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Hugo & Russell's Pharmaceutical Microbiology

8th Edition

Edited by
Stephen P Denyer
Norman Hodges
Sean P Gorman
Brendan Gilmore

WILEY-BLACKWELL

Hugo and Russell's

Pharmaceutical Microbiology

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Pharmaceutical Microbiology

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Preface to the eighth edition

We have been enthusiastic participants in the preparation of this 8th edition of Pharmaceutical Microbiology, a textbook which has again grown in size, reflecting advances in knowledge and the sustained relevance of microbiology in pharmacy. We have continued to develop the theme of recent editions, strengthening the connection between the basic sciences and clinical practice with an increased emphasis on pathogens and the host response, prescribing therapeutics and public health microbiology.

Once again, the editors must pay tribute to the willing efforts of our contributors, some of whom join us for the first time. So too must we thank our publishers for their support and expertise.

A book that outlasts its original editors is a tribute to their far-sightedness. It is with great sadness but much respect that the editors record the passing of Denver Russell in 2004. This edition is dedicated to him.

S.P. Denyer
B. Gilmore
S.P. Gorman
N.A. Hodges

Preface to the first edition

When we were first approached by the publishers to write a textbook on pharmaceutical microbiology to appear in the spring of 1977, it was felt that such a task could not be accomplished satisfactorily in the time available.

However, by a process of combined editorship and by invitation to experts to contribute to the various chapters this task has been accomplished thanks to the cooperation of our collaborators.

Pharmaceutical microbiology may be defined as that part of microbiology which has a special bearing on pharmacy in all its aspects. This will range from the manufacture and quality control of pharmaceutical products to an understanding of the mode of action of antibiotics. The full extent of microbiology on the pharmaceutical area may be judged from the chapter contents.

As this book is aimed at undergraduate pharmacy students (as well as microbiologists entering the pharmaceutical industry) we were under constraint to limit the length of the book to retain it in a defined price range. The result is to be found in the following pages. The editors must bear responsibility for any omissions, a point which has most concerned us. Length and depth of treatment were determined by the dictate of our publishers. It is hoped that the book will provide a concise reading for pharmacy students (who, at the moment, lack a textbook in this subject) and help to highlight those parts of a general microbiological training which impinge on the pharmaceutical industry.

In conclusion, the editors thank most sincerely the contributors to this book, both for complying with our strictures as to the length of their contribution and for providing their material on time, and our publishers for their friendly courtesy and efficiency during the production of this book. We also wish to thank Dr H.J. Smith for his advice on various chemical aspects, Dr M.I. Barnett for useful comments on reverse osmosis, and Mr A. Keall who helped with the table on sterilization methods.

W.B. Hugo
A.D. Russell

Part 1 Biology of microorganisms

1 Introduction to pharmaceutical microbiology

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1 Microorganisms and medicines

The opening paragraph of the previous edition of this book published in 2004 stated that 'despite continuing poverty in many parts of the world and the devastating effects of HIV and AIDS, the health of the world's population is progressively improving'. That trend has been sustained in recent years with the number of AIDS deaths reaching a peak in 2006 and the number of new HIV infections falling 16% between 2000 and 2008. During that same period life expectancy rose in 157 out of the 193 countries reporting data to the World Health Organization and declined in only 9. Much of this improvement is due to better nutrition and sanitation, but improved health care and the greater availability of effective medicines with which to treat common human and animal diseases are also major contributing factors. Substantial inroads have been made in both the prevention and treatment of cancer, cardiovascular disease and other major causes of death in Western society, and of infections and diarrhoeal disease that remain the big killers in developing countries. Several infectious diseases have been eradicated completely, and others from substantial parts of the world. The global eradication of smallpox in 1977 is well documented, and in 2011 rinderpest, the high-mortality cattle disease which, for centuries, has contributed to poverty and famine in Africa and Asia, will also formally be declared extinct; polio and guinea-worm infection are expected to follow in the next few years.

