elements generally needed for QSAR include: a verified bioassays database for the endpoint to be predicted; a set of chemical-physical parameters which describe the chemical structures so that the endpoint can be modeled in terms of these parameters; statistical techniques, that is, principally multivariate regression and discriminant analysis for weighing these parameters in a near-optimum fashion for the explanation of the endpoint; and computer technology to make it all practical (119). Using QSAR, Enslein (119) has analyzed 2066 chemical structures and found that the oral rat LD50 of almost 50% of the compounds examined was predicted within a factor of 2, and 95% within a factor of 8.

Obviously there are limitations for the QSAR approach to predict a complex toxic response in whole animals. These include a limited database on which to base a QSAR model, the temptation to extrapolate beyond the confines of the model, and the noise inherent in the bioassays on which the models are based (119). The results from QSAR have to be used with caution, and at this stage, QSAR is useful during the discovery stage and for prioritizing chemicals.

Cytotoxicity Tests

Recent interest has focused on the use of in vitro cytotoxicity assays as predictors of human acute toxicity. The relationship between acute toxicity and cytotoxicity is not only intuitively appealing, but its relevance may be explained by the *basal cytotoxicity concept* (113). This concept derives from classifying all chemical toxicity to humans into three categories:

(1) organizational or extracellular toxicity;
(2) organ-specific cytotoxicity; or
(3) basal cell toxicity or chemical injury to structures and functions which are common to all human cells.

Because the mechanism of action of most toxic chemicals is related to basic biochemical processes found in all cells, it is postulated that there should be some correlation between toxic concentrations determined in vitro as well as in vivo.

An international program, the Multicenter Evaluation of In Vitro Cytotoxicity (MEIC), organized by the Scandinavian Society of Cell Toxicology, was designed to evaluate the relevance of in vitro toxicity assays for human acute toxicity (36, 112, 114, 366). In this program, 29 laboratories tested 50 reference chemicals with known human toxicity (acute lethal blood concentrations as taken from clinical and forensic medicine handbooks) in 61 different in vitro assays (116). These assays comprised human and animal cell lines and primary cultures, fish cell cultures, other ecotoxicological systems, and cell free systems. In vitro IC50 values (geometric average 50% inhibitory concentrations) were compared to human mean lethal serum concentrations using multivariate partial least squares (PLS) analysis. To provide a benchmark for measuring the predictivity of in vitro assays to animal tests, rodent and human toxicity data were compared (115) and an evaluation of rodent LD50 values contrasted to human acute lethal dose was also conducted (116). Results indicated that rat and mouse LD50 values predicted human acute lethal doses with correlation coefficients (r2) of 0.61 and 0.65, respectively. In contrast, the various in vitro assay predictions of human lethal blood concentrations ranged from r2=0.69 (all human cell lines combined) to r2=0.34 (all plant systems combined). These correlative findings show that for the 50 reference chemicals studied, in vitro tests using human cell lines are equivalent to rodent LD50 determinations in predicting human lethal blood concentrations.

Although this simplistic approach ignores most metabolic and toxicokinetic aspects operative in the intact organism, the results support the continued evaluation of the basal cytotoxicity concept and suggest that the use of human cell lines in in vitro cytotoxicity assays as predictors of human acute toxicity should be given serious further consideration as alternative methods to acute lethality studies in rodents. Although substantial gains have been made, in vitro replacement methods for acute toxicity testing are difficult to define. Some investigators have proposed a battery of in vitro tests as a strategy to replace whole-animal acute toxicity tests (266).

ASSESSMENT OF EYE IRRITATION INDUCED BY CHEMICALS

The eye captures visible energy and converts the energy to neurosignals, which are transmitted to the intricate central nervous system in which they form neuroimages (vision). The importance of having this ability to perceive the external environment through vision is a giant step in the evolutionary process. In humans, vision along with hearing is vital for the development of speech, learning, and intelligence. Loss of vision can greatly curtail normal living.

There are three basic components of vision: optics, photoreceptors, and conducting nerves. All three components must function properly to form a clear and sharp neuroimage in the visual cortex. The optics of the eye (cornea, aqueous humor, iris, lens, and vitreous humor) must remain transparent and be able to refract and focus light on the right position on the photoreceptors. The photoreceptors (the cones and rods) of the retina must be able to undergo photolysis and convert light energy to neuropotential impulses. The optic nerves must be able to carry these neuroimpulses to the visual cortex.

Because the eye is constantly exposed to the external environment, the cornea must be protected from drying, dust, and microorganisms. The eyelids, the lacrimal system, and the somatosensory response of the cornea all work together to protect this outermost structure of the eye. Like other organs, the major portion of the eye is nourished by blood vessels. The retinal, circumcorneal, and uveal vessels also

nourish and help maintain the eye. These vessels are so arranged and constructed that they normally do not alter the transparency of the ocular optics. Nutrients reach the transparent

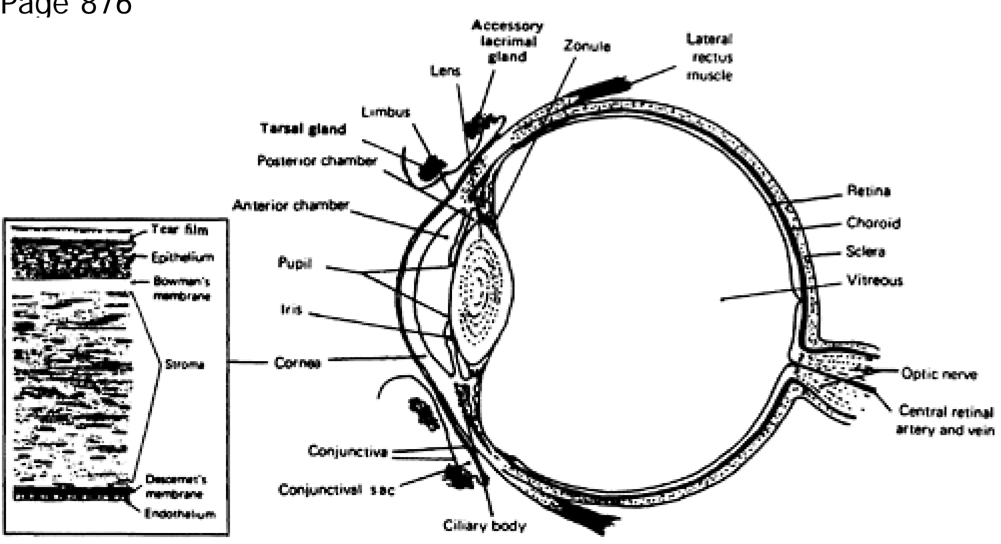

FIG. 18.4. Schematic illustration of the eye.

tissues of the eye via tears, the aqueous humor, and vitreous fluids.

Normal ocular functions are in delicate balance and are interdependent. Any traumatic insult, chemical or physical, can upset one or many of these ocular functions, thus creating a disturbance in vision. Depending on the extent of the traumatic injury (ranging from drying of the tear film to corneal ulceration or optic nerve damage), partial or complete loss of vision can result. Ocular injury not only can result from accidental physical trauma, but, also, from radiation and chemicals.

Chemicals can cause ocular damage locally by accidental exposure to the eye, or systemically by ingestion of chemicals such as food contaminants and drugs. Because many chemicals can produce ocular damage either locally or systemically (170, 180, 241, 308), it is important to test products for ocular effects before exposing workers during manufacturing and, ultimately, before subjecting consumers to products on the market. Ocular effects resulting from systemic exposure are beyond the scope of this chapter. This section focuses on eye irritation resulting from direct ocular contact.

Conducting ocular tests in humans is not only impractical but also unethical. Consequently, many methods and techniques have been developed over the years for testing ocular effects in animals. This section describes the methods for detecting potential eye irritants and discusses their limitations. In recent years, in vitro methods intended to replace eye irritancy tests in animals have evolved. The pros and cons of alternative methods are discussed.

Testing for potential eye irritancy is required for labeling and classification of chemicals by most regulatory agencies worldwide. The test protocol, interpretation of results, and classification scheme vary among countries. The differences among major industrial countries also are discussed.

Definition of Chemically Induced Eye Irritation and Corrosion

Irritation can be defined as reversible inflammatory changes in the eye and its surrounding mucous membranes following direct exposure to a material on the surface of the anterior portion of the eye. Corrosion is irreversible ocular tissue damage following exposure to a material. From a practical point of view, the distinction between reversible and irreversible changes sometimes is limited by the length of the observation period. Therefore, the term "eye corrosion" should be reserved for gross tissue destruction of the eye, which generally occurs rapidly following exposure. When interpreting results from an eye irritation study, one must take into consideration the biological significance of the ocular changes. For example, redness of the conjunctiva is considered a mild ocular effect. Even if it does not disappear completely within a specific time period

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(e.g., 21 days), one can hardly justify classifying the material as corrosive.

Normal Physiology and Anatomy of the Eye

A brief description of the normal physiology and anatomy of the eye is essential for understanding the development of eye irritation. Details can be found in a variety of textbooks and reviews (137, 238, 298).

Functionally, the eye can be divided into three basic parts (Figure 18.4). From posterior to anterior, they are:

- *Photoreceptors (retina):* The part of the eye that connects to the central nervous system via the optic nerve
- *Optics:* Structure that focuses visible light (image) onto the retina; it includes (from anterior to posterior) the cornea, iris, aqueous humor in the anterior chamber, the lens, and its related organelles such as the zonules and ciliary body (muscles), and the vitreous in the posterior chamber
- *Protective, lubricating, and nutritional structures:* These include the anterior eyelids and conjunctiva and associated secretory glands, the sclera and its outside layer (the fibrous tunic) and inside layer (uvea-vascular), and the ciliary body (secretory).

For chemically induced eye irritation, the main concern is generally on the directly exposed organelles such as the cornea, conjunctiva, and the iris. Effects on these structures can easily be detected by gross observation. If the chemical can penetrate deeper into the eye, other organelles also can be affected. Detection of the effects on these deeper structures requires special aids.

Cornea

The cornea is composed of, from anterior to posterior, the epithelium, Bowman's membrane, stroma, Descemet's membrane, and endothelium. The epithelium is about five cells deep in the transitional zones at the periphery. The basal cells are columnar, the other cells are squamous, and the cells between the two layers are polygonal (wing cells). The Bowman's membrane (12 μm) is an acellular layer of collagen and ground substance which provides a functional interface between the stroma and epithelium. An intact Bowman's membrane and the epithelial basal cell layer are vital to the regeneration of damaged epithelium. Damage to Bowman's membrane may predispose the cornea to fibrosis. The stroma consists of lamellae of collagen fibrils and fibroblasts supported by ground substances. The stroma forms most (nine-tenths) of the cornea and is limited on its inner surface by Decemet's membrane.

In addition to the organization of sheets of fibrils, other unique features such as proper hydration also contribute to corneal transparency. The Descemet's membrane (5–10 μm), like the Bowman's membrane, is an acellular layer which is the basement membrane of the endothelium. The endothelium is a single layer of cells which completely covers the posterior surface of the cornea. The cells are hexagonal with large nuclei. This layer of the cornea is particularly rich in the active transport enzyme adenine triphosphatase (ATPase). The maintenance of proper hydration of the cornea has been attributed to the activity of this enzyme, which catalyzes an active sodium-potassium pump (38, 203, 228). The limbus is a transitional region between the cornea and the sclera. This region, rich in vascularization, is the source of fluid and infiltration cells during corneal injury.

The epithelium and the overlying tear film provide the intrinsic protection for the cornea. Other layers have almost no intrinsic resistance to injury. Penetration into deeper layers of the cornea and other structures of the eye is limited by chemicals' solubility and lipophilicity. Chemicals that are lipophilic and water-soluble penetrate more rapidly and probably deeper into the eye than other chemicals.

The cornea is always covered with a film of tears, which consists of several oily and aqueous layers. Proper tear formation and drainage as well as the stability of the precorneal tear film are important for a normal precorneal optical surface, proper lubrication, nutrition for the cornea, removal of bacteria and debris from the cornea, and activity on the cornea. Reduction of tear formation can lead to a dry eye, mechanical friction, irritation, or infection. A discussion of the assessment of tear film formation, stability, and drainage is available (73).

The cornea is a powerful refractive biological optic. Its refractive power is dependent on its being transparent and on proper hydration. Maintenance of proper transparency and hydration is dependent on many mechanisms, for example, proper tear flow, absence of deposits and blood vessels, proper arrangement of collagen fibrils, unimpaired nutritional supply for the metabolic active pump (Na^+-K^+), and proper intraocular pressure. Decreased transparency or hydration can be a result of corneal scars (decreased corneal thickness) or corneal edema (increased corneal thickness). Corneal edema can be caused by epithelial damage, endothelial damage, increased intraocular pressure, lack of oxygen, or inhibition of the electrolyte balance pump (Na^+-K^+-activated ATPase), which is located mainly in the

endothelial membrane, but is also found in the epithelium. Methods for measuring corneal curvature, corneal thickness, intraocular pressure, blood/aqueous humor barrier, and corneal endothelium damages have been reviewed (73).

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Conjunctiva

The conjunctiva is part of the eyelid. It is the delicate membrane that lines the eyelid (palpebral conjunctiva) and covers the exposed surface of the eyeball (bulbar conjunctiva). Histologically, the conjunctiva is an aqueous, nonkeratinized epithelium with numerous mucussecreting cells. Accessory lacrimal glands are present in the conjunctiva, which contribute to the aqueous layer of a precorneal tear film. The Meibomian gland, a specialized sebaceous gland in the eyelid, secretes the outer oily layer of the tear film.

The main function of the eyelid is to protect the eye, especially the cornea, from external trauma through proper blinking reflexes and secretion of tears. Normal secretory and excretory functions of the tear are also important for normal optical function of the eye. The precorneal tear film can form an optically uniform layer over the microscopically irregular surface of the corneal epithelial cells. The tear flow continuously flushes cellular debris or foreign bodies from the eye, lubricates the corneal surface from mechanical friction caused by blinking, provides nutrients to the cornea, and induces antibacterial activities by proteolytic enzymes and immunoglobulin. All of these functions are important to maintain an optically intact corneal surface. Substances that affect the stability of the precorneal tear film by interfering with the secretory/excretory functions or with the blinking mechanism can cause serious damage to the cornea and may even cause corneal ulceration.

The nictitating membrane or the third eyelid is an important and prominent structure in many species of animals, including the rabbit, but is not as important in humans and nonhuman primates. It aids in protecting the conjunctiva and the cornea when the eyeball is retracted. The nictitating membrane, like the conjunctiva, also contains lacrimal glands and its secretion contributes to the aqueous layer of the precorneal tear film. In addition, the nictitating membrane helps to support the position of low eyelids and forms the lacrimal lake in the medial canthus. Vascularization in the conjunctiva generally consists of superficial and deep groups, mainly in the bulbar conjunctiva.

Three endpoints generally are associated with irritation in the conjunctiva: redness, chemosis, and discharge. In response to an irritant, the eyelids blink, the tear secretion increases, and the conjunctiva vessels dilate. Blinking and tearing (discharge) aid in removing the irritant from the eye, and tear flow also may reduce the acidity or basicity of the irritant. Vessel dilation may be triggered by histamine, prostaglandins, or other inflammatory mediators, resulting in an apparent increase in vascularity (redness) in the conjunctiva. If irritation is severe, the dilation of the vessel increases and vascular fluid and proteins leak into the conjunctiva resulting in edema (chemosis). If the edema is severe, bulging may hinder normal functioning of the eyelids.

Iris

The iris forms the pupil and functions in regulating the amount of light that may reach the retina. High-intensity light causes constriction of the diameter of the pupil and low-intensity light dilates it. It does so by two sets of muscles acting opposite each other to control the diameter of the pupil. These muscles, circulatory and radiating, are innervated by both the autonomic and sympathetic nervous systems. The set of muscles forms the distinct characteristic of iridic furrows of the iris.

The iris is anatomically located posterior to the cornea, and is a very vascular structure made of loose connective tissues, muscle, and pigmented cells. The amount of pigment in the iris varies. Heavily pigmented cells are found in most species, except albinos. Only a small amount of pigment is found in the albino rabbit eye. This is an advantage in ocular studies because it allows easier and better examination of the iridal vessels, lens, and retina.

The observation endpoints of local iridic injury are increased vascularity, edema (increased thickness of the stroma/swelling), reaction to light, aqueous flare, and gross destruction of tissue. These are the manifestations of an inflammatory process (iritis) responding to an irritant. Like the conjunctival vessels, the iridic vessels dilate and leak vascular fluid in response to irritants. Dilation of vessels and leakage cause edema and apparent changes in vascularity such as injection of iridic vessels (hyperemia). Aqueous flare is a result of protein leaking from the iridic vessels into the aqueous humor of the anterior chamber. Protein leakage into the anterior chamber alters the refractive index of the aqueous humors. Light beams entering the anterior chamber are scattered, giving the anterior chamber a cloudy appearance which contrasts with a clear appearance in normal eyes as a light beam passes through the pupil and the anterior chamber, for example, during examination with a slit lamp. This is called the aqueous flare or Tyndall phenomenon which is usually not noted during routine gross examination of the eye. In a more severe form of iritis, tissue destruction may result and nerve innervation may be disrupted, causing the pupil to be unresponsive to light. Failure to react to light, from a practical standpoint, is the most reliable observation of a severe iridic reaction since severe iritis is usually

accompanied by severe opacity in the cornea, which may obscure the visible detection of changes on the iris.

THE DRAIZE TEST

The Draize test was developed in 1944 by Draize et al. to study eye irritation (102). The test was based on the

original work of Freidenwald et al. (148). For years, the Draize test has been used as the animal test to identify human eye irritants. It is a simple and generalized test. It is easy to conduct and requires no special instruments. Even though simplicity is probably the main reason for its popularity, it is also the limitation of the test per se. Undeniably, the Draize test can adequately identify most of the moderate-to-severe human eye irritants, but the test may fail to detect mild or subtle ocular irritation even with proper modification.

In the original Draize test, a standard 0.1 ml or 0.1 g of test substance is applied to the conjunctival sac of an albino rabbit's eye. The eyelid is held together for a few seconds and then released. The degree or extent of opacity on the cornea, the redness on the iris, and the chemosis and discharge on the conjunctiva are scored subjectively according to an arbitrary scale at preselected intervals (1, 24, 48, and 96 h) after exposure. Scoring is based on the degree of effects caused by the testing substance. More emphasis is placed on the opacity of the cornea, which has a maximum score of 80, whereas emphasis is progressively less with other effects: conjunctival changes (maximum score of 20) and iritis (maximum score of 10) (62, 165, 232).

The Draize test has been a subject of controversy among animal rights groups (178, 313) and even in the scientific community (28, 62, 165, 172, 181, 232, 304, 374). It has been criticized on the bases of dose volume, use of animals as models, methods of exposure, irrigation, number of animals, observation and scoring including laboratory procedure variability, and interpretation of results, all of which are discussed below.

Dose Volume

The 0.1 ml dose volume used in the original test was based on the volume used earlier by Friedenwald et al. (148) to study the mechanism of acid- and base-induced ocular damage. This dose volume was selected arbitrarily as a standard volume for intraocular injection. Draize et al. (102) adopted it solely for convenience which unfortunately has set a seemingly unchangeable doctrine for years even though the 0.1 ml dose volume lacks a scientific basis and, in conjunction with the conjunctival dosing method, often overpredicts the eye irritancy of a chemical.

Proponents of the 0.1 ml dose volume argue that this dose is a maximized test for the worst case and that it can better predict human eye irritants. The Draize test is basically a safety test. Its main purpose is to predict what would happen to human eyes *within* the expected range of exposure. The 0.1 ml dose is out of the range of human exposure. The maximal volume the cul-de-sac of a rabbit's eye can hold is only 30–50 μl (246); thus, even though one desires a maximal test, the dose volume should not be more than 50 μl. Any volume over this absolute maximum simply falls from the eye. Furthermore, the worst case is not necessarily the best case. Constantly overrating eye irritation will have a desensitizing effect on consumers' and workers' awareness of potential eye irritation. This will defeat the purpose of eye irritancy testing which is to protect them.

There are no data to substantiate the argument that the 0.1 ml dose can better predict human eye irritants. On the contrary, in at least one survey, there was little correlation between human accidental exposure experience and data generated by the traditional 0.1 ml maximal dose. The survey did not support the general presumption that rabbit eyes are more sensitive than human eyes (68). Simply reducing the dose volume has produced data closer to eye irritation experienced in humans. For example, comparison of human eye irritation resulting from accidental exposure to many consumer products has revealed that lower dose volume predicts the eye irritancy potential much better than the 0.1 ml dose vol ume (147, 172). In one of the studies, the time needed for recovery from eye irritation in consumers or factory workers is compared with animal tests in monkeys and rabbits (147). This survey clearly demonstrated the modified Draize test (Federal Hazardous Substances Act (FHSA) protocol with a dose volume of 0.1 ml) was the poorest predicting test, whereas the low dose volume and the monkey tests were better predictors even though all three animal tests overpredicted the eye irritancy experienced in humans.

In 1977, a panel on eye irritancy test of the National Academy of Sciences (NAS), formed at the request of the Consumer Product Safety Commission (CPSC), recommended lowering the dose volume (267). Subsequently, even smaller dose volumes ranging from 0.003 to 0.03 ml were proposed by others because they predict human eye irritants more accurately, cause less pain to animals, and can discriminate slight-to-moderate eye irritants (172, 174, 380). Williams et al. (380) showed that direct corneal application in a dose volume of 0.01 ml increased the response on the cornea when compared with the standard 0.1 ml dose but did not change the response on the conjunctiva. These results in the absence of compounding effects of a high dose volume suggest that the lower dose volume is just as sensitive a method for eye irritancy testing as the higher dose volume.

Animal Models

As with other toxicological tests in animals, the primary issue of testing for ocular irritancy in animals is predictability for humans. Recognizing that there are anatomical, physiological, and biochemical differences

between human and animal eyes, researchers are confronted with the difficult task of selecting the appropriate animal model and suitable test conditions to identify potential human eye irritants. The corneal thickness of dogs and rhesus monkeys is similar to that of humans (approximately 0.5 mm) (232, 236, 247); rabbit corneal thickness is somewhat thinner (0.37 mm) (232). There is a lack of a recognizable Bowman's membrane in rabbits, but they have a well-developed nictitating membrane (an additional target tissue), thick fur around the eyes, loose eyelids susceptible to mild irritants, an ineffective tear drainage system, and a poorly developed blinking mechanism (267). There are also species differences in biochemistry (e.g., variation in enzyme content (216)) and different penetration rates of various substances (231).

Even though there are shortcomings and exceptions in predictability, the rabbit has been used for most eye irritancy studies. There are some obvious advantages for choosing the rabbit: a wide database, economy, availability, ease of handling, and large, unpigmented eyes suitable for various ophthalmological examinations. With some exceptions (171, 304), the rabbit eye is generally more sensitive to irritating materials than human or monkey eyes (25, 63). Thus, there are built-in safety factors for making extrapolation and assessment of hazard to humans.

In addition to rabbits, dogs and primates sometimes are used for ocular testing. Eye irritancy in primates generally is more closely correlated with the exposure experience in humans, although dogs also are suitable under certain circumstances (29). Because they are more expensive and less available, dogs and primates are only used occasionally to assess eye irritancy.

Regardless of which animal is used, the investigator should always have a good understanding of the animal eye being observed. Background ocular findings, if not observed prior to exposure, can be recorded falsely as chemically induced damage.

Methods of Exposure

Basically, there are two ways of applying a test sub stance to the eye: (a) applying the test material into the cul-de-sac of the conjunctiva or (b) applying it directly onto the cornea. The conjunctival exposure method has been adopted historically because of the ease of application. It also has been perceived as being accurate in dosing. However, some have experienced (29, 172) that conjunctival exposure is inappropriate under many circumstances, especially when the test material is a solid powder. The possibility exists that the test material will be trapped in the conjunctival sac, producing some undesirable mechanical effects and making the interpretation of the results more difficult. It is also evident that a considerable amount of the standard 0.1 ml or 0.1 g dose (especially as a solid powder) either falls or is blinked from the eye once the animal's eyelids are released. The claim that conjunctival dosing is more accurate may not be valid.

The corneal exposure method, on the other hand, mimics more closely the actual accidental exposure experience in humans. When assessing the hazard of most chemical accidents, this method should be considered except when the chemical is intended for pharmaceutical use (267). Applicators developed for the corneal exposure method have been used in some studies (25, 63). A more uniform corneal lesion was observed, resulting in less observation variability (25). For a study as specific as corneal wound healing, a corneal applicator is recommended (258). However, for hazard assessment, it is desirable to apply the test substance directly onto the cornea while the lids of the test eye are gently held open. The eyelids are closed for a second and then released to allow blinking; this action more closely mimics actual human exposure (172).

Irrigation

Washing the eye is a typical emergency remedy after accidental exposure to chemical substances. In experimental studies, the treated eye usually is irrigated 20–30 sec after exposure to the test substance. Water is rapidly but gently squeezed from a plastic bottle to produce a constant gentle stream of water irrigating the entire treated eye. Irrigation should last for at least 1 min.

The effect of irrigation on the interpretation of test results has been the subject of many studies (25, 27, 28, 37, 89, 140, 171, 173, 285, 322). While irrigation of the treated eye right after exposure can prevent or minimize eye irritation in rabbits, the effectiveness of irrigation is dependent on the chemical, the concentration, the time lag between exposure and initiation of the irrigation, and the volume of irrigation. Early washing (less than 1 min) generally is recommended to reduce irritation (89, 140, 173, 322), but in some cases, increased irritation has been observed after irrigation with water (153, 322). In other cases, ocular damage was almost instantaneous if irrigation did not begin after a few seconds (89).

Number of Animals

It is generally true in experimental studies that the larger the group size the more precise the test

results. Sometimes, the desired precision may be offset by animal-to-animal variabilities. Economic considerations also are important in determining the number of animals used in a test group. A balance between economic considerations and reliability of test results should determine the number of animals tested in a study.

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For eye irritation studies, a group size of 9 rabbits was recommended in the original Draize test, and group sizes of at least 6, 3, 3, and 4 rabbits have been recommended by the FHSA, Interagency Regulatory Liaison Group (IRLG), OECD, and NAS, respectively. The relationship of variability, classification, and group size is addressed in the literature (27, 173, 375). With larger group size, smaller variability has been noted (375), whereas with a decreased group size, lesser differentiation of irritancy has been suggested (27). Recognizing these facts, Guillot et al. (173) suggested a compromised approach. They suggested that with 3 rabbits in an initial study, there was a 96% chance that a positive or negative eye irritation result would be obtained. A similar conclusion was obtained in another study conducted with 67 petroleum products each with 6 rabbits (94). The eye irritation scores for the petroleum products based on all 6 rabbits were compared statistically with the scores from 2, 3, 4, or 5 animals. The comparison showed that a subsample size of 2, 3, 4, and 5 rabbits correctly classified (compared with the original 6 rabbits/test classification) the chemicals at 88, 93, 95, and 96% accuracy, respectively.

Observation and Scoring

Reversibility and severity are the two major criteria used to measure eye irritancy in the Draize test. Reversibility refers to the time needed for the ocular effects to disappear and for the eye to return to its normal state. To determine this reference time, treated eyes are examined periodically at 24-h intervals, on day seven after exposure, or at longer intervals if needed to establish reversibility (102). The observation period varies for different guidelines. For example, the FHSA uses 24-, 48-, and 72-h time spans (136); the OECD uses 1-, 24-, 48-, and 72-h, and, if needed, extended observations (276); and the NAS recommends 1, 3, 7, 14, and 21 days (267). The observation period should be flexible so that one can confidently assess the persistence of ocular effects and fully characterize the degree of involvement, because the onset and healing of ocular effects often are unpredictable (171).

The assessment of severity of different ocular effects is subjective. This subjective evaluation is the major source of error for intra- and inter-laboratory variation (374). Therefore, to minimize at least the intra-laboratory variability in scoring, uniformity in scoring techniques must exist among investigators regardless of which scoring system is followed. Pictorial references such as those prepared by the FDA (131) and the Consumer Product Safety Commission (CPSC) (85) can be extremely helpful in the standardization of scoring eye irritation.

The types of ocular effects observed in the Draize test involve the cornea, iris, nictitating membrane, and conjunctiva. A grading system (Table 18.8) was originally

Table 18.8 Scale of weighted scores for grading the severity of ocular lesions

Lesion	Score[a]
I. Cornea	
A. Opacity—degree of density (area which is most dense is taken for reading)	
Scattered or diffuse area—details of iris clearly visible	1
Easily discernible translucent areas, details of iris clearly visible	2
Opalescent areas, no details of iris visible, size of pupil barely discernible	3
Opaque, iris invisible	4
B. Area of cornea involved	
One-quarter (or less) but not zero	1
Greater than one-quarter—less than onehalf	2
Greater than one-half—less than threequarters	3
Greater than three-quarters—up to whole area	4
Score equals A×B×5	
Total maximum=80	
II. Iris	
A. Values	
Folds above normal, congestion, swelling. circumcorneal injection (any one or all of these or combination of any thereof), iris still reacting to light (sluggish reaction is positive)	1
No reaction to light; hemorrhage, gross destruction (any one or all of these)	2
Score equals A×5	
Total maximum=10	
III. Conjunctivae	
A. Redness (refers to palpebral conjunctivae only)	
Vessels definitely injected above normal	1
More diffuse, deeper crimson red, individual vessels not easily discernible	2

Diffuse beefy red	3
B. Chemosis	
Any swelling above normal (includes nictitating membrane)	1
Obvious swelling with partial eversion of the lids	2
Swelling with lids about half closed	3
Swelling with lids about half closed to completely closed	4
C. Discharge	
Any amount different from normal (does not include small amounts observed in inner canthus of normal animals)	1
Discharge with moistening of the lids and hairs just adjacent to the lids	2
Discharge with moistening of the lids and considerable area around the eye	3
Score equals (A+B+C)×2	
Total maximum=20	

From Reference 62.

a The maximum total score is the sum of all the scores obtained for the cornea, iris,and conjunctivae.

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Table 18.9 Grades for ocular lesions

Lesion	Grades	Lesion	Grades
Cornea		Conjunctivae	
No ulceration or opacity	0	Redness (refers to palpebral and bulbar conjunctivae excluding cornea and iris)	
Scattered or diffuse areas of opacity (other than slight dulling of normal luster), details of iris clearly visible	1*a*	Vessels normal	0
		Some vessels definitely injected	1
Easily discernible translucent areas, details of iris slightly obscured	2	Diffuse, crimson red, individual vessels not easily discernible	2*a*
Nacreous areas, no details of iris visible, size of pupil barely discernible	3	Diffuse, beefy red	3
		Chemosis	
Complete corneal opacity, iris not discernible	4	No swelling	0
Iris		Any swelling above normal (includes nictitating membrane)	1
Normal	0	Obvious swelling with partial eversion of lids	2*a*
Markedly deepened folds, congestion, swelling, moderate circumcorneal Injection (any of these separately or combined); iris still reacting to light (sluggish reaction is positive)	1*a*	Swelling of lids about half closed	3
		Swelling of lids more than half closed	4
No reaction to light, hemorrhage, gross destruction (any or all of these)	2		

a Lowest grade considered positive.

proposed by Draize et al. (102), and subsequently a number of modifications were proposed (85, 131, 267). In the Draize system, the intensity and area of involvement on the cornea are graded separately on a scale of 0–4. The product of the two scores is multiplied by 5 to obtain a weighted corneal score. The congestion, swelling, circumcorneal injection, hemorrhage, and iridic failure of reactions to light are graded collectively on a scale of 0–2, and the score is multiplied by 5 to obtain a weighted iridic score. The redness, chemosis, and discharge of the conjunctivae are graded on scales of 0–3, 0–4, and 0–3, respectively. The sum of the conjunctival scores is multiplied by 5 to obtain a weighted conjunctival score. Other lesions also are recorded, such as pannus (corneal neovascularization), phylyctena, and rupture of the eyeball.

In the guidelines set forth by the EPA, CPSC, FHSA, OECD, EEC, and Japan's Ministry of Agriculture, Forestry and Food (MAFF) (85, 109, 124, 136, 337), only the degree (intensity of cornea damage, iritis, and redness and chemosis [swelling]) of the conjunctivitis is scored (Table 18.9). The area involved on the cornea as well as the discharge of the conjunctiva are not taken into consideration in scoring. Various aids are used at times to facilitate or increase the resolution power of these observations. These aids include fluorescein staining and ophthalmoscopic or slit lamp microscopic examinations. A scoring system has been developed for the slit lamp and fluorescein staining examination (267) (Table 18.10). Other scoring systems have been proposed for lacrimation, blepharitis, chemosis, injection of conjunctival blood vessels, iritis, kerectasis, and corneal neovascularization (9).

Interpretation of Results

There are essentially four categories of data, generated by the Draize test, to be considered when interpreting the results of ocular testing:

(a) kind of ocular effects,
(b) severity,
(c) reversibility, and
(d) rate of incidence.

Weighting the scores in the original Draize test has, to some extent, to take the first category into consideration, yet it biases toward the cornea, one of the most critical ocular tissues. Severity is measured according to a graded scoring system, and reversibility is expressed as the time needed for the affected ocular tissue to return to the normal state. Incidence is the number of animals that show

some kind of ocular effect during the study. Interpretation of the data is a multiple and factorial undertaking. All four categories of data

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Table 18.10 Scoring criteria for ocular effects observed in slit lamp microscopy

Location of observations	Grades	Location of observations	Grades
Corneal observations		*Iridal observations (cont'd)*	
Intensity		Aqueous flare (Tyndall effect)	
Only epithelial edema (with only slight stromal edema or without stromal edema)	1	Slight	1
		Moderate	2
		Marked	3
Corneal thickness 1.5×normal	2	Iris hyperemia	
Corneal thickness 2×normal	3	Slight	1
Cornea entirely opaque so that corneal thickness cannot be determined	4	Moderate	2
		Marked	3
Area involved		Pupillary reflex	
≤25% of total corneal surface	1	Sluggish	1
>25% but <50%	2	Absent	2
>50% but <75%	3	Maximal iridal score	11
>75%	4	*Conjunctival observations*	
Fluorescein staining		Hyperemia	
≤25% of total corneal surface	1	Slight	1
>25% but <50%	2	Moderate	2
>50% but <75%	3	Marked	3
>75%	4	Chemosis	
Neovascularization and pigment migration		Slight	1
≤25% of total corneal surface	1	Moderate	2
>25% but <50%	2	Marked	3
>50% but <75%	3	Fluorescein staining	
>75%	4	Slight	1
Perforation	4	Moderate	2
Maximal corneal score	20	Marked	3
Iridal observations		Ulceration	
Cells in aqueous chamber		Slight	1
A few	1	Moderate	2
A moderate number	2	Marked	3
Many	3	Maximal conjunctival score	12

are somewhat interrelated; the individual scores do not represent an absolute standard for the irritancy of a material (273).

In one study, interpretation of eye irritation was not considered to be the major factor contributing to interlaboratory variability (374). This finding is not surprising, if one assumes that everyone adheres to the same interpretation criteria. However, the question is what are the appropriate criteria for interpreting eye irritation results that would have an impact on placing eye irritants into different categories? The individual scores do not represent an absolute standard for the irritancy of a material (275).

Many classification systems for eye irritants have been proposed. Some have been published in the literature (171, 173, 202, 267), and in various testing guidelines (109, 136, 337), and many others are used in individual laboratories. There is general agreement among investigators on how to classify test substances when no irritation is observed or when severe irritation or corrosion is seen, but there is little agreement on how to classify irritancy that falls between these two extremes. The manner in which data are evaluated directly affects the conclusions reached.

Because of the complexity of eye irritancy data and their interdependence, some investigators have chosen to simplify the interpretation to a pass-or-fail approach. For example, in the FHSA guideline (136), if four or more of the six test rabbits show ocular effects within 72 h after a conjunctival sac exposure (0.1 ml or 100 mg of the test material), the test material is considered to be a positive eye irritant. The ocular effects in consideration are "ulceration of the cornea (other than a fine stippling), corneal opacity (other than a slight deepening of the nor

mal luster), inflammation of the iris (other than deepening of folds), an obvious swelling with partial eversion of the lids, or a diffuse crimson red with individual vessels but not easily discernible." If only one of the six tested animals shows ocular effects within 72 h, the test is considered negative. If two or three of the six tested animals show ocular effects, the test is repeated. The test substance is considered to be a positive irritant if three or more animals show ocular effects in the repeated test; otherwise, the test is repeated. Any positive ocular effect observed in the third test automatically classifies the test substance as an irritant. A similar approach has been adopted in the IRLG guideline (192), but an option is given that declares a test positive when two or three of six rabbits tested show a positive ocular effect and the test is not repeated. The pass-or-fail interpretation is too simplistic, however, and it does not separate eye irritants, especially those that fall between the two extreme irritancy categories (from nonirritating to severely irritating). Gradation of potential eye irritation is important to denote an anticipated hazard and to convey to consumers or workers that a specific degree of precaution should be exercised whenever a potential exposure to the substance exists.
Green et al. (171) used a different approach. Eye irritancy was classified into four easily recognizable categories based on the most severe responder in a group:

- *Nonirritation:* Exposure of the eye to the material under the specified conditions causes no significant ocular changes. No tissue staining with fluorescein was observed. Any changes that did occur cleared within 24 h and were no greater than those caused by normal saline under the same conditions.
- *Irritation:* Exposure of the eye to the material under the specified conditions causes minor, superficial, and transient changes of the cornea, iris, or conjunctiva as determined by external or slit lamp examination with fluorescein staining. The appearance at any grading interval of any of the following changes was sufficient to characterize a response as an irritation: opacity of the cornea (other than a slight dulling of the normal luster), hyperemia of the iris, or swelling of the conjunctiva. Any changes cleared within 7 days.
- *Harmfulness:* Exposure of the eye to the material under specified conditions causes significant injury to the eye, such as loss of the corneal epithelium, corneal opacity, iritis (other than a slight infection), conjunctivitis, pannus, or bullae. The effect healed or cleared within 21 days.
- *Corrosion:* Exposure of the eye to the material under specified conditions results in the types of injury described in the previous category and also results in significant tissue destruction (necrosis) or injuries that adversely affect the visual process. Injuries persisted for 21 days or more.

This classification system has taken into consideration the kinds of ocular effects, the reversibility, and, to a certain extent, the qualitative severity, but not the incidence. The committee that revised the NAS publication 1138 (267) put forward a system of classification similar to that of Green et al. (171). The categories are named differently: inconsequential or complete lack of irritation, moderate irritation, substantial irritation, and severe or corrosive irritation. The classification also is based on the most severe responder, and incidence is not considered. A provision for repeating the test is given as an option to increase the confidence level in making a judgment in some borderline cases. This eye irritancy classification system has been widely adopted. One shortcoming of the NAS system is that too wide a spectrum is created for moderate irritancy, which may lead to overutilization of the cautionary term, *moderate*. Many investigators have experienced problems in interpreting results from fluorescein staining of the cornea when the NAS gradation system is used. The confusion arises mainly from the occasional artifacts inherent in fluorescein staining. Experience and sound scientific judgment are needed to properly interpret the fluorescein staining results (see the discussion on ophthalmological techniques).
Griffith et al. (172) disagreed with using the most severe responder for classification of eye irritancy, claiming that there was no epidemiological evidence to suggest that the most severe rabbit responder would correlate with the worst possible case of human exposure. Instead, these investigators used the median time for recovery for classification according to the same temporal criteria as in the NAS system. The underlying logic is that the incidence of responders is being considered indirectly.
The classification systems of Green et al. (171), Griffith et al. (172), and NAS (267) apparently have not taken into account the severity of irritancy. Although there is a perception of a direct relationship between severity and reversibility, if one examines the data of Griffith et al. (172), indeed, it can be shown that a direct correlation of median time to recovery and the severity of irritancy does occur.
Kay and Calandra (202) proposed yet another rating system based on the Draize scores, taking into account the extent and persistence of irritation and the overall consistency of the data. Another system was proposed by Guillot et al. (173). Here, the greatest mean irritation score within an observation period is identified. On the basis of this score, the test substance is classified into six categories, ranging

from nonirritating to maximum or extremely irritating. To maintain this initial rating,

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the data also must meet the arbitrary criteria for reversibility and frequency of occurrence, otherwise the rating is upgraded one category. The Kay and Calandra system has not been verified for correlation to human exposure experience, nor has it been compared with other classification systems. Guillot et al. (173) made an attempt to compare their rating with the OECD protocol. They claimed that one-third of the 56 materials tested could be classified into a lower category by the OECD protocol.

The most current modification of the OECD protocol (276) is an effort to minimize the number of animals used to produce data suitable for hazard classification. In this simplified scheme, a Draize eye test is conducted using one animal if severe effects are expected, or three animals if no severe eye irritation is anticipated. Scoring is based on ocular lesions that occur within 72 h of exposure and results are expressed in terms of the lesions and their reversibility (eye irritation) or irreversibility (eye corrosion). The EPA has recently revised its health effects test guidelines for acute eye irritation (126) to be more consistent with the OECD protocol. A revised EEC directive, based on the OECD approach, provides hazard classification corresponding to risk phrases (R 36—Irritating to eyes and R 41—Risk of serious damage to eyes). These risk phrases are assigned to the label of a chemical when two or more of the three animals exhibit scores within certain arbitrary numbers (110).

A summary of the current international classification systems and major features for eye irritancy testing is shown in Table 18.11. Despite such a range of classification schemes, there is little difference in the actual scoring system (basically adhering to the original Draize) (102).

SPECIAL OPHTHALMOLOGICAL TECHNIQUES

The Draize test is a generalized test concentrating on the effects of the material on the cornea, iris, and conjunctiva. Examination usually is performed under a hand light. Accurate observations are limited by the experience and training of the investigator. Subtle ocular changes may be missed. If these subtle changes are to be detected and ambiguous gross observations resolved, or if internal tissues (e.g., the lens and the retina) are to be examined, the investigator must rely on special techniques. Many such techniques have been developed over the years, most of which are more objective than the gross examination itself. A few comments on the fluorescein staining technique and several of the more objective methods are presented.

Fluorescein Staining for Corneal Damage

Fluorescein is a weak organic acid (Figure 18.5) and is only slightly soluble in water, but its sodium salt is moderately soluble in water. It is very efficient in absorbing ultraviolet light and emitting fluorescent light. The maximum absorption is 490 nm (excitation) in the violet region, and its maximum emission is 520 nm in the green region of the spectrum. Its un-ionized form is less fluorescent than its ionized form. At pH 7.4, fluorescein does not seem to bind to tissue and is nontoxic in animals, making it an ideal marker for an ocular fluid dynamics study. Because fluorescein is a deeply colored and highly fluorescent chemical, it can be detected at very low concentrations in biological tissues or fluid; however, its detection sensitivity often is limited by the background fluorescence of biological tissues.

Because sodium fluorescein is a polar molecule, it does not readily traverse lipophilic membranes but easily diffuses into aqueous medium. For example, if ulceration occurs on the cornea, the lipophilic membrane barrier is broken down and the fluorescein diffuses freely through the ulcerated area of the cornea and either is dissolved or suspended in the aqueous medium of the stroma. More detailed information on the chemical and biological properties of fluorescein is provided in two excellent reviews (235, 249).

Since its first use in studying the origin of aqueous humor secretion a century ago (111), fluorescein has become an important aid in ophthalmology. It has been used as a marker in detecting obstructions in the nasolacrimal drainage systems, for studying changes in the flow dynamics of different ocular fluids, for demonstrating leakage of retinal vessels in angiography, for estimating permeability of the cornea and lens, and for identifying ulcerations on the cornea (235). Among these, its use in detecting subtle changes on the corneal epithelium (80, 183) has been a routine procedure in animal eye irritation studies.

The corneal epithelium is a lipophilic barrier to sodium fluorescein, but such a barrier is broken when there is an ulceration or change in membrane structure. Some amount of fluorescein applied on the cornea will penetrate into the intercellular spaces of the stroma, which constitute a water-soluble layer of the stroma. When light is cast on the cornea, fluorescence is detected on the damaged area of the epithelium. Once the fluorescein enters the stroma, it eventually will pass through Descemet's membrane and the endothelium into the aqueous humor.

Fluorescein staining usually is accomplished by solution or impregnated paper strips. Fluorescein is commercially available in 2, 1, or 0.25% sodium salt solutions. Preservatives to minimize bacterial

contamination are common in these commercially available sol

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Table 18.11 Major feature of eye irritation tests and international classification schemes

Methodology	FHSA (CPSC, FDA, OSHA)	OECD	EPA (Modified OECD)	Canada (Modified OECD)	EU (EEC)
Initial considerations					
Screen for pH (<2 or >11.5)	NS	Yes	Same as OECD	Same as OECD	Same as OECD
Results from skin irritation	NS	Yes	Same as OECD	Same as OECD	Same as OECD
Results from validated alternatives	NS	Yes	Same as OECD	Same as OECD	Same as OECD
Number of animals:					
Screen for severe effects	NS	1	Same as OECD	Same as OECD	Same as OECD
Main Test	≥6	≥3	Same as OECD	Same as OECD	3
Volume administered	0.1 ml or 100 mg	0.1 ml or ≤100 mg	Same as OECD	Same as OECD	Same as OECD
Scoring times	1, 2, 3 d	1 h, 1, 2, 3 d (may be extended to assess reversibility)	1 h, 1, 2, 3 d (may be extended to assess reversibility ≤21 d)	1 h, 1, 2, 3 d (may be extended to assess reversibility)	1 h; 1, 2, 3 d
Minimal positive response:					
Corneal opacity	1	NS*a*	1	2.0*b*	≥2.0, <3.0*c*
Iritis	1	NS*a*	1	1.0*b*	≥1.0, <1.5*c*
Conjuctival					
redness	2	NS*a*	2	2.5*b*	2.5*c*
chemosis	2	NS*a*	2	2.5*b*	2.0*c*
Positive test	≥4 positive of 6 animals	NS	NS*a*		≥2 positive of 3 animals
Number of classes/label categories:					
Irritant	1 (reversible inflammatory effect)	Same as FHSA	Same as FHSA	1 (positive response requires labeling as a poisonous and infectious material)	1 (R 36: irritating to eyes) based on minimum positive response
Corrosive	1 (visible destruction or irreversible alterations)	Same as FHSA	Same as FHSA	NS	NS
Severe irritant	NS	NS	NS		1 (R 41: risk of serious damage to eyes) based on corneal opacity ≥3.0*c* and/or iritis ≥1.5*c*

NS, not specified.
a Individual scores do not represent an absolute standard for the irritant properties of a material.
b Mean of at least three animals.
c Mean of three scoring intervals and scores representing two or more animals.

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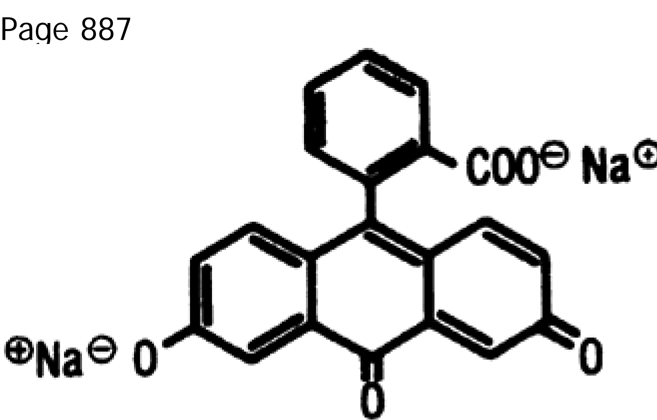

FIG. 18.5. Structure of fluorescein.

utions (71). A drop of the solution is instilled onto the eye and excessive fluorescein is flushed immediately with a sufficient amount of water. The eye then can be examined under a cobalt-filtered UV light for epithelial defects.

Fluorescein also is available in impregnated paper strips (210). These strips are free of contamination and easy to use. Moistened with collyria, a strip is touched lightly to the dorsal bulbar conjunctiva. The small amount of fluorescein should distribute uniformly on the cornea by either diffusion or blinking. Flushing is not usually necessary with the strips if applied properly. Nonetheless, if the strip touches the cornea, it becomes necessary for the cornea to be flushed with water before examination. Better results are obtained with the fluorescein-impregnated strip when examination is by slit lamp microscopy.

Fluorescein staining has two valuable applications in a routine eye irritation test. It can be used for screening eyes prior to the study to ensure that healthy eyes are being used. The other application is for the determination of total recovery from grossly observed damage on the cornea. Slight epithelial effects still can be detected by fluorescein even though they are not visible during gross observation. Even though most of these subtle effects on the cornea will disappear in a relatively short period of time, prolonged effects detected by fluorescein staining, but not by gross examination, should raise a concern over the healing process. However, when no gross lesions are detected at any time during a study (except for a few incidences of minor fluorescein staining on the cornea) one should not be overly concerned. If there are any effects on the cornea, they must be extremely minimal ones on the superficial epithelium for eye irritation to rate as nonirritating or inconsequential. If the staining is not an artifact, the minimal ocular effects detected under such circumstances should be readily reversible.

Although fluorescein staining can detect very subtle corneal epithelial changes, one can easily be misled by some very noticeable background staining. In addition, artifacts are quite common. For example, the apparent staining of the cornea can result from incomplete flushing of excessive fluorescein with water or even from reflected light. A strong jet of water during irrigation can cause mild damage to the cornea. Damage also can occur if the eye is not handled properly during gross examination. These changes are not related to the test compound but may be detected with fluorescein staining. Sometimes one may see haziness on the cornea after fluorescein staining even though a clear cornea is seen prior to fluorescein staining. Whether the hazy appearance of the cornea is a reflection of mild change or artifact depends on several factors. If the hazy appearance is also visible under a cobalt filter and is preceded by grossly visible lesions, it generally is considered to be a residual effect of mild severity that will disappear within a short time. However, if the hazy appearance is seen intermittently or is not preceded by ocular effects, it is likely an artifact. Proper training and experience are necessary to recognize artifacts and to obtain reliable, reproducible, and consistent results from fluorescein staining. In general, it is not necessary to stain lesions that are obvious and grossly evident. It is when lesions would otherwise go undetected by gross examination that fluorescein staining is of value.

Slit Lamp Microscopy

The slit lamp biomicroscope is an important instrument for studying ocular tissues, especially the cornea. As its name suggests, a slit lamp consists of a microscope that views optical sections of different layers of the cornea made by an intense light beam acting as a surgical knife or microtome, cutting through different layers of the eye. Many lesions that would remain undetected by gross examination can be observed with the slit lamp biomicroscope. Using recent models of slit lamp microscopes, one can observe not only the different layers of the cornea but, also, other transparent parts of the eye such as the aqueous humor, lens, and vitreous body.

The slit lamp biomicroscope consists of an illuminating light source and a microscope. Both components

are movable and adjustable, allowing the eye to be illuminated and observed from different angles and with different width and height adjustments of the slit light beam. An area of the cornea can simultaneously be illuminated and magnified by aligning the incidence of the light beam and the focus of the microscope. The light beam also can be directed at the area from different angles, providing several views of the same area.

Two types of slit images are used for illumination: parallelepiped and optical section (239). For the parallelepiped slit image, a rectangular light beam (approximately 1–2 mm wide and 5–10 mm high) is projected onto the cornea. The shape of the illuminated area is similar to a parallelepiped prism where the outer

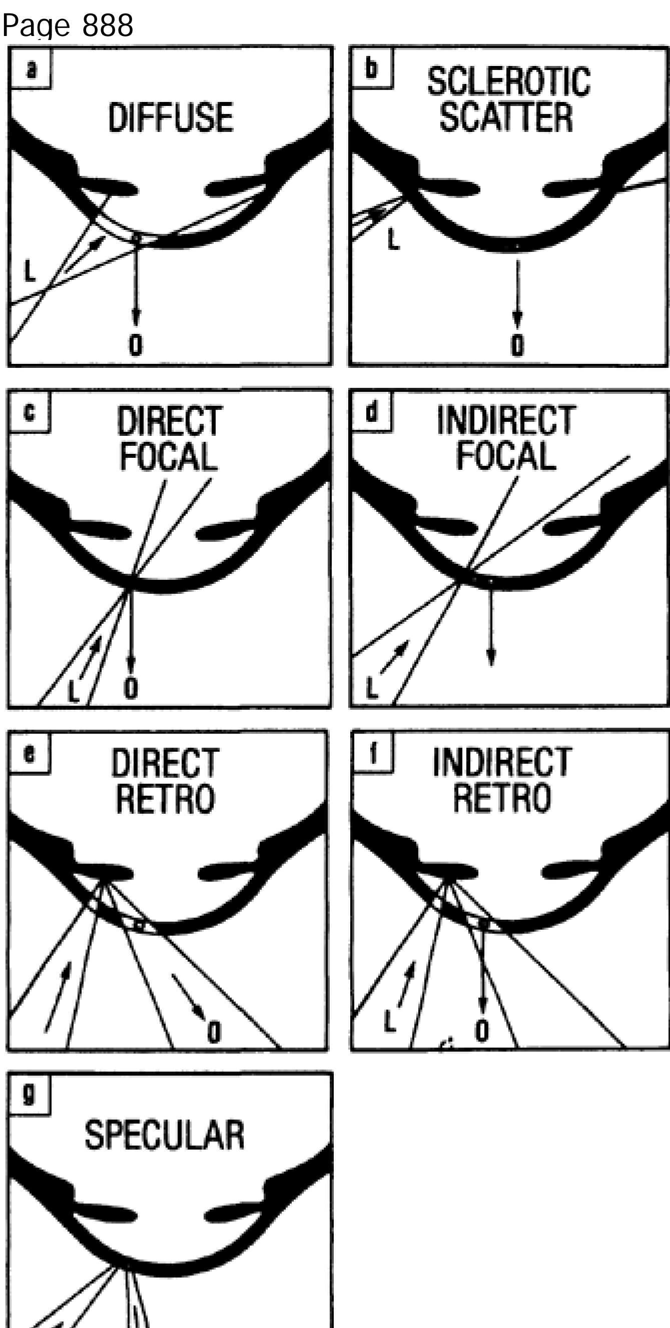

FIG. 18.6. Seven basic methods of illumination in slit lamp microscopy: (a) diffuse; (b) sclerotic scatter; (c) direct focal; (d) indirect focal; (e) direct retroillumination; (f) indirect retroillumination; (g) specular reflection. O=observer; L=illuminator light. Modified from Reference 239, with permission.

and inner surfaces are bent because of the shape of the cornea. For the optical section slit image, the width (20 μm) of the light beam is narrowed to its minimum and is projected onto the cornea, providing a sagittal view that is similar to a thin histological section.

There are several basic illumination techniques (Figure 18.6): diffuse illumination, sclerotic scatter illumination, direct and indirect focal illumination, direct and indirect retroillumination, and specular reflection (247, 346).

Diffuse Illumination

In diffuse illumination, a slightly out-of-focus wide beam is used to scan and localize any gross lesions of a large area of the eye. Usually the first step in examining the eye under a microscope is observing gross lesions and their extent of change. This technique is similar to observing the eye with a hand light, except that the observation is made under a microscope (Figure 18.6a).

Sclerotic Scatter Illumination

In sclerotic scatter illumination (Figure 18.6b), a narrow light beam is directed at the temporal limbus, and the microscope is focused centrally on the area of the cornea to be examined. The light reflected from the sclera will transmit within the cornea by total reflection. Under normal conditions nothing will be seen, but if even minor changes are present the reflected light will be obstructed and the damaged area (e.g., mild corneal edema) will be illuminated. This technique is useful for detecting minimal changes in the cornea.

Direct Focal Illumination

In direct focal illumination, the light beam and the microscope are focused sharply at the same point of interest in the same plane (Figure 18.6c). If a rectangular slit image is used for illumination and focused on the cornea, three general areas are seen when the parallelepiped is formed on the cornea: the epithelium (the anterior bright line), the stroma (the central clear marble-like area), and the endothelium (the posterior thin, bright line). If an optical section slit image is used for illumination, the corneal layers seen from anterior to posterior are a thin, bridge layer, a thin dark layer, a granular layer, and another thin bright layer. These correspond to the tear film, the epithelium, the stroma, and the endothelium, respectively. Altering the angle of incidence of the light beam decreases or increases the reflection. This allows for the detection of depth of the lesion. Opacities on the different layers can be detected easily as obstructions of the incident light beam.

Indirect Focal Illumination

Indirect focal illumination (Figure 18.6d) is accomplished by a narrow beam of light directed at an opaque area of the cornea. For example, changes in blood vessels at the cornea adjacent to the opaque area are illuminated and can be detected by focusing the microscope at these areas.

Direct and Indirect Retroillumination

In direct (Figure 18.6e) and indirect (Figure 18.6f) retroillumination, the light beam is directed at tissues behind the cornea, e.g., the iris or the fundus. The reflected light illuminates the area of interest of the corneal tissue and can be focused under the microscope. The microscope can be located directly on the path of the reflected light (direct retroillumination), thus permitting subtle changes to be observed against a con

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trasting background. Any optical obstruction by lesions such as scars, pigment, or vessels located along the reflection light path will appear as darker areas on a brighter background. Lesions such as corneal edema and precipitates that can scatter the reflection light will show up as a brighter area against a darker background. When the microscope is located off the reflection light path (indirect retroillumination), the corneal structure is observed against a dark background such as the pupil or iris. Indirect retroillumination is better for observing opaque structures, whereas direct illumination often is used to detect corneal edema and precipitates.

Specular Reflection Illumination

Specular reflection (Fig. 18.6g) is most useful in studying the endothelium and precorneal tear film. This technique makes use of the difference in refractive properties between the corneal surface and the adjacent medium of the posterior and anterior surfaces of the cornea. The microscope is focused on the cornea adjacent to the path of the incident slit light beam. By alternating the angle of incidence, a point can be reached such that a total reflection is obtained on the junction between the aqueous medium and the most posterior corneal surface, thus illuminating endothelial cell patterns and the Descemet's membrane. Similar techniques can be performed on the anterior corneal surface to visualize precorneal tear film.

Scoring System for Slit Lamp Examinations

By using slit lamp microscopic techniques, many subtle changes can be observed that would not otherwise be evident from the Draize test. A different scoring system must be developed to reflect such subtle changes. Baldwin et al. (8) proposed a scoring system for the cornea, anterior chamber, iris, and lens. Subsequently, NAS (267) developed a scoring system for slit lamp examinations that is similar to the Draize system in placing emphasis on the cornea, iris, and conjunctiva. Basically, in the NAS system, the intensity and the area involved are the two main criteria for scoring. Using this scoring system, the investigator must have a good understanding of the physiology of the normal eye. Like the Draize score, the NAS system is based on corneal effects; total maximal corneal score is 20 as compared with 11 and 15 for iridic and conjunctival scores, respectively. A detailed scoring scale and criteria are listed in Table 18.10.

Corneal Pachymetry

Because corneal transparency is so important to vision (over 70% of the Draize score is derived from assessment of damage to the cornea) objective procedures to quantify corneal effects are an important element in eliminating intra- and inter-laboratory variability in assessing the effects of ocular exposure to exogenous agents. Corneal transparency, thickness, and hydration are related in a linear fashion (179). Therefore, changes in corneal thickness can be used as an indicator of irritant affects, which may impair normal corneal hydration and transparency. When corneal thickness is measured appropriately, it can be used to objectively quantify swelling of the corneal stroma, which is a typical early irritant response. The measurement of corneal thickness is called pachymetry, which comes from the Greek words *pachys* (thick) and *metry* (the process of measuring).

One method for measuring corneal thickness uses an optical pachometer attached to a slit lamp microscope. Optical pachometers provide essentially indirect measurements of apparent corneal thickness based on displacement of light beams bouncing off the endothelial and epithelial surfaces of the cornea. The principles of this method have been described previously (73).

Various investigators (66, 81, 207, 251), using a variety of substances from different chemical classes, have reported that corneal thickness is significantly correlated with the Draize corneal score. Moreover, Kennah et al. (207) clearly demonstrated a substantial reduction in the coefficient of variation when comparing corneal swelling to Draize scores for various surfactants, alcohols, ketones, acetates, and aromatic chemicals.

Recent advances in human ophthalmological procedures to correct visual acuity (i.e., radial keratotomy, eximer-laser photorefractive keratectomy) have resulted in the development of improved devices to measure corneal thickness which guide the practitioner both before and after the procedure, and provide a means to measure the effectiveness of the treatment. The ultrasonic pachymeter is such a device (35, 264, 352, 363) and it may have useful application to in vivo ocular irritation testing.

The ultrasonic pachymeter is an instrument with a hand-held probe that emits an ultrasonic signal of fixed velocity. The probe is placed directly on the anterior surface of the cornea and after signal emission, a sensor directly measures the time difference between echoes of signal pulses reflected from the front and back surfaces of the cornea. This time differential is directly proportional to the thickness of the cornea via a function that is computed as the product of the time delay between the two echoes (in seconds) and the velocity of sound in the corneal tissue (in meters/second). The optical pachometer

indirectly equates displacement of incident light to corneal thickness, and the ultrasonic pachymeter provides a direct measurement.

Comparative evaluations of the sources of variability in human corneal thickness measurements using optical and ultrasonic (162, 182, 224, 289, 315) or between various ultrasonic devices (377) have been reported and discussed.

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Salz et al. (315) found that sources of variation include intra- and inter-session variation, inter-observer variation, left/right eye variation, and variations due to alternate settings of ultrasonic sound frequency. They reported that the optical pachymeter had significant inter-session variation, significant inter-observer variation, and significant differences in left and right eye thickness measurements, while the ultrasonic pachymeter demonstrated high reproducibility, no inter-observer variation, and no left/right eye variation.

The ultrasonic pachymeter has many desirable features such as a relatively low cost, portability, ease of operation, and use requiring less operator skill and training than the optical pachometer. When used in humans, a topical anesthetic is employed, because the tip of the measuring probe must be in contact with the corneal surface before a measurement can be taken. However, it has been reported that because of a lower corneal sensitivity in rabbits (75, 243) an anesthetic is not necessary before taking corneal thickness measurements.

Because the velocity of sound can vary in different tissue, accurate readings for absolute corneal thickness require that the ultrasonic sound frequency of the instrument be matched to the tissue of interest. The velocity of sound in human corneal tissue has been variously reported as 1502 m/s (265), 1586 m/s (307), and 1610 m/s (272). Salz et al. (315), in their human cornea comparison of optical to ultrasonic pachymetry, used an approximate velocity of 1590 m/s and found good agreement between the two measurement methods. The velocity of sound in cat (224), rabbit (74), and bovine (283) corneal tissue was found to be 1590 m/s, 1580 m/s, and 1550 m/s, respectively. Empirical methods to determine the velocity of sound in corneal tissue have been described (224, 283).

The utility of ultrasonic pachymetry in measuring corneal thickness changes in rabbits (230, 263) and rats (230), after treatment with ocular irritants, has been reported. The findings, albeit limited to a small number of chemicals, support the continued pursuit of this method as a relatively inexpensive, objective way to measure corneal irritant effects.

Confocal Microscopy

The confocal microscope is another instrument that can be used to measure corneal thickness, as well as provide high-resolution microscopic images to study the cellular structure within corneal tissue. The first confocal microscope was described by Minsky (244, 245) in a 1957 patent application. This device had a pinhole and a lens (objective and condenser) located on either side of the specimen to be viewed. The intent of the design was to eliminate any scattered light that might pass through the specimen, thus concentrating all light at a point source that was the focal point. The term confocal originated because the objective lens and the condenser lens were focused on the same specimen point. The image seen in a conventional light microscope includes the in-focus image in the *x, y* (horizontal) plane and the out-of-focus image above and below in the *z* (vertical) plane, the confocal microscope only focuses in the *x, y* plane. Indeed, de-focusing a confocal microscope makes the image totally disappear rather than appear blurred. Reducing the out of focus signal above and below the focal plane results in enhanced resolution. In contrast to the light microscope, which is focused by moving the objective, moving the specimen focuses the confocal microscope. This feature provides an optical sectioning capability that allows thick tissue sections such as the cornea to be viewed in vivo or in vitro in both the horizontal and vertical planes. Because of the point source light illumination, however, scanning the specimen is necessary to produce a full field of view with the confocal microscope. Scanned images can be viewed through a video monitor on a real-time basis, imported into a video cassette recorder, or stored as a digital image (291) for later viewing and analysis. For a complete review of the principles and applications of the scanning confocal microscope see Petroll et al. (292).

By successively scanning the cornea and capturing a series of optical sections it is possible to reconstruct a three-dimensional image of the tissue. Methods for three-dimensional imaging of rabbit cornea in vitro (196, 233, 293) and in vivo (129, 196, 250, 290), have been described. These methods have been used to characterize the changes in area and depth of corneal injury of surfactant-induced eye irritation in the rabbit (234) and to examine the relationship between area and depth of injury to corneal cell death (195).

Mauer et al. (234) used in vivo scanning confocal microscopy to qualitatively and quantitatively characterize the initial changes occurring after treatment with surfactants known to produce slight, mild, moderate, and severe corneal irritation. Materials were applied directly to the corneas of 6 rabbits per group at a dose of 10 μl with macroscopic (Draize) and microscopic evaluations beginning at 3 h after treatment and continuing periodically through day 35. Microscopic three-dimensional images were obtained from the surface epithelium to the endothelium and measurements made for surface epithelial cell size, epithelial layer thickness, total corneal thickness, and depth of keratocyte necrosis. The

average Draize scores at 3 h for the slight, mild, moderate, and severe irritants were 6.0, 39.3, 48.5, and 68.7, respectively. Confocal microscopic images at 3 h showed that corneal injury with the slight irritant was limited to the epithelium (cell size and thickness 59 and 82% of control). The mild irritant had removed the surface

epithelium, increased the corneal thickness to 158% of control, and produced keratocyte necrosis to a depth of 4.3 μm. With the moderate irritant the epithelium was markedly attenuated, the corneal thickness was 156% of controls, and keratocyte necrosis extended to a depth of 19 μm. For the severe irritant, the epithelium was significantly thinned, the corneal thickness was 166% of controls and keratocyte necrosis extended to a depth of 391 μm.

The use of confocal microscopy in studies designed to provide semi-quantitative information on the nature and depth of injury to the cornea after chemical treatment has the potential to serve as an important link to the development of physiologically relevant and mechanistically based in vitro alternatives to the Draize eye test (234).

LOCAL ANESTHETICS

For humane and scientific reasons, guidelines such as those established by the IRLG and the OECD provide options for using local anesthetics in eye irritation studies. Tetracaine, lidocaine, butacaine, proparacaine, and cocaine have all been examined for their usefulness in eye irritation studies, with the results being mixed and inconclusive. Most of these anesthetics can alleviate pain, but they also can inhibit or reduce the somatosensory area of the eye and the blinking reflex. Tear flow is reduced, causing the test substance to be trapped and remain undiluted on the cornea instead of being blinked from the eye or diluted and flushed away by the tear flow. Especially among higher primates, the blinking and tearing reflexes are important defense mechanisms against accidental exposure to any substance (181). Some local anesthetics can cause delay in corneal epithelial regeneration and loss of surface cells from the cornea (174). Some such as procaine, lignocaine, piperocaine, amylocaine, amethocaine, and cinchocaine are cytotoxic to cultured human cells, including conjunctival cells (90). However, at least one study has shown that a 0.5% tetracaine solution apparently had no effect on corneal healing (294). Further research is needed to reveal the interaction of local anesthetics and chemically induced ocular effects. Local anesthesia is sometimes useful to induce akinesia of the eyelid during eye examination.

Local anesthetics are desirable to alleviate pain, but one must be aware of the potential physical, chemical, physiological, and toxicological incompatibilities before considering their use.

HISTOLOGICAL APPROACHES

Histological examination of the eyes has been included routinely in subchronic and chronic toxicity studies, but because it is time-consuming and costly, it is performed only occasionally in eye irritation studies. Results may be no more informative than those from observations and measurements by other techniques. However, histological examination of ocular tissue can reveal the type of damage, tissues involved, and certain subtle changes in ocular tissue.

Both electron and light microscopic examinations have been used to evaluate local ocular injury (171, 197, 198, 308, 350, 354, 367, see Henkes and Canta [1973] (180) for review). Although such methods sometimes can reveal morphological changes of different parts of the cornea, conjunctiva, lens, and retina, as well as visual nerve degeneration, shortcomings are not uncommon. Among the problems are sectioning the precise lesion, problems in slide preparation, and subjective interpretation of observations. Another problem is that histological examination generally is made on dehydrated tissue (65), which makes some lesions, such as corneal edema, difficult to detect. However, histological examination of ocular tissues in local eye irritation studies has been considered an objective method because of its high sensitivity in detecting very mild ocular effects (181).

ALTERNATIVE METHODS FOR THE ASSESSMENT OF EYE IRRITATION

Humane and scientific concerns regarding the use of animals in toxicology have prompted development of many in vitro methods as potential alternatives for animal tests. One area that has seen considerable activity is the development of new methods for use as alternatives in the assessment of eye irritation. The use of firstgeneration screening tests (26, 30, 42, 46, 106–108, 127, 184, 205, 206, 248, 258, 261, 300, 317, 331, 344, 364) has laid the foundation for continuing research with more sophisticated methods, many of which use human ocular cells as their basis (31, 32, 130, 149, 150, 156, 191, 199, 209, 219, 222, 268, 295, 310, 312, 336, 348, 356, 357, 368, 381). Although the majority of procedures that use human cells are employed in studies of ocular disease, some are finding applications in eye irritation hazard assessment. For a review of this research, see Kruszewski (214).

Organizations such as the Johns Hopkins Center for Alternatives to Animal Testing, the European Center for the Validation of Alternative Methods (ECVAM), the Fund for Replacement of Animals in Medical Research in England (FRAME), and the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) have been active in promoting development of such alternative methods for animal testing.

Research on alternatives for assessing ocular irritancy has focused on development and validation of a number of in vitro tests. These methods can be grouped into functional categories according to test endpoint (146), which includes morphology, cell toxicity, cell and tissue physiology, inflammation/immunity, recovery/repair, and others (e.g., protein denaturation, computer-based structure-activity relationships, *Tetrahymena thermophila* motility, etc.). Most of these endpoints are indicators of cellular membrane integrity and/or cytotoxicity. Detailed reviews of these methods are beyond the scope of this chapter but are available (60, 306).

For the following discussion, the above classes of methods are grouped into four broad categories: ex vivo methods, cell-based assays, inflammation/immunity, and other methods.

Ex Vivo Methods

Several methods have been investigated which are based on morphological alterations to either whole eye tissue or isolated corneas. The enucleated perfused rabbit eye, enucleated chicken eye test, or isolated bovine or porcine cornea methods (17, 67, 117, 186–190, 237, 259, 260, 296, 382), are based on visible alterations of tissues as the endpoints. The effect of a chemical on the tissue is measured by corneal opacity, corneal thickness, or corneal fluorescein retention. These methods are complex, lack good correlation with in vivo results, and still require killing animals; however, eye tissue can be obtained from slaughterhouses, from animals processed for food (299).

The bovine corneal opacity and permeability (BCOP) test, developed by Gautheron et al. (154), is a more recent modification of the original isolated bovine procedure (260). In the bovine corneal opacity method, freshly excised bovine eyes obtained from a slaughterhouse are used. The test material is placed on the cornea for 30 sec, after which corneal lesions are scored. In the BCOP test two endpoints are determined:

(1) opacity, which is measured by change in light transmission passing through the cornea; and

(2) permeability to fluorescein dye, a measure of barrier function. This method is an improvement over the earlier procedures and has been used with a variety of chemicals and consumer product formulations to produce results that compare favorably when extrapolated to corneal effects in the rabbit eye test (69, 83, 155, 159, 302, 335, 358, 362). An expert working group has reported on the state of the art of the BCOP test (335).

Cell-Based Assays

Cytotoxicity Methods

These methods are based on cytotoxicity. Frazier et al. (146) grouped these methods under three categories according to the indicator of cytotoxicity. These are adhesion/cell proliferation (cellular growth), membrane integrity, and cell metabolism. Chemically induced cytotoxicity will interfere with cell growth and proliferation, which can be quantified by counting the number of viable cells, by assessing the adhesion or colony-forming ability of the cells, or by measuring the amount of macromolecules such as DNA, RNA, or protein. The endpoints investigated under the category of adhesion/cell proliferation include growth inhibition (BHK cells), colony-formation efficiency (BHK cells and SIRC cells), cell detachment (BHK cells), total protein (Balb/c 3T3 cells, BCL DI cells) (20, 270, 305, 330, 342, 370), or binding of certain dyes (the FRAME Kenacid Blue Test in 3T3-L1 cells) (79, 211), and a plant cell-derived pollen tube test (201). The cell lines generally are well characterized; some are corneal cell lines. Several of these procedures are discussed below.

Adhesion and proliferation. The *colony-formation efficacy test* involves establishing good viability and growth of cells, exposing BHK or SIRC cells to a test substance for a predetermined period of time (e.g., 1 h), washing the exposed cells, and reincubating for the time needed for cellular proliferation. After reincubation, the effect of the test substance on the ability of the cells to adhere and/or form colonies is determined by fixing and staining the cells. The number of functionally viable cells (i.e., with the ability to adhere and proliferate) in the chemically exposed group is compared with an unexposed control group. Ranking of ocular irritancy often is based on the IC50, the concentration of chemical at which functionally viable cells are only 50% of the unexposed control group. The correlation in ranking of irritancy of various surfactant and cosmetic formulations has been reported between this test and the in vivo rabbit eye irritancy test (39, 40, 158, 161, 271, 325).

The FRAME *Kenacid Blue (KB) Test* is based on the principle of a direct correlation between cell number, total protein, and the binding of certain dyes such as Kenacid Blue (Coomassie brilliant blue) (18, 79, 211, 330). Proliferating 3T3-L1 cells are exposed to a low concentration of chemical for 72 h (at least two cell cycles). Then exposed and unexposed cells are removed from the growth medium and dried in the air for 72 h, and the amount of protein in the cells is measured by the KB dye-binding assay. The method involves treating cells with a fixative (glacial acetic acid/ethanol/distilled water 1:50:49), then

staining with freshly prepared Kenacid Blue R solution, washing

the cells, and releasing the dye into a desorbing solution (approximately 10% potassium acetate in water/ethanol solution). The absorbency of the dye is measured at 570 nm against a cell-free desorbing solution. Absorbency correlates linearly with the amount of pro tein and the number of cells. This method has been used in projects sponsored by Cosmetic Toiletries and Fragrance Association (CTFA) and the Commission of European Communities (CEC). Correlation between in vitro ranking and in vivo Draize test ranking of salts, chlorides, and certain cosmetic formulations has been reported (9, 18, 158, 161).

The *pollen tube test* employs plant cells (201). It is essentially a cell proliferation assay. The endpoint is the amount of tube wall biomass *(Nicotiana sylvestris)* produced (through germination and growth) in vitro. After exposure to the test substance, the amount of biomass is determined by measuring optical density at 500 nm. Results are expressed as the concentration of test substance, which causes a 50% growth inhibition. Correlations between in vitro ranking and the in vivo Draize test ranking for certain cosmetic formulations and surfactant chemicals have been reported (158, 159, 161, 213).

Membrane integrity. Damaged membranes can cause a host of toxic responses including cell lysis and cell death, leakage of enzymes such as LDH, loss of certain cofactors such as Ca^{2+}, K^+, and NADPH, and alterations in substances being actively or passively transported across the membrane. Endpoints include dual-dye/fluorescent dye staining (LS cells, thymocytes), chromium isotope (^{51}Cr) release (RCE, SIRC, P815, YAC-1 cells), cell viability (L929 cells), hemolysis (bovine red blood cells), and transepithelial barrier integrity (MDCK or HCE-T cells) (100, 199, 206, 261, 317, 324, 325, 368). Alteration in the ability of substances being actively or passively transported across the membrane barrier (release or uptake) indicates membrane toxicity.

The *chromium isotope* (^{51}Cr) *release assay* has been used in several studies (39, 40, 161, 324, 325). In the presence of excess Cr in the growth medium, normal cells take up Cr, which binds to intracellular macromolecules. After changing to isotope-free growth medium, Cr-labeled macromolecules are transported actively across the membrane of normal cells into the extracellular medium at a slow rate. In cells exposed to cytotoxic agents (and if membrane integrity is altered), Cr-labeled macromolecules will diffuse passively across the membrane into the extracellular medium at a much higher rate. The increased release rate (difference between release rate of normal and chemically treated cells) of Cr-labeled macromolecules indicates membrane toxicity. Correlations have been reported between in vitro ranking and in vivo Draize test ranking for several surfactant (39, 40, 146) and certain cosmetic formulations (161).

The *Neutral Red Release (NRR) Test* also measures the cellular membrane integrity (303). It is based on the principle that living cells accumulate the dye (2-amino3-methyl-7-dimethyl aminophenazonium chloride) in lysosomes by an active metabolic process. If the membranes are damaged, the accumulated intracellular neutral red dye will be released into the extracellular medium. In this method, confluent 3T3-L1 cells are preloaded with neutral red dye, washed, replaced with fresh growth medium, and exposed to a high dose of the toxic substance for 1 min. The test substance is removed; the exposed and unexposed cells are washed and fixed with PBS, destained, and fixed with a glacial acetic acid/ethanol/distilled water (1:50:49) solution. The absorbance of the destained solution is measured at 540 nm against a cell-free destain reference solution. Results are expressed as NRR_{20}, NRR_{50}, or NRR_{80} (i.e., concentration of test substance, that causes 20%, 50%, or, 80% of the preloaded dye to be released). Correlations between in vitro ranking of certain classes of chemicals and certain cosmetic formulations and the in vivo Draize test ranking have been reported (158, 159, 161, 303).

A neutral red release method to test solid materials has been reported (365). The *agarose diffusion assay* uses a confluent monolayer of culture cells preloaded with the neutral red dye. The cells are overlaid with agarose. Test material placed on the agarose can diffuse through the agarose to the cells. Any damage caused by the test material to the cellular membrane will release the dye into the agarose. Scoring is based on the presence of a dye zone. (The size of the zone may or may not correlate with the degree of cytotoxicity). Correlations between in vitro ranking of certain cosmetic formulations or ingredients and the in vivo Draize test ranking have been reported (158, 159, 161, 365).

Membrane tight junctions between epithelial cells form a barrier for water movement across the corneal epithelium. When this barrier is damaged, water moves into the cornea, resulting in edema. The FRAME *fluorescein leakage (FL) test* is based on the principle that a damaged epithelial cell barrier will be more permeable to the nontoxic dye fluorescein than an undamaged barrier (327, 351). Canine kidney cells (MDCK cells, an epithelial cell line) are grown to confluence in tissue culture inserts. The confluent cell layer is washed, exposed to 100 μl of test substance for 1 min, and then the test substance is removed by washing with distilled water. The washed cells are placed in clean wells containing PBS medium.

Fluorescein (500 μl) in PBS (0.02%) is added to each cell insert. Cells are incubated for 1 h, and the leakage of fluorescein from the cells into the well is measured by absorbence at 492 nm. Results are expressed as FL20 and FL50 (concentration of test substance, which causes 20% and 50% of maximum leakage from inserts containing no cells) (327). A modification

of this method expresses results as FL10 and FL20 (concentration of test substance which causes 10% and 20% of maximum leakage) and measures recovery at 4, 24, 48, and 72 h after treatment (84). Comparison of in vitro response to the in vivo irritancy classification of various chemicals has been reported (84, 159, 327).

The *transepithelial permeability (TEP) and the transepithelial resistance (TER) assays* are barrier function tests based in the human corneal epithelial cell line, 10.014 pRSV-T (HCE-T cells) (199, 368, 369). This cell line has been used to develop a three-dimensional in vitro model of the human corneal epithelium (HCE-T model). HCE-T cells form a stratified culture when grown in serum-free medium on a collagen membrane at the air-liquid interface. Because it is a multilayer model, barrier function is a well-developed property in the HCE-T model which can be quantitatively measured by its retention of fluorescein (TEP assay) and the maintenance of high electrical resistance (TER assay). For these procedures, HCE-T cells are grown in 24 well plates to confluence (3–4 days) in low-calcium (0.15 mM CaCl2) keratinocyte growth medium (KGM), then airlifted in high-calcium (1.15 mM CaCl2) KGM until Day 7, when they are treated. Treatment consists of 100 μl of test substance in KGM for 5 min and then the test substance is removed by washing three times with D-PBS. The washed cells are placed in clean 24-well plates containing high-calcium KGM.

For the *TEP assay,* fluorescein (200 μl) in high-calcium KGM (0.02%) is added to each well. Cells are incubated for 30 min, and the leakage of fluorescein into the well is measured by absorbance at 490 nm. Results are expressed as FR85 (concentration of test substance, which causes fluorescein retention to decrease to 85% of control) (215).

For the TER assay, resistance measurements are taken using an epithelial voltohmeter, EVOM (World Precision Instruments) after treatment of the cells as per the TEP assay. The instrument probe is inserted into the culture insert and a reading is recorded after a 20-sec instrument stabilization period. Resistance is calculated based on an area of 0.64 cm2, which is the area of the culture insert. Results are expressed as R50 (concentration of test substance that causes the electrical resistance to decrease to 50% of control) (215). Correlations between these tests and the in vivo Draize test for various chemicals and certain cosmetic formulations have been reported (215).

Cellular metabolism. Even in viable cells, toxic substances can cause subtle metabolic changes in such cellular endpoints as plasminogen activator activity, ATP production, lysosomal activity, basic metabolic rate (measured as changes in acidic by-products), and amount of light produced by luminescent bacteria (43, 44, 64, 72, 204, 303, 328, 331). These changes can be quantified by techniques ranging from simple neutral red dye or 3-(4,5-dimethylthiazol-2-yl)-2,5-biphenyltetrazolium bromide (tetrazolium MTT) uptake into lysosomes (41, 43, 44, 200), silicon microphysiometer sensor for detecting changes in pH caused by acidic metabolic by-products (177), or output of light by luminescent bacteria (64).

The *neutral red uptake (NRU)* test is based on the principle that living cells absorb the dye and accumulate it in the lysosomes. Cytotoxic agents can disrupt the metabolic energy required for dye accumulation and thus decrease dye uptake. Such a change can be quantified by a simple method. A culture cell line (BALB/c3T3 mouse fibroblasts) is grown and exposed to various concentrations of the test substance for 24 h. After exposure, the medium is removed, and cells are washed with fresh growth medium. The neutral red dye is added and the cells are incubated for 3 h at 37°C. The medium is removed and the cells are washed and fixed with a formaldehyde-based fixative. The dye is extracted into an acetic acid/ethanol solution and the concentration of dye is measured spectrophotometrically at 540 nm. Results are expressed as either NR50, the concentration of test substance, that inhibits dye update by 50% compared to untreated cells, or HTD, the highest tolerated dose (41, 43, 44, 200). Correlations between in vitro ranking of several surfactant and certain cosmetic formulations and the in vivo Draize test ranking have been reported (39, 40, 120, 158, 159, 161).

The *MTT assay* measures the metabolic activity of mitochondria. It is based on the principle that only cells with respiring mitochondria can reduce significant amounts of MTT, a soluble, pale yellow dye, to the insoluble dark blue metabolite, MTT formazan (41). After incubating metabolically active cells (BALB/c3T3 mouse fibroblasts) with various concentrations of the test material for 24 h, cells are incubated with MTT for 3 h at 37°C in 96-well tissue culture plates. MTT formazan is solubilized in isopropanol and quantified by colorimetry. The concentration of MTT formazan as measured at 550 nm is related to the number of viable cells. Results are expressed as percentage difference from untreated cells. Correlations between in vitro ranking of certain cosmetic formulations and the in vivo Draize test ranking have been reported (158, 159, 161). MTT measurement in human epidermal keratinocytes after treatment with various chemicals, or personal care and cosmetic products has also been correlated to Draize test results (3, 128, 159, 301, 302).

The *microphysiometer* measures changes in the cellular metabolic rate. The changes are detected as small changes in the physical properties of the growth medium as a result of accumulation of acidic metabolic by-products (57, 177, 287). The results are expressed as concentration of test material, which causes a 50% drop in metabolic rate. Preliminary results are encouraging but limited to

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acidic metabolic by-products. Correlation between this test and the in vivo Draize test for various surfactants, household cleaning products, and cosmetic formulations has been reported (4, 57, 58, 70, 159).