The development of the many vaccines and other medicines that have been so crucial to the improvement in world health has been the result of the large investment in research by the major international pharmaceutical companies. This has led to the manufacture of pharmaceuticals becoming one of the most consistently successful and important industries in many countries, not only in the traditional strongholds of North America, Western Europe and Japan but, increasingly, in Eastern Europe, the Indian subcontinent and the Far East. Worldwide sales of medicines and medical devices are estimated to have exceeded $711 billion in 2007 (the latest year for which statistics are available), and in the UK pharmaceuticals was the industry sector with the largest trade surplus in 2007 having exports of £14.6 billion—a figure that translates into more than £235 000 for each employee in the industry. The growth of the pharmaceutical industry in recent decades has been paralleled by rising standards for product quality and more rigorous regulation of manufacturing procedures. In order to receive a manufacturing licence, a modern medicine must be shown to be effective, safe and of good quality. Most medicines

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consist of an active ingredient that is formulated with a variety of other materials (excipients) that are necessary to ensure that the medicine is effective and remains stable, palatable and safe during storage and use. While the efficacy and safety aspects of the active ingredient are within the domain of the pharmacologist and toxicologist respectively, many other disciplines contribute to the quality of the manufactured product as a whole. Analytical chemists and pharmacists take lead responsibility for ensuring that the components of the medicine are present in the correct physical form and concentration, but quality is not judged solely on the physicochemical properties of the product: microorganisms also have the potential to influence efficacy and safety.

It is obvious that medicines contaminated with potentially pathogenic (disease-causing) microorganisms are a safety hazard, so medicines administered by vulnerable routes (e.g. injections) or to vulnerable areas of the body (e.g. the eyes) are manufactured as sterile products. What is less predictable is that microorganisms can, in addition to initiating infections, cause product spoilage by chemically decomposing the active ingredient or the excipients. This may lead to the product being under-strength, physically or chemically unstable, or possibly contaminated with toxic materials. Thus, it is clear that pharmaceutical microbiology must encompass the subjects of sterilization and preservation against microbial spoilage, and a pharmacist with responsibility for the safe, hygienic manufacture and use of medicines must know where microorganisms arise in the environment, i.e. the sources of microbial contamination, and the factors that predispose to, or prevent, product spoilage. In these respects, the pharmaceutical microbiologist has a lot in common with food and cosmetics microbiologists, and there is substantial scope for transfer of knowledge between these disciplines.

Disinfection and the properties of chemicals (biocides) used as antiseptics, disinfectants and preservatives are subjects of which pharmacists and other persons responsible for the manufacture of medicines should be familiar, both from the perspective of biocide use in product formulation and manufacture, and because antiseptics and disinfectants are pharmaceutical products in their own right. However, they are not the only antimicrobial substances that are relevant to medicine: antibiotics are of major importance and represent a product category that regularly features among the top five most frequently prescribed. The term 'antibiotic' is used in several different ways: originally an antibiotic was defined as a naturally occurring substance that was produced by one microorganism that inhibited the growth of, or killed, other microorganisms, i.e. an antibiotic was a natural product, a microbial metabolite. More recently the term has come to encompass certain synthetic agents that are normally used systemically (throughout the body) to treat infection. The manufacture, quality control and, in the light of current concerns about resistance of microorganisms, the use of antibiotics, are other areas of knowledge that contribute to the discipline of pharmaceutical microbiology.

Commercial antibiotic production began with the manufacture of penicillin in the 1940s, and for many years antibiotics were the only significant example of a medicinal product that was made using microorganisms. Following the adoption in the 1950s of microorganisms to facilitate the manufacture of steroids and the development of recombinant DNA technology in the last three decades of the 20th century, the use of microorganisms in the manufacture of medicines has gathered great momentum. It led to more than 100 biotechnology-derived products on the market by the year 2000 with another 300 or more in clinical trials. While it is true to say that traditionally the principal pharmaceutical interest in microorganisms is that of controlling them, exploiting microbial metabolism in the manufacture of medicines is a burgeoning area of knowledge that will become increasingly important, not only in the pharmacy curriculum but also in those of other disciplines employed in the pharmaceutical industry. Table 1.1 summarizes these benefits and uses of microorganisms in pharmaceutical manufacturing, together with the more widely recognized hazards and problems that they present.