The *luminescent bacteria test (LBT)* is based on the principle that light emission occurs in certain strains of bacteria during metabolic activity. Thus, the amount of light produced by the bacteria reflects its metabolic rate. Cytotoxic agents which decrease the metabolic rate of the bacteria also will decrease light production. Light output from the luminescent bacteria *Phytobacterium phosphoreum* is measured after incubation of the bacteria with various concentrations of the test material. Results are expressed as the concentration of the test material that causes a 50% reduction in light output. Correlation between this test and the in vivo Draize test for a few chemicals and certain cosmetic and toiletry formulations has been reported (64, 91, 159, 161).

Cellular morphology. The *Balb/c3T3 cell/morphological assay* (43–45, 329) is a simple method that measures morphological changes in cultured cells. In this assay, chemicals are ranked on the basis of the highest tolerated dose (concentration) at which no morphological change associated with exposure to the chemical is observed by phase contrast microscopy.

Other cytotoxicity tests. Cell or tissue physiology also has been used as an endpoint for screening ocular irritants. Examples include electrical conductivity of epidermal tissue slices (284) and inhibition of contraction in rabbit ileum (258). In general, these tests have not been as extensively studied as the other methods because they all require killing animals. Some investigators have reported correlation with in vivo eye irritation tests for a few materials.

Inflammation/Immunity

Because ocular irritation is an inflammation process, inflammation/immunity has been evaluated as an endpoint for alternative irritancy tests (146). These tests include morphological observations of inflammatory/morphological changes in the chorioallantoic membrane (CAM) of developing chick embryos (212, 218, 220, 221, 297), in vitro macrophage dynamics (chemotaxis method using Balb/c3T3 cells and leukocyte chernotactic factors using the bovine corneal cup model) (344), and quantitative measurement of release of inflammation mediators such as histamine (rat peritoneal mast cells and bovine eye cup), serotonin (rat peritoneal mast cells) (76, 193), prostaglandins (rat vaginal tissue) (103, 104), and leukotriene C4 (bovine eye cup) (118).

Chorioallantoic Membrane

In these methods, the endpoints are the subjective evaluation of vascular changes (hemorrhage or obstruction) and necrosis of the CAM, the vascularized, respiratory membrane just underneath the egg shell. This membrane was suggested to be equivalent to the conjunctiva of the eye (220). It enjoys the advantage of a viable membrane with the vasculature needed for an inflammatory response. However, recent studies have shown that responses to irritants in the CAM methods are morphological, rather than inflammatory, alternations. There are three variations of the CAM method: the CAM, the CAM vascular assay (CAMVA), and the hen's egg test-CAM (HET-CAM) method. Using a method similar to Leighton et al. (220) for acids, alkali, and surfactants, Parish (288) reported a correlation between CAM and Draize results. Correlation between this test and the in vivo Draize test for various chemicals, surfactants, household cleaning products, cosmetic formulations, and pesticides has been reported (3–7, 39, 40, 47, 61, 158, 159, 161, 163). However, these methods require living embryos and results in ranking irritants have been mixed due to false positives probably attributable to embryo toxicity rather than local irritancy. The HET-CAM test has also been used in combination with the BCOP test (39, 40, 57, 361, 376). Irritancy is ranked according to the most severe response from the two tests. Correlation between this test and the in vivo Draize test for various surfactants, household cleaning products, and cosmetic formulations has been reported (39, 40, 57). A modification of the HET-CAM test (HET-CAM-TSA), which uses microscopic evaluation and a special test substance applicator, has also been reported (164).

Macrophage Chemotaxis

The underlying principle of this method is that leukocytes migrate to the site of inflammation. Cultured Balb/c3T3 cells are treated with potential irritants for different periods. The treated cells are washed and re-fed with fresh medium. The chemotactic factors in the re-fed medium are detected by placing the medium in the bottom wells of a microchemotaxis chamber, covering the wells with a polycarbonate membrane (5 μm pore size), and adding about 105 mouse peritoneal macrophages in the upper wells. The system is incubated for 4 h at 37°C. After staining, the number of macrophages that migrated through the membrane are counted. This method has not been routinely used and no information is available regarding extensive correlation to the in vivo Draize test.

Other Alternative Methods

Other approaches include the *Tetrahymena* motility assay (332), protein denaturation (168, 169), ocular wound healing test (197, 333), and quantitative structure-activity relationship analysis (119).

Tetrahymena Motility Assay

The motility assay evaluates the effect of the test material on the characteristic swimming pattern of the fresh water protozoa *Tetrahymena thermophila.* Motility is examined under a microscope following exposure to the test material for 2 min at 21°C. Results are expressed as the highest tolerated dose, the concentration or dilution of the test material, that is characterized by 90% normal cell motility (332). Correlations between this test and the in vivo Draize test for various chemicals, household cleaning products, and cosmetic formulations have been reported (3, 39, 40, 158, 159, 161, 332).

Protein Denaturation Test

Protein denaturation is the basis of the commercially available Irritection (Eytex) system. The test is based on the principle that opacity (precipitation of the specially organized protein in the cornea) is the major contribution to ocular irritancy in the Draize test. The degree of opacity and irritancy can be predicted by assessing the degree of aggregation of a synthetic (nonanimal) aqueous protein matrix that mimics the protein matrix of the cornea (168, 169). The Irretection (Eytex) assay tests solutions and insoluble, immiscible, or opaque materials by using a special semi-permeable membrane bullet over the responding protein matrix. Correlation between Eytex results and the in vivo rabbit eye test for various chemicals and cosmetic formulations has been reported (92, 97, 158, 159, 161, 229, 314).

Ocular Wound Healing Test

Although reversibility is a key criterion in the Draize test, only a few in vitro studies have evaluated healing and repair of ocular lesions (197, 333). In such studies cultured rabbit corneal cells are grown to multilayer confluency. A wound is created using a liquid nitrogen chilled probe and the test substance is added to the wounded cells. After 24 h of incubation, the cells are fixed and healing assessed by measuring the size (using computerized planimetry) of the remaining wound area not covered by cells as compared to the initial wound size (333). Although these tests may give information on cell migration and proliferation at the wound, a process needed for initial wound closure, the multilayer cell culture is not the same as an intact corneal epithelium with a basement membrane. This method has not been routinely used and no information is available regarding extensive correlation to the in vivo Draize test.

Quantitative Structure-Activity Relationship (QSAR)

Quantitative structure-activity relationship (QSAR) analysis, widely used to predict various physiological and biochemical activities of novel chemicals, also has been used to predict eye irritancy of structurally related chemicals. Using QSAR, Sugai et al. (345) examined the eye irritancy (opacity and conjunctivitis) of 131 chemically heterogeneous chemicals. The accuracy was 86.3% for classifying irritancy of the chemicals. In another study, overall accuracy of 91% was reported (119). More recent research involving QSAR analysis for eye irritancy has been reported (21, 22, 86, 87). Although this approach may provide useful information on structurally related chemicals, its current utility for formulated products is questionable. The same limitations that impact the use of QSAR for acute toxicity applications are also applicable to acute ophthalmic irritation (See Alternative Methods for LD50 Test, Quantitative Structure-Activity Relationship [QSAR] Analysis).

Evaluation and Validation Programs

Since eye irritation is a process involving multiple mechanisms (347), it is not surprising that development of alternative in vitro tests for eye irritancy is an extremely complex issue. Although the current thrust in method development and validation is encouraging, many issues remain. The Johns Hopkins Center for Alternatives to Animal Testing (CAAT)/European Research Group for Alternatives in Toxicity Testing (ERGATT) Workshop on Validation of Toxicity Testing Procedures recognized the need for mechanistic similarity between an in vitro assay and the in vivo animal model (14). For screening purposes, mechanistic similarity may not be necessary if the empirical correlation/predictability criterion is met. However, mechanistic similarity is generally required if the alternative method is to be a replacement test.

Important issues must be addressed before alternative ocular irritancy test methods can replace current in vivo animal tests. For example, insolubility of certain chemicals remains an issue. Testing extracts may partially resolve this problem. Many in vitro assays are not suitable for testing materials that change the physiological pH of the test system. A major problem is the lack of correlation between in vitro and in vivo data. Correlation appears to be good only within certain classes of chemicals. Predictability is further confounded when testing complex mixtures and total product formulations. A battery of assays could possibly minimize these issues. Most in vitro techniques do not establish reversibility, which is extremely important in classification of eye irritants (123, 202). These and other issues are apparent when reviewing the results of recent multi-laboratory evaluation/validation studies with alternative methods for ocular irritancy.

In the United States, a number of programs have been launched to evaluate alternative tests. Two such programs organized by the Soap and Detergent Associ

ation (SDA) and the Cosmetic Toiletry and Fragrance Association (CTFA) have reported on their findings (3, 39, 40, 158, 159, 161). The CTFA program was designed to evaluate the performance (correlation with Draize test results) of a set of in vitro tests on materials used in the cosmetic and toiletry industry.

In Phase I of the CTFA program, 25 alternative tests (representing 12 different endpoints) were used to test 10 different hydroalcoholic cosmetic and toiletry products (161). Results indicated that 6 of the 25 assays demonstrated the least disagreement with the Draize test for these products. Predictability generally was better for materials with low, rather than high, irritancy potential.

In CTFA Phase II, 30 alternative tests (representing 14 different endpoints) were used to test 18 different oil/water emulsion cosmetic and toiletry products (158). Results indicated that 16 of the 30 assays demonstrated the least disagreement with the Draize test for these products. As in Phase 1, predictability generally was better for materials with low, rather than high, irritancy potential.

In CTFA Phase III, 23 alternative tests (representing 41 different endpoints) were used to test 25 different surfactant-containing cosmetic and toiletry products (159). Results indicated that no single in vitro endpoint exhibited relative superiority with regard to prediction of the Draize test results for these products. Overall, predictability of the in vitro assays was better in Phase I (hydroalcoholic products) than in Phase II (oil/water emulsion products) or in Phase III (surfactant-containing products).

In Phase I of the SDA program, 14 alternative tests (mostly cytotoxicity) were used to test 8 household cleaning product ingredients or formulations (39). Six assays were selected as having the best agreement with Draize test results.

In SDA Phase II, 9 in vitro assays were used to test 23 household cleaning product ingredients or formulations, 8 of which were tested in Phase I (40). Phases I and II results indicated that the combination of product alkalinity and in vitro data provide useful information on the ocular irritancy potential of alkaline and acidic household cleaning product ingredients or formulations.

In SDA Phase III, 9 in vitro assays were used to test 22 household cleaning product ingredients or formulations (3). When only nonalkaline materials were considered, the correlation coefficients of the 9 methods were not significantly different from one another, although none of the tests could accurately predict the relative irritation potential of the irritant materials. Various evaluation and validation programs also have been conducted in Europe and Japan, all with mixed results (17, 48, 155, 282, 341).

A formal validation study on the HET-CAM and NRU cytotoxicity test was conducted in Germany to determine whether these tests were capable of identifying chemicals that are severe eye irritants (341). The study was carried out in 10 laboratories with 200 test materials under blind conditions. Results suggested that this combination of methods was adequate to identify severe irritants.

An evaluation of the BCOP test was sponsored by the European Directorate General (DGXI) (155). The study included 12 laboratories testing 52 chemicals with a wide range of structure, physical form, and irritancy potential. Results indicated that the BCOP correctly predicted whether a material would be an irritant or nonirritant for 44 of the 52 materials.

A European validation study was coordinated by the European Commission/British Home Office (EC/HO) (17). In this study, 9 Draize test alternatives were evaluated in 37 laboratories using 60 coded chemicals. The goal was to establish whether any of the alternative methods could replace the Draize test for all severely irritating materials, all severely irritating materials belonging to a specific chemical class, or materials with or without regard to chemical class. With the possible exception of predicting the irritancy potential of surfactants, none of the 9 tests met any of the pre-defined performance criteria.

In Phase I of a Japanese Cosmetic Industry Association validation trial, 12 Draize test alternatives in 20 laboratories were used to evaluate 9 cosmetic ingredients (282). Many of the assays handled the surfactant materials adequately.

The European Cosmetics Association (COLIPA) conducted a validation study evaluating 10 alternative methods in 34 laboratories using 55 test substances (23 ingredients, 32 formulations) selected as representative of those commonly used in the cosmetics industry (48). Using the criteria of reliability and relevance as defined in the study, preliminary results indicated that none of the methods could be considered a valid replacement for the Draize test across the full irritation scale, although several satisfied at least one criterion of reliability or relevance.

At present, none of the in vitro techniques appears able to replace eye irritation testing in animals. However, some methods are useful in screening for ocular irritancy potential and can aid in selection of a final product for further development. In fact, many of the tests described above have been adopted as routine screening tests for product development and are well documented in the scientific literature. As efforts continue to develop appropriate alternatives, prudent science necessitates continued use of animals in safety testing. Nonetheless, protocol refinements and reduction in the number of animals

should continue

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where appropriate while validated alternative methods are sought.
Protocol Refinement
Because it is generally agreed that in vitro techniques will not replace animal testing immediately, efforts should be made to reduce the number of animals used and to minimize their pain. The precision of an eye irritancy test is a function of the number of animals used. The question arises as to whether it is justified to use a large number of animals to increase precision. The answer is no, because there is seldom an advantage to testing eye irritancy with more than three to six animals. The largest variable in an eye irritancy test is among animals, and the test itself is designed to be a bioassay. Therefore, to use a large number of animals in hopes of achieving a higher level of precision is neither realistic nor scientifically sound. A statistical analysis of 155 Draize irritancy studies with six-rabbit scores has shown that reducing the number of animals to five-, four-, three-, or two-animal scores retains, respectively, a 98, 96, 94, or 91% agreement with an irritant classification of these chemicals based on the six-rabbit scores (349). The correlation coefficients for randomly selected subsets of five, four, three, or two scores were 0.998, 0.996, 0.992, and 0.984, respectively. The results of this study show that sufficient accuracy can be obtained by reducing the number of animals used in the Draize test. A combination of lower test substance dose volume (one-tenth the Draize test dose volume) and fewer animals (three) also has yielded good correlation with the standard Draize test (59).
Another proposal is to test only for skin irritation. If the material causes severe skin irritation, it is presumed to be severely irritating to the eye as well. Thus, the argument concludes, an eye irritation test is not needed. Extrapolation from skin to eye is not always valid. In at least one study of 60 severe skin irritants, only 39 also caused severe eye irritation; 15 caused mild or no ocular effects, and the other 6 caused moderate eye irritation (378, 379). Nonetheless, this approach has been proposed as one element of a tier system to prevent conducting an eye irritancy test when other potentially relevant information is available (194).
Many company guidelines specify that materials with extremely high or low pH do not need to be tested for eye irritancy. This approach is fully justified, especially for highly basic compounds. Alkali compounds generally cause more severe eye irritation than acidic compounds.
Tier Testing Strategies
A variety of tier testing strategies have been proposed to reduce the number of animals in eye irritation testing (23, 175, 185, 194). These strategies usually begin with a weight-of-evidence approach, in an effort to review existing information that would allow classification and labeling a material as a severe ocular irritant without animal testing, or to conduct testing with a reduced number of animals. An example of this approach is shown in Figure 18.7, a tier testing scheme proposed by the OECD to support the harmonization of eye irritation testing and classification (280). Stages 1–3 involve information on the physicochemical characterization of the test material and use decision points to preclude animal testing such as: if the test material has a high or low pH (<2 or >11.5); is a known corrosive or severe dermal irritant; or if relevant information from structure-activity relationships (SAR) is available. If the weight-of-evidence suggests that the test material is a severe irritant, it should be so labeled. If information suggests that the test material is not a severe eye irritant, conducting an alternative test is the next step. If the results of the alternative test are indicative of a severe response, the material is classified as a severe irritant. If not, then testing in one or two animals is necessary before a final evaluation can be made.
Current Regulatory Status of Eye Irritation Testing
As in acute oral toxicity testing, the current regulatory status of eye irritation testing is in a state of flux. Most international regulatory agencies are attempting both to reduce the number of animals necessary to assess the ocular hazard of a new substance and to harmonize classification and labeling schemes to accommodate some of the more modern testing approaches. For example, the use of the tier testing strategy (280) is a realistic approach that could minimize pain and suffering, reduce the number of animals without significantly compromising the safety of consumers and workers, and be harmonized to fit existing regulatory classification and labeling schemes.
The OECD has taken a lead role in coordinating the effort to harmonize regulatory strategies through its Program on Harmonization of Classification and Labeling Systems. Case studies are being conducted using existing rabbit eye irritation information submitted to various regulatory agencies under chemicals notification procedures. The data are then used to compare the sensitivity of various classification schemes. This ongoing project is attempting to define criteria for various degrees of eye irritation that are in the range of sensitivity of existing classification systems, and to explore ways to subdivide the effects for those systems that require such for labeling purposes (280).

For specific information on regulatory agency requirements for eye irritation testing, the reader is encouraged to check the most recent regulatory guidelines that apply.

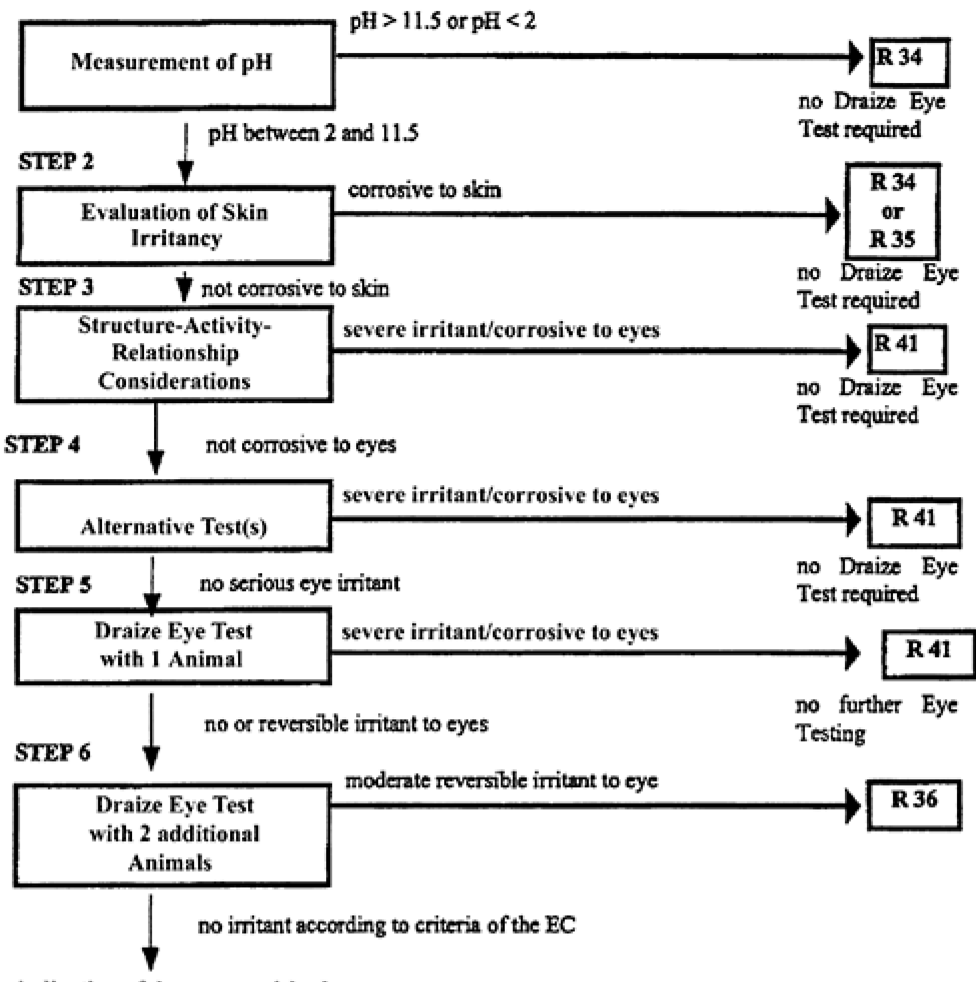

FIG. 18.7. Tier scheme for eye irritation testing. From Reference 280, with permission.

VALIDATION AND REGULATORY ACCEPTANCE OF ALTERNATIVE METHODS

The typical path for the emergence of new methods has historically comprised development and intra-laboratory assessment, peer review and publication, and, ultimately, peer acceptance of the method. Although this process has served us well, continued advancements in basic science, biotechnology, and engineering will undoubtedly result in a plethora of new methods intended as replacements for traditional animal tests such as acute toxicity and eye irritancy. The implementation of new and revised toxicological test methods should occur only if such methods have the capability to provide improved assessment of the potential toxic effects of chemicals and other agents on human and animal health and the environment. One way to assure that new methods represent an improvement over existing tests is through the process of formal validation followed by regulatory acceptance.

Validation has been described as *the process whereby the reliability and relevance of a procedure are established for a particular purpose* (15, 141). In this context, validation encompasses a number of inherent criteria and features that are necessary preliminaries to regulatory acceptance of an alternative method. In contrast

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Table 18.12 Criteria for a valid test

Necessary criteria	OECD Required	ECVAM Required	ICCVAM Required
Scientific and regulatory rationale including clear statement of proposed use	X	X	X
Relationship of test endpoint to biological effect of interest	X	X	X
Detailed protocol describing: materials needed; what is measured and how; acceptable performance criteria; how data will be analyzed; species to which results apply; classes of materials that can and cannot be assessed	X	X	X
Description of variability and reproducibility (also how biological variability may impact test results)	X	X	X
Demonstrated performance using coded reference chemicals; should include positives and negatives	X	X	X
Sufficient toxicity data to compare existing test with new test (data should be of acceptable quality and from relevant species)	X	X	X
Description of limitations	X		X
Data obtained using good laboratory practices (describe if not and indicate potential impact)	X	X	X
Supporting data available for review: protocols in public domain; methods and results published in peer-review journals; independent scientific review of methods and results	X	X	X

to the traditional path for methods development, the new paradigm supplements development, publication, and peer acceptance with several additional elements. These include pre-validation (optimization and protocol transfer), formal validation conducted in several laboratories (a blind trial), an independent review of the results, submission of the method and validation results to regulatory authorities, and regulatory review and approval.

Development of new and revised test methods to replace existing animal toxicology and safety tests has resulted in the formulation of a number of specific guidelines to be used when preparing a new method for validation and regulatory acceptance (11–13, 15, 56, 134, 141, 142–145, 166, 167). There has been recent movement by the OECD, ECVAM, and ICCVAM to formalize these guidelines and to put them into a harmonized, international regulatory context (16, 269, 274). A discussion of the guidelines follows.

Criteria for a Valid Test

Compliance with comprehensive criteria designed to assure that alternative test methods will meet the standards established for a proper validation study is the first and, unquestionably, the most important step in gaining regulatory acceptance for a new test. In recognizing the importance of such criteria, the OECD, ECVAM, and ICCVAM have each published a set of validation guidelines that are fairly consistent among the groups. A valid test can be defined as including a number of specific elements as summarized in Table 18.12.

For the most part, the criteria for a valid test simply represent good science. They require an appropriate rationale for the new method and an explanation as to how the test endpoint is related to the effect of interest, and they make sure that the methodology is adequately described in a protocol that includes information on the applicability and limitations of the test. Performance criteria must be established to allow an assessment of the inherent variability of the test and its performance in evaluating the chemical class of interest. Relevant in vivo data of sufficient quantity and quality must also be available to permit a comparison of the existing method to the new test. All data generation, collection, and reporting associated with the new test should be performed with adherence to GLP. Scientific credibility can be ensured through peer review of the test methodology, testing results, and subsequent published reports that place details of the new test and the attendant validation activities in the public domain.

If proper attention is given to these criteria and to their utilization in validation programs, appropriate recognition of new test methods by the scientific community will be facilitated. Having accomplished that, the poten

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FIG. 18.8. ICCVAM validation process. From Reference 269, with permission.

VALIDATION PROCESS

I. Test Development
II. Prevalidation/Test Optimization
   A. Preliminary planning
      1. Define basis and purpose of test
      2. Develop protocol
      3. Develop control values
      4. Develop data/outcome prediction model
   B. Activities
      1. Qualify and train laboratories
      2. Measure intra- and inter-laboratory reproducibility
      3. Identify limitations of test
III. Determine Readiness for Validation.
   A. Analyze test development and prevalidation data
   B. Standardize protocol
IV. Test Validation
   A. Form steering committee/management team
      1. Define purpose of validation study
      2. Design study
      3. Select participating laboratories
      4. Establish management evaluation and oversight procedures
   B. Pretest Procedures
      1. Implement data record keeping procedures
      2. Select reference chemicals
      3. Code and distribute reference chemicals
   C. Test Coded Chemicals
      1. Measure inter-laboratory performance
      2. Compile and evaluate data
   D. Evaluate Test
      1. Analyze and summarize test results
      2. Challenge data with prediction model
      3. Peer review of protocol and data
      4. Accept, revise, or reject model
V. Submission of Test for Regulatory Approval
   A. Prepare report
   B. Make supporting data available
   C. Prepare results for publication

tial for assuring international regulatory acceptance as later described will be greatly enhanced.

Validation Process

The OECD and ICCVAM have also made recommendations on what fundamental elements should be contained within the validation process. Figure 18.8 summarizes the validation process as described by ICCVAM.

The validation process starts with the development of a new test method for a relevant biological endpoint and proceeds through a step called prevalidation. The role of prevalidation in the development, validation, and acceptance of alternative methods has been described by Curren et al. (88). It is during the prevalidation phase that information is developed that defines the basis and purpose of the test, establishes its capabilities, limitations, and transferability between laboratories, and provides sufficient data to confirm whether the method correctly predicts the toxic endpoint of interest.

The tool used to predict the toxic endpoint of interest is called the prediction model. The requirements for an adequate prediction model have been described by Bruner et al. (55, 56) as containing:

1. A definition of the specific purpose for which the alternative method is to be used.
2. A definition of all the possible results that may be obtained from the alternative method.
3. An algorithm that defines how to convert results from the alternative method into a prediction of the in vivo toxicity.
4. An indication of the accuracy and precision of the in vivo toxicity endpoint obtained from the model.

Essentially, a prediction model converts the results from an alternative method into a prediction of in

vivo toxicity. For an alternative method to be considered reliable and relevant, it is important that the prediction model has high predictive power and is relatively insensitive to sources of variability. If these criteria are not met, the alternative method should not be pursued through the validation process because it will have little or no value to the toxicologist in making safety assessment decisions. Once the reliability and relevance of the new test method have been established, and the appropriate prediction model developed, a standardized protocol can be prepared and the test validation study initiated. For additional information on prediction models see Archer et al. (1).

The success of any validation study can be directly linked to the incorporation of several key elements into the study design. A number of these have been listed by Balls et al. (19) including:

1. A clear and unequivocal statement of what the validation study is designed to accomplish.
2. A well-defined plan for the study.
3. A sufficiently large set of test substances covering the relevant chemical classes, and the range of toxic endpoints to be evaluated.
4. Comparative in vivo data of high quality on all test substances to be used.
5. A clear description of how the alternative method can be used to predict an in vivo endpoint (the prediction model).

6. Agreed statistical procedures for testing whether the method can predict the in vivo endpoint of interest.
7. Agreed criteria to be met in order to show that an alternative method could successfully and safely replace the animal test.

Perhaps the most important of the listed elements are those which address sufficiency of test samples, quality of the in vivo data, and adequacy of the prediction model and subsequent statistical analysis of the resultant data. Without appropriate attention to these critical elements, a validation study can be doomed to failure.

Once a validation study has been completed, the results are put into the form of a report with supporting data and submitted for regulatory approval. Test method submissions must contain sufficient information for an independent peer-review panel to determine the validation status of the method, that is, its reproducibility, relevance, and limitations, and for agencies to assess the acceptability of the method for providing useful information for hazard or risk assessment.

Criteria for Regulatory Acceptance

Completion of a validation study does not automatically imply that a new test method is ready for regulatory acceptance. It is extremely important that the test fit into an existent regulatory structure and that it has a suitable regulatory rationale. The best way to ensure this is to involve regulatory agencies as early as possible in the design of any validation program. The importance of early liaison with regulators cannot be overemphasized.

The OECD and ICCVAM also provide guidance on the criteria and considerations for regulatory acceptance (Table 18.13). Once again, peer review is considered a significant element, along with adequately detailed information on the test method protocol and supporting documentation.

Demonstrating linkage between the new test and the existing method is critical, and must be put into the context of specific regulatory programs and agency authorities. Technical transfer of the new test method should be relatively straightforward and at a reasonable cost, and the test should be amenable to regulatory harmonization and international acceptance. The new test also should provide some advantage in the reduction, refinement, or replacement of animal usage.

Because regulatory acceptance is an integral part of the successful implementation of new test method development, all reasonable efforts should be made to accommodate dialogue with the appropriate regulatory authorities and to take whatever measures are necessary to ensure that new methods can be harmonized into current regulatory testing strategies.

Table 18.13 Criteria for regulatory acceptance

Necessary criteria	OECD Required	ICCVAM Required
Independent scientific review (by experts with knowledge of method and no financial interest)		X
Detailed protocol with SOPs and criteria to judge performance and results		X
Data adequately measures desired endpoint; links new test to existing test or effects in target species	X	X
Data adequately represents products or chemicals of interest to agency or regulatory program	X	X
Method generates data useful for risk assessment purposes	X	X
Data is as useful (or better) than that from existing methods	X	
Method is robust and able to transfer between properly staffed and equipped labs	X	X
Method is time- and cost-effective	X	X
Method can be harmonized with requirements of other agencies or international groups		X
Method is suitable for international acceptance		X
Method provides consideration for reduction, refinement, and replacement of animal use	X	X

Regulatory Approaches to Alternatives Acceptance

International health regulatory agencies often have unique responsibilities in their mission to protect human and animal health and the environment. Because an agency usually carries out its responsibility through activities that are specific to the nature of its regulatory authority, the information requirements for data to make hazard or risk assessments are primarily driven by regulatory mandate, and many times directly linked to the regulated entity, that is, industrial chemicals, pesticides, biologics, human or

veterinary drugs, cosmetics, consumer products, or chemicals in transport.

Most U.S. agencies have unique regulatory considerations that often result in different approaches to the way in which they request and use information to make hazard or risk assessments. These unique considerations will ultimately have an effect on the criteria used by an agency to approve new test methods. Moreover, other

international organizations that require toxicological testing have their own special considerations regarding test method acceptance and use. Because of these differences, cooperation among all health regulatory agencies is a critical link to the development and acceptance of new alternative methods. There is no agreed upon international process for health regulatory agencies to coordinate the review and acceptance of new alternative test methods. Further complicating the issue, in certain OECD member countries there is neither an understanding nor even a legal obligation that once an alternative test has been considered as sufficiently validated, it must be adopted for use.

ICCVAM recognizes that regulatory agency harmonization of approaches to test method acceptance is a critical aspect of the entire alternatives development process, and includes recommendations to enhance and facilitate the regulatory acceptance and use of new methods in its guidelines (269). Recommendations are provided in five areas: development and validation, regulatory review of new methods, intra- and interagency coordination and harmonization, communication, and international harmonization. The significant aspects of these recommendations are summarized below.

Development and Validation

As previously indicated, criteria for validation and regulatory acceptance must be taken into account in the planning and design stages of validation studies, and in so doing there should be continuing communication with the relevant regulatory agencies that will be asked to review and accept the methods. Likewise, regulatory agencies should be expected to evaluate new test methods using consistent, though flexible, validation criteria and should be involved in encouraging the development of new and innovative methods. Although both correlative and mechanistic tests can be validated and accepted, mechanistically based methods relevant to the biological effects of concern should be encouraged.

Regulatory Review of New Methods

An efficient and effective process that involves communicating with regulators at all stages prior to regulatory acceptance of alternative methods needs to be set up. As part of this process, agencies should establish internal central clearing systems for the evaluation of new or revised methods and for the periodic review of methods recommended by an agency to ensure their continued relevance. If agency personnel are not familiar with new methods, training should be instituted so that current efforts to incorporate validated alternatives into regulatory testing strategies can be continued and expanded. When questions arise about the acceptability of new or revised test methods, input from the relevant scientific community should be solicited. Many times, concurrent submission of data from new and existing methods will help facilitate regulatory acceptance and preclude these kinds of questions from being raised. If not, an agency-sponsored workshop is the best venue to exchange information.

Intra- and Interagency Coordination and Harmonization

Various regulatory agencies should be consistent in their processes and criteria for acceptance of new and revised toxicological test procedures. Interagency coordination is especially important for those test methods used by more than one agency. This coordination could be ensured by the formation of an interagency committee on test methods which would be a forum for information exchange and the vehicle for consistency in the review, evaluation, and acceptance of alternative test methods. When modifying existing test guidelines, each regulatory agency should solicit input from other agencies in an effort to harmonize testing guidelines to the extent possible. Such harmonization of testing guidelines may first require the harmonization of disparate hazard classification schemes.

Communication

Agency regulations and guidelines should be available to the public to facilitate a consistent and coordinated process of involvement and communication among researchers, method users, regulators, and the public. Exchange of information among these stakeholders will encourage the validation and acceptance of new alternative methods. Scientific journals, workshops, government publications (e.g., the *Federal Register*), and other means should be used to communicate the acceptance of new and revised test methods by regulatory agencies.

International Harmonization

Regulatory agencies in the United States should encourage international harmonization and make attempts to harmonize testing guidelines through international organizations. Where appropriate, this could be done by working with the OECD, the United Nations Committee of Experts on the Transport of Dangerous Goods organization, and other international groups.

From a regulatory perspective, the pieces are in place to foster the development of new and innovative methods that can be used to generate information to support the protection of human and animal health and the

environment. Through the continued efforts of researchers working with informed regulators the future of alternative methods development is encouraging; there now exists a recognized pathway for moving appropriately validated new methods from their inception stage to regulatory acceptance.

ACKNOWLEDGEMENTS

The authors acknowledge the contributions of Ping Kwong (Peter) Chan to the development of this chapter.

QUESTIONS

1. What is the importance of acute toxicity testing and is a precise LD50 value necessary to adequately define acute toxicity?

Answer: Acute toxicity testing is the way in which we define the intrinsic toxicity of a chemical, identify target organs, provide information for risk assessment of acute exposure, provide information for the design and selection of dose levels for more prolonged studies (i.e., subchronic, chronic), and, most importantly, provide information to clinicians for use in treatment of acute chemical poisoning. Information from acute toxicity testing is also used to provide insight into the mechanism of action of a chemical, to formulate safety measures during the early stages in the development of a new chemical, and for categorization and labeling purposes for handling and shipping chemicals. One should not confuse the concept of acute toxicity with the term, LD50. The LD50 is a statistically defined measure of acute toxicity but is only one of many ways to define it. Indeed, a precise LD50 is seldom required in acute toxicity testing, and its use is being de-emphasized to reduce the total number of animals and pain and suffering involved in their use. The LD50 is being replaced by more modern methods. The Up-and-Down Procedure and the Acute Toxic Class Method are alternatives to the LD50 test that can be used to estimate the median lethal dose and to provide hazard classification for labeling, respectively.

2. The following mortality data were obtained from an acute oral toxicity study:

Dose (mg/kg)	1	2	4	8	16	32
Mortality	0/10	1/10	3/10	4/10	7/10	10/10

Calculate the LD50, the SE of the LD50, the fiducial limits, and the slope of the dose-response curve.

		Probits		Probabilities expected *(P)*	Responses		
Log dose *(x)*	*n*	Observed	Expected		Observed	Expected	$\chi 2$
0.30	10	3.72	3.82	11.9	1	1.19	0.0344
0.60	10	4.48	4.36	26.1	3	2.61	0.0344
0.90	10	4.75	4.75	46.4	4	4.64	0.0789
1.20	10	5.52	4.45	67.4	7	6.74	0.1646
1.50	10	–	–	–	10	–	0.0307
							$\Sigma\chi 2=0.386$
							$df=2$

Answer: Procedure

1.	Log dose	0	0.3	0.6	0.9	1.2	1.5
2.	Probits	–	3.72	4.48	4.75	5.52	–

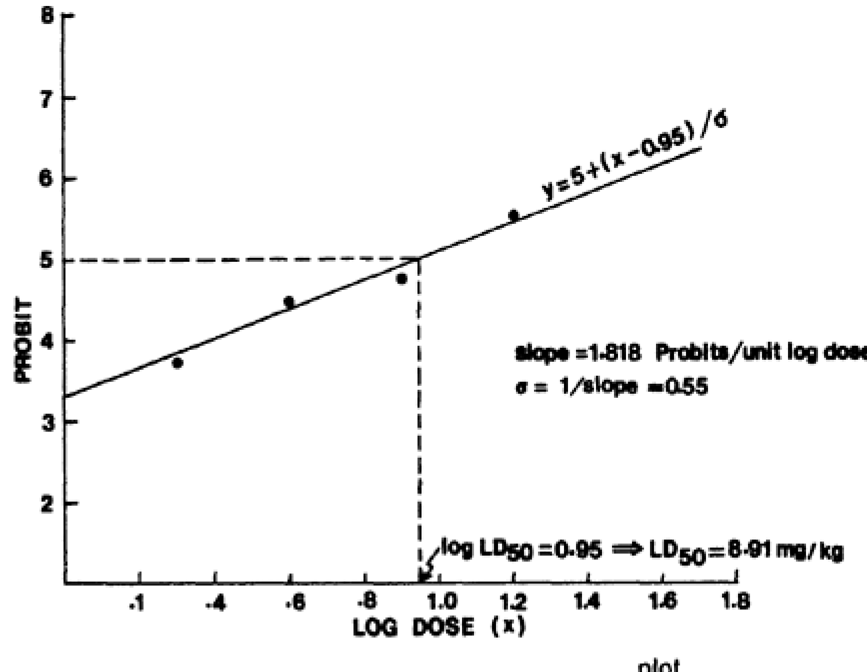
3.,4. Plot log dose versus probits (Figure 18.9) and fit the best point(s) to a straight line.

5. From the log-dose probit line, extrapolate the log LD50=0.95; then LD50=antilog 0.95=8.91 mg/kg body weight.

6. From the same line, calculate the slope as: (numbers of probit units)/unit log dose=2/11=1.818.

   Thus, $\sigma = \frac{1}{\text{slope}} = 0.55$ (Figure 18.9)

7. $\chi 2$ *test of goodness-of-fit.* Expected probability is converted from the expected probits. The test is conducted by converting each expected probit *(y)* back to the expected probability *(P)* and then to the number of expected responses *(E)* (i.e., multiply the expected probability *P* by *n*). The difference between expect



plot.

FIG. 18.9. Example of probit versus log-dose plot.

ed and observed number of responses will be used to calculate the $\chi 2$ statistic, but instead of using $\Sigma[(E-0)2/E]$, **the weighted value will be used, i.e.,** $\Sigma[(E-0)2/E(1-P)]$. The degrees of freedom *(df)* are $N-2$, where N is the number of dose levels used in the calculation of $\chi 2$. The critical $\chi 2$ for (4–2)=2 degrees of freedom is 6.0 at a $p=0.05$, and the calculated $\chi 2=0.386$, which is less than the critical value, indicating that the fitted line is adequate.

8. *Determination of precision of LD50 by weighting.* The SE of log $\mathrm{LD}_{50}=S_m=\sigma/\sqrt{\Sigma mW}=0.55/\sqrt{18.5}=0.129.$ The approx-imation of SE (LD50)=(10m) · *(Sm)*=8.91× 2.302×0.129=2.646. The precision of LD50=8.91±2.646 mg/kg.

Dose (mg/kg)	2	4	6	16
W	0.277	0.423	0.541	0.564
nW	2.77	4.23	5.41	5.64
nW			18.05	

9. *Fiducial limits.* Using the approximation formula, the fiducial limit calculated at the *F*=95% level is given by log LD50±1.96 *(Sm)*. Thus the lower log LD50 **limit=0.5−1.96× 0.129–0.697, and the** antilog 0.697=4.977. The upper log LD50 limit=0.95+1.96× 0.129=1.20=15.849. Antilogs of 0.697 and 1.2 give the fiducial LD50 limit 4.98 to 15.85 mg/kg.

3. Describe the basic elements needed to perform Quantitative Structure-Activity Relationship (QSAR) analysis and list the limitations of this method.

Answer: Basic elements needed to perform QSAR analysis include a reliable bioassay database for the predicted endpoint, a set of physical-chemical parameters that describe the chemical, structures of interest so the bioassay endpoint can be modeled in terms of these parameters, appropriate statistical methods to weigh these parameters in terms of the explanation of the endpoint, and associated computer technology to carry out the computations. Limitations of QSAR analysis in acute toxicity testing and eye irritancy include a limited database on which to base a QSAR model, uncertainties associated with extrapolating beyond the confines of the model, and biological variability (noise) in the bioassays on which the models are based.

4. Compare and contrast eye irritation and eye corrosion including in the discussion a description of the observation endpoints usually associated with irritancy in the three major tissues of the eye.

Answer: Eye irritation can be defined as reversible inflammatory changes in the eye and its surrounding mucous membranes following exposure to a material on the surface of the anterior portion of the eye. In contrast, corrosion represents irreversible tissue damage to the eye following exposure to a material. The amount of damage to each of the three major eye tissues, the cornea, the conjunctiva, and the iris, is what differentiates irritancy from corrosion. Gross tissue destruction that follows rapidly after exposure and persists for an extended period in any or all of these tissues is usually an indication of eye corrosion. Irritancy, however, can occur to various degrees. Assessment of injury is based on the presence and severity of: cloudiness (opacity) and swelling of the cornea; redness, edema (chemosis) and discharge in the conjunctiva; and increased vascularity, edema, absence of reaction to light, and cloudiness (aqueous flair) in the iris.

5. What are the Three Rs and how have they been implemented in acute toxicity and eye irritancy testing?

Answer: Three Rs refers to the reduction in the number of animals used, the refinement of testing methods to reduce the pain and suffering of animals, and the replacement of animals by other methods in testing procedures. Varying degrees of progress in the Three Rs have been made for acute toxicity and eye irritancy testing. Protocols requiring as few as six rats for acute toxicity or three rabbits for eye irritancy are being used in hazard assessment, although larger group sizes are usually required in regulatory requirements for labeling and classification of chemicals. At least one acute toxicity method, the fixed dose procedure, is based on classifying toxicity hazard using a pre-selected dose that is intended to be non-lethal, non-painful, and non-stressful, but toxicity-evident. Many company guidelines specify that materials with extremely high or low pH, e.g., <2 or >11.5, or which are severely irritating or corrosive to skin, do not need to be tested for eye irritancy, and the use of topical eye anesthetics before testing is also common practice. Quantitative StructureActivity Relationship (QSAR) analysis and various in vitro tests are being researched as replacement methods, but at the present, none appears able to replace in vivo testing in animals for acute toxicity and eye irritancy.

6. What are the basic requirements for the development, validation, and regulatory acceptance of new alternative methods?

Answer: An understanding of the nature and use of the animal or other test intended for replacement is a prerequisite to the development of a new method. The new method is built around this knowledge, which must include supporting information that explains how the new test endpoint is related to the effect of interest. If the new method is intended for regulatory approval, there should be early dialog with the appropriate regulators to assure the method fits the existing regulatory scheme. Once a new test has been developed for a specific use it undergoes a prevalidation (optimization) phase. During prevalidation, the purpose and basis of the test are defined, a test protocol is standardized, a database is developed, inter- and intra-laboratory reproducibility is assessed, and a prediction model is generated from the database. The prediction model must explain the toxic endpoint of interest in terms of the new test output(s). Next, a validation study is designed to test the new method with a sufficiently large set of coded substances covering a relevant chemical class and a range of toxic endpoints. High-quality in vivo data should be available on these substances for comparative purposes. The validation study should be conducted among a sufficient number of laboratories to further assess inter- and intra-laboratory variability of the standardized protocol. Testing results should be analyzed using appropriate statistical methods that challenge the data with the prediction model. All methods, results, and supporting data should be peer reviewed, prepared for publication, and submitted for regulatory approval.

REFERENCES

1. Archer, G., Balls, M., Bruner, L.H., Curren, R.D., Fentem, J.H., Holzhutter, H., Liebsch, M., Lovell, D.P., and Southee, J.A. (1997): The validation of toxicological prediction models. *ATLA,* 25:505–516.
2. Ashford, J.R. (1959): An approach to the analysis of data for semiquantal responses in biological assay. *Biometrics,* 156:573–581.
3. Bagley, D., Booman, K.A., Bruner, L.H., Casterton, P.L., Demetrulias, J., Heinze, J.E., Innis, J.D., McCormick, W.C., Nuen, D.J., Rothenstein, A.S., and Sedlak, R.I. (1994): The SDA alternatives program phase III: Comparison of in vitro data with animal eye irritation data on solvents, surfactants, oxidizing agents and prototype cleaning products. *J. Toxicol. Cutan. Ocul. Toxicol.,* 13:127–155.
4. Bagley, D.M., Bruner, L.H., DeSilva, O., Cotton, M., O'Brien, K. A.F., Uttley, M., and Walker, A.P. (1992): An evaluation of five potential alternatives in vitro to the rabbit eye irritation test in vivo. *Toxicol. in Vitro*, 6:275–284.
5. Bagley, D.M., Rizvi, P.Y., Kong, B.M., and DeSalva, S.J. (1988): In: *Alternative Methods in Toxicology, Vol. 6, Progress in In Vitro Toxicology.* edited by A.M.Goldberg, pp. 131–138. Mary Ann Liebert, New York.
6. Bagley, D.M., Rizvi, P.Y., Kong, B.M., and DeSalva, S.J. (1991): Factors affecting use of the hens' egg chorioallantoic membrane as a model for predicting eye irritation potential. I. *J. Toxicol. Cutan. Ocul. Toxicol.,* 10:95–104.
7. Bagley, D.M., Rizvi, P.Y., Kong, B.M., and DeSalva, S.J. (1991): Evaluation of the vascular components of the chorioallantoic membrane assay as a model for eye irritation potential. II. *J. Toxicol. Cutan. Ocul. Toxicol.,* 10:105–114.
8. Baldwin, H.Q., McDonald, T.D., and Beasley, C.H. (1973): Slit-lamp examination of experimental animal eyes. 11. Grading scales and photographic evaluation of induced pathological conditions. *J. Soc. Cosmet. Chem.,* 24:181–195.
9. Ballantyne, B., and Swanston, D.W. (1974): The irritant effects of dilute solutions of dibenzoxyazepine(CR) on the eye and tongue. *Acta Pharmacol. Toxicol.,* 35:412–423.
10. Balls, M. (1991): Why modification of the LD50 test will not be enough. *Lab. Animals,* 25:198–206.
11. Balls, M. (1995): Scientific validation: A crucial and unavoidable prerequisite to the acceptance of new tests and testing strategies. *ATLA,* 23:474–479.
12. Balls, M. (1995): Defining the role of ECVAM in the development, validation and acceptance of alternative tests and testing strategies. *Toxicol. in Vitro*, 9:863–869.
13. Balls, M., and Blaauboer, B.J. (1994): The validation of replacement alternative methods. In: Proceedings of the ECVAM opening symposium, edited by M.Balls and B.J.Blaauboer. *Toxicol. in Vitro,* 9:789–869.
14. Balls, M., Blaauboer, B.B., Brusick, D., Frazier, J., Lamb, D., Pemberton, M., Reinhardt, C., Roberfroid, M., Rosenkranz, H., Schmid, B., Speilemann, H., Stammati, A.L., and Walum, E. (1990). Report and recommendations of the CAAT/ERGATT workshop on the validation of toxicity test procedures. *ATLA,* 18:313–328.
15. Balls, M., Blaauboer, B., Brusick, D., Frazier, J., Lamb, D., Pemberton, M., Reinhardt, C., Roberfroid, M., Rosenkranz, H., Schmid, B., et al. (1990): Report and recommendations of the CAAT/ERGATT workshop on the validation of toxicity test procedures. *ATLA,* 18:313–337.

16. Balls, M., Blaauboer, B., Fentem, J.H., Bruner, L., Combes, R.D., Ekwall, B., Fielder, R.J., Guillouzo, A., Lewis, R.W., Lovell, D. P., et al. (1995): Practical aspects of the validation of toxicity test procedures. The report and recommendations of ECVAM Workshop 5. *ATLA*, 23:129–147.
17. Balls, M., Botham, P.A., Bruner, L.H., and Spielman, H. (1995): The EC/HO international validation study on alternatives to the Draize eye irritation test. *Toxicol. in Vitro*, 9:871–929.
18. Balls, M, and Clothier, R.H. (1992): *Cytotoxicity Assays for Intrinsic Toxicology*, edited by G.Jolles and A.Cordier, pp. 31–52. Academic Press, New York.
19. Balls, M., De Klerck, W., Baker, F., van Beek, M., Bouillon, C., Bruner, L., Carstensen, J., Chamberlain, M., Cottin, M., Curren, R., et al. (1995): Development and validation of non-animal tests and testing strategies: The identification of a coordinated response to the challenge and the opportunity presented by the 6th Amendment to the Cosmetic Directive (76/768/EEC). The report and recommendations of ECVAM Workshop 7. *ATLA*, 23:398–409.
20. Balls, M., and Homer, S.A. (1985): The FRAME Inter-Laboratory Programme on In Vitro Cytotoxicology. *Food Chem. Toxicol.*, 23:209–213.
21. Barratt, M.D. (1995): A quantitative structure-activity relationship for the eye irritation potential of neutral organic chemicals. *Toxicol. Lett.*, 80:69–74.
22. Barratt, M.D. (1997): QSARS for the eye irritation potential of neutral organic chemicals. *Toxicol. in Vitro*, 11:1–8.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_906.html[2010/10/28 18:49:07]

23. Barratt, M.D., Castell, J.V., Chamberlain, M., Combes, R.D., Dearden, J.C., Fentem, J.H., Gerner, I., Giuliani, A., Gray, T.J.B., Livingston, D.J., et al. (1995): The integrated use of alternative approaches for predicting toxic hazard. *ATLA,* 23:410–429.
24. Bass, R., Gunzel, P., Henschler, D., et. al. (1982): LD50 versus acute toxicity. *Arch. Toxicol.,* 51:183–186.
25. Battista, S.P., and McSweeney, E.S. (1965): Approaches to a quantitative method for testing eye irritation. *J. Soc. Cosmet. Chem.,* 16:199–301.
26. Baum, J.L., Niedra, R., Davis C., and Yoe, D. (1979): Mass culture of human corneal endothelial cells. *Arch. Ophthalmol.,* 97:1136–1140.
27. Bayard, S., and Hehir, R.M. (1976): Evaluation of proposed changes in the modified Draize rabbit irritation test. *Toxicol. Appl. Pharmacol.,* 37:186.
28. Beckley, J.H. (1965): Comparative eye testing: Man versus animal. *Toxicol. Appl. Pharmacol.,* 7:93–101.
29. Beckley, J.H. (1965): Critique of the Draize eye test, now and then: Eighteen, nine or six rabbits. *Am. Perf. Cosmet.,* 80:51–54.
30. Bell, M., Holmes, P.M., Nisbet, T.M., Uttley, M., and Van Abbe, N.J. (1979): Evaluating the potential eye irritancy of shampoos. *Int. J. Cosmet. Sci.,* 1:123.
31. Benson, M.T., Shepherd, L., Rees, R.C., and Rennie, I.G. (1992): Production of interleukin-6 by human retinal pigment epithelium in vitro and its regulation by other cytokines. *Curr. Eye Res.,* 1:173–179.
32. Bernstein, P.S., Lloyd, M.B., O'Day, W.T., and Bok, D. (1992): Effect of phytanic acid on cultured retinal pigment epithelium: An in vitro model for Refsum's disease. *Exp. Eye Res.,* 55:869–878.
33. Bliss, C.I. (1934): The method of probits-A correction. *Science,* 79:409–410.
34. Bliss, C.I. (1964): Insecticide assays. In: *Statistics and Mathemat-ics in Biology,* edited by O.Kempthorne, T.A.Bancroft, J.W. Gowen, and J.L.Lush, pp. 345–360. Hofner, New York.
35. Bohnke, M., Chavanne, P., Gianotti, R., and Salathe, R.P. (1996): High-precision, high-speed measurement of eximer laser keratectomies with a new optical pachymeter. *Ger. J. Ophthalmol.,* 5:338–342.
36. Bondesson, I., Ekwall, B., Hellberg, S., Romert, L., Stenberg, K., and Walum, E. (1989): MEIC. A new international multicenter project to evaluate the relevance to human toxicity of in vitro cytotoxicity tests. *Cell Biol. and Toxicol.,* 5:331–347.
37. Bonifield, C.T., and Scala, R.A. (1965): The paradox in testing for eye irritation. A report on thirteen shampoos. *Proc. Sci. Sect. Toilet Goods Assoc.,* 43:34–43.
38. Bonting, S.L., Simon, K.A., and Hawkins, N.M. (1961): Studies on sodium-potassium-activated adenosine triphosphatase. 1. Quantitative distribution in several tissues of the rat. *Arch. Biochem.,* 95:416–423.
39. Booman, K.A., Cascieri, T.M., Demetrulias, J., Driedger, A., Griffith, J.F., Grochoski, G.T., Kong, B., McCormick, W.C. 3rd, North-Root, H., Rozen, M.G., and Sedlak, R.I. (1988): In vitro methods for evaluating eye irritancy of cleaning products. Phase 1: preliminary assessment. *J. Toxicol. Cutan. Ocul. Toxicol.,* 7:173–185.
40. Booman, K.A., De Prospo, J., Demetrulias, J., Driedger, A., Griffith, J.F., Grochoski, G.T., Kong, N., McCormick, W.C. 3rd, North-Root, H., Rozen, M.G., and Sedlak, R.I. (1989): The SDA alternatives program: Comparison of in vitro data with Draize test data. *J. Toxicol. Cutan. Ocul. Toxicol.,* 8:35–49.
41. Borenfreund, E., Babich, H., and Martin-Alguacil, N. (1988): Comparison of two in vitro cytotoxicity assays—The neutral red (NR) and tetrazolium MTT tests. *Toxicol. in Vitro,* 2:1–6.
42. Borenfreund, E., and Borrero, O. (1984): In vitro cytotoxicity assays: Potential alternatives to the Draize ocular irritancy test. *Cell Biol. Toxicol.,* 1:33–39.
43. Borenfreund, E., and Puerner, J.A. (1984): A simple quantitative procedure using monolayer culture for cytotoxicity assays (HTD/NR90). *J. Tissue Culture Meth.,* 9:7–9.
44. Borenfreund, E., and Puerner, J.A. (1985): Toxicity determined in vitro by morphological alterations and neutral red absorption. *Toxicol. Lett.,* 24:119–124.
45. Borenfreund, E., and Shopsis, C. (1984): Toxicity monitored with a correlated set of cell culture assays. *Xenobiotica,* 15:705–712.
46. Borenfreund, E., Shopsis, C., Barrero, O., and Sathe, S. (1983): In vitro alternative irritancy assays: Comparison of cytotoxic and membrane transport effects of alcohols. *Ann. NY Acad. Sci.,* 407:416–419.
47. Boue-Grabot, M., Bernardin, G., Chaumond, S., and Pinon, J.F. (1995): Alternative methods: Hen's egg chorioallantoic membrane and in vitro cytotoxicity—Complementary approach. *Int. J. Cosmet. Sci.,*

17:207–215.
48. Brantom, P.G., Bruner, L.H., Chamberlain, M., De Silva, O., Dupuis, J., Earl, L.K., Lovell, D.P., Pape, W.J.W., Uttley, et al. (1997): A summary report of the COLIPA international validation study on alternatives to the Draize rabbit eye irritation test. *Toxicol. in Vitro*, 11:141–179.
49. British Toxicology Society (1984): A new approval to classification of substances and preparations on the basis of their acute toxicology. *Human Toxicol.*, 3:85–92.
50. Bross, I.D.J. (1958): How to use RIDIT analysis. *Biometrics*, 14:18–38.
51. Brownlee, K.A., Hodges, J.L., and Rosenblatt, M. (1953): The up-and-down method with small samples. *J. Am. Stat. Assoc.*, 48:262–277.
52. Bruce, R.D. (1984): An up-and-down procedure for acute toxicity testing. In: *Acute Toxicity Testing: Alternative Approaches*, edited by A.M.Goldberg, p. 184. Mary Ann Leibert, New York.
53. Bruce, R.D. (1985): An up-and-down procedure for acute toxicity testing. *Fund. Appl. Toxicol.*, 5:151–157.
54. Bruce, R.D. (1987): A confirmatory study of the up-and-down method for acute toxicity testing. *Fund. Appl. Toxicol.*, 8:97–100.
55. Bruner, L.H., Carr, G.J., Chamberlain, M., and Curren, R.D. (1996): Validation of alternative methods for toxicity testing. *Toxlcol. in Vitro*, 10:479–501.
56. Bruner, L.H., Carr, G.J., Chamberlain, M., and Curren, R.D. (1996): No prediction model, no validation study. *ATLA*, 24:139–142.
57. Bruner, L.H., Kain, D.J., Roberts, D.A., and Parker, R.D. (1991): Evaluation of seven in vitro alternatives for ocular safety testing. *Fund. Appl. Toxicol.*, 17:136–149.
58. Bruner, L.H., Miller K.R., Owicki, J.C., Paree, J.J.W., and Muir, V.C. (1991): Testing ocular irritancy in vitro with silicon microphysiometer. *Toxicol. in Vitro*, 5:277–284.
59. Bruner, L.H., Parker, R.D., and Bruce, R.D. (1992): Reducing the number of rabbits in the low-volume eye test. *Fund. Appl. Toxicol.*, 19:330–335.
60. Bruner, L.H., Shadduck, J., and Essex-Sorlie, D. (1991): Alternative methods for assessing the effects of chemicals in the eye. In: *Dermal and Ocular Toxicology: Fundamentals and Methods*, edited by D.W.Hobson. pp. 585–606. CRC Press. Boca Raton, FL.
61. Budai, P., Somlyay, I.M., Varnagy, L.E., and Varga, T. (1995): Comparison of in vitro (HET–CAM) and in vivo (Draize) irritation tests using different pesticides. *Meded Fac. Landbouwkd. Toegepaste Biol. Wetenschap Univ. Gent.*, 60:593–597.

62. Buehler, E.V. (1974): Testing to predict potential ocular hazards of household chemicals. In: *Toxicology Annual*, edited by C.L. Winek, pp. 53–69. Marcel Dekker, New York.
63. Buehler, E.V., and Newman, E.A. (1964): A comparison of eye irritation in monkeys and rabbits. *Toxicol. Appl. Pharmacol.*, 6:701–710.
64. Bulich, A.A., Tung, K.K., and Scheibner, G. (1990): The luminescent bacteria toxicity test: Its potential as an in vitro alternative. *J. Biolumin. Chemilumin.*, 5:71–77.
65. Burnstein, N.L. (1980): Corneal cytotoxicity of topically applied drugs, vehicles, and preservatives. *Surv. Ophthalmol.*, 25:15–30.
66. Burton, A.B.G. (1972): A method for the objective assessment of eye irritation. *Food Cosmet. Toxicol.*, 10:209–217.
67. Burton, A.B.G., York, M., and Lawrence, R.S. (1981): The in vitro assessment of severe eye irritant. *Food Cosmet. Toxicol.*, 19:471–480.
68. Calabrese, E.J. (1983): Ocular toxicity. In: *Principles of Animal Extrapolation*, p. 400. John Wiley, New York.
69. Casterton, P.L., Potts, L.F., and Klein, B.D. (1996): A novel approach to assessing eye irritation using the bovine corneal opacity and permeability assay. *J. Toxicol. Cutan. Ocul. Toxicol.*, 15:147–163.
70. Catroux, P., Rougier, A., Dossou, K.G., and Cottin, M. (1993): The silicon microphysiometer for testing ocular toxicity in vitro. *Toxicol. in Vitro*, 7:465–469.
71. Cello, R.M., and Lasmanis, J. (1958): *Pseudomonas* infection of the eye of the dog resulting from the use of contaminated fluorescein solution. *J. Am. Vet. Med. Assoc.*, 132:297.
72. Chan, K.Y. (1986): Chemical injury to an in vitro ocular system differential release of plasminogen activator. *Curr. Eye Res.*, 5:357–562.
73. Chan, P.K., and Hayes, A.W. (1985): Assessment of chemically induced ocular toxicity: A survey of methods. In: *Toxicology of the Eye, Ear and Other Special Senses*, edited by A.W.Hayes. Raven Press, New York.
74. Chan, T., Payor, S., and Holden, B.A. (1983): Corneal thickness profiles in rabbits using an ultrasonic pachymeter. *Invest. Ophthalmol. Vis. Sci.*, 24:1408–1410.
75. Chan-Ling, T., Tervo, K., Tervo, T., Vannas, A., Holden, B.A., and Eranko, L. (1987): Long-term neural degeneration in the rabbit following 180° limbal incision. *Invest. Ophthalmol. Vis. Sci.*, 28:2083–2088.
76. Chasin, M., Scott, C., Shaw, C., and Persico, F. (1979): A new assay for the measurement of mediator release from rat peritoneal mast cells. *Int. Srch. Allergy Appl. Immunol.*, 58:1–10.
77. Choi, S.C. (1971): An investigation of Wetherill's method of estimation for the up-and-down experiment. *Biometrics*, 27:961–970.
78. Clark, A.J. (1933): *Mode of Action of Drugs on Cells*. Williams and Wilkins, Baltimore.
79. Clothier, R.H., Hulme, L., Ahmed, A.B., Reeves, A.L., Smith, M., and Balls, M. (1988): In vitro cytotoxicity of 150 chemicals to 3T3-L1 cells. assessed by the FRAME Kenacid Blue method. *ATLA*, 16:84.
80. Cohen, I.J. (1983): Use of fluorescein in eye injuries. *J. Occup, Med.*, 5:540.
81. Conquet, P.H., Durand, G., Laillier, J., and Plazonnet, B. (1977): Evaluation of ocular irritation in the rabbit: Objective versus subjective assessment. *Toxicol. Appl. Pharmacol.*, 39:129–131.
82. Cornfield, J. (1964): Measurement and composition of toxicities: The quantal response. In: *Statistics and Mathematics in Biology*, edited by O.Kempthorne, T.A.Bancroft, J.W.Gowen and J. L.Lush, pp. 327–344. Hofner, New York.
83. Cottin, M., Dossou, K.G., DeSilva, O., Tolle, M., Roguet, R., Cohen, C., Catroux. P., Delabarre, I., Sicard, C., and Rougier, A. (1994): Relevance and reliability of in vitro methods in ocular safety assessment. *In Vitro Toxicol.*, 7:277–282.
84. Cottin, M., and Zanvit, A. (1997): Fluorescein leakage test: A useful tool in ocular safety assessment. *Toxicol. in Vitro*, 11:399–405.
85. Consumer Product Safety Commission (CPSC) (1976): Illustrated guide for grading eye irritation by hazardous substances. Directorate for Engineering and Science, Washington, D.C.
86. Cronin, M.T. (1996): The use of cluster significance analysis to identify asymmetric QSAR data sets in toxicology. An example with eye irritation data. *SAR QSAR Environ. Res.*, 5:167–175.
87. Cronin, M.T., Basketter, D.A., and York, M.A. (1994): Quantitative structure-activity relationship (QSAR) investigation of a Draize eye irritation database. *Toxicol. in Vitro*, 8:21–28.
88. Curren. R.D., Southee, J.A., Spielmann, H., Liebsch, M., Fentam, J.H., and Balls, M. (1995): The role of prevalidation in the development, validation and acceptance of alternative methods. *ATLA*, 23:211–

217.
89. Davies, R.G., Kynoch, S.R., and Liggett, M.P. (1976): Eye irritation tests—An assessment of the maximum delay time for remedial irrigation. *J. Soc. Cosmet. Chem.*, 27:301–306.
90. Dawson, M., and Mustafa, A.F. (1985): Use of cultured human conjunctival and other cells to assess the relative toxicity of six local anesthetics. *Food Chem. Toxicol.*, 23:305–308.
91. Decker, D., Harper, R., and Rehfeldt, T. (1994): Evaluation of the Microtox system as a predictor of ocular irritancy of toiletry products. *In Vitro Toxicol.*, 7:83–88.
92. Decker, D., Stemp, M., and Harper, R. (1993): Evaluation of the Eytex system for use as a predictor of ocular irritancy: II Conditioners and styling aids. *J. Toxicol. Cutan. Ocul. Toxicol.*, 12:371–380.
93. DePass, L.R. (1989): Alternative approaches in median lethality (LD50) and acute toxicity testing. *Toxicol. Lett.*, 49:159–170.
94. DeSousa, D.J., Rosue, A.A., and Smolon, W.J. (1984): Statistical consequences of reducing the number of rabbits utilized in eye irritation testing. Data on 67 petrochemicals. *Toxicol. Appl. Pharmacol.*, 76:234–242.
95. Dews, P.B., and Berkson, J. (1964): On the error of bioassay with quantal response. In: *Statistics and Mathematics in Biology*, edited by O.Kempthorne, T.A.Bancroft, J.W.Gowen, and J.L.Lush, pp. 361–370. Hofner, London.
96. Diem, K. (1968): *Documenta Geigy Scientic Tables*, 6th ed. Geigy Pharmaceutical Division, Geigy Chemical Corp., Ardsley, New York.
97. Dierickx, P.J. and Gordan, V.C. (1990): The EYTEX- system and the neutral red uptake inhibition assay in cultured Hep G2 cells as alternative methods for in vivo eye irritation following the EEC protocol. *ATLA*, 17:325–333.
98. Dixon, W.J. (1965): The up-and-down method for small samples. *J. Am. Stat. Assoc.*, 60:967–978.
99. Dixon, W.J., ed., (1981). BMDP Statistics Software. University of California Press, Berkeley.
100. Douglas, H.J., and Spillman, S.D. (1983): Product safety testing. In: *Alternative Methods in Toxicology. Vol. 1* pp. 205–230, edited by A.M.Goldberg. Mary Ann Liebert, New York.
101. Doull, J. (1980): Factors influencing toxicology. In: *Cassarett and Doull's Toxicology: The Basic Science of Poisons*, edited by J. Doull, C.D.Klaassen, and M.O.Amdur, pp. 70–83. Macmillan, New York.
102. Draize, J.H., Woodward, G., and Calvery, H.O. (1944): Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *J. Pharmacol. Exp. Ther.*, 82:377–390.
103. Dubin, N.H., Diblasi, M.C., Thomas, C.L., and Wolff, M.C. (1984): Development of an in vitro test for cytotoxicity in vaginal

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_908.html[2010/10/28 18:49:08]

tissue effect of ethanol on prostanoid release. In: *Alternative Methods in Toxicology, Vol. 2. Acute Toxicity Testing Alternative Approaches,* edited by A.M.Goldberg, pp. 127–137. Mary Ann Liebert, New York.
104. Dubin, N.H., Wolff, M.C., Thomas, C.L., and DiBlasi, M.C. (1985): Prostaglandin production by rat vaginal tissues in vitro in response to ethanol, a mild mucosal irritant. *Toxicol. Appl. Pharmacol.,* 78:458–463.
105. Dunnett, C.W. (1968): Biostatistics in pharmacological testing. In: *Selected Pharmacological Testing Methods,* edited by A.Burger, pp. 7–18. Edward Arnold, London.
106. Edelhauser, H.F., Gonnerings, R., and Van Horn, D.L. (1978): Intraocular irrigation solutions: A comparative study of BBS plus and lactated Ringer's solution. *Arch. Ophthalmol.*, 93:516.
107. Edelhauser, H.F., Van Horn, D.L., Hundink, R.A., and Schultz, R.O. (1975): Intraocular irrigating solutions: Their effect on the corneal endothelium. *Arch. Ophthalmol.*, 93:648.
108. Edelhauser, H.F., Van Horn, D.L., Schultz, R.O., and Hundink, R.A. (1976): Comparative toxicity of intraocular irrigating solution on the corneal endothelium. *Am. J. Ophthalmol.*, 81:473–481.
109. EEC (1983): Methods for the determination of toxicity. EEC Directive 79/831 Annex V, Part B. Brussels.
110. EEC (1992): Acute toxicity (eye irritation). EEC guideline for testing of chemicals No. B.5. Brussels.
111. Ehrlich, P. (1882): Uber provocirte flurescenzer-Scheinungen am Auge. *Dtsch. Med. Wochenschr.*, 2:21.
112. Ekwall, B. (1992): Features and prospects of the MEIC cytotoxicity evaluation project. *AATEX,* 1:231–237.
113. Ekwall, B. (1994): The basal cytotoxicity concept. In: *Alternative Methods in Toxicology and the Life Sciences, Vol. 11, The World Congress on Alternatives and Animal Use in the Life Sciences, Education, Research, Testing,* edited by A.M.Goldberg and A.F.M.van Zupthen. Mary Ann Liebert, New York.
114. Ekwall, B., Bondesson, I., Castell, J.V., Gomez-Lechon, M.J., Hellberg, S., Hogberg, J., Jover, R., Ponsoda, X., Romert, L., Stenberg, K., and Walum, E. (1989): Cytotoxicity evaluation of the first ten MEIC chemicals: acute lethal toxicity in man predicted by cytotoxicity in five cellular assays and by oral LD50 tests in rodents. *ATLA,* 17:83–100.
115. Ekwall, B., Clemedsen, C., Crafoord, B., Ekwall, B., Hallander, S., Walum, E., and Bondesson, I. (1998): MEIC evaluation of acute systemic toxicity. Part V: Rodent and human toxicity data for the 50 reference chemicals. *ATLA,* 26(Suppl. 2):571–616.
116. Ekwall, B., Walum, E., Clemedsen, C., Barile, F.A., Castano, A., Clothier, R.A., Dierickx, P., Ekwall, B., Ferro, M., Fiskesjo, G., Garza-Ocanas, L., Gomez-Lechon, M.J., Gulden, M., Hall, T., Isomaa, B., Kahru, A., Kerszman, G., Kristen, U., Kunimoto, M., Karenlampi, S., Lewan, L., Loukianov, A., Ohno, T., Persoone, G., Romert, L., Sawyer, T.W., Shrivastava, R., Segner, H., Stammati, A., Tanaka, N., Valentino, M., Walum, E., and Zucco, F. (1998): MEIC evaluation of acute systemic toxicity. Part IV: Prediction of human toxicity by rodent LD50 values and results from 61 in vitro tests. *ATLA,* 26(Suppl. 2):617–658.
117. Elgebaly, S.A., Gilles, C., Forouhar, F., Hashem, M., Baddour, M., O'Roucke, J., and Kreuter, D.L. (1988): An in vitro model of leukocyte mediated injury to the corneal epithelium. *Curr. Eye. Res.*, 7:403–410.
118. Elgebaly, S.A., Nabawi, K., Kerbert, N., O'Tourke, J., and Kruetzer, D.L. (1985): *Invest. Ophthalmol. Vis. Sci.*, 26:320.
119. Enslein, K. (1988): An overview of structure-activity relationships as an alternative to testing in animals for carcinogenicity, mutagenicity, dermal and eye irritation and acute oral toxicity. *Toxicol. Ind. Health*, 4:479–498.
120. EPA (1983): Pesticide programs: Good laboratory practice standards: Final rule. *Fed. Register*, 48(230):53945–53969, November 29.
121. EPA (1983): Toxic substance control: Good laboratory practice standards: Final rule. *Fed. Register*, 48(230):53921–53944, November 29.
122. EPA (1984): EPA fact sheet: Background on acute toxicity testing for chemical safety, August 1984.
123. EPA (1984): Data requirements for pesticide registration: Final rule. 40 CFR Part 158. *Fed. Register*, Oct. 24:42855–42905.
124. EPA/FIFRA (1982): Pesticide Assessment Guidelines . Subdivision F, Hazard evaluation: Human and domestic animals. PB83–153916. Office of Pesticide Programs. U.S. EPA. Reproduced by the National Technical information Service, U.S. Department of Commerce, Springfield, VA.

125. EPA/TSCA (1984): Health effects test guidelines. PB84 Office of Pesticides and Toxic Substances, U.S. EPA. Reproduced by NTIS, U.S. Department of Commerce, Springfield, VA.
126. EPA/OPPTS (1998): Health effects test guidelines. Acute eye irritation. 870.2400. Office of Prevention, Pesticides and Toxic Substances. U.S. EPA, Washington, D.C.
127. Ernst, R., and Ardetti, J. (1980): Biological effects of surfactants IV. Effects of non-ionic and amphoterics on Hela cells. *Toxicology,* 15:233.
128. Esperson, R.J., Olsen, P., Nicolaisen, G.M., Jensen, B.L., and Rasmussen, E.S. (1997): Assessment of recovery from ocular irritancy using a human tissue equivalent model. *Toxicol. in Vitro*, 11:81–88.
129. Essepian, J.P., Rajpal, R.K., Azar, D.T., New, K., Antonacci, R., Shields, W., and Stark, W. (1994): The use of confocal microscopy in evaluating corneal wound healing after eximer laser surgery. *Scanning,* 16:300–304.
130. Eurell, T.E., and Meacham, S.H. (1994): In vitro evaluation of ocular irritants using tissue isoelectric focusing protein profiles from human, rabbit, and bovine corneal specimens. *Toxicol. Methods,* 4:66–75.
131. FDA (1976): Illustrated guide for grading eye irritation by hazardous substances. FDA, Washington, D.C.
132. FDA (1983): Final report on acute studies workshop. Sponsored by the U.S. Food and Drug Administration on November 9, 1983.
133. *Federal Register* (1980): 45:33063, May 19.
134. Fentem, J.H., Prinsen, M.K., Spielmann, H., Walum, E., and Botham, P.A. (1995): Validation-lessons learned from practical experience. *Toxicol. in Vitro*, 9:857–862.
135. Ferdinard, W. (1976): *The Enzyme Molecule*. John Wiley, New York.
136. FHSA (1979): Regulations under the Federal Hazardous Substance Act. Chapter 11. Title 16. Code of Federal Regulations.
137. Fine, B.S., and Yanoff, M. (1972): *Ocular Histology: A Text and Atlas*. Harper and Row, New York.
138. Finney, D.J. (1971): *Probit Analysis,* 3rd ed. Chapters 3 and 4, Cambridge University Press, Cambridge, United Kingdom.
139. Fisher, R.A., and Yates, F. (1963): *Statistical Tables for Biological, Agricultural and Medical Research,* 6th ed., edited by Oliver and Boyd Ltd., Edinburgh, Scotland.
140. Floyd, E.P., and Stockinger, H.G. (1958): Toxicity studies of certain organic peroxides and hydroperoxides. *Am. Ind. Hyg. Assoc. J.*, 19:205–212.
141. Frazier, J.M. (1990): OECD environment monograph no. 36: Scientific criteria for validation of in vitro toxicology tests. OECD, Paris.
142. Fraizer, J.M. (1990): Validation of in vitro models. *J. Am. Coll. Toxicol.*, 9:355–359.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_909.html[2010/10/28 18:49:08]

143. Fraizer, J.M. (1992): Validation of in vitro toxicity tests. In: *In Vitro Toxicity Testing: Applications to Safety Evaluations*, edited by J.M.Frazier. Marcel Dekker, New York.
144. Frazier, J.M. (1994): The role of mechanistic toxicology in test methods validation. *Toxicol. in Vitro*, 8:787–791.
145. Frazier, J.M. (1995): Interdisciplinary approach to toxicity test development and validation. *Toxicol. in Vitro*, 9:8925–8949.
146. Frazier, J.M., Gad, S.C., Goldberg, A.M., and MaCulley, J.P. (1987): A critical evaluation of alternatives to acute irritation testing. In: *Alternative Methods in Toxicology, Vol. 4,* edited by J.M.Frazier, S.C.Gad, A.M.Goldberg, and J.P.MaCulley. Mary Ann Liebert, New York.
147. Freeberg, F.E., Griffith, J.F., Bruce, R.D., and Bay, P.H.S. (1984): Correlation of animal test methods with human experience for household products. *J. Toxicol. Cutan. Ocul. Toxicol.*, 1(3):53.
148. Friedenwald, J.S., Hughes, W.F., and Hermann, H. (1944): Acid-base tolerance of the cornea. *Arch. Ophthalmol.*, 31:279–283.
149. Fulcher, S., Foulks, G.N., Wilkerson, M., Cobo, L.M., Houston, L.L., and Hatchell, D. (1993): Suppression of human corneal epithelial proliferation with breast carcinoma immunotoxin. *Cornea*, 12:391–396.
150. Gabourel, J.D., Bradley, J.M.B., and Acott, T.S. (1990): Antagonism of retinol-induced RNA synthesis: Assessment of retinoid toxicity in cultured retinal pigment epithelium. *J. Toxicol. Cutan. Ocul. Toxicol.*, 9:251–263.
151. Gad, S.C., Smith, A.C., Cramp, A.L., Gavigan, F.A., and Derelanko, M.J. (1984): Innovative designs and practices for acute systemic toxicity studies. *Drug Chem. Toxicol.*, 7:423–434.
152. Gaddum, J.H. (1983): Reports on biological standards III. Methods of biological assay depending on a quantal response. *Spec. Rep. Ser. Med. Res.*, No. 813, London.
153. Gaunt, I.F., and Harper, K.H. (1964): The potential irritancy to rabbit eye mucosa of certain commercially available shampoos. *J. Soc. Cosmet. Chem.*, 15:209–230.
154. Gautheron, P., Dukic, M., Alix, D., and Sina, J. (1992): Bovine corneal opacity and permeability test: An in vitro assay of ocular irritancy. *Fund. Appl. Toxicol.*, 18:442–449.
155. Gautheron, P., Giroux, J., Cottin, M., Audegond, L., Morilla, A., Mayordomo-Blanco, L., Tortajada, A., Haynes, G., Vericat,J. A., Pirovano, R., Gillio Tos, E., Hagemann, C., Vanparys, P., Deknudt, G., Jacobs, G., Prinsen, M., Kalweit, S., and Spielmann, H. (1994): Interlaboratory assessment of the bovine corneal opacity and permeability (BCOP) assay. *Toxicol. in Vitro*, 8:381–392.
156. Geppetti, P., Del Bianco, E., Cecconi, R., Tramontana, M., Romani, A., and Theodorsson, E. (1992): Capsaicin releases calcitonin gene-regulated peptide from the human iris and ciliary body in vitro. *Regul. Pept.*, 41:83–92.
157. Gettings, S.D. (1991): The current status of in vitro test validation (evaluation) in the United States. *ATLA*, 19:432–436.
158. Gettings, S.D., DiPasquale, L.C., Bagley, D.M., Casterton, P.L., Chudkowski, M., Curren, R.D., Demetrulias, J.L., Feder, P.I., Galli, C.L., Gay, R., Glaza, S.M., Hintze, K.L., Janus, J., Kurtz, P.J., Lordo, R.A., Marenus, K.D., Moral, J., Muscatiello, M., Pape, W.J.W., Renskers, K.J., Roddy, M.T., and Rozen,M. G. (1994): The CTFA evaluation of alternatives program: An evaluation of in vitro alternatives to the Draize primary eye irritation test. (Phase II) oil/water emulsions. *Food Chem. Toxicol.*. 32:943–976.
159. Gettings. S.D., Lordo, R.A., Hintze, K.L,. Bagley, D.M., Casterton, P.L., Chudkowski, M., Curren, R.D., Demetrulias, J.L., DiPasquale, L.C., Earl, L.K., Feder, P.I., Galli, C.L., Glaza, S.M., Gordon, V.C., Janus, J., Kurtz, P.J., Marenus, K.D., Moral, J., Pape, W.J.W., Renskers, K.J., Rheins,L. A.. Roddy, M.T., Rozen, M.G., Tedeschi, J.P., and Zyracki, J. (1996): The CTFA Evaluation of Alternatives Program: An evaluation of potential in vitro alternatives to the Draize primary eye irritation test. (Phase III) Surfactant-based formulations. *Food Chem. Toxicol.*, 34:79–117.
160. Gettings, S.D., and McEwen, Jr., G.N. (1990): Development of potential alternatives to the Draize eye test: The CTFA evaluation of alternatives program. *ATLA*, 17:317–324
161. Gettings, S.D., Teal, J.J., Bagley, D.M., Demetrulias, J.L., Dipasquale, L.C., Hintze, K.L., Rozen, M.G., Weise, S.L., Chudkowski, M., Marenus K.D., Pape, J.W., Roddy, M.T., Schnetzinger, R., Silber, P.M., Glaza, S.M., and Kurtz, P.J. (1991): The CTFA Evaluation of Alternatives Program: An evaluation of in vitro alternatives to the Draize primary eye irritation test (Phase 1) hydroalcoholic, formulations: (Part 2) Data analysis and biological significance. *In Vitro Toxicol.*, 4:247–288.
162. Giasson, C., and Forthomme, D. (1992): Comparison of central corneal thickness measurements between optical and ultrasound pachymeters. *Optom. Vis. Sci.*, 69:236–241.
163. Gilleron, L., Coecke, S., Sysmans, M., Hansen, E., Van Oproy, S., Marzin, D., Van Cauteren, H., and

Vanparys, P. (1996): Evaluation of a modified HET–CAM assay as a screening test for eye irritancy. *Toxicol. in Vitro*, 10:431–446.
164. Gilleron, L., Coecke, S., Sysmans, M., Hansen, E., Van Oproy, S., Marzin, D., Van Cauteren, H., and Vanparys, P. (1997): Evaluation of the HET–CAM–TSA method as an alternative to the Draize eye irritation test. *Toxicol. in Vitro*, 11:641–644.
165. Giovacchini, R.P. (1972): Old and new issues in the safety evaluation of cosmetics and toiletries. *CRC Crit. Rev. Toxicol.*, 1:361–378.
166. Goldberg, A.M., Frazier, J.M., Brusick, D., Dickens, M.S., Flint, O., Gettings, S.D., Hill R.N., Lipnick, R.L., Renskers, K.J., Bradlaw, J.A., et al. (1993): Framework for validation and implementation of in vitro toxicity tests. *In Vitro Cell. Dev. Biol.*, 29A:688–692.
167. Goldberg, A.M., Epstein, L.D., and Zurlo, J. (1995): A modular approach to validation: A work in progress. *In Vitro Toxicol.*, 8:431–435.
168. Gordon, V.C., and Bergman, H.C. (1987): EYTEX: An in vitro method for evaluation of ocular irritancy. In: *Alternative Methods in Toxicology, Vol. 5, Approaches to Validation*, edited by A.M. Goldberg, pp. 293–302. Mary Ann Liebert, New York,
169. Gordon, V.C., and Kelly, C.P. (1989): An in vitro method for determining ocular irritation. *Cosmet. Toilet.*, 104:69–73.
170. Grant, W.M. (1974): *Toxicology of the Eye,* 2nd ed. Charles C. Thomas, Springfield, Illinois.
171. Green, W.R., Sullivan, J.B., Hehir, R.M., et al. (1978): A systematic comparison of chemically-induced injury in the albino rabbit and rhesus monkey. Soap and Detergent Association, New York.
172. Griffith, J.F., Nixon, G.A., Bruce, R.D., et al. (1980): Dose-response studies with chemical irritants in the albino rabbit eye as a basis for selecting optimum testing conditions for predicting hazard to human eye. *Toxicol. Appl. Pharmacol.*, 55:501–513.
173. Guillot, J., Gonnet, J.F., and Clement, C. (1982): Evaluation of the ocular irritation potential of 56 compounds. *Food Chem. Toxicol.*, 20:573–582.
174. Gunderson T., and Liebman, S.D. (1944): Effect of local anesthetics on regeneration of corneal epithelium. *Arch. Ophthalmol.*, 31:29–33.
175. Gupta, K.C., Chambers, W.A., Green, S., Hill, R.N., Hurley, P. M., Lambert, L.A., Liu, P.T., Lowther, D.K., Seabaugh, V. M., Springer, J.A., and Wilcox, N.L. (1993): An eye irritation test protocol and an evaluation and classification system. *Food Chem. Toxicol.*, 31:117–121.

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176. Gurland, J., Lee, L., and Dahm, P.A. (1960): Polychotomous quantal response in biological assay. *Biometrics,* 16:382–398.
177. Hafeman, D.G., Parce, W.J., and McConnell, H.M. (1988): Light addressable potentiometric sensor for biochemical system. *Science*, 240:1182–1185.
178. Harriton, L. (1981): Conversation with Henry Spira: Draize test activist. *Lab. Anim.*, 10:16–22.
179. Hedbys, R.O., and Mishima, S. (1966): The thickness-hydration relationship of the cornea. *Exp. Eye Res.*, 5:221–228.
180. Henkes, H., and Canta, L.R. (1973): Drug-induced disorders of the eye. In: *Proceedings of the European Society for the Study of Drug Toxicity*, edited by W.A.M.Duncan, pp. 146–153. Elsevier, North-Holland, New York.
181. Heywood, R., and James, R.W. (1978): Towards objectivity in the assessment of eye irritation. *J. Soc. Cosmet. Chem.*, 29:25–29.
182. Hitzenberger, C.K., Drexler, W., and Fercher, A.F. (1992): Measurement of corneal thickness by laser doppler interferometry. *Invest. Ophthalmol. Vis. Sci.*, 33:98–103.
183. Holland, M.C. (1964): Fluorescein staining of the cornea. *JAMA*, 188:81.
184. Hull, D.S. (1979): Effects of epinephrine, benzalkonium chloride, and intraocular miotics on corneal endothelium. *S. Afr. Med. J.*, 2:1390–1381.
185. Hurley, P.M., Chambers, W.A., Green, S., Gupta, K.C., Hill,R. N., Lambert, L.A., Lee, C.C., Lee, J.K., Liu, P.T., Lowther,D. K., Roberts, C.D., Seabaugh, V.M., Springer, J.A., and Wilcox, N.L. (1993): Screening procedures for eye irritation. *Food Chem. Toxicol.*, 31:87–94.
186. Igarashi, H. (1987): The opacification of bovine isolated cornea by surfactants and other chemicals: A process of protein denaturation. *ATLA,* 15:8–19.
187. Igarashi, H., Katsuta, Y., Matsuno, H., Nakazato, Y., and Kawasaki, T. (1989): Carbachol-induced opacity of porcine isolated corneas. *ATLA,* 16:322–330.
188. Igarashi, H., Katsuta, Y., Matsuno, H., Nakazato, Y., and Kawasaki, T. (1989): Opacification test by using pig isolated cornea and its application to a test of corneal opacity induced by befunolol hydrochloride. *J. Toxicol. Sci.,* 14:91–103.
189. Igarashi, H., Katsuta, Y., Nakazato, Y., and Kawasaki, T. (1991): The opacifying effects of carteolol HCl and benzalkonium chloride on porcine isolated corneas. *ATLA,* 19:344–351.
190. Igarashi, H., and Northover, A.M. (1987): Increase in opacity and thickness induced by surfactants and other chemicals in the bovine isolated cornea. *Toxicol. Lett.*, 39(2–3):249–254.
191. Immonen, I., Siren, V., Stephens, R.W., Lietso, K., and Vaheri, A. (1993): Retinoids increase urokinase-type plasminogen activator production by human retinal pigment epithelial cells in culture. *Invest. Ophthalmol. Vis. Sci.*, 34:2062–2067.
192. IRLG (Interagency Regulatory Liaison Group) (1981): Recommended guidelines for acute eye irritation test.
193. Jacaruso, R.B., Bartlett, M.A., Carson, S., and Trombetta, L.D. D. (1985): Release of histamine from rat peritoneal cells as an in vitro index of irritation potential. *J. Toxicol. Cutan. Ocul. Toxicol.*, 4:39–48.
194. Jackson, J., and Rutty, D.A. (1985): Ocular tolerance assessment-integrated tier policy. *Food Chem. Toxicol.*, 23: 309–310.
195. Jester, J.V., Li, H., Petroll, W.M., Parker, R.D., Cavanagh, H. D., Carr, G.J., Smith, B., and Maurer, J.K. (1998): Area and depth of surfactant-induced corneal injury correlates with cell death. *Invest. Ophthalmol. Vis. Sci.*, 39:922–936.
196. Jester, J.V., Petroll, W.M., Garana, R.M.R., Lemp, M.A., and Cavanagh, H.D. (1992): Comparison of in vivo and ex vivo cellular structure in rabbit eyes detected by scanning confocal microscopy. *J. Microsc.*, 165:169–181.
197. Jumblatt, M.M., Fogle, J.A., and Neufeld, A.H. (1980): Cholera toxin stimulates adenosine 3′5′-monophosphate synthesis and epithelial wound closure in the rabbit cornea. *Invest. Ophthalmol. Vis. Sci.*, 19:1321–1329.
198. Jumblatt, M.M., and Neufeld, A.H. (1981): Characterization of cyclic AMP-mediated wound closure of the rabbit corneal epithelium. *Curr. Eye Res.*, 1:189–195.
199. Kahn, C.R., Young, E., Lee, I.H., and Rhim, J.S. (1993): Human corneal epithelial primary cultures and cell lines with extended life span. *Invest. Ophthalmol. Vis. Sci.*, 34:3429–3441.
200. Kalweit, S., Gerner, I., and Spielmann, H. (1987): Validation project of alternatives for the Draize eye test. *Mol. Toxicol.*, 1:579–603.
201. Kappler, R. and Kristen, U. (1987): Photometric quantitation of in vitro pollen tube growth: A new method suited to determine the cytotoxicity of various environmental substances. *Environ. Exp. Bot.*,

27:305–309.
202. Kay, J.H. and Calandra, J.C. (1962): Interpretation of eye irritation tests. *J. Soc. Cosmet. Chem.*, 13:281–289.
203. Kaye, G.I., and Tice, L.W. (1966): Studies on the cornea. V Electron microscopic localization of adenosine triphosphatase activity in the rabbit cornea in relation to transport. *Invest. Ophthalmol.*, 5:22–32.
204. Kemp, R.B., Cross D.M., and Meredith R.W.J. (1986): Adenosine triphosphate as an indicator of cellular toxicity. *Food Chem. Toxicol.*, 24:465–466.
205. Kemp, R.B., Meredith, R.W., and Gamble, S.H. (1985): Toxicity of commercial products on cells in suspension culture: A possible screen for the Draize eye irritation test. *Food Chem. Toxicol.*, 23:267–270.
206. Kemp, R.B., Meredith, R.W., Gamble, S., and Frost, M. (1983): A rapid cell culture technique for assessing the toxicity of detergent-based products in vitro as a possible screen for eye irritancy in vivo. *Cytobios*, 36:153.
207. Kennah, H.E., Hignet, S., Laux, P.E., Dorko, J.D., and Barrow, C.S. (1989): An objective procedure for quantitating eye irritation based on changes in corneal thickness. *Fundam. Appl. Toxicol.*, 12:258–268.
208. Kennedy, G.L., Jr., Ferenz, R., and Burgess, B.A. (1986): Estimation of acute oral toxicity in rats by determination of the approximate lethal dose rather than the LD50. *J. Appl. Toxicol.*, 6:145–148.
209. Khaw, P.T., Sherwood, M.B., MacKay, S.L.D., Rossi, M.J., and Shultz, G. (1992): Five-minute treatments with fluorouracil, floxuridine, and mitomycin have long-term effects on human tendon's capsule fibroblasts. *Arch. Ophthalmol.*, 110:1150–1154.
210. Kimura, S.J. (1951): Fluorescein paper: Simple means of insuring use of sterile fluorescein. *Am. J. Ophthalmol.*, 34:466.
211. Knox, P., Uphill, P.F., Fry, J.R., Benford, J., and Balls, M. (1986): The FRAME multicentre project on cytotoxicology. *Food Chem. Toxicol.*, 24:457–463.
212. Kong, B.M., Viau, C.J., Rizvi, P.Y., and DeSalva, S.J. (1987): The development and evaluation of the chlorioallantoic membrane (CAM) assay. In: *Alternative Methods in Toxicology, Vol. 5, Approach to Validation*, edited by A.M.Goldberg, pp. 59–73. Mary Ann Liebert, New York.
213. Kristen, U., Hoppe, U., and Pape, W. (1993): The pollen tube growth test: A new alternative to the Draize eye irritation test. *J. Soc. Cosmet. Chem.*, 44:153–162.
214. Kruszewski, F.H. (1998): Human cells as in vitro alternatives for ocular toxicity studies. *Comm. Toxicol.*, 6:221–233.
215. Kruszewski, F.H., Walker, T.L., and DiPasquale, L.C. (1997): Evaluation of a human corneal epithelial cell line as an in vitro model for assessing ocular irritation. *Fundam. Appl. Toxicol.*, 36:130–140.

216. Kuhlman, R.E. (1959): Species variation in the enzyme content of corneal epithelium. *J. Cell. Comp. Physiol.*, 53:313–326.
217. Kuijpers, M.H.M., and Walvoort, H.C. (1991): Discomfort and distress in rodents during chronic studies. In: *Animals in Biomedical Research*, edited by C.F.M.Hendriksen and H. W.B.M.Koeter, pp. 281–285. Elsevier, Amsterdam.
218. Lawrence, R.S., Groom, M.H., Ackroyd, D.M., and Parish,W. E. (1986). The chorioallantoic membrane in irritation testing. *Food Chem. Toxicol.*, 24:497–502.
219. Lee, D.A., Shapourifar-Tehrani, S., Stephenson, T.R., and Kitada, S. (1991): The effects of fluorinated pyrimidines FUR, FudR, FUMP, and FdUMP on human tendon's fibroblasts. *Invest. Ophthalmol. Vis. Sci.*, 32:2599–2609.
220. Leighton. J., Nassaurer, J., and Tehoa, R. (1985): The chick embryo in toxicology, An alternative to the rabbit eye. *Food Chem. Toxicol.*, 23:293–298.
221. Leupke, N.P. (1985): Hen's egg chorioallantoic membrane test for irritation potential. *Food Chem. Toxicol.*, 23:287–291.
222. Li, L., and Hoffman, R.M. (1991): Eye tissues grown in 3-dimensional histoculture for toxicological studies. *J. Cell. Pharmacol.*, 2:311–316.
223. Lieberman, H.R. (1983): Estimating LD50 using the probit technique: A basic computer program. *Drug Chem. Toxicol.*, 6:111–116.
224. Ling, T., Ho, A., and Holden, B.A. (1986): Method of evaluating ultrasonic pachymeters. *Am. J. Optom. Physiol Optics*, 63:462–466.
225. Lipnick, R.L., Cotruvo, J.A., Hill, R.N., Bruce, R.D., Stitzel, K. A., Walker, A.P., Chu, I., Goddard, M., Segal, L., Springer, J.A., and Myers, R.C. (1995): Comparison of the up-and-down, conventional LD50, and fixed dose acute toxicity procedures. *Food Chem. Toxicol.*, 33:223–231.
226. Litchfield, J.T., and Wilcoxon, F. (1949): A simplified method of evaluating dose-effect experiments. *J. Pharmacol. Exp. Ther.*, 96:99–115.
227. Lorke, D. (1983): A new approach to practical toxicity testing. *Arch. Toxicol.*, 54:275–287.
228. Maeda, K., and Sakagudin, K. (1965): Studies on sodium-potassium-activated adenosine triphosphatase in the cornea. Electron-microscopic observations on the rat cornea. *Jpn. J. Ophthalmol.*, 9:195–199.
229. Martin, S.A., Roy, T.A., Saladdin, K.A., Fleming, B.A., and Mackerer, C.R. (1994): Safety evaluation of petroleum products using an in vitro eye irritation test battery. *Toxicol. in Vitro*, 8:715–717.
230. Martins, T., Pauluhn, J., and Machemer, L. (1992): Analysis of alternative methods for determining ocular irritation. *Food Chem. Toxic.*, 30:1061–1067.
231. Marzulli, F.N. (1965): New data on eye and skin tests. *Toxicol. Appl. Pharmacol.*, 7:79–85.
232. Marzulli, F.N., and Simmon, M.E. (1971): Eye irritation from topically applied drugs and cosmetics: Preclinical studies. *Am. J. Optom.*, 48:61–79.
233. Masters, B., and Paddock, S. (1990): In vitro confocal imaging of the rabbit cornea. *J. Micros.*, 158:267–274.
234. Mauer, J.K., Li, H., Petroll, W.M., Parker, R.D., Cavanagh, H. D., and Jester, J.V. (1997): Confocal microscopic characterization of initial corneal changes of surfactant-induced eye irritation in the rabbit. *Toxicol. Appl. Pharmacol.*, 143:291–300.
235. Maurice, D.M. (1967): The use of fluorescein in ophthalmological research. *Invest. Ophthalmol.*, 6:465–477.
236. Maurice, D.M., and Giardini, A.A. (1951): A simple optical apparatus for measuring the corneal thickness, and the average thickness of the human cornea. *Br. J. Ophthalmol.*, 35:169–177.
237. Maurice, D.M., and Singh, T. (1986): A permeability test for acute corneal toxicity. *Toxicol. Lett.*, 31:125–130.
238. McCaa, C.S. (1985): Anatomy, physiology and toxicology of the eye. In: *Toxicology of the Eye, Ear, and Other Special Senses*, edited by A.Wallace Hayes, pp. 1–15. Raven Press, New York.
239. McDonald, T.O., Baldwin, H.A., and Beasley, C.H. (1973): Slit-lamp examination of experimental animal eyes. I. Techniques of illumination and the normal eye. *J. Soc. Cosmet. Chem.*, 24:163–180.
240. Mehlman, M.A., Pfitzer, E.A., and Scala, R.A. (1989): A report on methods to reduce, refine, and replace animal testing in industrial toxicology laboratories. *Cell Biol. Toxicol.*, 5:349–357.
241. Meier-Ruge, W. (1973): Eye toxicity. In: *Proceedings of the European Society for the Study of Drug Toxicity, Vol. 14*, edited by W.A.M.Duncan, pp. 133–145. Elsevier, North Holland, New York.
242. Miller, L.C. (1964): The quantal response in toxicity tests. In: *Statistics and Mathematics in Biology*, edited by O.Kempthorne, T.A. Bancroft, J.W.Gowen, and J.L.Lush, pp. 315–326. Hofner, New York.

243. Millodot, M., Lim, C.H., and Ruskell, G.L. (1978): A comparison of corneal sensitivity and nerve density in albino and pigmented rabbits. *Ophthal. Res.*, 307.
244. Minsky, M. (1988): Memoir on inventing the confocal scanning microscope. *Scanning*, 10:128–138.
245. Minsky, M. (1961): U.S. patent no. 30313467. Microscopy Apparatus. December 19, 1961.
246. Mishima, S. (1981): Clinical pharmacokinetics of the eye. *Invest. Ophthalmol. Vis. Sci.*, 21:504.
247. Mishima, S., and Hedbys, B.O. (1968): Measurement of corneal thickness with the Haag-Streit pachometer. *Arch. Ophthalmol.*, 80:710–713.
248. Mishima, S., and Kudo, T. (1967): In vitro incubation of rabbit cornea. *Invest. Ophthalmol. Vis. Sci.*, 6:329–339.
249. Mishima, S., and Maurice, D.M. (1971): In vivo determination of the endothelial permeability to fluorescein. *Acta Soc. Ophthalmol. (Japan)*, 765:236–243.
250. Moller-Pedersen, T., Li, H.F., Petroll, W.M., Cavanagh, H.D., and Jester, J.V. (1998): Confocal microscopic characterization of wound repair after photorefractive keratectomy. *Invest. Ophthalmol. Vis. Sci.*, 39:487–501.
251. Morgan, R.L., Sorenson, S.S., and Castles, T.R. (1987): Prediction of ocular irritation by corneal pachymetry. *Food Chem. Toxicol.*, 25:609–613.
252. Morton, D.B. (1995): The post-operative care of small experimental animals and the assessment of pain by score sheets. In: *Proceedings of Animals in Science Conference Perspective on their Use, Care and Welfare*, edited by N.E.Johnston, pp. 82–87. Monash University, Victoria, Australia.
253. Morton, D.B. (1997): A scheme for the recognition and assessment of adverse effects. In: *Animal Alternatives, Welfare and Ethics*, edited by L.F.M.van Zutphen and M.Balls, pp. 235–241. Elsevier, Amsterdam.
254. Morton, D.B., Burghardt, G., and Smith, J.A. (1990): Critical anthropomorphism, animal suffering and the ecological context. *Science and Ethics*, 20:13–19.
255. Morton D.B., and Griffiths, P.H.M. (1985): Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment. *Veterinary Record*, 116:431–436.
256. Morton, D.B., and Townsend, P. (1995): Dealing with adverse effects and suffering during animal research. In: *Laboratory Animals an Introduction for Experimenters*, edited by A.A.Tuffery, pp. 215–231. John Wiley & Sons, Ltd., Chichester, United Kingdom.

257. Moses, R.A., Parkinson, G., and Schuchardt, R. (1979): A standard large wound of the corneal epithelium in rabbits. *Invest. Ophthalmol. Vis. Sci.*, 18:103–106.
258. Muir, C.K. (1983): The toxic effect of some industrial chemicals on rabbit ileum in vitro compared with eye irritancy in vivo. *Toxicol. Lett.*, 19:309.
259. Muir, C.K. (1984): A simple method to assess surfactant-induced bovine corneal opacity in vitro: Preliminary findings. *Toxicol. Lett.*, 22:199–203.
260. Muir, C.K. (1985): Opacity of bovine cornea in vitro induced by surfactants and industrial chemicals compared with ocular irritancy in vivo. *Toxicol. Lett.*, 24:157–162.
261. Muir, C.K., Flower, C., and Van Abbe, N.J. (1983): A novel approach to the search for in vitro alternatives to in vivo eye irritancy testing. *Toxicol. Lett.*, 18:1–5.
262. Muller, H., and Kley, H.P. (1982): Retrospective study on the reliability of an "approximate LD50" determined with a small number of animals. *Arch. Toxicol.*, 51:189–196.
263. Myers, R.C., Ballentyne, B., Christopher, S.M., and Chun, J.S. (1998): Comparative evaluation of several methods and conditions for the in vivo measurement of corneal thickness in rabbits and rats. *Toxicol. Meth.*, 8:219–231.
264. Nagy, Z.Z., Suveges, I., and Nemeth, J. (1995–1996): Interoperative pachymetry during eximer photorefractive keratectomy. *Acta Chir. Hung.*, 35:217–223.
265. Nakajima, A., Kimura, T., and Yamazaki, M. (1967): Applications of ultrasound in biometry of the eye. In: *Ultrasonics in Ophthalmology Diagnostic and Therapeutic Applications*, edited by R. E.Goldberg and L.K.Sarin, pp. 124–144. W.B.Saunders, Philadelphia.
266. Nardone, R.M. (1989): The LD50 test and in vitro toxicology strategies. *Acta Pharmacol. Toxicol. (Copenhagen)*, 52(Suppl 2):65–79.
267. NAS Committee for Revision of NAS Publication 1138 (1977): Dermal and eye toxicity tests. In: *Principles and Procedures for Evaluating the Toxicity of Household Substances*, pp. 41–54. National Academy of Sciences, Washington, D.C.
268. Nguyen, K.D., and Lee, D.A. (1993): In vitro evaluation of antiproliferative potential of topical cyclo-oxygenase inhibitors in human tendon's fibroblasts. *Exp Eye Res.*, 57:97–105.
269. NIEHS. (1997): Validation and regulatory acceptance of toxicological test methods: A report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods. NIH Publ. No. 97–3981. Research Triangle Park, North Carolina.
270. North-Root, H., Yackovich, F., Demetrulias, J., Gacula, M., Jr., and Heinze, J.E. (1982): Evaluation of an in vitro cell toxicity test using rabbit corneal cells to predict the eye irritation potential of surfactants. *Toxicol. Lett.*, 14:207–212.
271. North-Root, H., Yackovich, F., Demetrulias, J., Gacula, M., Jr., and Heinze, J.E. (1985): Prediction of the eye irritation potential of shampoo using the in vitro SIRC cell toxicity test. *Food Chem. Toxicol.*, 23:271–273.
272. Nover, A., and Glanschneider, D. (1965): Untersuchungen uber die fortpflanzungsgeschwindigkeit und absorptiondes ultraschalls im Gewebe. Experimentelle beitrage zur ultraschalldiagnostik intraocular tumoren. *Albtecht von Graefes Arch. Klin. Exp. Ophthamol.*, 168:304–321.
273. OECD (1984): Data interpretation guides for initial hazard assessment of chemicals. OECD, Paris.
274. OECD (1996): Final report of the OECD workshop on harmonization of validation and acceptance criteria for alternative toxicological test methods. ENV/MC/CHEM/TG(96)9. OECD, Paris.
275. OECD (1981): OECD guidelines for testing of chemicals. OECD, Paris.
276. OECD (1987): OECD guideline for testing of chemicals. Guideline 405: Acute eye irritation/corrosion. OECD, Paris.
277. OECD (1992): OECD guideline for testing of chemicals. Guideline 420: Acute oral toxicity—Fixed dose method. OECD, Paris.
278. OECD (1996): OECD guideline for testing of chemicals. Guideline 423: Acute oral toxicity—Acute toxic class method. OECD, Paris.
279. OECD (1998): OECD guideline for testing of chemicals. Guideline 425: Acute oral toxicity—Up and down procedure. OECD, Paris.
280. OECD (1999): OECD series on testing and assessment. Detailed review on classification systems for eye irritation/corrosion in OECD member countries. ENV/JM/MONO(99)4. OECD, Paris.
281. OECD Test Guidelines (1981): Decision of the council concerning mutual acceptance of data in the assessment of chemicals. Annex 2. OECD Principles of Good Laboratory Practices. OECD, Paris.
282. Ohno, Y., Kaneko, T., Kobayaashi, T., Inoue, T., Kuroiwa, Y., Yoshida, T., Momma, J., Hayashi, M., Akiyama, J., Atsumi, T., Chiba, K., Endo, T., Fujii, A., Kakishima, H., Kojima, H., Masamoto, K., Masuda,

M., Matsukawa, S., Ohkoshi, K., Okada, J., Sakamoto, K., Takano, K., and Tanaka, A. (1994): First-phase validation of the in vitro eye irritation tests for cosmetic ingredients. *In Vitro Toxicol.*, 7:89–94.
283. Oksala, A., and Lehtinen, A. (1958): Measurement of the velocity of sound in some parts of the eye. *Acta Ophthalmologica*, 36:633–639.
284. Oliver, G.J.A., and Pemberton, M.A. (1985): An in vitro epidermal slice technique for identifying chemicals with potential for severe cutaneous effects. *Food Chem. Toxicol.*, 23:229–232.
285. Olson, K.J., Dupree, R.W., Plomer, E.T., and Rerve, V. (1962): Toxicological properties of several commercially available surfactants. *J. Cos. Cosmet. Chem.*, 13:469–476.
286. Paget, G.E., and Thomson, R. (1979): *Standard Operation Procedures in Toxicology.* MTP Press, Lancaster, United Kingdom.
287. Parce, J.W., Owicki, J.C., Keresco, K.M., Sigal, G.B., Wada, H. G., Muir, V.C., Bouse, L.J., Ross, K.I., Sikie, B.I., and McConnell, H.M. (1989): Detection of cell healing agents with a silicon biosensor. *Science*, 246:243–247.
288. Parish, W.E. (1985): Ability of in vitro (corneal injury-eye organ-and chorioallantoic membrane) tests to represent histopathological features of acute eye inflammation. *Food Chem. Toxicol.*, 23:215–227.
289. Patel, S., and Stevenson, R.W.W. (1994): Clinical evaluation of a portable ultrasonic and a standard optical pachometer. *Optom Vis. Sci.*, 71:43–46.
290. Petroll, W.M., Cavanagh, H.D., and Jester, J.V. (1993): Three-dimensional imaging of corneal cells using in vivo confocal microscopy. *J. Micros.*, 170:213–219.
291. Petroll, W.M., Cavanagh, H.D., Lemp, M.A., Andrews, P.M., and Jester, J.V. (1992): Digital image acquisition in in vivo confocal microscopy. *J. Micros.*, 165:61–69.
292. Petroll, W.M., Jester, J.V., and Cavanagh, H.D. (1994): In vivo confocal imaging: General principles and applications. *Scanning*, 16:131–149.
293. Petroll, W.M., Jester, J.V., and Cavanagh, H.D. (1996): Quantitative three-dimensional confocal imaging of the cornea in situ and in vivo: System design and calibration. *Scanning*, 18:45–49.
294. Pfister, R.R., and Burstein, N. (1976): The effects of ophthalmic drugs, vehicles, and preservatives on corneal epithelium: A scanning electron microscope study. *Invest. Ophthalmol.*, 15:246–258.
295. Polansky, J.R., Fauss, D.J., Hydorn, T., and Bloom, E. (1990): Cellular injury from sustained versus acute hydrogen peroxide exposure in cultured human corneal endothelium and human lens epithelium. *CLAO J.*, 16:S23–S28.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_913.html[2010/10/28 18:49:11]

296. Price. J.B., and Andrews, I.J. (1985): The in vitro assessment of eye irritancy using isolated eyes. *Food Chem. Toxicol.*, 23:313–315.
297. Price, J.B., Barry, M.P., and Andrews, I.J. (1986): The use of the chick chorioallantoic membrane to predict eye irritants. *Food Chem. Toxicol.*, 24:503–505.
298. Prince, J.H., Diesem, C.D., Eglitis, I., and Ruskell, G.L. (1960): *Anatomy and Histology of the Eye and Orbit in Domestic Animals.* Charles C Thomas, Springfield, Illinois.
299. Prinsen, M.K., and Koeter, H.B.W.M. (1993): Justification of the enucleated eye test with eyes of slaughterhouse animals as an alternative to the Draize eye irritation test with rabbits. *Food Chem. Toxicol.*, 31:69–76.
300. Protty, C., and Ferguson, T.F.M. (1976): The effects of surfactants upon rat peritoneal mast cells in vitro. *Food Cosmet. Toxicol.*, 14:425.
301. Rachui, S.R., Robertson, W.D., Duke, M.A., and Heinze, J. (1994): Predicting the ocular irritation potential of surfactants using the in vitro Skin2 model ZK 1200. *J. Toxicol. Cutan. Ocul. Toxicol.*, 13:215–220.
302. Rachui, S.R., Robertson, W.D., Duke, M.A., Paller, B.S., and Ziets, G.A. (1994): Predicting the ocular irritation potential of cosmetics and personal care products using two in vitro models. *In Vitro Toxicol.*, 7:45–52.
303. Reader, S.J., Blackwell, V., O'Hara, R., Clothier, R.H., Griffin, G., and Balls, M. (1989): A vital dye release method for assessing the short-term cytotoxic effects of chemicals and formulations. *ATLA*, 17:28–33.
304. Reiger, M.M., and Battista, G.W. (1964): Some experiences in the safety testing of cosmetics. *J. Soc. Cosmet. Chem.*, 15:161–172.
305. Reinhardt, C.A., Pelli, D.A., and Zbinden, G. (1985): Interpretation of cell toxicity data for the estimation of potential irritation. *Food Chem. Toxicol.*, 23:247–252.
306. Rhode, B.H. (1992): In vitro methods in ophthalmic toxicology. In: *Ophthalmic Toxicology,* edited by G.C.Y.Chiou, pp. 106–165. Raven Press, New York.
307. Rivera, A., and Sanna, G. (1962): Determiniazione della velocita degli ultrasuoni nei tessuti oculari di uomo et di maiale. *Annali di Ottalmologia e Clinica Oculistics*, 88:675–682.
308. Roeig, D.L., Hasegawa, A.I., Harris, G.J., Lynch, K.L., and Wang, R.I.H. (1980): Occurrence of corneal opacities in rats after acute administration of 1-alpha-acetylmethadol. *Toxicol. Appl. Pharmacol.*, 56:155–163.
309. Roll, R., Hoffer-Bosse, T., and Kayser, D. (1986): New perspectives in acute toxicity testing of chemicals. *Toxicol. Lett.,* (Suppl. 31):86.
310. Romani, A., Puccioni, M., Tramontana, M., Del Bianco, E., and Geppetti, P. (1992): Release of proinflammatory neuropeptide (calcitonin gene-regulated) from capsaicin-sensitive, nerve fibers of the iris and ciliary body in humans. *Chibret Int. J. Ophthalmol.*, 9:3–9.
311. Rosiello, A.P., Essigmann, J.M., and Wogan, G.N. (1977): Rapid and accurate determination of the median lethal dose (LD50) and its error with a small computer. *J. Toxicol. Environ. Health*, 3:797–809.
312. Rothman, B., Despins, A., Webb, D., Taylor, D., Sundarraj, N., O'Rourke, J., and Kreutzer, D. (1991): Cytokine regulation of C3 and C5 production by human corneal fibroblasts. *Exp. Eye Res.*, 53:353–361.
313. Rowan, A. (1981): The Draize test: Political and scientific issues. *Cosmet. Tech..* 3(7):32–48.
314. Roy, T.A., Saladdin, K.A., and Mackerer, C.R. (1994): Evaluation of the EYTEX system as a screen for eye irritatancy of petroleum products. *Toxicol. in Vitro*, 8:197–198.
315. Salz, J.J., Azen. S.P., Berstein, J., Caroline, P., Villasenor, R.A., and Schanzlin, D.J. (1983): Evaluation and sources of variability in the measurement of corneal thickness with ultrasonic and optical pachymeters. *Ophthal. Surg.*, 14:750–754.
316. SAS User's Guide: Statistics. SAS Institute Inc. Cary, North Carolina.
317. Scaife, M.C. (1982): An investigation of detergent action on cells in vitro and possible correlations with in vivo data. *Int. J. Cosmet. Sci.*, 4:179.
318. Scaife, M.C. (1985): An in vitro cytotoxicity test to predict the ocular irritation potential of detergents and detergent products. *Food Chem. Toxicol.*, 23:253–258.
319. Schlede, E., Mischke, U., Diener, W., and Kayser, D. (1994): The international validation study of the acute toxic class method (oral). *Arch. Toxicol.*, 69:659–670.
320. Schlede, E., Mischke, U., Roll, R., and Kayser, D. (1992): A national validation study of the acute toxic class method—An alternative to the LD50 test. *Arch. Toxicol.*, 66:455–470.
321. Schutz, E., and Fuchs, H. (1982): A new approach to minimizing the number of animals used in

acute toxicity testing and optimizing the information of test results. *Arch. Toxicol.*, 51:197–220.
322. Seabaugh, V.M., Osterberg, R.E., Hoheisel, C.A., Murphy, J.C., and Bierboer, G.W. (1976): A comparative study of rabbit ocular reactions of various exposure times to chemicals. *Society of Toxicology, Fifteenth Annual Meeting*, Atlanta.
323. Selling, J., and Ekwall, B. (1985): Screening for eye irritancy using cultured Hela cells. *Xenobiotica*, 15:8–9.
324. Shadduck, J.A., Everitt, J., and Bay, P.H.S. (1985): Use of in vitro cytotoxicity to rank ocular irritation of six surfactants. In: *Alternative Methods in Toxicology, Vol. 3, In Vitro Toxicology*, edited by A.M.Goldberg, pp. 641–649. Mary Ann Liebert, New York.
325. Shadduck, J.A., Render, I., Everitt, J., Meccoli, R.A., and Essex-Sorlie, D. (1987): An approach to validation: Comparison of six materials in three tests. In: *Alternative Methods in Toxicology, Vol. 5, In Vitro Toxicology*, edited by A.M.Goldberg, pp. 75–78. Mary Ann Liebert, New York.
326. Shapiro, H. (1956): Setting and dissolution of the rabbit cornea in alkali. *Am. J. Ophthalmol.*, 42:292–298.
327. Shaw, J.A., Clothier, R.H., and Balls, M. (1990). Loss of transepithelial impermeability of a confluent monolayer of Madin-Darby canine kidney (MDCK) cells as a determinant of ocular irritancy potential. *ATLA*, 18:145–151.
328. Shopsis, C. (1984): Inhibition of uridine uptake in cultured cells: A rapid, sublethal cytotoxicity test. *J. Tissue Culture Meth.*, 9:19.
329. Shopsis, C., Borenfreund, E., Walberg, J., and Stark, D.M. (1985): A battery of potential alternatives to the Draize test: Uridine uptake inhibition, morphological cytotoxicity, macrophage chemotaxis, and exfoliative cytology. *Food Chem. Toxicol.*, 23:259–266.
330. Shopsis, C., and Eng, B. (1985): Rapid cytotoxicity testing using a semi-automated protein determination on cultured cells. *Toxicol. Lett.*, 26:1–8.
331. Shopsis, C., and Sathe, S. (1984): Uridine uptake inhibition as a cytotoxicity test: Correlation with the Draize test. *Toxicology*, 29:195–206.
332. Silverman, J.T., and Pennisi, S. (1987): Evaluation of *Tetrahymena thermophila* as an in vitro alternative to ocular irritation studies in rabbits. *J. Toxicol. Cutan. Ocul. Toxicol.*, 6:33–42.
333. Simmons, S.J., Jumblatt, M.M., and Neufeld, A.H. (1987): Corneal epithelial wound closure in tissue culture. An in vitro model of ocular irritancy. *Toxicol. Appl. Pharmacol.*, 88:13–23.
334. Sina, J. (1994): Validation of the bovine corneal opacitypermeability assay as a predictor of ocular irritation potential. *In Vitro Toxicol.*, 7:283–289.
335. Sina, J., Gautheron, P., Casterton, P., Evans, M.G., Harbell, J. W., Curren, R.D., Earl, L., and Bruner, L. (1998): Report from

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_914.html[2010/10/28 18:49:12]

the bovine corneal opacity and permeability technical workshop, November 3–4, 1997, Gaithersburg, Maryland. *In Vitro and Molec. Toxicol.*, 11:315–353.
336. Smyth, R.J., Nguyen, K., Ahn, S.S., Panck, W.C., and Lee, D.A. (1993): The effects of Photofrin on human tendon's capsule fibroblasts in vitro. *J. Ocular Pharmacol.*, 9:171–178.
337. Society of Agricultural Chemical Industry (1985): Agricultural Chemicals Laws and Regulations. Japan(II) (English translation).
338. Society of Toxicology Animal in Research Committee (1989): SOT position paper comments on LD50 and acute eye and skin irritation tests. *Fund. Appl. Toxicol.*, 13:621–623.
339. Society of Toxicology of Canada (1985): Position paper on the LD50. Adopted at the STC annual meeting on December 3, 1985.
340. Sperling, F. (1976): Nonlethal parameters as indices of acute toxicity: Inadequacy of the acute LD50. In: *New Concepts in Safety Evaluation*, edited by M.A.Mehlman, R.E.Shapiro, and H. Blumenthal, pp. 177–191. Hemisphere, Washington, D.C.
341. Spielmann, H., Kalweit, S., Liebsch, M., Wirnsberger, T., Gerner. I., Bertram-Neis, E., Krauser, K., Kreiling, R., Miltenburger, H.G., Pape, W., and Steiling, W. (1993): Validation study of alternatives to the Draize eye irritation test in Germany: Cytotoxicity testing and HET–CAM test with 136 industrial chemicals. *Toxicol. in Vitro*, 7:505–510.
342. Stark, D.M., Borenfreund, E., Walberg, J., and Shopsis, C. (1985): Comparison of several alternative assays for measuring potential toxicants. In vitro toxicology: A progress report from the Johns Hopkins Center for Alternatives to Animal Testing. In: *Alternative Methods in Toxicology, Vol. 3*, edited by A.M.Goldberg. pp. 371–390. Mary Ann Liebert, New York.
343. Stark, D.M., Shopsis, C., Borenfreund, E., and Babich, H. (1986): Progress and problems in evaluating and validating alternative assays in toxicology. *Food Chem. Toxicol.*, 24:449–455.
344. Stark, D.M., Shopsis C., Borenfreund, E., and Walberg, J. (1983): *Alternative Approaches to the Draize Assay: Chemotoxicity, Cytology, Differentiation and Membrane Transport Studies in Product Safety Evaluation*, edited by A.M.Goldberg, p. 179. Mary Ann Liebert, New York.
345. Sugai, S., Murata, K., Kitagaki, T., and Tomita, I. (1990): Studies on eye irritation caused by chemicals in rabbits. I. A quantitative structure-activity relationship approach to primary eye irritation of chemicals in rabbits. *J. Toxicol. Sci.*, 15:245–262.
346. Sugar, J. (1980): Corneal examination. In: *Principles and Practice of Ophthalmology, Vol. 1*, edited by G.A.Peyman, D.R.Sanders, and M.F.Goldberg, pp. 393–395. W.B.Saunders, Philadelphia.
347. Swanton, D.W. (1983): Eye irritancy testing. In: *Animals and Alternatives in Toxicity Testing*, edited by M.Balls, R.J.Ridell, and A.N.Worden, p. 337. Academic Press, London.
348. Takahashi, N., and Ikoma, N. (1990): The cytotoxic effect of 5-fluorouracil on cultured human conjunctival cells. In: *Ocular Toxicology*, edited by S.Lerman and R.C.Tripathi, pp. 157–166. Marcel Dekker, New York.
349. Talsma. D.M., Leach, C.L., Hatoum, N.S., Gibbons, R.D., Roger, J.C., and Garvin, P.J. (1988). Reducing the number of rabbits in the Draize eye irritancy test: A statistical analysis of 155 studies conducted over 6 years. *Fund. Appl. Toxicol.*, 10:146–153.
350. Tanaka, N., Ohkawa, T. , Hiyama, T., and Nakajima, A. (1982): Evaluation of ocular toxicity of two beta blocking drugs, cereteolol and practolol, in beagle dogs. *J. Pharmacol. Exp. Ther.*, 224:424–430.
351. Tchao, R. (1988): Trans-epithelial permeability of fluorescein in vitro as an assay to determine eye irritants. In: *Alternative Methods in Toxicology, Vol. 6. Progress in In Vitro Toxicology*, edited by A.M.Goldberg, pp. 271–283. Mary Ann Liebert, New York.
352. Terry, M.A., and Ousley, P.J. (1996): Variability in corneal thickness before, during, and after radial keratotomy. *J. Refract. Surg.*, 12:700–704.
353. Thompson, W.R. (1947): Use of moving averages and interpolation to estimate median effective dose. *Bacterial. Rev.*, 11:115–145.
354. Tonjum, A.M. (1975): Effects of benzalkonium chloride upon the corneal epithelium: Studies with scanning electron microscopy. *Acta Ophthalmol.*, 53:358–366.
355. Trevan, J.W. (1927): The error of determination of toxicity. *Proc. R. Soc. Lond.*, 101B:483–514.
356. Tripathi, B.J., Tripathi, R.C., and Kolli, S.P. (1992): Cytotoxicity of ophthalmic preservatives on human corneal epithelium. *Lens Eye Toxicol. Res.*, 9:361–375.
357. Tripathi, B.J., Tripathi, R.C., and Millard, C.B. (1990): Epinephrine-induced toxicity of human trabecular cells in vitro. In: *Ocular Toxicology*, edited by S.Lerman and R.C.Tripathi, pp. 141–156. Marcel Dekker, New York.
358. Ubels, J.L., Erickson, A.M., Zylstra, U., Kreulen, C.D., and Casterton, P.L. (1998): Effect of

hydration on opacity in the bovine corneal opacity and permeability (BCOP) assay. *J. Toxicol. Cutan. Ocul. Toxicol.*. 17:197–220.
359. Van den Heuvel, M.J., Clark, D.G., Fielder, R.J., Koundakjian, P.P., Oliver, G.J.A., Pelling, D., Thomlison, N.J., and Walker. A.P. (1990). The international validation of a fixed dose procedure as an alternative to the classical LD50 test. *Food Chem. Toxicol.*, 28:469–482.
360. Van den Heuvel, M.J., Dayan, A.D., and Shillaker, R.O. (1987): Evaluation of the BTS approach to the testing of substances and preparations for their acute toxicity. *Human Toxicol.*, 6:279–291.
361. Van Erp, Y.H.M., and Weterings, P.J. (1990): Eye irritancy screening for classification of chemicals. *Toxicol. in Vitro*, 4:267.
362. Vanparys, P., Deknudt, G., Sysmans, M., Teuns, G., Coussement, W., and VanCauteren, H. (1993): Evaluation of the bovine opacity-permeability assay as an in vitro alternative to the Draize eye irritation test. *Toxicol. in Vitro*. 7:471–476.
363. Villasenor, R.A., Santos, V.R., Cox, K.C., Harris, D.F., Lynn, M., and Waring, G.O. (1986): Comparison of ultrasonic corneal thickness measurements before and during surgery in the prospective evaluation of Radial Keratotomy (PERK) Study. *Ophthalmol.*, 93:327–330.
364. Walberg, J. (1983): Exfoliative cytology as a refinement of the Draize eye irritancy test. *Toxicol. Lett.*, 18:49.
365. Wallin, R.F., Hume R.D., and Jackson, E.M. (1987): The agarose diffusion method for ocular irritancy screening: Cosmetic products. Part 1. *J. Toxicol. Cutan. Ocul. Toxicol.*, 6:239–250.
366. Walum, E., Clemedsen, C., and Ekwall, B. (1994): Principles for the validation of in vitro toxicology test methods. *Toxicol. in Vitro*, 8:807–812.
367. Waltman, S.R., and Kaufman, H.E. (1970): In vivo studies of human corneal and endothelial permeability. *Am. J. Ophthalmol.*, 70:45–47.
368. Ward, S.L., Kahn, C.R., Walker, T.L., and Dimitrijivch, S.D. (1994): Response and recovery of a human corneal epithelial cell line to chemical insult. *Invest. Ophthalmol. Vis. Sci.*, 35:1942.
369. Ward, S.L., Walker, T.L., and Dimitrijivch, S.D. (1997): Evaluation of chemically induced toxicity using an in vitro model of human corneal epithelium. *Toxicol. In Vitro*, 11:121–139.
370. Watanabe, M., Watanabe K., Zuzuki, K., Nikaide, O., Ishii, I., Konishi, H., Tanaka, N., and Sugahara, T. (1989): Use of primary rabbit cornea cells to replace the Draize rabbit eye irritancy test. *Toxicol. in Vitro*, 3:329–334.
371. Waud, D.R. (1972): On biological assays involving quantal responses. *J. Pharm. Exp. Ther.*, 183:577–607.

372. Weil, C.S. (1952): Tables for convenient calculation of median effective dose (LD50 or ED50) and instruction in their use. *Biometrics*, 8:249–263.
373. Weil, C.S. (1983): Economical LD50 and slope determinations. *Drug Chem. Toxicol.*, 6:595–603.
374. Weil, C.S., and Scala, R.A. (1971): Study of intra- and interlaboratory variability in the results of rabbit eye and skin irritation tests. *Toxicol. Appl. Pharmacol.*, 19:276–360.
375. Weltman, A.S., Sharber, S.B., and Jurtshuk, T. (1968): Comparative evaluation and influence of various factors on eye irritation tests. *Toxicol. Appl. Pharmacol.*, 7:308–319.
376. Weterings, P.J., and Van Erp, Y.H.M. (1987): In vitro toxicology approaches to validation. In: *Alternative Methods in Toxicology, Vol 5*, edited by A.M.Goldberg. Mary Ann Liebert, New York.
377. Wheeler, N.C., Morantes, C.M., Kristensen, R.M., Petit, T.H., and Lee, D.A. (1992): Reliability coefficients of three corneal pachymeters. *Am. J. Ophthalmol.*, 113:645–651.
378. Williams. S.J. (1984): Prediction of ocular irritancy potential from dermal irritation test results. *Food Chem. Toxicol.*, 22: 157–161.
379. Williams, S.J. (1985): Changing concepts of ocular irritation evaluation: Pitfalls and progress. *Food Chem. Toxicol.*, 23:189–193.
380. Williams, S.J., Grapel, G.J., and Kennedy, G.I. (1982): Evaluation of ocular irritancy: Potential intralaboratory variability and effect of dosage volume. *Toxicol. Lett.*, 12:235–241.
381. Williams, D.E., Nguyen, K.D., Shapourifar-Tehrani, S., Kitada, S., and Lee, D.A. (1992): Effects of Timolol, Betaxolol, and Levobunolol on human tendon's fibroblasts in tissue culture. *Invest. Ophthalmol. Vis. Sci.*, 33:2233–2241.
382. York, M., Lawrence, R.S., and Gibson, G.B. (1982): An in vitro test for the assessment of eye irritancy in consumer products—preliminary findings. *Int. J. Cosmet. Sci.*, 44:223.

Chapter **19**

SHORT-TERM, SUBCHRONIC, AND CHRONIC TOXICOLOGY STUDIES

Nelson H.Wilson, Jerry F.Hardisty, and Johnnie R.Hayes

Principles and Methods of Toxicology,
Fourth Edition, edited by A.Wallace Hayes.
Taylor & Francis, Philadelphia © 2001.

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REPEATED DOSE STUDIES AND HAZARD (SAFETY) ASSESSMENT

Repeated dose toxicity studies are conducted to screen for potential adverse effects of a chemical, using labora-tory animals as surrogates for a target species, most often the human. Repeated dose studies may be of varying duration, generally 1 to 4 weeks for short-term studies, 3 months for subchronic studies, and 6–12 months for chronic studies. Many variables associated with the health of the test species are monitored in short-term, subchronic, and chronic toxicity studies, resulting in the ability to detect a variety of adverse effects. Sometime during their career, most toxicologists are involved in the design, performance, or review of data from these toxicology studies. This results from the central role played by these studies in the safety assessment of Pharmaceuticals, pesticides, food additives, and other chemicals. It has been suggested that subchronic data alone may be sufficient to predict the hazard of long-term, low-dose exposure to a compound (50). Even though this may be true for compounds where adequate structure-activity relationships exist, it generally is not true when compounds have completely unknown toxicity or when structure-activity relationships predict a poten-tial adverse effect. For certain chemicals or mixtures, results from a short-term or a subchronic toxicity study may represent the most sophisticated toxicology data available. With many chemicals, a subchronic study is critical to the design of longer-term hazard assessment studies.

It is essential that toxicologists become familiar with the scientific principles upon which repeated dose toxicology studies are based and understand the method-ology used to perform these studies. This chapter provides an introduction to these studies and some of the principles upon which they are based. A typical hazard assessment program is illustrated in Figure 19.1. However, in the practice of toxicology, there is no such thing as a "typical" hazard assessment

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Test Material Identification
Chemical Characterization
Literature Review
Structure/Activity Assessment
Short-Term Animal Studies (Acute/Short-Term Repeated Dose)
In vitro Genetic Toxicology
Metabolism/Initial Pharmacokinetics
Subchronic Toxicity
Reproductive/Teratology
Chronic Toxicity
Oncogenicity
Special Studies

FIG. 19.1. Schematic representation of a typical hazard assessment program illustrating a step-by-step, tiered approach and the interactions between the various elements. The approach presented here is not the only approach that could be used.

program. Each program is unique, based upon the indi-vidual material being tested and its intended use.

The first step in any hazard assessment is determination of the material to be studied and the purpose of the assessment. Both these factors will influence the final design of the program. At the time of selection of the material to be tested, its intended use in the marketplace will have been defined, dictating, in most cases, factors such as exposure route and the types of studies required.

The next step is the chemical and physical characteriza-tion of the material or mixture to be investigated. Knowl-edge of the major component(s) and any associated contaminants is required for almost every phase of the hazard assessment program. The more detailed the chemical and physical characterization, the greater the assurance that there will not be unexpected interferences from impurities.

Once the toxicologist has information concerning the chemical nature of the *test material*, the scientific litera-ture is searched to determine what, if anything, is known about its biological activity. If no information is available, then the potential biological activity of

Table 19.1 Objectives of short-term repeated dose studies

- Determine adverse effects of the test compound at doses low enough to allow survival of most animals, as opposed to acutely toxic doses
- Determine adverse effects over a longer exposure (dosing) period than used in acute studies
- Determine dose response for adverse effects following repeated dosing and identify no observable adverse effect level (NOAEL)
- Identify target organs
- Provide data concerning species differences, if any, in sensitivity to potential adverse effects
- Provide initial data for comparative risk assessment
- Determine need for specialized endpoints to be assessed in longer-term studies

chemicals with similar chemical structure should be ascertained. Based upon the chemical characterization data and any toxicology information available in the scientific literature, the toxicologist can then perform a structure-activity assessment. This will aid in developing the hazard assessment program and the specific designs for each of the toxicology studies.

In most cases, the first series of studies will be short-term toxicity studies. The initial short-term toxicity study is generally a single dose, *acute* toxicity study. Results from acute toxicity studies are used to estimate dosages to be used in short-term repeated dose studies and obtain initial data useful in assessing relative toxicity. A number of regulatory agencies have made recommendations concerning designs for acute studies as discussed in the acute toxicity chapter of this text. Additional variables may be added to these recommended designs based upon the structure-activity assessment and litera-ture review, if warranted.

Data obtained from short-term repeated dose studies are generally required for the successful design of subchronic toxicity studies. Similarly, data from subchronic studies are essential for design of chronic studies. Table 19.1 lists objectives for short-term repeated dose toxicity studies. Table 19.2 lists examples of data obtained from these studies that are useful in the design of subsequent toxicity studies. As is discussed later, one of the difficult decisions for a toxicologist designing a toxicology study is selection of the dose range to be used. Dose response relationships for toxicological endpoints evaluated in short-term toxicology studies are critical to the successful design of subsequent toxicity studies.

Although not always available, data from genetic toxicology and metabolism studies are useful adjuncts to data from short-term repeated dose studies. For

Table 19.2 Data obtained from short-term repeated dose studies that are useful in the design of subsequent studies

- Palatability of test material/diet mixture, if dosing is by feed
- Body weight response patterns
- Physical observations
- Observed behavior changes
- Clinical pathology
- Gross necropsy
- Histopathology
- Identification of target organs
- Dose responses

instance, if the in vitro phase of the genetic toxicology program has indicated that the test material has genotoxic potential, it may be dropped from consideration for further development and further testing may be unnecessary. On the other hand, in vivo genetic toxicology studies can be added to the design of subchronic or chronic toxicology studies. This can, in many cases, reduce the number of animals used in the overall safety assessment and conserve other resources. If infor-mation concerning the metabolism and pharmacokinetics of the test material is known, it should be used in the design of repeated dose toxicity studies. For example, if pharmacokinetic studies indicate a potential for accumulation of the test material from multiple dosing, this is an important consideration in deciding the most appropriate dose range. Also, because different species may metabolize the test material differently, this infor-mation is important in selection of appropriate animal species and strains for subsequent testing. The major objectives of subchronic toxicity studies are presented in Table 19.3.

In some cases, subchronic toxicity testing may com-plete the data required for a hazard assessment. In other cases, as illustrated in Figure 19.1, data from subchronic studies are used in the design of additional studies, includ-ing chronic toxicity studies. Data from subchronic toxicity studies are useful in the design of oncogenicity and reproduction studies as well as any “special” studies that may be either

warranted by the results of other studies or requested by a regulatory agency.

As noted above, data from a short-term repeated dose study should be available before initiation of subchronic toxicity testing and, generally, data should be available from a subchronic study before proceeding with chronic testing. The major utility in progression from shorter-term to longer-term studies is to insure, to the extent possible, that proper dose ranges are selected. Requirements for a scientifically valid short-term repeated dose study are similar to those of subchronic

Table 19.3 Objectives of subchronic toxicology studies

- Identify adverse effects not detected in shorter-term acute or repeated dose studies
- Provide additional information on adverse effects identified in short-term studies
- Identify observable effect level and the NOAEL
- Provide data for dose selection and other study design features for chronic toxicity and other longer-term studies
- Confirm and/or identify target organs or sites of action
- Provide data to determine if specialized endpoints are required
- Provide a basis for species selection for additional studies, if required, and for data extrapolation to humans
- Provide information for regulatory agencies in support of the safety of the test material
- Provide risk assessment data

toxicity studies. Therefore, the general aspects of short-term studies will not be discussed separately. However, three aspects of short-term repeated dose studies need to be mentioned.

First, as implied by the description "short-term repeated dose study," these studies are of shorter duration than subchronic toxicity studies. Generally, the duration of these studies is either 14 or 28 days with compound administered daily. A 28-day study can pro-duce more valuable information than a 14-day study because the longer-term exposure increases the probabil-ity of detecting more slowly developing adverse effects-assuming the dose-range tested is similar.

Second, dose selection is somewhat dependent upon the purpose of the study. If the short-term dosing is designed to produce information to be used in the design of a subchronic study, then dosing should be high enough to ensure any potential adverse effect is observed. Higher doses are generally used in short-term studies to deter-mine target organs and it may not be as critical to ascer-tain a no observable adverse effect level (NOAEL). However, it is always useful to have a NOAEL in a toxicology study. If no further studies are anticipated with the compound, a dose range that includes a NOAEL becomes more important. Because little information other than acute toxicity data is generally available, it may be necessary to run a more comprehensive range of dose levels in short-term repeated dose studies. Four, five, or more dose groups are generally used in short-term studies. This increases the chances of defining the dose response and should increase the confidence of dose range selection for subsequent studies.

A third aspect of short-term studies that is different from subchronic studies is the number of animals per group. Although fewer animals may be used, 10 animals of each gender in each dose group is the recommendation for short-term rodent studies. Additional animals may be required for non-routine endpoints, such as a group added to ascertain the reversibility of an adverse effect upon cessation of dosing. Additional animals also may be added to the high-dose group if potential com-pound-related mortality is expected.

Many other factors influence the design of all repeated dose toxicity studies. Before initiation of a study, it must be decided if the specific chemical or chemical mixture—that is, the test material—to be used in the study is appropriate based on its purity and other chemi-cal characteristics. Appropriate animal models must be chosen, the correct route of exposure must be selected, and the study duration must be decided. Control and treatment groups and their doses have to be selected. Variables to be evaluated must be selected to maximize the probability of detecting potential adverse effects.

Regulatory Requirements

National and international regulatory bodies have issued guidelines for the design and conduct of repeated dose toxicity studies. Even though study designs pro-scribed by these various guidelines have similar characteristics, some differences between the require-ments have existed within and between regulatory agencies. This has resulted, in some cases, in the duplication of studies to ensure guidelines of different agencies are satisfied. The agencies themselves and the regulated community have considered this duplication of the use of animals and other resources undesirable. As a result, regulatory authorities worldwide are attempting to harmonize their guidelines to ensure toxicity studies conducted under a single set of regu-lations will be universally acceptable. In the United States, the Environmental Protection Agency (EPA) issued a single set of harmonized toxicity testing guide-lines in 1997 (17) to blend the requirements of the separate guidelines previously promulgated through the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), the Toxic Substances Control Act (TSCA), and the Organization for Economic Cooperation and Develop-ment (OECD).

OECD, an international organization of 29 countries including the United States, has issued its own toxicity testing guidelines (54). Each member country has agreed to accept studies conducted according

to these guidelines. In 1991, regulatory authorities and trade organizations from the United States, Japan, and the European Union initiated a cooperative effort through the International Conference on Harmonization (ICH) to produce

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guidance documents concerning requirements for safety studies with pharmaceutical products. ICH guidance documents generally address specific issues related to toxicity testing, for example, the duration of chronic toxicity testing (43), and are not detailed guidelines for protocol design.

Each regulatory harmonization effort discussed above has been useful. However, a well-planned, scientifically valid, adequately conducted, repeated dose study should satisfy the requirements of any regulatory agency. The scientific foundation for toxicological hazard assessment is continually expanding and to compromise this foun-dation by pursuing standard checklist protocols is irrational, wasteful, and unscientific. Today, regulations generally recognize the importance of scientific judgment and encourage discussions between the regulated com-munity and the regulator about alternative study designs. Investigator and regulator each share in the obligation to do a scientifically sound study.

This chapter focuses on hazard assessment for chemicals that are small molecules. Today, biotechnology is a rapidly developing field. Hazard assessments for biologicals—for example, recombinant proteins—are a special case that will not be discussed in detail in this chapter. Suffice it to say that guidelines for testing such materials are under development. The hazard assessment for a biological is tailored specifically to the compound and may be more or less comprehensive than a classical assessment with a small-molecule compound. However, many of the principles and methods of individual studies with small molecules are applicable to testing with biotechnology products. The toxicologist responsible for investigating the safety of these materials should become familiar with the current guidelines and suggestions for their testing.

The balance of this chapter relates to the typical design and conduct of subchronic and chronic repeated dose toxicity studies. The information provided should suffice to satisfy most regulatory guidelines. Variables deter-mined in subchronic and chronic toxicity studies are essentially identical, the major difference being the sched-ule for data collection. Table 19.4 compares the minimum requirements for subchronic and chronic toxicity studies using rodents as described in the EPA (17), U.S. Food and Drug Administration (FDA) (23), and OECD (54) testing guidelines. For repeated dose study designs intended to satisfy guidelines of a particular regulatory authority, it is recommended that the reader:

(1) use the testing guidelines of that authority as a starting point during the design of a study,
(2) refine the design based upon the compound of interest,
(3) document the design in a detailed, written protocol, and
(4) discuss the protocol with a representative of the regulatory authority prior to initiating a study.

Good Laboratory Practice Regulations

In addition to regulations and guidelines concerning the design of repeated dose studies, U.S. and international regulatory authorities have issued good laboratory prac-tice (GLP) regulations or principles concerning the manner in which all nonclinical hazard assessment studies (e.g., animals studies) are to be conducted, documented, and reported (11, 19, 36, 46–48, 56, 60). Thes e G designed to ensure the quality of the study data and report. Requirements from each of the regulatory agencies are similar. GLPs set standards for the *test system,* laboratory organization, personnel, facilities, equipment, operations, and record keeping. They require that studies be conducted according to written protocols (see Table 19.5) and validated, written standard operating procedures (SOPs). Chemical analyses are required to characterize the *test article* and control article adminis-tered during the studies, and study procedures and data must be clearly and completely documented and reported. Regulatory agencies inspect testing laboratories to assure GLP compliance. Safety studies not conducted according to GLPs will not be accepted by regulatory agencies, and some versions of GLPs, including those of the U.S. regu-latory agencies, provide for disqualification of labora-tories that are in major noncompliance with GLP requirements. For these reasons, laboratories conducting studies for regulatory submission generally take great care to comply with GLPs.

STUDY DESIGN

Chemical and Physical Characterization of the Test Material

It may seem that selection of the test material for repeated dose toxicity testing should require little involve-ment by the toxicologist because it is usually provided by a chemist or product manager. However, several import-ant factors must be considered by the toxicologist to insure that a study will adequately assess the potential toxicity of the chemical and be accepted by regulatory agencies. Obviously, one of these factors is that the batch or lot of test material should be representative of the chemical intended to be tested. Adequate chemical and physical characterization is essential for this deter-mination. Several regulatory agencies and organizations have issued guidelines to describe the information needed and some of the methodology to be used to characterize a chemical (16, 24, 42, 55).

Table 19.4 Subchronic and chronic rodent oral toxicity studies on various regulatory guidelines

	EPA OPPTS Guidelines		FDA Redbook		OECD Guidelines	
	Subchronic (14)	Chronic (15)	Subchronic (22)	Chronic (23)	Subchronic (57)	Chronic (53)
Study Duration	≥90 days	≥12 mo.	≥90 days	≥12 mo.	≥90 days	≥12 mo.
No. of Treated Groups						
standard study	≥3	≥3	≥3	≥3	≥3	≥3
limit test*a*	1	1	NA	NA	1	NA
No. of Negative Control Groups*b*						
untreated control	1	1	1	1	1	1
vehicle control	1	1	1	1	1	1
No. of Animals/Gender/Group′	≥10	≥20	≥20	≥20	≥10	≥20
Age of Animals (at start of study)	≤8–9 weeks	≤8 weeks	≤6 weeks	~6 weeks	<9 weeks	asap*f*
Body Weight Measurement						
frequency through 13 weeks	weekly	weekly	weekly	weekly	weekly	weekly
frequency after 13 weeks	NA	every 4th wk	NA	monthly	NA	every 4th wk
Feed Consumption Measurement						
frequency through 13 weeks	weekly	weekly	weekly	weekly	weekly	weekly
frequency after 13 weeks	NA	monthly	NA	monthly	NA	every 3rd mo
Observations						
for mortality and morbidity (times/day)	2	2	2	2	2	2
for general condition (times/day)	1	1	n	n	1	1
for detailed clinical findings (frequency)	weekly	weekly	daily	daily	weekly	daily
Neurotoxicity Evaluation (at term)*d*	y	y	n	n	y	n
Ophthalmology						
no. animals pretest	AA	AA	AA	AA	AA	n
no. animals/gender/high dose and control every 3 months*e*	NA	n	NA	AS	NA	n
no. animals/gender/high dose and control at term*e*	AS	10	AS	AS	AS	n
Hematology and Clinical Chemistry (no./gender/group)	AS	10	10	10	AS	10
intermediate time(s)	n	6 mo	n	every 3rd mo	n	3 & 6 mo*g*
term	y	y	y	y	y	y
Urinalysis (no./gender/group)	AS	10	NA	NA	AS	10
intermediate time(s)	n	n	n	n	n	3 & 6 mo
term	o	y	n	n	o	y

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	EPA OPPTS Guidelines		FDA Redbook		OECD Guidelines	
	Subchronic (14)	Chronic (15)	Subchronic (22)	Chronic (23)	Subchronic (57)	Chronic (53)
Gross Necropsy and Tissue Collection	AA	AA	AA	AA	AA	AA
Organ Weights (no/gender/group at term)	AS	AS	AS	AS	AS	10
adrenals, kidneys, liver	y	y	y	y	y	y
brain	y	y	n	n	y	y
testes/ovaries	y/y	y/y	y/n	y/n	y/y	y/y
epididymides, heart, uterus	y	y	n	n	y	n
spleen	y	y	n	n	y	n
thymus	y	n	n	n	y	n
Histopathology						
all tissues (all high-dose and control animals)	y	y	y	y	y	y
all tissues (all animals killed or died on study)	y	y	y	y	n	y
target tissues and gross lesions (All animals)	y	y	y	y	y	y
selected tissues (all intermediate-dose animals)	n	n	n	y	n	n

NA=not applicable; y=yes/required; n=no/not required; o=optional; AA=all animals; AS=all survivors

a If a test at one dose level of at least 1000 mg/kg body weight/day produces no observed adverse effects and if toxicity would not be expected based upon data from structurally related compounds, then a full study using three dose levels may not be considered necessary.

b Contingent upon the route of exposure in the test article treated groups.

c Extra animals must be added for interim sacrifices.

d Not required if similar data are available from other studies or if other clinical signs are noted to an extent that would interfere with evaluation.

e Animals in intermediate groups are to be evaluated if treatment related findings are noted in the high-dose group.

f As soon as possible after weaning and acclimation, e.g. ≤6–8 weeks of age.

g No clinical chemistry at three months.

Table 19.5 Protocol contents required by FDA good laboratory practices regulations

- Title and study objective
- Identification of test and control articles
- Identification of sponsor and testing facility
- Justification of test system (animal model)
- Test system information (number, body weight, gender, source, species/strain, age, method of identification, etc.)
- Description of study design and methods for control of bias, such as random assignment of animals to treatment groups, processing of clinical pathology samples in replicates, etc.
- Animal husbandry information
- Dosing information, including dose form preparation and route of administration
- Methods by which degree of absorption of the test and control articles by the test system will be determined, if necessary
- Types and frequencies of assays, analyses, and measurements to be made
- Description of statistical methods
- Records to be maintained

A safety assessment should include a partnership between the toxicologist and a chemist who understands the chemical characteristics of the test material. An adequate chemical characterization of the test material should be provided to the toxicologist, including methods of synthesis, precursors used in the synthesis and any solvents and manufacturing aids used in the manufacturing process. In addition, the methods of purification should be provided, to allow the toxicologist to determine the potential for occurrence of residues and impurities that could produce adverse effects. This provides the toxicologist with information needed to determine if any specific residues or impurities should be specifically targeted for additional analytical determinations.

The specific information required by the toxicologist may vary with each test material, and the required data may be different from that used to establish manufactur-ing and quality control specifications. It is essential, however, that the manufacturing and quality control specifications and procedures meet the specific regulatory requirements for toxicity testing. For instance, good manufacturing practices (GMP), which are somewhat similar to GLPs, are required for some chemicals (21, 30, 32, 33, 40). The chemist should insure that GMP records exist, if they are required.

Prior to initiation of a repeated dose study, the toxicologist should carefully review the chemical and physical characterization data. Chemical characteriza-tion should include a quantitative assessment of the major components, with associated accuracy and precision information, and at least a qualitative analysis of minor components. Ideally, the toxicologist should be provided with a mass balance for the test material.

Before initiation of a repeated dose toxicity study, chemical analyses should be conducted to ensure that the test material, in bulk form and after mixing with any carrier, is stable over the range of anticipated con-centrations and for the maximum period of use during the study. According to GLPs, this evaluation can be con-ducted concurrently with the study, but detection of insta-bility during the study could invalidate the results. If the material is stable only under certain conditions—for example, frozen storage—special arrangements for its storage should be made to eliminate instability problems with the test material, resulting in lower than expected doses and exposure of the animals to degradation pro-ducts. The products of degradation may either have their own unique toxicity or alter the toxicity of the test material. This may make it impossible to correctly inter-pret data from the study.

The toxicologist will have assessed the chemical characterization of the test material at the initial stages of the hazard assessment. More complete information may become available before initiation of repeated dose toxicity studies. Sometimes the chemical synthesis or other production methods may change between the initial assessment and the start of subchronic or chronic studies, and, it may be necessary to reassess the chemical characterization before initiation of these studies. Unanticipated changes in the chemistry of the test material or mixture may necessitate changes in the toxicity study design.

The test material should be as similar as possible to the chemical to which humans will be exposed, with every attempt made to ensure the test material is either identical to the final commercial product or represen-tative of the anticipated final product. This may not always be possible with commercial products because large-scale production facilities are usually not avail-able during this phase of a safety assessment. When such facilities become available, it may be possible to bridge between the final commercial product and the test material by chemical analysis. "Bridge chemistry" should identify any

differences between the chemical that was tested and the commercial pro-duct, allowing a determination of the toxicological bioequivalence of the test material and final commer-cial product.

If a "new" impurity of unknown toxicity is detected in a drug product after chronic animal studies have been con-ducted, the second International Conference on Harmon-ization (42) has proposed the following:

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1. If intended human use is short term:
• Single-dose toxicology comparison of old and new test substance
• Repeated dose, four-week comparison of old and new test substance
• Mutagenicity tests
2. If intended use is long term, with a high dose of the active compound:
• Single-dose toxicology comparison of old and new test substance
• Repeated dose, three-month subchronic com-parison of old and new test substance
• Mutagenicity tests (if results of the mutagenicity test are positive, carcinogenicity testing of the impurity may be considered)
3. If the product is to be given to women of childbearing age:
• Consider the need for a comparative embryotoxicity and teratogenicity (segment II) study in one suitable species

The test material should have not only the same chemical characteristics as the material of commerce, but should also have the same physical characteristics. If the test material is a solid intended to be used as a powder, it should be administered to the test animals in powder form. Particle size of the powder should be similar for the test material and the material to which humans will be exposed. If humans will be exposed to the material in solution, a solution of the material should be used in the repeated dose study. Analyses should be conducted to ensure that the composition of the test material falls within the limits of anticipated or known product specifications. Physical specifications will allow the toxicologist, often in consultation with the chemist, to determine the most appropriate method of adding the test material to the dosing matrix, such as the diet or drinking water (see Route of Exposure discussion).

It is important for the toxicologist to ensure an adequate supply of test material is available before initiating a toxicity study. A single lot of the test material should be used throughout the subchronic or chronic study, whenever possible. If the repeated dose study is part of a series of studies in a safety assessment program, it is desirable to use a single lot of test material for the entire program. This reduces the probability of encountering inconsistent results in different studies with the same test material, resulting from inter-lot differences in chemical/physical characteristics. If a single lot of test material is not available in sufficient quantity to complete a study or studies, multiple lots may be used, with chemi-cal characterization of each new lot required to insure it meets all specifications and reasonably duplicates pre-vious lots.

The FDA has issued guidelines addressing the chemis-try data requirements for direct food additives and gen-erally recognized as safe (GRAS) petitions (24). ICH has issued guidelines for chemistry requirements for drug candidates during preclinical *hazard* assessment (42). Although the toxicologist may not have as complete a data package as described above before the initiation of a repeated dose study, sufficient data must be available to meet GLP regulations.

FDA GLPs state that, "The identity, strength, purity, and composition or other characteristics which will appropriately define the test or control article shall be determined for each batch and shall be documented. Methods of synthesis, fabrication, or derivation of the test and control articles shall be documented…." (35).

EPA GLP statements concerning the requirements for chemical characterization of the test material are essentially identical to those of FDA (12). The OECD GLP guidelines for chemical characterization state, "For each study, the identity, including batch number, purity, composition, concentrations, or other characterizations to appropriately define each batch of the test or reference items should be known" (56).

Further information on the chemical characterization of the test material is provided by the FDA in its proposed revision to the guidelines for toxicity studies, "The com-position of the test substance should be known: Infor-mation should include the name and quantities of all major components, known contaminants and impurities, and the percentage of unidentifiable materials. The test substance in toxicology studies should be the same sub-stance that the petitioner intends to market"(25).

Route of Exposure and Method of Test Material Administration

The anticipated human route of exposure to the test material dictates the route of exposure for most subchronic and chronic toxicity studies. Unintended routes of human exposure also should be considered during selection of exposure route. The most common routes of exposure in these studies are dietary, oral (gavage or capsule), dermal, and inhalation. Less frequently, test materials are administered in the drinking water or parenterally by intravenous, subcutaneous, intraperitoneal or other types of injection. Other types of administration include direct implant and *parenteral* infusion using implanted or external pumps.

Frequently, there are several potential routes of human exposure to a single chemical. For example, consumer exposure to a pesticide may occur by dietary consumption of food crops containing residues of the chemical. Farm

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worker exposure to the same pesticide may occur by either inhalation or dermal routes during application and harvesting. In such cases, subchronic testing may be required to assess the effects of exposure by all three routes. Emphasis is generally placed upon the route by which the most widespread human exposure would occur, with chronic testing usually conducted using only that route of exposure. Several of the more common routes of exposure are discussed in detail below.

Capsule Administration

If expected human exposure is by the oral route, a solid test material (e.g., a dry powder) can be administered by capsule. Unformulated bulk test material can be given to some large animals, such as dogs and cats, in gelatin capsules inserted into the esophagus manually or with the aid of a mechanical device designed to prevent the animal from biting the individual administering the capsule. Capsules are available in a wide range of sizes and can hold, depending on the density of the test material, as much as 1.5 g. The amount of test material that can be administered is limited by the capacity of each capsule and the practical number of capsules that can be administered at one time. For smaller species, such as rodents, this method is impractical and the test material must be administered orally as a solution or suspension in an appropriate vehicle.

Oral Gavage

Another common route of oral administration is oral *gavage* by *intubation*. This technique may be used for rodent and nonrodent species. For oral gavage, a solution or suspension of the test material is deposited into the stomach via the esophagus using an intubation tube attached to a graduated syringe or other device. A test material is added to an appropriate vehicle, usually aqueous. If the material is not readily soluble, a suspension may be prepared. For suspensions, a thickener such as methylcellulose, carboxymethylcellulose, or gum tragacanth is added to water to increase the viscosity, and the test material is homogeneously suspended in the vehicle. A wetting agent such as Tween 80 can be used to increase the suspendability of the material. Although aqueous vehicles are preferred, it is possible to use an oil vehicle for lipid-soluble materials. Food oils, such as corn oil, may be used but mineral oils must be avoided. It should be remembered that food oils add to normal dietary caloric intake and, at high volumes, oils may inter-fere with absorption of fat-soluble nutrients, such as fat-soluble vitamins. Also, absorption of the test material may be quite different with an oil vehicle compared to an aqueous vehicle and this may be highly volumedependent.

The volume of test solution or suspension administered can influence gastric emptying time. Because gastric emptying time may affect gastrointestinal uptake and bioavailability, it generally is preferred that the dose levels of the test substance be administered on a con-stant-volume (ml/kg), variable-concentration (mg/ml) basis. For dosing using a constant volume, 10 ml/kg body weight is most commonly used as the upper limit. At 10 ml/kg, a 260 g rat would receive a dose volume of 2.6 ml, a 35 g mouse would receive 0.35 ml, and a 10 kg dog would receive 100 ml. Dosing usually is conducted once daily but may be performed more frequently to mimic the intended human dosing regimen or if concen-tration and/or pharmacokinetics considerations limit the achievable single-dose exposure to less than the amount desired.

Daily exposure by oral gavage results in a *bolus dose*. Administration via the diet or drinking water results in a more constant exposure throughout the duration of the study that is more dependent on feeding patterns than dosing schedule. Because rodents are nocturnal, the peak periods for activity and feeding behavior are the end of the light and dark periods (i.e., just before lights-on or just after lights-off). If high volumes of dosing solution (i.e., 20–50 ml/kg) are to be administered to rodents and/or if the presence of feed may interfere with the absorption of the test substance, consideration should be given to dosing animals during late morning or in the afternoon. For nonrodents, timing of dosing relative to feeding (i.e., before or at a certain time after daily feeding) accomplishes the same purpose. In any case, the timing of gavage dose(s) in relation to feeding should be stated clearly in the study protocol.

Administration of a test chemical as an undiluted liquid, as a diluted liquid, as a dissolved solid, or as a solid in suspension generally results in accurate delivery of the intended dose to each of the animals in a repeated dose study when exposure is by oral gavage. To deliver an accurate dose of test chemical in solution or suspension, the chemical must be mixed with solvent or suspending agent at the proper concentration which can be calculated using the intended dose level and the dose volume of the solution/suspension to be adminis-tered to the animals. This calculation is illustrated in Figure 19.2. Obviously, this same calculation applies to solutions or suspensions for other exposure routes, for example, administration by capsule, dermally, or parenterally.

Use of a vehicle to carry the test material into the ani-mal has been mentioned several times in the

preceding discussion. Choice of vehicle is a critical decision for toxicology studies. Obviously, the vehicle should be nontoxic at the dose (volume) administered, and should not act in an additive or synergistic manner to enhance the toxicity of the test material, nor should it interfere with the expression of any potential toxicity. Because such interactions are not always predictable, it is

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• Formula

CONCENTRATION (mg test material/ml solution or suspension)=

$$\frac{\text{INTENDED DOSE LEVEL (mg test material/kg body weight/interval)}}{\text{DOSE VOLUME (ml solution or suspension/kg body weight/interval)}}$$

• Example

The concentration of a solution intended to deliver a daily 1500 mg/kg dose level of test material A to rats at a dose volume of 5 ml/kg body weight (BW) is calculated as follows:

$$\text{Concentration (mg A/ml)} = \frac{\text{1500 mg A/kg BW/day}}{\text{5 ml solution/kg BW/day}}$$

$$= \text{300 mg A/ml solution}$$

As indicated in the following, a 300 gram rat would receive 1.5 ml of this solution each day and, therefore, would receive the intended daily dose level. First, calculating the volume of solution administered to the rat:

0.3 kg body weight x 5 ml solution/kg BW/day=1.5 ml/day

Then, calculating the amount of test material A in that volume:

1.5 ml of solution/day x 300 mg A/ml solution=450 mg A/day

Finally, calculating the dose level resulting from administration of that amount of test material:

450 mg A/day÷0.3 kg BW=1500 mg A/kg BW/day

FIG. 19.2. Calculation of test material concentration in a solution or suspension.

imperative that vehicle controls be used in all oral gavage toxicology studies (and in virtually every other type of administration). However, even use of a vehicle control may not always compensate for the administration of vehicle in the groups receiving the test material. Interac-tions between vehicle and test materials can be encountered and must be considered during the design of studies because vehicles may produce physiological/ nutritional alterations that may affect the toxicity of the test material, especially in longer-term studies. For instance, an oil vehicle may result in poor absorption of fat-soluble nutrients; food oil vehicles add to the caloric intake of the animal and may produce effects in longer-term studies; absorption of fat-soluble materials from oil vehicles can be slower than when the same material is administered in an aqueous matrix. The result-ing change in the toxicokinetics of the test material can produce profound differences in toxicity. Because toxicity testing generally uses exaggerated doses compared to human exposure, it is sometimes difficult to obtain solutions. In such cases, suspensions are sometimes used, which, again, can affect toxicokinetics and influence toxicity. For these reasons, among others, great care must be utilized in selecting an appropriate vehicle for admin-istration of test materials to animals.

Dermal Application

For a drug, cosmetic, industrial chemical, or pesticide with dermal exposure potential for humans, dermal appli-cation is the most appropriate exposure route for repeated dose studies. The test material is applied to a defined area of skin from which the hair has been removed. Removal of hair by both shaving and depilatories can alter skin permeability to applied materials. Because the test animal can ingest some of a dermal dose during grooming, it is common practice to cover the test material application site by wrapping the trunk of the animal with a semi-occlusive material (e.g., gauze strips) during the exposure period. Occasionally, if exaggerated dermal absorption is desired, the application site is covered with an occlusive material (e.g., rubber dam). Either method of wrapping, occlusion or semi-occlusion, maintains the test material in contact with the skin. Wrapping also increases the accuracy of the administered dose because

on unoccluded animals, dry test materials may fall off the application site and liquid materials may run off or evap-orate from the site. Restraint and/or "Elizabethan" collars are also sometimes used to prevent tampering with the wrap or to prevent oral ingestion if the application site is unoccluded. However, use of restraint or collars stresses the animals and, therefore, may alter the outcome of a study. To avoid unnecessary stress, the animals should be trained to accept such procedures before actual dosing is initiated.

Parenteral Administration

A pharmaceutical intended for administration to humans by injection should be administered to the test animals by the same parenteral route. A common type of parenteral exposure is subcutaneous injection. To avoid irritation and other potential effects, such as fibrotic reactions, the specific site of injection should be changed daily. It is recommended that the dosing vehicle be aqueous since oil vehicles may track back along the needle path and be deposited in the lipophilic skin and hair. As with oral gavage exposure, absorption from an oil vehicle may differ compared with an aqueous vehicle. For subcutaneous administration, care must be taken to ensure the material is deposited subcutaneously rather than intradermally or intramuscularly. This can be accomplished by lifting the skin of the animal to form a pocket and injecting the dosage into the subcutaneous pocket.

Other common routes of parenteral exposure include intravenous (iv), intramuscular (im), and intraperitoneal (ip) injection. Special considerations are required for each of these routes. For iv administration, the test material must be soluble in an aqueous vehicle since physiological saline is the usual vehicle. Air bubbles must be cleared from the delivery system—the needle, catheter and/or syringe, before injecting the material intravenously. The dosing solution for iv administration must be at a physiological pH and must not be extremely irritating or corrosive. Repeated use of the same injection site must be avoided. To prevent inadvertent delivery of the test material into the muscle or other surrounding tissue, care must be taken to insure that the needle tip is in the lumen of the vein before injecting the test material. Proper place-ment of the needle can be assessed by drawing a small amount of blood back into the delivery device. If no blood is observed, the needle is not located in the lumen of the vein. For im and ip routes of exposure, proper placement of the needle tip also must be assessed before adminis-tering the dose material. In contrast to iv exposure, draw-ing back on the delivery device without obtaining blood is required prior to administering material by the im route. Similarly, drawing back on the device without obtaining blood or intestinal contents prior to injecting test material is necessary for correct ip adminstration. Most other con-siderations for im and ip exposure are similar to those for iv administration for example, the material must not be corrosive or irritating, repeated injections at the same site should be avoided, and so on.

Continuous infusion may be used to simulate a con-stant human exposure or, for intravenous infusion of poorly soluble test substances, to deliver a sufficiently large daily dose for safety assessment. Infusions may be administered using either implanted or external pumps. Implanted pumps may be battery-operated mech-anical pumps (nonrodents) or osmotic pumps (rodents and nonrodents); both of which permit unrestricted movement of the animal. Practical considerations of vol-ume and weight of implanted pumps restrict their use to very soluble and/or potent test substances that can be administered slowly over a long period. External pumps may be connected to rodents and nonrodents using tethers and swivels or affixed to nonrodents as "back packs." External pumps allow for infusion of larger vol-umes of test substance but also require more waste sub-stance because of the larger "dead space" of the tether catheter. Their use may be problematic for subchronic or chronic studies because of their relatively long duration. The patency of the catheter for implanted and external pumps must be confirmed. The dose received by each animal can be determined by measuring the weight of the pump and solution prior to initiation and following completion of exposure, or by direct meas-urement of the infused volume by syringe markings or by using calibrated pumps displaying the infusion rate. Study designs often incorporate measurement of blood concentrations of the test material and its metabolytes.

Implantation

Subcutaneous or intramuscular implantation is often used for evaluating biopolymers for medical devices or prostheses. In addition, test materials have been embedded in special matrices that allow continuous, sus-tained release for weeks or months after subcutaneous implantation. Demonstration of the stability of the test substance in the matrix, both during preparation of the pellet and after implantation, is required when using this technology. Determination of plasma concentrations of the test material and/or its metabolites can be used to confirm proper dosing. Because of size limitations, these pellets are useful only for delivery of very potent substances, such as hormones and biological proteins.

Dietary Administration

Dietary administration would be appropriate for a food additive or a pesticide that has potential to become a residue in or on food crops. This type of administration, although frequently used in subchronic and chronic toxicity studies, is less accurate than most other routes of dose delivery primarily because of differences between

body weight and feed consumption of individual animals, which result in variable compound consumption. A further complication in rodent studies is that these ani-mals tend to add body fat and not lean body mass as they age. Therefore, lipophilic compounds have a larger mass of fat into which they can partition. This may actually decrease the effective dose. In addition, fat does not have significant detoxication enzymes. Therefore, as dose is increased to account for increased body weight in older rodents, detoxication capacity may be stressed resulting in unanticipated toxicity. This complication is not unique to dietary exposure studies and will affect the effective dose of a test material in rodents exposed by most other routes. Although potential variability in effective dose is important to interpretation of the results of a study, no attempt is generally made to compensate for this varia-bility during preparation of the test diet or calculation of the administered dose in repeated dose toxicity studies. Spillage or soiling of feed, which occurs fairly frequently with some test animals, is a factor that contributes varia-bility to the calculation of dietary dose. Significant spill-age or soiling must be considered when measuring feed consumption to be used in calculation of dietary concen-tration or compound consumption. Dietary adminis-tration can be conducted by either of two methods:
(1) adjusting the dietary concentration of the test material to account for changing body weight and feed consumption, or
(2) feeding a constant concentration in the diet.
Routinely adjusting the dietary concentration of a test material based upon the changing body weight and feed consumption of the animal provides reason-ably good control over the delivered dose during a repeated dose study. Dietary concentration is usually adjusted weekly during a subchronic study. In a chronic study, diet concentration is usually adjusted weekly during the first 13-14 weeks (especially for rodents because of the rapid growth of the animals during this period) and biweekly or sometimes monthly thereafter. Using this method, the mean feed consumption and body weight of a dose group during a given study interval are used to calculate the dietary concentration to be fed to that group during the following interval. There are several ways to calculate the concentration. One formula for this calculation and an example of its use are presented in Figure 19.3. A caution in using this approach in quickly growing, young rats is that the concentration may increase dramatically during the study. Sometimes the target concentrations become too high to formulate the mix homogeneously or they reach a level that becomes unpalatable to the animals.
Feeding a constant concentration of the test material in the diet throughout the study is the second form of dietary administration. This method provides less control over the administered dose level because it is a function of the amount of feed (and, therefore, test material) con-sumed and body weight gained by each animal. The con-sumed dose, or compound consumption, of each individual animal can be calculated using its feed con-sumption and body weight. The mean of the individual animal compound consumptions represents the com-pound consumption for each dose group. A commonly used formula for the calculation of individual compound consumption is illustrated in Figure 19.4.
When feeding a constant concentration in a rodent study, the toxicologist must be aware that the compound consumption may vary significantly for an individual ani-mal or group of animals during the course of the study. For example, compound consumption during the first week of a 13-week rat study in which the test compound is fed at a constant dietary concentration is frequently more than twice the compound consumption during the last week of the study. This variation results from the rapid decrease in feed consumption relative to body weight (g feed/kg body weight/day) by young rats during the first several months of life. This variability in com-pound consumption is illustrated in the example given in Figure 19.4.
Drinking Water
Water-soluble test substances can be offered as a mixture in the drinking water, providing adequate dosage can be achieved. This route may be preferred when it mimics human exposure conditions of constant exposure, compared with event-oriented exposure such as pill taking. Spillage can be a significant problem when admin-istering material in drinking water and recovery of spilled water is usually not feasible. Another complication with drinking water administration is that evaporation of water and/or volatilization of the test material can occur from the tip of the drinking (sipper) tube, resulting in alteration of the concentration of the material in the water. Use of a sipper tube containing a ball bearing tip minimizes this problem.
Assessment of the Adequacy of Test Material Preparations
Whatever the route of exposure, it is critical to deter-mine if the test material is delivered to the animals at the intended doses. For dietary studies, test diets should be prepared before initiation of a repeated

dose study, using the intended diet preparation method. It must be shown that this preparation method yields diets con-taining the appropriate amounts of homogeneously mixed test material. Chemical analysis of samples taken from several locations in each test diet preparation should be conducted to determine if the proper concentrations of test material have been achieved and to assess the

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Formula

- CONCENTRATION (mg test material/kg diet)=

$$\frac{\text{INTENDED DOSE LEVEL (mg test material/kg body weight/day)}}{\text{PROJECTED FEED CONSUMPTION (kg feed /kg body weight/day)}}$$

where projected feed consumption (PFC) for a study week is based on body weight (BW) and absolute feed consumption (AFC) data from the previous week and is calculated as follows:

PFC (kg/kg/day) for weekn=

$$\frac{\text{AFC week}_{n-1}\text{ (kg)}}{7\text{ days}} \div \left[\text{BW end of week}_{n-1}\text{ (kg)} + \frac{\text{BW gain during week}_{n-1}\text{ (kg)}}{2}\right]$$

- Example

In a subchronic rat study, the mean body weight of the males in the 15 mg/kg/day dose group at the beginning of week 11 is 520 g. At the end of week 11, the mean weight for these males is 540 g. Mean feed consumption of these animals is 154 g during the 7 days of week 11. The dietary concentration intended to deliver a 15 mg/kg/day dose level of compound A to this group of rats during week 12 of the study is calculated as follows:

PFC (week 12)

$$= \frac{0.154\text{ kg feed}}{7\text{ days}} \div \left[0.540\text{ kg BW} + \frac{0.020\text{ kg BW gain}}{2}\right]$$

= 0.04 kg feed/kg BW/day

and, therefore

Concentration (week 12)

$$= \frac{15\text{ mg A/kg BW/day}}{0.04\text{ kg feed/kg BW/day}}$$

= 375 mg A/kg feed

Note that, because their body weight and feed consumption differ, the diet concentrations for males and females in the same dose group will generally differ throughout the study.