Looking ahead to the second decade of the 21st century, it is clear that an understanding of the physiology and genetics of microorganisms will also become more important, not just in the production of new therapeutic agents but in the understanding of infections and other diseases. Genetic techniques such as ribotyping are becoming increasingly used to identify cross-infection, reduce transmission and optimize management of hospital-acquired infections, e.g. those due to *Clostridium difficile*, and, because of the traditional breadth of their science education and their accessibility to the public, pharmacists are not infrequently called upon to explain the terminology and concepts of genetics and other biological sciences to both work colleagues and patients. Several of the traditional diseases that were major causes of death before the antibiotic era, e.g. tuberculosis and diphtheria, are now re-emerging in resistant form—even in developed countries—adding to the problems posed by infections in which antibiotic resistance has long been

Table 1.1 Microorganisms in pharmacy: benefits and problems

Benefits or uses	Related study topics	Harmful effects	Related study topic
The manufacture of: antibiotics steroids therapeutic enzymes polysaccharides products of recombinant DNA technology	Good manufacturing practice Industrial 'fermentation' technology Microbial genetics	May contaminate non-sterile and sterile medicines with a risk of infection	Non-sterile medicines: Enumeration of microorganisms in the manufacturing environment (environmental monitoring) and in raw materials and manufactured products Identification and detection of specific organisms
Use in the production of vaccines	Quality control of immunological products		Sterile medicines: Sterilization methods Sterilization monitoring and validation procedures Sterility testing Assessment and calculation of sterility assurance Aseptic manufacture
As assay organisms to determine antibiotic, vitamin and amino acid concentrations	Assay methods		
To detect mutagenic or carcinogenic activity	Ames mutagenicity test		
		May contaminate non-sterile and sterile medicines with a risk of product deterioration	Enumeration, identification and detection as above, plus: Characteristics, selection and testing of antimicrobial preservatives
		Cause infectious and other diseases	Immunology and infectious diseases Microbial biofilms Characteristics, selection and use of vaccines and antibiotics Infection and contamination control Control of antibiotic resistance Alternative strategies for antimicrobial chemotherapy

(*continued*)

Table 1.1 (*continued*)

Benefits or uses	Related study topics	Harmful effects	Related study topic
		Cause pyrogenic reactions (fever) when introduced into the body even in the absence of infection	Bacterial structure Pyrogen and endotoxin testing
		Provide a reservoir of antibiotic resistance genes	Microbial genetics

a problem, and those like Creutzfeldt–Jakob disease, West Nile virus and severe acute respiratory syndrome (SARS) that have only been recognized or have changed in character in recent years.

Not only has the development of resistance to established antibiotics become a challenge, so too has the ability of microorganisms to take advantage of changing practices and procedures in medicine and surgery. Microorganisms are found almost everywhere in our surroundings and they possess the potential to reproduce extremely rapidly; it is quite possible for cell division to occur every 20 minutes under favourable conditions. These characteristics mean that they can adapt readily to a changing environment and colonize new niches. One feature of modern surgery is the ever-increasing use of plastic, ceramic and metal devices that are introduced into the body for a wide variety of purposes, including the commonly encountered urinary or venous catheters and the less common intraocular lenses, heart valves, pacemakers and hip prostheses. Many bacteria have the potential to produce substances or structures that help them to attach to, and grow as biofilms over, the surfaces of these devices, even while combating the immune system of the body. Thus, colonization often necessitates removal and replacement of the device in question—often leading to great discomfort for the patient and substantial monetary cost to the healthcare service. It has been estimated that, on average, a hospital-acquired infection results in an extra 14 days in hospital, a 10% increase in the chance of dying and an additional healthcare cost per patient of between £1700 and £4120. The development of strategies for eliminating, or at least restricting, the severity or consequences of these device-related infections is a challenge for pharmacists and microbiologists within the industry, and for many other healthcare professionals.

In addition to an improved understanding of the mechanisms of antibiotic resistance, of the links between antibiotic resistance and misuse, and of the factors influencing the initiation of infections in the body, our insights into the role of microorganisms in other disease states have broadened significantly in recent years. Until about 1980 it was probably true to say that there was little or no recognition of the possibility that microorganisms might have a role to play in human diseases other than clear-cut infections. In recent years, however, our perception of the scope of microorganisms as agents of disease has been changed by the discovery that *Helicobacter pylori* is intimately involved in the development of gastric or duodenal ulcers and stomach cancer; by the findings that viruses can cause cancers of the liver, blood and cervix; and by the suspected involvement of microorganisms in diverse conditions like chronic fatigue syndrome and Alzheimer's disease. These, and other conditions like Bell's palsy, atherosclerosis and multiple sclerosis, were amongst 16 chronic diseases suspected of having infectious origins that were named in a 2005 report published by the American Academy of Microbiology.