FIG. 19.3. Calculation of adjusted diet concentration to yield constant dose level.

homogeneity of the dietary admixtures. If the results of these analyses indicate the anticipated concentrations were not achieved or the distribution of test material in the diet was not homogenous, the diet preparation method should be revised and retested. Diet preparation must be validated before the study can be initiated.

During the pre-study homogeneity determinations, additional diet samples should be collected and analyzed to show that, within the range of concentrations to be used in the study, the test material is stable in the diet. These samples should be stored under animal room conditions and under frozen conditions for the maximum period of time during which the diet will be used or stored. For a study in which dietary admixtures will be prepared and fed once per week, stability of the test material in the diet would commonly be assessed for samples stored under animal room conditions for at least seven and fourteen days. This allows estimation of the degradation rate at room temperature. Analysis of frozen diet samples stored for several intervals is also advisable. Demonstration of stability under frozen storage conditions makes chemical analysis of diet samples immediately after collection during the toxicity study unnecessary and also validates the possibility of confirming analytical results by reanalysis of stored frozen samples, if needed. Adequate stability of the test material in the diet should be demonstrated prior to initiation of the study.

Even though the adequacy of the diet preparation method and stability of the test material in the diet have been demonstrated, it is important to monitor diet preparation during the study. For each diet preparation

Formula

$$\text{COMPOUND CONSUMPTION (mg test material/kg body weight/day)} =$$

$$\underset{\text{(mg test material/kg feed)}}{\text{CONCENTRATION}} \times \underset{\text{(kg feed/kg body weight/day)}}{\text{RELATIVE FEED CONSUMPTION}}$$

where relative feed consumption (RFC) for a study week is based on body weight (BW) and absolute feed consumption (AFC) during mat week and is calculated as follows:

$$\text{RFC (kg/kg/day)} = \frac{\text{AFC (kg)}}{\text{7 days}} \div \left[\text{BW start of week (kg)} + \frac{\text{BW gain during week (kg)}}{2}\right]$$

Example

In a subchronic rat study, a group of males is fed test material A at a constant dietary concentration of 2% (w/w). The body weight of one animal in this group is 175 g at the start of week 1 and 225 g at the end of week 1. Its feed consumption during the 7 days of week 1 is 168 g. Subsequently, this rat weighs 490 g at the start and 510 g at the end of week 13; and its feed consumption during that week is 1% g. The compound consumption of mis rat for each week is calculated as follows:

$$\text{RFC (week 1)} = \frac{\text{0.168 kg feed}}{\text{7 days}} \div \left[\text{0.175 kg BW} + \frac{\text{0.050 kg BW gain}}{2}\right]$$

$$= \text{0.120 kg feed/kg BW/day}$$

$$\text{RFC (week 13)} = \frac{\text{0.196 kg feed}}{\text{7 days}} \div \left[\text{0.490 kg BW} + \frac{\text{0.020 kg BW gain}}{2}\right]$$

$$= \text{0.056 kg feed/kg BW/day}$$

and, therefore:

Compound Consumption (week 1) = 2% test material A x RFC (week 1)
= 2 g A7100 g feed x 0.120 kg feed/kg BW/day
= 20,000 mg A/kg feed x 0.120 kg feed/kg BW/day
= 2400 mg A/kg BW/day

Compound Consumption (week 13) = 2% test material A x RFC (week 13)
= 20,000 mg A/kg feed x 0.056 kg feed/kg BW/day
= 1120 mg A/kg BW/day

FIG. 19.4. Calculation of compound consumption resulting from constant diet concentration.

during the first several weeks of the study, concentrations of the test material in the diets should be assessed. Subsequent analysis of diet preparations every two to four weeks will add assurance that diets were prepared properly. More frequent analysis, for example, weekly throughout the study, is even more desirable.

For routes of exposure other than dietary, the principles cited above also apply. Concentration, homogeneity and stability of the test material in solvents or suspending agents must be determined for studies using oral, dermal, inhalation, or other routes of exposure when the test material is to be administered in solution, suspension, or as an aerosol. Suspensions represent a special case because care must be taken to ensure that the suspensions do not settle and become nonhomogenous during administration to the test species. For inhalation studies, the concentration of gas, aerosol, or particulates to which the animals are exposed should also be assessed using appropriate analytical methods.

Duration of Exposure

As stated previously, the duration of subchronic toxicity studies involves exposure of the test species to a chemical during a significant portion of its lifetime. Classically, these studies are conducted for 90 consecutive days or approximately 13 weeks. Chronic toxicity studies in rodents most commonly involve exposure for a major portion of their life span, generally 12 months, although studies of shorter duration (i.e., 6-9 months) are considered acceptable by some groups (43). Rodent chronic toxicity studies are sometimes combined with lifetime oncogenicity studies to achieve efficiencies during some

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of the study procedures, for example, diet preparation. During these combination studies, the animals in the chronic toxicity segment are generally studied during the first 6–12 months, and then are terminated. Those in the oncogenicity segment continue on study generally for at least 24 months. In nonrodents—for example, dogs and non-human primates, which are longer lived than rodents—6–12 months represent a significantly smaller portion of their life span but is currently considered an adequate duration of exposure to detect chronic effects.

Daily test material administration during a repeated dose toxicity study can be continuous, intermittent, or repeated. In most dietary and drinking water studies, the animals have free access to diets or water containing the test material throughout the study and exposure is essentially continuous, although influenced by diurnal variation. In dermal or inhalation studies, exposure to the test material is intermittent, generally four to six hours per day. When the route of administration is intravenous infusion, exposure may be either continuous or intermittent. With bolus dose parenteral administration or oral gavage, test material administration is generally once or, at most, a few times each day. Labor-intensive methods of administration, such as oral gavage, are sometimes done only during the standard work week, that is, five days per week. This is not recommended because two days of non-exposure each week during the study may be sufficient to allow modification or reversal of toxic responses.

Dose Groups

The minimum number of groups receiving test material in a repeated dose toxicity study is generally three (low, mid-, and high dose). The high-dose level should produce evidence of toxicity, but should not result in more than 10 percent mortality. The mid-dose level should produce no more than slight toxicity and the low-dose level should produce no toxicity yielding a NOAEL. As previously stated, a short-term (two to four weeks) repeated dose study should be conducted to aid in the selection of doses for subchronic testing. For test materials where a dose response has not been well defined during a short-term repeated dose study, additional dose groups may be required in the subchronic study to insure the range of desired responses—that is, no toxicity to significant toxicity—is achieved. However, it is sometimes difficult to completely satisfy these criteria. Before selecting doses for a chronic toxicity study, a subchronic study that defines no-effect and effect levels should be completed.

Limit Studies

For test materials that possess very low potential for toxicity, the inclusion of only one test material dose group in a repeated dose study is sometimes acceptable. A study with this design is termed a *limit study*. Limit testing is inappropriate for materials with anticipated high human exposure. The dose level for the test material group in dietary and dermal limit studies is normally at least 1000 mg/kg/day. Another type of limit study involves utilization of the maximal exposure level under the conditions of the study. For example, suppose the majority of toxicology data concerning a lipophilic drinking water contaminant has been collected using oral administration in a corn oil vehicle and additional data are desired using a water vehicle. The limited water solubility of the test material may result in the maximal dose being significantly lower than the dose used in the corn oil gavage studies. However, the test material can be tested as a saturated water solution and such a study may reveal the test material to be either more or less toxic in water than in the oil vehicle. The data are relevant to the assessment of hazard associated with exposure in the drinking water because the maximal possible exposure by this route of administration was tested. Thus, while a limit study may not define the "complete" toxicology of a test material, it can define the "practical" toxicology of the material.

Control Groups

Adequate controls are essential to successful toxicity studies of all types, including repeated dose studies. Studies should contain at least one control group for comparison with the groups receiving the test material. The control group should be treated identically to the treated groups except the control group should receive no test material. Control groups can be either negative or positive controls.

Negative control groups are intended to demonstrate the normal state of the animal for comparison to data from the groups treated with the test material. They also provide an opportunity to compare baseline data for the current study to baseline data from previous studies. There are several types of negative controls. If the test material is dissolved or suspended in a vehicle for administration, a vehicle control group should receive, by the same route of exposure, the maximum amount of solvent or suspending agent administered to any of the test material groups. If the test material is administered in the diet, an untreated control group should receive the same diet without test material. For test materials administered undiluted, a sham control group should receive the same physical treatment as the treated groups, for example, insertion of an intubation tube with or without delivery of an innocuous substance like water, administration of empty capsules, or injection of physiological saline.

Positive control groups are intended either to demonstrate susceptibility of the animal to a specific toxicity or to compare the response of test material-treated animals to that of animals treated with a chemical that produces a known toxicity similar to the test material. If a positive control group is included in a study design, at least one negative control group should also be included. Positive control groups are infrequently used in repeated dose toxicity testing. However, if the chemical structure of a test material suggests that it may possess a specific toxicity—for example, neurotoxicity—it may be important to demonstrate that the species and strain selected for testing is susceptible to that toxicity.
A positive control that is sometimes useful in repeated dose studies is the reference control. This control consists of a material that is chemically or physically similar to the test material but has either a comprehensive toxicology database associated with it or a history of use without adverse effects. Inclusion of a reference control group allows a comparison between reference and test material within the same study. This can assist in identifying any effects related to the general characteristics of the reference material. For instance, oral administration of a poorly absorbed oil can decrease the absorption of fat-soluble vitamins. If the test material is known or suspected to produce this effect, use of a reference material, such as mineral oil, can be useful. This would distinguish effects related to vitamin depletion from effects produced directly by the test material. Additionally, if the test material were to add substantially to the caloric intake, a reference control diet isocaloric to the test diet would be useful, especially in longer-term studies. A reference control group also may be useful to compare the degree of anticipated toxicity of the test material to a reference material of known toxicity. For example, it could be important to demonstrate that the hepatotoxicity of a test material intended for use as an anesthetic is significantly less severe than that produced by an anesthetic already in use.

Animal Models

To increase the probability of testing in a species that may respond to the test material in a manner similar to humans, two species are generally used. Routinely, one rodent species and one nonrodent species are utilized. Rats and dogs are the generally preferred species for most routes of exposure. The rabbit is preferred for dermal exposure. Mice, hamsters, miniature swine, guinea pigs, non-human primates, and a few other species are used on occasion in these studies. Many factors should be carefully considered during the selection of the most appropriate species and strain for testing with a specific

Table 19.6 Selection criteria for species and strain in repeated dose studies

- Requirements by regulatory agencies
- Metabolism of test material in a manner similar to humans
- Availability of historical control data
- Most sensitive species and strain
- Responsiveness of particular organs and tissues to specific toxicities
- Availability of the species and strain
- Availability of appropriate animal housing and husbandry
- Experience of the laboratory in the use of the species and strain

chemical. Some of these factors are summarized in Table 19.6.

Toxicokinetics

Ideally, selection of an animal model for repeated dose toxicity studies should be based upon the similarity between toxicokinetics of the test chemical in that species and strain to its toxicokinetics in humans. This selection criterion assumes that these factors are known in potential test animals and in humans, though often, these data are unavailable during the initial phase of a hazard assessment. Although the metabolism of a chemical may be understood in one strain of one species of laboratory animal before initiation of repeated dose testing, it is seldom known in several species and strains. With the exception of Pharmaceuticals, the metabolism of a chemical in humans is almost never known before initiation of a subchronic or chronic study. Consequently, similarity in metabolism between humans and animal models is seldom the initial basis for selection of test species and strain. This may change, however. Currently, human microsomes and systems that express specific human detoxification enzymes are commercially available. This opens the possibility of having in vitro data concerning human metabolism before initiating a hazard assessment.

Sensitivity to Test Material

Another commonly used criterion for selection of the animal species and strain for repeated dose testing is sensitivity to the test material. As a conservative approach to the extrapolation of toxic effects seen in animals to humans, the animal model selected should be the most sensitive to the effects of the chemical. Data required for this decision are often not available until

a significant portion of the total hazard assessment program for the chemical has been completed. Acute and short-term repeated dose studies may reveal information concerning species sensitivity. However, relative sensitivity of different species and strains frequently only can be determined following completion of longer-term studies with their more comprehensive endpoints. Nevertheless, sensitivity to the chemical should be considered during selection of the test animal. For example, differences in the sensitivity of particular organs and tissues to toxic compounds among different species should be considered, and strains that have aberrant metabolic pathways, especially those associated with detoxification, should not be used except in special cases. For instance, the Gunn rat does not produce certain glucuronides (63) and would not be an appropriate animal model for a hazard assessment. Cats are deficient in their ability to produce glucuronides but can produce sulfate conjugates.

Although the concurrent control is the most important source of data for comparison, availability of historical control data for the variables evaluated during repeated dose toxicity testing is an important consideration in selecting the test species and strain. These data are frequently useful in determining the significance of a finding when comparison of data from treated and concurrent control groups suggests a potential treatment-related effect. Historical data concerning growth, feed consumption, clinical pathology, and other variables are often useful in interpreting findings from a subchronic or chronic study. Historical histopathology data are of particular importance due to their subjective nature. Although published data can be useful, historical data from the laboratory at which the study is being conducted are more applicable. Most laboratories have historical databases for commonly used species and strains. If less common species are being considered, the availability of historical data should be assessed before final selection.

Other Animal Model Considerations Involved in Study Design

After consideration of the above criteria, pragmatic considerations are necessary during selection of a species and strain. The animals should be obtained from a reputable, reliable supplier who will guarantee their health and will arrange expeditious and controlled shipment of the animals to the laboratory. The supplier should maintain careful records concerning the animal colony and maintain a healthy colony, providing disease-free animals because it is often not possible to treat for disease once a study has begun. The quantity of available test material may influence the selection of the animal model. For example, it may be necessary to select a rodent species if the amount of test material available is insufficient for long-term administration to a larger species such as the dog or non-human primate. Capabilities of the testing laboratory should be considered during test animal selection. The laboratory must have appropriate caging and other equipment and must be able to maintain the proper environmental conditions in the animal room. In addition, the laboratory conducting the study should have experience with use of the chosen species in toxicology studies. This can avoid problems associated with species specific physiology and anatomy.

Age of Animals

Age of animals used in subchronic and chronic toxicity studies is relatively standard. For rodents, initiation of test material administration at six weeks of age will satisfy virtually all guidelines for testing. Dogs should be approximately four to six months of age at initiation of exposure to the chemical. Precise age of non-human primates is frequently not known; however, age can be approximated by experienced suppliers. For non-human primates and other less commonly used species, young animals generally should be used.

Pre-study Health Assessment

To the extent possible, it should be ensured that each animal included in a repeated dose study is in good health. The animals must not have been previously used for any other type of experimental procedures. An exception is sometimes made for non-human primates, which may occasionally be used for more than one study, with a reasonable period between studies to ensure any residual test material is absent. These animals should undergo extensive health screening, including clinical pathology, between studies.

For rodent studies, enough animals of each gender should be obtained to allow culling of those with conditions that could either interfere with completing the study or be interpreted as treatment related at completion of the study. It is good practice to obtain at least 10% more animals than will be required to fill the study groups. Minimally, pre-test physical examination and body weight measurement should be conducted to assess the health of each animal before study initiation. Pre-test ophthalmological examination and clinical pathology evaluations are advisable. Animals in poor health or exhibiting ocular or other defects should be eliminated from consideration for the study.

To insure the toxicologist is aware of any infection that the animals may be exposed to during the

study, a sentinel group is often maintained in the room with the study animals. For rodents, this group normally contains five to ten animals of each gender. Serum antibody titers are assessed at the initiation of the study and at the termination of the in-life phase of the study. If necessary, antibody titers and/or other evidence of infection can also be obtained from these animals during the study without

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Table 19.7 Serum antibody analyses in rodents

Rat	Mouse
Sendai virus	Sendai virus
Pneumonia virus of mice	Pneumonia virus of mice
Reovirus type III	Reovirus type III
Mycoplasma pulmonis	*Mycoplasma pulmonis*
Lymphocytic choriomeningitis virus	Lymphocytic choriomeningitis virus
Mouse adenovirus FL/K87	Mouse adenovirus FL/K87
Mouse polio virus Hantaan virus	Mouse polio virus Hantaan virus
Encephalitozoon cuniculi	*Encephalitozoon cuniculi*
Cilia associated respiratory bacillus	Cilia associated respiratory bacillus
Rat parvovirus-IFA	Mouse parvovirus-IFA
Rat coronavirus/ sialodacryoadenitis virus	Murine hepatitis virus
Kilham rat virus	Minute virus of mice
Toolan H-I virus	Ectomelia virus
	Mouse pneumonitis virus
	Polyomavirus
	Mouse thymic virus
	Epizootic diarrhea of infant mice virus
	Mouse cytomegalovirus

disturbing the animals on test. A relatively complete list of antibody analyses used in rodent species is presented in Table 19.7.

Health assessment of nonrodent species by the supplier is generally more comprehensive than rodents. This reduces the need to obtain many extra nonrodents. However, it is good practice to conduct procedures after receipt of nonrodents to make sure their health has not changed before use in a subchronic or chronic toxicity study.

Number of Animals

To satisfy most regulatory guidelines, a minimum of 10–20 rodents of each gender should be included in each control and test material dosed group in a repeated dose study. For nonrodents, the minimum number of animals of each gender in each group is four. However, the minimum number of animals is frequently exceeded in an attempt to allow for unexpected mortality or to increase the sensitivity of the study. Twenty rodents or four-to-six nonrodents of each gender per group are often used as the base number of animals for the study. Some study designs include an interim necropsy at one or more intervals for detection and evaluation of the progression of potential effects during the study. Other designs may contain treated animals that will be maintained without exposure after the termination of the main study groups to determine the reversibility of any adverse effects. Still other designs include satellite groups for special purposes, for example, toxicokinetic determinations or untreated sentinel animals used to monitor the health of the study animals. The base number of animals placed on study at its initiation should be increased by the number of animals to be used for these enhanced study designs.

Individual Animal Identification

Before assignment to the repeated dose toxicity study, each animal must be provided a unique identification number. This number will be associated with the animal throughout the study and will be used to identify specimens, tissues, and data from the animal after the in-life portion of the study is completed. Therefore, this number must stay with the animal continuously during the study so that there is no chance of misidentification. It is not adequate to simply attach the animal identification to each cage because animals may escape from their cages or may be placed in the wrong one during cage-changing operations. Unique identification numbers can be placed on the animals by a number of methods. Whatever the identification method, it should remain permanent and readable for the duration of the study. Older methods used for rodents included toe clipping, where a small portion of the toe was removed in a specified coded manner, and ear punching, where holes were punched through the ears in a specified coded manner. These methods are less acceptable today because more precise and humane methods have become widely available. Currently, the use of a numbered tag attached to an ear or tattoos placed on the tail or ear are commonly used methods for large and small animal identification. A newer method that has gained considerable acceptance in recent years involves subcutaneous implantation of a miniature electronic device that can be read by a hand held scanner.

Randomization of Animals

After culling all animals that do not meet the study criteria, such as those that do not pass physical examination or are not within specified body weight boundaries, and assigning unique identification numbers to the remaining animals, the next step is to randomize them into the various study groups. This is a critical step in the study to ensure the greatest ability to detect statistical differences between the groups in the study without bias. Although a number of randomization methods have been devised, some more appropriate than others, one of the most popular is the utilization of random number tables. After randomization into the various study groups, some method should be employed to determine if the animals are truly randomized based upon a variable critical to the study. The most commonly used variable is body weight. Statistical analysis of mean body weight data is conducted to show that there are no statistically signifi

cant intergroup differences in mean body weight, and, hopefully, other variables, at the initiation of the study. It is not uncommon to find that the mean body weight of the animals in one of the study groups is significantly different from one or more of the other groups. In cases where there may be a significant difference between the mean body weights of any of the study groups, the animals are again randomized into study groups and the process repeated. Randomization must be conducted independently for each gender because of body weight differences between males and females.

Animal Husbandry

Proper care and maintenance of animals in a repeated dose toxicity study is essential not only for ethical reasons but also to minimize mistakenly attributing adverse findings to the test material. The Animal Welfare Act (AWA), enforced by the Animal, Plant and Health Inspection Service (APHIS) of the U.S. Department of Agriculture, mandates standards for acceptable handling, care, treatment, and transportation of many species, including most laboratory species except mice, rats, and birds (61). In its *Guide for the Care and Use of Laboratory Animals,* the Institute of Laboratory Animal Resources (ILAR) of the National Research Council has published guidelines that are widely accepted as standards for laboratory animal husbandry (44). From their arrival at the laboratory, animals must be maintained in an appropriately controlled environment and provided an adequate quantity and quality of feed and water and housed in clean cages of appropriate design. They should be acclimated to the study room conditions for at least one week before study initiation (see Chapter 16).

Environmental Factors

Temperature and humidity should be controlled within limits specified in the documents referenced above. Table 19.8 is taken from the ILAR document and contains the recommended temperature ranges for various laboratory animal species (44). Low humidity can result in drying of the mucous membranes and eyes of laboratory animals, and high humidity can result in growth of bacterial and fungal populations that result in respiratory distress and dermal involvement, such as ringworm. In addition, urine and excreta may not dry as readily, thereby increasing room odor. Relative humidity of 30–70% is considered acceptable by ILAR for most laboratory species (44).

Adequate ventilation is a key factor in maintaining good animal health during a toxicology study. Establishing a positive room air pressure reduces possible exposure of animals to test materials being used in other animal rooms. When more air is forced into a room than can be completely cleared by exhaust systems, it flows through the cracks, around the door, and the partial pressure of air in the room becomes positive with respect to the hallway or area outside the room. Ventilation should be homogeneous throughout the room; this generally is controlled by adjustable diffusers and the ventilation of all rooms in a facility must be "balanced" periodically to provide the same relative airflow and positive pressure with regard to hallways. In general, 10–15 fresh air changes per h is considered acceptable but this range is highly dependent on a number of factors, for example, the number of animals in the room.

Common lighting schedules used are 12 h of continuous light and 12 h of darkness for rats, mice, dogs, and monkeys, and 14 h of light and 10 h of dark for hamsters. This schedule allows the animals to become acclimated to a light cycle. This stimulates a constant pattern of secretion of thyroid hormones, ACTH, and growth hormone. Regulated lighting cycles are necessary in reproduction studies because rodents enter continuous estrus under conditions of constant light without darkness. Because high-intensity fluorescent light can cause blindness in albino rodents, current practice is to limit their exposure to high-intensity light to times when observations are collected by providing dual-intensity (high-low) lighting systems.

Animal Caging

In the United States, rodents are commonly housed one per cage during subchronic and chronic toxicity studies. In other countries, rodents are frequently multiply housed during these studies because it is believed that multiple housing increases survival and decreases background pathology. Multiple caging of animals can produce problems associated with unique identification and trauma to the animals from fighting, and multiply housed rodents are more susceptible to transmitted disease and other health concerns. Furthermore, a multiply caged rodent that dies on study may sustain tissue destruction from cannibalism. It also is not possible to determine individual feed consumption when multiple animals are housed in a single cage, resulting in the loss of important data

Table 19.8 ILAR recommended dry-bulb temperatures for common laboratory species

	Dry-Bulb Temperature	
	°C	°F
• Mouse, rat, hamster, gerbil, guinea pig	18–26	64–79

• Rabbit	16–22	61–72
• Cat, dog, non-human primate	18–29	64–84
• Farm animals and poultry	16–27	61–81

because it is not possible to correlate body weight with individual feed intake. In addition, if the test material is fed as part of the diet, it is not possible to calculate actual exposure doses for individual rodents in the absence of individual feed consumption data. Although some of the same problems exist for nonrodents, multiple housing of some species (e.g., non-human primates and dogs) on a regular or continuous basis during the study is accepted practice to permit the social interaction and exercise considered necessary for these species.

Rodents generally are housed in metal (stainless or galvanized steel) or plastic (polyethylene, polypropylene, or polycarbonate) cages. Metal caging or floor pens are used for dogs. Minimum cage sizes for all species are stipulated in the ILAR publication (44) and, for nonrodents, in the AWA (61). Compliance is monitored by federal and state health agencies. Because minimum sizes for cages are stipulated, only caging type remains to be decided between solid floor cages or pens and suspended-floor cages.

Solid floor caging requires bedding to be added to the cage to absorb and contain waste materials; this may introduce dust. Sawdust and chips of some conifers induce hepatic cytochrome P450 monooxygenase activity, which may affect the outcome of the study. In addition, this type of caging allows animals to have access to their waste. Shoebox-style cages used for rodents may clear the atmosphere at a slower rate than suspended wire cages.

Cages with suspended wire floors also have disadvantages. Traumatic foot and leg injuries can occur, particularly with smaller animals. Plantar foot pad lesions are common in long-term studies of rats housed in wire bottom cages, especially in heavy males. This type of cage also exposes the animal to room drafts.

Most gradients in light, temperature, or airborne products in an animal room will occur vertically. Animals within groups should be distributed in cage racks so that members of each study group are present equally at all vertical caging levels. This practice, and the practice of periodically changing the relative position of each cage rack within the room, avoids confounding treatment group with cage position. Documentation of environmental conditions and of cage/rack rotation is essential.

Cleaning cages at frequent intervals is essential. Poor husbandry may result in skin lesions, alopecia, or the appearance of signs and behavior that may be interpreted as a possible effect of the test article.

Diets

The influence of diet and nutrition on the toxicity of xenobiotics is another important aspect of the design of toxicology studies. Because the diet fed the animals during toxicology studies can influence the results, the decision made by the toxicologist concerning what diet to feed the animals may have a profound impact upon the outcome of the studies. The diet fed during a study should have been designed for the study species. Although diets can be custom-made, they are generally obtained from commercial suppliers who should be reputable and capable of supplying information concerning basic diet composition and nutritional information. Although not feasible for a long-term study (e.g., 12 months), the same lot of diet should be used for the entire study whenever possible, and used before its expiration date. It should be stored under appropriate conditions to maintain nutritional value, minimize insect and rodent infestations, and ensure it is not contaminated by environmental chemicals.

Commercial diets are available in either ground “powder” or pelleted form. When the test material is to be incorporated into the feed, a powdered diet is generally used. Use of powdered diet also facilitates the determination of feed consumption. Pelleted diets are most frequently used when test material administration is by routes other than dietary. A powdered diet can be pelleted after a test material has been added, which reduces the dust from the diets. Pelleted diets also decrease the potential exposure of animal room personnel to the test material. However, the heat and pressure involved during the pelleting process may cause degradation of test materials sensitive to these conditions.

Diets used in toxicology studies are of two basic compositions. Currently, diets made from natural ingredients are most commonly used, but semi-purified diets made from refined macronutrients, such as protein and carbohydrate, and micronutrients, such as vitamin and mineral mixes, are sometimes used. Each has particular advantages and disadvantages that must be carefully considered by the toxicologist.

Natural diets are formulated from unrefined plant and animal products to meet the nutritional requirements of a particular species. In closed formula diets, the manufacturer does not provide the exact proportions of the constituents. The diets are formulated based upon nutritional specifications without emphasis on consistency of specific ingredients between lots. Plant materials contain a number of “non-nutritive” components that can effect various physiological and biochemical functions, including detoxification and metabolic activation, in the test animal. Because these components may vary with plant species, strain, growing conditions, and site, individual lots of closed-formula diets may differ in

these constituents. Open-formula diets are formulated with constant quantities of specified ingredients, resulting in a more consistent composition than closed-formula diets. An example is the NIH-07 rodent diet, which has been relatively well characterized (59). Open-formula diets have advantages for long-term studies because of their consistent formulation. An additional consideration -

concerning both open- and closed-formula natural diets is their potential to contain contaminants, such as pesticides, heavy metals, and mycotoxins. To overcome this problem, some manufacturers provide diets that have been assayed for certain potential contaminants to insure they are below stated specifications. It is highly recommended that these "certified" diets be used in toxicology studies. A disadvantage of natural commercial diets is that their nutritional composition cannot be readily altered. It is possible to supplement these diets but not to remove constituents. An important advantage of natural diets is their long history of use and the resulting large quantities of historical control data.

Semi-purified diets are made from refined macroconstituents such as protein, carbohydrate and fiber, and micronutrient mixes containing individual minerals and vitamins and a defined fat source such as corn oil. Their constituents can be varied to design diets for specific nutritional purposes and allow for the inclusion of test materials that may provide nutrient activity or result in nutritional deficits. Nutrient composition can be reproduced exactly from lot to lot of semi-purified diet. As opposed to natural ingredient diets, semi-purified diets do not contain pesticides, mycotoxins, and other constituents that may alter the animal's response to the test material. However, a major problem with semi-purified diets is a lack of historical data from their use in long-term studies. A large number of different dietary compositions are currently in use and data obtained from one semi-purified diet may not extrapolate to another. Even with the most commonly used semi-purified diet, the AIN-76A (1), there are insufficient data to determine its impact on long-term toxicology studies, especially carcinogenicity studies (26). Although these diets can be utilized in subchronic toxicity studies, the data obtained may not be as useful as that from studies with natural diets, especially when these data are used to design longer-term studies with natural diets. While it may be necessary to use semi-purified diets with specific test materials, care must be taken if they are to be used in a safety assessment.

Dietary restriction has been shown to prolong the life of rats and mice. There is some interest in employing restricted food intake (~20%) in chronic toxicity and carcinogenicity studies to prolong the lifespan, and therefore the exposure period, of the animals. If feed restriction is planned, it may be appropriate to employ this regimen in the shorter-term studies also. Utilization of feed restriction in toxicity studies should be discussed with the appropriate regulatory agency before it is included in a safety assessement program.

Drinking Water

Drinking water free of contaminants that could interfere with the objectives of the study should be available to the animals during repeated dose toxicity studies. Water is frequently provided to the rodent and nonrodent animals through automatic watering systems in which a common water supply is piped to the animal cages and each cage contains a valve that allows the animal ad libitum access to the water. Water bottles are another, more labor-intensive method of providing water to the rodents and some nonrodents. For this method, each bottle is fitted with a stopper containing a sipper tube through which the animal can drink and, which, is suspended on the cage. A third method, generally only used with nonrodents, is to provide the animals with water in a drinking bowl. Any of these is acceptable as long as procedures are in place to insure that the animals are provided an adequate supply of potable water.

In-Life Evaluations

Physical Examination

Several variables are routinely evaluated during the treatment phase of subchronic toxicity studies. Each animal should be observed twice daily at least four hours apart (A.M. and P.M.) for overt signs of toxicity, moribundity, and mortality. During these A.M. and P.M. observations, the cage of each animal should be opened to permit unobstructed observation. In addition, each animal should be removed from its cage for a complete physical examination at least once a week. These examinations should include detailed observations for approximate time of onset of any changes, degree and duration of changes involving the skin, fur, eyes, mucous membranes, respiratory function, circulatory system, autonomic and central nervous systems, somatomotor function, and general behavior. A study-specific or SOP-specific glossary of clinical terms and descriptive criteria for each finding is recommended, with simple and descriptive terms, and using a minimum of medical or diagnostic terminology.

Body Weight Measurement

It is recommended that body weight be measured at least once a week, even though biweekly or monthly measurement after the first three months of a chronic toxicity study is acceptable to most regulatory agencies. Weekly measurement is recommended because body weight is one of the most sensitive indicators of the condition of an animal if it is monitored frequently and carefully during a study. Rapid and/or marked body weight loss is usually a harbinger of ill health or death. Rapid body weight loss can be due to either decreased feed or water consumption, disease, or specific toxic effects.

Feed Consumption

In rodents, feed consumption generally is measured once a week during subchronic studies and the first three

months of chronic studies. After the third month of a rodent chronic study, feed consumption may be measured less frequently, that is, biweekly or monthly. For nonrodents, in which the quantity of feed required usually does not allow weekly feeding, feed consumption is evaluated for shorter intervals, often once or twice per day. Accurate measurement of feed consumption is essential for studies in which the test material is administered in the diet. As discussed earlier in this chapter, feed consumption and the dietary concentration of the test material are used to calculate the dose of test material consumed by the animals in such studies. Some species, especially the mouse and the non-human primate, frequently soil or waste feed; which makes accurate measurement of consumption difficult. In these species, feed consumption measurement can be attempted using either a feed container designed to minimize wastage or by attempting to estimate feed wastage. Limitations of such data should be considered in evaluating test material consumption and the significance of any apparent differences between feed consumption in test material-treated and control animals. Feed consumption measurement is another means of monitoring animal well-being. Animals that are ill or suffering adverse effects from exposure to the test material frequently will exhibit significantly decreased feed intake.

Ophthalmological Examination

Ophthalmological examination of all test animals should be conducted before initiation and at the completion of the test material administration period. This evaluation should be conducted by a veterinary ophthalmologist experienced in the observation of the species used for the study.

Clinical Pathology

Clinical pathology variables such as hematology, clinical chemistry, and urinalysis are important indicators of general health and toxicity and are assessed at termination of a subchronic or chronic study. In addition, pretest and interim (typically at 4 weeks in subchronic studies and at 13 weeks in chronic studies) clinical pathology may be conducted to allow evaluation of progression of any treatment-related effects noted at termination of the study. In rodents, clinical pathology determinations are usually conducted for 10 animals of each gender in each group. For nonrodents, clinical pathology should be done for all animals.

Sample collection. Proper sample collection and handling are critical to completion of a meaningful clinical pathology evaluation. Whenever possible, the method of sample collection should be the same throughout the study and should be one that distributes variance—such as run-to-run variation in an enzyme assay—equally across groups. Samples generally are collected according to either a totally random design or a stratified random design. A stratified random design ensures that approximately the same number of animals of each gender and from each group are sampled within any block of time or during any set of assays.

Repeated blood sampling of rodents can be accomplished by serial collection from the same animals by non-terminal procedures (such as puncture of the orbital sinus or jugular vein), or by collection from the abdominal aorta or vena cava at termination of subgroups of animals. Because of practical restraints on the frequency and volume of blood collection in rodents, pretest studies often are not performed. Repeated collection of adequate volumes of blood usually is not a problem for nonrodents and pretest clinical pathology is often included in studies using these animals.

The effect of repeated blood sampling on the animals and on the sample volume that can be reliably obtained at each sampling interval should be considered. Sample volumes should be sufficient both to conduct the assays indicated in the protocol and, if possible, to provide a reserve sample for any necessary repeat test. However, significant reduction of blood volume (more than 10%) by blood collection should be avoided.

Plasma or serum to be used for clinical evaluation should be clear and straw-colored. Red or pink plasma suggests that some hemolysis has occurred either as a result of pathology or as an artifact of the sample collection/preparation procedures. Severe artifactual hemolysis may alter the results for some of the clinical variables to be evaluated. Slight hemolysis, commonly observed in serum and plasma collected from rodents by orbital sinus or jugular venipuncture, generally is acceptable for clinical pathology studies as long as historical laboratory ranges have been established for blood collected by these methods. If unusual or unexpected results are obtained, aliquots of serum or plasma can be "spiked" with ascending amounts of test material to determine if it interferes with the assay.

Clinical chemistry, hematology, and urinalysis. Clinical pathology should include determination of a number of serum or plasma chemistry and hematology variables which should assess electrolyte balance, protein and carbohydrate metabolism, and organ function. An acceptable list of clinical chemistry variables is shown in Table 19.9. Additional variables should be assessed, as appropriate, to address other anticipated effects of the test material, for example, serum cholinesterase levels in the

case of carbamate or organophosphate insecticides. Assays designed for assessment of clinical chemistry in humans must be validated for use with the species used in the toxicology studies. Typical hematological variables assessed during repeated dose testing are shown in Table 19.10. The reader is referred to the chapter concerning clinical pathology in

Table 19.9 Clinical chemistry variables normally obtained in repeated dose studies*a.b*

• glucose*	• potassium*
• urea nitrogen*	• chloride*
• creatinine*	• bilirubin (total)
• total protein*	• cholesterol
• albumin	• triglycerides
• globulin	• alkaline phosphatase*
• albumin/globulin ratio	• aspartate aminotransferase
• inorganic phosphorus	• alanine aminotransferase*
• calcium	• gamma glutamyl transferase*
• sodium*	• ornithine carbamyl transferase*

*a*Based, in part, upon the recommendations of Referene 23.
b This list does not include all clinical chemistry variables that could be obtained. Additional variables could be added dependent upon the test material. priority be given those assays marked with an asterisk (*). When the blood volume obtained for analysis is small, the FDA recommends

Table 19.10 Hematology and urinalysis variables generally determined in a repeated dose study

Hematology	Urinalysis
• hematocrit	• appearance
• hemoglobin	• urine volume
• erythrocyte count	• specific gravity
• mean corpuscular volume	• pH
• mean corpuscular hemoglobin	• glucose
• mean corpuscular hemoglobin concentration	• protein
•	• microscopic evaluation of urinary sediment
• total leukocyte count	
• differential leukocyte count	
• reticulocyte count	
• platelet count	
• prothrombin time	

this text for a more detailed discussion of these clinical chemistry and hematology determinations, and two excellent veterinary texts available for further reference (45, 49).

Urinalysis is often included in the clinical pathology evaluation and may be important, especially for test materials that are nephrotoxins. Urinalysis variables typically evaluated are listed in Table 19.10. Urinalysis however, is frequently of limited value because collection of satisfactory urine samples is fraught with technical difficulties. Urine generally is collected in containers or tubes from troughs or trays placed below the cages in which the animals are housed and, therefore, steps must be taken to minimize fecal contamination. Because urine is frequently collected during an extended period, for example, overnight, bacterial growth in the sample is a concern. Collection of the sample on ice can reduce bacterial growth but presents its own technical challenges. Care should be taken that water be either freely available to the animals throughout the urine collection period or withdrawn at the appropriate time before collection, and the sample must not be inadvertently contaminated by feed or drinking water spilled by the animal. Because of these difficulties, the utility of urinalysis should be discussed with an experienced veterinary clinical pathologist prior to its inclusion in the design of a repeated dose toxicity study. If urinalysis is conducted, its limitations must be kept in mind when the data are reviewed.

Post-Mortem Evaluations

One of the more definitive assessments of toxicological effects conducted during a repeated dose study is the macroscopic and microscopic examination of tissues and organs from treated and control animals. In typical subchronic and chronic studies, samples of approximately 50 tissues and organs are collected during the necropsy of each animal. Table 19.11 presents a list of tissues that are commonly collected for potential histopathological examination.

Necropsy

Necropsy of an animal is conducted when it dies during the study, when it is killed during the study for humane reasons (e.g., in cases of moribundity), or when it is killed at a scheduled interval (interim sacrifice or termination of the study). Necropsy should be completed as quickly as possible after the death of an animal to avoid *autolysis* that can interfere with the subsequent microscopic examination of its tissues. Autolysis is defined as the enzymatic self-digestion of cells or tissues that occurs after death.

It is an especially important consideration for animals that die during the study because their death may not be discovered for a significant period of time. Animals found to be moribund (i.e., about to die) during the study should be terminated for humane reasons and to avoid tissue autolysis.

During necropsy, tissues and organs are systematically removed and macroscopically visible abnormalities are noted, including changes in color, shape, size, or consistency of a tissue. The documentation of an abnormality in the necropsy records should include its location and a clear description of the change, using nondiagnostic terminology. Completeness of the examination during necropsy and the quality of the description of abnormalities are critical to the determination of pathological effects. An abnormality in a tissue can only be prepared for microscopic examination if it was collected and accurately described during necropsy. Because of the central role that the necropsy plays in detecting effects in a repeated dose toxicity study, it is extremely important

that necropsy technicians are highly trained and experienced in the necessary techniques.

After collection, tissue samples are usually preserved by immersion in an appropriate fixative, commonly 10% neutral buffered formalin. In some cases, particularly for organs such as testes or eyes, special fixatives may be used (62). To ensure adequate fixation, the volume of fixative should be at least 10 times the volume of tissues. Certain organs, for example, the lung and urinary bladder, are frequently filled with fixative prior to immersion to improve fixation. During collection of large numbers of tissues from many animals, it is possible to inadvertently miss a tissue. Therefore, it is highly advisable to inventory and document the samples as they are placed into the fixative containers. This inventory will be invaluable during subsequent preparation of the tissues for microscopic examination and in reconstructing the study during post-study auditing of the data. In addition, it must be ensured that the identity of each tissue is clearly maintained while in fixative. This is not a problem for tissues that are large or have distinctive morphology. To ensure subsequent identification of extremely small tissues and those with indistinct morphology, they are frequently placed in labeled plastic cassettes or cloth bags prior to being placed in the fixative container.

Organ Weights

Collection of terminal body weight and organ weights for all animals during necropsy is normal practice in repeated dose toxicity studies. Minimally, weights should be recorded for the brain, liver, kidneys, testes, and adrenal glands. Frequently, other organs such as the thyroid/parathyroid, ovaries, spleen, thymus, uterus, epididymis, heart, or lungs are also weighed. Consideration should be given to the residual blood that may remain in organs such as the spleen, heart, and lungs, which may be variable between animals due to the method of sacrifice and blood collection. Organs should be weighed as soon as possible after removal from the animal and trimmed free of fat and connective tissue prior to weighing and placed into fixative immediately thereafter.

It is common practice to normalize organ weights by expressing them relative to body weight and brain weight. Relative organ weights are used to eliminate the influence of normal variation in animal growth on the interpretation of organ weight data. However, normalized organ weight data should be reviewed with the knowledge that they have some limitations. Expressing organ weights relative to body weight can yield apparent, but artificial, treatment-related effects on organ weights in studies where the test material affects body weight gain, although organ weights normalized to brain weights help overcome this problem because test materials that alter body weight generally do not alter brain weight. The best practice is to consider all three types of data, that is, actual organ weight and organ weight relative to body and brain weight. Histopathological data are often used to help assess the significance of apparent differences between organ weights of test material-treated and control animals.

Microscopic Pathology

Microscopic examination of the tissues and organs of treated and control animals is one of the most time-consuming laboratory functions in toxicity studies. In nonrodent studies, sections of all tissues and organs from all animals should be prepared for microscopic examination. Generally, in rodent studies, only tissues and organs from the controls and high-dose group animals and animals that were killed or died during the study are examined microscopically. For the other treatment groups, only a few major organs (e.g., liver and kidney, and any other organs in which macroscopic abnormalities were noted at necropsy or in which test material-related effects are detected in high-dose animals) are examined. Although initial histopathological examination of control and high-dose tissues followed by examination of other doses is typical in rodent studies, simultaneous histopathological examination of all tissues from all animals is not uncommon. This practice yields the most expeditious completion of the histopathological evaluation phase of a study because sequential examinations are not required. Simultaneous examination also reduces the inter-group variability in diagnoses that might occur when tissues from intermediate groups are examined considerably after completion of the evaluation of the control and high-dose groups. Such variability can lead to incorrect conclusions concerning treatment relationship of lesions noted in the intermediate dose groups. In a draft of its guidelines for safety studies, the FDA has proposed that tissues from all dose groups, not just the high-dose and controls, be subjected to histopathological examination (27).

Routinely, tissues are prepared for light microscopic examination by embedding in paraffin, sectioning at 5–7 microns, and staining with hematoxylin and eosin (H&E) stain (Figure 19.5). Special stains, such as stains for the presence of fat (Oil Red O) or connective tissue (trichrome stain) may be used for some tissues. Use of a protein-specific stain (Mallory-Heidenhain) is illustrated in Figure 19.6. If desired, representative samples of selected tissues may be frozen at necropsy and stored for biochemical or immunohistochemical analyses or specially prepared for electron microscopy. In the histology laboratory,

it is important that tissues be prepared according to standardized procedures especially with respect to type of section (i.e., cross, longitudinal), location, and orientation on the microscope slide. The his-

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tology technician must review the observations recorded during necropsy of the animals to ensure that all grossly observed lesions are properly sectioned and mounted on the slide for subsequent microscopic examination. Whenever possible, samples of lesions prepared for examination should include the lesion and portions of surrounding "normal" tissue.

Histopathological examination of the tissues requires specialized training and is performed by a pathologist trained and experienced in the evaluation of toxicologic pathology. The pathologist must be familiar with the normal features and naturally occurring lesions that can be observed microscopically in tissues from laboratory animals. The pathologist's responsibility, however, is more than evaluation of the tissues and accounting for all the lesions reported at necropsy. The pathologist should be an integral part of the protocol design team to provide input into many factors, for example, selection of the species/strain to be used, clinical pathology variables to be evaluated, and others. The pathologist should also provide guidance concerning the list of tissues to be collected to ensure that they are processed, stained, and evaluated in a manner that satisfies the study objective.

It is critical that the pathologist review the data generated during the in-life and necropsy phases of the study before proceeding with the histopathological evaluation. Results of clinical observations, clinical chemistry and hematology determinations, organ weight measurements, and necropsy examinations can lead the pathologist to focus on particular organs as potential targets for toxicity during the microscopic examination. For example, increased liver weight should lead to a more careful examination for hepatocellular hyperplasia or hypertrophy, and elevated serum creatinine along with a necropsy description of the surface of the kidneys as "rough" should result in a more thorough examination for nephropathy.

Some have suggested that knowledge of in-life and necropsy findings will bias the pathologist, causing a more stringent examination of the potentially affected tissue. Similarly, some are concerned that knowledge of the dose level administered to the animal during the study will bias the pathologist, resulting in a more thorough examination of the tissues from animals that received the test material. The second situation, in particular, could result in a higher incidence and/or severity of microscopic findings in treated animals compared with controls, a higher incidence that is simply an artifact of the thoroughness of the microscopic examination. One way to prevent potential bias is to keep the pathologist ignorant of other study findings and of the identity of the animal until histopathological examination of the tissues has been completed. Because this so-called "blinded reading" does prevent bias but may also prevent the pathologist from identifying certain subtle, dose-related changes, blinded reading is not recommended for routine histopathological evaluations and should only be conducted in special situations.

Pathological changes in cellular or subcellular structure can occur either spontaneously, such as with aging, or as a result of exposure to a chemical. Deciding which changes are significant and what severity should be assigned to a change during histopathological examination is quite subjective. Because of this, it is possible that different pathologists looking at the same tissues will produce different diagnoses. It is even possible that, during histopathological evaluation of a large number of tissues that extends over a number of months, the criteria for diagnosis of the same finding by a single pathologist will change somewhat. The variability that results from the subjective nature of histopathological evaluation is unavoidable. To minimize its effect on the results of toxicity studies, several procedures can be useful. First, during each "reading period," the pathologist should examine tissues from a small subset of the study animals. Each subset should contain approximately equal numbers from each control and treated group to be examined. Second, if a potential target organ is identified over an extended period of time, the pathologist should re-examine that organ from all animals during a compressed reading period of a few days or less to assess to what extent, if any, "diagnostic drift" occurred over time. Third, informal peer consultation concerning unusual or subtle tissue changes observed during examination of the tissues can be conducted to arrive at a consensus diagnosis. In addition, a formal peer review process involving

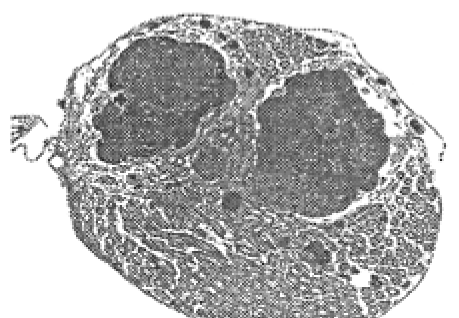
(1) re-examination of target organs,

(2) reexamination of a representative percentage of other tissues from the study, and

(3) review of interpretation of the pathology findings can be conducted by a second pathologist to ensure consistency in diagnosis and grading of tissue changes and accuracy of the pathology conclusions (37).

The objective of the histopathological evaluation is the same as the objective of all other determinations during a repeated dose toxicity study—to detect adverse effects that could be relevant to humans or any other target species exposed to the test compound. Because of certain idiosyncrasies, some animals

and strains are not useful for this purpose. In some cases, the animal exhibits a high spontaneous rate of pathology in a particular organ that prevents detection of any compound-induced increase in that rate. For example, severe testicular pathology occurs spontaneously in a very high percentage of old



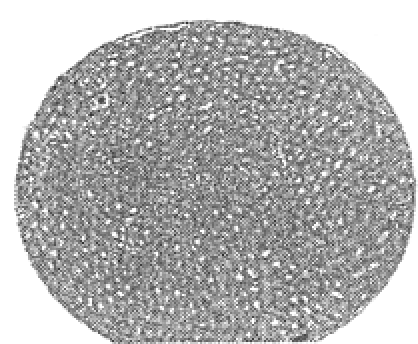


FIG. 19.5. Photomicrographs of Hematoxylin & Eosin stained testes from Fischer 344 rats (2.5 x magnification). The testis on the left exhibits an interstitial cell tumor. The testis on the right is without pathological changes.

male Fischer 344 rats (see Figure 19.5). Therefore, this strain of rat is generally not useful for the detection of testicular effects. In other cases, a pathological change occurs in the laboratory animal only in response to test-compound exposure, but that pathology would not be expected to occur in the target species. An example of this is light hydrocarbon nephropathy that occurs only in male rats. Many hydrocarbons produce this nephropathy, for example, d-Limonene and unleaded gasoline. It is caused by accumulation of a male rat-specific protein, alpha-2-microglobulin, in the renal tubule following exposure to these chemicals. This nephropathy is characterized by hyaline droplets in the cytoplasm (Figure 19.6), granular casts in the lumen (Figure 19.7) and cellular regeneration in the tubules. Because humans do not produce alpha-2-microglobulin, this gender- and species-specific pathological finding has no relevance to human hazard assessment and the male rat is not a suitable model for human renal effects related to light hydrocarbon exposure. For both examples cited, however, the test animal is perfectly acceptable for use as a human surrogate in toxicity testing as long as the limitations imposed by its idiosyncrasies are taken into account.

Even though the idiosyncratic situations discussed above do occur occasionally, the pathology that occurs in most laboratory animals and most tissues is considered relevant to the assessment of hazard in humans. The histopathological examination of tissues in a subchronic or chronic toxicity study can yield a vast array of diagnoses. A detailed discussion of possible chemicalrelated pathological findings is beyond the scope of this chapter. For detailed descriptions of methods for and diagnosis of veterinary toxicological pathology, the reader is referred to a review by Hardisty (37) and two comprehensive texts on the subject (2, 38).

Additional Endpoints for Repeated Dose Toxicology Studies

Evaluation of special endpoints can be added to repeated dose toxicology studies to maximize the utilization of animal resources, minimize the time and cost of a hazard assessment, and obtain additional data. Care should be used in selecting these endpoints to ensure valid methodology is used and that the data will be accepted by regulatory agencies. Draft guidelines for safety assessment of direct food additives and color additives suggest data concerning the immunotoxic and neurotoxic potential of the test material should be generated during subchronic toxicity studies (27). To insure the methodology and data presentation is acceptable to the regulatory agencies, meetings should be held with the appropriate agency during design of the study.

It is possible, through the addition of special determinations, to make a subchronic or chronic toxicity study so complicated that the main objectives are jeopardized. All the ramifications of the addition of special endpoints to a study design, including practical considerations such as daily workload, should be considered to ensure that basic study endpoints are not compromised. Rather than overwhelming the capabilities of the testing laboratory, conduct of a separate study designed to evaluate the special endpoints may be preferable. This is not to say that special evaluations should never be added to subchronic or chronic studies; with appropriate consideration of the possible complications, they can be and often are.

If a question still remains whether additional endpoints should be added to short-term repeated dose study or to longer-term studies, the following should be considered. If these endpoints were added to short-term studies, then the data would be available to aid in design of longer-term

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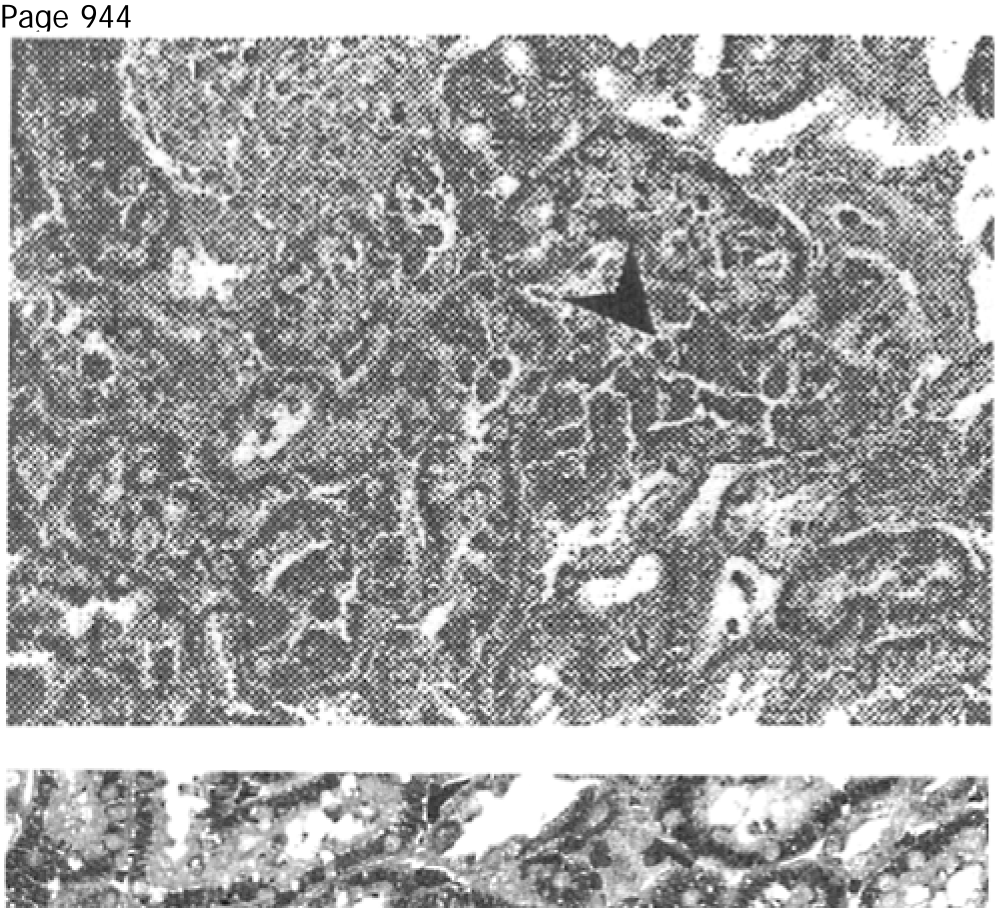

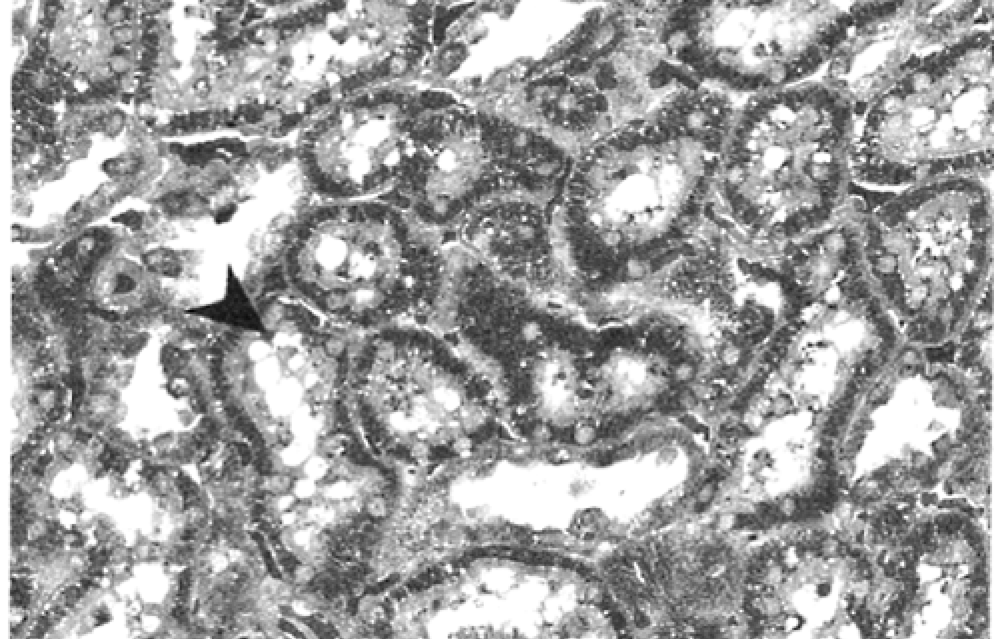

FIG. 19.6. Photomicrographs of kidneys from Fischer 344 male rats stained for protein using Mallory-Heidenhain stain (100 x magnification). The kidney tubules on the top are stained heavily and contain many hyaline droplets (arrow). The kidney tubules on the bottom exhibit normal levels of protein staining (arrow).

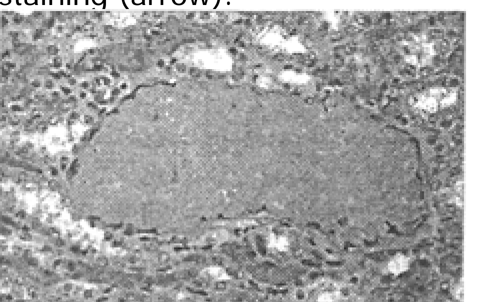

FIG. 19.7. Photomicrographs of Hematoxylin & Eosin stained kidneys from Fischer 344 male rats (100 x magnification). The lumen of one tubule in the kidney on the left is obstructed by a large accumulation of granular casts. The kidney on the right is without pathological changes.

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studies. However, if the particular variable to be evaluated appears only after longer exposure periods, any change might not occur in short-term studies. The most conservative approach is to add the endpoints to each study type. The following paragraphs provide examples of how data concerning non-standard toxicological endpoints may be obtained from classical repeated dose toxicity study designs.

Genetic Toxicology

Certain in vivo and in vivo/in vitro genetic toxicology data may be obtained during or at the termination of a repeated dose toxicity study. Addition of these endpoints to a study could decrease the number of animals used in a hazard assessment and shorten its duration. In vivo genetic toxicology studies increase the value of a hazard assessment. Because they use the route of administration by which humans are exposed to the test material and all processes of absorption, distribution, metabolism, excretion, and DNA repair are intact, these in vivo studies provide important information that cannot be obtained from in vitro studies using cellular systems.

A number of in vivo genetic toxicology assays are in use, some of which are suitable for incorporation into repeated dose toxicity assays. For example, the authors have incorporated the bone marrow micronucleus assay into a classic subchronic study design. During most repeated dose toxicity studies, bone marrow smears are made (see Table 19.11), in essentially the same manner as those used for the in vivo micronucleus assay (39). These slides can be stained with acridine orange and the polychromatic erythrocytes analyzed for micronuclei. Use of bone marrow slides from the repeated dose study has several advantages:

(1) it incurs no additional time and cost for collecting the samples,
(2) it allows the assay to be conducted on animals exposed to the test material for long periods of time,
(3) it eliminates the need for resources to conduct an independent study, and
(4) it does not interfere with the histopathological assessment of the tissues.

It may be possible to use bone marrow slides from repeated dose toxicity studies for the bone marrow chromosomal aberration assay (4). This is another in vivo genetic toxicology assay that could provide additional data without an independent study being conducted. Another such genetic toxicology assay is the in vivo/in vitro unscheduled DNA synthesis assay (51), in which freshly isolated hepatocytes are used to determine unscheduled DNA synthesis. Although this assay is

Table 19.11 Tissues collected for histopathology in repeated dose studies

Adrenals	Mammary gland (females)
Aorta	Muscle (thigh)
Bone marrow smear	Nose
Brain	Ovaries
Cecum	Pancreas
Colon	Pharynx
Duodenum	Pituitary
Epididymides	Prostate
Esophagus	Rectum
Eyes	Salivary gland (submandibular)
Femur and bone marrow	Sciatic nerve
Gallbladder (when present)	Seminal vesicles
Heart	Skin
Ileum	Spinal cord (cervical, thoracic, lumbar)
Jejunum	Spleen
Kidneys	Sternum and bone marrow
Lacrimal gland	Stomach
Larynx	Testes
Lesions	Thymus
Liver	Thyroid with parathyroid
Lungs	Trachea
Lymph nodes	Urinary bladder
(mandibular and mesenteric)	Uterus

not compatible with histopathological use of the liver, as few as two to three extra animals per group are all that are required. One problem associated with incorporation of additional endpoints, such as genetic toxicology, into a repeated dose study design is that the laboratory conducting the study must have valid assay methodology. This is not always the case.

Not all in vivo genetic toxicology assays are completely suitable for incorporation into repeated dose

studies. For instance, the in vivo sister chromatid exchange assay requires the administration of deoxybromouridine to the animals. This compound can compromise the classical endpoints used in toxicity studies, including histopathology. It is possible, however, to incorporate extra animals to assess specific endpoints while not compromising the main study animals.

Neurotoxicity

It is possible to incorporate neurotoxicity screening into repeated dose toxicity study designs. In fact, in its draft version of its food additive toxicology testing recommendations, the FDA has suggested that neurotoxicity screening be incorporated into these study designs (27). EPA has similar recommendations (14, 15).

Neurotoxicity screening is designed to determine if the test material has the potential to produce adverse effects on the nervous system. Screening is conducted to determine if additional, more sophisticated, neurotoxicity

testing is required. The first indication of a requirement for neurotoxicity testing may come from the structureactivity assessments. However, the database for neurotoxicity structure activity assessments is not as extensive as for certain other types of toxicity and may not provide useful insight into the need for neurotoxicity testing. Drugs and pesticides that target the nervous system are a well-known exception. For instance, there is no question that a new organophosphate insecticide will require neurotoxicity testing. For most compounds, it will be necessary to develop data through empirical testing.

Most classical repeated dose study designs contain elements that may provide some information on neurotoxicity potential. These include cage-side observations of the animals, physical examinations, and measurement of variables, such as food consumption, that may relate to behavior modifications during the in-life phase of the study. Additional information is obtained during histopathological examination of the structures of the nervous system collected at necropsy, such as the brain and spinal cord. The FDA believes, however, that these procedures, as well as others, should be specifically included into the design of repeated dose toxicity protocols (27). Table 19.12 lists the design elements recommended by the FDA for a neurotoxicity screen. Specific behavioral and neurotoxicity tests exist to provide most of the requested data, and the particular test designs should be chosen based upon their validity, history of use, lack of undue stress to the animal, and the

Table 19.12 FDA draft criteria for a neurotoxicity screen as a component of short-term and subchronic studies

- Histopathological examination of tissues representative of the nervous system, including the brain, spinal cord, and peripheral nervous system
- Quantitative observations and manipulative test to detect neurological, behavioral, and physiological dysfunctions. These may include:
  - general appearance
  - body posture
  - incidence and severity of seizure
  - incidence and severity of tremor, paralysis, or other dysfunction
  - level of motor activity and arousal
  - level of reactivity to stimuli
  - motor coordination
  - strength
  - gait
  - sensorimotor response to primary sensory stimuli
  - excessive lacrimation or salivation
  - piloerection
  - diarrhea
  - polyuria
  - ptosis
  - other signs of neurotoxicity deemed appropriate

experience of the laboratory with the specific test. Care should be taken to perform these procedures on all the treatment groups and controls in the study. A concern is that some of these tests may stress the animals and produce changes in the traditional variables measured during a toxicity study. If this were to happen, it is assumed that the control group would also demonstrate these changes, which may or may not be true. Therefore, a conservative approach would be the addition of extra animals to the study that would be subjected to the manipulative procedures for neurotoxicity screening and not be used in the traditional phases of the study. For a more complete discussion of the FDA recommendations, the reader is referred to the draft guidelines for safety of direct food additives (27).

Immunotoxicity

Rapid advances have been made during the last 20 years in respect to detection of immunotoxicity. The two major forms of immunotoxicity are immunosuppression and hyperactivity of the immune system. Immunosuppression results in a reduction in the animal's resistance to infection and potential increase in susceptibility to tumorigenesis by a suppression of critical immunological responses. Hyperactivity of the immune system can result in autoimmune diseases and increased sensitivity to allergic disorders. Determination of the mechanisms associated with these disorders can be extremely complex because of the large number of biochemical, cellular, and physiological factors that can be effected as well as the cellular interactions required to mount an immunological defense. The detection of potential immunological changes is less complex and a number of tests exist that can provide warning of a

potentially immunotoxic compound.

The FDA has published draft recommendations for the inclusion of immunotoxicity evaluations in repeated dose toxicity studies (27). It suggests that such evaluations be conducted in rodents. Immunotoxicology testing procedures were divided into two broad categories—Type I tests are those assays that do not require the study animals to be treated with an agent that presents an immunological challenge. Type II Tests are assays that require the study animals to be challenged with an agent that elicits an immune response, such as antigens, vaccines, infectious agents, or tumor cells. Because Type I tests do not require manipulation of animals, they can be included in the routine assays done during a repeated dose toxicity study. Because Type II tests do require treatment of the animals with an immunological challenge, these animals are not suitable for evaluations conducted during toxicity studies. Therefore, additional animals must be included in the study design.

Table 19.13 lists the immunotoxicology evaluations that the FDA suggests should be included in Type I tests.

Table 19.13 FDA draft recommendation for type I immunotoxicity test that can be included in repeated dose toxicity studies

Type I Test

Hematology	Clinical Chemistry
• white blood cell counts	• total serum protein
• differential white blood cell counts	• albumin
• lymphocytosis	• albumin-to-globulin ratio
• lymphopenia	• serum transaminases
• eosinophilia	

Histopathology
- lymphoid tissues
- spleen
  - lymph nodes
  - thymus
  - Peyer's patches in gut
  - bone marrow
  - cytology (if needed)[a]
  - prevalence of activated macrophages
  - tissue prevalence and location of lymphocytes
  - evidence of B-cell germinal centers
  - evidence of T-cell germinal centers
- necrotic or proliferative changes in lymphoid tissues

a More comprehensive cytological evaluation of the tissues would not be done unless there is evidence of potential immunotoxicity from the preceding evaluations.

Generally, these evaluations are those currently recommended for repeated dose toxicity studies with the exception of a more comprehensive histopathology of lymphoid tissues. Inclusion of these evaluations into current study designs should have no impact on the validity of the study. Inclusion of the expanded Type I tests listed in Table 19.14 would require more planning during the study design phase and assurance that the laboratory performing the study would be capable of performing the assays. The expanded Type I test generally would only be conducted after consultation with the FDA. Type II immunotoxicity tests recommended by FDA could be done as a component of a repeated dose toxicity study, but they appear to be more appropriately conducted as independent studies and are beyond the scope of this discussion.

Miscellaneous Other Endpoints

There are a wide variety of additional endpoints that could be evaluated in repeated dose studies. In part, any additional endpoints would depend upon the questions to be addressed and the creativity of the toxicologist designing the studies. For instance, the increased availability of electron microscopy makes this endpoint a more viable option today than in previous decades. Development of an increased number of histochemical assays, especially those employing specific antibodies, makes

Table 19.14 FDA draft recommendation for expanded type I immunotoxicity test that can be included in repeated dose toxicity studies

Hematology	Serum Chemistry
• Flow cytometric analysis	• Serum protein electrophoresis
• B-lymphocytes	• albumin
• T-lymphocytes	• α-globulin
• T-lymphocyte subsets	• β-globulin
• TH & TS	• γ-globulin
• Immunostaining of blood or spleen fraction	• Quantification of γ-globulin
• B-lymphocytes	• IgG, IgM, IgA, IgE
• T-lymphocytes	• Complement
	• Cytokines
	• -IL-2, IL-1, γ-interferon
	• Auto-antibodies
	• Anti-parietal cell antibodies

Histopathology
- Immunostaining of B-lymphocytes in spleen and lymph nodes with polyclonal antibodies to IgG

- Immunostaining of T-lymphocytes and subsets with monoclonal or polyclonal antibodies
- Micrometric measurements of germinal centers and periarteriolar lymphocyte sheath of the spleen and follicles and germinal centers of lymph nodes

In vitro analysis of functional capacity of specific immune cells

- Activity of natural killer cells
- Mitogenic stimulation of B- and T-lymphocytes
- Macrophage phagocytic index
- Stem cell assays

the preserved wet tissues, embedded tissues, and the histopathology slides obtained from these studies valuable for future use. In many cases, the need for additional endpoints is unknown until the initial results of the study are known, at which point the preserved materials then become a valuable resource. For instance, if it is found that the liver from a study contains vacuoles and the toxicologist suspects these to be fat vacuoles, it is possible to use special lipid stains to determine if the vacuoles are lipid. Other special stains exist for a variety of purposes. An example of a protein-specific stain, MalloryHeidenhain, is illustrated in Figure 19.6.

If the toxicologist believes the histopathological data indicate a particular compound is producing cellular proliferation, there are a number of approaches to investigate this hypothesis. A standard method to determine cellular proliferation is by injecting the animal with 3H-thymidine and measuring the incorporation of the radiolabel into cellular DNA by methods such as radioautography. However, if the study has been terminated, this is not possible. Also, the toxicologist may not want to administer radiolabel to the animals for a number of reasons, even if the in-life portion of the study has not been terminated. If the evidence of increased cellular prolifer

ation occurs in tissues with a relatively high rate of normal proliferation, such as the gut mucosa, it is possible to determine the mitotic index by counting mitotic figures in cells from slides previously prepared for histopathological analysis. Alternatively, it is now possible to immunostain for specific proteins associated with cellular proliferation in preserved tissues. Again, the particular additional endpoints added to the toxicology study should address a specific issue.

DATA ANALYSIS AND INTERPRETATION

Compilation and Summarization of Study Data

Once the data have been collected from a repeated dose toxicity study, the next steps involve data analysis, interpretation, and reporting. These data are derived either from the measurement of a variable associated with the test animal—for example, body weight, feed consumption, serum enzyme activity—or from observation of the animal, for example physical examination findings, macroscopic and microscopic pathology, and so on. The frequency, number, and variety of these measurements and observations in repeated dose toxicity studies yield an extremely large volume of data that must be organized and summarized prior to analysis. Historically, individual animal data were recorded manually and then were compiled either manually or following entry into a computer. Some specialized data are still handled manually today; however, most routine data are collected, compiled, and statistically analyzed electronically using custom or commercially available computer programs.

For quantitative data such as body weights and feed consumption, individual animal data are used to calculate the mean values with a measure of statistical variation for each treated or control group. For other types of quantitative data such as the number of animals exhibiting a behavioral effect or the number of animals in which a particular finding is determined histopathologically, the incidence of the observation in each treated and control group—that is, the number of animals affected as a fraction of the total number of animals observed—is presented. Some data, like leukocyte differential counts and results of microscopic examination of urinary sediment, cannot be effectively summarized using a group mean or incidence value. Summarization of these types of data involves listing the individual animal data in their appropriate groups.

Determination of Treatment-Related Effects

Data from the treatment groups are compared with data from the control group to determine if any treatment related effects have occurred. In virtually all cases, the data from one group of animals will differ somewhat from the data for any other group. Therefore, differences between the sets of data that are potentially related to treatment must be differentiated from spurious occurrences and from normal biological variation. This is accomplished by two methods. The first is simple examination of the data and detection of differences worthy of further consideration based upon the experience of the toxicologist and comparison with historical data. The second method uses statistical tests to detect differences for which the probability that the difference occurred by chance is low. These methods should always be used in combination. Although it is an extremely powerful and useful tool, statistical analysis alone should not be used to detect treatment-related effects because, as stated by the FDA, "… statistical outliers are not always biological outliers and a "significant" statistical test ($p<0.05$) does not always indicate biological significance" (27).

Differences between the data from the control and treated animals that are detected using the methods cited above may indicate either an adverse effect associated with the test material, physiological adaptation to the test material, or normal biological variation unrelated to the test material. Determination of the significance of differences between treated and control groups is based on a number of factors that are frequently considered in combination with each other. These are listed in Table 19.15 and discussed in the following paragraphs.

Dose-related Trend

One of the best indicators of an effect related to treatment is the presence of a dose-related trend in the data, that is, the magnitude of the effect varies directly with the dose level. Such an effect is reflective of the basic principle of toxicology stated by Paracelsus in the 16th century and often paraphrased as, "The dose makes the poison" If a difference from controls is noted in two or more dose groups and the severity or incidence of the difference increases as the dose level of the test material increases, it is probably a treatment-related effect. When a difference from controls is noted only

Table 19.15 Factors considered to determine the significance of differences between treated and control groups

- Dose-related trends
- Reproducibility
- Related findings

- Magnitude and type of difference
- Occurrence in both genders

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in animals receiving the highest dose level of the test material, it may or may not be treatment-related and other factors must be considered in determining its significance. Differences from control data in test material-treated animals are probably not associated with treatment if a dose-related trend is not observed. Because of this, as stated previously, selection of an appropriate range of dose levels is extremely important and facilitates data interpretation.

Reproducibility of Effect

Another reliable indicator of a treatment-related effect is its reproducibility. If a difference from controls is noted in the treated animals at multiple intervals during a study, the difference is likely related to treatment. Further weight is given to the determination of the treatment relationship if the same difference is noted in other independent studies in which the test material was administered to the same species and, even more weight is given if the difference is observed in a second species. The absence of reproducibility, especially in the same species, is an indication that the difference may have occurred by chance.

Correlated Findings

Another consideration in the assessment of significance of an intergroup difference is the presence of related findings. For example, an elevation in the activity of serum alanine aminotransferase in treated animals when compared with the control group is probably related to treatment if there is an elevation in serum aspartate aminotransferase with concomitant hepatic necrosis. If no correlation with other findings is observed, the elevation may be of no significance or, at least, its significance must be determined considering other factors.

Magnitude and Type of Inter-group Difference

The magnitude and type of difference observed between treated and control data may also give an indication of its potential association with test material administration. For example, a doubling of an organ weight in treated animals compared with controls should be considered more likely to be treatment-related than a 10% increase, even if the smaller increase is statistically significant. Furthermore, a fairly large decrease in the activity of serum alanine aminotransferase in treated animals is generally assigned limited clinical significance whereas an increase of the same magnitude in the activity of this enzyme may be considered indicative of a toxic effect. It is obvious that the assessment of the significance of a change on the basis of its magnitude or type requires knowledge of normal trends and ranges for the data.

Gender Differences

Determination of the significance of an apparently treatment-related effect is also influenced by whether or not the difference occurs in animals of both genders. Since treatment-related effects often occur in both genders, a difference from controls that is noted only in treated animals of one gender may not be associated with the test material. It must be remembered, however, that there are cases where one gender or the other is more sensitive to the toxicity of a chemical and, therefore, only the sensitive gender will exhibit the effect at a given dose. For this reason, a difference should not be considered insignificant solely on the basis of its absence in one gender. Male rats are well known for their greater capacity to detoxify certain compounds because of their higher activity of P450-dependent monooxygenases. Therefore, they may demonstrate less toxicity to these compounds than females. However, if the metabolic product is more toxic than the parent compound, they may demonstrate higher sensitivity than females (see Chapter 3). This gender difference is not seen in a number of other species, including humans (52).

STUDY REPORT

After the data from a study have been analyzed, a report is prepared. Depending upon the intended use of the report, it may contain various levels of detail. For example, reports prepared for submission to a regulatory agency in support of the safety of a chemical will generally contain much more detail (including all individual animal data) than research reports intended for publication or that will be used by an organization or individual only to give guidance for future testing. Whatever the purpose of the report, it should be written in sufficient detail to permit peer review of the conduct and conclusions of the study and to allow the study to be reproduced.

Report Content

All reports, regardless of their purpose, should contain certain common elements that are essential to adequately describe the conduct and results of a study. The report should clearly state the objective(s) of the study. It should precisely define the test material, indicate the test species used, and describe the methods and equipment employed to collect and analyze the data. Protocol deviations and an assessment of their impact upon the study should be presented. The report should present the data

pertinent to the study objective(s) in a form that facilitates its review and should discuss these data in the depth required to support the conclusion(s) of the study. The discussion

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should describe any treatment-related effects observed and should explain how the various factors described above were used to determine the significance of any differences between treated and control animals in the study. Finally, the conclusion(s) drawn from the results of the study should be clearly and concisely stated.

Retrospective Report Audits

After the study has been completed and reported, retrospective audits of the study are frequently conducted. The manufacturer of the test material may audit the study prior to submitting it to a regulatory agency in support of registration or approval of the test material for its intended use. The regulatory agency to which the study report was submitted may also conduct an audit. Regulators also audit study reports to assess compliance of the testing laboratory with GLP regulations. Whatever the reason for retrospective auditing of the study, the process is essentially the same. The raw data are inventoried to ensure that they have been properly maintained. They are reviewed and compared with the study report to ensure the report accurately and completely presents the methods used and the data collected. Any deviations from laboratory standard operating procedures, the study protocol, or GLP regulations that occurred during conduct and documentation of the study should have been clearly explained in the report. Individual animal data should support summary tables and discussion of the results should be consistent with the individual and summary data.

Retrospective auditing is of great value to all parties involved. The manufacturer of the test material can feel comfortable that it will receive no surprises during a subsequent audit by the regulatory agency. The regulator will be more confident about the quality of the study if the data have been audited. If the regulator is confident about the data, his or her review will proceed more smoothly and, therefore, regulatory decisions will be expedited.

REGULATIONS CONCERNING GENERATION AND USE OF DATA FROM REPEATED DOSE TOXICITY STUDIES

Almost every industrialized country in the world has regulations governing the introduction, transportation, and use of new pesticides, food additives, human and animal drugs, and other chemicals. Many of these countries also have regulations governing medical devices, workplace exposure to chemicals, the introduction of industrial chemicals into commerce, and the disposition of chemical waste.

There is general uniformity in the objectives of these laws, that is, not to impede the beneficial use of chemicals, but at the same time to ensure their safety in use. Even though the regulatory agencies can agree, in broad terms, on one framework of toxicity testing guidelines, the toxicologist must become familiar with the details of particular guidelines to fulfill his or her role as a bridge between scientific and regulatory concerns. Several regulations governing repeated dose toxicity and other types of toxicity testing are briefly described below.

United States Laws and Regulatory Guidelines

Federal Food, Drug and Cosmetic Act (FFDCA)

FFDCA (29) as amended by the Food and Drug Administration Modernization Act of 1997 (38), controls the introduction of human and animal drugs, direct food additives, indirect food additives (such as packaging materials), and components of cosmetics. In the case of new human or animal drugs, safety and efficacy must be established for a particular therapeutic application before the FDA grants approval for marketing. The approval process is comprehensive and involves two sequential phases. For the investigational new drug (IND) phase, industry is required to file preclinical toxicity data with the FDA before investigation of the safety and potential therapeutic value of a drug in limited numbers of humans. When the efficacy and safety of the drug in the treatment of a particular disease is established through extensive clinical trials, these data together with additional animal toxicity data are provided to the agency as part of a new drug application (NDA) or new animal drug application. The NDA is reviewed by the FDA with respect to safety and claims of efficacy of the drug, and is either approved or disapproved, or deficiencies in the data cited.

The summary of a NDA must address benefit/risk relationships, clinical data, nonclinical pharmacology and toxicology, human pharmacokinetics, bioavailability, and microbiology. It must also contain information on pharmacological class, scientific rationale and clinical benefits, and chemistry and manufacturing (34).

For food additives, industry must show that a material either intended for direct addition to food, such as a preservative or flavoring agent, or having indirect contact with food, such as a packaging or can-coating material, is safe for its intended use. Results from a hazard assessment are submitted to the FDA for review as part of a food additive petition. If the data demonstrate the safety of the chemical, a

regulation is published allowing the chemical to be used for a particular purpose in food or in contact with food. In 1982, the FDA published *Toxicological Principles for the Safety Assessment of*

Direct Food Additives and Color Additives Used in Food (23). A revised version of this document has recently been released by the FDA (Toxicological Principles for the Safety of Food Ingredients, Redbook 2000) (27). This so-called "Redbook" delineates the nature of evaluations necessary to determine food additive safety and provides a basic scheme for scientifically sound decisions for the development of the safety assessment. These guidelines include a priority-setting system that increases the efficiency of the food additive safety assessment.

FFDCA also provides an alternative method by which materials can be approved for use in or on food, the generally recognized as safe (GRAS) process (31). For a material to be considered GRAS, its safety evaluation must satisfy three basic requirements. First, a group of experts qualified by scientific training and experience must conclude that the material, when used as intended, is safe for human consumption. The sponsor of the potential additive convenes this expert group and the group operates without regulatory oversight. Second, the information considered by the expert group during the GRAS review must be common knowledge, that is, available in the scientific literature. Third, within the scientific community there must be general agreement with the conclusion of the expert group. Final GRAS approval does not require regulatory review; however, many producers of such materials petition FDA to affirm their GRAS status prior to marketing them.

There are no requirements for the FDA to review cosmetic formulations for safety prior to marketing. Even though the FFDCA only requires that the cosmetics be free of any "poisonous and deleterious" substances, responsible suppliers of ingredients for use in cosmetics and manufacturers of final products conduct toxicity studies relevant to the specific cosmetic.

FFDCA also addresses the concentrations of pesticides permitted in foods in the United States. Under FFDCA Section 408, the EPA establishes maximum allowable concentrations for pesticide residues in raw agricultural commodities—food crops, eggs, raw milk, and meat. Under FFDCA Section 409, EPA also establishes a maximum allowable concentration for a pesticide in processed food, if processing concentrates the residue of the pesticide in the raw agricultural commodity. These maximum concentrations are referred to as *tolerance levels* or tolerances. Human exposure resulting from consumption of foods containing tolerance levels of a pesticide must not exceed the maximum permissible intake of the pesticide established by EPA under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). Tolerances for most foods are enforced by FDA, and those for meat, poultry, and some egg products are enforced by the Food Safety and Inspection Service (FSIS) within the U.S. Department of Agriculture (USDA).

Federal Insecticide, Fungicide and Rodenticide Act

FIFRA was administered initially by USDA and is now administered by the EPA (10). Under FIFRA, EPA is responsible for registration of pesticides for use in the United States. This act requires extensive toxicity testing to be conducted in mammalian, avian, and aquatic species to support the safety of a pesticide. Detailed guidelines for toxicity study design and reporting have been issued by EPA (17). Toxicity data submitted in an application for registration of a pesticide are reviewed by toxicologists in the Office of Pesticide Programs at EPA. Other data specifically required by FIFRA as a condition of pesticide registration include product chemistry, residue chemistry, environmental fate, reentry protection, spray drift, plant protection, non-target insects, and product performance.

Food Quality Protection Act

The Food Quality Protection Act (FQPA) was signed into law in 1996 and amends sections of both FFDCA and FIFRA (13). It is intended to establish more consistent regulation of pesticides and to protect human health using an approach that places increased emphasis on the scientific evaluation of pesticide safety data. Among other provisions affecting pesticide registration and tolerance setting, it

(1) mandates special considerations for protection of infants and children,

(2) requires determination of aggregate pesticide exposure from all sources (e.g., food, home use, drinking water, etc.),

(3) requires summation of exposures from multiple chemicals that exhibit a common mechanism of toxicity,

(4) expedites approval of pesticides considered to be most safe, and

(5) requires periodic reevaluation of tolerances for registered pesticides to be certain that the data supporting registration remain acceptable and complete by current standards.

The methods to be used for implementation of several of the provisions of the FQPA are still being developed, and some (such as the means for special protection of children) are the subject of considerable controversy within the scientific community. EPA is considering input concerning resolution of these controversial issues from pesticide manufacturers, agricultural trade organizations, academia,

consumer protection groups, and the public prior to finalizing their approach. Significant progress must be made soon, however, because FQPA mandates that EPA complete review of the tolerances for 33% of all currently registered pesticides by August 1999, of

an additional 33% by August 2002, and of the balance by August 2006.

Toxic Substances Control Act

The Toxic Substances Control Act (TSCA) is administered by the Office of Pollution Prevention and Toxics within EPA and is a complex and far-reaching law that affects industrial chemicals existing in commerce as well as new chemicals in the United States (20). One of the first requirements of TSCA was the compilation of an inventory of chemicals that were active in commerce in the years 1977–1979 and determination of the need for toxicity testing of these chemicals.

Manufacturers or importers of industrial chemicals that are considered new under the TSCA definition are required to notify the EPA at least 90 days before the manufacture or import of a new chemical. The act requires that certain information regarding the new chemical be submitted to the EPA for review in a premanufacture notification (PMN). Although the act does not require toxicity testing to be conducted on a new chemical prior to manufacture, it does require submission of all existing health and safety data for the new chemical so that its risk to health or the environment can be assessed. If the EPA determines in its review that the new chemical does present an unreasonable risk, one of several actions it may take is to require that the chemical be tested for specific toxic effects. The EPA may also issue a testing rule requiring that a specified chemical or chemical mixture be tested for certain toxic effects. The EPA has issued test standards for the conduct of toxicity studies on chemicals or chemical mixtures for which testing will be required under TSCA. These standards are the same guidelines used for pesticide testing under FIFRA (17).

Transportation Act

Regulations promulgated by the U.S. Department of Transportation (DOT) require that materials shipped in interstate commerce be labeled and contained in a manner consistent with the degree of hazard they present (5). DOT requires that acute toxicity data for chemicals be used to place them into “packing groups,” with labeling requirements for chemicals based upon this packing group.

The Coast Guard

Prior to importing a chemical into the United States, the Coast Guard requires a set of acute mammalian toxicity data for the chemical (6). This acute toxicity profile should minimally include the following: acute oral toxicity, acute dermal toxicity, and skin and eye irritation studies.

Consumer Product Safety Act

Prior to passage of the Consumer Product Safety Act (CPSA), the classification and testing for acute toxicity of household products was conducted under regulations promulgated by the FDA, which administered the Federal Hazardous Substances Act (FHSA). The function of administering FHSA now resides with the Consumer Product Safety Commission (CPSC). If results obtained in acute oral or dermal toxicity tests conducted using methods outlined in the *Code of Federal Regulations* (CFR) for hazardous substances meet prescribed criteria of toxicity, labeling and packaging as prescribed in the regulation must be used (3).

Occupational Safety and Health Act

This law, administered by the Occupational Safety and Health Administration (OSHA) of the U.S. Department of Labor, is designed to assure safety in the workplace (58). No requirements exist in this law for manufacturers to test substances for toxicity prior to their use in the workplace. The impact of the TSCA has an overlapping effect, in that occupational exposure to new chemicals is considered in premanufacture notices. As indicated previously, specific test requirements under TSCA affect new or existing chemicals that are manufactured or processed in the United States.

Resource Conservation and Recovery Act

The Resource Conservation and Recovery Act (RCRA) authorizes the EPA to institute a national program to control hazardous waste defined as “solid, liquid, semi-solid or gaseous waste that may cause increased mortality or serious illness, or may cause substantial hazard to the health or the environment when improperly managed” (18). The main purpose of these regulations is to control the generation, storage, treatment, transportation, disposal, record-keeping, and reporting of hazardous waste. RCRA places the primary responsibility of identifying and managing hazardous waste on the waste generators. Other persons or institutions involved in waste disposal and management also have an obligation to know if the waste is hazardous. The degree of toxicity, concentration, migration to the environment, persistence or degradation in the environment, bioaccumulation in the ecosystem, types of improper management, quantities of waste, past human and environmental damage records, and other factors are all taken into consideration.

International Laws and Regulatory Guidelines

The United States has not been alone in developing laws to protect humans and their environment from

possibly dangerous effects of new industrial chemicals in the

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marketplace. In the European Union, the Council of Ministers of the European Economic Community (EEC) has issued a directive concerning laws, regulations, and administrative procedures which relate to the classification, packaging, and labeling of dangerous substances (7). An amendment to this directive was adopted later to protect humans and their environment from potential risks that might arise through the marketing of new chemicals (8). It requires that a new chemical be subjected to a base set of tests to define its physical and chemical properties, mammalian toxicity, and ecotoxicological effects. The base set of mammalian studies consists of the following: acute oral LDso, acute dermal LDso, acute inhalation LCso (if applicable), skin and eye irritation, and dermal sensitization. A manufacturer or importer is required to furnish the appropriate authorities in his or her EEC member state with a notification containing, in part, the results of these tests with the new chemical. Such notification must be filed 45 days before marketing in the member state in which it is to occur.

The amendment to the EEC directive is the counterpart of the U.S. TSCA. There are, however, some differences, not the least of which is in the approach to toxicity testing. The EEC requires only notification of a new chemical prior to marketing whereas TSCA demands that the notification, in the case of a domestic manufacturer, be given to the EPA at least 90 days before manufacture.

The EEC has also provided guidance on the evaluation of safety and efficacy of drugs. It has now adopted guidance notes for efficacy, testing of pharmacokinetics in humans, and bioavailability of drugs for long-term use, as well as a number of more specific activity groups including cardiac glycosides, oral contraceptives, topical corticosteroids, nonsteroidal anti-inflammatories, antimicrobials, anticonvulsants, antianginals, and chronic peripheral arterial disease agents. Safety guidance notes also were adopted for single-dose and repeated dose toxicity, pharmacokinetic metabolism, mutagenic potential, carcinogenic potential, and reproduction studies (9).

As discussed earlier in this chapter, with many countries establishing their own regulations for safety assessment, it became more likely that manufacturers would have to perform several different versions of the same tests to satisfy the requirements of different countries. In an attempt to avoid unnecessary and wasteful duplication of work, the Organization for Economic Cooperation and Development (OECD) produced a set of toxicity testing guidelines that allow tests to be carried out in a similar manner in different countries (54). The OECD package, which has been revised 10 times to implement improvements to individual study designs, includes guidelines on acute oral, dermal, and inhalation studies; eye and skin irritation and skin sensitization studies; subchronic oral, dermal, and inhalation studies; and teratogenicity, carcinogenicity, and chronic and combined chronic/carcinogenicity studies. Results from studies conducted according to OECD guidelines are generally fully acceptable to the various regulatory bodies throughout the world.

To facilitate more universal acceptance of data generated to support approval and use of pharmaceutical products, the International Conference on Harmonization (ICH) has developed guidance documents concerning the efficacy, quality, and safety of these drugs. The ICH is made up of regulatory authorities and trade organizations from the United States, Japan, and the European Union. Instead of providing detailed instructions for study design or conduct, the ICH guidances address specific issues related to testing, for example, the duration of chronic toxicity testing, definition of an acceptable battery of genotoxicity studies, required elements of chemical stability testing of new drug products, and so on (41). Because of ICH efforts, many hindrances to the approval and use of valuable Pharmaceuticals around the world have been removed.

Regulatory Internet Sites

Many of the regulatory agencies and organizations worldwide that have responsibility for protection of humans from the hazardous effects of chemicals maintain Internet websites. These websites usually include information concerning the history, structure, and specific responsibilities of these organizations. The sites also generally contain or reference the statutes and guidelines under which the organizations operate. Internet addresses for a number of informative regulatory sites are listed in Table 19.16.

ACKNOWLEDGMENTS

The third edition of this text contained one chapter concerning short-term and subchronic toxicity studies and a second concerning chronic toxicity studies. Because many elements of short-term, subchronic, and chronic toxicity testing are similar, the decision was made to combine these subjects into a single chapter in the fourth edition. Some information in this chapter is derived entirely or only with slight modification from the chronic toxicity chapter in the third edition. This note is written to acknowledge the efforts of the authors of that chapter, Drs. Kent R.Stevens and Louis Mylecraine.

QUESTIONS

1. *Better Belly* is a new drug entity intended to mitigate the irritating properties of aspirin in the human

gut. Because of its intended use, human exposure to this

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Table 19.16 Internet Addresses for regulatory websites

Regulatory Subject	Site Address (http://)
International:	
European Union	www.eurunion.org/legislat/index.htm
EU Testing Guidelines for Medicinal Products	dg3.eudra.org/eudralex/index.htm
Organization for Economic Cooperation and Development	www.oecd.org
OECD Testing Guidelines	www.oecd.org/ehs/test/testlist.htm
International Conference on Harmonization	www.ich.org
ICH Guidance Documents	www.ich.org/ich5.html
United States:	
Food and Drug Administration	www.fda.gov
FDA Center for Food Safety and Applied Nutrition	vm.cfsan.fda.gov/list.html
FDA Center for Drug Evaluation and Research	www.fda.gov/cder
Environmental Protection Agency	www.epa.gov
EPA Office of Pollution Prevention and Toxic Substances	www.epa.gov/internet/oppts
EPA Office of Pesticide Programs	www.epa.gov/pesticides
Toxic Substances Control Act	www4.law.cornell.edu/uscode/15/ch53.html
Federal Insecticide, Fungicide and Rodenticide Act	www.law.cornell.edu/uscode/7/ch6.html
Federal Food, Drug and Cosmetic Act	www.fda.gov/opacom//laws/fdcact/fdctoc.htm
Food Quality Protection Act	www.epa.gov/oppfeadl/fqpa
Food and Drug Administration Modernization Act of 1997	www.fda.gov/opacom/backgrounders/modact.htm
EPA Testing Guidelines	www.epa.gov/opptsfrs/home/guidelin.htm
FDA Guidance Documents	www.fda.gov/cder/guidance/index.htm

compound will range from a few days to a few months. The intended treatment population includes males and females from 4 to 90+years of age. You are designated to be the toxicologist responsible for generating the toxicity data necessary to get *BetterBelly* to the marketplace. What steps/studies would you include in the hazard assessment program for this compound? When you propose your plan to FDA, what questions would you ask them concerning approval of this compound?

2. It has been determined that the hazard assessment program for *BetterBelly* should include subchronic toxicity testing. In a short-term, repeated dose study with this compound in rats at 500 mg/kg/day, oral gavage administration resulted in mortality in 40% of the animals. These rats also exhibited marked body weight loss, convulsions followed by prostration, urinary incontinence, and enlarged and discolored kidneys. At 100 mg/kg/day, no mortality occurred in the study and the only adverse signs observed were mild and reversible body weight loss, lethargy, urinary incontinence, and twitching of the extremities. At 20 mg/kg/day, neither mortality nor adverse signs were noted. Efficacy testing revealed that *BetterBelly* is effective for its intended use at 2 mg/kg/day. Pharmacokinetic determinations indicate that *BetterBelly* is excreted almost entirely in the urine and its serum half-life is 120 minutes. What dose levels would you select for the subchronic toxicity study? Why? What concentrations of dosing solutions would be required to accurately deliver these dose levels to the rats? In addition to the standard variables, what special endpoints would you include for investigation during the subchronic study?

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Table 19.17 Selected results from the subchronic toxicity study of *Better Belly* in rats

Variable/Finding/Interval	Treatment Group—Males				Treatment Group—Females			
	Control	Low	Mid	High	Control	Low	Mid	High
Body weight (g)								
Week 4	200±7	205±11	192±10	173±9a	112±8	105±8	110±5	98±6a
Week 13	403±13	410±15	371±11a	350±17a	201±12	202±10	198±13	175±10a
Hepatocellular hypertrophy (rats with finding/total rats)								
Week 4	0/10	2/10	0/10	6/10a	1/10	0/10	1/10	4/10
Week 13	3/20	8/20a	2/20	15/20a	2/20	3/20	1/20	12/20a
Renal tubule hyperplasia (rats with finding/total rats)								
Week 4	1/10	2/10	4/10	7/10a	0/10	0/10	2/10	2/10
Week 13	4/20	5/20	12/20a	17/20a	3/20	2/20	3/20	12/20a

a Statistically significantly different from controls ($p<0.05$).

3. The results in Table 19.17 were obtained during the subchronic study with *BetterBelly.* In what dose groups is there a treatment-related effect on body weight? Are liver and kidney target organs for *BetterBelly* toxicity? If so, in which dose groups is there a treatment-related effect? Based on the data in the table, what is the NOAEL for this study?

REFERENCES

1. American Institute of Nutrition (1977): Report of the American Institute of Nutrition Ad Hoc Committee on Standards for Nutritional Studies. *J. Nutr.*, 107:1340–1348.
2. Boorman, G.A., Eustis, S.L., Elwell, M.R., Montgomery, C.A., Jr., and MacKenzie, W.F. (1990): *Pathology of the Fischer Rat—Reference and atlas.* Academic Press, San Diego.
3. Consumer Products Safety Commission (CPSC): 16 CFR Parts 1015–1402, *Consumer Product Safety Act.* Office of the Federal Register, National Archives and Records Administration, U.S. Government Printing Office, Washington DC.
4. Datta, P.K., Friger, H., and Schleiermacher, E. (1970): The effect of chemical mutagens on the mitotic chromosomes of the mouse in vivo. In: *Chemical Mutagenesis in Mammals and Man*, edited by F.Vogel and G.Rohrborn, pp. 194–213, Springer-Verlag, New York.
5. U.S. Department of Transportation (DOT): 49 CFR Part 173, *Shippers-General Requirements for Shipments and Packaging*. Office of the Federal Register, National Archives and Records Administration, U.S. Government Printing Office, Washington DC.
6. DOT: 49 CFR Part 176, *Carriage by Vessel.* Office of the Federal Register, National Archives and Records Administration, U.S. Government Printing Office, Washington, DC.
7. European Economic Community (EEC) (1967): Council Directive 677548/EEC, Official Journal of the European Community, Brussels.
8. EEC (1979): Sixth Amendment to Council Directive 79/831/EEC, Official Journal of the European Community, Brussels.
9. EEC (1987): Council Recommendation 87/176/EEC, Official Journal of the European Community. Brussels.
10. U.S. Environmental Protection Agency (EPA)/FIFRA: 40 CFR 152–186, *Federal Insecticide, Fungicide and Rodenticide Act*, Office of the Federal Register. National Archives and Records Administration, U.S. Government Printing Office, Washington DC.
11. EPA/FIFRA: 40 CFR Part 160, *Good Laboratory Practice Standards.* Office of the Federal Register, National Archives and Records Administration, U.S. Government Printing Office, Washington, DC.
12. EPA/FIFRA: 40 CFR Part, *Good Laboratory Practice Standards*, p. 150, Office of the Federal Register, National Archives and Records Administration, U.S. Government Printing Office, Washington, DC.
13. EPA/FQPA: Public Law 104–170, *Food Quality Protection Act.* Office of the Federal Register, National Archives and Records Administration, U.S. Government Printing Office, Washington, DC.
14. EPA/OPPTS (1998): *90-Day Toxicity in Rodents*, OPPTS Test Guideline 870–3100. U.S. Government Printing Office, Washington, DC.
15. EPA/OPPTS (1998): *Chronic Toxicity*, OPPTS Test Guideline 870–4100. U.S. Government Printing Office, Washington, DC.
16. EPA/OPPTS (1998): *OPPTS Test Guidelines*, Series 830, Physical Chemical Properties. U.S. Government Printing Office, Washington, DC.
17. EPA/OPPTS (1998): *OPPTS Test Guidelines*, Series 870, Health Effects U.S. Government Printing

Office, Washington, DC.
18. EPA/RCRA: 40 CFR Parts 240–271, *Resource Conservation and Recovery Act*. Office of the Federal Register, National Archives and Records Administration, U.S. Government Printing Office, Washington, DC.
19. EPA/TSCA: 40 CFR Part 792, *Good Laboratory Practice Standards*. Office of the Federal Register, National Archives and

Records Administration, U.S. Government Printing Office, Washington, DC.
20. EPA/TSCA: 40 CFR Parts 700–799, *Toxic Substances Control Act*. Office of the Federal Register, National Archives and Records Administration, U.S. Government Printing Office, Washington, DC.
21. European Union (EU) (1991): *Good Manufacturing Practices—Medicinal and Veterinary Products.* Directives 91/356/EEC and 91/412/EEC. Commission of the European Communities, Brussels.
22. U.S. Food and Drug Administration (FDA) (1982): *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food*, Appendix II, pp. 19–21. Bureau of Foods, FDA. National Technical Information Service, Springfield, Virginia.
23. FDA (1982): *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food.* Center for Food Safety and Applied Nutrition, FDA, Washington, DC.
24. FDA (1983): *FDA Guidelines for Chemistry and Technological Data Requirements for Direct Food Additives and GRAS Food Ingredients.* Bureau of Foods, FDA, Washington, DC.
25. FDA (1993): *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food "Redbook II,"* p. 61. Center for Food Safety and Applied Nutrition, FDA, Washington, DC.
26. FDA (1993): *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food*, "Redbook II," p. 86. Center for Food Safety and Applied Nutrition, FDA, Washington, DC.
27. FDA (2000): *Toxicological Principles for the Safety of Food Ingredients, Redbook 2000,* Center for Food Safety and Applied Nutrition, FDA, Washington, DC.
28. FDA (1997): Public Law 105–115, *Food and Drug Administration Modernization Act of 1997.* U.S. Department of Health and Human Services, Public Health Service, FDA, Rockville, Maryland.
29. FDA: 21 USC 301 et seq.. *Federal Food Drug and Cosmetic Act, as Amended, and Related Laws.* U.S. Department of Health and Human Services, Public Health Service, FDA, Rockville, Maryland.
30. FDA: 21 CFR 110, *Current Good Manufacturing Practice in Manufacturing, Packaging or Holding Human Food.* Office of the Federal Register, National Archives and Records Administration, U.S. Government Printing Office, Washington, DC.
31. FDA: 21 CFR 170, *Food Additives.* Office of the Federal Register, National Archives and Records Administration, U.S. Government Printing Office, Washington, DC.
32. FDA: 21 CFR 210, *Current Good Manufacturing Practice in Manufacturing, Processing, Packaging or Holding of Drugs; General.* Office of the Federal Register, National Archives and Records Administration, U.S. Government Printing Office, Washington, DC.
33. FDA: 21 CFR 211, *Current Good Manufacturing Practice for Finished Pharmaceuticals.* Office of the Federal Register, National Archives and Records Administration, U.S. Government Printing Office, Washington, DC.
34. FDA: 21 CFR 314, *Applications for FDA Approval to Market a New Drug or an Antibiotic Drug.* Office of the Federal Register, National Archives and Records Administration, U.S. Government Printing Office, Washington, DC.
35. FDA: 21 CFR Part 58, *Good Laboratory Practice for Nonclinical Laboratory Studies*, p. 253. Office of the Federal Register, National Archives and Records Administration, U.S. Government Printing Office, Washington, DC.
36. FDA: 21 CFR Part 58, *Good Laboratory Practice for Nonclinical Laboratory Studies.* Office of the Federal Register, National Archives and Records Administration, U.S. Government Printing Office, Washington, DC.
37. Hardisty, J.F., and Eustis, S.L. (1990): Toxicological pathology: A critical stage in study interpretation. In: *Progress in Predictive Toxicology*, edited by D.B.Clayson, I.C.Nunro, P.Shubik, and J.A.Swenberg, Elsevier Science Publishers, New York.
38. Haschek, W.M., and Rousseaux, C.G. (1991): *Handbook of Toxicologic Pathology.* Academic Press, San Diego, California.
39. Heddle, J.A., Hite, M., Kirkhart, B., Larson, K., MacGregor, J.T., Newell, G.W., and Salamone, M.F. (1983): The induction of micronuclei as a measure of genotoxicity. *Mutat. Res.*, 123:61–118.
40. HPB (1998): *Good Manufacturing Practices (GMP) Guidelines—1998 Edition.* Health Protection Branch, Ottawa, Canada.
41. ICH (1994–1998): *Harmonized Tripartite Guidelines.* ICH Secretariat, Geneva.
42. ICH (1996–1997): *Harmonized Tripartite Guideline—Quality.* ICH Secretariat, Geneva.
43. ICH (1998): *Harmonized Tripartite Guideline—Duration of Chronic Toxicity Testing in Animals (Rodent and Non-Rodent Toxicity Testing).* ICH Secretariat, Geneva.

44. ILAR (1996): *Guide for the Care and Use of Laboratory Animals.* National Academy Press, Washington, DC.
45. Jain, N.C. (1986): *Schalm's Veterinary Hematology*, 4 ed., edited by N.C.Jain. Lea & Febiger, Philadelphia.
46. Japan/MAFF (1984): *Good Laboratory Practice Standards for Toxicological Studies in Agricultural Chemicals.* Agricultural Production Bureau, Ministry of Agriculture, Forestry and Fisheries, Japan.
47. Japan/MITI (1984): *Good Laboratory Practice Standards Applied to Industrial Chemicals.* Basic Industries Bureau, Ministry of International Trade and Industry, Japan.
48. Japan/MOHW (1982): *Good Laboratory Practice Standards for Safety Studies on Drugs.* Pharmaceutical Affairs Bureau, Ministry of Health and Welfare, Japan.
49. Loeb, W.F., and Quimby, F.W. (1989): *The Clinical Chemistry of Laboratory Animals.* Pergamon Press, New York.
50. McNamara, B.P. (1976): Concepts in health evaluation of commercial and industrial chemicals. In: *New Concepts in Safety Evaluation,* edited by M.A.Mehlman, R.E.Shapiro, and H. Blumenthal, pp. 61–140. Hemisphere, Washington, DC.
51. Mirsalis, J.C., and Butterworth, B.E. (1980): Detection of unscheduled DNA synthesis in hepatocytes isolated from rats treated with genotoxic agents: An In Vivo-In Vitro assay for potential carcinogens and mutagens. *Carcinogenesis,* 1:621–625.
52. Mugford, C.A., and Kedderis, G.L. (1998): Sex-dependent metabolism of xenobiotics. *Drug Metab. Rev.*, 30:441–498.
53. OECD (1981): *Chronic Toxicity Studies—OECD Guidelines for Testing of Chemicals.* Test Guideline 452. OECD, Paris.
54. OECD (1981–1998): *OECD Guidelines for Testing of Chemicals,* Section 4. Health Effects. OECD, Paris.
55. OECD (1981–1998): *OECD Guidelines for Testing of Chemicals,* Section 1, Physical Chemical Properties. OECD, Paris.
56. OECD (1997): *OECD Principles of Good Laboratory Practice.* OECD, Paris.
57. OECD (1998): *Repeated Dose 90-Day Oral Toxicity Study in Rodent—OECD Guidelines for Testing of Chemicals,* Test Guideline 408, OECD. Paris.
58. Occupational Safety and Health Administration (OSHA): 29 CFR Parts 1910, 1915, 1918 and 1926, *Occupational Safety and Health Act.* Office of the Federal Register, National Archives and Records Administration, U.S. Government Printing Office, Washington, DC.

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59. Rao, G.N., and Knapka, J.J. (1987): Contaminant and nutrient concentrations of natural ingredient rat and mouse diet used in chemical toxicology studies. *Fund, and Appl. Tox.*, 9:324–238.
60. UK/DHSS (1986): *Good Laboratory Practice—The United Kingdom Compliance Programme*. Department of Health and Social Security, London.
61. U.S. Department of Agriculture (USDA): 9 CFR 1–3, *Animal Welfare Act.* Office of the Federal Register, National Archives and Records Administration, U.S. Government Printing Office, Washington, DC.
62. World Health Organization (WHO) (1978): *Principles and Methods for Evaluating the Toxicity of Chemicals*, part 1, pp. 178–198, WHO, Geneva.
63. Zakim D., Hochman, Y., and Vessey, D.A. (1985): Methods for characterizing the function of UDP-Glucuronyltransferases. In: *Biochemical Pharmacology and Toxicology, Vol. I*, edited by D. Zakim and D.A.Vessey, p. 189. John Wiley & Sons, New York.
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Gary M.Williams and Michael J.Iatropoulos
Principles and Methods of Toxicology,
Fourth Edition, edited by A.Wallace Hayes.
Taylor & Francis, Philadelphia © 2001.

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CHEMICALS WITH CARCINOGENIC ACTIVITY

Cancer is one of the leading causes of death, and it can result from exposure to exogenous chemicals (275). Thus, in the toxicological assessment of chemicals, testing for Carcinogenicity constitutes one of the most important evaluations (Table 20.1).

A large database on the carcinogenic activities of chemicals in rodents has accrued (87, 177) as a consequence of over 80 years of basic research and the output from national testing programs in several countries, particularly the United States and Japan. Under the aegis first of the U.S. National Cancer Institute (NCI), and subsequently of the U.S. National Toxicology Program

Table 20.1 Regulations or agreements under which carcinogenicity testing may be required

Legislation/guidance	Agency	Agents of concern
Commission Directive 414 EEC (1991)	EU	Plant protection products
Commission Directive 67 EEC (1993)	EU	Risk assessment for new notified substances
Commission Regulation 1488 EEC (1994)	EU	Risk assessment for existing substances
Dangerous Substances Directive (1967; amended 1992)	EU	Industrial chemicals
Food, Drug and Cosmetics Act (1906, 1938, amended 1992)/FDA Red Book II	US FDA	Food, medicines, cosmetics, food additives, color additives, animal and feed additives, medical devices
Federal Hazardous Substances Act (1960; amended 1988)	US CPSC	Toxic household products
Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (1948, amended 1978)	US EPA	Pesticides
Guidance on Toxicology Study Data for Application of Agriculture Chemical Registration (1985)	MAFF, Japan	Agricultural chemicals
Guideline for Toxicity Testing of Chemicals (1990)	MHW, Japan	Chemicals
Pharmaceutical Affairs Law (1980)/Guidelines for Toxicity Studies of Drugs Manual (1990)	MHW, Japan	Medicines
Guidelines for Toxicity Studies of New Animal Drugs (1988)	MHW, Japan	Animal medicines
Pesticide Registration Directive (1991)	EU	Pesticides
Technical Requirements for the Registration of Pharmaceuticals for Human Use (1995)	ICH	Medicines
Toxic Substances Control Act (TCSA) (1976, amended 1992)	US EPA	Hazardous chemicals not covered by other laws, includes premarket review

Note. EEC, European Economic Communities; EU, European Union; MAFF, Ministry of Agriculture, Forestry and Fisheries; MHW, Ministry of Health and Welfare; ICH, International Conference on Harmonizations; US CPSC, U.S. Consumer Product Safety Commission; US EPA, U.S. Environmental Protection Agency; US FDA, U.S. Food and Drug Administration.

(NTP), routine rodent cancer bioassays (RGBs), mainly in mice and rats, have been conducted on about 300 chemicals (106).

The definitive test for animal carcinogenic activity is the RGB, which in its various forms is detailed here and elsewhere (115, 168, 207, 260, 261, 274). The present method for the RGB is exemplified by that currently in use by the NTP (16). This method was refined from basic procedures developed in the 1960s largely by the pharmaceutical industry under guidance from the U.S. Food and Drug Administration (U.S. FDA) and by the NCI Bioassay Program (33, 34, 48, 273). The extensive use of the RGB has led to the recognition that its results cannot be unquestioningly extrapolated to humans (172, 274), for which, of course, carcinogenic activity is established by epidemiological studies. Available mechanistic procedures to be discussed assist in assessment of potential human hazard.

Carcinogenic Activity

A committee of the International Federation of Societies of Toxicologic Pathologists has adopted the definition of a chemical carcinogen as a "substance that causes a cell or group of normal cells, which would not otherwise have shown this property, to change its biological behavior and demonstrate progressive growth of a malignant character" (66). Thus, the carcinogenic or oncogenic activity of chemicals is best defined by the finding of unequivocal evidence in a strain of animal of either gender that a test substance (TS) causes types of malignant neoplasms not seen in controls, clearly indicating ab initio induction of neoplasia. A marginal increase of a very rare malignancy of a certain type and site under some circumstances may incriminate a chemical substance. Another generally used criterion is an increase in the incidence of the types of malignant

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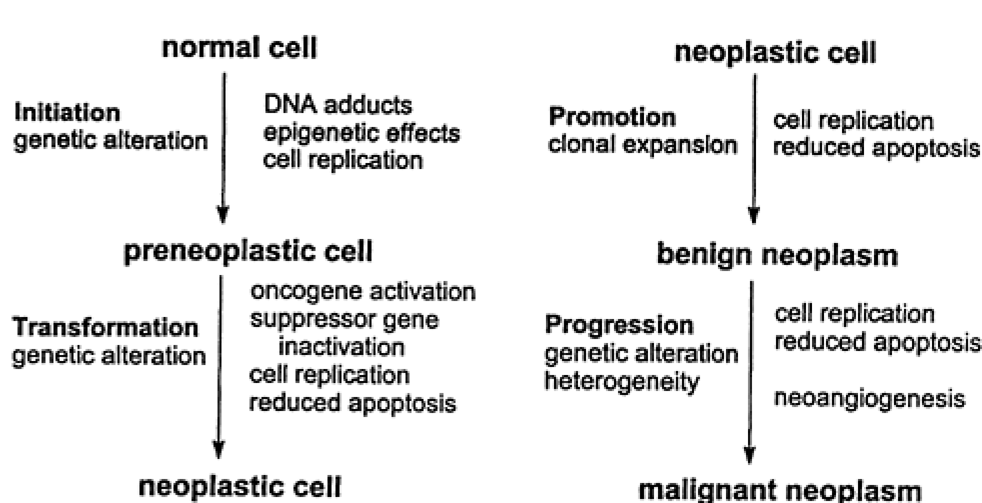

FIG. 20.1. Sequences of oncogenesis.

neoplasms that occur in controls. The malignant neoplasms can be of any histological type, epithelial or mesenchymal, and while a clear increase in malignant neoplasms is most persuasive, a combination of malignant and benign neoplasms of the same cell type of origin is generally accepted as reflecting carcinogenic activity (114, 164). The evidence of malignancy is best established by the presence of invasion or metastasis, but for most rodent neoplasms, the diagnosis of malignancy is made histologically on evidence of cellular atypia; thus some diagnoses are controversial, as discussed in the sections on anatomic pathology and on cancer hazard evaluation. The finding of an increase in only benign neoplasms, especially if the type of neoplasm is not established to be premalignant, does not constitute sufficient evidence for carcinogenicity, but does provide limited evidence. In addition to these criteria, an increase in the multiplicity of neoplasms above that in controls or a reduction in the latency period for development of neoplasms has also been considered. While these may indicate an influence of the chemical on tumor development, they are less definitive evidence of carcinogenic activity. Rarely, an increase in the overall incidence of neoplasms without increase in any specific type of neoplasm has been proposed as evidence of carcinogenic activity, but this is highly questionable (115). These criteria apply to findings in any species/strain/gender. A strong effect in one gender of one species may be sufficient to implicate a chemical as a carcinogen, but obviously findings in more than one gender or species strengthen the evidence.

Of course, each expert body applies criteria as it sees fit, often influenced by a concern for hazard identification in the interests of public health protection. Ultimately, in assessing human risk, the reason for which animal tests are done, scientific judgment must be used in evaluating all the information available, including importantly mechanism (or mode) of action.

Types of Carcinogens

Widely varied chemical structures have exhibited carcinogenic activity in rodents (87, 116, 121, 122). This reflects the fact that the multistep process of oncogenesis (Figure 20.1) can be influenced by chemicals in various ways, mainly involving either chemical reactivity in producing neoplastic transformation or epigenetic modulation of cell growth facilitating neoplastic development. Thus, chemicals can give rise to increases in neoplasms through a variety of mechanisms, which have been broadly characterized as DNA reactive or epigenetic (283, 288). The types of chemicals that can be assigned to these categories are given in Tables 20.2 and 20.3 and have been discussed in detail elsewhere (288). Carcinogens are, of course, both naturally occurring and man-made.

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Table 20.2 Classification of chemicals with carcinogenic activity

A DNA-Reactive	
1. Activation-independent	Alkylating agents Nitrogen mustards, chlorambucil Epoxides: ethylene oxide
2. Activation-dependent	Alphatic halides: vinyl chloride Aromatic amines, heterocyclic amines, aminoazo dyes and nitro-aromatic compounds: Monocyclic *o*-toluidine, 2-amino-l-methyl-6-phenyl-imidazo[4,5-b]pyridine(PhIP), polycyclic 4-aminobiphenyl, benzidine, dimethylaminoazobenzene, 1-nitropropane Polycyclic aromatic hydrocarbons: benzo[a]pyrene *N*-Nitroso compounds: dialkyl, dimethylnitrosamine; cyclic-N-nitrosonornicotine Triazines, hydrazines, azoxymethane, methyl-azoxymethamol Mycotoxins: aflatoxin B1 Pharmaceuticals: cyclophosphanide, pheacetin, tamoxipen
3. Inorganica	Metals: beryllium, cadmium, chromium, nickel, silica Minerals: asbestos
B. Epigenetic	
1. Promoter	Liver enzyme-inducer type hepatocarcinogens; chlordane, DDT, pentachlorophenol, phenobarbital, polybrominated biphenyls, polychlorinated biphenyls Saccharin
2. Endocrine modifier	Hormones: estrogens, diethylstilbestrol, atrazine, cholor-S-triazines Estrogens Atrazine Diethylstilbestrol Antiandrogens: finasteride, vinclozolin Antithyroid thyroid tumor enhancers Thyroperoxidase inhibitors: amitrole, sulfamethazine Thyroid hormone conjugation enhancers: phenobarbital, spironolactone Gastrin-elevating inducers of gastric neuroendocrine tumors: omeprazole, lansoprazole, pantoprazole
3. Immunosuppressor	Purine analogs Cyclosporin
4. Cytotoxin	Mouse forestomach toxicants: butylated hydroxyanisole, propionic acid, diallyl phthalate, ethyl acrylate Rat Nasal toxicants: chloracetanilide herbicides Rat renal toxicants: potassium bromate, nitrilotriacetic acid $\alpha 2\mu$-Globulin nephropathy inducers: *d*-limonene, *p*-dichlorobenzene
5. Peroxisome proliferator	Hypolipidemic fibrates: ciprofibrate, clofibrate, gemfibrozil Phthalates: di(2-ethylhexyl)phthalate(DEHP), di(isononyl)phthalate(DINP) Lactofen
C. Unclassified	Acrylamide, acrylonitrile, dioxane, furfural, methapyrilene, sugar alcohols

a Some are categorized as genotoxic because of evidence for damage of DNA; others may operate through epigenetic mechanisms such as alterations in fidelity of DNA polymerase.

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Table 20.3 Classification of chemicals and mixtures judged to be carcinogenic to humans by the International Agency for Research on Cancer

DNA-Reactive	
Aflatoxins	Cyclophosphamide
4-Aminobiphenyl	Ethylene oxide[a]
2-Aminonaphthalene	Melphalan
5-Azacytidine	MOPP (nitrogen mustard, vincristine, procarbazine, and prednisone)
Benzidine	
Betel quid with tobacco	
N,N-Bis(2-chloroethyl)-2naphthylamine (chlornaphazine)	Nickel and nickel compounds
	Phenacetin-containing analgesic mixtures
Bis(chloromethyl) ether	
1,4-Butanediol dimethanesulfonate (myleran)	Soot
	Sulfur mustard
Chlorambucil	Tamoxifen
I-(2-Chloroethyl)-3-(4-methylcyclo hexyl)-1-nitrosourea (methyl-CCNU)	Thiotepa
	Tobacco smoke and products
Chromium compounds, hexavalent	Triethylenethiophosphoramide (treosulphan)
Coal tars	Vinyl chloride
Epigenetic	
Azathioprine	Oral contraceptives, combined
Cyclosporin A	2,3,7,8-Tetrachloro-dibenzo*p*-dioxin (TCDD)
Postmenopausal therapy	
Unclassified	
Alcoholic beverages	Diethylstilbestrol
Arsenic and arsenic compounds	Mineral oils
Benzene	Nickel and nickel compounds
	Shale oils

a Based on evidence for a relevant mechanism in humans.

Of the many chemicals with carcinogenic activity in RGBs, few are associated with cancer in humans (Table 20.3). Most of these are of the DNA-reactive type, indicating the importance of this mechanism in human hazard. In support of this, several DNA-reactive carcinogens have been active in primates (237), including transplacentally (203). Apart from cigarette smoke and mycotoxins, virtually all DNA-reactive carcinogens occur as occupational or therapeutic exposures, which are substantial compared to general environmental exposures. The few human epigenetic carcinogens are mainly Pharmaceuticals, and these are associated with cancer increase only at therapeutic exposures that produce the cellular effect that underlies their carcinogenicity in rodents, mainly immunosuppression or hormonal effects. Thus, a primary objective of cancer hazard assessment is to identify chemicals with DNA reactivity (289).

Because of the significance of DNA reactivity and the reliability with which it is identified, few agents of this type are proposed for uses in which there is any appreciable human exposure, apart from chemotherapeutic alkylating agents. In contrast, agents that elicit rodent tumor increases by epigenetic mechanisms are widely used; for example, more than 80 medicines in current use are tumorigenic in the RGB (46). Importantly, no epigenetic carcinogen has been active in primates (224, 237). Accordingly, an important aspect of carcinogen testing is to elucidate any mode of action that might lead to a tumor increase in an RCB in order to guide mechanistic research for informed hazard evaluation (289).

Potency

The magnitude of the carcinogenic activity in rodents of chemicals with respect to dose varies more than 10-million-fold (87). The most extensive system for expressing numerical indices of carcinogenic potency is the Carcinogenic Potency Data Base (87), which uses TD50 values, defined as the daily dose rate required to halve the probability of an experimental animal of remaining tumor free at the end of its standard life span (196). A simplified method proposed for use in the regulatory setting is the T25, defined as the chronic dose rate in milligrams per kilogram body weight per day that will give 25% of the animals tumors at a specific site, after correction for the spontaneous incidence, within the standard lifetime of that species (55). Also, for DNA-reactive carcinogens, there is a general relationship between carcinogenicity and DNA binding, which can be expressed as the chemical binding index (CBI) (157).

REQUIREMENTS FOR TESTING

Testing of chemicals in experimental animals for carcinogenic activity is done to assess potential human

cancer hazard under conditions to which humans might be exposed. The U.S. federal government has enacted numerous laws covering requirements for carcinogenicity testing of substances for which there is human or environmental exposure (Table 20.1). Other countries have similar provisions (Table 20.1). For Pharmaceuticals and food additives, both direct and indirect, circumstances requiring carcinogenicity studies have been agreed upon by the International Conference on Harmonization (available on FDA/Center for Drug Evaluation and Research web site, http://www.fda.gov/cder), which applies in the United States, European Union, and Japan. Cosmetics, as with foods, usually do not require carcinogenicity testing.

The requirements for testing of biopharmaceuticals present specific issues (223), for which FDA/Center for Biologies Evaluation and Research has provided guidance (http://www.fda.gov/cber). Growth factors and immunosuppressive antibodies are noted as raising concern for carcinogenic potential. Normal hormones or growth factors that are intended to correct deficiency states, such as insulin, may not need to be tested, unless they are administered by a route that results in substantial exposure of tissues that would not be exposed to the endogenous protein. Modified proteins with new biologic properties may require testing, since, for example, it is known that a modified insulin with affinity for the insulin-like growth factor-1 receptor produced mammary tumors in rats (51). Also, a fragment of parathyroid hormone with bone-trophic activity is widely rumored to have produced bone neoplasms in rats. Certain modifications of proteins to improve bioavailability, such as with conjugation to polyethylene glycol, have not raised carcinogenicity issues. As yet, carcinogenicity testing of transgenes used in gene therapy is not required.

An emerging aspect of regulatory concern is photochemicalcarcinogenicity (129). Indications for the possible need for photochemicalcarcinogenicity testing are (a) long-term exposure of the skin to chemicals that can undergo photoactivation, (b) alteration of the structure of the epidermis, (c) sensitization of the skin to ultraviolet radiation (UVR), and (d) exacerbation of suspected UVR-induced carcinogenesis (75).

Thus, for the use of many types of chemicals, proscribed RGBs are required. Nevertheless, expedited approaches to assessment of potential carcinogenic activity are valuable to obtain data quickly and cheaply with minimal use of experimental animals in order to appreciate potential hazard before extensive development or exposures of humans.

SYSTEMATIC APPROACH TO TESTING

The goal of a systematic approach to testing is to obtain reliable data for hazard assessment and evaluation of the TS at the earliest possible stage. An approach that incorporates the mechanistic concepts described above is the decision-point approach (DPA) (274), which is presented here as a general framework for the concept (Table 20.4). An expedited approach to testing is needed not only for the large reservoir of existing untested chemicals, but also for the host of new molecular (or chemical) entities (NMEs), both small and large (e.g., protein) molecules, that are being synthesized, particularly in the pharmaceutical industry, using combinatorial chemical synthesis.

Table 20.4 Decision point approach in carcinogen testing

Stage A. *Structure of chemical*

1. Possible electrophiles
2. Relation to known carcinogens

Stage B. *Short-term genotoxicity assays*

1. Bacterial mutagenesis hepatocyte, DNA repair
2. Other

Decision point 1: Evaluation of findings in stages A and B.

Stage C. *Assays for epigenetic effects*

1. Cultured cells
   - Mitogenesis
   - Induction of cytochrome P450
   - Peroxisome proliferation
   - Gap junction protein downregulation
   - Inhibition of cell-cell communication
   - Altered gene expression
2. In vivo
   - Increased cell proliferation
   - Induction of cytochrome P450
   - Peroxisome proliferation
   - Hormone perturbation
   - Gap junction protein downregulation
   - Enhancement of preneoplastic lesions
   - Immunosuppression

Decision point 2: Evaluation of results from stages A through C.

Stage D. *In vivo assays*

1. Genotoxicity
   - DNA binding

2. Limited bioassays: Preneoplastic lesions—rat liver, mouse skin, mouse lung, rat breast; transgenic mice

Decision point 3: Evaluation of results from stages A-C and selected tests in stage D.

Stage E. *Carcinogenicity bioassays*

1. Accelerated bioassays
2. Long-term bioassays

Decision point 4: Final evaluation of all results and cancer hazard assessment.

Implications of Chemical Structure

From the classes of DNA-reactive carcinogens given in Table 20.2, the types of electrophiles that are involved in chemical reactivity and hence DNA binding are well known (Figure 20.2). Such molecular features also have

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Electrophiles

carbonium ion **1** nitrenium ion **2** diazonium ion **3** aziridinium ion **4** episulphonium ion **5**

Precursors

alkyl esters of phosphonic acid; alkyl esters of sulphonic acid; N-(hydroxymethy) derivatives; monohaloalkenes; aromatic and aliphatic substituted primary alkyl halides; aromatic nitro groups; aliphatic nitro groups; N- and S-mustards; urethane derivatives (carbamates); N-chloramines; alkyl N-nitrosamines; aromatic azo groups; β-propiolactones; β-propiosultones; arene oxides; aromatic, mono, and dialkyl amino groups; alkene oxides; alkyl hydrazines; aromatic and aliphatic aziridinyl derivatives; $C(X)_4$ X=H, F, Cl, Br, or I halogenated methanes; 1,2-dibromoalkanes

FIG. 20.2. Structure of reactive electrophiles and precursors.

been referred to as structural alerts (8). In general, there is a relationship between DNA binding and carcinogenicity (157).

Among both DNA-reactive and epigenetic carcinogens, numerous classes have structural features in common within the class. The presence of one of these features in an NME of unknown carcinogenicity suggests potential activity. The U.S. Food and Drug Administration has grouped food additives into classes by chemical structure, estimating their potential toxicity. These structural classes are used for assignment to levels of concern. Additives with functional groups of high probable toxicity are assigned to category C. Additives of intermediate or unknown probable toxicity are assigned to category B, and compounds of low probable toxicity are assigned to category A (260). Artificial intelligence systems for assessing potential toxicities related to structures have been developed (141, 161).

Genotoxicity Assays

In Vitro

A large number of assays for various genetic endpoints are available (280, see Chapter 17), and all regulatory agencies have specific recommendations or requirements, which may extend beyond the intent to predict potential carcinogenicity. For pharmaceuticals, a core battery including both in vitro and in vivo assays has been agreed upon by the International Conference on Harmonization (ICH) (44).

The results of genotoxicity assays are usually interpreted in terms of potential carcinogenicity of the TS. However, the predictivity of most assays (i.e., the percentage of positives that prove to be carcinogens) is generally limited to DNA-reactive carcinogens as a consequence of the fact that DNA alteration is a molecular basis of mutagenicity. Exceptions are assays designed specifically for epigenetic effects (see Assays for Epigenetic Effects section), including cell transformation, which appears to respond to both DNA-reactive and epigenetic agents (150). The *Salmonella* microsome mutagenesis assay (87) is required in all testing batteries and has perhaps the highest predictivity, about 80% (298). Nevertheless, about 20% of bacterial mutagens are noncarcinogens. The ability of *Salmonella* mutagenicity to differentiate carcinogens from noncarcinogens is not increased by certain other standard in vitro assays, such as mammalian cell mutagenicity and chromosome aberrations often included in testing batteries (298). To reinforce assurance of the predictiveness of a positive *Salmonella* mutagenicity finding, in the DPA (Table 20.4, stage B), another well-established assay with a defined protocol, the hepatocyte/DNA repair assay (287), is recommended in addition to *Salmonella* mutagenesis because positive results in the two combined provide essentially perfect predictivity for detection of DNA-reactive carcinogens (286). The latter assay affords the valuable feature that it can be conducted with human cells. Clear positive results in both of these assays, therefore, raise serious concerns for many uses of such a chemical. If testing at this level, however, yields equivocal findings, in vivo assays for DNA reactivity are available.

In Vivo

In vivo assays are undertaken, as indicated in Table 20.4, stage D, if there is a suspicion of DNA reactivity for the NME that is not resolved by in vitro assays. The availability of radiolabeled chemical allows measurement of DNA binding (157). Otherwise, assays for DNA damage that can be applied include the in vivo/in vitro hepatocyte/DNA repair assay (21), 32P postlabeling (199), and the alkaline single-cell gel electrophoresis (COMET) assay (67). Positive results in these assays, if considered insufficient, direct the need for a radiolabeled chemical binding assay, which should include demonstration of an adducted DNA base.

Since the only agents that represent human cancer hazards apart from hormones and immunosuppressants are DNA reactive chemicals (see Cancer Hazard and Risk Assessment), a 4 week study for DNA adduct formation, if negative, could obviate the need for an RCB.

In vivo mutagenicity assays such as the mouse (rat) bone marrow micronucleus assay and rat bone marrow chromosome aberration assay are usually done as part of standard batteries. Newer models for in vivo mutagenicity include transgenics such as the Muta mouse and Big Blue transgenic mouse (175). The latter allow for detection of mutations in other tissues in addition to bone marrow and can provide information on the molecular nature of induced mutations. These assays, if negative, provide further evidence of lack of in vivo DNA reactivity of a chemical and combined with a negative adduct study, further preclude the need for an RCB.

Assays for Epigenetic Effects

The assays in Table 20.4, stage C are applied selectively depending on the properties of the NME, that is, chemical structure, biologic or pharmacologic action and toxicity. They provide evidence for an epigenetic mechanism that may result in an increase in neoplasms in chronically exposed rodents. Many can be conducted in cultured cells, particularly hepatocytes, although even when applied in vivo, the

experiments are of short duration, except for assays for promoting activity, which are described further in the section on limited carcinogenicity bioassays. Positive results for the TS indicate a potential tumorigenic

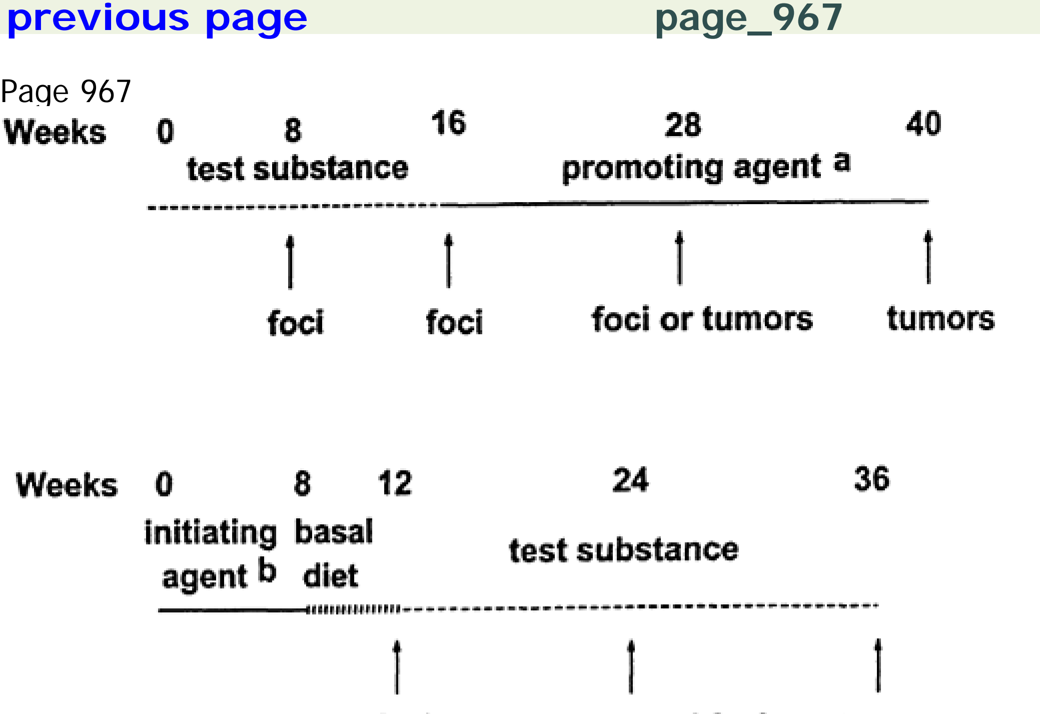

FIG. 20.3. Limited bioassays for initiation and promotion in rodent liver.

effect, in which case the carcinogenic potency of established tumorigens with a similar mechanism and organotropism provides a guide to whether a favorable risk assessment can be made for the NME.

Genomic technologies are becoming available for screening for effects on gene expression and function, including microarray hybridization methods ("DNA Chips") and proteomics. If expression of specific genes, such as acyl coenzyme A (CoA) oxidase (peroxisome proliferators) or cytochrome P450s (liver promoters), can be linked to epigenetic carcinogenesis, then these methods will have utility for screening.

Limited Carcinogenicity Bioassays

Limited carcinogenicity bioassays (LCB) are based on either neoplasms or established pre-neoplastic lesions as their endpoint (60, 61, 284). These can be applied as initiation assays, in which the TS is tested for its ability to induce the endpoint lesion, or as promotion assays, in which the TS is administered after an agent known to induce the endpoint lesion to determine the ability of the TS to enhance development of the lesion (240).

Initiation/Promotion

In early experimental studies of initiation of skin carcinogenesis, initiation was achieved with a single exposure (11). Although this is possible with potent DNA-reactive agents, repeated exposure is required for an adequate assay for initiation. Since promotion requires an even longer time for expression, more extensive exposure, up to 6 months, is required for an adequate bioassay. Essentially, an assay for initiating activity is directed largely toward assessing potential in vivo genetic activity of the TS, whereas the assay for promoting activity assesses an epigenetic mechanism. Accordingly, assays for promoting activity are also appropriate in the DPA at stage C in vivo assays for epigenetic effects (Table 20.4).

The most extensively validated and used model for an LCB is rat liver (53, 60, 61, 226). This is a consequence of the extensive capability of chemical biotransformation in the liver and the availability of sensitive and reliable markers for preneoplastic lesions. A typical study design for initiation and promotion is shown in Figure 20.3.

Other commonly used LCBs are the mouse skin papilloma, the mouse lung adenoma, and the rat mammary-gland tumor assays (274). These have advantages for specific types of agents; for example, mouse skin is very responsive to polycyclic aromatic hydrocarbons.

Positive findings for initiation are highly indicative of potential carcinogenic activity (123). In fact, Williams et al. (294) calculated that initiation/promotion can be

as sensitive as chronic bioassay in detecting carcinogenic activity. Promoting activity also suggests a likelihood of carcinogenic activity (123). In either case, it is possible to establish dose-response data and no-effect levels for design of chronic bioassays or risk assessment.

Transgenic Mice

Another type of LCB, the use of which is expanding rapidly, is the transgenic mouse model (56), in which the principal genetic targets are specific oncogenes (H-ras model) or tumor suppressor genes (p53 model), or the entire genome in DNA-repair-deficient animals (XPA-deficient model). Four models are receiving the greatest attention: the p53 heterozygous mouse (p53+/−), the Tg-AC mouse, the CB6FI-Tg-H-ras2 mouse and the XPA−/− mouse (234). Other models, such as Min, Eμ-pim-1, ARF-deficient, and double mutant p53/XPC, have shown responsiveness to carcinogens but are being used mainly for mechanistic research. The studied transgenic models have responded appropriately to a number of carcinogenic and noncarcinogenic agents and have been introduced as alternatives to the RGB in mice and accepted as providing evidence of carcinogenicity (35, 234).

Three of the commonly used models are based upon alterations in genes that are relevant to many human and rodent neoplasms—the p53 tumor suppressor gene and the H-ras oncogene—while the XPA−/− model provides an enhanced response to DNA damage as a consequence of the absence of nucleotide excision repair. Each model has specific features. Mice heterozygous for p53 differ in response depending upon the strains used as parents. For example, the C57BL, which is most widely used (52), is resistant to hepatocarcinogenesis, whereas the C3H is highly susceptible. The Tg-AC model carries a v-Ha-ras oncogene fused to the promoter of C-globin gene in the FVB/N mouse strain (151), a strain not commonly used in toxicology and that is susceptible to audiogenic seizure. The Tg-H-ras2 mouse carries five to six copies of human c-Ha-ras gene integrated in tandem array in the genome of Fj mice of transgenic male C57BL/6J mice and normal female BALB/cByJ mice (212), which again are strains not very susceptible to hepatocarcinogenesis. Currently, the p53+/− and Tg-AC mice are widely available, whereas the Tg-H-ras2 is available only in Japan where it was developed. Although any route of exposure can be used with these models, most data have been obtained by the oral route for the p53+/− and Tg-H-ras2, whereas skin painting is the preferred route for the Tg-AC. In these models, tumors can be elicited within 6 months with few or no tumors in controls. Beyond 6 months, these animals begin to develop genetically determined tumors in high incidence as follows: the p53+/− with a C57BL parent develops lymphomas and sarcomas (159); the Tg-AC, odontogenic tumors, erythrocytic leukemia, and salivary-gland and ovarian tumors (158, 159); and the H-ras2, benign and malignant lung tumors, splenic hemangiosarcomas, and forestomach papillomas (212). Therefore, evaluation of increases in any of these tumor types must consider whether the increase is attributable to induction of neoplasia as opposed to acceleration of development. The XPA-deficient mice do not show appreciably increased incidences of spontaneous neoplasms (only some liver neoplasms in C3H-derived strain) (50, 268).

All the models respond primarily to DNA-reactive carcinogens, although the Tg-AC model has the potential to identify epigenetic skin carcinogens (230), and the Tg-H-ras-2 has also responded to epigenetic carcinogens (297). A mouse model engineered to contain the human growth hormone gene under the control of the human heat-shock protein 70 (Hsp 70) promoter has been demonstrated to respond to toxicants of which some act as epigenetic carcinogens (210).

The p53+/− mouse in the few studies available so far clearly responds with accelerated development of thymic lymphomas (e.g., phenolphthalein), but compared to the wild-type background has not exhibited an accelerated response to DNA-reactive carcinogens targeting liver (e.g., diethylnitrosamine) (139), mammary gland (e.g., 7,12-dimethylbenz[a]anthracene) (131a), or colon (e.g., 1,2-dimethylhydrazine). This may reflect the fact that p53 mutation is not an early event in murine carcinogenesis for some tissues. If established, this situation strongly diminishes the usefulness of the p53 model for chemical screening.

For all these models, evaluation may be enhanced by measurement of cell proliferation in critical target organs.

Other Models

The newborn mouse is also used as an LCB (70). In this model, newborn mice of any strain are administered intraperitoneal injections or oral intubations of TS at days 8 and 15 after birth and then held for observation for up to 1 year of age. The model exhibits high sensitivity to DNA-reactive carcinogens, but is unlikely to respond to epigenetic agents because of the limited exposure that is provided.

An interesting new approach is the use of avian eggs for an in ovo carcinogenicity assay (IOCA) (61).

TS is injected into the egg white of fertilized turkey or quail eggs prior to incubation. The embryonic liver is removed 3–4 days before hatching for the evaluation of preneoplastic lesions. This assay has the advantage of being defined as an in vitro method for carcinogenicity testing.

Table 20.5 Accelerated carcinogenicity bioassay

Initiation segment (IS)		Promotion segment (PS)	
Control, 16 weeks		Control, 24 weeks	
TS, 16 weeks		Promoter, 24 weeks	
		Liver	PB
		Kidney	NTA
		Bladder	NTA
		Stomach	BHA
		Lung	BHT
		Breast	DES
Initiator, 10 weeks		TS, 24 weeks	
Liver	DEN		
Kidney	EHEN		
Bladder	BHBN		
Stomach	MNU		
Lung	DMN		
Breast	DMBA		
TS, 16 weeks		TS, 24 weeks	

Note. TS, test substance; DEN, diethylnitrosamine; EHEN, *N*-ethyl-hydroxy-ethylnitrosamine;mine;BHBN,N-butyl-N-(4-hydroxybutyl)nitrosamine; MNU, methylnitrosourea; DMN, dimethylnitrosamine; DMBA, 7,12-dimethylbenz[a]anthracene; PB, phenobarbital; NTA, nitrilotriacetic acid; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; DES, diethylstilbestrol.

Accelerated Cancer Bioassay

The accelerated cancer bioassay (ACB) model can be used to develop data on carcinogenicity when (a) there is not a requirement for a RGB, (b) there is an urgent need to obtain data, or (c) as an alternative RGB for one species.

The ACB is essentially a composite of six or more LCBs for rodent organs in which carcinogenicity has been found for known human carcinogens: the liver, lung, kidney, urinary bladder, stomach, and mammary gland. The protocol involves two segments, a first in which the TS is administered for 16 weeks in an initiating segment followed by promoters for the target organs and a second part in which the TS is administered in a promotion segment for 24 weeks after exposure to initiating agents for the target organs (Table 20.5). The TS is also given alone for 40 weeks, to assess carcinogenicity.

The ACB has a number of advantages as follows: (a) It takes less time than the RCB, as the name implies; (b) it provides mechanistic data on initiation/ promotion; and (c) the animals exhibit much less age-related pathology at termination because they are less than 1 year of age. Of course, the chief limitation is that the ACB is not as comprehensive as the RCB, although it has been calculated that because of the promoting stimulus, initiation is as sensitive as a chronic bioassay (294).

RODENT CANCER BIOASSAY

Historically, the RCB has been conducted in rats and mice, although other species may be used, as discussed in the section on animals and their environment. For Pharmaceuticals, experts in Europe question the value of a mouse RCB (267). Alternatives to the mouse RCB that are now being evaluated are the transgenic or neonatal mouse bioassays and the ACB in mice, as discussed in the section on limited bioassays.

The RCB was developed for testing of small molecules, but also is being used for testing of large biological molecules. Such biomolecules were introduced into medicine beginning with the hormone insulin and now include oligodeoxynucleotides, genes, recombinant human proteins, humanized monoclonal antibodies, blood products, vaccines, and cellular therapies. Each of these presents specific issues in application of the RCB. The International Conference on Harmonization has produced a framework for preclinical safety evaluation of biotechnology-derived Pharmaceuticals (223, 265). In the systematic approach discussed here, genetic toxicology studies unfortunately are not particularly informative for biopharmaceuticals because the large molecules may not enter the cells used in assays, particularly bacteria. Moreover, it is exceedingly unlikely that such macromolecules would be genotoxic. Customized approaches are usually required for chronic preclinical safety assessment of biotechnology-derived Pharmaceuticals and genetically engineered food products (265). Some of these products are intended for intravenous administration, which is a difficult route of administration for an RCB. Immunogenicity presents an additional complication with proteins, since with the development of neutralizing antibodies the biological activity of the protein can be abrogated, and the chronic antigenic

stimulation can compromise carcinogenicity testing. Guidances concerning the design of chronic/carcinogenicity studies are generally based on the clinical indication or in-use exposure. Exceptional with these products is the possibility of using relevant but nontraditional species or the use of animal models of disease (223). Any testing should, of course, be done in a species in which the molecule has biological activity. For an immunogenic protein, one solution to this problem is to test a homologous rodent molecule in the corresponding species.

Design

Most RCBs are done to meet regulatory requirements, as listed in Table 20.1; otherwise, there are more efficient approaches to carcinogen identification, as discussed in the section on systematic approach to testing. An RCB

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performed for regulatory requirements must follow guidelines (63, 66, 261, 265), and, in particular, the regulations for Good Laboratory Practices (described below), although those conducted by the NTP do not strictly meet this criterion. Detailed descriptions of standard procedures for an RGB with chemicals have been published (168, 207, 274). Aspects of the design, conduct, analysis, evaluation, reporting, and interpretation are given next.

Feeding Procedures

During the past 20 years, increased variability in body weights, survival and incidences of spontaneous tumors in Sprague-Dawley and Fischer 344 strain rats and CD-I and B6C3Fi mice have been noted (3, 29, 137). Specifically, increased body weights, decreased survival, and increased spontaneous tumor incidence can confound and even jeopardize the interpretation of an RGB (19, 135, 137, 183, 200, 222, 238).

Thus far, most RGBs have been conducted using ad libitum (AL) feeding. To overcome the problem of overeating with AL feeding, two solutions have been proposed (29, 137, 183, 238); one is referred to as caloric optimization diet (COD) and consists of limiting caloric intakes to 50–80% of AL consumption (29, 94, 95), while the other is called the diet-restricted (DR) model, in which animals are given diets limited in offered quantity of feed sufficient to produce a 15% reduction in body weight compared to the AL controls (1, 183). In addition to these procedures, the use of weight-matched groups that are fed in such a way that their mean body weight is matched with that of the high exposure AL group has been evaluated (1, 183) but is not currently being used. The COD and DR procedures clearly improve the health and survival of animals, reducing the occurrence of age-related pathology, such as chronic progressive nephropathy and myocardiopathy in rats (113) and incidence of certain spontaneous neoplasms (113). Another approach to over-coming the overeating problem is simply to use a strain that does not present this difficulty (see the section on species and strain).

In the past, dietary control was routinely used in testing of oral contraceptives (132). Recently, an emphasis has been placed on the use of dietary control for all medicinal and chemical products (3). Differences in the incidence of neoplasia between AL-fed and DR rodents are shown in Table 20.6 for the two commonly used rat strains, Sprague-Dawley and Fischer 344, and the B6C3F] hybrid mouse. In both rats and mice, and especially the latter, hepatocellular neoplasia in both genders is reduced in DR groups. Similarly, pancreatic (both acinar and islet) neoplasia (especially in male rats), pituitary (especially in female rats) neoplasia, and adrenomedullary (especially in male Sprague-Dawley rats) neoplasia reduction with DR was present in both species. In general, these decreases in DR groups are due to changes in metabolism and hormonal homeostasis induced by DR. In addition, certain decreases in the DR groups are only present in one species (thyroid C-cell, mammary gland, pulmonary, ovarian, uterine, and hematopoietic), one strain (thyroid C-cell neoplasia in male F344 rats), or one gender (pulmonary adenoma in male mice). The mechanism of these decreases cannot be explained currently. Available information, however, on the impact of either COD or DR on response to well-studied carcinogens generally reveals a reduced response. Accordingly, detection of carcinogenic effects may be masked at sites sensitive to tumor reduction by body weight gain inhibition. Analyzing tumor incidence within body weight strata can reduce bias introduced by weight differences (see the section on statistical analyses).

Groups

Animals must be assigned to groups using randomization procedures. Randomization eliminates bias, but if there is another source of variation, such as gender, cage position, and order of euthanasia at termination, then a stratified randomization is more appropriate. This involves separate randomization within each level of stratifying variable, such as body weight or cage position (152). For further information, see the section on statistics.

The use of two independent control groups helps to control for biological variability in incidences of commonly occurring cryptogenic neoplasms that may occur in only one group. The usual group size is 50–60, which permits detection of neoplasms with incidences in the range of 5–10%. Groups of 75 can be used to allow for an interim kill, as described later in the statistical analyses section. An interim kill of control and exposed animals is useful in that it provides information on time to tumor. This information is not otherwise available when the neoplasia is not life-threatening and there are no resultant unscheduled deaths with neoplasms (80, 152). Finally, since the diagnosis of proliferative lesions can be controversial, a short recovery segment (1–3 months) is desirable, because induced neoplastic lesions should progress while nonneoplastic proliferative lesions may regress. To monitor for intercurrent diseases (see the section on animals and their environment), a satellite group of six or nine is used.

The groups typically consist of a high-dose group (see the section on dose selection) and one or two

lower doses. Many laboratories, including the NTP, favor a second group at half the high dose to provide sufficiently exposed animals in the event that the high dose impairs adequate long-term survival. Other laboratories space doses by

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Table 20.6 Comparative percent incidence of neoplasia under conditions of ad libitum (AL) feeding and diet restriction (DR) regimens in 104-week-old rodents

	F344 rats				S-D rats				B6C3F1 Mice			
	AL*a*		DR*a*		AL*b*		DR*c*		AL*d*		DR*e*	
Types of neoplasia	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
Hepatocellular adenoma	4	<1	2	0	5	<1	0	2	29	30	16	14
Hepatocellular carcinoma	2	0	0	0	2	0	2	0	26	16	12	4
Pancreatic acinar adenoma	6	0	0	0	0	1	0	0	0	2	0	0
Pancreatic islet adenoma	12	2	4	0	8	9	4	4	2	0	0	0
Pheochromocytoma	21	4	5	0	23	5	2	0	0	2	0	0
Pituitary adenoma	49	42	12	15	62	85	45	77	2	8	0	2
Thyroid C-cell adenoma	17	8	8	2	7	6	4	8	0	0	0	0
Thyroid follicular adenoma	0	0	0	0	4	2	0	0	2	6	4	2
Mammary-gland fibroadenoma	4	57	6	0	2	54	0	15	0	0	0	0
Mammary-gland carcinoma	0	4	0	0	<1	26	0	30	0	0	0	0
Skin papilloma	6	0	2	0	2	0	2	0	0	0	0	0
Skin fibroma	10	2	2	2	2	1	0	1	1	2	1	0
Pulmonary adenoma	4	4	2	0	<1	1	0	0	22	6	12	4
Leydig-cell adenoma	89	NA	61	NA	7	NA	6	NA	0	NA	0	NA
Ovarian cystadenoma	NA	2	NA	0	NA	0	NA	0	NA	6	NA	0
Ovarian granulosa neoplasia	NA	4	NA	0	NA	1	NA	0	NA	0	NA	0
Uterine polyps	NA	14	NA	8	NA	6	NA	0	NA	1	NA	0
Mononuclear-cell leukemia	62	42	26	0	0	0	0	0	0	0	0	0
Lymphoma	0	0	0	0	2	1	0	0	14	24	4	10

Note. F344, Fischer 344 rats; S-D, Sprague-Dawley rats; B6C3F1 mice (C57BL/6N+C3H/Hen)F1; NA, nonapplicable; the average number used by species/strain/gender was in excess of 750 animals; AL, ad libitum; DR, diet restriction (see text).

*a*Modified from References 183 and 29.

b Modified from References 167 and 29.

c Modified from Reference 29.

d Modified from References 232 and 183.

e Modified from Reference 183.

one-third or one-fourth. Since generally only TSs that are not DNA reactive should advance to an RGB, a valuable third group is one at the no-effect level (NEL) for any epigenetic effect identified at higher doses that may lead to tumorigenesis. This NEL should yield a cancer NEL, which is valuable for risk assessment. If quantitative risk assessment is envisioned, even more dose levels may be needed.

Duration

With the commonly used strains of rats the anticipated life span is 24–30 months, and 18–24 months for mice. The usual duration for both a rat and mouse RGB is 24 months, although for a mouse RGB, 18 months is acceptable, especially if survival is compromised (33). Exposure should be daily (7 days/week) and should start shortly after weaning. Exposure groups are not allowed to live longer than control groups. If a high-dose group experiences high mortality (greater than 50%) due to exposure, it should be terminated; the other exposure groups and controls should continue until 24 months are completed. In general, survival should not be less than 50% for mice at 15 months and rats at 18 months, or 25% for mice at 18 months and rats at 24 months (33).

GOOD LABORATORY PRACTICE

As regards the RGB, the main intent of Good Laboratory Practice (GLP) is the maintenance of integrity of a complex system of data management. This presupposes that the study is designed, conducted, evaluated, and reported according to standard operating procedures and that records are maintained in a manner that ensures a comprehensive and independent review (251, 259). GLP must be conducted in such a way that all data can be validated.

GLP is a global process, which is internationally harmonized, with de facto reciprocal recognition of national GLP programs. This occurs through planned publications of the International Conference on Harmonization (ICH) since 1990 (43, 231). The purpose of ICH is to make continuous recommendations on ways to achieve harmonization of technical guidelines and requirements for medicinal product approval. In addition, the U.S. Food and Drug Administration (FDA) and the U.S. Environmental Protection Agency (EPA) have relied in the past on bilateral memoranda of understanding (MOU). Currently, both agencies are negotiating a mutual recognition agreement (MRA) with the European Union. It is conceivable that all MOUs will be replaced by MRAs. At the time of this writing, MRAs exist between Germany, Netherlands, and the United Kingdom. In a GLP study, the study director plays the critical roles of moderator, catalyst, and gatekeeper, responsible for the integrity of study data. As with any process, the management of the GLP process has three components; these are planning (organizing, goal setting, prioritizing, and scheduling), operating (implementing, conducting), and controlling (monitoring, evaluating, and taking of remedial action). Here the importance of the test facility manager is crucial. The test facility manager has the ultimate responsibility for ensuring that all work from all studies is carried out in accordance with GLP principles. The specific responsibilities of the test facility manager include appointment and, if needed, replacement of the study director, the commitment of adequate and trained human resources to each study, the appropriateness of housing for the experimental animals, and scientists and equipment dedicated to the conduct of a study and the assurance of the integrity of the TS.

At the core of the GLP process are information transfer and data acquisition systems and programs. Documented and validated performances (using a standard data set) of these systems and programs are requirements in GLP. Manual systems are subject to standard operating procedures (SOP) review and approval. Computer-based systems (both hardware and software) are subject to validation. The validation methods must address common features relating to system definition, documentation and management. These methods must be clear, specific, operational, and must be periodically reviewed. Validation is the responsibility of the end user. The validation control system includes (a) system definition, (b) test protocol, (c) validation testing, (d) performance evaluation, (e) operational procedures, and (f) validation report. The basic objectives of the test protocol should include determination of accuracy, reliability, performance, and reporting of activities and errors. There are systems such as statistical analysis system (SAS), vendor-supplied graphics, word processor, spreadsheet applications, and calculator programs, which ordinarily have a validation control system provided by the vendor. For these, documentation is needed of the source of codes and formulas. For other programs requiring a control system, validation of hardware, the manufacturer's name, model, serial number, configuration options, peripheral devices, memory boards sensors, interface boards, controlling devices, communication links, and references to system documentation are needed. For other software, development resources (e.g., compilers), function libraries, source-code management tools, and debugging utilities are needed.

All raw data must be properly identified and stored, eventually in study notebooks that ensure the

integrity of the data. All data must be entered in a permanent and legible manner. Any changes to data must be dated and identified as changes, with reasons noted for any change, as well as the identification of the scientist making

the change. Further, the professionals responsible for data entry, verification, and review should also be identified. Finally, the documentation of the data so described must be maintained at all times, and secure audit trails must be created for authorized changes in the database and also in the study notebook (251). In histopathology, raw data are by definition the tissues on glass slides and their respective blocks, requiring a specific trail leading from sampling records during necropsy and trimming records after necropsy (15, 259). To achieve proper material, tissues are prepared according to standardized procedures with respect to location, type of section (e.g., cross), and orientation on the slide. In addition, slides of lesions should include both lesion and surrounding "normal tissue." Any failure to adhere to this regimen is a form of censorship and results in noncompliance. It is the duty of the study pathologist to integrate clinical (cage-side observation), structural (gross and microscopic), and functional (cellular and biochemical) data. Failure to integrate the three types of data will result in compromise of data and loss of data integrity. The pathologist must keep the study director apprised of events as they occur. The responsibilities of the study pathologist include keeping account of all the lesions reported at necropsy and performing microscopic evaluation of the normal and abnormal tissue changes. Thus, the pathologist ensures that appropriate tissues are collected, processed, and evaluated in a manner that satisfies the objective of the study. The pathologist also functions in a quality control capacity for the morphologic aspects of a carcinogenicity study (e.g., if a compound discolors adipose tissue, the pathologist ascertains whether all adipose tissues of exposed animals are discolored or whether the intensity is more pronounced in the high-dose group, etc.). Finally, the pathologist ensures consistency in diagnosis, integration of data, and grading of pertinent lesions, avoiding diagnostic drifts and censorship, as discussed in the section on pathology.

For a facility to be GLP compliant, specified environmental conditions (see the section on animals and their environment) must be maintained and monitored through a program applied at appropriate intervals.

The final regulatory end-product for every study is the compliance statement of the final report, which is signed by the study director. In this section of the report, all modifications, deviations, and amendments to the protocol should be listed. A second page with a quality assurance (QA) statement is signed by the QA auditor. During the last 20 years of GLP implementation, several common findings of importance (listing deficiencies in form FD-483) have been compiled by various agencies. The deficiencies noted were in all subparts of the FDA regulations (subparts A-J, 21 Code of Federal Regulation (CFR) 58.10–58.190). Those pertaining to pathology are detailed in the section on anatomic pathology.

Clearly, implementation of GLPs has benefited the conduct of chronic toxicity and carcinogenicity studies in many ways. These include (a) improved documentation, (b) refinement in bioavailability/bioequivalance information, (c) crystallization of the thinking process in safety assessment, and (d) strong, science-based regulated professionalism.

HEALTH AND SAFETY PROCEDURES

A comprehensive, rigorously followed health and safety plan is necessary for the proper conduct of an RCB. The fact that the substance is being tested for such activity makes it, in effect, a suspect carcinogen, although information in the Material Safety Data Sheet (MSDS) (e.g., genotoxicity, reproductive toxicity) influences the stringency of the handling procedures instituted, from receipt of the chemical through disposal of animal waste and processing of tissues for histopathological examination. All measures are subject to quality control.

The safety plan (178, 190) must address the responsibility within management for development and adherence of the plan; medical surveillance for employees; employee training; safe handling practices for the chemical; animal handling; general laboratory safety; safe personnel practices; safe work-area practices (e.g., spill control and decontamination); handling of air, liquid, and solid wastes; monitoring of workers and physical equipment; emergency control; record keeping; the design of facilities; and the pollution potential. Applicable regulations of the Occupational Safety and Health Administration (OSHA) provide only a minimum structure from which to work, and lessening of the hazard within the particular facility must be addressed individually and with ingenuity. Laboratory directors must appreciate that chemicals may penetrate protective clothing and travel a considerable distance from their point of use (189, 214, 215, 216, 254, 257).

No safety measure is unique to an RCB; it is the degree of adherence to such procedures that distinguishes the conduct of these studies from all others. It is beyond the scope of this section to address each individually. A few examples of aspects that are often inadequately addressed include the following: (a) use of a properly ventilated cage dumping area or an enclosed animal bedding disposal cabinet to prevent inhalation of contaminated dust and aerosols by employees; (b) an air-handling

system that provides decreasing gradations of air pressure from clean corridor to the animal rooms to the dirty corridor and that is periodically tested under such stress as several doors being opened at one time or with all possible chemical hoods in operation; (c) main

tenance personnel as well as scientific supervisors that follow the same rules as technicians for personal protection; (d) storage facilities that protect the integrity of the chemicals over an extended period of time during which unused material may be held and the immediate containers checked for deterioration; (e) a "breathable air" line available for use with an air-supplied respirator in the TS preparation areas; and (f) workers, including weekend staff, who are familiar with emergency safety instructions within the laboratory and know whom to notify in the event of various types of potential emergency situations.

ANIMALS AND THEIR ENVIRONMENT

The use of animals in research is subject to national regulations. In the United States, the use of rats, mice, and birds is not regulated. Guidelines for other animal use have been provided by the U.S. Department of Health and Welfare for Care and Use of Laboratory Animals (178, 179, 249), the latest amendment of the U.S. Congress Animal Welfare Act (245), the U.S. Public Health Service Policy of Humane Care and Use of Laboratory Animals (179), the U.S. Department of Agriculture Animal Welfare Rules (248), the Animal Welfare Act of the National Research Council (182), and the U.S. EPA Health Effects Test Guidelines (258). Institutional responsibilities include making available all protocols for review by a committee and providing veterinary care. More extensive coverage is provided in Chapter 16.

Species and Strain (Genotype)

For the rat RGB, several strains have been widely used. The NTP generally uses the inbred Fischer F344, while the pharmaceutical and chemical industries have favored the outbred Sprague-Dawley or Wistar. For the mouse RCB, industry generally favors the CD-I strain, whereas the NTP uses the B6C3Fi hybrid, which is the first-generation cross between male C3H and female C57BL/6 strains.

Both rat and mouse strains differ substantially in their background of tumors and their susceptibility to induction of tumors (30). For example, the F344 has very high incidence of several neoplasms (Table 20.7). Among mouse strains, those derived from the C3H, that is, B6C3F1, have a high incidence of liver tumors. Thus, male C3H/HeJ mice are highly susceptible to induction of liver cancer whereas those of the C57BL strain (6J or 10J sublines) are resistant; the B6C3F] hybrid is intermediate. Furthermore, when A/J mice (high lung tumor susceptibility) were crossed with C3HeB/FeJ (C3H) mice (high liver tumor susceptibility), the relative frequency of lung tumors was A/J>Hybrid>C3H and of liver tumors was C3H>Hybrid>A/J. For both tumor types, the incidences were higher in males than in females. The comparative percent incidence of the principal spontaneous neoplasms from five different strains of rats and mice is given in Table 20.7.

Several factors that influence response to carcinogens differ among genotypes. Notably, biotransformation activities differ considerably for certain chemicals. In addition, gender-dependent differences in xenobiotic metabolism are most pronounced in rats. The differences involve mainly cytochrome P450s (CYP), sulfotransferases, glutathione transferases, and glucuronyltransferases (176, 184).

A troublesome feature of the F344 rat and B6C3F1 mouse is that their average survival has progressively decreased and increases have occurred in the incidences of liver tumors in female and male mice, pituitary tumors in female mice and thyroid tumors, adrenal pheochromocytomas and leukemias in male rats, and mammary tumors in female rats (201, 243). Some of these tumor increases are positively correlated with excessive body weight (243), due to overeating. A detailed discussion of this issue is presented earlier in this chapter, in the feeding procedures section. Likewise, in Charles River Sprague-Dawley rats decreases in survival have been reported (136, 137). These problems may be due to breeding practices. Currently, the Wistar strain does not appear to present this problem (197).

Feed and Water

Four major types of diets are available, as follows: (a) natural-product, unrefined, largely cereal-based formulations unusually referred to as "chow-type" diets, such as NIH-07 (Purina 5018 or Purina 5001); (b) semipurified diets, formulated from refined nutrient ingredients, such as AIN76A (with sucrose) or modified AIN 76A (with dextrose); (c) open-formula diets, such as NIH-31 (Purina 7017), which are formulated to contain researcher-specified quantities of nonproprietary ingredients; and (d) chemical-defined diets, which are individually specialized (142, 182). Industry generally favors the open formula diet. The NTP has introduced a new diet, NTP-2000, designed to reduce certain spontaneous pathologies in the F344 rat.

Details about various regimens of feed availability are given earlier, in the discussion of Feeding Procedures.

Caging and Stratification

Following randomization (see section on groups), animals can be caged individually or with more than

one animal in each cage, each having advantages and disadvantages. Individually caged animals tend to

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Table 20.7

Comparative percent incidence of pertinent neoplasia in different strains of rats and mice (104 weeks old)

	F344 rats*a*		S-D rats*b*		Wistar rats*c*		B6C3F1 rnice*d*		CD-I mice*e*	
Types of neoplasia	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
Hepatocellular adenoma	4	<1	5	<1	1	2	29	30	26	5
Hepatocellular carcinoma	2	0	2	0	<1	<1	26	16	10	1
Pancreas islet adenoma	12	2	8	9	4	2	2	0	<1	<1
Pancreas islet carcinoma	3	0	<1	5	<1	<1	0	0	0	0
Pancreas acinar adenoma	6	0	1	0	13	<1	2	0	<1	0
Pheochromocytoma	21	4	23	5	10	2	0	2	<1	<1
Adrenocortical adenoma	0	2	3	0	8	9	<1	0	1	<1
Pituitary adenoma	49	42	62	85	34	55	2	8	0	5
Thyroid C-cell adenoma	17	8	7	6	6	8	0	0	0	0
Thyroid follicular adenoma	0	0	4	2	2	1	2	6	1	<1
Mammary-gland fibroadenoma	4	57	2	54	3	36	0	0	<1	1
Mammary-gland carcinoma	0	4	<1	26	1	13	0	0	0	6
Skin fibroma	10	2	2	<1	5	1	1	2	<1	<1
Skin papilloma	6	0	2	0	2	<1	0	0	<1	0
Pulmonary adenoma	4	4	<1	<1	<1	0	22	6	15	15
Preputial-gland neoplasia	10	NA	>1	NA	<1	NA	<1	NA	<1	NA
Leydig-cell neoplasia	89	NA	7	NA	11	NA	0	NA	1	NA
Clitoral-gland neoplasia	NA	14	NA	<1	NA	<1	NA	<1	NA	0
Uterine polyps	NA	14	NA	6	NA	16	NA	1	NA	<1
Ovarian neoplasia	NA	6	NA	1	NA	8	NA	6	NA	1
Mononuclear-cell leukemia	62	42	0	0	<1	<1	0	0	2	2
Lymphoma	0	0	2	1	3	5	14	24	8	22
Forestomach papilloma	0	2	<1	<1	0	<1	4	2	<1	<1
Scrotal mesothelioma	5	NA	1	NA	2	NA	0	NA	0	NA

Note. F344, Fischer 344 rats; S-D, Sprague-Dawley rats; B6C3F1), mice (C57BL/6N+C3H/HeN)F1; CD-I, ICRCr: CD-I mice; NA, nonapplicable; the average number used by species/strain/gender was in excess of 750 animals.

a Modified from References 183 and 29.
b Modified from References 167 and 29.
c From References 271, 14, and 197.
d Modified from References 232 and 183.
e Modified from Reference 160.

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overeat. Multiple-animal caging leads to a conflict for hierarchy in the cage and consequent cage differences. Group-caged female mice develop pseudopregnancy, which results in uterine decidual reactions. Also, group caging of mice produces almost a doubling of the lymphoma incidence in both males and females (236). In general, group-caged rodents demonstrate higher survival rates and lower background pathology (206). For inhalation and dermal administration studies, single housing is required.

All cages used should be either metallic (stainless or galvanized steel) or plastic (polycarbonate, polyethylene, or polypropylene), with a minimum stipulated cage size (249). The floor of the cages can either be solid or suspended. Solid-floor cages require bedding, that should not possess enzyme induction properties, such as hardwood bedding (269).