Clearly, a knowledge of the mechanisms whereby microorganisms are able to resist antibiotics, colonize medical devices and cause or predispose humans to other disease states is essential in the development not only of new antibiotics, but of other medicines and healthcare practices that minimize the risks of these adverse situations developing.

2 Scope and content of the book

Criteria and standards for the microbiological quality of medicines depend upon the route of administration of the medicine in question. The vast majority of medicines

that are given by mouth or placed on the skin are non-sterile, i.e. they may contain some microorganisms (within limits on type and concentration), whereas all injections and ophthalmic products must be sterile, i.e. containing no living organisms. Products for other anatomical sites (e.g. nose, ear, vagina and bladder) are often sterile but not invariably so (Chapter 22). The microbiological quality of non-sterile medicines is controlled by specifications defining the concentration of organisms that may be present and requiring the absence of specific, potentially hazardous organisms. Thus the ability to identify the organisms present, to detect those that are prohibited from particular product categories, and to enumerate microbial contaminants in the manufacturing environment, raw materials and finished product are clearly skills that a pharmaceutical microbiologist should possess (Chapters 2–6). So, too, is a familiarity with the characteristics of antimicrobial preservatives that may be a component of the medicine required to minimize the risk of microbial growth and spoilage during storage and use by the patient (Chapters 17 and 19).

For a sterile product the criterion of quality is simple: there should be no detectable microorganisms whatsoever. The product should, therefore, be able to pass a test for sterility, and a knowledge of the procedures and interpretation of results of such tests is an important aspect of pharmaceutical microbiology (Chapter 21). Injections are also subject to a test for pyrogens; these are substances that cause a rise in body temperature when introduced into the body. Strictly speaking, any substance which causes fever following injection is a pyrogen, but in reality the vast majority are of bacterial origin, and it is for this reason that the detection, assay and removal of bacterial pyrogens (endotoxins) are considered within the realm of microbiology (Chapter 22).

Sterile medicines may be manufactured by two different strategies. The most straightforward and preferred option is to make the product, pack it in its final container and sterilize it by heat, radiation or other means (terminal sterilization, Chapter 21). The alternative is to manufacture the product from sterile ingredients under conditions that do not permit the entry of contaminating organisms (aseptic manufacture, Chapters 17 and 23); this latter option is usually selected when the ingredients or physical form of the product render it heat- or radiation-sensitive. Those responsible for the manufacture of sterile products must be familiar with the sterilization or aseptic manufacturing procedures available for different product types, and those who have cause to open, use or dispense sterile products (in a hospital pharmacy, for example) should be aware of the aseptic handling procedures to be adopted in order to minimize the risk of product contamination.

The spoilage of medicines as a result of microbial contamination, although obviously undesirable, has as its main consequence financial loss rather than ill health on the part of the patient. The other major problem posed by microbial contamination of medicines, that of the risk of initiating infection, although uncommon, is far more important in terms of risk to the patient and possible loss of life (Chapters 7 and 17). Infections arising by this means also have financial implication of course, not only in additional treatment costs but in terms of product recalls, possible litigation and damage to the reputation of the manufacturer.

The range of antimicrobial drugs used to prevent and treat microbial infections is large; for example, a contemporary textbook of antimicrobial chemotherapy lists no fewer than 43 different cephalosporin antibiotics that were already on the market or the subject of clinical trials at the time of publication. Not only are there many antibiotic products, but increasingly, these products really have properties that make them unique. It is far more difficult now than it was, say, 25 years ago, for a manufacturer to obtain a licence for a 'copycat' product, as licensing authorities now emphasize the need to demonstrate that a new antibiotic (or any new medicine) affords a real advantage over established drugs. Because of this range and diversity of products, pharmacists are now far more commonly called upon to advise on the relative merits of the antibiotics available to treat particular categories of infection than was the case hitherto (Chapters 11, 12, 14 and 15). A prerequisite to providing this information is a knowledge not only of the drug in question, but of the infectious disease it is being used to treat and the factors that might influence the success of antibiotic therapy in that situation, e.g. the potential of the infecting organism to grow as a biofilm in which it is protected both from antibiotics and from the immune system by extracellular polymers or slime layers and, as a consequence, is much more difficult to eradicate (Chapters 7 and 8).