Rotation of cages and racks periodically should be used to balance known confounding sources of variability such as proximity to dual-intensity fluorescent light (148, 202). Consequently, rodents within groups should be distributed in cage racks in such a way as to be present equally at all vertical levels of caging. A thorough documentation of such cage and rack rotation is mandated.

Ventilation, Temperature, Humidity, and Emergency Power

Environmental stress to test animals must be minimized, particularly with mice, which are easily stressed even when maintained under conventional housing conditions and handled in the usual manner. The incidence of tumors in mice infected with viruses can be increased by chronic stress (205, 221). Standards for care are detailed in the *Guide for the Care and Use of Laboratory Animals* (182). These include (a) 10–15 fresh air changes per hour in each animal room; (b) air pressure adjusted so that the animal rooms are slightly positive to the "dirty" corridor and negative to the "clean" one, with minimal crossovers between the corridors; (c) all air adequately filtered before it enters the animal facility, and diluted or filtered after it leaves to prevent possibly toxic concentrations of the test chemical from entering the outside air (a process that is particularly important with inhalation studies because of the large amounts of chemical used); (d) temperature and humidity maintained within those ranges reported to be optimal, that is, 23.3±1.1°C (74±2°F) and a relative humidity of 40±5% in rat and mouse rooms; and (e) automatic control systems that record both temperature and humidity at least three or four times per day. Control of lighting is essential. Usually, a 12-h continuous light interval per day is used for both rats and mice (14-h light day for hamsters). Moreover, high (only during cage-side observation time of approximately 4 h)-low (20 h) intensity of fluorescent light is recommended in order to avoid blindness caused by high-intensity light. In addition, because the light and temperature gradients occur vertically, a cage-rack rotation is mandated (182). An emergency power source is essential to maintain operation of storage freezers and refrigerators, lighting, tissue processors, and some degree of air-conditioning, as well as air handling, during power failure or when personnel are unable to reach the facility. The emergency power and alarm systems should be tested on a regular schedule. In a GLP-compliant facility, all these conditions must be monitored.

Also important is control of pests by adequate facility design and sanitary procedures. Pesticides must not be allowed to contaminate the animal rooms, feed rooms, or cage washing areas and accordingly should be dispensed only in closed traps in limited areas. Detergents and cleaning agents for use on floors, cage washers, and other equipment must be nonvolatile and must not leave a residue.

DOSE SELECTION STUDIES FOR DIOASSAY

Dose Selection: The Maximum Tolerated Dose

Dose setting can be based upon a number of end-points, including toxicity, toxicokinetics, saturation of absorption, and maximum feasible dose (73). Generally, it has been expected that the high dose level in an RCB should be a toxicity-based dose, the maximum tolerated dose (MTD) (229), which is also referred to as the minimum toxic dose (or the minimally toxic dose used by NTP) (100). The various methods for selection of the high-dose level have been reviewed by a working group on dose selection convened by the International Life Sciences Institute (73). Testing at the MTD has the virtue that a negative outcome is compelling, but creates problems in the interpretation of positive findings, because of specific high-dose effects, as discussed with rodent cancer bioassay evaluation.

The first widely used definition of the MTD was formulated by the National Cancer Institute (229) as follows: "The MTD is defined as the highest dose of the test agent during the chronic study that can be predicted not to alter the animals' normal longevity from effects other than carcinogenicity." This definition does not stipulate that any toxicity be produced, and hence a slight, but significant, reformulation was introduced by the U.S. Interagency Staff Group on Carcinogens (181, 262) as follows: The MTD is "the highest dose which when given for the duration of the chronic study is just high enough to elicit signs of minimal toxicity without

significantly altering animals' normal lifespan due to effects other than carcinogenicity." The MTD so defined is a dose used in the chronic study (7), which, of necessity, is selected from subchronic studies (normally 90-day). The ICH defines the MTD as the dose predicted from a range finding study to produce minimum toxicity over the course of the carcinogenicity study (262). Such toxicity may be predicted from alterations in physiological function that would be expected to alter survival, toxicity to target organs, significant alterations in clinical pathological parameters or suppression of weight gain of no more than 10% relative to controls calculated as the difference between the starting weights and those at the end of the study (181, 262). A fortuitously selected target MTD should in the RGB study suppress weight gain by 10% or slightly greater and produce only minimal other toxicities. This, however, infrequently happens, and issues arise when the high dose is either below or above what is considered by a regulatory agency to be an MTD.

Toxicokinetic endpoints have gained acceptance for dose setting for Pharmaceuticals (42, 76). A dose that produces saturation of absorption is considered an MTD, and a dose that produces a plasma concentration that is 25 times greater than human exposure is considered pragmatic (124). The endpoints are easier to establish.

Toxicodynamic endpoints can be used to establish a high dose that will produce a cellular effect beyond which the validity of the study would be compromised. An example is induced toxicity, which would be expected to become more severe with aging in rats as they develop chronic progressive nephropathy.

To identify the target MTD or high dose, subchronic studies are performed, as described next. If the target MTD (or the high dose) cannot be established on the criteria discussed, it is usually recommended that the high dose level or limit dose not exceed 5% in the feed (115, 169, 191), which translates into approximately 3–4 g/kg/day for rats and 7–8 g/kg/day for mice. This limit appears to be based upon concern for the nutritional impact of high proportions of TS. Certainly, some TSs at high exposure interfere with nutritional elements, such as impairment of vitamin K function by butylated hydroxytoluene (37, 127), but such problems can be overcome by nutritional supplements. Nevertheless, reduced feed intake, and hence caloric intake, will affect the outcome of the RGB, usually reducing tumor incidence (1, 29, 183).

In the United States, for Pharmaceuticals, the dose selection should be submitted to the FDA Center for Drug Evaluation and Research Carcinogenicity Assessment Committee, which strongly favors a toxicity-based endpoint. Such consultation is not yet practiced in Europe.

The selection of other dose levels is discussed in the section on rodent cancer bioassay groups.

Subchronic Study

The MTD is usually identified from results of a 90-day study, using the route of administration to be used in the RGB. It is unusual that a tested dose would qualify as the predicted MTD, and therefore interpolation is usually made. When genders show different MTDs, then based on kinetic study results, different sets of doses are administered.

Kinetic Studies

Before starting an RGB, it is helpful, and in the case of medicinal products, it is a requirement (76, 124), to determine the time course of exposure and the relationship between the concentration of exposure and effect. For this purpose, multiple-dose toxicokinetic studies are conducted (23, 42, 124). Plasma protein binding of the TS in rodents and humans should be determined before initiation of the carcinogenicity studies. Should binding exceed 80%, it is advisable to express exposure (plasma TS concentration) in terms of the free fraction (26, 276). In addition, tissue distribution and accumulation data can be valuable (25, 198, 225), but this is more difficult and requires other techniques.

Furthermore, accurate and useful extrapolation from rodents to humans presupposes understanding of histokinetic and xenodynamic considerations. For example, smaller species (e.g., rodents) have a higher xenobiotic metabolic rate per kilogram of body weight, faster rate of tissue distribution, and shorter tissue half-life (111). Tissue volume directly affects the volume of distribution of xenobiotics, bioavailability, half-life, and systemic clearance (13, 130, 140). In general, with increasing age, as will occur over the course of a carcinogenicity study, body weight increases, as does the proportion of adipose tissue, which has a lower metabolic rate than skeletal muscle mass (82). Both influence the outcome of kinetic studies. In dose-proportional linear kinetics, half-life and clearance are independent of xenobiotic concentration. In contrast, in nonlinear kinetics, these parameters are dependent, because the various processes (e.g., absorption, distribution, metabolism, and excretion) can become saturated (153).

The toxicokinetic component of a carcinogenicity study often entails satellite groups run in parallel and consists of at least three rodents per group per gender per time interval, with interim kills at least every

6 months. The time-course factor is very important in explaining how the various concentrations accumulate and relate to different exposures (76). The animals are maintained and exposed under identical conditions with the main study. Approximately 4 blood samples are taken from each animal over the duration of the RGB, with the blood removed not exceeding 15% of the total blood volume.

The parameters examined may include (a) the maximum achieved concentration (Cmax), the minimum concentration (Cmin), the time to Cmax (rmax), and the area under the flood concentration versus time curve (AUC) for TS and all metabolites (109). Only the AUC is required to establish exposure (124). In an RGB, together with measuring the systemic concentration of exposure (AUC), determination of the total (cumulative) amount of exposure over time is also essential so that exposure can be related to induced effects (124, 198). In addition, if indicated from chronic toxicity data, target site (tissue) compound levels (or major metabolites) can be measured, but, as noted, this requires separate assay validation and a more extensive study design to acquire appropriate tissues. Target site compound levels potentially connect the target site of both chronic toxicity and carcinogenicity. For accurate extrapolation of exposure over time, human time equivalent (HTE) values are employed. These values amount at 12 months of exposure to a HTE of 38 years for the mouse and 34 years for the rat, and at 24 months to a HTE of 76 and 64 years for the mouse and rat, respectively (109, 111).

Pharmacokinetic data are critical, not only for delineating blood levels, but also for understanding the effects of exposure—that is, if the parent or a metabolite is the main active moiety, or even if the plasma concentration reflects the cellular site of action, and which is the interspecies concentration (blood)-response (site) relationship (109, 171, 193). Furthermore, metabolism can also distort concentration-response homeostasis. Here, at high exposures, together with absorption and elimination being saturable (i.e., capacity limited), intermediary metabolism can also significantly target tissue bioavailability through transformation, which is especially important in interspecies extrapolation (27, 86, 225, 296). Moreover, metabolic saturation and activation of secondary routes of metabolism should also be taken into consideration (25, 47, 85, 93). Knowledge of metabolic behavior (e.g., species differences in biotransformation enzymes) of the xenobiotic under test is the best foundation for interpreting the mechanism(s) of neoplasia and for extrapolating to humans.

QUALITY CONTROL OF THE TEST SUBSTANCE

The test substance should be of a high quality and stability, and should be manufactured in the same way and contain the same concentration of impurities as the final product. Impurities in excess of 0.1% should be individually identified. For Pharmaceuticals, the preclinical and clinical final product tested should be preferably the same. The product should have a well-defined and described scale-up process (125).

Most medicines will be formulated with excipients and, accordingly, the noncarcinogenicity of these must be established. In other situations, as with agricultural chemicals and cosmetics, the technical-grade product or a representative technical grade of active ingredients is tested. In some instances, complex mixtures are tested, as with polychlorinated biphenyls. This aspect is discussed further with complex mixtures.

Test Substance

Chemicals or Small Molecules

In some situations, it is desirable or necessary to test pure chemicals, as with candidate medicines or food additives. With isomeric compounds, if the two enantiomers exhibit inversion in vivo or have the same biological activity, they are considered one entity and carcinogenicity of the racemate is appropriate. If the biological activity is not the same, then carcinogenicity testing of the active enantiomer is indicated.

Biopharmaceuticals

Biopharmaceuticals include recombinant human proteins, humanized monoclonal antibodies, oligodeoxynucleotides, and genes. Recombinant human proteins are being produced by a variety of techniques, often in bacteria where glycosylation does not occur. Quality control of biopharmaceuticals, as with small molecules, is necessary to establish that they are actually what they are expected to be at all times during the study. For this purpose, all relevant information, such as synonyms, trade names, and structural and molecular formulas and weights, as well as methods of analysis and chemical and physical properties of the pure substance, should be provided.

Impurities or Contaminants

It is highly recommended that all TS should be pure chemicals of analytical grade, since even traces of impurities of <1% can have confounding effects resulting in neoplasms. An example of this would be o-toluenesulfonamide (OTS), an impurity of saccharin, present in the early carcinogenicity studies (173). Impurities can occur in the starting materials to be used in the formulation, or in materials used in the manufacturing process of the TS (69, 131, 266). Impurities in excess of 0.1% should be thoroughly identified. If the TS with the identified impurity is intended for long-term use, such as with a high dose of active compound, then multiple-dose toxicity (up to 3 months) and mutagenicity studies should be

performed. If the results of the mutagenicity assays are positive, carcinogenicity testing of the impurity may be considered (125).

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Preparation of Dose
The most essential aspects in preparing a TS for dosing are identity of all ingredients, homogeneity of the product, particle size, stability of all active ingredients, and vehicle (carrier) to be used (69, 131). All batches of dosage preparations must be analyzed for concentration to confirm accurate preparation prior to use.

Homogeneity of the TS-diet mixture and stability of the TS in diet under the intended in-study conditions should be established prior to the start of RGB. During the conduct of the RGB, samples of the TS-diet mixture are taken approximately every 6 months for analysis and correction if necessary. It is recommended that the same batch be used for the entire RGB. This presupposes that there is a complete method of analysis for the active ingredients at hand. Quality control data as described earlier should be provided, and monitoring should continue throughout the duration of the study.

TEST SUBSTANCE OF ADMINISTRATION
The route of administration should be appropriate to potential human exposure and reflect knowledge about comparative bioavailability. Thus, comparative absorptive, dispositional, metabolic, and excretion data are essential and should be used in designing the 2-year RGB.

The most common route is the oral, followed by parenteral exposure and inhalation. The selection of a delivery system is crucial for all routes, as it has the potential to significantly affect bioavailability. Second, producing uniform and homogenous TS exposure throughout the bioassay is equally important. This requires the availability of a validated analytical methodology (124, 225).

For some TS there is a rationale for prenatal exposure, but generally exposure is begun postweaning at 4–6 weeks of age.

Oral
The oral route of administration is most frequently used, especially for medicines and food additives for which ingestion is the usual route of exposure of humans. For oral administration, the TS can be admixed in the feed or given by intragastric (ig) instillation (gavage). Comparable systemic exposures can be achieved with either, but with the ig route, the bolus dose results in a higher blood Cmax (143). The concentration of TS in diet is adjusted to compensate for changes in body weight. During the rapid growth phase the concentrations are adjusted biweekly at first, then weekly, and later monthly after the growth plateau has been reached. Here TS stability and homogeneity data are essential. Periodically during the study, samples of TS-diet mixtures should be analyzed to confirm desirable concentrations and enable corrective action to be implemented. In general, this is the most cost-efficient mode of exposure with most TS.

Intragastric (ig) administration affords the most precise oral delivery of TS. For this route of administration, a vehicle is required, such as corn or olive oil. For a TS that cannot be dissolved in water or oils, a suspension in Tween 80 or carboxymethylcellulose can be used. For a TS that is unstable or volatile in diet, microencapsulation can be used. Oils as vehicle will prolong residence time in the stomach compared to water (23). Corn oil given ig is reported to increase the body weights of male rats, but to have an opposite effect on females (99). Also, the incidence of some background tumors, such as mononuclear-cell leukemia and pancreatic acinar-cell tumors, can be affected. For this reason, a second control group (without gavage) is strongly recommended. For more details, see the section on feeding procedures. In general, the TS volume in rats is 3–5 ml/kg body weight. In mice a volume of up to 10 ml/kg has been shown to be desirable, as this enables more accurate measurement of the dose. In addition, dilution enhances absorption and decreases local irritation, especially with weak bases or acids, which comprise the majority of TS (17). Unscheduled deaths should be closely monitored at necropsy by inspection of the lungs and trachea for TS. If deaths are due to gavage accidents, then a monitoring program should be implemented. In general, the baseline rate for accidents per technician should not exceed 1 per 10,000 gavages.

Dermal
Under certain circumstances of dermal human exposure, TS are delivered via this route either by topical application or injection. The sensitivity of this route of exposure depends on the fact that the skin is also capable of biotransformation, albeit not to the same degree as that of the liver or intestinal tract (23). Dermal application is usually to the superior dorsal area of the back (interscapular), where the skin is clipped at least weekly, 24 h prior to application (149). In dermal studies, animals are routinely housed singly to minimized ingestion by other cage mates. TS (0.25–1 ml) is applied topically over the clipped area at intervals (e.g., 2 times or 3 times weekly) to allow for recovery (especially when the TS is irritating). Skin penetration varies with species, chemistry of TS, and vehicle (219), Thus, one should consider, in dermal dosing by weight or surface area, to the fact that rats are large enough to vary

significantly in size, whereas mice are not. Moreover, effects at the site of dermal injection should be monitored carefully.

Inhalation (Intratracheal)

With some TS, chronic inhalation is indicated. Routinely, under these conditions, animals are exposed 6 h/day during the day (104). After exposure, the animals can either remain in the chamber or be taken to another room. Either way, constant airflow through the chamber during and after exposure prevents build up of ammonia. Single cage occupancy is recommended to avoid grooming and licking-based ingestion. The cages should be rotated within the chamber periodically. Inhalation exposure is an expensive, labor-intensive route of administration, requiring frequent monitoring of achieved air concentrations. Sampling should be done from several fixed locations in the chamber after documenting homogeneity of the test atmosphere (162).

Nose-only or head-only exposure units are available for rodents (228). Their major advantages include minimization of external contamination and effective monitoring of respiratory parameters. The major disadvantages include restraining the animals, leading to alteration of many physiological parameters and entailing excessive manpower requirements.

Intratracheal exposure can be conducted under anesthesia to permit chronic delivery (up to 2 years) directly into the bronchial passages. Here, a second control group (with the anesthetic) should be added and exposure should be limited to once or twice weekly (227).

Parenteral

In special cases, or when the TS is destroyed in the gastrointestinal tract, intraperitoneal, intramuscular, or subcutaneous routes of administration are employed. Here, new factors such as molecular size and pH have the potential to affect absorption and cause irritation (9). Again, if a carrier is used, then a second control group without carrier is needed. The exposure regimen here is limited to two or three times per week. In using these routes, the potential exists for local tumor formation due to physical factors (207).

Multigenerational/Transplacental/Perinatal

Experimental transplacental carcinogenesis has been extensively studied, mainly in rats and mice (239), but also in primates (203, 237). The design of experiments ranges from multigenerational, involving exposure of germ cells of one or both parents and subsequently the progeny, to exposure of embryonal or fetal cells (4, 239). It has frequently been discussed whether such exposures should be included in an RGB, but so far, it is accepted that a conventional RGB beginning at 4 to 6 weeks of age can identify carcinogens that might have activity in developmental stages.

CLINICAL AND PATHOLOGICAL EXAMINATION

The RGB is not an extended chronic toxicity study; standard chronic toxicity assays (i.e., 6 and 9 or 12 months, depending upon requirements) involve more clinical observations than are necessary or appropriate for the RGB. In the RGB, animals should be observed at the beginning and at the end of each work day so that unwell animals can be euthanized before they become moribund or are lost to autolysis (or cannibalization, if multiple caging is used). Real-time automated programs for carcinogenicity studies have been developed (188), allowing for monitoring, at least biweekly, the appearance, location, and growth of palpable cutaneous or subcutaneous masses.

Standard parameters measured in the RGB include body weight, health status, and mortality.

Body Weight and Survival

In RGBs using an MTD, controls frequently show greater weight gain and often poorer survival than the high-dose group, in which body weight is reduced both by toxicity as well as secondary effects resulting in reduced feed consumption or energy utilization (156).

Intercurrent Diseases

Laboratory strains of rats and mice are susceptible to a variety of diseases, both genetic and acquired. The genetically determined conditions include predisposition to development of neoplasms, as discussed with species and strain. Also, pathologies such as amyloidosis in mice and chronic progressive nephropathy in rats are common. These conditions increase with age and complicate long-term studies. Acquired diseases, such as sialoadenitis and murine hepatitis, can be minimized by proper animal husbandry. Many strains of mice harbor *Helicobacter hepaticus* in the gastrointestinal tract. Infection with this organism can lead to an increased incidence of liver neoplasms, particularly in male mice (91). Satellite sentinel animals are included in all chronic studies to effectively monitor intercurrent acquired diseases.

Clinical Pathology

Several regulatory agencies have suggested the monitoring of continuous variables, including hematology, clinical chemistry, urinalysis, and organ weights (128, 260). It must be realized that, in spite of initial randomization, aged rodents are no longer homogeneous because of nonrandom attrition

and development of diseases in the latter portion of the in-life phase. Although

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such measures may be helpful at times, they need not be implemented without a specific reason; for example, hematology smears assist in the diagnosis of leukemia.

Anatomic Pathology

Pathology is not only an integral part of the protocol design, but plays a pivotal role throughout the conduct, evaluation, and interpretation of carcinogenicity studies (16), as discussed in Good Laboratory Practice. In general, emphasis is placed on routine methods, but special pathology methods may be required depending on the target organs. Whatever can be anticipated from previous subchronic and chronic studies should be utilized in the protocol for pathology. Furthermore, the pathologist must correlate clinical observations, body weight gain and survival patterns, clinical pathology data, and other data with gross and microscopic changes. The general and specific GLP and statistical considerations are given in separate sections elsewhere in this chapter.

All animals euthanized for humane reasons and those found dead should be submitted to a complete necropsy. At scheduled necropsies, the pathology team should be prepared for potential outcomes by participating in a prenecropsy briefing, where all known clinicopathological correlations are discussed. At necropsy, body weights are obtained. Examination of all recorded palpable masses constitutes an initial procedure with examination of all body orifices and skin. A ventral midline incision, with reflection of the skin so that subcutaneous tissues are exposed, initiates the opening of the abdominal cavity, followed by the thoracic and finally the cranial cavities. All gross lesions are described as to their location, size, shape, consistency, and color. In general, organs should be examined in situ as well as after removal from the animal. Any artifactual tissue damage (e.g. crushing or tearing of tissues) must be avoided, but if this is not possible, damage should be minimized and noted. Tissues should be cleaned by rinsing in physiologic saline solution (tap water is not acceptable because the low osmolarity damages cells). Alimentary-tract hollow organs (because of their rapid rate of autolysis) are opened to avoid tissue autolysis and examined (their anatomic integrity if possible should be maintained). Lungs are instilled (with 10% neutral buffered formalin, ~4 ml in rats and ~2 ml in mice) with care not to overinflate, and if indicated, also the urinary bladder (with formalin ~0.2–0.5 ml), after which the trachea and urethra, respectively, are ligated to maintain the inflated state. Lesions/neoplasms are dissected to include regional lymph nodes, if possible, and a small portion of surrounding (normal) tissue. At least 55 standard tissues and lesions are sampled, trimmed, and pro cessed for histopathological examination, including brown adipose tissue, blood and bone marrow smears (28, 79, 163, 207). Any significant deviation from any of these procedures amounts to censoring, which potentially compromises the integrity of the study (see GLP and statistical analyses sections). Organ weights are usually not taken in carcinogenicity studies because of variability of weights caused by disease, neoplasms, or body-weight fluctuations. In some cases selected organs are weighed (260, 261), usually the adrenals, brain, heart, kidneys, liver, lungs, spleen, testes (with epididymides), and uterus (including horns). Where organ weights are taken, organ-to-body-weight percentage values are recommended (79).

The preparation of routine microscopic slides should be in accordance with a standard operating procedure (SOP). Each slide should be matched with blocks and routinely taken tissues or grossly observed findings. Special methods (e.g., quantification of proliferating cell nuclear antigen [PCNA]), compatible with formalin fixation, should be either described in detail in the protocol or be part of an SOP. Recently, these methods have shown utility, since they are capable of identifying proliferation (PCNA) and preneoplasia and early neoplasia (112, 294). During microscopic examination, an "open" slide evaluation is recommended (107, 108, 186). It consists of evaluation of the concurrent control group(s) first and, subsequently, the highexposure groups. After this the rest of the exposure groups are evaluated and the presence or absence of an exposure response pattern is established. Lastly, the findings can be compared to in-house historical or published control data to minimize subjectivity and diagnostic drift. "Open" evaluation is preferably conducted by one pathologist. If the study entails more than 1000 animals, then one pathologist may read the males and a second the females, to reduce the length of time to complete the histopathologic evaluation. "Open" evaluation is also performed when quantitation is performed (e.g., PCNA). Under certain circumstances, a re-evaluation of certain tissue-specific lesions is needed. Then a "blinded" microscopic examination of selected target tissues may be performed, in which all slides are reevaluated by the pathologist in a blinded manner in a random sequence. Also, the valuable practice of peer review can be utilized. This consists of an independent examination by a second pathologist of all tissues from a representative sampling of randomly selected animals of both genders from the control and high-dose groups and a representative sampling of proliferative lesions to substantiate the data from the initial evaluation for neoplasms and other proliferative lesions. Here it is also recommended that the evaluation be "open," and that all changes

(not only microscopic) are taken into consideration.

It is vital that all pathology data are recorded in a consistent manner and are depicted as individual data in appendices. A summary of all exposure-related data should be in tables. An appendix depicting all missing tissues is highly desirable. Neoplastic diagnoses should adhere to an accepted nomenclature of histopathologic terms, according to recommendations made by the Society of Toxicologic Pathologists (Guides for Toxicologic Pathology, STP/ARP/AFIP, Washington, DC), with particular care to distinguish between proliferative non-neoplastic lesions, benign neoplasms, and malignant neoplasms (65, 66, 88, 90, 164). The pathologist must integrate all clinical, structural (gross and microscopic), and functional (cellular and biochemical) data. Moreover, the pathologist ensures proper accounting of gross and microscopic lesions and changes, as detailed in the sections covering Good Laboratory Pratice and statistical analyses. In pathology, the most common deficiencies in Good Laboratory Practice are as follows: (a) Gross observations are not fully available and the exposure related ones are not compared side-by-side with microscopic findings; (b) gross microscopic pathology data do not match and no explanation is provided; (c) organ weights in notebook and report do not match; (d) termination dates before completion of the study are not in the final report and no explanation is given; (e) differences between forms of data recording by the pathologist and the SOPs are not explained; (f) individual animal data in the notebook and data in tabular form in the report on the corresponding animal are not the same and no explanation is provided; (g) there is a lack of uniformity in pathology nomenclature and no explanation is provided; (h) there is lack of lesion accountability and important tissues are missing without any explanation being given; (i) the method of slide evaluation is not stated (e.g., open, peer-reviewed, etc.); and (j) lack of initialing and dating in various records such as gross data, tissue trimming, microscope evaluation, tissue recuts, and so on. Each of these is important and, if no explanation is provided, can compromise the RGB.

RODENT CANCER BIOASSAY EVALUATION

Tumor Increases or Decreases

If the RGB has been conducted properly, it should provide adequate evidence to assess whether exposure has lead to increases or decreases in the incidence of specific well-defined neoplasms. A conclusion of lack of carcinogenic activity requires survival of adequate numbers of animals given sufficient relevant exposures, usually the MTD, with no evidence of tumor increases according to the criteria discussed in the section on chemicals with carcinogenic activity.

A conclusion for a positive outcome is generally less rigorous. That is, in the interest of conservative hazard identification, a clear increase in tumors is often accepted as valid in spite of artifacts in the study such as excessively reduced weight gain or poor survival. Nevertheless, the possibility must be considered that the tumor increases are a consequence of the toxicity of the treatment conditions (89), and not a chemical action of the TS.

A critical aspect in the evaluation is the interpretation of pathological diagnoses. Findings of increases in malignant neoplasms are universally accepted as evidence of carcinogenic activity. Combining malignant and benign neoplasms of the same cell type of origin is also widely accepted, and guidelines have been published for the NTP procedures (164). In evaluating tumor increases, attention must be paid to pathology in the tissue that is the site of tumors because cell injury and compensatory cell proliferation can facilitate tumor development (see the section on cancer hazard and risk assessment).

The most appropriate comparison of an experimental group is with its matched control or controls, where two groups are used (83). Nevertheless, in arriving at a conclusion of tumor increase, it is important to consider historical control data (98), especially if control groups differ. Differences between controls and exposed groups can be analyzed by a variety of statistical methods, as discussed next. Whether any true increases in neoplasms have relevance for human cancer hazard is discussed in the section on cancer hazard and risk assessment.

A controversial issue is the interpretation of tumor decreases in the RGB (45), which are quite frequent (101). One consideration is that the reduction should not be attributable to nonspecific weight gain suppression, as discussed in the feeding procedures section. A variety of mechanisms for specific anticarcinogenesis have been delineated, as discussed in the section on anticarcinogenesis.

Statistical Analyses

An expert biostatistician should be involved in the RGB. The methods ultimately employed can be diverse and only some general guidance is provided here. More extensive coverage is provided in Chapter 7.

Statistics are employed in all aspects of the study, that is, design, conduct, evaluation, analysis, and interpretation. The object of statistics is to aid an investigator in the interpretation of RBC results by providing a quantitative measure (p value) of the likelihood that an increased tumor incidence is due to

random variability. Other than exposure, two other factors can underlie an increase in neoplasia: One is bias, which is a systematic difference other than what is caused by exposure, and the other is chance, a random difference. It is indeed

highly desirable to avoid bias and to minimize (and control) chance, although it cannot be completely excluded (i.e., identically exposed animals do not respond identically—the biological reality). The probability of chance can be statistically measured: the smaller the probability, the higher the confidence (152). A further complicating factor is that the multiplicity of tumor sites examined in a typical RGB increases the likelihood that an increased tumor incidence at any single given site is due to chance.

A one-sided (one-tailed) *p* value, is the probability of getting by chance an exposure effect in a specified direction as great as or greater than that observed (68). A two-sided (two-tailed) *p* value is the probability of getting by chance an exposure difference (effect) in either direction that is as great as or greater than that observed. The calculation of a *p* value of <0.05 implies that the effect could have occurred by chance less than 1 time in 20. Theoretically at least, randomization eliminates nonsystematic bias. But if there is another major source of variation (e.g., gender of the same strain, or batch of the same strain), then a stratified randomization is more appropriate. To achieve that, separate randomization within each level of the stratifying variable (e.g., cage position, order of weighting, or order of killing at termination) must be performed (152). The power of any statistical measurement refers to the probability that the subject test will correctly detect a difference, when such a difference truly exists (80).

There are three aspects that determine the nature of collected data: the biological system, the study design instrumentation, and methodologies applied. Censoring any of these must be minimized. In general, exposure variables are independent, while effect variables are dependent. (80, 152). Representative samples should be appropriately collected and be of sufficient size. In addition, samples should be accurate (of high quality) and precise (reproducible). The pattern of distribution of data in the sample is very important, because it shows their central tendency and dispersion. There is the Gaussian distribution (the most common), in which two-thirds of all values are within one standard deviation. Others include binomial and chi-square patterns of distribution. In order to successfully combine data for analysis, stratification is applied (e.g., early, late, or total deaths).

In order to enhance detection exposure effects, the exposure-related trend is employed (233). Furthermore, if there are differences in survival, there is a need for age adjustment to avoid bias. Certain statistical procedures used in the analysis of tumor data require specification of the context of observation is taken into account—that is, whether the condition (neoplasm) is assumed to have caused death, or death was an incidental finding (195). If the study pathologist concludes that it is not possible to determine the context of observation, then alternative methodology must be used (78). Because of the multiplicity of tumor sites examined it may also be necessary in certain cases to employ a statistical adjustment for multiple comparisons.

When animal weights differ across dose groups, as is usually the case with high-dose testing, such differences can contribute to differences in tumor incidence (see feeding procedures). Analyzing tumor incidence within body weight strata can reduce the bias resulting from weight differences (84).

In the past, the single most important statistical consideration in the analysis of bioassays was a simple quantal response: that is, either neoplasia did occur or it did not occur. Presently, the mechanisms underlying neoplasia induced by chemicals are more fully understood and must be given individual consideration. These mechanisms include effects on survival rate, body weight gain, age at first tumor, time-to-tumor, patterns (trends) of tumor incidence, tumor multiplicity, rates of proliferation at target sites, presence of markers of preneoplasia or early neoplasia, and exposure response. As recommended in the study design section, at least one interim kill should be included, and possibly also a short (1–3 months) recovery segment before final termination.

In an RGB, the time course of adverse effects (or risks) is of importance. Consequently, life-table methods are employed to compare survival curves and/or survival time until neoplasms develop (40, 41, 195). For continuous, normally distributed outcome measures, group means are typically compared against the control mean using a one-way analysis of variance (ANOVA) followed by Dunnett's method for multiple comparisons, which is a powerful post hoc test (54). Moreover, a square-root transformation may be necessary in some cases to stabilize the variance. To make all possible pairwise comparisons, Tukey's multiple-comparison procedure may be used (72). To assess the course of exposure response (its linearity), ordinary least-squares regression analysis can be used, fitting the outcome level versus exposure and squared exposure terms. For incidence of specific site neoplasia comparing all test groups, the Pearson chi-square test followed by pairwise comparisons of each exposure group with control, adjusted for multiple comparisons, can be applied (77). For incidence trend analysis, the Cochran-Armitage test, partitioning the chi-square statistic into the overall trend and departure from linearity (*p* nonlinear), can be tested (71). Furthermore, survival data can be used by applying log-rank test for

both homogeneity and exposure-related trend (96, 97). Neoplastic data can be analyzed using a survival-adjusted trend test discriminating fatal, incidental, and palpable neoplasms (195). For rare neoplasms, a *p* value of <0.025 and for common neoplasms

Table 20.8 Classification of carcinogens

IARC	HWC	EPA	IFSTP
Group 1	Group I	Group A	Group 1
Group 2A	Group II	Group BI	Group 2a
Group 2B	Group III	Group 82	Group 2b
—	—	Group C	—
Group 3	Group IV	Group D	Group 3 a
—	—	—	Group 3b
—	—	—	Group 3c
Group 4	Group V	Group E	—

Note: IARC=International Agency for Research on Cancer, (116). HWC=Health and Welfare Canada, (103). EPA=U.S. Environmental Protection Agency (250). IFSTP=International Federation of Societies of Toxicologic Pathologists, Faccini et al. (66).—, not applicable. Group I=Group I=Group A=Group I=The agent is carcinogenic to humans; there is sufficient evidence in man showing a positive relationship between cancer and human exposure; chance, bias, and confounding variables can be reasonably ruled out. Group 2A=Group II=Group B1=Group 2a=The agent is probably carcinogenic to humans; there is limited evidence in man showing a positive relationship between cancer and human exposure; but chance, bias, and confounding variables cannot be ruled out; there is sufficient evidence of carcinogenicity in animals, i.e., the genotoxic agent is sufficiently in strong evidence of carcinogenicity in animals, i.e., the agent has caused benign and malignant neoplasms in two independent studies or there is a positive relation between cancer and animal exposure. Group 2B=Group III=Group B2=Group 2b=The agent is possibly carcinogenic to humans; there is either limited evidence or absence of data in man; there is either sufficient or limited and weak evidence of carcinogenicity in animals, i.e. presence of other relevant data, genotoxic agents that cause only benign tumors or increases in certain spontaneous neoplasms; Group C=The agent is possibly carcinogenic to humans, there is either absence of data in man, or limited evidence of carcinogenicity in animals, i.e., agents that cause only benign tumors, or neoplasm incidence increases are marginal and not consistent; Group 3=Group IV=Group D=Group 3a=The agent is not classifiable as to its carcinogenicity in man; in Group V the data are inadequate for evaluation, or these agents cannot be classified in other groups; in IFSTP Group 3a, the experimental data of epigenetic carcinogens show threshold level within the range of human exposure. Group 3b=The experimental data of epigenetic carcinogens show threshold level beyond the range of human exposure. Group 3c=The experimental data of epigenetic carcinogens show that their mechanism of action is not applicable in humans. Group 4=Group IV=Group E=The agent is probably not carcinogenic to humans; there is evidence suggesting lack of carcinogenicity in man (even if it is inadequate) and animals (negative animals studies); in IFSTP Group 4, the suspected carcinogens have not been sufficiently tested.

a p value of <0.05 are appropriate levels of significance (Table 20.7) (96).

The interpretation of analyzed data is the final critical step of the whole process. Of importance here is the existence of extensive historical control data, both published and unpublished, for the specific species and strains used (97, 99, 147, 189). The operational concepts here involve the biological and statistical data differences between control and exposed groups. Important here is the nature of these differences and the main reason for the differences. The final interpretation should be based on both biological and statistical consideration (66, 265). If a statistical test falsely detects a significant neoplastic effect when none truly exists, a false positive outcome is the result. This constitutes a type 1 error. Conversely, if a statistical test fails to identify a true biological effect (e.g. a small increase in the incidence of a rare tumor), then a type 2 error has occurred. In both cases, by providing an explanation for the differences (causality) and demonstrating the proof for the underlying mechanism, both types of error are minimized. Statistical considerations are very important, but they should not be a substitute for sound biological judgement.

Finally, any statistical evaluation of RCB data should take into account the following eight factors: (a) exposure-effect relationship; (b) incidence of proliferation and preneoplastic and early neoplastic markers at the target site of neoplasia; (c) presence of gender and species similarities or differences at the target sites; (d) convergence in target sites of nonproliferative chronic toxicity and neoplasia; (e) combined neoplasia increases in tissues affected by chronic toxicity; (f) neoplasms of similar histogenetic target sites in other genders or species; (g) concurrent and historical control data, and (h) relative survival of control and exposed groups.

BIOASSAY REPORTING

The final critical step of the RCB is the study final report. The final report consists first of an introductory section containing the compliance statement signed by the study director, followed by the quality assurance and study identification statements. The study identification statement contains the study title and number, the test substance, the testing facility, the sponsor, the study director, and the principal investigator of all study aspects, the exact specific study timetable, and approved signatures from all final report authors. This should include the study director and the investigators of all aspects of the study (e.g., analytical, toxicokinetic, duringlife, pathologist, etc.). The first part of the final report itself consists of the summary. This is an abstract of the entire study. It contains, in this order, an introduction, a listing of the materials and methods, the results, and the conclusions. The summary is followed by a summary table that depicts all pertinent findings in tabular form.

After the summary section, there is an extensive introduction into the origin and purpose of the RGB, followed by listings of test animals, test materials, methods, results, discussion, conclusions, and references. All sections should fully describe all methods used and all data obtained. All individual data should be in appropriate appendices (e.g., analytical, body weight, necropsy, and microscopic data). All relevant summary data should be in tables (e.g., analytical, body weight, etc.). Numerical incidences should precede percent incidences. Tables and graphs presenting special issues and arguments are recommended in the text (text tables or text graphs). Appropriate statistical analysis of correlation of survival patterns, clinical observations, body weight gain pattern, and toxicokinetic data with gross and microscopic

findings should be conducted. All of these considerations have been discussed in other sections in detail. An effective way of summarizing the findings is a format used in Europe known as the tabulated study report (TSR). All relevant data are presented in standardized tabular form without narrative. This corresponds to the tables of the final report described here.

CLASSIFICATION OF EVIDENCE OF CARCINOGENICITY

Completed RGBs must be reported to the regulatory agency under whose purview they were performed (33, 48, 63, 255). The results are then subject to evaluation and classification.

In the United States, the results of RGBs on Pharmaceuticals tested under Investigational New Drug applications (INDs) approved by FDA Center for Drug Evaluation and Research (CDER) are submitted to the Reviewing Division, which then evaluates them often through the CDER Carcinogenicity Assessment Committee (CAC). The final interpretation of the results will appear in the labeling of the medicine, if approved. The FDA normally describes the RCB data without comment on human relevance, except to note multiples of exposure in the rodents compared to humans. The CAC consists of a chair, an executive secretary, and members from several divisions: The Office of Epidemiology and Biostatistics, the Office of Testing and Research, and the Office of Pharmaceutical Sciences. The Reviewing Division (of the FDA) notifies the sponsor when a CAC meeting is scheduled after all RCB studies are submitted.

The U.S. Environmental Protection Agency (EPA) has been using an alphabetical/numerical classification ranging from group A, human carcinogen (based on animal data), to group E, noncarcinogen (192, 255, 258), but has proposed to convert to a narrative classification (257, 258), which allows incorporation of mechanistic data, similar to the International Federation of Societies of Toxicologic Pathologists (IFSTP) classification, described later. At the U.S. EPA, an ad hoc CAC of the EPA Science Advisory Committee evaluates the submitted dossier.

RGBs conducted by the NTP are reviewed by a peer review panel and published as technical reports, which are submitted to the National Institute of Environmental Health Sciences (NIEHS) and subsequently to the U.S. EPA, FDA, and OSHA for regulatory action (255, 256). The NTP uses a classification system of no, limited (some), or clear evidence of Carcinogenicity (183). The NTP also publishes a biennial report on carcinogenesis, the most recent of which appeared in 2000 (249a).

In Europe and Japan, similar classification schemes are used by various health boards and the Commission for Proprietary Medicinal Product of the European Medicine Evaluation Agency (48, 55, 62, 64).

The International Agency for Research on Cancer (IARC) convenes working groups several times each year to evaluate groups of chemicals with published Carcinogenicity data. The findings are published as monographs with evaluations of the experimental and human data. The grouping ranges from group 1, carcinogenic to humans, to group 4, evidence suggesting lack of Carcinogenicity.

The IFSTP (66) has proposed a classification as follows: 1, carcinogens for man based on epidemiological data; 2, genotoxic carcinogens for animals based on experimental data; 3, epigenetic carcinogens for animals based on experimental data; and 4, suspected carcinogens insufficiently tested. This is the only classification that explicitly incorporates mechanistic distinction.

Some of the classification schemes are depicted in Table 20.8. The IARC reviews are the most comprehensive, since they include all chemicals with published reports, and review all chemical and biologic data relevant to risk assessment.

CANCER HAZARD AND RISK ASSESSMENT

In cancer risk assessment, the first step is hazard identification, which involves the RCB to identify exposure-related tumors (see the section on rodent cancer bioassay evaluation). Using dose-response data from the bioassay and potential human exposure, a cancer risk is assessed (180), often involving allomorphic scaling (253). To identify a potential human cancer hazard, as for example following the IFSTP recommendations, the RCB results must be interpreted together with other mechanistic data. If the agent is clearly genotoxic, that implies a potential hazard (279), as discussed later in the section on types of cancer hazards. On the other hand, as discussed earlier, it is now recognized that epigenetic carcinogens may elicit their effects only in particular rodent species, as for $\alpha 2\mu$-globulin nephropathy inducers, or only at high toxic doses, as for nitrilotriacetic acid. Such effects are considered irrelevant to human hazard (252) or can be subjected to a margin-of-exposure (MOE) risk assessment (256, 258). The mechanism of carcinogenesis for epigenetic (nongenotoxic) agents is complex, involving a variety of secondary organ and tissue target sites, with indirect interference with the organ/tissue homeostasis. Disruption of endocrine, paracrine, nervous, and immune systems is often involved in the pathogenesis of neoplasia induced by such agents. Accordingly, the carcinogenetic effects of these agents are species, gender, and tissue specific.

Table 20.9 Examples of neoplastic effects in rodents with limited significance for human safety

Neoplastic effect	Pathogenesis (agents)
Renal tubular neoplasia in male rats	*α*2μ-Globulin nephropathy/hydrocarbons (*d*-limonene, *p*-dichlorobenzene)
Hepatocellular neoplasia in rats and mice	Peroxisome proliferation (clofibrate, phthalate esters, phenoxy agents) Phenobarbitol-like promotion
Urinary-bladder neoplasia in rats	Crystalluria, carbonic anhydrase inhibition, urine pH extremes, melamine, saccharin, carbonic anhydrase inhibitors, dietary phosphates
Hepatocellular neoplasia in mice	Enzymatic-metabolic activation (in part unknown)/phenobarbital-like promotion
Thyroid follicular-cell neoplasia in rats	Hepatic enzyme induction, thyroid enzyme inhibition/oxazepam, amobarbital, sulphonamides, thioureas
Gastric neuroendocrine-cell neoplasia mainly in rats	Gastric secretory suppression, gastric atrophy induction (cimetidine, omeprazole, butachlor)
Adenohypophysis neoplasia in rats	Feedback interference/neuroleptics (dopamine inhibitors)
Mammary-gland neoplasia in female rats	Feedback interference/neuroleptics, antiemetics, antihypertensives (calcium channel blockers), serotonin agonists, anticholinergics, exogenous estrogens
Pancreatic islet-cell neoplasia in rats	Feedback interference/neuroleptics
Harderian-gland neoplasia in mice	Feedback interference/misoprostol (PGEi), nalidixic acid, aniline dyes
Adrenal medullary neoplasia in rats	Feedback interference (lactose, sugar alcohols)
Forestomach neoplasia in rats and mice	Stimulation of proliferation/butylated hydroxyanisole, phthalate esters, proprionic acid
Lymphomas in mice	Immunosuppression/cyclosporin
Mononuclear-cell leukemia in rats (mainly F344)	Immunosuppression (in part unknown)/furan, iodinated glycerol
Splenic sarcomas in rats	Methemoglobinemia (in part unknown)/dapsone
Osteomas in mice	Feedback interference (calcineurin, in part unknown)/cyclosporin, misoprostol, proestrogens
Leydig-cell testicular neoplasia in rats	Feedback interference/lactose, sugar alcohols, H2 antagonists, carbamazepine, vidarabine, isradipine, dopaminergics, finasteride
Leydig-cell testicular neoplasia in mice	Feedback interference (proestrogens, finasteride, methoxychlor, cadmium)
Endometrial neoplasia in rats	Feedback interference (proestrogens, dopamine agonists)
Uterine leiomyoma in mice	Feedback interference (*β*1-antagonists)
Mesovarial leiomyoma in rats (occasionally in mice)	Feedback interference (*β*2-agonists)
Ovarian tubulostromal neoplasia in mice	Feedback interference (cytotoxic agents, nitrofurantoin)

Most of these secondary mechanisms involve interference with proliferation, disruption of hormonal feedback pathways, inhibition of the trophic activity in tissues including long-standing tissue ischemia, immune surveillance dysfunction, sustained exaggerated pharmacological effect, inhibition of enzymatic reaction/ activation in cells, modulation of apoptosis, and sustained accumulation of normally low occurring level of endogenous products. All these effects result in sustained cellular toxicity, leading to compensatory proliferation, which is a common pathway through which agents with diverse cellular effects ultimately induce neoplasia (31, 38, 112). The effects that lead to compensatory cellular proliferation usually require high levels of exposure and exhibit thresholds. It is perhaps for this reason that of the NCI/NTP rodent carcinogens tested, 6% had increased tumor rates that were limited to the top dose for all sites of carcinogenicity (100). Examples of neoplastic effects with limited significance for human hazard are given in Table 20.9, and some mechanisms and tumor findings are described in more detail next.

Mechanisms Not Indicative of Cancer Hazard to Humans

For several neoplastic responses in rodents, sufficient mechanistic information has accrued to support the general conclusion that the underlying mechanisms for agents that are not DNA reactive in the target tissue are species specific and do not operate in humans.

Rat Kidney **α2μ**-*Globulin Nephropathy-Mediated Increases in Kidney Neoplasms*

Various xenobiotics induce kidney tumors in male rats, mainly F344, which excrete α2μ-globulin in the urine. This protein is associated with hyaline droplet formation, atypical hyperplasia of the epithelium of the P2 segment of the proximal tubules and neoplasia. Male rats (especially F344) are very proteinuric compared to humans, and no human renal protein is similar to α2μ-globulin (252). Accordingly, the U.S. EPA (252) concluded that renal tubule tumors produced as a result

of the α2μ-globulin accumulation mechanism are not an appropriate endpoint for human hazard identification. Likewise, an IARC working group concluded that an agent that acts solely through α2μ-globulin nephropathy in the production of renal cell tumors alone in male rats is not a cancer hazard to humans (204).

Rat Stomach Acid Secretion Suppression-Mediated Neuroendocrine Neoplasm (Carcinoid)

Hyperplasia and neoplasia of gastric neuroendocrine cells (enterochromaffin-like cells) are stimulated by gastrin in rats and to a lesser degree in mice. Elevations of gastrin are elicited by reduced hydrochloric acid production, which can be caused either by gastric anti-secretory medicines such as proton pump inhibitors (lansoprazole, omeprazole, pantoprazole) or H2 antagonists (loxtidine or cimetidine) (18, 170). Agents that cause gastric atrophy—for example, alachlor and butachlor—have also elicited this neoplasm (235). Rats have a high density of gastric neuroendocrine cells (NE), achieve high levels of gastrin (over 1000 pg/ml), and are very responsive to elevation of gastrin (241). With most of the agents, female rats are more susceptible than males.

NE cell tumors have been observed in patients with multiple endocrine neoplasia syndrome (MEN-1), associated with elevated gastrin, but not with antiulcer therapy (170). Significant NE cell proliferation in humans is seen only with gastrin levels above 400 pg/ml, and this can be controlled.

Rat Urinary Bladder Luminal-Milieu-Modification-Based Increases in Transitional Cell-Neoplasms

Many studies have used rat models for urothelial neoplasia. Rats have been shown to be more sensitive than mice to urothelial damages, apparently because the rat bladder lacks tight junctions, rendering the superficial urothelial layer ineffective as an intraluminal barrier, leaving the underlying layers vulnerable to chronic stimulation (110, 144). Moreover, rats have more intraluminal proteins, silicate precipitation, crystal formation, and urolithiasis than humans. In particular, rats, unlike humans, develop calcium phosphate urinary precipitates (204). Thus rats are more prone to chronic cell damage to the bladder urothelium, which results in cell proliferation and neoplasia (31). This effect does not occur in humans (31, 57, 291). An IARC working group has concluded that production of bladder cancer in rats under conditions of formation of calcium phosphate-containing urinary precipitates is not predictive of cancer hazard to humans (204).

Rodent Liver Peroxisome Proliferator-Mediated Increases in Liver Neoplasms

Widely varied xenobiotics elicit increases in rodent liver tumors associated with increased numbers of per oxisomes (120). Rodents are more susceptible to induction of hepatic peroxisome proliferation than primates or humans (120, 290), apparently because of high expression of the peroxisome proliferator activated receptor of the class (PPARα) in rodent liver (242). Perhaps related to this, it has been reported that in rat hepatocytes, peroxisome proliferators enhance DNA synthesis and suppress apoptosis, whereas in human hepatocytes, DNA synthesis was suppressed and apoptosis enhanced (194). While the mechanisms of carcinogenicity of these agents is not fully understood, none is associated with cancer in humans, and an IARC group has recommended that a tumor response in mice or rats secondary only to peroxisome proliferation could modify the evaluation of carcinogenicity (120).

Rodent Thyroid-Pituitary Disruption-Mediated Thyroid Tumor

Few DNA-reactive carcinogens elicit thyroid tumors, probably because bioactivation is minimal in this gland. On the other hand, thyroid-pituitary disruption is a common mechanism of thyroid carcinogenesis in rodents, particularly rats (236). Reduced thyroid hormone levels, through either inhibition of synthesis by antithyroid agents (e.g., amitrole) or increased clearance as a result of enhanced conjugation (e.g., phenobarbital), can lead to feedback increase of thyroid stimulating hormone levels, which produce thyroid follicular-cell hypertrophy, hyperplasia, and eventually neoplasia. Species differ in their susceptibility to this disruption, with the rat being particularly sensitive (59). Several inducers of liver thyroid hormone conjugation (which often is associated with increased liver tumors) in rats do not affect mice (270). No nonradioactive chemical exposure is known to cause thyroid follicular neoplasms in humans (204). Accordingly, it has been concluded that chemical-specific data on thyroid effects in rodents can be applied to risk assessment (105), and that agents that cause thyroid neoplasia through an adaptive hormonal mechanism belong to a different category from those acting through genotoxic effects or involving pathological response to tissue injury (204).

Mechanisms Probably Not Indicative of Cancer Hazard to Humans

Several mechanisms of epigenetic tumorigenesis in rodents appear not to be relevant to human cancer hazard (6).

Rat Testes Hormone Disruption-Mediated Leydig-Cell Neoplasms

Leydig- or interstitial-cell neoplasms occur spontaneously in high incidence (>80%) in aged F344 rats

(102). These tumors are invariably benign. The human counterpart is extremely rare, and no agent that produces increases in rat testicular tumors (i.e., cimetidine, hydralazine, gemfibrozil, carbamazepine, vidarabine, isradipine, exogenous gonadotropins, luteinizinghormone releasing hormone [LHRH] analogs, flutamide, ergolines, and finasteride) has been associated with induction of this or any other neoplasm in humans. Therefore, the data suggest that nongenotoxic compounds that induce Leydig-cell neoplasms in rats do not indicate a human cancer hazard (36).

Rodent Hormone Disruption-Mediated Mammary and Adenohypophysis Neoplasia

The occurrence of mammary neoplasms and prolactinomas in female rats (mainly) and mice after exposure to neuroleptics, antiemetics, antihypertensives, calcium channel blockers, serotonin agonists, exogenous estrogens, or anticholinergics is species specific (185, 217, 277, 289). The triggering mechanism for this consists of sustained prolactin (PRL) elevation. PRL is controlled by dopamine. In contrast to its action in humans, PRL is luteotrophic in rats and mice, leading to progesterone elevation and sustained stimulation of proliferation of mammary epithelium (209). These neoplasms are accompanied by an increase in serum PRL, contrary to mammary neoplasia in humans (208, 220).

Tumors of Questionable Significance to Human Cancer Hazard

Tumors of questionable significance for hazard assessment (6) are those whose pathogenesis may be unique to rodents, in some cases due to their cell type of origin, and for which no association with human cancer hazard has been established. If one of these is the only tumor increased in the RGB, such a finding should not be taken as evidence of human cancer hazard.

Mouse Bladder "Mesenchymal Lesion"

This lesion occurs in the trigone area of the bladder and has been known under various names for sometime (133). Recently the lesion was called "mesenchymal tumor" (20, 92). The lesion has been found in mice given agents that bind to progesterone receptors (165). Persuasive evidence has been provided (133, 134) that the lesion represents a decidual reaction of mesenchymal cells carrying or developing progesterone receptors. No known counterpart of this lesion has ever been described in humans, and therefore its significance is questionable.

Mouse Histiocytic Sarcoma

This neoplasm of histiocytes affects mainly the liver and uterus in mice. A comparable lesion has not been reported in humans and no agent established to produce an increase in only this neoplasm is associated with cancer development in humans (244).

Mouse Ovary Tubular Adenoma

This is a benign neoplasm with tubular, stromal, or mixed components, and occurs mainly in mice (5, 24). Tubulostromal adenomas have been observed with cytotoxic agents but are not seen in other laboratory animals or humans and are considered irrelevant for human safety assessment (5, 24).

Rat Granular-Cell Tumor

A proliferative lesion of "granular" cells with granular eosinophilic cytoplasms occurs in the vaginal-cervical regions of female Sprague-Dawley, Donryu, and Wistar rats (39, 218). This lesion is probably under hormonal influence, mainly estrogen. Granular cell aggregates occur rarely in the vulva of women, but there is no evidence to suggest that the pathogenesis is similar to that of rat granular-cell tumors. Thus, this lesion is considered probably not relevant for humans.

Rat Mammary-Gland Fibroadenoma

This is a benign neoplasm with a minor glandular epithelial component and a predominant pericanalicular type of proliferation of connective tissue. It bears no resemblance to the common intracanalicular type of fibroadenoma seen in women (208, 209), which is hormonally responsive (10). Fibroadenoma is the most common breast neoplasm in all the major rat strains and does not progress to malignancy. Thus, combining fibroadenomas and carcinomas is inappropriate and the tumor by itself is of questionable significance.

Rat Mesovarial Leiomyoma

Smooth-muscle tumors of the ovarian suspensory ligament have developed in female rats after long exposures to beta2-adrenoceptor stimulant medicines (138). This neoplasm is rare in humans, and the agents that have induced it in rats (i.e., soterenol, mesuprine, zinterol, terbutaline, reproterol, salbutamol) are not associated with cancer in humans.

Rat Mononuclear-Cell Leukemia

This neoplasm occurs in high incidence (about 60% in males and 40% in females) in F344 rats (102). Mononuclear-cell leukemia (MCL), also referred to as large granular lymphocyte leukemia (LGL), is a spontaneously occurring lethal neoplasm that first develops in the spleen and then in the liver, lungs, lymph nodes, and bone marrow. It occurs at over 18 months of age. No agent

has been demonstrated to reproducibly induce this neoplasm and hence nothing is known about its pathogenesis. MCL is induced by DNA-reactive agents such as elthylene oxide. In addition, a number of chemicals that are not implicated in human cancer (furan, C.I. direct blue 15, iodinated glycerol, diisononyl phthalate, and dimethylmorpholinophosphoramidate) are known to be associated with increased incidences (58, 155).

Rodent Forestomach Squamous-Cell Carcinoma

A number of DNA-reactive agents have induced neoplasms of the forestomach in rodents (146), usually through a direct effect. The rodent forestomach is a portion of the stomach between the esophagus and glandular stomach, lined by squamous epithelium, and does not exist in humans. Nongenotoxic agents such as butylated hydroxyanisole, propionic acid, and HMG CoA-reductase inhibitors have produced increases in this neoplasm. The epigenetic mechanism appears to involve chronic irritation leading to a promoting action, which requires high exposure, as shown for butylated hydroxyanisole (291, 293). None of these epigenetic agents has been associated with cancer in humans.

Flat Scrotal Tunica Vaginalis Mesothelioma

This mesenchymal lesion, which includes hyperplasia and neoplasia (166), arises from the serous membranes of the scrotal tunica vaginalis testes. It is common (about 3%) in F344 rats, (102). Since the scrotal lesion often arises in association with testicular tumors, especially Leydig-cell tumors, which assume large size, there may be an element of physical initiation involved in the pathogenesis. Chemicals that produce increases in this tumor (e.g., acrylamide, potassium bromate, pentachlorophenol) are chemically diverse and no mode of action has been established. None of these is associated with mesothelioma in humans or with any other cancer.

Types of Cancer Hazards

Formerly, all rodent carcinogens were considered to be potential human cancer hazards. This concept was embodied in the Delaney clause to the 1958 Federal Food, Drug, and Cosmetic Act, section 409(3)(A), which provided that no chemical determined to be carcinogenic in either humans or animals could be allowed as a food or color additive, regardless of concentration. Likewise, the U.S. EPA cancer principles of 1970 stated that no level of exposure to a chemical carcinogen should be considered toxicologically insignificant for humans. Subsequently, expanded understanding of mechanisms of carcinogenesis has led to refinements of hazard assessment. Notably, in Europe, governmental agencies have not been required by legislatures to impose standards of no exposure for carcinogenic agents and have used more flexible approaches than those imposed in the United States (278). Beginning in 1992, the I ARC accepted data on mechanisms as being relevant to evaluation of the carcinogenic risk of an agent to humans (118), and this is being elaborated upon (204). Recently, the distinction between DNA-reactive and epigenetic carcinogens has been explored in detail by an international group of experts, drawing on comprehensive reviews of mechanisms of 10 prototype carcinogens (126).

Currently, in assessing potential human cancer hazard, regulatory agencies often refer to findings implicating an agent as a "genotoxic carcinogen" usually with only an operational definition (32) that the chemical produced positive results in genotoxicity tests. All chemicals that are reliably positive in a variety of tests are in fact DNA reactive and thus belong to that category of carcinogen, as discussed in the section on types of carcinogens. Most chemicals, however, are positive in some tests, like phenobarbital and DES (116, 123), sometimes because of intrinsic spurious positive results (19), and thus the issue becomes the question of which tests are to be accepted as evidence of genotoxicity. A scientifically sound approach is to define genotoxicity as a mechanism of carcinogenesis; that is, a genotoxic carcinogen is one that forms molecular lesions (such as DNA adducts) that lead to mutations or one that produces mutations in the cells that are the precursors of tumors induced by the agent. Under this definition, genotoxic carcinogens would likely be confined to DNA-reactive agents fulfilling the criterion for carcinogenic activity of an agent that induces malignant tumors not seen in controls. Such chemicals are generally multispecies carcinogens, which induce tumors in high yield with short latent periods and often in several organs. For agents of this type, assumption of human hazard is well founded (279), although evidence is accruing that even DNA-reactive carcinogens have thresholds (294). Moreover, some of the underlying mechanisms of these agents are species specific and do not operate in humans because of functional or toxicokinetic differences. Epigenetic carcinogens, in contrast, are generally not relevant to human hazard, as evidenced by the few associated with human cancer (Table 20.3), in spite of the large number to which humans are regularly exposed (87, 122). The lack of relevance stems from the fact that their effects are either rodent specific or require high and long-duration exposure in rodents in order to elicit the cellular effect leading to carcinogenicity. Epigenetic mechanisms that are either not indicative of a cancer hazard to humans or probably not indicative have

been discussed already. These should be taken into account in formulating any risk assessment.

Cancer Risk Assessment

It is becoming increasingly accepted that for epigenetic agents, even if some cancer hazard is presumed, a safety margin can be established. For plasma concentration versus time pharmaceuticals, this has been referred to as the safety factor, which is the ratio of the area under the concentration vs. time curve (AUC) in rodents for the highest noncarcinogenic dose (nontumorigenic effect level, i.e., NTEL) to the human AUC at the therapeutic dose (12, 32). In 1996, the Federal Food, Drug, and Cosmetic Act was amended by removing the zero-risk provision of the Delaney clause and replacing it with a new standard of "a reasonable certainty of no harm" (33). The new standard applies to pesticide residues in both raw and processed foods, allowing the presence of some residues that have been shown to cause cancer in animals (246, 247). In the new U.S. EPA draft cancer assessment guidelines, the safety margin is referred to as margin of exposure (MOE) (192). The FDA Center for Food Safety and Nutrition has published a "threshold of regulation" procedure for indirect food additives (263, 264). In an analysis of human risk for carcinogenic veterinary drugs present as tissue residues, Galer and Monro (81) argue that human intake of up to 100/ig/person/day presents no cancer risk, similar to the assessments of Williams (282) and Munro et al. (174). In a further refinement, Williams (285) proposed a carcinogen safe exposure level (SEL) as the no-effect level for the molecular/cellular effect that is the basis for carcinogenicity divided by a safety margin, similar to uncertainty factors. This is actually more conservative than using the NTEL, since the NEL for molecular effects is lower.

For genotoxic or DNA-reactive agents, authorities regulate such agents either by prohibiting human exposure or using a linear no-threshold model for quantitative risk assessment (250, 255, 295). In the European Union, it is proposed to incorporate potency considerations, including T25 values, and to classify all carcinogens in three potency groups (55). However, evidence is accruing that even DNA-reactive carcinogens have thresholds (294), and accordingly, the SEL concept just described can also be applied to DNA-reactive carcinogens using a NEL for DNA binding (285).

INTERACTIVE CARCINOGENESIS

Interactive carcinogenesis comprises the enhancement or inhibition of carcinogenesis by combined, concurrent, or sequential exposures to more than one carcinogen or a carcinogen and noncarcinogen (281). Various types of interaction between chemicals have been described, including syncarcinogenesis, tumor promotion, cocarc inogenesis, and anticarcinogenesis. In the testing of complex mixtures, these types of interactions can influence the outcome (Figure 20.4).

Syncarcinogenesis

Syncarcinogenesis is the enhancement of carcinogenesis produced by concurrent or sequential administration of two carcinogens, usually of the DNA-reactive type. This interaction in the case of DNA-reactive carcinogens represents a summation of the genetic effects of the agents. Usually the enhancement occurs in a target organ where both carcinogens produce a tumor effect.

Promotion

Tumor promotion is the enhancement of tumor development by a second agent given after an initiating carcinogen (11), when a sufficient interval has been allowed for acute molecular effects, such as DNA adducts, to be processed. If the second agent is administered when molecular lesions are still present, the enhancement may be due to cocarcinogenesis (discussed next). Promoting agents essentially facilitate clonal expansion of initiated cells and their evolution into neoplasms. The selective growth of preneoplastic populations can be achieved either by an enhanced rate of cell proliferation in the tissue or a decreased rate of apoptosis in incipient neoplasms.

Promoting agents are usually assumed to be noncarcinogens, but in fact, most are weak carcinogens under some circumstance, probably because they facilitate tumor development from cryptogenically initiated cells that are the source of spontaneous tumors. Thus, most, if not all, promoters are epigenetic carcinogens (Table 20.2). An essential characteristic of a promoting agent is that it is not DNA reactive and is not an initiating agent; otherwise, the enhancement is likely to be due to syncarcinogenesis.

Cocarcinogenesis

Cocarcinogenesis is the enhancement by a noncarcinogen of the carcinogenicity of a carcinogen when administered prior to or concurrently with the carcinogen or when given shortly after a carcinogen at a time when molecular damage is still present. Cocarcinogens may enhance the uptake of the carcinogen, enhance its tissue localization, increase the proportion that is bioactivated, or enhance the induced neoplastic transformation, usually by transiently increasing cell proliferation. Cocarcinogens do not act as pro

		carcinogen[1]		Tumor yield
Syncarcinogenesis	carcinogen[2]	carcinogen[2]	carcinogen[2]	↑
Promotion		recovery	promoter	↑
Co-carcinogenesis	co-car[1]	co-car[2]	co-car[3]	↑
Anticarcinogenesis	inhibitor	blocking agent	suppressing agent	↓
Photochemical carcinogenesis		photoactive chemical / + UV radiation		- / +

FIG. 20.4. Types of interactive carcinogenesis.

moters, although most promoters have cocarcinogenic activity, often due to enhancement of cell proliferation.

Anticarcinogenesis

Anticarcinogenesis is the reduction of the carcinogenicity of an agent by a previously, concurrently, or subsequently administered agent, usually a noncarcinogen, although certain epigenetic carcinogens, such as phenobarbital, are effective anticarcinogens. Three operational pathways are recognized as follows: inhibitors that prevent formation of carcinogens, blocking agents that counteract effects of carcinogens, and suppressing agents that suppress tumor development (272).

Photochemical Carcinogenesis

A specific type of interactive carcinogenesis is photochemical carcinogenesis, which is the combined skin carcinogenicity of a chemical and ultraviolet light (75, 129). Photochemical carcinogenicity can result from several types of interaction between the chemical, ultravioltet radiation (UVR), and the skin. Some chemicals, such as psoralens, can be photoactivated to DNA-reactive chemical species. Others, such as fluoroquinolones, can undergo photoactivation to generate secondary reactive molecular species such as reactive oxygen (230a). Also, some chemicals can affect the structure of skin, for example, thinning of the epidermis in the case of retinoids, to sensitize the skin to effects of UVR radiation. Finally, immunosuppression can enhance skin carcinogenesis (145).

Photochemical carcinogenicity studies are often required for topically applied medicines and even for some oral medicines, as well as for topically applied cosmetics and consumer products. The test species is usually the SKH1 albino hairless mouse, which has the advantage that it does not require hair clipping and allows easy detection of UVR-induced squamous-cell papillomas and carcinomas. In a typical protocol (49, 74, 213), the test substance is applied before UVR (at 290–400 nm by a UV solar simulator) on Monday, Wednesday, and Friday and after UVR on Tuesday and Thursday for 40 weeks, followed by a 12-week observation period without exposure. This pattern of exposure allows detection of photoactivated chemicals, as well as those that may modulate photocarcinogenesis. Typically, separate groups are administered low and high UVR in addition to the TS. The endpoints of evaluation include tumor incidence (prevalence), multiplicity (yield), and latency (time to tumor).

Complex Mixtures

Most chemical exposures involve mixtures rather than single agents. Yet the scientific data for these mixtures are generated almost entirely from studies of individual

agents. Mixtures are comprised of chemicals with several isomers, chemicals with major contaminants, hazardous waste in solid or liquid form, and air pollutants. The mixtures can either have a common source (e.g., tobacco smoke, aluminum production, coal tars) or be formulated deliberately (coal gasification, footware processing work exposure). Several such mixtures are recognized as human carcinogens, such as tobacco smoke and coal tars. A daunting challenge has been to ascertain the role of individual components in the carcinogenicity of such mixtures (117). To accomplish this, complex mixtures can be fractionated and characterized into chemically defined entities, which can be individually tested. Nevertheless, in the mixture, individual chemicals can enhance or inhibit the activity of others, as discussed earlier.

The most relevant information for cancer hazard identification and risk assessment for cancer of humans involves a combination of mixture characterization/ exposure data from experimental systems and epidemiological data from humans (117). Characterization and definition of exposure, identification of the source, and distribution and occurrence of the mixture, as well as its composition and physicochemical properties, are essential. Here the most common method of fractionation is the one that partitions complex mixtures into organic and inorganic constituents. This is followed until active agents are separated from inactive ones. Chemical characterization of all biologically active ingredients is pursued. Important in experimental systems is the identification of in vitro/in vivo effects and rodent carcinogenicity data. In addition, definitions of dosimetry, toxicokinetics, bioavailability, disposition, and molecular and biochemical effects are also needed. The comparative potency method (CPM) is one that is based on data correlation from all in vitro and in vivo experimental assays, with human potency estimates (2, 22). What is being compared is a known human risk complex mixture with a CPM from a similar complex with incomplete data (154). CPM for cancer risk estimation is not validated yet, and consequently its utility is still limited.

Comparative Potency Method

The comparative potency method (CPM) has been applied to estimate human lung cancer risk from coke oven emissions, roofing tar, and cigarette smoke condensate, all combustion products of complex mixtures. Each of these complex mixtures was compared using standard protocols, which included an in vivo mouse skin initiation/promotion study, and two in vitro studies, such as mouse lymphoma and *Salmonella typhimurium* (Ames) mutagenicity assays. The result of the three mixtures showed agreement among assays (Ames activity was highest with cigarette smoke condensate), and thus it was possible to use CPM for cancer risk estimation (22, 154).

One of the major limitations of CPM is due to the chemical changes of the complex mixtures over time. Such changes come about through environmental biodegradation, photo-oxidation, volatilization, migration (to groundwater), or adsorption (to solid soil particles). Another limitation is the inappropriateness of short-term in vivo or in vitro assays to the assessment of some complex mixtures, such as diesel particulates, which may not be accessible for in vitro systems.

Toxic Equivalency Factor

The toxic equivalency factor (TEF) was first developed to assess the toxicological interactions of specific complex mixtures, that is, polychlorinated dibenzodioxins and dibenzofurans. For this purpose, a number of short-term assays were utilized, including enzyme induction, receptor binding, and cell keratinization studies. All are helpful in predicting carcinogenic responses. The reference substance was 2,3,7,8-TCDD, which was assigned the value of 1 (22).

Based on this, the TEF was determined by multiplying the substance concentration with the relative toxicity from the short-term assays for each mixture ingredient—that is, the product is the TEF. The TEF is very helpful with chemically related compounds in a mixture. The process has been applied to polycyclic aromatic hydrocarbons and polychlorinated biphenyls. However, results of several studies have also shown that for specific responses, the TEFs for some halogenated aromatic hydrocarbon mixtures are nonadditive (211).

ACKNOWLEDGMENTS

We thank Barbara Iatropoulos and Katey Bateman for preparation of the text. Also, we gratefully acknowledge the thoughtful comments by many colleagues in the chemical and pharmaceutical industries. The preparation of this chapter was made possible by support provided to the Medicine, Food, and Chemical Safety Program at New York Medical College.

QUESTIONS

1. What are the pertinent mechanisms of carcinogenesis operational in rodents and man?
2. What are the mechanisms of carcinogenesis operational only for rodents? Explain why.
3. Which neoplasms are of questionable significance to human cancer hazard? Explain why.

4. What are the types of cancer hazard?
5. What constitutes adequate exposure in a rodent cancer bioassay?

REFERENCES

1. Abdo, K.M,, and Kari, F.W. (1996): The sensitivity of the NTP bioassay for carcinogen hazard evaluation can be modulated by dietary restriction. *Exp. Toxicol. Pathol*, 48:129–137.
2. Albert, R.E. (1985): The comparative potency method: An approach to quantitative cancer risk asessment. In: *Methods for Estimating Risk of Chemical Injury: Humans and Non-Human Biota and Ecosystems*, edited by V.B.Vonk, G.C.Butler, D. G.Hoel, and D.B.Peakall, pp. 281–287. John Wiley and Sons, New York.
3. Allaben, W.T., Turturro, A., Leakey, J.E.A., Seng, J.E., and Hart, R.W. (1996): FDA Points-to-consider documents: The need for dietary control for the reduction of experimental variability within animal assays and the use of dietary restriction to achieve dietary control. *Toxicol. Pathol.*, 24:776–781.
4. Alexandrov, V.A., Popovich, I.G., Anisimov, V.N., and Napalkov, N.P. (1989): Influence of hormonal disturbances on transplacental and multigeneration carcinogenesis in rats. In: *Perinatal and Multigeneration Carcinogenesis*, edited by N.P. Napalkov, J.M.Rice, L.Tomatis and H.Yamasaki, pp. 35–49. IARC Science Publ. No. 96, Lyon, France.
5. Alison, R.H., and Morgan, K.T. (1987): Ovarian neoplasms in F344 rats and B6C3F] mice. *Environ. Health Perspect.*, 73:91–106.
6. Alison, R.H., Capen, C.C., and Prentice, D.E. (1994): Neoplastic lesions of questionable significance to humans. *Toxicol. Pathol.*, 22:179–186.
7. Apostolou, A. (1990). Relevance of maximum tolerated dose to human carcinogenic risk. *Regul. Toxicol. Pharmacoi*, 11:68–80.
8. Ashby, J., and Tennant, R.W. (1991): Definitive relationships among chemical structure, carcinogenicity and mutagenicity for 301 chemicals tested by the U.S. NTP. *Mutat. Res.*, 257:229–306.
9. Ballard, B.E. (1968): Biopharmaceutical consideration in subcutaneous and intramuscular drug administration. *J. Pharm. Sci.*, 57:357–378.
10. Bartow, S.A. (1994): The breast. In: *Pathology*, edited by E.Rubin and J.L.Farber, pp. 73–992, J.B.Lippincott, Philadelphia.
11. Berenblum, I. (1974): *Frontiers of Biology: Carcinogenesis as a Biological Problem*, edited by A.Neuberger and E.L.Tatum. North Holland, Amsterdam.
12. Bergman, K., Olofsson, I.-M., and Sjoeberg, P. (1998): Dose selection for carcinogenicity studies of pharmaceuticals: Systemic exposure to phenacetin at carcinogenic dosage in the rat. *Regul. Toxicol. Pharmacoi*, 28:226–229.
13. Bischoff, K.B. (1975): Some fundamental considerations of the applications of pharmacokinetics to cancer chemotherapy. *Cancer Chemother. Rep.*, 59:777–793.
14. Bomhard, E., and Rinke, M. (1994). Frequency of spontaneous tumours in Wistar rats in 2-year studies. *Exp. Toxicol. Pathol.*, 46:17–29.
15. Boorman, G.A., Montgomery, C.A., Jr., Eustes, S.L., Wolfe, M. J. McConnell, E.E., and Hardesty, J.F. (1985): Quality Assurance in pathology for rodent carcinogenicity studies. In: *Handbook of Carcinogen Testing*, edited by H.Milman and E.Weisburger, pp. 345–357. Noyes, Park Ridge, NJ.
16. Boorman, G.A., Maronpot, R.R., and Eustis, S.L. (1994): Rodent carcinogenicity bioassay: Past, present and future. *Toxicol Pathol*, 22:105–111.
17. Borowitz, J.L., Moore, P.F., Yim, G.K.W., and Miya, T.S. (1971): Mechanism of enhanced drug effects produced by dilution of the oral dose. *Toxicol. Appl. Pharmacoi*, 19:164–168.
18. Brunner, G.H.G., Lamberts, R., and Creutzfeldt, W. (1990): Efficacy and safety of omeprazole in the long-term treatment of peptic ulcer and reflux oesophagitis resistant to ranitidine. *Digestion*, 47:64–68.
19. Brusick, D., Albertini, R., McRee, D., Peterson, D., Williams, G., Hanawalt, P., and Preston, J. (1998): Genotoxicity of radio frequency radiation. *Environ. Mol. Mutagen.*, 32:1–16.
20. Butler, W.H., Cohen, S.H., and Squire, R.A. (1997): Mesenchymal tumors of the mouse urinary bladder with vascular and smooth muscle differentiation. *Toxicol. Pathol*, 25:268–274.
21. Butterworth, B.E., Ashby, J., Bermudez, E., Casciano, D., Mirsalis, J., Probst, G., and Williams, G. (1987): A protocol and guide for the in vivo rat hepatocyte DNA repair assay. *Mutat. Res.*, 189:123–133.
22. Calabrese, E.J. (1991): *Multiple Chemical Interactions.* Lewis, Chelsea, MI.
23. Caldwell, J., Gardner, I., and Swales, N. (1995): An introduction to drug disposition: The basic principles of absorption, distribution, metabolism and excretion. *Toxicol Pathol*, 23:148–157.
24. Capen, C.C., Beamer, W.G., Tennent, B.J., and Stitzel, K.A. (1995): Mechanisms of hormone-mediated carcinogenesis of the ovary in mice. *Mutat. Res.*, 333:143–151.
25. Cayen, M.N. (1995): Considerations in the design of toxicokinetic programs. *Toxicol Pathol*, 23:148–157.

26. Cayen, M.N., and Black, H.E. (1993): Role of toxikokinetics in dose selection for carcinogenicity studies. In: *Drug Toxicokinetics*, edited by P.G.Welling and F.A.de la Iglesia, pp. 69–83. Marcel Dekker, New York.
27. Chappell, W.R., and Mordenti, J. (1991): Extrapolation of toxicological and pharmacological data from animals to humans. *Adv. Drug Res.*, 20:2–116.
28. Chengelis, C.P., Gad, S.C., and Holston, J. (1995): *Regul Toxicol.* Raven Press, New York.
29. Christian, M.S., Hoberman, A.M., Johnson, M.D., Brown, W. R., and Bucci, T.J. (1998): Effect of dietary optimization on growth, survival, tumor incidences and clinical pathology parameters in CD Sprague-Dawley and Fischer-344 rats: A 104-week study. *Drug Chem. Toxicol*, 21:97–117.
30. Clayson, D.B., and Kitchin, K.T. (1999): Interspecies differences in response to chemical carcinogens. In: *Carcinogenicity*, edited by K.T.Kitchin, pp. 837–880. Marcel Dekker, New York.
31. Cohen, S.M., and Ellwein, L.B. (1991): Genetic errors, cell proliferation, and carcinogenesis. *Cancer Res.*, 51:6493–6505.
32. Contrera, J.F., Jacobs, A.C., Prasanna, H.R., Mehta, M., Schmidt, W.J., and DeGeorge, J.J. (1995): A systemic exposure-based alternative to the maximum tolerated dose for carcinogenicity studies of human therapeutics. *J. Am. Coll. Toxicol*, 14:1–10.
33. Contrera, J.F., Jacobs, A.C., DeGeorge, J.J., Chen, C., Choudary, J., DeFelice, A., Fairweather, W., Farrelly, J. Fitzgerald, G., Goheer, A., Jordan, A., Lin, D., Lin, K., Kelly, R., Meyers, L., Osterberg, R., Prasanna, H.R., Resnick, C., Sheevers, H., and Sun, J. (1996): Carcinogenicity testing and the evaluation of regulatory requirements for pharmaceuticals. *Fed. Reg., U.S.* Department of Health and Human Services, Public Health Service, Docket No. 96D-0235.
34. Contrera, J.F., Jacobs, A.C., and DeGeorge, J.J. (1997): Carcinogenicity testing and the evaluation of regulatory requirements for pharmaceuticals. *Regul. Toxicol. Pharmacoi*, 25:130–145.
35. Contrera, J.F., and DeGeorge, J.J. (1998): In vivo transgenic bioassays and assessment of the carcinogenic potential of pharmaceuticals. *Environ. Health Perspect.*, 106(suppl. 1):71–80.
36. Cook, J.C., Klinefelter, G.R., Hardisty, J.F., Sharpe, R.M., and Foster, P.M.D. (1999): Rodent Leydig cell tumorigenesis: A review of the physiology, pathology, mechanisms, and relevance to humans. *Crit. Rev. Toxicol*, 29:169–261.
37. Cottrell, S., Andrews, C.M., Clayton, D., and Powell, C.J. (1994): The dose-dependent effect of BHT (butylated hydroxytoluene) on

Page 994
vitamin K-dependent blood coagulation in rats. *Food Chem. Toxicol.*, 32(7):589–594.
38. Counts, J.L., and Goodman, J.I. (1995): Principles underlying dose selection for, and extrapolation from, the carcinogen bioassay: Dose influences mechanism. *Regul. Toxicol. Pharmacol.*, 21:418–421.
39. Courtney, C.L., Hawkins, K.L., Meierhenry, E.F., and Graziano, M.J. (1992): Immunohistochemical and ultrastructural characterization of granular cell tumors of the female reproductive tract in two aged Wistar rats. *Vet. Pathol.*, 29:86–89.
40. Cox, D.R. (1972): Regression models and life tables. *J.R. Stat. Soc.*, 13:187–220.
41. Cutler, S.J., and Ederer, F. (1958): Maximum utilization of life table method in analyzing survival. *J. Chron. Dis.*, 8:699–712.
42. Dahlem, A.M., Allerheiligen, S.R., and Vodicnik, N.J. (1995): Concomitant toxicokinetics: Techniques for interpretation of exposure data obtained during the conduct of toxicology studies. *Toxicol. Pathol.*, 23:170–178.
43. D'Arcy, P.F., and Harron, D.W.G. (1992): *Proceedings of the First International Conference of Harmonization.* Brussels, Belgium, 1991.
44. D'Arcy, P.F., and Harron, D.W.G. (1996): *Proceedings, Third International Conference on Harmonization.* Yokohama, Japan, 1995, Greystone Books, Antrim, North Ireland.
45. Davies, T.S., and Monro, A. (1994): The rodent carcinogenicity bioassay produces a similar frequency of tumor increases and decreases: Implications for risk assessment. *Regul. Toxicol. Pharmacol.*, 20:281–301.
46. Davies, T.S., and Monro, A. (1995): Marketed human Pharmaceuticals reported to be tumorigenic in rodents. *J. Am. Coll. Toxicol.*, 14:90–107.
47. Dedrick, R.L. (1986): Interspecies scaling of regional drug delivery. *J. Pharmaceut. ScL*, 175:1047–1052.
48. DeGeorge, J.J., and Contrera, J.F. (1996): A regulatory perspective of the guidance on the utility of two rodent species. In: *Proceedings, Third Internatinal Conference on Harmonization.* Yokohama, Japan, 1995, edited by P.F.D'Arcy and D.W. G. Harron, pp. 274–277, Greystone Books, Antrim, North Ireland.
49. De Gruijl, F.R., and Forbes, P.D. (1995): UV-induced skin cancer in a hairless mouse model. *BioEssays*, 17:651–660.
50. De Vries, A., von Oostrom, C.T.M., Dortant, P.M., Beems, R.B., van Kriejl, C.F., Capel, P.J.A., and van Steeg, H. (1997): Spontaneous liver tumours and benzo[a]pyrene-induced lymphomas in XPA-deficient mice. *Mol. Carcinogen.*, 19:46–53.
51. Dideriksen, L.H., Joregensen, L.N., and Drejer, K. (1992): Car¬ cinogenic effect on female rats after 12 months administration of the insulin analogue B (10)Asp. *Diabetes*, 41(suppl. 1):143A (abstr.).
52. Donehower, I., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Jr., Butel, J.S., and Bradley, A. (1992): Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumors. *Nature*, 356:215–221.
53. Dragan, Y.P., Rizvi, T, Xu, Y.-H., Hully, J.R., Bawa, N., Campbell, H.A., Maronpot, R.R., and Pilot, H.C. (1991): An initiation-promotion assay in rat liver as a potential complement to the 2-year carcinogenesis bioassay. *Fundam. Appl. Toxicol.*, 16:525–547.
54. Dunnett, C.W. (1955): A multiple comparison procedure for comparing several treatments with a control. *J. Am. Stat. Assoc.*, 50:1096–1122.
55. Dybing, E., Sanner, T., Roelfzema, H., Kroese, D., and Tennant, R.W. (1997) T25: A simplified carcinogenic potency index: Description of the system and study of correlations between car¬ cinogenic potency and species/site specificity and mutagenicity. *Pharmacol. Toxicol.*, 30:272–279.
56. Eastin, W.C., Haseman, J.K., Mahler, J.F., and Bucher, J.R. (1998): The National Toxicology Program evaluation of genetically altered mice predictive models for identifying carcinogens. *Toxicol. Pathol.*, 26:461–584.
57. Ellwein, L.G., and Cohen, S. (1990): The health risk of saccharin revisted. *Crit. Rev. Toxicol.*, 20:311–326.
58. Elwell, M.R., Dunnick, J.K., Hailey, J.R., and Haseman, J.K. (1996): Chemicals associated with decreases in the incidence of mononuclear cell leukemia in the Fischer rat. *Toxicol. Pathol.*, 24:238–245.
59. Emerson, C.H., Cohen, J.H. III., Young, R.A., Alex, S., and Fan, S.-L. (1990): Gender-related differences of serum thyroxine-binding proteins in the rat. *Acta Endocrinol.*, 123:72–78.
60. Enzmann, H., Bombard, E., Iatropoulos, M.J., Ahr, H.J., Schlueter, G., and Williams, G.M. (1998): Short- and intermediate-term carcinogenicity testing—A review: Part 1—The prototypes mouse skin tumour assay and rat liver focus. *Food Chem. Toxicol.*, 36:979–995.
61. Enzmann, H., Iatropoulos, M.J., Brunnemann, K.D., Bomhard, E., Ahr, H.J., Schlueter, G., and

Williams, G.M. (1998): Shortand intermediate-term carcinogenicity testing—A review: Part 2—Available experimental models. *Food Chem. Toxicol.*, 36:997–1013.
62. European Economic Communities. (1967): Directive 67/548/EEC with amendments and adaptions: Annex VI. *Criteria for Classification of Carcinogenic Substances*, Brussels, Belgium.
63. European Economic Communities. (1983): *Note for Guidance Con¬ cerning the Application of Chapter I (E) of Part 2 of the Annex to Directive 75/398/EEC, With a View to the Granting of a Marketing Authorization of a New Drug.* Brussels, Belgium.
64. European Economic Communities. (1988): *Directive 88/379/EEC With Amendments: Annex I Criteria for Classification of Carcinogenic Substances.* Brussels, Belgium.
65. Faccini, J.M., Abbott, D.P., and Paulus, G.J.J. (1990): *Mouse Histopathology, A Glossary for Use in Toxicity and Carcinogenicity Studies.* Elsevier, Amsterdam.
66. Faccini, J.M., Butler, W.R., Friedmann, J.-C., Hess, R., Reznik, G.K., Ito, N., Hayashi, Y., and Williams, G.M. (1992): IFSTP guidelines for the design and interpretation of the chronic rodent carcinogenicity bioassay. *Exp. Toxicol. Pathol.*, 44:443–456.
67. Fairbairn, D.W., Olive, P.L., and O′Neill, K.L. (1995): The comet assay: A comprehensive review. *Mutat. Res.*, 339:37–59.
68. Feinstein, A.R. (1975): Clinical biostatistics. *Clin. Pharmacol. Ther.*, 17:499–513.
69. Fitzgerald, J.M., Boy, V.F., and Manus, A.G. (1984). Formulation of insoluble and inmiscible test agents in liquid vehicles for toxicity testing. In: *Chemistry for Toxicity Testing,* edited by C.W.Jameson and D.B.Walters, pp. 83–90. Butterworth, Stoneham, MA.
70. Flammang, T.J., von Tungeln, L.S., Kadlubar, F.F., and Fu, P.P. (1997): Neonatal mouse assay for tumorigenicity: Alternative to the chronic rodent bioassay. *Regul. Toxicol. Pharmacol.*, 26:230–240.
71. Fleiss, J.L. (1981): *Statistical Methods for Rates and Proportions*, 2nd ed., pp. 145–146. John Wiley and Sons, New York.
72. Fleiss, J.L. (1986): *The Design and Analysis of Clinical Experiments*, pp. 58–59. John Wiley and Sons, New York.
73. Foran, J.A. (Ed.). (1997): *Principles for the Selection of Doses in Chronic Rodent Bioassays.* ILSI Press, Washington, DC.
74. Forbes, P.D., Sambuco, C.P., and Davies, R.E. (1993): Photocarcinogenesis safety testing. *J. Am. Coll. Toxicol.*, 12:417–424.
75. Forbes, P.D., and Sambuco, C.P. (1998): Assays for photocarcinogenesis: Relevance of animal models. *Int. J. Toxicol.*, 17:577–588.

76. Frantz, S.W., Beatty, P.W., English, J.C., Hundley, S.G., and Wilson, A.G.E. (1994): The use of pharmacokinetics as an interpretive and predictive tool in chemical toxicology testing and risk assessment: A position paper on the appropriate use of pharmacokinetics in chemical toxicology. *Regul. Toxicol. Pharmacol*, 19:317–337.
77. Gabriel, K. (1966). Simultaneous test procedures for multiple comparisons on categorical data. *J. Am. Stat. Assoc.*, 61:1081–1096.
78. Gabriel, K.R. (1978): A simple method of multiple comparison of means. *J.Am. Stat. Assoc.*, 73:724–729.
79. Gad, S.C. (1996): Histologic and clinical pathology in the safety assessment and development of new therapeutic agents. *Scand. J. Lab. Anim. Sci.*, 13:325–334.
80. Gad, S.C., and Weil, C.S. (1986): *Statistics and Experimental Design for Toxicologists*, pp. 1–17. Telford Press, Caldwell, NJ.
81. Galer, D.M., and Monro, A.M. (1998): Veterinary drugs no longer need testing for carcinogenicity in rodent bioassays. *Regul Toxicol. Pharmacol.*, 28:115–123.
82. Garby, L., Garrow, J.S., Jorgensen, B., Lammert, O., Madsen, K., Sorensen, P., and Webster, J. (1988): Relationship between energy expenditure and body composition in man: Specific energy expenditure in vivo of fat and fat-free tissue. *Eur. J. Clin. Nutrition*, 42:301–305.
83. Gart, J.J., Chu, K.C., and Tarone, R.E. (1979): Statistical issues in the interpretation of chronic bioassay tests for carcinogenicity. *JNCI*, 62:957–974.
84. Gaylor, D.W., and Kodell, R.I. (1999): Dose-response trend tests for tumorigenesis, adjusted for body weight. *Toxicol. Sci.*, 49:318–323.
85. Gehring, P.J., Watanabe, P.G., and Park, C.N. (1978): Resolution of dose response toxicity data for chemicals requiring metabolic activation. Example—Vinyl chloride. *Toxicol. Appl. Pharmacol.*, 44:581–591.
86. Gillette, J., Weisburger, E.K., Kraybill, H., and Kelsey, M. (1985): Strategies for determining the mechanisms of toxicity. *Clin. Toxicol.*, 23(1): 1–78.
87. Gold, L.S., and Zeiger, E. (1996): *Handbook of Carcinogenic Potency and Genotoxicity Data Bases.* CRC Press, Boca Raton, FL.
88. Gopinath, C., Prentice, D.E., and Lewis, D.J. (1987): *Atlas of Experimental Toxicological Pathology.* MTP, Lancaster, UK.
89. Grasso, P., Sharratt, M., and Cohen, A.J. (1991): Role of persistent, non-genotoxic tissue damage in rodent cancer and relevance to humans. *Annu. Rev. Pharmacol. Toxicol.*, 31:253–287.
90. Greaves, P.C. (1990): *Histopathology of Preclinical Toxicity Studies.* Elsevier, Amsterdam.
91. Hailey, J.R., Haseman, J.K., Bucher, J.R., Raadovsky, A.E., Malarkey, D.E., Miller, R.T., Nyska, A., and Maronpot, R. R. (1998): Impact of *Helicobacter hepaticus* infection in B6C3Fi mice from twelve National Toxicology Program two-year carcinogenesis studies. *Toxicol. Pathol*, 26:602–611.
92. Halliwell, W.H. (1998): Submucosal mesenchymal tumors of the mouse urinary bladder. *Toxicol. Pathol.*, 26:128–136.
93. Halpert, J.R., Guengerich, F.P., Bend, J.R., and Correia, M.A. (1994): Selective inhibitors of cytochrome P450. *Toxicol. Appl. Pharmacol*, 124:163–175.
94. Hart, R.W., Keenan, K.P., Turturro, A., Abdo, K.M., Leakey, J., and Lyn-Cook, L. (1995): Caloric restriction and toxicology. *Fundam. Appl. Toxicol.*, 25:184–195.
95. Hart, R.W., Neumann, D.A., and Robertson, R.T. (Eds.). (1995): *Dietary Restriction: Implications for the Design and Interpretation of Toxicity and Carcinogenicity Studies.* ILSI Press, Washington, DC.
96. Haseman, J.K. (1984): Statistical issues in the design analysis and interpretation of animal carcinogenicity studies. *Environ. Health Perspect.*, 58:385–392.
97. Haseman, J.K. (1990): Use of statistical decision rules for evaluating laboratory animal carcinogenicity studies. *Fundam. Appl. Toxicol.*, 14:637–648.
98. Haseman, J.K., Huff, J., and Boorman, G.A. (1984): Use of historical control data in carcinogenicity studies in rodents. *Toxicol. Pathol.*, 2:126–135.
99. Haseman, J.K., and Rao, G.N. (1992): Effects of corn oil, time-related changes, and inter-laboratory variability on tumor occurrence in control Fischer 344 (F344/N) rats. *Toxicol. Pathol.*, 20:52–60.
100. Haseman, J.K., and Lockhart, A. (1994): The relationship between use of the maximum tolerated dose and study sensitivity for detecting rodent carcinogenicity. *Fundam. Appl. Toxicol.*, 22:382–391.
101. Haseman, J.K., and Johnson, L. (1996): Analysis of National Toxicology Program rodent bioassay data for anticarcinogenic effects. *Mutat. Res.*, 350:131–141.
102. Haseman, J.K., Hailey, J.R., and Morris, R.W. (1998): Spontaneous neoplasm incidences in Fischer

344 rats and B6C3Fi mice in two-year carcinogenicity studies: A National Toxicology Program update. *Toxicol. Pathol.*, 26:428^41.
103. Health and Welfare Canada. (1989): *Guidelines for Canadian Drinking Water Quality: Supporting Documentation*, Part 1, pp. 1–5. Health and Welfare, Ottawa, Canada.
104. Hesseltine, G.R., Wolff, R.K., Hanson, R.L., Mauderly, J.L., and McClellan, R.O. (1984): *Effect of day versus night inhalation exposure on lung burdens of gallium oxide in rats.* Inhalation Toxicology Research Institute Annual Report, Albuquerque, NM.
105. Hill, R.N., Crisp, T.M., Hurley, P.M., Rosenthal, S.L., and Singh, D.V. (1998): Risk assessment of thyroid follicular cell tumors. *Environ. Health Perspect.*, 106:447–457.
106. Huff, J. (1999): Value, validity, and historical development of carcinogenesis studies for predicting and confirming carcinogenic risks to humans. In: *Carcinogenicity*, edited by K.T.Kitchin, pp. 21–123. Marcel Dekker, New York.
107. Iatropoulos, M.J. (1984): Appropriateness of methods for slide evaluation in the practice of toxicologic pathology. *Toxicol. Pathol.*, 12:4–5.
108. Iatropoulos, M.J. (1988): Society of Toxicologic Pathologists position paper: “Blinded” microscopic examination of tissues from toxicologic or oncogenic studies. In:*Carcinogenicity*, edited by H.C.Grice and J.L.Ciminera, pp. 133–135, Springer Verlag, New York.
109. Iatropoulos, M.J. (1993): Comparative histokinetic and xenodynamic considerations in toxicity. In: *Drug Toxicokinetics*, edited by D.G.P.Welling and F.A.de la Iglesia, pp. 245–266. Marcel Dekker, New York.
110. Iatropoulos, M.J., Newman, A.J., Dayan, A.D., Brughera, M., Scampini, G., and Mazue, G. (1994): Urinary bladder hyperplasia in the rat: Non-specific pathogenetic considerations using a beta-lactam antibiotic. *Exp. Toxicol. Pathol.*, 46:265–274.
111. Iatropoulos, M.J., Williams, G.M., Wang, C.–X., and Karlsson, S.H. (1996): New histopathologic and histokinetic methods in preclinical safety studies. *Scand. J. Lab. Anim. Sci.*, 13:339–343.
112. Iatropoulos, M.J., and Williams, G.M. (1996): Proliferation markers. *Exp. Toxicol. Pathol.*, 48:175–181.
113. Imai, K., Yoshimura, S., Yamaguchi, K., Matsui, E., Isaka, H., and Hashimoto, K. (1990): Effects of dietary restriction on age-associated pathological changes in F-344 rats. *J. Toxicol. Pathol.*, 3:209–221.

file:///E|/0203362837__gigle.ws/0203362837__gigle.ws/0203362837/0203362837/files/page_995.html[2010/10/28 18:50:02]

114. Interdisciplinary Panel on Carcinogenicity (IPC/AIHC). (1984): Criteria for evidence of chemical carcinogenicity. *Science*, 225:682–687.
115. International Agency for Research on Cancer. (1980): *Long-Term and Short-Term Screening Assays for Carcinogens: A Critical Appraisal*, IARC Monographs, Supplement 2, Lyon, France.
116. International Agency for Research on Cancer. (1987): *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Preamble.* IARC Tech. Rep. No. 87/001, Lyon, France.
117. International Agency for Research on Cancer. (1990): *Complex Mixtures and Cancer Risk.* IARC Science Publ. No. 104, Lyon, France.
118. International Agency for Research on Cancer. (1992): *Mechanisms of Carcinogenesis in Risk Identification.* IARC Science Publ. No. 116, Lyon, France.
119. International Agency for Research on Cancer. (1992): *Solar and Ultraviolet Radiation.* IARC Tech. Rep. No. 55, Lyon, France.
120. International Agency for Research on Cancer. (1995): *Peroxisome Proliferation and Its Role in Carcinogenesis, Views and Expert Opinions of an IARC Working Group.* IARC Tech. Rep. No. 24, Lyon, France.
121. International Agency for Research on Cancer. (1996): *Directory of Agents Being Tested for Carcinogenicity.* IARC Science Publ. No. 134, Lyon, France.
122. International Agency for Research on Cancer. (1997): *IARC Monographs on the Evaluation of Carcinogenic risks to Humans*, vols. 1–69. IARC, Lyon, France.
123. International Agency for Research on Cancer. (1999): *The Use of Short- and Medium-Term Test for Carcinogens and Data on Genetic Effects in Carcinogenic Hazard Evaluations.* IARC Tech. Rep. No. 146, Lyon, France.
124. International Conference on Harmonization. (1994): *Guideline HI/5081 on Toxicokinetics.* Commission of the European Communities, Directorate-General III, Industry: Industrial Affairs III: Consumer Goods Industries-Pharmaceuticals, Brussels, Belgium.
125. International Conference on Harmonization. (1996): *Impurities in New Drug Products: ICH Harmonized Tripartite Guideline.* Washington, DC.
126. International Expert Panel on Carcinogen Risk Assessment (IEPCRA). (1996): The use of mechanistic data in the risk assessments of ten chemicals: An introduction to the chemical-specific reviews. *Pharmacol. Ther.*, 71:1–5.
127. International Programme on Chemical Safety (IPCS). (1996): Butylated hydroxytoluene. *Toxicological Evaluation of Certain Food Additives and Contaminants*, pp. 3–86. Prepared by the Expert Committee on Food Additives (JECFA) at the 4th joint meeting of FAO/WHO. WHO, Geneva.
128. International Workshop. (1992): Clinical pathology testing in preclinical safety assessment. *Toxicol. Pathol*, 20:469–543.
129. Jacobs, A., Avalos, J., Brown, P., and Wilkin, J. (1999): Does photosensitivity predict photococarcinogenicity? *Int. J. Toxicol.*, 18:191–198.
130. Jain, R.K., Gerlowski, L.E., Weissbrod, J.M., Wang, J., and Pierson, R.N., Jr. (1981): Kinetics of intake, distribution and excretion of zinc in rats. *Ann. Biomed. Eng.*, 9:345–361.
131. Jameson, C.W. (1984): Analytical chemistry requirements for toxicity testing of environmental chemicals. In: *Chemistry for Toxicity Testing*, edited by C.W.Jameson and D.B.Walters, pp. 3–14. Butterworth, Stoneham, MA.
131a. Jerry, D.J., Butel, J.S., Donehower, L.A., Paulson, E.J., Cochran, C., Wiseman, R.W., and Medina, D. (1994): Infrequent p53 mutations in 7,12-dimethylbenz(a)anthracene induced mammary tumors in BALB/c and p53 hemizygous mice. *Mol. Carcinog.*, 9:175–183.
132. Jordan, A. (1992): FDA requirements for nonclinical testing of contraceptive steroids. *Contraception*, 46:499–509.
133. Karbe, E. (1999): "Mesenchymal tumor" or "decidual-like reaction"? *Toxicol. Pathol*, 27:354–362.
134. Karbe, E., Hartmann, E., George, C., Wadsworth, P., Harleman, J., and Geiss, V. (1998): Similarities between the uterine decidual reaction and the "mesenchymal lesion" of the urinary bladder in aging mice. *Exp. Toxicol Pathol*, 50:4–6.
135. Keenan, K.P. (1996): The uncontrolled variable in risk assessment: Ad libitum overfed rodents—Fat, facts and fiction. *Toxicol Pathol*, 24:376–383.
136. Keenan, K., Smith, P., Hertzog, P., Soper, K., Ballam, G., and Clark, R. (1994): The effects of overfeeding and dietary restriction on Sprague-Dawley rat survival and early pathology biomarkers of aging. *Toxicol Pathol*, 22:300–331.
137. Keenan, K.P., Laroque, P., Ballam, G.C., Soper, K.A., Dixit, R., Mattson, B.A., Adams, S.P., and

Coleman, J.B. (1996): The effects of diet, ad libitum overfeeding, and moderate dietary restriction on the rodent bioassay: The uncontrolled variable in safety assessment. *Toxicol. Pathol*, 24:757–768.
138. Kelly, W.A., Marler, R.J., and Weikel, J.H. (1993): Drug-induced mesovarial leiomyomas in the rat—A review and additional data. *J. Am. Coll Toxicol*, 12:13–22.
139. Kemp, C.J. (1995): Hepatocarcinogenesis in p53-deficient mice. *Mol. Carcinogenesis*, 12:132–136.
140. King, F.G., Dedrick, R.L., and Farris, F.F. (1986): Physiological pharmacokinetic modeling of cisdichlorodiamine platinum(II) (DDP) in several species. *J. Pharmacokin. Biopharmaceut.*, 14:131–155.
141. Klopman, G., and Rosenkranz, H.S. (1994): Approaches to SAR in Carcinogenesis and mutagenesis. Prediction of carcinogenicity/mutagenicity using MULTICASE. *Mutat. Res.*, 305:33–46.
142. Knapka, J.J. (1979): Laboratory animal feed. *Science*, 204:1367–1368.
143. Komulainen, H. (1996): Pharmacokinetic experiments in animals—Needs and application of data. *Scand. J. Lab. Anim. Sci.*, 23:315–316.
144. Kunze, E., and Chowaniec, J. (1990): Tumors of the urinary bladder. In *Pathology of Tumours in Laboratory Animals*, vol. I, *Tumors of the Rat*, pp. 345–373. IARC Science Publ. No. 99, Lyon, France.
145. Kripke, M.L. (1994): Ultraviolet radiation and immunology: Something new under the sun—Presidential address. *Cancer Res.*, 54:6102–6105.
146. Kroes, R., and Wester, P.W. (1986): Forestomach carcinogens: Possible mechanisms of action. *Food Chem. Toxicol*, 24:1083–1089.
147. Krewski, D., Smythe, R.T., Dewanji, A., and Colin, D. (1988): Statistical tests with historical controls. In: *Carcinogenicity*, edited by H.C.Grice and J.L.Ciminera, pp. 23–38. Springer Verlag, New York.
148. Lai, Y.L., Jacoby, R., and Jonas, A. (1978): Age related and light associated retinal changes in Fischer rats. *Invest. Ophthalmol Visual Sci.*, 17:634–638.
149. Lavbelin, G., Roba, J., Roncucci, R., and Parmentier, R. (1975): Carcinogenicity of 6-aminochrysene in mice. *Eur. J. Cancer*, 11:327–334.
150. Le Boeuf, R.A., Kerckaert, G. Aardema, M., Gibson, D., Brauninger, R., and Isfort, R. (1996): The pH 6.7 Syrian hamster embryo cell transformation assay for assessing the carcinogenic potential of chemicals. *Mutat. Res.*, 356:85–127.
151. Leder, A., Kuo, A., Cardiff, R.D., Sinn, E., and Leder, P. (1990) v-Ha-ras transgene abrogates the initiation step in mouse skin

tumorigenesis: Effects of phorbol esters and retinoic acid. *Proc. Natl. Acad. Sci. USA*, 87:9178–9182.
152. Lee, P. (1988): Assumptions in analyses of the bioassay; A statistician's view. In: *Carcinogenicity*, edited by H.C.Grice and J.L.Ciminera, pp. 1–10. Springer Verlag, New York.
153. Leung, H.W., and Paustenbach, D.J. (1988): Application of pharmacokinetic to derive biological exposure indexes from threshold limit values. *Am. Ind. Hyg. Assoc.*, 49:445–450.
154. Lewtas, J. (1988): Genotoxicity of complex mixtures: Strategies for the identification and comparative assessment of airborne mutagens and carcinogens from combustion sources. *Fundam. Appl. ToxicoL*, 10:571–589.
155. Lington, A.W., Bird, M.G., Plutnick, R.T., Stubblefield, W.A., and Scala, R.A. (1997): Chronic toxicity and carcinogenic evaluation of diisonoyl phthalate in rats. *Fundam. Appl. ToxicoL*, 36:79–89.
156. Long, G.G., Symanowski, J.T., and Roback, K. (1998): Precision in data acquisition and reporting of organ weights in rats and mice. *ToxicoL PathoL*, 26:316–318.
157. Lutz, W.K. (1986): Quantitative evaluation of DNA binding data for risk estimation and for classification of direct and indirect carcinogens. *J.Cancer Res. Clin. OncoL*, 112:85–91.
158. Mahler, J.F., Stokes, W., Mann, P.C., Takaoka, M., and Maronpot, R.R. (1996): Spontaneous lesions in aging FVB/N mice. *ToxicoL PathoL*, 24:710–716.
159. Mahler, J.P., Flagler, N.D., Malarkey, D.E., Mann, P.C., Haseman, J.K., and Eastin, W. (1998): Spontaneous and chemically-induced proliferative lesions in Tg.AC transgenic and p53-heterozygous mice. *ToxicoL PathoL*, 26:501–511.
160. Maita, K., Hirano, M., Harada, T., Mitsumori, K., Yoshida, A., Takahashi, K., Nakashima, N., Kitazawa, T., Enomoto, A., Inui, K., and Shirasu, Y. (1988): Mortality, major cause of moribundity, and spontaneous tumors in CD-I mice. *ToxicoL PathoL*, 16:340–349.
161. Matthews, E.J., and Contrera, J.F. (1998): A new highly specific method for predicting the carcinogenic potential of Pharmaceuticals in rodents using enhanced MCASE QSAR-ES software. *Regul. ToxicoL PharmacoL*, 28:242–264.
162. McClellan, R.O., and Hobbs, C.H. (1986): Generation, characterization and exposure systems for test atmospheres. In: *Safety Evaluation of Chemicals*, edited by W.E.Lloyd, pp. 41-^8. Hemisphere, Washington, DC.
163. McConnell, E.E. (1983): Pathology requirements for rodent two year studies. I. A review of current procedures. *ToxicoL PathoL*, 11:60–64.
164. McConnell, E.E., Solleveld, H.A., Swenberg, J.A., and Boorman, G.A. (1986): Guidelines of combining neoplasms for evaluation of rodent carcinogenesis studies. *JNCI*, 76:283–289.
165. McConnell, R.F. (1989): General observations on the effects of sex steroids in rodents with emphasis on long-term oral contraceptive studies. In: *Safety Requirements for Contraceptive Steroids*, edited by F.Michael, pp. 211–229. Cambridge University Press, New York.
166. McConnell, R.F., Westen, H.H., Ulland, B.M., Bosland, M.C., and Ward, J.M. (1992): Proliferative lesions of the testes in rats with selected examples from mice. In: *Guides for Toxicologic Pathology*, pp. 1–25. STP/ARP/AFIP, Washington, DC.
167. McMartin, D.N., Sahota, P.S., Gunson, D.E., Hsu, H.H., and Spaet, R.H. (1992): Neoplasms and related proliferative lesions in control Sprague-Dawley rats from carcinogenicity studies. Historical data and diagnostic considerations. *ToxicoL PathoL*, 20:212–225.
168. Milman, H.A., and Weisburger, E.K. (1985): *Handbook of Carcinogen Testing.* Noyes, Park Ridge, NJ.
169. Ministry of Health and Welfare. (1989): *Guidelines for toxicity studies required for applications for approved to manufacture (import) drugs: Carcinogenicity study*. Tokyo, Japan.
170. Modlin, I.M., and Sachs, G. (1998): *Age Related Diseases: Biology and Treatment*, pp. 242–245. Schnetztor-Verlag, Konstanz, Germany.
171. Monro, A. (1992): What is an appropriate measure of exposure when testing drugs for carcinogenicity in rodents? *ToxicoL Appl. PharmacoL*, 112:171–181.
172. Monro, A. (1993): How useful are chronic (life-span) toxicology studies in rodents in identifying Pharmaceuticals that pose a carcinogenic risk to humans? *Adv. Drug React. ToxicoL Rev.*, 12:5–34.
173. Munro, I.C. (1977): Considerations in chronic testing: The chemical, the dose, the design. *J. Environ. PathoL ToxicoL*, 1:183–197.
174. Munro, I.C., Ford, R.A., Kennepohl, E., and Sprenger, J.G. (1996): Thresholds of toxicological concern based on structure-activity relationships. *Drug Metab. Rev.*, 28:209–217.
175. Morrison, V., and Ashby, J. (1994): A preliminary evaluation of the performance of the Muta mouse (bac Z) and Big Blue (bac I) transgenic mouse mutation assays. *Mutagenesis*, 9:367–376.

176. Mulder, G.J. (1986): Sex differences in drug conjugation and their consequences for drug toxicity. Sulfation, glucuronidation and glutathione conjugation. *Chem. Biol. Interact.*, 57:1–15.
177. National Cancer Institute. (1994): *Survey of Compounds Which Have Been Tested for Carcinogenic Activity.* NIH Publ. No. 94–3765, Washington, DC.
178. National Institutes of Health. (1981): *NIH Guidelines for the Laboratory Use of Chemical Carcinogens.* NIH Publ. No. 81–2385, Washington, DC.
179. National Institutes of Health. (1986): *Humane Care and Use of Laboratory Animals.* NIH Publ. No. 86–23, Washington, DC.
180. National Research Council. (1983): *Risk Assessment in the Federal Government: Managing the Process.* National Academy Press, Washington, DC.
181. National Research Council. (1993): *Use of Maximum Tolerated Dose in Animal Bioassay s for Carcinogenicity.* National Academy Press, Washington, DC.
182. National Research Council. (1996): *Guide for the Care and Use of Laboratory Animals.* National Academy Press, Washington, DC.
183. National Toxicology Program. (1997): *Effect of dietary restriction on toxicology and carcinogenesis studies in F344/N rats and B6C3F*1 *mice.* NTP Tech. Rep. 460. NIH Publ. No. 97–3376. Research Triangle Park, NC.
184. Nelson, D.R., Koymans, L., Kamatski, T., Stegeman, J.J., Feyereisen, R., Waxman, D.J., Waterman, M.R., Gotoh, O., Coon, M.J., Estabrook, R.W., Gunsalus, I.C., and Nebert, D.W. (1996). P450 superfamily: Update on new sequences, gene mapping ascession numbers and nomenclature. *Pharmacogenetics*, 6:1–42.
185. Neumann, F. (1991): Early indicators for carcinogenesis in hormone sensitive organs. *Mutat. Res.*, 248:341–356.
186. Newberne, P.M., and dela Iglesia, F.A. (1985): Philosophy of blind slide reading in toxicologic pathology. *ToxicoL PathoL*, 13:225.
187. Newberne, P.M., and Sotnikov, A.V. (1996): Diet: the neglected variable in chemical safety evaluations. *ToxicoL PathoL*, 24:746–756.
188. Noble, J.F. (1984): Automated data acquisition systems in the 80s and beyond. II. Operation. In: *Toxicology Laboratory Design and Management for the 80s and Beyond*, edited by A.Tegeris, pp. 143–158. Karger, New York.

189. Office of Science and Technology Policy. (1985): Chemical carcinogens: A review of the science and its associated principles. *Fed. Reg.*, March 14:10371–10442.
190. Office of Technology Assessment. (1987): *Identifying and Regulating Carcinogens, A Background Paper*. U.S. Congress, Washington, DC.
191. Organization for Economic Cooperation and Development. (1981): *Adopted Guidelines for Testing of Chemicals*, Section 4—Health effects, Number 451—Carcinogenicity Studies. Paris.
192. Page, N.P., Singh, D.V., Farland, W., Goodman, J.I., Conolly, R. B., Anderson, M.E., Clewell, H.J., Frederick, C.B., Yamasaki, H., and Lucier, G. (1997): Implementation of EPA revised cancer assessment guidelines: Incorporation of mechanistic and pharmacokinetic data. *Fundam. Appl. Toxicol.*, 37:16–36.
193. Peck, C.C., Barr, W.H., and Benet, L.Z. (1992): Opportunities for integration of pharmacokinetics, pharmacodynamics and toxicokinetics in rational drug design. *J. Pharm. Sci.*, 81:605–610.
194. Perrone, C.E., Shao, L., and Williams, G.M. (1998): Effect of rodent hepatocarcinogenic peroxisome proliferatiors on fatty acyl-CoA oxidase, DNA synthesis, and apoptosis in cultured human and rat hepatocytes. *Toxicol. Appl. Pharmacol*, 150:277–286.
195. Peto, R., Pike, M.C., Day, N.E., Gray, R.G., Lee, P.N., Parish, S., Peto, J., and Wahrendorf, J. (1980): Guidelines for simple, sensitive significance tests for carcinogenic effects in long-term animal experiments. In: *Long-Term and Short-Term Screening Assays for Carcinogens: A Critical Appraisal*, edited by R.Montesano, H. Bartsch, and L.Tomatis, pp. 311–426. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Suppl. 2. Lyon, France.
196. Peto, R., Pike, M.C., Bernstein, L., Gold, L.S., and Ames, B.N. (1984): A proposed general convention for the numerical description of carcinogenic potency of chemicals in chronic-exposures animal experiments. *Environ. Health Perspect.*, 58:1–8.
197. Poteracki, J., and Walsh, K.M. (1998): Spontaneous neoplasms in control Wistar rats: A comparison of reviews. *Toxicol. Sci.*, 45:1–8.
198. Powles, P. (1996): Interpretation of data from toxicokinetic studies. *Scand. J. Lab. Anim. Sci.*, 23:317–323.
199. Randerath, K., Reddy, M.V., and Gupta, R.C. (1981): 32P-postlabeling test for DNA damage. *Proc. Natl. Acad. Sci. USA*, 78:626–6129.
200. Rao, G.N., Piegorsch, W.W., and Haseman, J.K. (1987): Influence of body weight on the incidence of spontaneous tumors in rats and mice of long-term studies. *Am. J. Clin. Nutr.*, 45:252–260.
201. Rao, G., Haseman, J., Grumbein, S., Crawford, S., and Eustis, S. (1990): Growth, body weight, survival and tumor trends in (C57Bl/6xC3H/NeN) Fl (B6C3F1) mice during a nine year period. *Toxicol. Pathol*, 18:71–77.
202. Reuter, J., and Hobbelen, H. (1977): The effect of continuous light exposure on the retina in albino and pigmented rats. *Physiol. Behav.*, 18:939–944.
203. Rice, J.M., Williams, G.M., Palmer, A.E., London, W.T., and Sly, D.L. (1981): Pathology of gestational choriocarcinoma induced in patas monkeys by ethylnitrosourea given during pregnancy. *Placenta Suppl.*, 3:223–230.
204. Rice, J.M., Baan, R.A., Blettner, M., Genevois-Chameau, C., Grosse, Y., McGregor, D.B., Partensky, C., and Wilbourn, J. D. (1999): Rodent tumors of urinary bladder, renal cortex, and thyroid gland in IARC monographs evaluation of carcinogenic risk to humans. *Toxicol. Sci.*, 49:166–171.
205. Riley, V. (1975): Mouse mammary tumors: Alteration of incidence as apparent function of stress. *Science*, 189:465–467.
206. Riley, V. (1981): Psychoneuroendocrine influences on immune-competence and neoplasia. *Science*, 212:1100–1109.
207. Robens, J.F., Calabrese, E.J., Piegorsch, W.W., Schueler, R.L., and Hayes, A.W. (1994): Principles of testing for Carcinogenicity. In: *Principles and Methods of Toxicology*, 3rd ed., edited by A. W.Hayes, pp. 697–728. Raven Press, New York.
208. Rosai, J. (1981): Breast. In: *Ackerman's Surgical Pathology*, pp. 1098–1149, C.V. Mosby, St. Louis, MO.
209. Russo, J., Russo, I.H., Rogers, A.E., Van Zwieten, M.J., and Gusterson, B. (1990): Tumours of the mammary gland. In: *Pathology of Tumours in Laboratory Animals*, vol. I, *Tumors of the Rat*, edited by V.S.Turusov and U.Mohr, pp. 47–78. IARC Science Publ. No. 99, Lyon, France.
210. Sacco, M.G., Zecca, L., Bagnasco, L., Chiesa, G., Parotini, C., Bromley, P., Cato, E.M., Roncucci, R., Clerici, L.A., and Veggoni, P. (1997): A transgenic mouse model for the detection of cellular stress inducted by toxic inorganic compounds. *Nature Biotechnol*, 15:1392–1397.
211. Safe, S. (1997/1998): Limitations of the toxic equivalency factor approach for risk assessment of

TCDD and related compounds. *Teratogen. Carcinogen. Mutagen.*, 17:285–304.
212. Saitoh, A., Kimura, M., Takahashi, R., Yokoyama, M., Nomura, T., Izawa, M., Sekiya, T., Nishimura, S., and Katsuki, M. (1990): Most tumors in transgenic mice with human c-Ha-ras gene contained somatically activated transgenes. *Oncogene*, 5:1195–1200.
213. Sambuco, C.P., Davies, R.E., Forbes, P.D., and Hoberman, A. M. (1991): Photocarcinogenesis and consumer product testing: Technical aspects. *Toxicol. Methods*, 1:75–83.
214. Sansone, E.B., and Losikoff, A.M. (1978): Contamination from feeding volatile test chemicals. *Toxicol. Appl. Pharmacol.*, 46:703–708.
215. Sansone, E.B., and Tewari, Y.B. (1978): Penetration of protective clothing materials by l,2-dibromo-3-chloropropane, ethylene dibromide, and acrylonitrile. *J. Am. Ind. Hyg. Assoc.*, 39:921–922.
216. Sansone, E.B., and Tewari, Y.B. (1978): The permeability of laboratory gloves to selected solvents. *J. Am. Ind. Hyg. Assoc.*, 39:169–174.
217. Sarkar, D.K., Gottschall, P.E., and Meites, J. (1982): Damage to hypothalamic dopaminergic neurons associated with development of prolactin-secreting pituitary tumors. *Science*, 218:684–686.
218. Sasahara, K., Ando-Lu, J., Nishiyama, K., Takahashi, M., Yoshida, M., and Maekawa, A. (1998): Granular cell foci of the uterus in Donryu rats. *J. Comp. Pathol.*, 119:195–199.
219. Scheuplein, R.J., and Blank, I.H. (1971): Permeability of the skin. *Physiol. Rev.*, 51:702–743.
220. Schyve, P.M., Smithline, F., and Metzger, H.Y. (1978): Neuroleptic-induced prolactin level elevation and breast cancer. *Arch. Gen. Psychiatry*, 35:1291–1301.
221. Seifter, E., Rettura, G., Zisblatt, M., Levenson, S.M., Levine, N., Davidson, A., and Seigter, J. (1973): Enhancement of tumor development of physically-stressed mice inculated with an oncogenic virus. *Experientia*, 29:1379–1882.
222. Seilkop, S.K. (1995): The effect of body weight on tumor incidence and Carcinogenicity testing in B6C3F] mice and F344 rats. *Fundam. Appl. Toxicol.*, 24:247–259.
223. Serabian, M.A., and Pilaro, A.M. (1999): Safety assessment of biotechnology-derived pharmaceuticals: ICH and beyond. *Toxicol. Pathol.*, 27:27–31.
224. Sieber, S.M., and Adamson, R.H. (1978): Long-term studies on the potential Carcinogenicity of artificial sweeteners in non-human primates. In: *Health and Sugar Substitutes*, edited by B. Guggenheim, pp. 266–271. Karger, Basel, Switzerland.
225. Shah, V.P., Midha, K.K., Dighe, S., McGilveray, I.J., Skelly, J. P., Yacobi, A., Layloff, T., Viswanathan, C.T., Cook, C.E., McDowall, R.D., Pittman, K.A., and Spector, S. (1992): Analytical methods validation—Bioavailability, bioequivalence and pharmacokinetic studies. *J. Pharmaceut. Res.*, 81:309–312.

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226. Shirai, T., Hirose, M., and Ito, N. (1999): Medium-term bioassays in rats for rapid detection of the carcinogenic potential of chemicals. In: *The Use of Short- and Medium-Term Tests for Carcinogenic Hazard Evaluation,* edited by D.B.McGregor, J.M. Rice, and S.Venitt, pp. 251–271. IARC Publ. No. 146, Lyon, France.
227. Smith, D.M., Rogers, A.E., and Newberne, P.M. (1975): Vitamin A and benzo(a)pyrene carcinogenesis in the respiratory tract of hamsters fed a synthetic diet. *Cancer Res.,* 35:1485–1488.
228. Smith, D.M., Ortiz, L.W., Archuleta, R.F., Spalding, J.F., Tillery, M.I., Ettinger, H.J., and Thomas, R.G. (1981): A method of chronic nose-only exposures of laboratory animals to inhaled fibrous aerosols. In: *Inhalation Toxicology and Technology,* edited by H.P.Leong, pp. 89–105. Ann Arbor Science, Ann Arbor, MI.
229. Sontag, J.R., Page, N.P., and Safiotti, U. (1976): *Guidelines for Carcinogen Bioassay in Small Rodents.* DHHS Publication (NIH) 76–801, National Cancer Institute, Bethesda, MD.
230. Spalding, J.W., French, J.E., Tice, R.R., Furedi-Machek, M., Haseman, J.K., and Tennant, R.W. (1999): Development of a transgenic mouse model for carcinogenesis bioassays: Evaluation of chemically induced skin tumors in Tg.AC mice. *Toxicol. Sci.,* 49:241–254.
230a. Spratt, T.E., Schultz, S.S., Levy, D.E., Chen, D., Schliiter, G., and Williams, G.M. (1999): Different mechanisms for the photo-induced production of oxidative damage by fluoroquinolones differing in photostability. *Chem. Res. Toxicol.,* 12:805–815.
231. Stringer, C. (1992): Safety workshop-ICH 1. *Regul. Affairs J.,* 3:350–356.
232. Tamano, S., Hagiwara, A., Shibata, M., Kurata, Y., Fukushima, S., and Ito, N. (1988): Spontaneous tumors in aging B6C3Fi mice. *Toxicol. Pathol,* 16:321–326.
233. Tarone, R.E. (1975): Tests for trend in life table analysis. *Biometrika,* 62:679–682.
234. Tennant, R.W., Stasiewicz, S., Mennear, J., French, J.E., and Spalding, J.W. (1999). Genetically altered mouse models for identifying carcinogens: In: *The Use of Short- and Medium-Term Tests for Carcinogens and Data on Genetic Effects in Carcinogenic Hazard Evaluation,* edited by D.B.McGregor, J.M.Rice, and S. Venitt, pp. 23–148. IARC Science Publ. No. 146, Lyon, France.
235. Thake, D.C., Iatropoulos, M.J., Hard, G.C., Hotz, K.J., Wang, C.–X., Williams, G.M., and Wilson, A.G.E. (1995): A study of the mechanism of butachlor-associated gastric neoplasms in Sprague-Dawley rats. *Exp. Toxicol. Pathol.,* 47:107–116.
236. Thomas, G.A., and Williams, E.D. (1991): Evidence for and possible mechanisms of non-genotoxic carcinogenesis in the rodent thyroid. *Mutat. Res.,* 248:357–370.
237. Thorgiersson, U., Dalgard, D., Reeves, J., and Adamson, R. (1994): Tumor incidence in a chemical carcinogenesis study of nonhuman primates. *Regul. Toxicol. Pharmacol.,* 19:130–151.
238. Thurman, J.D., Bucci, T.J., Hart, R.W., and Torturro, A. (1994): Survival, body weight, and spontaneous neoplasms in ad libitum—fed and food-restricted Fischer-344 rats. *Toxicol. Pathol.,* 22:1–9.
239. Tomatis, L. (1989): Overview of perinatal and multigeneration carcinogenesis. In: *Perinatal and Multigeneration Carcinogenesis,* edited by N.P.Napalkov, J.M.Rice, L.Tomatis, and H. Yamasaki, pp. 1–15. IARC Science Publ. No. 96, Lyon, France.
240. Tsuda, H., Park, C.B., and Moore, M.A. (1999): Short- and medium-term carcinogenicity tests. In: *The Use of Short- and Medium-Term Tests for Carcinogenic Hazard Evaluation,* edited by D.B.McGregor, J.M.Rice, and S.enitt, pp. 203–249. IARC Publ. No. 146, Lyon, France.
241. Tuch, K., Ockert, D., Hauschke, D., and Christ, B. (1992): Comparison of the ECL-cell frequency in the stomachs of 3 different rat strains. *Pathol. Res. Pract.,* 188:672–675.
242. Tugwood, J.D., and Elcombe, C.R. (1999): Predicting carcinogenicity: Peroxisome proliferators. In: *Carcinogenicity,* edited by K.T.Kitchin, pp. 337–360. Marcel Dekker, New York.
243. Turturro, A., Duffy, P., Hart, R., and Allaben, W.T. (1996): Rationale for the use of dietary control in toxicity studies—B6C3F1 mouse. *Toxicol. Pathol.,* 24:769–775.
244. Turusov, V.S. (1994): Histiocytic sarcoma. In: *Pathology of Tumours in Laboratory Animals,* vol. II, *Tumors of the Mouse,* edited by V.S. Turusov and U. Mohr, pp. 671–680. IARC Science Publ. No. III, Lyon, France.
245. U.S. Congress. (1985): *Animal Welfare Act.* CFR 9, Parts 1, 2, 3, Washington, DC.
246. U.S.Congress. (1996): *Food Quality Protection Act.* Public Law 104–170 of August 3, 1996. Washington, DC.
247. U.S. Congress. (1998): *Food Quality Protection Act Amendment.* Public Law 105–324 of October 30, 1998. Washington, DC.
248. U.S. Department of Agriculture. (1989): *Animal Welfare Rules.* CFR 9, Parts 1, 2. Washington, DC.
249. U.S. Department of Health and Welfare. (1977): *Guide for Care and Use of Laboratory Animals.*

Publl. No. NIH 77–23. Washington, DC.
249a. U.S. Department of Health and Human Services (2000): *Ninth Report on Carcinogens.* PHS, NTP, Research Triangle Park, NC.
250. U.S. Environmental Protection Agency. (1986): Guidelines for carcinogen risk assessment. *Fed. Reg.*, 51:33992–34005.
251. U.S. Environmental Protection Agency. (1990): *Good Automated Practices*, Draft 12–28–90. Scientific Systems Staff, Office of Information Resources Management. U.S. EPA, Washington, DC.
252. U.S. Environmental Protection Agency. (1991): *Alpha 2u-globulin: Association with chemically induced renal toxicity andneoplasia in the male rat. Risk Assessment Forum.* EPA/625/3–91/019F. U.S. EPA, Washington, DC.
253. U.S. Environmental Protection Agency. (1992): A cross-species scaling factor for carcinogen risk assessment based on equivalence of mg/kg3/4/day: Notice. *Fed. Reg.*, 57:24152–24173.
254. U.S. Environmental Protection Agency. (1992): Guidelines for exposure assessment. *Fed. Reg.*, 57:22888–22938.
255. U.S. Environmental Protection Agency. (1996): Proposed guidelines for carcinogen risk assessment. *Fed. Reg.*, 61:17960–18011.
256. U.S. Environmental Protection Agency. (1996): Proposed guidelines for ecological risk assessment; Notice. *Fed. Reg.*, 61:47052–47631.
257. U.S. Environmental Protection Agency. (1997): *Exposure Factors Handbook.* Office of Research and Development, EPA/ 600/P-95–002A, Washington, DC.
258. U.S. Environmental Protection Agency. (1998): *Health Effects Test Guidelines.* OPPTS 870.4200 and OPPTS.4300, EPA 712-C-98–211 and 212. Washington, DC.
259. U.S. Food and Drug Administration. (1978): Good Laboratory Practices for Nonclinical Laboratory Studies, Code of Federal Regulations, Title 21, Part 58. *Fed. Reg.*, 43:59986–60025.
260. U.S. Food and Drug Administration. (1993): *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food.* Redbook II. Washington, DC.
261. U.S. Food and Drug Administration. (1993): Advisory committee for protocols for safety evaluation, panel on carcinogenesis: Report on cancer testing in the safety of food additives and pesticides. *Toxicol. Appl. Pharmacol.*, 20:419–438.
262. U.S. Food and Drug Administration. (1994): International Conference on Harmonization; Draft guideline on dose selection for carcinogenicity studies of Pharmaceuticals. *Fed. Reg.*, 59:9752–9760. Available at http://www.ifpma.org/ich5s.html
263. U.S. Food and Drug Administration. (1994): *General Principles for Evaluating the Safety of Compounds Used in Food-Producing Animals.* Center for Veterinary Medicines, Washington, DC.

264. U.S. Food and Drug Administration. (1995): Food additives: Threshold of regulation for substances used in food contact articles. *Fed. Reg.*, 60:36582–36596.
265. U.S. Food and Drug Administration. (1997): International Conference on Harmonization: Guidance on preclinical safety evaluation of biotechnology-derived Pharmaceuticals. *Fed. Reg.*, 62:61515–61519. available at http://www.iben.gov
266. U.S. Food and Drug Administration. (1997): International Conference on Harmonization: Guidelines on impurities in new drug products. *Fed. Reg.*, 62:27454–27461.
267. van Oosterhout, J.P.J., van der Laan, J.W., de Waal, E.J., Olejniczak, K., Hilgenfeld, M., Schmidt, V., and Bass, R. (1997): The utility of two rodent species in carcinogenic risk assessment of Pharmaceuticals in Europe. *Regul. Toxicol. Pharmacoi*, 25:6–17.
268. van Steeg, H., Klein, H., Beems, R.B., and van Kreijl, C.F. (1998): Use of DNA repair-deficient XPA transgenic mice in short-term carcinogenicity testing. *Toxicol. Pathol.*, 26:742–749.
269. Vessel, E.S. (1967): Induction of drug metabolizing enzymes in liver microsomes of mice and rats by softwood bedding. *Science*, 157:1057–1058.
270. Viollon-Abadie, C., Lassere, D., Debruyne, E., Nicod, L., Carmichael, N., and Richert, L. (1999): Phenobarbital, ^-napthoflavone, clofibrate, and pregnenolone-16a-carbonitrile do not affect hepatic thyroid hormone UDP-glucuronosyl transferase activity, and thyroid gland function in mice. *Toxicol. Appl. Pharmacoi.*, 155:1–12.
271. Walsh, K.M., and Poteracki, J. (1994): Spontaneous neoplasms in control Wistar rats. *Fundam. Appl. Toxicol.*, 23:65–72.
272. Wallenberg, L.W. (1985): Chemoprevenlion of cancer. *Cancer Res.*, 45:1–8.
273. Weisburger, E.K. (1983): History of the bioassay program of Ihe Nalional Cancer Inslilule. *Prog. Exp. Tumor Res.*, 26:187–201.
274. Weisburger, J.H., and Williams, G.M. (1984): Bioassay of carcinogens: In vilro and in vivo lests. In: *Chemical Carcinogenesis*, ACS Monograph 182, 2nd ed., vol. 2, pp. 1323–1373. American Chemical Society, Washington, DC.
275. Weisburger, J.H., and Williams, G.M. (1995): Causes of cancer. In: *American Cancer Society Textbook of Clinical Oncology*, edited by G.P.Murphy, W.Lawrence, Jr., and R.E.Lenhard, Jr., pp. 10–39. ACS, Atlanta, GA.
276. Welling, P.G. (1993): Pharmacokinetic principles. In: *Drug Toxicokinetics*, ediled by P.G.Welling and F.A.de la Iglesia, pp. 69–83. Marcel Dekker, New York.
277. Welsch, C.W., and Nasagawa, H. (1977). Prolactin and murine mammary tumorigenesis: A review. *Cancer. Res.*, 37:951–963.
278. Whysner, J., and Williams, G.M. (1992): International cancer risk assessment: The impact of biologic mechanisms. *Regul. Toxicol. Pharmacoi.*, 15:41–50.
279. Williams, G.M. (1987): Definition of a human cancer hazard. In: *Nongenotoxic Mechanisms in Carcinogenesis*, Banbury Report 25, pp. 367–380. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
280. Williams, G.M. (1989): Methods for evaluating chemical genotoxicity. *Annu. Rev. Pharmacoi. Toxicol.*, 29:189–211.
281. Williams, G.M. (1989): Interactive Carcinogenesis in the liver. In: *Liver Cell Carcinoma*, Falk Symposium 51, edited by P.Bannasch, D.Keppler, and G.Weber, pp. 197–216. Kluwer, Boston. MA.
282. Williams, G.M. (1990): Screening procedures for evaluating the potential carcinogenicity of food-packaging chemicals. *Regul. Toxicol. Pharmacoi.*, 12:30–40.
283. Williams, G.M. (1992): DNA reactive and epigenetic carcinogens. *Exp. Toxicol. Pathol.*, 44:457–464.
284. Williams, G.M. (1999): Chemical-induced preneoplastic lesions in rodents as indicators of carcinogenic activity. In: *The Use ofShortand Medium-Term Tests for Carcinogens and Data on Genetic Effects in Carcinogenic Hazard Evaluations*, edited by D.B. McGregor, J.M.Rice, amd S.Venitt, pp. 185–202. IARC Publ. No. 146, Lyon, France.
285. Williams, G.M. (1999): Mechanistic considerations in cancer risk assessment. *Inhal. Toxicol.*, 11:549–554.
286. Williams, G.M., Laspia, M.F., and Dunkel, V.C. (1982): Reliability of the hepatocyte primary culture/DNA repair test in testing of coded carcinogens and non carcinogens. *Mutat. Res.*, 97:359–370.
287. Williams, G.M., Mori, H., and McQueen, C.A. (1989): Structure-activity relationships in the rat hepatocyte DNA-repair test for 300 chemicals. *Mutat. Res.*, 221:263–286.
288. Williams, G.M., and Weisburger, J.H. (1991): Chemical Carcinogenesis. In: *Toxicology, The Basic Science of Poisons*, edited by M.O.Amdur, J.Doull, and C.D.Klaassen, pp. 127–200. Pergamon, New

York.
289. Williams, G.M., Iatropoulos, M.J., and Weisburger, J.H. (1996): Chemical carcinogen mechanisms of action and implications for testing methodology. *Exp. Toxicol. Pathol.*, 48:101–111.
290. Williams, G.M., and Perone, C. (1996): Mechanism-based risk assessment of peroxisome proliferating rodent hepatocarcinogens. In: *Peroxisomes: Biology and Role in Toxicology and Disease*, edited by J.K.Reddy, T.Suga, G.P.Mannaerts, P.B.Lazarow, and S.Subramani, pp. 554–572. New York Academy of Sciences, vol. 804, New York.
291. Williams, G.M., Karbe, E., Fenner-Crisp, P., Iatropoulos, M.J., and Weisburger, J.H. (1996): Risk assessment of carcinogens in food with special consideration of non-genotoxic carcinogens. *Exp. Toxicol. Pathol.*, 48:209–215.
292. Williams, G.M., Iatropoulos, M.J., Jeffrey, A.M., Luo, F.Q., Wang, C.-X., and Pittman, B. (1999): Diethylnitrosamine exposure-response for DNA ethylation, hepatocellular proliferation and initiation of Carcinogenesis in rat liver display non-linearities and thresholds. *Arch. Toxicol.*, 73:394–402.
293. Williams. G.M., Iatropoulos, M.J., and Whysner, J. (1999): Safety assessment of butylated hydroxyanisole and butylated hydroxytoluene as antioxidant in food additives. *Food Chem. Toxicol*, 37:1027–1038.
294. Williams, G.M., Iatropoulos, M.J., and Jeffrey, A.M. (2000): Mechanistic basis for nonlinearity and thresholds in rat liver Carcinogenesis by the DNA-reactive carcinogens 2-acetyl aminofluorene and diethylnitrosamine. *Toxicol. Pathol.*, 28:388–395.
295. Wiltse, J., and Dellarco, V.L. (1996): U.S. Environmental Protection Agency guidelines for carcinogen risk assessment: Past and future. *Mutat. Res.*, 365:3–15.
296. Wolfe, F.J. (1980): Effect of overloading pathways on toxicity. *J. Environ. Pathol. Toxicol.*, 3:113–134.
297. Yamamoto, S., Urano, K., Koizumi, H., Wakana, S., Hioki, K., Mitsumori, K., Kurokawa, Y., Hayashi, Y., and Nomura, T. (1998): Validation of transgenic mice in carrying the human prototype c-Ha-ras gene as a bioassay model for rapid carcinogenicity testing. *Environ. Health Perspect.*, 106:57–69.
298. Zeiger, R. (1998): Identification of rodent carcinogens and non-carcinogens using genetic toxicity tests: Premises, promises, and performance. *Regul. Toxicol. Pharmacoi.*, 28:85–95.

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Chapter **21**

Principles of Clinical Pathology for Toxicology Studies

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Principles and Methods of Toxicology,
Fourth Edition, edited by A.Wallace Hayes.
Taylor & Francis, Philadelphia C 2001.

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Clinical pathology is an integral component of preclinical safety assessment and toxicity studies designed to identify target organs and establish dose-response relationships. In the context of these studies, clinical pathology usually consists of relatively routine hematology, clinical chemistry, and urinalysis tests. The majority of the parameters evaluated are identical to those used in human and veterinary medicine because the fundamental physiology and pathophysiology of blood and major organ systems are similar in most mammalian species. There are, of course, species differences for reference ranges, some methodologies, the value or appropriateness of individual tests, and interpretation of findings. Selection of tests for a toxicology study is dependent upon several factors, including study objectives, test species, regulatory requirements, and characteristics of the test material.

Clinical pathology tests are best characterized as screening tools to identify general metabolic or pathologic processes and target tissues. Although specific diagnoses and precise mechanisms for a toxic effect are infrequently identified, test results narrow the possibilities and help direct further studies. Clinical pathology tests also provide one measure for determining the biological importance of effects associated with administration of a test material. Alterations in clinical pathology test results are typically not the only evidence of adverse or pathologically significant toxicologic effects. In-life clinical observations and/or anatomical pathology findings usually corroborate pathologically meaningful laboratory findings.

Interpretation of clinical pathology data from a toxicology study is considerably different from the assessment of data from an individual patient suffering from an unknown illness. The most obvious difference is that data from groups of treated subjects, receiving increasing dose levels of a test material, are compared with data from a group of age-, weight-, and sex-matched control subjects that are concurrently exposed to the same environmental and experimental conditions. For larger laboratory animals (e.g., rabbits, dogs, monkeys), pretreatment clinical pathology data for each individual are also

available for comparison with posttreatment results. Finally, clinical pathology results from a toxicology study can be correlated with carefully recorded in-life observations, necropsy observations, organ weight data, and histopathologic findings for an extensive list of tissues. Given the uniformity of the animals studied and the analytical precision of modern clinical pathology instrumentation, identification of very subtle effects on clinical pathology results, which would not be apparent for an individual

patient, is the norm. One of the most challenging aspects of data interpretation for a toxicology study is differentiating potentially harmful toxic effects from minor changes representing subtle homeostatic or metabolic responses to benign effects of the test material or to study-related procedures. Proper interpretation of clinical pathology results from a toxicology study requires not only an understanding of the tests themselves, but knowledge of species differences, study design, unique studyrelated procedures, clinical observations, anatomical pathology findings, and the test material. Interpretation of one test result is frequently dependent upon the results of another test, and pattern recognition is essential.

This chapter addresses (a) experimental design considerations including test selection, timing and frequency of testing, sources of variability in clinical pathology test results with emphasis on preanalytical factors, and quality control, (b) basic principles of clinical pathology data interpretation including the use and misuse of reference ranges, and (c) the characteristics and interpretation of routine hematology, clinical chemistry, and urinalysis tests used in toxicology studies. For in-depth descriptions of clinical pathology tests, including methods, the reader is referred to references 8, 14, 63, 68, 72, 73, and 84.

EXPERIMENTAL DESIGN CONSIDERATIONS

The value of clinical pathology in toxicology studies is heavily influenced by the experimental design. Selection and timing of appropriate tests, consideration of unique study procedures, reduction of sources of variation, proper sample collection and handling, and controlled analytical technique are all factors that ultimately determine the worth of clinical pathology test results.

Test Selection

The selection of appropriate clinical pathology tests for a toxicology study is first dependent upon the objective or purpose of that study. If the objective is simply to screen a number of similar chemical entities for potential hepatocellular toxicity, it may be sufficient to limit the laboratory evaluation to a single test such as alanine aminotransferase activity. On the other hand, if the study is part of the package of studies required to support governmental approval of a new drug or other chemical entity with potential human exposure (e.g., food additives, pesticides, chemicals used in manufacturing), there are several tests required or recommended in study guidelines published by the presiding governmental regulatory agencies (e.g., U.S. Food and Drug Administration, FDA; U.S. Environmental Protection Agency, EPA; Japanese Ministry of Health and Welfare) or professional standards organization (e.g., the European Organization for Economic Co-operation and Development, OECD) (60). Although there are many similarities between the various published guidelines with respect to recommended clinical pathology tests, there are also several differences and instances of ambiguous or inappropriate testing requirements.

In an effort to encourage global harmonization of regulatory guidelines, a Joint Scientific Committee for International Harmonization of Clinical Pathology Testing was formed in 1992 to provide recommendations for clinical pathology testing of laboratory animals used in regulated safety assessment and toxicity studies. The committee was comprised of representatives from ten different professional organizations, located throughout the world, with scientific expertise in animal clinical pathology and was independent of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. The committee prepared a document (135) listing minimum recommendations for clinical pathology testing in regulated safety assessment and toxicity studies. These recommendations are described in the following paragraphs.

With respect to hematology, the core recommended tests are total white blood cell (WBC) count, absolute differential WBC count, red blood cell (RBC) count, hemoglobin concentration, hematocrit (or packed cell volume), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), evaluation of RBC morphology, and platelet count. The importance of calculating absolute WBC differential counts from the total WBC count and the relative (%) WBC differential counts was stressed. The method for evaluation of RBC morphology was not defined, but most laboratories prepare blood smears and examine the RBCs microscopically for morphologic characteristics such as variations in size (anisocytosis, microcytosis, or macrocytosis), color (polychromasia), shape (poikilocytosis), and hemoglobin content (hypochromasia). Other laboratories may choose to evaluate RBC morphology with the use of automated measurements such as MCV, MCH, MCHC, red cell distribution width (RDW), and hemoglobin distribution width (HDW). For those laboratories using automated measurements, it is prudent to routinely prepare blood smears in the event the data indicate a need to examine the cells microscopically.

Although not routinely recommended, absolute reticulocyte counts and bone marrow cytologic

examinations may be indicated by other hematology findings. For example, test material-induced anemia is an indication for performing absolute reticulocyte counts to assess whether the anemia is regenerative or non-regenerative. Unexplained nonregenerative anemia, leukopenia, thrombocytopenia, and pancytopenia are indications for performing bone marrow cytologic examinations. It was therefore recommended that blood

smears be prepared for possible reticulocyte counts and bone marrow smears be made at termination for possible cytologic examination. If a laboratory has the ability to perform automated reticulocyte counts, it may wish to do these routinely as an alternative to preparing reticulocyte count smears.

Prothrombin time (PT) and activated partial thromboplastin time (APTT) [or appropriate alternatives such as the Thrombotest (55)] and platelet count are the core recommended tests for assessment of hemostasis. If blood volume limitations are a concern (e.g., multiple blood collections for a rat study), it may be necessary to perform PT and APTT only at study termination.

Current regulatory guidelines recommend performing limited hematology tests on some or all animals at set intervals during a carcinogenicity/oncogenicity study (e.g., weeks 26, 52, 78, and 104). This approach to identifying leukemogenic test materials is very insensitive compared with the microscopic examination of multiple tissues from animals that died during the study, were terminated because of poor health, or survived to the terminal kill. For carcinogenicity/oncogenicity studies, the only procedure recommended by the Joint Scientific Committee is to make blood smears for all animals at unscheduled kills (e.g., moribund animals) and at the terminal kill. If necessary, the smears can be used as an adjunct to histopathology for the identification of hematopoietic neoplasia. For example, if the histopathologist is unsure whether leukocytic infiltrates in multiple tissues from an animal represent leukemia or a leukemoid response (i.e., marked leukocytosis secondary to an inflammatory stimulus), the blood smear can be examined to help differentiate between the two conditions. It may also be possible to diagnose a specific type of leukemia from the blood smear (e.g., lymphocytic leukemia versus granulocytic leukemia).

With respect to clinical chemistry, the core recommended tests are glucose, urea nitrogen, creatinine, total protein, albumin, globulin (calculated from total protein and albumin), cholesterol, calcium, sodium, potassium, and selected tests of hepatocellular and hepatobiliary health and function. Measurement of at least two scientifically appropriate tests for hepatocellular evaluation (e.g., alanine aminotransferase, aspartate aminotransferase, sorbitol dehydrogenase, glutamate dehydrogenase, or total bile acids) and at least two scientifically appropriate tests for hepatobiliary evaluation (e.g., alkaline phosphatase, gamma glutamyltransferase, 5′-nucleotidase, total bilirubin, or total bile acids) was recommended. Because there are several acceptable tests used to evaluate hepatic health and function, the Joint Scientific Committee decided it was appropriate to give each laboratory the freedom to choose those tests that best met their individual needs and with which they had the most experience. For example, glutamate dehydrogenase is commonly evaluated in Europe, but no commercial kit for this enzyme assay is available in the United States.

The core recommended urinalysis tests, performed on an overnight sample (i.e., approximately 16-h collection), are an assessment of urine appearance (color and turbidity), volume, specific gravity or osmolality, pH, and either the quantitative or semiquantitative determination of total protein and glucose.

The Joint Scientific Committee listed several tests that are specifically not recommended for routine use in animal toxicity and safety studies. These tests include ornithine decarboxylase, ornithine carbamoyltransferase, lactate dehydrogenase, creatine kinase, serum or plasma protein electrophoresis, microscopic examination of urine sediment, and urinary mineral and electrolyte excretion (e.g., urine sodium, potassium, chloride, calcium, or inorganic phosphorus excretion). Although ornithine decarboxylase appears in the test lists of several regulatory guidelines, it has no value as a diagnostic clinical chemistry test (19). This enzyme may have been included in the original FDA guidelines by mistake, and the error was repeated by other organizations. The FDA may have intended to include ornithine carbamoyltransferase, a liver-specific enzyme involved in the urea cycle that enjoyed limited popularity as a diagnostic test in the late 1970s. This enzyme never demonstrated a clear diagnostic advantage over other more common liver enzymes (e.g., alanine aminotransferase) and is rarely measured in today's laboratories. Lactate dehydrogenase is very similar to aspartate aminotransferase, and the use of another nonspecific enzyme is not considered beneficial. Creatine kinase may be helpful for evaluating test materials that cause muscle injury but is not considered necessary for the great majority of test materials, especially if aspartate aminotransferase is already part of the clinical chemistry test list. Blood collection techniques that potentially damage muscle and contaminate the blood sample (e.g., cardiac puncture and retro-orbital plexus collection) can diminish the value of measuring muscle enzyme activities by increasing variability. As a diagnostic test for patients, serum or plasma protein electrophoresis is used to evaluate large, unexplained increases or decreases in globulin concentration. With respect to increased globulin concentration, the goal of protein electrophoresis is to rule out a monoclonal gammopathy caused by some cancers of lymphoid origin (e.g., plasma cell

myeloma). Monoclonal gammopathies and large, unexplained decreases in globulin concentration are rare in toxicity and safety studies, and the routine use of protein electrophoresis is inappropriate. Microscopic examination of urine sediment may be helpful for screening test materials that are known to cause severe renal or bladder toxicity, but histopathology is a more sensitive tool for detecting lesions of the kidney and bladder. In part, this is because

the collection of high-quality urine specimens from many animals at one time is very difficult. On rare occasions, examination of urine sediment may be valuable for detecting the presence of crystals specific for a test material. Measurement of urinary mineral or electrolyte excretion may be appropriate for test materials that are known to affect renal function (e.g., diuretics) or bone metabolism (e.g., parathyroid hormone), but as routine screening tests, these are inappropriate. If serum/plasma mineral and electrolyte concentrations are greatly affected by a test material and other causes for these findings are ruled out (e.g., vomiting, diarrhea, renal failure), an assessment of the renal handling of the mineral or electrolyte in question may then be valuable.

The recommendations of the Joint Scientific Committee and the requirements listed in the various regulatory guidelines should be viewed as a minimum database for screening the potential toxicity of a test material that will undergo extensive evaluation in studies of varying duration with multiple species before regulatory approval. There are many additional tests that may be appropriate to perform, depending upon the study objectives and known characteristics of the test material. Platelet function tests (e.g., platelet aggregation and bleeding times) may be appropriate for evaluating drugs that target platelets. Analysis of methemoglobin concentration or the enumeration of erythrocyte Heinz bodies can be valuable for assessing oxidative injury caused by a compound. Activity of plasma or red blood cell cholinesterase is a measure of exposure to organophosphates or carbamates. Activity of brain cholinesterase is a measure of the toxic effect of these compounds. Determination of urinary enzyme activities and characterization of urinary protein excretion can be used for screening related compounds known or suspected to cause renal toxicity (29, 77, 117). Various hormones may be measured when endocrine dysfunction is suspected, and lipoprotein analysis may help define effects on lipid metabolism. Serum inorganic phosphorus and chloride concentrations are commonly measured as part of standard chemistry profiles in the United States and can be helpful when assessing changes in calcium and sodium concentrations, respectively. Determination of alkaline phosphatase and creatine kinase isoenzyme activities may help differentiate the origin of increases in total activity of these enzymes. The list of potentially valuable clinical pathology tests is long and growing. The key is to use the tests judiciously. That is, a nonstandard test should be used for a specific purpose and only after the test has been proven useful for the species in question.

Frequency and Timing of Testing

Frequency and timing of clinical pathology testing are dependent upon study objectives, study duration, the bio logical activity of the test material, and the species tested. The Joint Scientific Committee made minimum recommendations (135) that may be modified because of these factors.

With respect to regulated, acute, or single-dose toxicity studies, it is interesting to note that the regulatory guidelines have no clinical pathology requirements. Traditionally in these studies, the animals are dosed once and killed and necropsied following a 2-week observation period. Although the purpose of many of these studies is to determine appropriate dose levels for future repeated-dose studies, a great deal can be learned about potential toxicities of the test material because the dose levels are frequently higher than those administered in the repeated-dose studies. If clinical pathology tests are utilized, it is a mistake to wait until the end of the observation period to obtain samples. Two weeks following the administration of near-lethal doses of carbon tetrachloride or mercuric chloride to rats, standard clinical pathology tests will fail to recognize the effects of these compounds on the liver and kidney, respectively, because of the regenerative capacity and large functional reserve of these tissues. Although there is no single optimal time for all test materials, a general guideline for clinical pathology testing following an acute dose is to obtain samples between 24 and 72 h postdose. Occasionally, specific clinical pathology tests may be most appropriate within hours postdose because the test material has a very short half-life or a limited duration of action (e.g., some cytokines, peptides, and organophosphates). Following administration of acute renal toxins, urinary enzyme activities tend to be greatest in the first 24 to 48 h postdose. Alternatively, the effects of some test materials (e.g., cytotoxic chemotherapeutic agents) may take longer to reach their peak. For these test materials, it may be best to wait several days before performing clinical pathology tests or to take samples at multiple times in order to distinguish the time of greatest effect (e.g., days 4, 7, 10, and 14).

Limited blood volume in mice dictates that blood sample collection is usually practical only when the animals are killed. Because the blood volume of a 30-g mouse is less than 2 ml, it is difficult to acquire a full milliliter of blood from a single mouse, regardless of the blood collection technique. If several tests are required or desired (e.g., full hematology and clinical chemistry profiles), it may be necessary to specify certain animals for hematology tests and others for clinical chemistry tests. A preferred alternative is to limit the number of clinical chemistry tests to those that are the most relevant as

screening tools for major organ toxicity (e.g., urea nitrogen or creatinine, alanine aminotransferase or another liver-specific enzyme, total protein, and albumin). Pooling of blood samples is inappropriate for clinical pathology testing.

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Prestudy clinical pathology testing is not recommended for rats because of the relatively large number of animals per group, the homogeneity of the population, and the risk of adversely affecting the health of young animals due to blood loss or the blood collection procedure. For repeated-dose studies in rats, testing should at least be done at study termination. Interim testing may not be necessary for long-duration studies (e.g., a 13-week study) if testing were done in short-duration studies (e.g., a 4-week study) that used dose levels not substantially lower than those of the long-duration studies. On the other hand, interim testing (e.g., a week 6 testing point during a 13-week study) can be beneficial for interpretation of subtle effects. Clinical pathology testing is not recommended for rodents after 52 weeks because naturally occurring geriatric disease conditions (e.g., ulceration and infection of mammary gland tumors, chronic progressive nephropathy, pituitary tumors disrupting normal endocrine function) obscure meaningful interpretation of laboratory data.
For repeated-dose studies in dogs and monkeys, testing should be done before initiation (i.e., prestudy or baseline), at least once during the study, and at study termination. Animals shipped from a supplier should have several days to acclimate to the new environment before baseline testing is performed. The baseline data are important for screening out animals with potential health problems or results that are notably different from those of their peers. Findings that may signal possible subclinical health problems and that are commonly cited for eliminating animals from study consideration include low values for erythrocyte parameters (e.g., red blood cell count, hemoglobin concentration, hematocrit, and mean corpuscular volume), high white blood cell and neutrophil counts, low total protein and albumin concentrations, high globulin concentration, and high liver enzyme activities. There may be a specific need to eliminate otherwise healthy animals because they have findings that might complicate interpretation of test material-related effects. For example, beagles that are heterozygotes for factor VII deficiency have slightly prolonged prothrombin times. Even though these animals are clinically normal, it may be inappropriate to include one in a study evaluating a product that targets coagulation. One might choose to exclude an animal with a normally low neutrophil count from a study of a chemotherapeutic drug because it may be more difficult to determine if, or how much, the drug is impacting neutrophil production. By the same token, if an animal has an unusually high neutrophil count at a baseline interval simply because of excitement or fear during blood collection (i.e., physiological leukocytosis), then a subsequent postdose decrease in the neutrophil count, when the animal is less fearful of various procedures, could be overinterpreted as caused by the test material.
The advantage of using test animals such as dogs and monkeys with greater blood volume enabling more frequent clinical pathology evaluations is offset by the low number of animals in each treatment group (often four or less per sex per group) and the increased variability between animals for many tests. The lower the number of animals studied, the more desirable it is to have more than one baseline interval. Because monkeys tend to exhibit greater variability than dogs, it can be helpful for data interpretation to have more than one baseline interval for studies using monkeys, regardless of the number of animals per sex per group. An additional benefit to multiple baseline intervals is that the animals become more accustomed to the blood collection procedures, and variability caused by excitement or fear is generally reduced. In some range-finding studies, it is not unusual to have only one animal per sex per group. In essence, each animal serves as its own control. Using at least two baseline intervals affords an appreciation for normal day-to-day, intra-animal variability. Finally, two baseline intervals are generally desirable for animals that have been surgically manipulated (e.g., placement of indwelling intravenous catheters) to avoid using animals with iatrogenic complications.
The timing and number of clinical pathology intervals for repeated-dose studies in dogs and monkeys are often dictated by the test material. For studies of 6 weeks duration or less, an interim testing interval is sometimes recommended within 7 days of initiation of dosing. The primary purpose for this early interval is to detect transient increases in serum enzyme activities that may be absent at later intervals (34). This information can be very important for the clinical trials. For studies with cytotoxic chemotherapeutic agents, the number and frequency of hematology intervals are often considerable because one common objective is to identify the nadir for circulating leukocyte counts and the timing of hematopoietic recovery. For example, a single-dose study of a chemotherapeutic agent in dogs might require hematology tests twice before initiation of treatment and at days 4, 7, 10, 14, 21, and 28 and clinical chemistry tests twice before initiation of treatment and at days 7 and 28.
Urinalysis testing should be conducted at least once during a repeated-dose study. It is best to conduct the urinalysis testing at the same time as other clinical pathology tests. Although not stated in the Joint Scientific Committee's document, urinalysis testing for mice is impractical and not recommended as a routine test.

Sources and Control of Preanalytical Variation

Although most toxicology studies are relatively well controlled, there are many study design and procedural

factors that affect variability of the data and impact clinical pathology evaluation and interpretation. In order to identify subtle (and in some cases, not so subtle) effects on clinical pathology test results, preanalytical sources of variation should be reduced whenever possible within the limitations of the study. Sources of variation can be loosely categorized as physiological, procedural, and artifactual. Physiological sources of variation include differences associated with age, strain, sex, diet, fasted condition, excitement or fear, stress, and time of blood collection. Procedural sources of variation include order of sample collection (i.e., group order versus randomization), blood collection site and technique, and anesthesia. Causes of artifactual or spurious results include poor-quality specimens (e.g., partially clotted hematology samples; hemolyzed serum/plasma samples), inappropriate use of an anticoagulant, improper sample storage, and iatrogenic blood loss.

Initiation of treatment for most regulated toxicology studies occurs when the animals are relatively young and still in a growth phase (e.g., rats 6–8 weeks old and beagles 4–6 months old). As the animals mature, several clinical pathology parameters are affected. Typical changes in most species include increasing red blood cell count, hemoglobin concentration, hematocrit, absolute neutrophil count, total protein and globulin concentration and decreasing reticulocyte count, mean corpuscular volume, absolute lymphocyte count, alkaline phosphatase activity, and inorganic phosphorus concentration. Although these and other age-related changes may be subtle, they are sufficient to evoke false conclusions if interpretation of posttreatment data were based solely on comparisons with pretreatment or baseline data collected more than a week or two earlier. Age-related changes are just one of many factors that make concurrent control groups an absolute necessity for most toxicology studies. As previously mentioned, the variability of a number of parameters becomes much greater for aging rodents, especially those over 1 year of age. This variability significantly reduces the likelihood of drawing meaningful conclusions from clinical pathology data collected in the latter half of chronic rodent studies.

Strain differences, especially for rodents, are important to consider when evaluating clinical pathology data. Differences for hematology parameters tend to be the most obvious. For example, Fischer 344 rats tend to have lower leukocyte counts than those of Sprague-Dawley rats but are also more predisposed to developing large, granular lymphocytic leukemia (86, 119). An important difference has recently been identified for red blood cells of cynomolgus monkeys *(Macaca fasicularis)* based on geographical origin. Cynomolgus monkeys from China and contiguous areas such as Vietnam have much larger, but fewer, red blood cells than cynomolgus monkeys from Indonesia, the Philippines, or Mauritius (16). The differences are so great that reference ranges for red blood cell count and mean corpuscular volume for these animals may not overlap. Interpretation of hematologic effects could easily be compromised if monkeys from these different geographical origins were used in the same toxicology study or perhaps different studies of the same test material. Although not yet reported, it is likely that there are other differences for clinical pathology test results between these populations of cynomolgus monkeys.

Diet clearly affects many clinical pathology parameters, and standard diets are necessary to avoid small differences that might be misinterpreted. Comparison of data from animals fed purified or unusual diets with data from animals fed standard diets (e.g., historical reference ranges) should be done with caution. Some species, such as the rabbit, are prone to the effects of atherogenic diets and exhibit very high cholesterol concentrations when fed these diets. The amount of protein in the diet is known to affect urea nitrogen concentration but likely has subtle effects on other parameters over time. Because some diets, especially for dogs, are more prone than others to cause false positive results for fecal occult blood, diet can be an important factor when assessing the potential of a test material to cause gastrointestinal ulceration or hemorrhage.

Much has been published on the effects of fasting animals prior to blood collection for clinical pathology (1, 76, 85, 87, 124), and differences of opinion exist concerning this practice, especially with respect to rodents. While most laboratories routinely fast dogs and monkeys overnight prior to blood collection, procedures for rodents differ among laboratories. Historically, fasting has been encouraged in clinical practice as a means of reducing the variability of certain parameters, most conspicuously glucose concentration, so that the physician can more readily compare the results from a single patient with reference ranges in order to formulate a differential diagnosis. In toxicology studies, because the concurrent control group is much more relevant for comparison purposes than are historical reference ranges, the key principle is to treat all of the groups the same with respect to conditions prior to blood collection.

Fasting of mice for longer than a few hours is not encouraged because mice reduce their water consumption when fasted and rapidly become dehydrated. Not only does dehydration affect many

clinical pathology parameters (e.g., erythrocyte count, serum protein concentrations, urea nitrogen concentration), it makes blood collection more difficult and reduces the volume of blood collected. Because some laboratories prefer to fast animals before killing (in part to reduce hepatocyte glycogen stores and improve histopathologic detection of hepatocellular injury or change) and because blood collection from mice is often a terminal procedure prior to

sacrifice, mice are sometimes fasted for a limited time before blood collection and kill (e.g., four h). If this is done, care must be taken to keep the period of fasting similar for all animals even though the killing of many animals may take several hours because of necropsy procedures.

Most laboratories in the United States prefer to fast rats overnight. Although one frequently cited reason for this is to reduce variability for certain parameters such as glucose, perhaps the most important reason is to "standardize" the conditions for all animals. If animals in the high-dose group are eating poorly, providing all animals with access to feed before blood collection can have the effect of comparing fed animals (i.e., the control animals) to fasted animals (i.e., the high-dose animals). Since there are several differences for clinical pathology parameters between fed and fasted animals, it is more difficult to determine if differences between the control and high-dose groups are due to an effect of the test material or simply to differences in overnight feed consumption. When compared with rats having access to feed, fasted rats tend to have lower white blood cell counts; lower urea nitrogen, cholesterol, triglyceride, calcium, and bilirubin concentrations; and lower alanine aminotransferase and alkaline phosphatase activities (76, 87).

Excitement/fear and stress can have pronounced effects on clinical pathology test results. Excitement and fear are associated with acute endogenous catecholamine release ("fight or flight" phenomenon), and stress is associated with endogenous corticosteroid release. Effects of catecholamines are immediate but short-lasting (e.g., less than 30 min). Effects of corticosteroids tend to be more gradual and long-lasting. The most obvious changes observed in very excited or frightened animals are increased leukocyte counts and glucose concentrations. These changes are observed occasionally with the overexcited beagle and with unanesthetized monkeys that are not used to handling for blood collection. Monkeys may also react to the presence of several people in the animal room performing additional study-related procedures at the same time as blood collections. Endogenous corticosteroids affect leukocyte counts, but somewhat differently than catecholamines. Whenever possible, clinical pathology testing should be delayed for at least a week following shipping or surgical procedures to avoid stress-related changes.

Blood samples should be collected in a manner that minimizes the possibility of temporal biases. Examples of time-related biases include differences between morning and afternoon results (circadian effects), differences caused by delayed separation of serum from clotted blood, and day-to-day differences (may be preanalytical or analytical). These biases can be eliminated or at least minimized by randomization of the animals for blood collection and the use of procedures that enable blood collection and sample processing over the shortest reasonable amount of time. An alternative to true randomization is a structured pattern of bleeding such that one animal from each group is bled in succession. Once samples have been collected, they should be analyzed in the same order as the blood collection. Rearranging the samples back to group order has the potential of causing false positive findings that are actually due to analytical drift. For example, a small drift in the analysis of chloride by ion-selective electrode (e.g., an increase of 2 mmol/L over 60 samples) may be sufficient to produce a statistically higher mean chloride concentration for the high-dose animals if the groups are sufficiently large (e.g., 15 animals/sex/group), the control animals are analyzed first, and the high-dose animals are analyzed last. If animals must be bled over 2 days because of laboratory or necropsy capacity issues, the problem of day-to-day variability can be reduced by collecting and analyzing samples from the males on one day and the females on the other.

Randomization of animals for blood collection is occasionally impractical (e.g., if timed collections must follow intravenous administration of the test material). When animals must be sampled by group, it is better to sample the high-dose group immediately before or after the control group than to sample the animals in consecutive group order (i.e., control, low-dose, mid-dose, high-dose). If it is necessary to sample the animals in consecutive group order, then procedures must be in place to analyze or process the samples in a similar time frame. When control animals are bled 1 h or more before the high-dose animals, analysis of the hematology samples and separation of the chemistry samples should not be delayed until all of the groups are bled. Such a delay can result in differences between the control and high-dose groups that are due solely to time-related, in vitro changes. This is most likely to occur for rodents when blood collection is one of the terminal procedures and several hours are necessary to bleed, sacrifice, and necropsy all of the animals. If hematology analysis of whole blood from the control animals is delayed, it is possible that the last group bled will appear to have increased platelet counts relative to those of the control group because platelet counts decrease over time as a result of spontaneous platelet aggregation. If clotted blood from the control group is allowed to stand 1 or 2 h longer than that from the high-dose group before separation of serum, the high-dose group will often

have statistically significant differences for several chemistry parameters, including higher glucose concentration, lower potassium and inorganic phosphorus concentrations, and lower aspartate aminotransferase and lactate dehydrogenase activities. These differences result from changes in the control animal samples—that is, the consumption of glucose by erythrocytes, and the

release of cell constituents by erythrocytes, leukocytes, and platelets. Circadian effects are another potential source of variation when blood collection is protracted. For example, rodents bled early in the morning can have slightly higher leukocyte counts than those bled in the afternoon during their normal period of inactivity.

Collection site, collection technique, and use of anesthesia are perhaps the most commonly cited procedural influences on clinical pathology test results. Many investigators have analyzed differences in data resulting from these variables (6, 33, 75, 76, 86, 88, 92, 94, 104, 112, 118, 121, 126), especially for the rat. But while differences in the results do exist (e.g., total leukocyte counts in samples from the retro-orbital venous plexus are higher than those from larger vessels such as the abdominal aorta or posterior vena cava; glucose concentrations in samples from the abdominal aorta are higher than those from other sources), the principal message from these works is that laboratories should use the technique with which they have had good success obtaining high-quality samples and with which they are most comfortable. Use of an unfamiliar or unpracticed blood collection technique introduces unnecessary variability and spurious results. With appropriate instruction and practice, any of the commonly used techniques can generate adequate results. To optimize the value of the data, however, a single method of collection should be used throughout a study or series of comparable studies. For example, anesthesia tends to decrease interanimal variability for clinical pathology results from monkeys, especially their cell counts and electrolyte concentrations. If pretreatment or baseline blood collection is performed on animals anesthetized for physical examinations or other baseline procedures, the relatively heterogeneous data obtained from unanesthetized animals during treatment may cause confusion or be misinterpreted. Similar problems can occur when interim blood samples from rodents are collected from the tail or retro-orbital venous plexus, and terminal blood samples are collected from the heart or posterior vena cava. Small differences observed between control and treated animals at one interval may be masked at another interval because of increased preanalytical variability. The cause of the variability is often related to differences in bleeding technique proficiency and not the method itself (88). Blood collection from dogs, monkeys, and rabbits is facilitated by relatively easy access to large vessels (e.g., jugular, cephalic, and saphenous veins for dogs; femoral, cubital, and saphenous veins for monkeys; ear and jugular veins for rabbits). Blood collection from mice is complicated by size and volume limitations, and terminal procedures are often used (e.g., cardiac puncture or sampling of the posterior vena cava or abdominal aorta at necropsy). There are many acceptable methods for blood collection from rats, and the choice of technique depends on a number of factors, not the least of which is frequency of opportunities to collect blood from rats. If a laboratory performs enough studies on rats to require blood collection every day or two, then it is probably worthwhile for some of the technical staff to become proficient at collecting blood from the jugular vein (105). Although every bleeding technique has procedural advantages and disadvantages, this technique offers the greatest number of advantages if mastered. Several high-quality blood samples can be collected from one animal, directly from a large vessel with needle and syringe, without anesthesia or expensive equipment, and without damaging important structures such as the eye or heart. Because there are no time-consuming ancillary procedures (e.g., warming the tail to dilate the tail vein or anesthesia), samples can be collected quickly, and it is possible to accurately collect timed samples (e.g., at 1, 5, 10, and 15 min postdose) from a single animal. As with most techniques, blood collection from the jugular vein requires regular practice to remain proficient.