While there was a belief among some commentators a generation ago that infectious disease was a problem that was well on the way to permanent resolution owing to the development of effective vaccines and antibiotics, such complacency has now completely disappeared. Although cardiovascular and malignant diseases are more frequent causes of death in many developed countries, infectious diseases remain of paramount

importance in many others, and they account for approximately 23% of the global 58 million deaths per year, with respiratory infection, HIV/AIDS, diarrhoeal disease, tuberculosis and malaria being the prime causes. The confidence that antibiotics would be produced to deal with the vast majority of infections has been replaced by a recognition that the development of resistance to them is likely to substantially restrict their value in the control of certain infections (Chapter 13). Resistance to antibiotics has increased in virtually all categories of pathogenic microorganisms and is now so prevalent that there are some infections, particularly among those acquired in hospitals, for which, it is feared, there will soon be no effective antibiotics. The UK National Audit Office estimated that the cost to the National Health Service of treating hospital-acquired infections exceeded £1 billion in 2007 and the scale of the problem is such that dramatically increasing attention is being paid to infection control procedures that are designed to minimize the risk of infection being transmitted from one patient to another within a hospital (Chapter 16). The properties of disinfectants and antiseptics, the measurement of their antimicrobial activity and the factors influencing their selection for use in hospital infection control strategies or contamination control in the manufacturing setting are topics with which both pharmacists and industrial microbiologists should be familiar (Chapters 18 and 20). Another consequence of increasing antibiotic resistance is that the public are becoming better informed about the need to preserve the antibiotics we presently possess and to avoid their unnecessary use. This has stimulated interest in alternative forms of antimicrobial chemotherapy including the use of materials of plant origin, e.g. tea tree oil, and novel strategies like phage therapy (Chapter 27).

It has long been recognized that microorganisms are valuable, if not essential, in the maintenance of our ecosystems. Their role and benefits in the carbon and nitrogen cycles in terms of recycling dead plant and animal material and in the fixation of atmospheric nitrogen are well understood. The uses of microorganisms in the food, dairy and brewing industries are also well established, but until the late 20th century advances in genetics, immunology and biotechnology, their benefits and uses in the pharmaceutical industry were far more modest. For many years the production of antibiotics and microbial enzyme-mediated production of steroids were the only significant pharmaceutical examples of the exploitation of metabolism of microorganisms. The value of these applications, in both monetary and healthcare terms, has been immense. Antibiotics currently have estimated global sales of $42 billion per annum, and by this criterion they are surpassed as products of biotechnology only by cheese and alcoholic beverages, but the benefits they afford in terms of improved health and life expectancy are incalculable. The discovery of the anti-inflammatory effects of corticosteroids had a profound impact on the treatment of rheumatoid arthritis in the 1950s, but it was the use of enzymes possessed by common fungi that made cortisone widely available to rheumatism sufferers. The synthesis of cortisone by traditional chemical methods involved 31 steps, gave a yield of less than 0.2% of the starting material and resulted in a product costing, even in 1950s terms, $200 per gram. Exploiting microbial enzymes reduced the synthesis to 11 steps and the cost rapidly fell to $6 per gram.

Apart from these major applications, however, the uses of microorganisms in the manufacture of medicines prior to 1980 were very limited. Enzymes were developed for use in cancer chemotherapy (asparaginase) and to digest blood clots (streptokinase), and polysaccharides also found therapeutic applications (e.g. dextran, used as a plasma expander). These were of relatively minor importance, however, compared with the products that followed the advances in recombinant DNA technology in the 1970s. This technology permitted human genes to be inserted into microorganisms, which were thus able to manufacture the gene products far more efficiently than traditional methods of extraction from animal or human tissues. Insulin, in 1982, was the first therapeutic product of DNA technology to be licensed for human use, and it has been followed by human growth hormone, interferon, erythropoietin, blood clotting factors and many other products (Chapter 25). DNA technology has also permitted the development of vaccines which, like that for the prevention of hepatitis B, use genetically engineered surface antigens rather than whole natural virus particles, so these vaccines are more effective and safer than those produced by traditional means (Chapters 10 and 24).

All these developments, together with miscellaneous applications in the detection of mutagenic and carcinogenic activity in drugs and chemicals and in the assay of antibiotics, vitamins and amino acids (Chapter 26), have ensured that the role of microorganisms in the manufacture of medicines is now well recognized, and that a basic knowledge of immunology (Chapter 9), gene cloning and other biotechnology disciplines (Chapter 25) is an integral part of pharmaceutical microbiology.