Inappropriately collected or prepared samples increase variability of the data by introducing spurious or artifactual results. Fibrin or clot formation in a hematology sample always results in a spuriously low platelet count but may also cause low erythrocyte and leukocyte counts. Small clots may form because of an insufficient amount of anticoagulant (potassium ethylenediamine tetraacetic acid [K EDTA] is the anti-coagulant of choice for hematology samples), inadequate mixing of the blood with anticoagulant, or poor blood collection technique with exposure of the blood to substances from traumatized tissue. Excessive anticoagulant can cause dilutional errors for cell counts and prolonged coagulation times. In addition to the dilutional error, manual packed cell volumes are further lowered because of shrinkage of the erythrocytes. The use of an inappropriate anticoagulant can cause spurious results and must be avoided. Intentional or accidental exposure of clinical chemistry samples to K EDTA results in very high potassium concentrations, very low calcium and magnesium concentrations due to chelation, and very low activities of enzymes such as alkaline phosphatase and creatine kinase that use magnesium as a cofactor (41). Trisodium citrate, the anticoagulant of choice for the coagulation assays, also chelates divalent cations and would additionally increase sodium concentration if incorrectly used for the clinical chemistry sample.

Hemolysis can be caused by poor technique for sample collection, sample transport, or serum/plasma separation, and hemolysis can result in spuriously increased or decreased test results by two principle mechanisms: release of erythrocyte constituents, and interference with test methodology (41, 50, 53, 80, 81).

Techniques and procedures should be chosen that eliminate in vitro hemolysis to the greatest extent possible.
As previously indicated, prolonged contact between serum and clotted blood causes spurious changes that can be controlled by prompt separation of the serum (143). Although most analytes are relatively stable for a reasonable amount of time (73, 97, 125), unnecessary delay or storage of samples before analysis should be avoided. Ideally, hematology samples should be analyzed within a few hours of collection and no later than 24 h after collection. If samples for coagulation tests cannot be run on the day of collection, the plasma should be frozen at −20°C and thawed only once before analysis (38). If samples for clinical chemistry tests cannot be run on the day of collection, the serum or plasma (lithium heparin is the recommended anticoagulant when plasma is used for clinical chemistry) should be refrigerated or frozen overnight. If there will be a long delay before analysis or if a desired analyte is relatively labile, then the samples should be frozen at −70°C.
There are several other sources of variation for clinical pathology data, but the impact of each on data interpretation in toxicology studies is generally minimized by the inclusion of age and sex-matched control groups exposed to the same environmental conditions and undergoing the same experimental procedures. Occasionally overlooked are the effects of procedures that may differ between the control and treated groups. Because toxicology studies in larger animals frequently include blood collections for analyses other than clinical pathology (e-g-> toxicokinetic measurements or detection of antibody directed at a peptide test material), it is imperative that control animals be bled in a similar manner to the treated animals. The number and volume of blood samples for these tests can have a significant effect on hematology and clinical chemistry results (108), and data interpretation is seriously compromised if control animals do not undergo the same procedures. Even when control animals are bled in a like manner, iatrogenic blood loss can complicate data interpretation (67).

Analytical Variation and Quality Control

In addition to sources of variation that occur before sample analysis, the analytical procedure itself is a source of variation. Analytical variation is minimized by an active quality control system within the clinical pathology laboratory. Detailed discussions of quality control systems are available in many textbooks (14, 100, 138). At a minimum, the quality control system should include initial verification that a new method satisfies the goals of the laboratory for accuracy and precision for the analyte being measured; standard operating procedures for all laboratory functions necessary for analysis and reporting of test results; documentation of routine instrument calibration procedures; documentation of routine and nonroutine instrument maintenance; documentation of appropriate personnel training; proper labeling of all reagents, controls, standards, calibrators, and other chemicals in the laboratory; routine analysis of quality control specimens and review of quality control data for detection of systematic errors; routine review of "patient" data for detection of random errors; standard procedures for responding to and documenting "out-of-control" situations; and participation in some form of external quality control or proficiency testing with "unknown" samples for analysis.
Analytical variation is affected by the accuracy and precision of a test procedure. Accuracy is a measure of the extent to which the mean estimate of a quantity approaches its true value, and precision is a measure of the agreement among replicate measurements (i.e., the reproducibility of a test result). Accuracy is generally determined in a formal way when a new method is introduced into the laboratory, and it is continually reassessed, albeit somewhat crudely, by means of proficiency testing. Most analytical procedures exhibit a small amount of systemic bias or inaccuracy that is either constant or proportional. In other words, the mean estimate of the quantity of analyte is always in error in the same direction, either higher or lower, than the true quantity. Precision of a test can be assessed within a single run (e.g., 20 consecutive analyses of the same sample for within-run precision) or from day to day (e.g., the same sample analyzed several days in a row for between-run precision) and is reflected by a test's coefficient of variation (CV). The CV expresses the error or variability of replicate test results as a percentage of the mean value (i.e., [standard deviation/mean] x 100); the lower the CV, the greater is the test's precision or repeatability.
Although accuracy and precision are both desirable, test procedures can be accurate but imprecise or inaccurate but precise. In the context of most toxicology studies, where results from groups of treated animals are compared with results from concurrent control groups and their own pretreatment results, good precision is more valuable than good accuracy. This is in contrast to the clinical setting, where the physician or veterinarian evaluates individual patients under less controlled conditions and uses, by necessity, broad historical reference ranges for making decisions. An imprecise test, regardless of accuracy, is less able to detect small differences between the control and treated groups. If the true

mean glucose concentrations for 4 control dogs and 4 treated dogs are 100 and 115mg/dl, respectively, but the standard deviations for the groups are large because of imprecision, then it is unlikely that the observed difference between the means (i.e., 15 mg/dl) will be considered a real difference. A precise test, regardless of a systemic bias or inaccuracy,

is better able to identify small test material-related differences between the groups. In the same example, if the glucose test had a positive bias but was more precise, the means of the two groups might be 110 and 125 mg/dl with lower standard deviations. The improved precision permits a more accurate interpretation of the same 15-mg/dl difference between group means.

Clinical pathology laboratories that test animal samples frequently use tests, especially standard clinical chemistry tests found in routine chemistry panels, that are optimized for accuracy using human samples. It is likely that many of these tests have small systemic biases when used for animal specimens. In most cases, however, the excellent precision afforded by using standardized commercial reagent kits, standards, and calibrators is preferable to time-consuming, costly efforts to optimize the accuracy of a test for different animal species by using "home-brew" materials that undermine precision. Occasionally there are standard chemistry tests that do not work well for certain species, such as albumin for rabbits, and these tests require specialized methods. Immunoassays utilizing monoclonal or polyclonal antibodies raised against human substances (such as hormones) are frequently inappropriate for use on animal specimens, and hematology analyzers that enumerate and differentiate blood cells must be modified for animal blood because of differences in cell morphology.

For human hospital laboratories, federal regulations allow each laboratory to set its own policies for assaying control materials as long as at least 2 control samples of different concentrations (i.e., normal and abnormal levels) are assayed every 24 h. The data from each quality-control analysis are used to make decisions about the validity of the patients' data. In the setting of preclinical toxicology studies, it is advisable to assay at least two control samples with each study run. In other words, if a laboratory is scheduled to run samples from two or more regulated toxicology studies (e.g., studies needed to make submissions to regulatory agencies and using Good Laboratory Practices), then quality control samples should be assayed with each of the studies, and the results should be maintained with the study data. If results from a control sample are found to exceed the allowable limits for one or more analytes, then steps must be taken to resolve the problem. Actions that may be taken include, but are not limited to, the following: check for obvious problems such as reagent levels, clots, or mechanical fault; repeat the assays on control samples using fresh aliquots; repeat the assays using newly reconstituted control samples; recalibrate the instrument for the analyte(s) in question, then reassay the control samples; change the reagents, recalibrate, and reassay the control samples; and perform maintenance, recalibrate, and reassay the control samples.

PRINCIPLES OF DATA INTERPRETATION

Interpretation of data from toxicology studies begins with the identification of differences between control and treated groups and ends with an assessment of toxicological or biological relevance. Or in other words, are there real differences between the groups, and if so, are those differences bad? Interpretation of clinical pathology data requires an understanding of each test's characteristics, species differences, and principles of internal medicine. Factors that influence the interpretation of a potential effect include study design and conditions, clinical observations, other clinical pathology results, anatomical pathology findings, and the test material itself. Interpretations of many clinical pathology findings are interdependent, and pattern recognition is critical. After range-finding studies have been conducted, appropriate dose selection usually precludes large, dramatic effects on clinical pathology test results. The most common effects are relatively mild and often appear to be secondary to small alterations in metabolic or homeostatic mechanisms. However, test materials causing significant damage to liver, kidney, or hematopoietic tissue can produce marked changes in clinical pathology results. Effects on clinical pathology results are rarely the only evidence of biologically important or adverse toxic effects. Clinical observations or anatomical pathology findings usually corroborate biologically important laboratory findings.

Statistical Comparisons

Statistical analysis of clinical pathology data is commonly performed in toxicology studies, and it often results in identification of several statistically significant differences between control and treated groups. However, all effects caused by a test material need not be statistically significant, and all statistically significant differences do not necessarily represent true or lexicologically significant effects. If used, statistical tests should be viewed simply as a tool to help identify differences between groups and not as the principal justification for decisions concerning potential test material effects (20, 26). It is important to remember that the power of a statistical test is affected by the number of animals per group. Fewer test subjects increases the likelihood that statistical tests will fail to identify a true effect. Since the number of animals/per group is usually quite small for studies with dogs or monkeys (e.g., 4/sex/group, or less), it is imperative that the data for each animal at the different test intervals be examined to look

for patterns of change over time among the treated animals that are absent among the control animals. As the number of animals per group increases, the frequency of identifying statistically significant differences of very small magnitude

increases. In rat studies with 15 or more animals per sex per group, it is common to observe statistically significant differences that have little or no effect on the health of the animals and are not lexicologically relevant.

Is an Apparent Difference Real?

When faced with an apparent difference between control and treated groups, the first answer the investigator must determine is whether or not that difference represents a true effect of the test material or is an incidental finding. Many factors can influence the answer, not the least of which is the size of the difference. A large difference is obviously more likely to be real than a small one. Additional factors that favor a difference being test material-related include dose dependency, consistency over time, consistency between sexes, correlation with clinical observations (e.g., low chloride concentration and emesis), correlation with other clinical pathology findings (e.g., low hematocrit and high reticulocyte count), correlation with anatomical pathology findings (e.g., high globulin concentration and lymph-node hyperplasia), presence in a large number of animals (e.g., 15/sex/group versus 3/sex/group), and consistency with previously identified effects of the test material or related compounds. With large animals, it is necessary to make certain that an apparent difference was not present before treatment was initiated. The chronology of an apparent difference is also important for interpretation. For example, following a single administration of most test materials, it is more likely that a real difference will occur within a few days rather than only after 2 weeks. Following repeated administration of most test materials, it is more likely that a real difference will occur after a few months of treatment rather than only after several months. In other words, it is somewhat unusual that a real difference at 6 months will be completely absent at 3 months.

With regard to specific tests and test species, the amount of expected analytical, interanimal, and intra-animal variability can influence the interpretation of apparent differences. For example, because alanine aminotransferase activity has much greater interanimal and intra-animal variability for monkeys than for dogs, a relatively modest difference for this enzyme between control and treated groups is less likely to be a true effect for monkeys. Interanimal variability increases dramatically for older rodents (e.g., >52 weeks of age) because of naturally occurring disease conditions, and small differences between groups are less likely to represent true effects. Procedural factors such as the route of test material administration, blood collection technique, and randomization for blood collection also affect variability and must be considered. For example, continuous intravenous infusion increases interanimal variability for several tests (e.g., white blood cell count, hematocrit, serum proteins), and interpretation of small differences in results of these tests is difficult. Blood collection by cardiac puncture affects variability of muscle enzymes such as creatine kinase and aspartate aminotransferase. The potential for spurious findings associated with lack of randomization for blood collection was discussed previously.

Is a Real Difference Bad?

If a difference between control and treated groups is clearly real, the next answer the investigator must determine is whether or not that difference represents a bad or adverse effect. Does the finding itself (e.g., low hemoglobin concentration) or the condition that caused the finding (e.g., blood loss from gastrointestinal erosions/ulcerations) represent a toxic effect that compromises the animal's health? The answer is often ambiguous and quite subjective. Many of the factors previously considered are important, and the size of the difference is clearly a focal point. Although a large difference for a given parameter is more likely to be adverse than a small difference, it is clearly not that simple, and it is not possible to define set limits for each test that represent an adverse effect. The same magnitude of change for a given test can have completely different connotations depending upon the mechanism for that change, correlative findings, the test species, the study design and procedures, and the test material itself (e.g., a clinical pathology parameter may be the target for the pharmacological activity of a drug). Urea nitrogen may be markedly increased because of dehydration (e.g., mice that have been fasted too long before blood collection or animals that refuse to drink water containing test material), but only mildly increased in the early stages of renal toxicity. Increased alanine aminotransferase activity associated with histopathological evidence of hepatocellular degeneration and necrosis is likely to be more lexicologically important than the same level of increase for which there are no correlative findings. Because of species differences for interanimal variability, a threefold increase for alanine amino-transferase activity for dogs (i.e., treated vs. control group means) is more likely to represent an adverse condition than the same increase for monkeys. A 10% decrease in hemoglobin concentration is less likely to represent an adverse condition in animals that were bled repeatedly during a study or that received the test material by continuous intravenous infusion than in animals that were not bled repeatedly or were treated by oral gavage. Very high neutrophil counts would normally reflect an

adverse condition, but if the test material was a granulocyte

colony-stimulating factor, the high counts would be a desirable effect.

Some tests measure analytes that are critical to good health (e.g., neutrophil count, hemoglobin concentration, glucose concentration, calcium concentration, potassium concentration), and correlative in-life observations (e.g., bacterial infections, lethargy, weakness, weight loss) may help determine whether an observed effect on such a test has impacted the animals' health. Other tests measure analytes that are markers for effects best evaluated by histopathological examination (e.g., alkaline phosphatase, urea nitrogen). All routine tests can be altered by more than one process or mechanism, and some mechanisms have worse implications than others. For example, increased urea nitrogen in mice associated with poor feed and water consumption is likely of less concern than increased urea nitrogen because of proximal tubular necrosis. When determining the biological or toxicological significance of an effect on a clinical pathology test result, consideration must be given to the analyte's normal function for maintaining health, correlative findings that may better define the overall impact of the test material on health, and the mechanism that brought about the change.

Reference Intervals

Like statistical comparisons, historical reference intervals can be used as a tool when assessing apparent differences between control and treated groups. Unfortunately, the value of reference intervals for data interpretation is sometimes overestimated, and the potential for their improper use is great (61, 131, 134). It is tempting to invoke historical reference intervals when trying to decide whether apparent differences for clinical pathology results are true effects or whether they are adverse effects. While historical reference intervals can be helpful for establishing some perspective concerning what is typical or expected, the conditions of every toxicology study are unique, and it is inappropriate to use a reference interval as the primary reason for dismissing an apparent difference between control and treated animals as being incidental or biologically insignificant. It can be equally inappropriate to use a reference interval as the primary reason for determining that an apparent difference is real or adverse. As detailed in the previous sections, there are many other factors to consider when evaluating the nature of an apparent test material effect.

Reference intervals (often incorrectly referred to as reference ranges or normal ranges) are constructed with values obtained from reference individuals. In most clinical pathology laboratories used for toxicology studies, the reference individuals are control animals from previous studies and clinically healthy animals that have not received treatment (e.g., monkeys or dogs that have clinical pathology tests performed before initiation of dosing). In human medicine, the National Committee for Clinical Laboratory Standards recommends that reference intervals be estimated by the nonparametric method and that a minimum of 120 values from reference individuals be used (93). Test results from the reference individuals are subjected to statistical treatment such that rare values at both ends of the distribution are eliminated. For example, if the lowest and highest 2.5% of the values are eliminated, the resulting reference interval represents the central 95% of the distribution of values. If the distribution is gaussian, the interval corresponds to the mean±1.96 SD. By definition, when the central 95% is used as the reference interval, 5% of the results from "normal" individuals (1 of 20) are outside of the reference interval for any given test. Clearly, a value outside of the reference interval does not necessarily indicate an abnormality (36).

The suitability of a historical reference interval for data interpretation for a given study is a function of the parameters or *partitioning factors* that define the reference population used to construct the interval. There are many potential partitioning factors with respect to toxicology studies, and their importance is sometimes overlooked. The most commonly used partitioning factors are species, strain, sex, and age. In other words, a typical reference population might be defined as male Fischer 344 rats from 8 to 10 weeks of age. However, many other partitioning factors can influence the reference interval and make it broader, narrower, higher, or lower. These factors include animal supplier, site of blood collection, use of anesthetic, type of anesthetic, diet, fasting status, time of sample collection, and sample matrix (e.g., serum or plasma). If control animals are used for the reference population, additional partitioning factors include route of administration (e.g., dietary, oral gavage, or intravenous infusion), vehicle or control material administered (e.g., sterile water or corn oil in a gavage study; sterile saline or 5% dextrose in an intravenous infusion study), and whether or not the animals were bled repeatedly for toxicokinetic analyses or multiple clinical pathology intervals. Finally, there are laboratory considerations including instrumentation or technique used to analyze the specimen and sample storage conditions (e.g., storage temperature and time interval between collection and analysis). Ideally, whenever a new instrument or reagent system is introduced, a new reference interval is constructed. In practice, laboratories often rely on evidence that demonstrates relative consistency between the old and new analytical methods to

avoid complete replacement of old intervals.
The number of partitioning factors used obviously has a direct impact on the number of reference individuals

available for each reference interval. With the exceptions of reference intervals for dogs and monkeys tested before treatment is initiated (e.g., male beagles, 4 to 6 months old, XYZ supplier, jugular vein, no anesthetic, fasted), it can be very difficult obtaining enough data for meaningful reference intervals. Because of this problem, laboratories often ignore many of the partitioning factors and "lump" together the data from control animals of dissimilar types of studies or from samples that have been handled differently. The result is broader reference intervals with less relevance to data interpretation.

Even when reference intervals are appropriate for a given study (i.e., the partitioning factors match the study animals, conditions, and procedures), it is wrong to assume that a difference between control and treated groups represents a true effect or an adverse effect simply because the mean of the treated group falls outside of the reference interval. Concurrent control animal data for a given study are never a perfect reflection of the historical reference data; that is, they do not exhibit the same means and distribution. The mean for the control group may fall anywhere within the reference interval, and the distribution of results for the control group is almost always narrower than that of the reference interval. Depending on the location of the control group data within the reference interval, a very small, incidental difference for the treated group may fall outside of the reference interval and a relatively large, adverse test material-induced difference may fall within the reference interval. There are many situations when a small difference, within the reference interval, represents a very significant adverse effect. For example, anemia and hypoproteinemia can be masked by dehydration, and the loss of an entire subpopulation of lymphocytes is not necessarily enough to cause absolute lymphocyte count to fall below the reference interval. When reference intervals are broad, as occurs when few partitioning factors are used or normal interanimal variability is great (e.g., alkaline phosphatase activity for monkeys), significant toxicity can occur without test results exceeding the limits of the intervals. Investigators must understand the limitations of reference intervals with respect to data interpretation. By themselves, reference intervals do not determine whether or not an apparent difference is real or adverse. They are simply an adjunct to sound scientific judgment.

Regardless of the many pitfalls affecting their use for data interpretation, historical reference intervals do have other important functions. They serve as a nonspecific measure of quality control that can detect changes over time in assays, study conditions, or animal characteristics. For example, it may be noticed that liver enzyme activity in mice has increased over time. The cause of this finding might be traced to changes in handling practices or animal supplier. Reference intervals also serve as a nonspecific measure of analyte variability. The cause of seemingly excessive variability might be inadequate assay precision, nonstandardized preanalytical procedures, or true interanimal variability. Finally, reference intervals can be valuable when very few animals are used for small investigational studies with no concurrent control group. Although pretreatment baseline data are more relevant to interpretation of potential effects, it may be discovered that the few animals acquired for the study have preexisting problems or are atypical with respect to historical data.

HEMATOLOGY TESTS AND INTERPRETATION

The hematology tests recommended and most often performed for toxicology studies evaluate erythrocytes, leukocytes, platelets, and coagulation. Technological advances by manufacturers of hematology and coagulation analyzers, along with a growing interest in supporting customers who must evaluate samples from a variety of animal species, have increased the availability of highly sophisticated instruments to perform these tests with precision and accuracy suitable for preclinical safety assessment studies. The automated methods are a necessity. Manual methods are unacceptably imprecise, labor-intensive, and slow. Many currently marketed hematology instruments are capable of measuring or calculating all of the routinely recommended tests and more from less than 150 μl of anticoagulated whole blood. The instruments typically utilize a combination of two or more principles including electrical aperture impedance, laser light scatter, and differential staining characteristics to determine cell number (RBC, WBC, and platelet counts), cell size and size distribution (MCV, RDW, mean platelet volume [MPV], and platelet distribution width [PDW]), and cell type (WBC differential count). They measure hemoglobin concentration directly and calculate MCH and MCHC; some also calculate HDW. Because the blood cells of laboratory animal species differ morphologically from human blood cells, instruments and the instrument software used for human samples are generally inappropriate for use without modifications. Fortunately, instrument manufacturers are producing instruments with the necessary modifications to permit accurate and precise analysis of blood from a variety of laboratory animals species. Modifications have also been made for coagulation analyzers that measure the time required for fibrin clot formation in a plasma sample, to which reagent has been added, by detecting changes in conductivity, physical resistance, light scatter, or optical density. Most instruments can now be

programmed to permit detection of clot formation at times much faster (e.g., prothrombin time for dogs) or

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slower (e.g., prothrombin time for guinea pigs) than are typically observed for human samples.

Erythrocytes

Effects on erythrocyte parameters typically reflect a change in the balance between red blood cell production and red blood cell loss or destruction. In addition, changes in plasma volume (e.g., dehydration or volume expansion) can indirectly affect erythrocyte parameters. Although mechanisms for effects on erythrocyte parameters may be obvious, such as hemorrhage from gastric ulceration, they are often relatively obscure. But even when the exact mechanism is unclear, the effects can usually be described in terms of broad mechanistic categories and impact on health.

Anemia

Anemia is defined clinically as the condition characterized by a hemoglobin concentration below the lower reference limit. RBC count and hematocrit may or may not be proportionately lower, depending on the cause of the anemia and whether or not cell size and hemoglobin content are affected. Low hemoglobin concentration reduces the oxygen-carrying capacity of blood, which in turn may result in clinical signs such as pallor, weakness, exercise intolerance, tachycardia, or tachypnea. In many toxicology studies, a treated group has lower means for RBC count, hemoglobin concentration, and hematocrit than those for the control group, but the differences are less than those necessary to cause clinical signs and are not indicative of anemia. For example, reductions from control values for these parameters up to approximately 10% are relatively mild and probably do not have an adverse effect on the health of the animal. Reductions from approximately 10% to 25% may be considered a moderate effect and may or may not be clinically adverse. Reductions of more than 25% are marked and clearly represent a clinically adverse anemic condition. Keep in mind that while a 5% reduction in hemoglobin concentration may be insufficient to adversely affect health, the cause of the reduction (e.g., liver toxicity) may be very adverse. Unless the differences for RBC count, hemoglobin concentration, and hematocrit are quite large, it is preferable to simply discuss the magnitude of the differences between the control and treated groups and avoid using the term *anemia*. In this chapter, however, *anemia* is used for the purposes of discussing mechanisms.

Anemias are broadly categorized as regenerative or nonregenerative. Regenerative anemias are characterized by an appropriate erythropoietic response to reduced circulating erythrocyte mass or hemoglobin concentration, and nonregenerative anemias are characterized by the absence of an appropriate erythropoietic response.

Regenerative anemias result from two general causes: abnormal blood loss (hemorrhage), and accelerated erythrocyte destruction (hemolysis). Following acute blood loss or hemolysis, it takes approximately 3 to 4 days for new erythrocytes, called reticulocytes, to increase in number in peripheral blood. Reticulocytes are larger and slightly more basophilic than mature erythrocytes when stained with the Romanowsky-type stains (e.g., Wright or Giemsa stain) typically used for assessing red blood cell morphology and doing manual WBC differential counts. During a strong regenerative response, erythrocyte morphology is described by the terms *anisocytosis* (variable size) and *polychromasia* (variable color) because of the increased numbers of reticulocytes. Greater numbers of nucleated red blood cells, also called metarubricytes, and Howell-Jolly bodies (small pieces of nuclear material not cleared from the erythrocyte) may be observed during a regenerative response, but these may also increase with some nonregenerative conditions. Reticulocytes can be counted with an automated reticulocyte counter or counted manually by staining blood with a vital stain such as new methylene blue before making a blood film. For proper interpretation, the absolute reticulocyte count (i.e., reticulocytes/μ1) should always be determined by multiplying the relative reticulocyte count (i.e., percent of erythrocytes) by the RBC count. Relative reticulocyte counts can be increased in severely anemic animals even though the absolute reticulocyte counts are no different than for normal animals. In this case, the erythropoietic response is not appropriate for the degree of anemia and indicates inadequate RBC production is at least partially responsible for the anemic condition. During a strong regenerative response, the increased number of "young" erythrocytes will usually result in higher MCV and lower MCHC because these relatively large cells have lower hemoglobin concentration. In practice, higher MCV is more commonly observed than lower MCHC. During a strong regenerative response, erythrocyte production can increase six- to eightfold over normal levels. As long as the accelerated erythropoiesis is able to match the erythrocyte loss or destruction, the anemia will not become worse. If erythropoiesis exceeds the loss or destruction, or if their cause is eliminated, the anemia will be reversed.

In toxicology studies, mild nonregenerative anemias are more common than regenerative anemias. If normal erythrocyte production is reduced for any reason, or if erythropoiesis does not increase in

response to decreased erythrocyte survival, circulating erythrocyte mass will decrease over time. In nonregenerative anemia, erythrocyte morphology is characterized by the absence of anisocytosis and polychromasia. Most often, the

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erythrocytes appear normocytic (normal size) and normochromic (normal color). Absolute reticulocyte count is unchanged or decreased, depending on whether the problem is simply an inability to adequately respond to increased needs or, more significantly, failure of erythropoiesis. Mean corpuscular volume is typically unchanged or mildly decreased.

The most common hematology findings in preclinical toxicology studies are mildly decreased RBC count, hemoglobin concentration, and hematocrit without a corresponding increase in absolute reticulocyte count and without an obvious mechanism for the effect. The decreases are usually no more than about 10% from the values for the respective control group (e.g., mean control group hematocrit=44%; mean high-dose group hematocrit=40%) and less than what would be classified as anemia. Slightly lower MCV (e.g., control mean= 56 fl; high-dose mean=54 fl) may also be observed, especially in rodents. In conjunction with these minor effects, the animals may exhibit mild reductions in body weight or body weight gain and less frequently, feed consumption. They may have other clinical signs of poor health, including dull haircoat, poor grooming habits, and decreased activity. Potential concurrent effects on other clinical pathology test results include mildly decreased total protein and albumin. Although specific mechanisms for these changes are typically not identified, they suggest a generalized reduction of anabolic processes. Anything that affects the normally brisk pace of RBC production (in humans, approximately 100 billion new cells/day), will ultimately be reflected in the test results. It is possible that decreased physical activity and correspondingly decreased tissue oxygen demand may contribute to reduced erythropoiesis. These relatively mild, nonspecific findings for circulating erythrocyte mass are identified most frequently in rat studies where the number of animals per sex per group is usually high (e.g., 10 animals per sex per group), the dose levels used are typically higher than those for dog or monkey studies, and the normal interanimal variability of hematology data is relatively low. In addition, because the circulating life spans of mouse and rat erythrocytes (approximately 25 to 40 and 45 to 65 days, respectively) (68) are shorter than those for dogs (approximately 100 to 120 days) and nonhuman primates (varies with species; for rhesus monkeys, approximately 85 to 100 days) (68), similar reductions in RBC production will first become apparent for mice and rats.

Nonregenerative Anemias

There are several other causes of reduced RBC production, and they are distinguished from one another by their severity, the presence of morphologically distinct erythrocytes, and other findings that identify the etiology. Test materials may affect RBC production directly or indirectly. Direct inhibition of erythropoiesis, if sustained, will result in a gradually developing, severe anemia because senescent erythrocytes are not replaced. Indirect effects on erythropoiesis are more common in toxicology studies and result from toxic effects on other tissues or organ systems. Indirect effects on the erythron tend to be relatively mild and are not as toxicologically important as the effects on the primary target tissue.

Direct injury to pluripotent hematopoietic stem cells or their stromal microenvironment causes failure of blood cell production, resulting in the condition called *aplastic anemia* (109, 137). Aplastic anemia is characterized by varying degrees of pancytopenia (i.e., decreased erythrocytes, leukocytes [primarily neutrophils], and platelets) and hypocellular bone marrow. If the animal survives long enough, the anemia becomes severe. Typically, however, decreased resistance to bacterial infections causes severe illness or death before the anemia is life-threatening. Irradiation is a classic model of stem cell injury and is used therapeutically as part of the process for bone marrow transplantation. In addition to irradiation, several chemicals and drugs are known to cause aplastic anemia in humans. These include benzene, toluene, lindane, pentachlorophenol, chloramphenicol, phenylbutazone, penicillamine, gold salts, and acetazolamide. Chemotherapeutic drugs such as alkylating agents (e.g., busulfan and cyclophosphamide), anti-metabolites (e.g., fluorouracil and methotrexate), and cytotoxic antibiotics (e.g., doxorubicin and daunorubicin) have the potential to cause aplastic anemia because of their pharmacological activity. Normally, however, their effects are reversible following completion of each treatment cycle. In toxicology studies with test materials such as these, decreased WBC and platelet counts are typically recognized earlier in the study (generally 7–10 days after initiation of treatment) than decreased RBC counts because the circulating life spans for neutrophils (12–24 h) and platelets (7–10 days) are much shorter than that for erythrocytes. On the other hand, reticulocyte counts can be a sensitive indicator of hematopoietic injury and may be better than WBC or platelet counts for identifying the onset of toxic effect and subsequent recovery. The value of assessing reticulocyte counts when testing antineoplastic drugs is most apparent in rodent studies because the normally high reticulocyte counts for young rodents facilitate identification of decreases while their normally low neutrophil counts make neutropenia more difficult to recognize. Furthermore, reticulocyte counts are not compromised by poor blood collection technique that may cause increased interanimal variability for platelet count. The

timing of sample collection following administration of a test material that temporarily interrupts hematopoiesis will dictate the findings in peripheral blood and can impact interpretation. During the peak effect, reticulocytes, neutro

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phils, monocytes, eosinophils, platelets, and often lymphocytes are decreased in number, and histopathologic examination of the bone marrow reveals hypocellularity. Once the effect ends, hematopoietic tissue usually mounts a strong recovery, sometimes called a rebound effect, that is characterized peripherally by reticulocytosis, thrombocytosis, and sometimes neutrophilia. Increased extramedullary hematopoiesis in the spleen, especially for rodents, is often apparent before the bone marrow repopulates. Because the time to peak effect and the duration of effect vary for different test materials, it is necessary to perform hematology tests at multiple intervals for proper interpretation.

Pure red cell aplasia is rarely observed in toxicology studies, even though several drugs are known to cause the disorder in humans (46). Because drug-induced pure red cell aplasia is usually an idiosyncratic condition and may be immune mediated, recognition of this toxic effect is low in animal studies using a limited number of test subjects. Furthermore, it would be very difficult to prove that uncomplicated, nonregenerative anemia in a single animal was a direct effect of the test material.

Chronic inflammatory diseases (44, 48, 49) and significant kidney (23), liver (99), and endocrine dysfunction (e.g., hypothyroidism and hypoadrenocorticism) (45) negatively affect erythropoiesis and erythrocyte survival, and all of these conditions can be associated with a mild to moderate, normochromic, normocytic anemia. With chronic inflammatory conditions, the principle cause of reduced erythropoiesis is thought to be decreased availability or transfer of iron to developing erythrocytes. True iron deficiency, most commonly associated with chronic blood loss or inadequate dietary iron, is relatively rare in toxicology studies, and, in contrast to the anemia of chronic disease, is characterized by microcytic (low MCV), hypochromic (low MCHC) erythrocytes. With renal disease, reduced erythropoiesis is attributed to decreased renal production of erythropoietin and the effects of "uremic toxins." Liver failure is sometimes associated with acanthocytosis, a morphological abnormality of erythrocytes characterized by several blunt cytoplasmic projections resembling pseudopodia. The acanthocytes are thought to result from accumulation of free, nonesterified cholesterol in the RBC membrane. Acanthocytes are relatively inflexible and are eventually removed from circulation by cells of the mononuclear phagocyte system. Small reductions in circulating erythrocyte mass are observed with hypothyroidism and may result from reduced basal metabolic rate and cellular requirements for oxygen. Changes in thyroid metabolism may play a role in the mild erythrocyte effects observed in some animals with reduced food consumption because caloric malnutrition can result in decreased TS and decreased responsiveness to TB, which may in turn lead to reduced production of erythropoietin.

Megaloblastic anemia is a nonregenerative anemia characterized by macrocytic erythrocytes, asynchronous maturation of cytoplasm and nucleus in hematopoietic precursors, and hypersegmented or "giant" neutrophils (2). In humans, it is associated with a variety of disorders that cause folate or vitamin 812 deficiency (e.g., sprue, alcoholic cirrhosis, and pernicious anemia) and drugs (e.g., methotrexate) that impair DNA synthesis. Macrocytosis results because developing erythrocytes undergo fewer divisions before maturation. Megaloblastic anemia is rarely identified in laboratory animals, perhaps due to differences in uptake and metabolism of folate and vitamin B12. On the other hand, nonhuman primates have been used frequently as animal models for folate and vitamin B12 deficiency (142).

Finally, nonregenerative anemia is usually a feature of leukemia because the proliferating neoplastic hematopoietic cells compete with normal hematopoietic cells for nutrients and space in the bone marrow and spleen. It is not unusual in carcinogenicity studies to observe severe anemia secondary to naturally occurring leukemia in a few animals.

Regenerative Anemias

Blood loss can occur secondary to a variety of conditions or study-related procedures and should always be considered when a decrease in circulating erythrocyte mass is accompanied by a similar decrease in serum protein concentration. The source of the blood loss may be identified by clinical observations (e.g., dermal ulceration, melena, epistaxis), necropsy findings (e.g., gastrointestinal ulceration, cystitis), or other laboratory tests (e.g., fecal and urine occult blood tests). Blood loss associated with serial blood collection for pharmacokinetic investigations, clinical pathology tests, or other study-specific requirements must always be accounted for when interpreting changes in erythrocyte parameters. Blood loss typically results in increased polychromasia, MCV, and reticulocyte count unless the condition is very acute (i.e., less than the 3–4 days necessary for the regenerative response to build up) or there are complicating factors affecting erythropoiesis.

There are a number of potential mechanisms for test material-induced hemolysis, but the three most common mechanisms observed in toxicology studies are direct damage to the red blood cell membrane, oxidation of hemoglobin resulting in Heinz body formation, and immune-mediated red blood cell

destruction (90). Each has specific characteristics, and they are relatively easy to differentiate.
Test materials administered intravenously are those most likely to be associated with direct damage to the red blood cell membrane. The lipid bilayer of the cell membrane is sensitive to test materials with detergent-like properties, and intravascular hemolysis can occur rapidly

during treatment when the red blood cells are exposed to high concentrations of the test material at the site of injection or infusion. If the amount of released hemoglobin exceeds the carrying capacity of circulating haptoglobin, unbound hemoglobin passes through the glomerulus and is excreted in the urine. Visible hemoglobinuria (red-tinged urine) may be observed within a few hours of treatment, and a regenerative response is detectable in the blood within 3 or 4 days of treatment. Intravenously administered test materials that cause extensive intravascular hemolysis are usually associated with local damage to endothelium. The effect of the vascular damage may be observed grossly (e.g., tail lesions in rodents treated via the tail vein) or histologically. Additional histological evidence of intravascular hemolysis is the presence of hemoglobin pigment within renal tubular epithelial cells and hemoglobinuric nephrosis. Administration of a very hypotonic solution, as might occur if sterile water is inappropriately chosen as the vehicle for a low concentration solution, is another potential cause of acute intravascular hemolysis. Water passively enters red blood cells because of the ionic concentration gradient, and the cells swell and rupture. Hypertonic solutions typically do not cause hemolysis.

Heinz bodies are particles of irreversibly denatured hemoglobin attached to the interior of the red blood cell membrane. They result when test materials with oxidative properties cause disulfide bonds to form from the sulfhydryl groups of hemoglobin. Red blood cells containing Heinz bodies may be removed from circulation by cells of the mononuclear phagocyte system (extravascular hemolysis), or they may become morphologically distinct (e.g., ghost cells and blister cells) following selective removal of the Heinz bodies. Although Heinz bodies can be difficult to detect with the Romanowskytype stains, they stain prominently with vital stains such as methylene blue, crystal violet, or brilliant cresyl blue used for manual reticulocyte counts, and the number of affected cells can be determined in the same manner as that for manual reticulocyte counts. The size and number of Heinz bodies are dependent on the causative agent, the dose, and the time after exposure. Acute exposure to a potent oxidative agent typically causes an acute anemia characterized by the observation of many affected red blood cells containing a single, large Heinz body, or, less frequently, multiple, small Heinz bodies. Ghost cells, blister cells, and other morphologic abnormalities may be present. Reticulocytosis develops after 3 to 4 days. As is true for most causes of significant, acute extravascular hemolysis, splenomegaly and extramedullary hematopoiesis are common findings. Bilirubinemia and bilirubinuria are possible but less common correlative findings. Chronic, low-level exposure to an oxidizing agent usually does not cause anemia. Although absolute reticulocyte count and MCV may be slightly increased, hematocrit and hemoglobin concentration are relatively unchanged or only slightly reduced. The number of red blood cells containing Heinz bodies is low, and they may go unrecognized if not looked for specifically. Identification of test materials that cause Heinz body formation is particularly important clinically because the most common human erythrocyte enzyme deficiency, glucose 6-phosphate dehydrogenase deficiency, makes affected individuals particularly susceptible to oxidant-induced hemolysis (7).

Test materials that cause Heinz body formation have the potential to cause methemoglobinemia and vice versa (91). Methemoglobin is hemoglobin in which the iron has been reversibly oxidized from the ferrous state (Fe^{2+}) to the ferric state (Fe^{3+}); it is incapable of carrying oxygen. The clinical signs of methemoglobinemia, therefore, are those of hypoxia. Mucous membranes become cyanotic at methemoglobin concentrations above 10%. Lethargy and weakness occur at concentrations of approximately 30% or more. At greater than 80%, methemoglobinemia may be fatal. Blood containing a high concentration of methemoglobin appears brown. Accurate and efficient measurement of methemoglobin concentration can be accomplished with dedicated instruments called hemoximeters. These multiwavelength, microprocessor-controlled photometers are designed to measure hemoglobin pigments such as carboxyhemoglobin and methemoglobin as well as the percentage of hemoglobin oxygenation. Samples collected for methemoglobin determination should be analyzed quickly (e.g., within 30 to 60 min) because methemoglobin is normally reduced by the erythrocyte enzyme methemoglobin reductase. The combination of a sensitive, precise instrument and a well-executed toxicology study allows detection of test materialinduced methemoglobin formation at concentrations well below those necessary to cause clinical signs (35). Among laboratory animals, the mouse is a poor model for studying the potential of test materials to cause methemoglobinemia because it has very high activity of methemoglobin reductase (115). Test material-induced methemoglobin in mice is quickly reduced to hemoglobin and therefore more difficult to detect.

Immune-mediated red blood cell destruction has been associated with many drugs (98) but is largely an idiosyncratic phenomenon and is therefore detected infrequently in preclinical toxicology studies. When it is observed, there are typically only one or two animals affected, and they may or may not be in the high-dose group. In contrast to hemolytic conditions that occur immediately upon exposure to the test

material, immune-mediated hemolysis is typically not observed until the test material has been administered for at least a week. There are three general mechanisms for

immune-mediated hemolysis: The test material acts as a hapten bound to the red blood cell membrane; the test material elicits an antibody response and the resulting antigen-antibody complex binds to the red blood cell; and the test material causes the immune system to mistakenly recognize normal red blood cell membrane antigens as foreign, and true autoantibodies are produced. On rare occasion, immune-mediated hemolysis is complement mediated and intravascular. More commonly, cells of the mononuclear phagocyte system, especially in the spleen, recognize antibodycoated red blood cells and either phagocytize the entire cell (extravascular hemolysis) or remove a portion of its membrane, creating morphologically distinct cells called spherocytes. In a blood film, spherocytes appear smaller and denser than other red blood cells; they are perfectly round and lack central pallor. While small numbers of spherocytes can be observed with other conditions, they are the predominant morphologic feature of immune- mediated hemolytic anemia. Autoagglutination of red blood cells will sometimes occur, especially if the antibody response is primarily immunoglobulin M (IgM), and may be observed in the blood film. Alternatively, autoagglutination may be detected in a wet mount of fresh blood diluted with saline. Direct antiglobulin tests may be used to confirm the presence of antibody or complement on red blood cells, but species-specific anti- immunoglobulin or anticomplement must be used. Animals with test material-induced immune-mediated hemolytic anemia become severely anemic with repeated administration of the test material. However, they usually exhibit a strong regenerative response and will nearly always recover if administration of the test material is stopped. Confirmation that the anemia was induced by the test material can be accomplished by rechallenging the animal following recovery. Upon rechallenge, hemolysis should be evident within a day or two. Immune-mediated hemolytic anemia, unrelated to test material administration, is a common sequela of the large granular lymphocyte leukemia observed in a high percentage of Fischer 344 rats older than 1 year of age (119).

Whenever regenerative anemia is identified in a nonhuman primate study, consideration must be given to the possibility of hemolysis associated with the hemotropic parasite *Plasmodium*. Many imported monkeys, even though they are captive bred, harbor subclinical infections with the organism that causes malaria (39). The readily identifiable intracellular organism is frequently observed in blood films from healthy animals that have no signs of anemia. Parasitemia, however, is inconsistent or cyclical, and multiple blood samples may be examined from an animal before the organism is identified. On rare occasion, administration of a test material or the stress of shipment and study-related pro cedures precipitates a hemolytic crisis. When this occurs, the parasitemia is usually quite obvious.

An infrequent cause of hemolysis in toxicology studies is mechanical fragmentation or microangiopathic hemolysis. This form of hemolysis typically occurs when red blood cells are forced to pass through small, fibrin-obstructed vessels in highly vascular tissue. The resulting fragmented red blood cells are called schizocytes (helmet cells) and are easily identified microscopically. Disseminated intravascular coagulation (DIG) is the best example of a condition causing microangiopathic hemolysis, but widespread vascular injury in tissues such as lung, liver, or intestine also causes some degree of fragmentation. These conditions, especially DIG, are generally so severe that an animal may fail to mount a significant regenerative response before it dies or is humanely killed.

Leukocytes

The differential WBC count enumerates neutrophils, lymphocytes, monocytes, eosinophils, and basophils in peripheral blood. Increased numbers of these cells in circulation are termed neutrophilia, lymphocytosis, monocytosis, eosinophilia, and basophilia, respectively. Neutropenia, lymphopenia, monocytopenia, and eosinopenia refer to decreases; normal basophil numbers are so low that decreases are not recognized. Leukocytosis and leukopenia are less specific terms that indicate increased and decreased total WBC count, respectively. When interpreting and reporting differential WBC count results, it is essential to evaluate the absolute cell counts (i.e., cells/^1). Relative or percent counts are simply a means for determining the absolute counts and have little or no inherent value for assessing an animal's condition. A 50% neutrophil count in a dog could be normal or represent neutrophilia or neutropenia, depending on the total WBC count.

Neutrophils and lymphocytes are the most numerous cell types in peripheral blood, and toxicological effects on leukocytes usually involve them. While direct effects on these two cell lines can and do occur (136), indirect effects, in response to study-related procedures or test material effects on other tissues, are much more common.

Physiological Leukocytosis

Excited or frightened animals may exhibit a physiological leukocytosis secondary to endogenous catecholamine release. Increased heart rate, blood pressure, and muscular activity shift cells from the marginal leukocyte pool (i.e., cells that adhere to the endothelium of small vessels or are sequestered in

vascular beds of tissues like the spleen) to the circulating leukocyte pool. The total WBC count may double in number. The specific cell type

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responsible for the majority of the increase varies with species because of species-dependent differences in normal distribution of leukocyte types. Neutrophilia is the most obvious change for dogs, while lymphocytosis is most conspicuous for rats. The physiological leukocytosis observed for primates is fairly evenly distributed between neutrophils and lymphocytes. Because physiological leukocytosis is most frequently observed in animals that are not accustomed to handling or blood collection, it is not unusual for a few animals to have notably high WBC counts only at the first blood collection interval for a study. Recognition of this phenomenon is critical in studies using few animals (usually dogs or monkeys) and only one pretreatment interval. Overinterpretation of the data might lead to the false conclusion that a test material has a myelosuppressive effect because posttreatment WBC counts, determined when the animal is less excited, may be much lower than pretreatment counts. By the same token, if the test material is an antineoplastic drug, and myelosuppression is a critical endpoint, strong consideration should be given to acquiring at least two pretreatment hematology samples to facilitate proper data interpretation.

Steroid- or Stress-Induced Leukocyte Response

This leukocyte pattern occurs following exogenous corticosteroid administration or when stressful conditions result in increased production of endogenous corticosteroids. It is characterized primarily by mature neutrophilia (immature neutrophils, such as bands, are absent), lymphopenia, and eosinopenia. Monocytosis may or may not be present. It is relatively unusual to observe this pattern for an entire group of animals on study, even though study-related procedures or administration of the test material appear to create conditions typically considered stressful. However, individual animals, especially those in moribund condition resulting from toxicity, often exhibit this pattern.

Neutrophilia and Neutropenia

Although their primary function is to protect the host from bacterial infections, neutrophils are a common component of many nonseptic inflammatory lesions as well. It is therefore not unusual to observe increased neutrophil counts secondary to a variety of inflammatory conditions resulting from test material toxicity or study-related procedures (e.g., chronic catheterization or repeated injections), with or without the involvement of an infectious agent. If severe enough, degeneration and necrosis of most tissues result in inflammation and a systemic neutrophil response. Hemolysis, for example, can be a potent stimulus for neutrophilia. In addition, various cytokines with therapeutic potential have been identified and produced that affect neutrophil kinetics directly and may result in mild to marked neutrophilic leukocytosis. The term *left shift* refers to an increased number of immature neutrophils (e.g., band neutrophils) in circulation, usually in response to an inflammatory lesion with a significant demand for neutrophils. Lesions that cause left shifts or marked neutrophilia are almost always easily identified, either by physical examination or at necropsy, and often involve invasion of damaged tissues by bacteria. The term *degenerative left shift* describes the combination of a normal or decreased absolute neutrophil count with more immature than mature neutrophils. It generally reflects a very severe infection as might occur with aspiration pneumonia, gastrointestinal perforation, or septicemia associated with bacterial contamination of an indwelling intravenous catheter. In conditions such as these, "toxic neutrophils" may be observed. These neutrophils have distinct morphological characteristics including cytoplasmic basophilia, vacuolation, and granulation and the presence of Dohle bodies (small, bluish-gray cytoplasmic inclusions made of aggregated rough endoplasmic reticulum).

Unless the test material is a potent cytotoxic agent (e.g., a conventional chemotherapeutic), the observation of severe neutropenia, with or without a left shift or toxic neutrophils, is generally limited to one or two individuals that have severe complications secondary to test material toxicity or study-related procedures. Leukocyte effects of cytotoxic agents, on the other hand, are typically observed in most or all of the animals in groups receiving toxic dose levels. Because mice and young rats normally have very low neutrophil counts (e.g., less than 1000//zl), recognition of neutropenia is more difficult for these species than for others. In addition, while a neutrophil count below 500/μl is generally considered an indicator of great risk for bacterial infection in humans, monkeys, and dogs, the same is not true for rodents.

Single or short-term administration of a potent cytotoxic agent is usually characterized by neutropenia within a few days to a week of treatment, followed by a recovery that may include the presence of immature neutrophils for a short time and may result in a rebound neutrophilia. Detection of these changes is dependent on the timing and frequency of hematology testing. Similarly, the appearance of the bone marrow, whether hypocellular or hypercellular, is also dependent on timing of sample collection. Hematology findings for test materials that directly injure pluripotent stem cells or rapidly dividing committed precursor cells are usually characterized by decreased numbers of reticulocytes,

platelets, neutrophils, monocytes, eosinophils, and possibly lymphocytes. Selective damage of granulocyte precursors without affecting erythropoiesis or thrombopoiesis is unusual.

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Lymphocytosis and Lymphopenia
Lymphocytes are responsible for a wide variety of immune system functions. There are several subpopulations of lymphocytes, but they are indistinguishable by light microscopy. Lymphocytes are relatively long-lived compared with other leukocytes and have the ability to leave the vascular system through venules in lymph nodes and eventually reenter the blood via the thoracic duct. Lymphocytosis is an uncommon test material-related effect, although it may be observed in conjunction with chronic inflammatory lesions, especially in rodents, or with administration of test materials that are antigenic and elicit an immune response by the test animals. Physiological leukocytosis should be considered whenever lymphocytosis is present in only a few animals.
Lymphopenia is most frequently observed as part of the stress- or steroid-induced leukocyte response. Moribund animals are commonly lymphopenic. Cytotoxic test materials often cause decreased absolute lymphocyte count, but the magnitude of the decrease is usually less prominent than that for neutrophils. In rodents, however, it may be easier to detect the effect on lymphocytes because of the normally high number of lymphocytes compared with neutrophils. During recovery from effects of cytotoxic test materials, lymphocyte counts may remain decreased longer than neutrophils and typically do not exhibit a prominent rebound response. Because of the many subpopulations of lymphocytes, it is difficult to gauge the biological importance of small decreases in absolute lymphocyte count. Selective reduction or elimination of a subpopulation may occur (e.g., the human immunodeficiency virus effect on CD4+ lymphocytes) without greatly affecting the total lymphocyte count. Evaluation of the overall health of the animals and findings that might suggest immunosuppression are essential correlates that help to place the importance of small changes into context.
Monocytes, Eosinophils, and Basophils
Absolute counts for these cell types are generally quite low (e.g., < 1000/+l), and effects on these cells are infrequently observed in toxicology studies. As with neutrophils and lymphocytes, increases in circulating numbers of these cells are generally secondary phenomena unless the test material is a hematopoietic growth factor or other cytokine (e.g., interleukin-5 increases eosinophil count) that directly stimulates cell production. Eosinophils are part of the body's defense against helminthic parasite infections, and eosinophilia is occasionally observed in nonhuman primates with a heavy parasite load. Eosinophilia is also observed secondarily to some hypersensitivity reactions. A primary function of monocytes is phagocytosis and digestion of large particulate matter, such as senescent cells and necrotic cell debris. Monocytosis may occur secondarily to any condition with substantial tissue destruction, such as widespread inflammation, tumor-associated necrosis, or hemolytic anemia. Because of their normally low circulating numbers, decreases in these cell types are often difficult to detect. However, eosinopenia and monocytopenia are sometimes identified following administration of chemotherapeutic agents, and eosinopenia is occasionally observed as part of the stress-induced leukocyte pattern. Effects on basophil counts are extremely rare.
Leukemia
In most, if not all, 2-year carcinogenicity studies using rodents, a small percentage of the animals will develop leukemia, a neoplastic proliferation of a hematopoietic cell line. Although leukemias are often characterized by markedly elevated white blood cell counts and the presence of neoplastic cells (e.g., blasts) in circulation, some animals with leukemia exhibit neither of these prominently, and the diagnosis is best made by histopathological examination of tissues infiltrated with the neoplastic cells. The odds of correctly identifying animals with leukemia are much greater by doing routine histopathology than by doing periodic examinations of the blood at regularly scheduled intervals. Even when neoplastic cells are present in peripheral blood, it is often difficult to determine from which cell line they were derived (e.g., granulocytic, lymphocytic, myelomonocytic, etc.) because immature, anaplastic, or blast-stage cells from different cell lines can be indistinguishable by light microscopy using standard staining techniques.
The most commonly observed leukemia in laboratory animals is large granular lymphocyte leukemia of Fischer 344 rats (119). As many as 30–40% of Fischer 344 rats may develop this leukemia, also known as mononuclear cell leukemia, in the second year of a carcinogenicity study. The neoplasm appears to arise in the spleen and commonly infiltrates other tissues, particularly the liver. Affected rats consistently develop an immune-mediated hemolytic anemia and often exhibit hyperbilirubinemia and elevated liver enzyme activities in the serum. Neoplastic cells in peripheral blood appear as large, immature lymphocytes and may contain prominent azurophilic granules. Erythrophagocytosis by the neoplastic cells is occasionally observed.
Platelets
When blood vessels are damaged, platelets quickly adhere to the subendothelium, undergo a shape

change, and begin to aggregate, forming a primary platelet plug that is sufficient to control bleeding from minor injuries to small vessels. These activated platelets secrete a var-

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iety of substances that stimulate vasoconstriction and promote fibrin formation. Fibrin serves to cement the aggregated platelets into a stable hemostatic plug. Healthy endothelial cells in close proximity to the damaged vessel release inhibitors of platelet aggregation and fibrin formation in order to limit the size of the clot.

Thrombocytosis is typically asymptomatic, although extremely high platelet counts may increase the risk of thrombosis. Signs of thrombocytopenia include petechial and ecchymotic hemorrhages, most commonly observed in mucous membranes or at mucocutaneous sites, epistaxis, melena, menorrhagia, and prolonged bleeding from small wounds such as venipuncture sites. These signs typically do not occur spontaneously unless the platelet count is very low (e.g., less than 20,000/jul) or there is some type of hemostatic challenge (e.g., surgery) (9). Test materials that affect platelet function have the potential to cause the same clinical signs as marked thrombocytopenia, but the tendency to do so is much less because of the complexity of platelet function and the presence of alternative or redundant pathways in vivo for the various platelet functions. Platelet function tests such as bleeding time and platelet aggregation may be beneficial when evaluating safety of therapeutic agents related to coagulation and platelet function, but it is important to recognize the considerable analytical and interanimal variability for these tests. Group results may be less meaningful than assessment of results from individual animals before and after test material administration.

Thrombocytosis

Unless the test material is a hematopoietic growth factor (e.g., thrombopoietin), increased platelet count is almost never a primary effect. The terms *reactive* or *secondary thrombocytosis* have been used to describe the increased platelet count observed in conjunction with generalized bone marrow stimulation as observed with hemolytic anemia, blood loss, and many types of acute and chronic inflammation. Release of cytokines such as interleukin-6 and interleukin-11 may be at least partially responsible for the increased platelet production (141) in some of these conditions. Acute, transient thrombocytosis may occur in association with physiological leukocytosis because catecholamine-induced splenic contraction releases platelets into circulation that were sequestered within the sinusoids of the spleen. A rebound thrombocytosis typically follows significant thrombocytopenia caused by test materials such as chemotherapeutic agents that reversibly inhibit platelet production or injure megakaryocytes. The increased platelet counts observed in toxicology studies are generally small and not likely to have any biological significance.

Thrombocytopenia

Decreased platelet count is a relatively common spurious finding associated with difficult venipuncture or inadequate anticoagulation of the blood sample and subsequent in vitro platelet aggregation. When this occurs, platelet clumps can usually be observed at the feathered edge of the blood film. Although most typically a problem detected for individual mice and rats, spuriously low platelet counts can sometimes appear group related because animals receiving the test material may be more difficult to bleed as a result of poor health, dehydration, or small size relative to the control animals.

When not a spurious finding, decreased platelet count results from decreased production or increased consumption of platelets. Test materials that reduce erythroid and myeloid cell production, such as chemotherapeutic agents, frequently inhibit platelet production as well. Moderately to markedly reduced platelet counts tend to occur a few days after obvious reductions in neutrophil and reticulocyte counts because the circulating life span of platelets is about 7 to 10 days. Thrombocytopenia due to increased consumption of platelets can occur secondarily to acute lesions of highly vascular tissues such as the gastrointestinal tract or result from extensive hemorrhage, especially from multiple sites. If lesions affecting blood vessels are severe and widespread, disseminated intravascular coagulation may develop, and platelet counts will be markedly decreased. Immunemediated thrombocytopenia, like immune-mediated hemolytic anemia, has been associated with many drugs (54) but is largely an idiosyncratic phenomenon and is therefore detected infrequently in preclinical toxicology studies. When observed, there are typically only one or two animals affected, and these animals may or may not be in the high-dose group. Immune-mediated thrombocytopenia and immune-mediated hemolytic anemia may occur together. Antiplatelet antibody is very difficult to detect, and the best evidence that thrombocytopenia is immune mediated may come from a rechallenge exposure with the test material following cessation of treatment and recovery. Upon rechallenge, platelet count should drop acutely if the mechanism is immune-mediated destruction.

Bone-Marrow Smear Evaluation

The most important aspect of bone marrow smear evaluation is understanding when it is indicated. Although preparation of smears is advisable for most regulated, repeated-dose toxicology studies,

evaluation of those smears is usually unnecessary. The standard hematology tests provide considerable information con-

earning bone marrow function, and if results from these tests are unaffected, it is very unlikely that bone marrow evaluation will provide any additional knowledge concerning potential significant test material effects on the hematopoietic system. Even when results of hematology tests are affected by administration of a test material, bone marrow evaluation has no benefit if mechanisms for the hematology findings are already evident. For example, regenerative anemias indicate a normal erythropoietic response to hemorrhage or hemolysis, and bone marrow evaluation would simply confirm the presence of erythroid hyperplasia. Neutrophilic leukocytosis in response to an inflammatory condition is normal, and bone marrow evaluation would only confirm granulocytic hyperplasia. The main indications for bone marrow smear evaluation in toxicology studies are moderate to marked nonregenerative anemia, leukopenia, or thrombocytopenia (or any combination of the three) with no apparent etiology. If the effects observed in peripheral blood are relatively small, it is less likely that bone marrow smear evaluation will be beneficial. The primary objective of the bone marrow smear evaluation is to assess the relative numbers of precursor cells, their morphologic appearance, and whether they are developing or maturing normally. Miscellaneous findings such as increased iron stores, plasma cell hyperplasia, or excessive cytophagia may also be recognized.

There are multiple approaches to bone marrow smear evaluation. Regardless of the approach taken, the findings must be interpreted in conjunction with peripheral blood test results. The most simplistic and least informative bone marrow evaluation is to determine the myeloid: erythroid (M:E) ratio by counting at least 500 cells and differentiating the granulocytic cells from the erythroid cells. An increased M:E ratio may indicate granulocytic hyperplasia, erythroid hypoplasia, or both. If the animal has a high neutrophil count and its hematocrit is normal, then an increased M:E ratio likely indicates granulocytic hyperplasia. If the animal has a normal neutrophil count and a nonregenerative anemia, then an increased M:E ratio likely indicates erythroid hypoplasia. In both cases, the outcome of the M:E ratio could have been predicted from the peripheral blood results. If the animal is neutropenic and has a nonregenerative anemia, an increased M:E ratio only indicates that there are relatively more granulocytic cells than erythroid cells. It does not help to understand the underlying problem.

The most time-consuming and labor-intensive bone marrow evaluation is to perform a bone marrow cell differential count by differentiating the cell type of at least 500 cells. When completed, the M:E ratio can be calculated from the results. This evaluation yields more information but at a very high cost. Differential counts provide numerical information concerning the relative numbers of different precursor cells and whether a cell line is maturing normally. Unusual or abnormal morphological characteristics of the cells must be described separately.

The most cost-effective and informative approach to bone marrow evaluation is the subjective cytological examination. In this approach, the bone marrow smear is examined in much the same manner as a morphologic pathologist examines a histologic section of liver, and a diagnosis or interpretation is recorded. The person performing the examination, usually a veterinary pathologist or clinical pathologist, assesses the quality and cellularity of the smear, the presence and relative number of precursors for each of the three major cell lines (erythrocytes, granulocytes, and platelets), and the maturation of each of the cell lines. Abnormal morphology is noted, as well as unusual numbers or characteristics of other cell types such as lymphocytes, plasma cells, monocytes, macrophages, and mast cells. A diagnosis or interpretation is rendered based on the examination of the smear and the results of the peripheral blood tests. Because bone marrow smears are relatively poor indicators of the actual cellularity of the bone marrow, it is also prudent to consider the histopathological findings for sections of sternum, rib, or possibly femur. While these sections are inadequate for evaluating individual cell types and abnormal cell morphology, they can provide a good assessment of overall cellularity. Ultimately, the goal of the bone marrow smear evaluation is to determine if administration of the test material has negatively impacted the number and/or maturation of hematopoietic cell precursors (103). For example, the absence of megakaryocytes indicates thrombocytopenia is due to failure of platelet production rather than increased platelet consumption peripherally. Increased numbers of normal-appearing megakaryocytes indicate thrombocytopenia is due to a consumptive process.

Coagulation

The clotting mechanism or "cascade" has traditionally been divided into two pathways. In vivo, the intrinsic pathway begins with the activation of zymogen factor XII following exposure to negatively charged subendothelial components such as collagen. Factors XI, IX, and VIII are also part of the intrinsic pathway. The extrinsic pathway begins with the activation of zymogen factor VII following exposure to tissue factor (also called tissue thromboplastin) expressed by cells deep in the vessel wall. Both pathways share the same terminal sequence of events including activation of factor X, con-

version of prothrombin to thrombin, and conversion of fibrinogen to fibrin. It is thought that the extrinsic pathway is the primary initiator of coagulation in vivo (70). The intrinsic and extrinsic pathways are routinely evaluated by the activated partial thromboplastin time (APTT) and one-stage prothrombin time (OSPT or PT), respectively. The activated coagulation time (ACT) test is a simple, rapid measure of the intrinsic pathway that does not require a coagulation analyzer (17, 107, 140). These coagulation assays are relatively insensitive and nonspecific. Activity of a single clotting factor must be reduced to approximately 30% of normal before noticeably prolonged times are detected for an individual animal. When the results from groups of animals are com pared, statistically significant differences are occasionally observed that are smaller than what would generally be considered an important change for an individual animal (e.g., less than 2 s of difference between the means for the control and high-dose groups). The toxicological sig nificance of differences such as these is sometimes difficult to determine. While they clearly do not represent an effect likely to be associated with a bleeding diathesis for indi vidual animals, they may be an indication of an important change in coagulation homeostasis. It may be valuable to design a longer study or increase the dose level to see if the effect is repeatable, dose-related, and associated with clinical signs.

Under the conditions of most toxicology studies, where animals are exposed to high concentrations of a test material for a prolonged period of time, any major effect on the production of a clotting factor will likely result in a clinically obvious bleeding diathesis. The adminis tration of vitamin K antagonists such as dicumarol or the ingestion of synthetic or poorly absorbed fat substi tutes is associated with bleeding and prolonged APTT and PT because the fat-soluble vitamin K is required by the liver for production of functional forms of factors II, VII, IX, and X. In theory, PT will be affected before APTT because factor VII has the shortest half-life of the clotting factors. Although nearly all the clotting factors are synthesized by the liver, APTT and PT are insensitive measures of liver function. Because of the liver's large functional reserve, liver injury must be rela tively severe before coagulation times are noticeably affected. In addition to the previously mentioned thrombocytopenia, disseminated intravascular coagula tion is characterized by depletion of all clotting factors, including fibrinogen, and moderately to markedly pro longed coagulation times. In many cases of disseminated intravascular coagulation, the plasma samples fail to clot during the coagulation assays.

Similarly to platelet count, coagulation times can be spuriously prolonged by difficult blood collection or poor collection technique. The combination of low platelet count and prolonged coagulation times, in an otherwise healthy animal, is an indication of poor sample quality. Inherited factor VII deficiency affects a small number of laboratory beagles (38). These animals can usually be distinguished during pretreatment screening by a PT that is 2 or 3 s longer than those of the other animals acquired for the study. Although the deficiency rarely causes a clinical problem, it would be inappropriate to use these animals if the test material is known or suspected to affect coagulation. PT and APTT can both be artifactually prolonged because of excessive sodium citrate anticoagulant in the plasma sample (79, 96). This could occur if insufficient blood volume is added to standardized collection tubes or if the animal's hematocrit is elevated because of hemoconcentration (e.g., dehydration) or drug-induced polycythemia. Normal coagulation times vary from one laboratory animal species to another. Among the notable differences are the relatively fast PTs for dogs (e.g., 6 to 8 s) and slow PTs for guinea pigs (e.g., 30 to 40 s).

CLINICAL CHEMISTRY TESTS AND INTERPRETATION

Routinely performed clinical chemistry tests provide information concerning hepatocellular and biliary integrity and function, renal function, carbohydrate, lipid, and protein metabolism, and mineral and electrolyte balance. Modern clinical chemistry analyzers require very small sample volumes, and complete biochemical profiles can be obtained from rats at multiple intervals within a study without compromising the health of the animals because of excessive blood collection. Less than 250 µl of serum is needed to perform as many as 20 tests. Few of the common test methods require modification for testing animal samples.

Hepatocellular and Hepatobiliary Integrity and Function

Many routine clinical chemistry tests can be affected by liver toxicity because of the liver's critical metabolic, synthetic, and excretory functions and the abundant enzymatic machinery needed to perform these functions (110, 120). Conversely, a significant loss of liver tissue with little or no detectable change in routine tests is possible because of the liver's large functional reserve. No single test is superior for detecting all of the various types of liver toxicity, but the pattern of abnormal findings in a battery of tests may help characterize the location and severity of liver lesions (21).

Liver Enzymes

Many enzymes normally present within hepatocytes exhibit increased serum activity following hepatocellular

injury with degeneration and necrosis. The utility of a particular enzyme depends on factors such as relative specificity to liver, intrahepatic location, intracellular location, the concentration gradient between cell and serum, serum half-life, in vitro stability, and economy of measurement (12, 13, 74). The most frequently used enzymes to assess hepatocellular injury are alanine aminotransferase (ALT), aspartate aminotransferase (AST), sorbitol dehydrogenase (SDH), glutamate dehydrogenase (GDH), and lactate dehydrogenase (LDH). Each has advantages and disadvantages. The aminotransferases, ALT and AST, were formerly referred to as serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT), respectively, and are the most commonly measured of all serum enzymes. Although sometimes included under the heading of liver function tests, serum activities of hepatic enzymes do not evaluate liver function.

In general, ALT is the most useful enzyme for detection of hepatocellular injury in the majority of laboratory animal species. Although the enzyme is present in many tissues, its greatest concentration in most species is within hepatocytes, and for practical purposes, significant elevations of serum ALT activity result only from release of ALT by hepatocytes. Species for which ALT is less useful because of relatively low hepatocyte concentrations include the guinea pig (28) and large domestic animals such as pigs, goats, sheep, cows, and horses (13, 30, 78). Because the enzyme is primarily cytosolic, and its concentration within the cell is up to 10,000 times greater than that in the serum, ALT may enter the serum in any condition that sufficiently alters cell membrane permeability. In addition to simple leakage from degenerating or necrotic cells, there may be other mechanisms for movement of the enzyme across the cell membrane because high serum activities of ALT are occasionally observed with no apparent cell death. The magnitude of serum activity elevation is proportional to the number of affected hepatocytes and is not necessarily indicative of the reversibility of the lesion. However, the greatest elevations result from severe lesions affecting a large portion of the liver. As a general guideline, activities for ALT in excess of 200 IU/L are usually accompanied by histopathologic evidence of hepatocellular injury, while activities below this level may or may not have correlative findings. Following an acute but reversible hepatotoxic event, serum ALT activity rises relatively rapidly, peaks within 1 or 2 days, and then declines over the next few days. Significant hepatotoxicity can go undetected if clinical pathology tests are delayed for 1 or 2 weeks following a single administration of test material. Prolonged elevations following a single insult may reflect increased production of ALT in regenerative liver tissue or continued loss of ALT from cells in close proximity to the primary lesion that undergo degenerative changes as a result of the altered microenvironment.

Increased serum ALT activity does not always indicate primary hepatocellular injury. Biliary disease or toxicity and bile duct obstruction may cause increased serum ALT activity at least in part due to the effect of retained bile salts on the cell membranes of neighboring hepatocytes. Muscle damage, if severe and extensive, can increase serum ALT activity in the absence of hepatic injury (123, 133). Increased intracellular activity of ALT as a result of induction will cause serum ALT activity to increase proportionately. Drugs such as corticosteroids and anticonvulsants appear to induce ALT production. Because these drugs may also have pathologic effects on hepatocytes, it may be difficult to determine whether an elevation is due to enzyme induction or drug-induced disease.

Interpretation of potential effects on serum ALT activity in monkeys may be complicated by the presence of subclinical, enzootic hepatitis A infection (111). Transiently increased serum ALT activity correlates with seroconversion to the virus and periportal inflammation. Because animals entering a facility may not have been exposed to the virus previously, it is possible to observe sporadic, high ALT activities (e.g., up to approximately 300 IU/L) for a few individual monkeys during the course of a toxicology study. Some facilities choose to bank serum collected from monkeys before a study is initiated for possible serologic testing to help clarify ambiguous study findings for ALT activity. Interpretation of serum ALT activities for mice is complicated by considerable interanimal variability, much of which is probably due to the effects of handling (122). In toxicology studies using mice, it is not unusual for a few animals, including the control animals, to have much higher serum ALT activities than those of the majority (e.g., 200 IU/L vs. 40 IU/L). The cause of these high activities is thought to be physical damage to the liver, especially when mice are handled by grasping the body. Unfortunately, if the only animals affected happen to belong to the high-dose group, it may be difficult to rule out an effect of the test material.

Serum AST and LDH activities tend to parallel serum ALT activity with respect to liver damage, but these enzymes are much less liver specific because of high concentrations in other tissues, especially muscle. Rodents tend to exhibit increased interanimal variability for these enzymes, some of which may be due to contamination with muscle tissue during blood collection procedures such as cardiac puncture and

rupture of the retroorbital plexus. There is little advantage to determining both of these enzyme activities, and AST is generally preferred. Elevations in serum AST activity caused by hepatotoxicity are usually less pronounced than concurrent elevations in serum ALT activity. Since a portion

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of intracellular AST is located in mitochondria, a more severe injury may be necessary for release of like quantities of AST. As with ALT, drugs such as corticosteroids and anticonvulsants may induce production of AST.

Decreased serum activities of ALT and AST are occasionally observed in toxicology studies. Among the potential causes for these findings are decreased hepatocellular production or release of the enzymes, inhibition or reduction of the enzymes' activity, and interference with the enzyme assay. The most widely recognized of these causes involves an effect on pyridoxal 5′-phosphate (vitamin Be), a coenzyme cofactor required for full catalytic activity of the aminotransferases. If a test material negatively affects this cofactor, directly or indirectly, serum aminotransferase activities decrease (31, 37, 132). Because the aminotransferase assays can be run with or without additional pyridoxal 5′-phosphate, a test material-related effect on pyridoxal 5′-phosphate can be identified by correction of an apparent decrease in aminotransferase activity when the assay is repeated with additional cofactor. Regardless of the mechanism involved, decreased serum activities of the aminotransferases have not been shown to correlate with toxicologically significant effects on the liver.

Serum SDH and GDH activities have been recommended as good indicators of hepatic toxicity in laboratory animal species (21, 40, 135) because increased serum activities are liver specific and relatively sensitive. SDH is a cytosolic enzyme, and GDH is mitochondrial. Elevations in serum SDH activity generally return to baseline levels faster than for other liver enzymes because of a short serum half-life. The addition of either of these tests to a standard clinical chemistry profile is a good choice if potential liver toxicity is of particular interest. The major drawback for both, however, is the assay. Although GDH is commonly used in Europe and both enzymes have been used in veterinary medicine for assessing liver injury in large domestic animals, simple test kits and standard automated procedures are unavailable because these enzymes lack popularity in human medicine in the United States.

Several enzymes that originate from hepatocytes and biliary epithelial cells are increased in serum as a result of increased production following intrahepatic or extrahepatic cholestasis or in conjunction with biliary hyperplasia. These include serum alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), leucine aminopeptidase (LAP), and 5′-nucleotidase (5′-N). Of these, the most commonly measured are ALP and GGT.

Serum ALP activity results from a mixture of membrane-localized isoenzymes, the product of different genes, and isoforms, the product of posttranslational modifications. In humans, at least four genes have been identified that code for different ALP isoenzymes: tissue nonspecific (found in liver, bone, and kidney), intestinal, placental, and germ cell. In most laboratory animals, only two genes have been identified: tissue nonspecific and intestinal. Although the ALP activities originating in liver, bone, and kidney are the product of the same gene and therefore are isoforms, they can be distinguished because of differences in degree of posttranslational glycosylation and tissue of origin and have classically been referred to as isoenzymes (64, 65). Dogs have a unique isoform, known as the corticosteroid-induced ALP isoenzyme, that is a hyperglycosylated form of the intestinal ALP isoenzyme. The contribution of each isoenzyme to the total serum ALP activity is dictated by tissue production and serum half-life.

In normal dogs, serum ALP activity is a combination of the liver, bone, and corticosteroid-induced isoenzyme activities. The relative amount of corticosteroid-induced ALP activity is small compared with the others, and it is completely absent for most dogs. In young puppies, the bone isoenzyme may be responsible for up to 95% of total serum ALP activity, while in adult dogs, the liver isoenzyme is the predominant form. Intestinal and kidney ALP isoenzymes are virtually absent in dog serum because of extremely short serum half-lives, and diseases affecting these tissues do not increase serum ALP activity. In normal rats, serum ALP activity is a combination of the liver, bone, and intestinal isoenzyme activities. The intestinal isoenzyme activity increases after feeding and is reduced with fasting (130). Because toxicology studies are routinely initiated with young, growing animals, it is common for total serum alkaline phosphatase to decrease during the course of the study as a result of reduced osteoblast activity (the origin of bone ALP) in the maturing animals.

In spite of the number of ALP isoenzymes, serum ALP activity is most frequently considered a measure of cholestasis. It is a sensitive indicator of cholestasis in the dog and usually increases well before other markers such as GGT and total bilirubin. Because of cell swelling and pressure obstruction of small bile ductules, primary hepatocellular toxicities often cause enough intrahepatic cholestasis to elevate serum ALP activity. Periportal lesions result in higher activities than do centrilobular lesions. Extrahepatic cholestasis, as might occur with pancreatitis, biliary calculi, or complications of bile duct cannulation, stimulates higher serum ALP activity than intrahepatic cholestasis. The degree of elevation, however, is rarely sufficient for differentiating primary hepatocellular toxicity from primary biliary toxicity. In contrast

to the dog, the value of serum ALP activity for distinguishing cholestatic lesions in monkeys is reduced because of marked interanimal variability.

Although the lack of specificity is sometimes considered a fault of ALP, the potential for detecting increases in serum ALP activity due to effects on bone is actually

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a benefit of this test. Elevations of serum ALP activity can be the first indication of a toxic effect on bone formation. Elevations of serum ALP activity due to increased osteoblast activity tend to be less pronounced than those due to cholestasis (e.g., no greater than two- to threefold higher than control animals). If the test material is administered for sufficient duration, the effect on ALP activity is usually accompanied by clinical or histopathological evidence of effects on bone.

Drugs such as anticonvulsants and corticosteroids can induce production of the liver ALP isoenzyme, with or without evidence of hepatobiliary disease. Following corticosteroid administration to dogs, liver ALP activity increases within a few days, while the corticosteroid-induced ALP activity does not increase noticeably for about 10 days (113). Although an increase in corticosteroid-induced ALP activity has been observed in dogs with a variety of chronic disease conditions (65) and may be related to increased endogenous corticosteroid release, this isoenzyme has not been closely evaluated in toxicology studies using dogs.

The measurement of serum GGT activity has gained popularity because it is more specific than ALP and was shown to be effective in certain models of biliary toxicity in the rat (82). Although the highest tissue concentrations of this membrane-localized enzyme are in the kidney and pancreas, serum elevations have been reported only with hepatobiliary toxicity and following induction by drugs that stimulate microsomal enzyme production (56, 110). Unlike ALP, GGT is not affected by bone growth or disease. Furthermore, its serum activity is less likely to increase secondary to primary hepatocellular toxicity or intrahepatic cholestasis due to hepatocellular swelling. In rodents, serum GGT activity is often undetectable, and even small increases may be significant.

Serum LAP and 5′-N activities have been investigated as alternatives to ALP and GGT but have not found general acceptance. In some models of liver toxicity, 5′-N appears to be more sensitive than ALP or GGT (21, 22).

The sensitivity or predictive value of any liver enzyme is largely dependent on the models of hepatotoxicity used to make those determinations. Acknowledgment of this fact is at least partially responsible for the practice of including multiple liver enzymes in the clinical chemistry test panels for toxicology studies. The absence of change in liver enzyme activities does not necessarily rule out the possibility of hepatotoxicity or hepatic dysfunction. Elevations may be missed because of poor timing for clinical pathology testing, and excessive variability within the control group (especially for mice and monkeys) may obscure an effect on treated groups. Furthermore, serum liver enzyme activities are not hepatic function tests. The liver can be dysfunctional in the absence of significant cholestasis or ongoing hepatocellular degeneration and necrosis. Animals with end-stage liver cirrhosis, for example, can exhibit normal serum enzyme activities. In contrast to the liver enzymes, serum total bilirubin concentration is primarily a liver function test. In the absence of hemolysis, hyperbilirubinemia indicates liver dysfunction.

Bilirubin

Bilirubin results from the breakdown of heme by cells of the mononuclear phagocyte system. Hemoglobin from senescent erythrocytes accounts for approximately 85% of all serum bilirubin. When macrophages release bilirubin into circulation, it is known as free, unconjugated, prehepatic, or indirect bilirubin. It is water insoluble and circulates bound to albumin. Hepatocytes efficiently remove unconjugated bilirubin from plasma and prepare it for removal from the body by a four-step process that includes uptake, conjugation, secretion, and excretion. Secretion of conjugated bilirubin across the canalicular membrane is the rate-limiting step in the process, and small amounts of conjugated or direct bilirubin escape into plasma. Conjugated bilirubin is not bound to albumin and is freely filtered through the glomerulus. In most species, conjugated bilirubin is completely reabsorbed by renal tubular epithelial cells unless the amount of filtered bilirubin is excessive. In the dog, the renal threshold is low and traces of bilirubin are normal in concentrated urine.

Even though the liver is a frequent target organ, increased total bilirubin concentration, whether due to conjugated bilirubin, unconjugated bilirubin, or both, is a relatively uncommon finding in toxicology studies because of the large functional reserve of the liver. In the dog, a 70% hepatectomy will not increase total bilirubin concentration.

Conjugated hyperbilirubinemia occurs as a result of impaired secretion of bilirubin, cholestasis, or both. Because bilirubin secretion is the rate-limiting step, any disease that damages enough hepatocytes can potentially increase serum conjugated bilirubin concentration. Periportal lesions cause higher serum bilirubin concentrations than do centrilobular lesions, and extrahepatic cholestasis causes higher serum bilirubin concentration than does intrahepatic cholestasis. When increased bilirubin concentration results from a cholestatic process, particularly in the dog, serum ALP activity is elevated.

Unconjugated hyperbilirubinemia occurs almost exclusively as a result of relatively severe, acute

hemolysis. If hepatocytes cannot process the large amount of unconjugated bilirubin produced by macrophages during a hemolytic episode, serum bilirubin concentration increases. A hemolytic event sufficient to overload a normal liver always produces other findings indicative of hemolysis. It is possible, however, for

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relatively modest hemolysis to cause unconjugated hyperbilirubinemia if hepatic function is already compromised. Although a number of nonhemolytic, unconjugated hyperbilirubinemia syndromes are known, these syndromes are usually due to hereditary defects in the uptake and conjugation of free bilirubin.

Unconjugated (or indirect) bilirubin can be differentiated from conjugated (or direct) bilirubin by the Van den Bergh test. The test is used clinically to help distinguish prehepatic causes of hyperbilirubinemia, such as hemolysis, from hepatic or posthepatic causes such as hepatitis or biliary obstruction. In well-designed toxicology studies, the combination of clinical observations, other laboratory data (e.g., hematocrit or liver enzyme activities), and anatomical pathology findings (e.g., hemosiderin accumulations in splenic macrophages or periportal hepatocellular necrosis) are usually more than sufficient to determine the primary mechanism for any observed hyperbilirubinemia. Laboratory determination of direct and indirect bilirubin is rarely necessary and should not be included as part of the routine panel of tests performed in toxicology studies.

Decreased serum bilirubin concentration is occasionally associated with administration of test materials that induce microsomal enzyme production (56). Human patients receiving phenobarbital therapy have lower serum bilirubin levels than the general population as a whole (69). Enzyme induction apparently enhances the metabolism and excretion of bilirubin and could potentially mask an otherwise elevated bilirubin level.

Bile acids are synthesized from cholesterol by hepatocytes, conjugated to an amino acid, secreted into the biliary system, and excreted into the intestine where they facilitate fat absorption. There is an efficient enterohepatic circulation of bile acids with most of the reabsorption occurring at the level of the ileum. Portal blood conveys the bile acids to the liver for uptake, reconjugation, and resecretion. Any toxicity of the liver has the potential to alter one of the steps in bile acid metabolism and cause increased serum bile acid concentration. Like bilirubin concentration, bile acid concentration is a measure of a hepatic function. Although not commonly used in toxicology studies, serum bile acid concentration is considered a sensitive and specific test for hepatobiliary disease (24). Alone, however, increased serum bile acid concentration does not discriminate between different types of hepatic lesions.

Miscellaneous Parameters

The liver is wholly or partially responsible for the synthesis of many substances including glucose, cholesterol, urea nitrogen, and a variety of proteins. Severe hepatocellular dysfunction can cause decreased serum urea nitrogen concentration, hypoglycemia, hypocholesterolemia, hypoproteinemia (especially hypo albuminemia), and prolonged coagulation times. On the other hand, liver disease can also result in hypercholesterolemia and hyperglobulinemia. The patterns of change caused by different types of liver toxicity, whether primary or secondary (e.g., hypoxia-induced centrilobular necrosis), are varied, but often overlapping. Examination of the entire biochemical profile, along with other clinical pathology and anatomical pathology findings, is necessary to properly evaluate potential liver toxicity.

Renal Function

Serum urea nitrogen and creatinine concentrations, in conjunction with urine specific gravity or urine osmolality, are the most common tests used to evaluate renal function (10, 52, 83, 101, 116). Although easy and inexpensive to perform, these tests are relatively insensitive to small effects on the kidney, and there are a number of nonrenal causes for changes in their results.

Urea is synthesized by the liver from ammonia that is absorbed from the intestine or produced by endogenous protein catabolism. Urea is freely filtered through the glomerulus and excreted in urine. Some urea is reabsorbed passively with water at the level of the proximal tubule; the amount reabsorbed is inversely related to rate of urine flow through the tubule. Serum urea nitrogen concentration is therefore affected by the rate of urea production, the glomerular filtration rate (GFR), and flow rate of urine through the renal tubule. Increased serum urea nitrogen concentration, termed azotemia, is categorized as prerenal, renal, or postrenal.

Prerenal azotemia occurs as a result of increased urea synthesis or decreased renal blood flow. Increased urea synthesis results from consumption of high-protein diets or conditions that increase protein catabolism such as starvation, fever, infection, tissue necrosis, and high gastrointestinal hemorrhage. Decreased renal blood flow, which decreases GFR, results from conditions such as dehydration (the most common cause of increased urea nitrogen in toxicology studies), cardiovascular disease, or shock. Changes in serum urea nitrogen concentration caused by increased urea synthesis are typically small. Changes caused by decreased renal perfusion may also be small, but if GFR is severely affected, the increase in urea nitrogen is indistinguishable from that which would occur due to primary renal failure. The causes of prerenal azotemia typically do not affect the ability of the kidney to

concentrate urine. If dehydration is the cause of increased serum urea nitrogen concentration, urine specific gravity and urine osmolality will usually be increased, and urine volume will be decreased, because the kidneys attempt to conserve water. It is not unusual to observe very small, but statistically significant, differ

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