2 Fundamental features of microbiology

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1 Introduction

Microorganisms differ enormously in terms of their shape, size and appearance and in their genetic and metabolic characteristics. All these properties are used in classifying microorganisms into the major groups with which many people are familiar, e.g. bacteria, fungi, protozoa and viruses, and into the less well known categories such as chlamydia, rickettsia and mycoplasmas. The major groups are the subject of individual chapters immediately following this, so the purpose here is not to describe any of them in great detail but to summarize their features so that the reader may better understand the distinctions between them. A further aim of this chapter is to avoid undue repetition of information in the early part of the book by considering such aspects of microbiology as cultivation, enumeration and genetics that are common to some, or all, of the various types of microorganism.

1.1 Viruses, viroids and prions

Viruses do not have a cellular structure. They are particles composed of nucleic acid surrounded by protein; some possess a lipid envelope and associated glycoproteins, but recognizable chromosomes, cytoplasm and cell membranes are invariably absent. Viruses are incapable of independent replication as they do not contain the enzymes necessary to copy their own nucleic acids; as a consequence, all viruses are intracellular parasites and are reproduced using the metabolic capabilities of the host cell. A great deal of variation is observed in shape (helical, linear or spherical), size (20–400 nm) and nucleic acid composition (single- or double-stranded, linear or circular RNA or DNA), but almost all viruses are smaller than bacteria and they cannot be seen with a normal light microscope; instead they may be viewed using an electron microscope which affords much greater magnification.

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Viroids (virusoids) are even simpler than viruses, being infectious particles comprising single-stranded RNA without any associated protein. Those that have been described are plant pathogens, and, so far, there are no known human pathogens in this category, although human hepatitis D virus shares some features in common with viroids, and may have originated from them.

Prions are unique as infectious agents in that they contain no nucleic acid. A prion is an atypical form of a mammalian protein that can interact with a normal protein molecule and cause it to undergo a conformational change so that it, in turn, becomes a prion and ceases its normal function. Prions are the agents responsible for transmissible spongiform encephalopathies, e.g. Creutzfeldt–Jakob disease (CJD) and bovine spongiform encephalopathy (BSE). They are the simplest and most recently recognized agents of infectious disease, and are important in a pharmaceutical context owing to their extreme resistance to conventional sterilizing agents like steam, gamma radiation and disinfectants (Chapter 21).

1.2 Prokaryotes and eukaryotes

The most fundamental distinction between the various microorganisms having a cellular structure (i.e. all except those described in section 1.1 above) is their classification into two groups—the prokaryotes and eukaryotes—based primarily on their structural characteristics and mode of reproduction. Expressed in the simplest possible terms, prokaryotes are the bacteria and archaea (see section 1.2.1), and eukaryotes are all other cellular microorganisms, e.g. fungi, protozoa and algae. The crucial difference between these two types of cell is the possession by the eukaryotes of a true cell nucleus in which the chromosomes are separated from the cytoplasm by a nuclear membrane. The prokaryotes have no true nucleus; they normally possess just a single chromosome that is not separated from the other cell contents by a membrane. Other major distinguishing features of the two groups are that prokaryotes are normally haploid (possess only one copy of the set of genes in the cell) and reproduce asexually; eukaroyotes, by contrast, are usually diploid (possess two copies of their genes) and normally have the potential to reproduce sexually. The capacity for sexual reproduction confers the major advantage of creating new combinations of genes, which increases the scope for selection and evolutionary development. The restriction to an asexual mode of reproduction means that the organism in question is heavily reliant on mutation as a means of creating genetic variety and new strains with advantageous characteristics, although many bacteria are able to receive new genes from other strains or species (see section 6.1 and Chapter 3). Table 2.1 lists some distinguishing features of the prokaryotes and eukaryotes.

1.2.1 Bacteria and archaea

Bacteria are essentially unicellular, although some species arise as sheathed chains of cells. They possess the properties listed under prokaryotes in Table 2.1 but, like viruses and other categories of microorganisms, exhibit great diversity of form, habitat, metabolism, pathogenicity and other characteristics. The bacteria of interest in pharmacy and medicine belong to the group known as the eubacteria. The other subdivision of prokaryotes, the archaea, have no pharmaceutical importance, and although formerly considered largely to comprise organisms capable of living in extreme environments (e.g. high temperatures, extreme salinity or pH) or organisms exhibiting specialized modes of metabolism (e.g. by deriving energy from sulphur or iron oxidation or the production of methane) they are now known to occur in a wide variety of habitats.

The eubacteria are typically rod-shaped (bacillus), spherical (cocci), curved or spiral cells of approximately 0.5–5.0 mm (longest dimension) and are divided into two groups designated Gram-positive and Gram-negative according to their reaction to a staining procedure developed in 1884 by Christian Gram (see Chapter 3). Although all the pathogenic species are included within this category, there are very many other eubacteria that are harmless or positively beneficial. Some of the bacteria that contaminate or cause spoilage of pharmaceutical materials are saprophytes, i.e. they obtain their energy by decomposition of animal and vegetable material, while many could also be described as parasites (benefiting from growth on or in other living organisms without causing detrimental effects) or pathogens (parasites damaging the host). Rickettsia and chlamydia are types of bacteria that are obligate intracellular parasites, i.e. they are incapable of growing outside a host cell and so cannot easily be cultivated in the laboratory. Most bacteria of pharmaceutical and medical importance possess cell walls (and are therefore relatively resistant to osmotic stress), grow well at temperatures between ambient and human body temperature, and exhibit wide variations in their requirement for, or tolerance of, oxygen. Strict aerobes require atmospheric oxygen, but for strict anaerobes oxygen is toxic. Many other bacteria would be described as facultative anaerobes (normally growing best in air but can grow without it) or microaerophils

Table 2.1 Distinguishing features of prokaryotes and eukaryotes

Characteristic	Eukaryotes	Prokaryotes
Size	Normally > 10 µm	Typically 1–5 µm
Location of chromosomes	Within a true nucleus separated from the cytoplasm by a nuclear membrane	In the cytoplasm, usually attached to the cell membrane
Nuclear division	Exhibit mitosis and meiosis	Mitosis and meiosis are absent
Nucleolus	Present	Absent
Reproduction	Asexual or sexual reproduction	Normally asexual reproduction
Chromosome number	>1	1
Mitochondria and chloroplasts	May be present	Absent
Cell membrane composition	Sterols present	Sterols absent
Cell wall composition	Cell walls (when present) usually contain cellulose or chitin but not peptidoglycan	Walls usually contain peptidoglycan
Ribosomes	Cytoplasmic ribosomes are 80S	Ribosomes are smaller, usually 70S
Flagella	Structurally complex	Structurally simple
Pili	Absent	Present
Fimbriae	Cilia	Present
Storage compounds	Poly-β-hydroxybutyrate absent	Poly-β-hydroxybutyrate often present

(preferring oxygen concentrations lower than those in normal air).

1.2.2 Fungi

Fungi are structurally more complex and varied in appearance than bacteria and, being eukaryotes, differ from them in the ways described in Table 2.1. Fungi are considered to be non-photosynthesizing plants, and the term *fungus* covers both yeasts and moulds, although the distinction between these two groups is not always clear. Yeasts are normally unicellular organisms that are larger than bacteria (typically 5–10 µm) and divide either by a process of binary fission (see section 4.2 and Figure 2.1a) or budding (whereby a daughter cell arises as a swelling or protrusion from the parent that eventually separates to lead an independent existence, Figure 2.1b). *Mould* is an imprecise term used to describe fungi that do not form fruiting bodies visible to the naked eye, thus excluding toadstools and mushrooms. Most moulds consist of a tangled mass (mycelium) of filaments or threads (hyphae) which vary between 1 and over 50 µm wide (Figure 2.1c); they may be differentiated for specialized functions, e.g. absorption of nutrients or reproduction. Some fungi may exhibit a unicellular (yeast-like) or mycelial (mould-like) appearance depending upon cultivation conditions. Although fungi are eukaryotes that should, in theory, be capable of sexual reproduction, there are some species in which this has never been observed. Most fungi are saprophytes with relatively few having pathogenic potential, but their ability to form spores that are resistant to drying makes them important as contaminants of pharmaceutical raw materials, particularly materials of vegetable origin.

1.2.3 Protozoa

Protozoa are eukaryotic, predominantly unicellular microorganisms that are regarded as animals rather than plants, although the distinction between protozoa and fungi is not always clear and there are some organisms

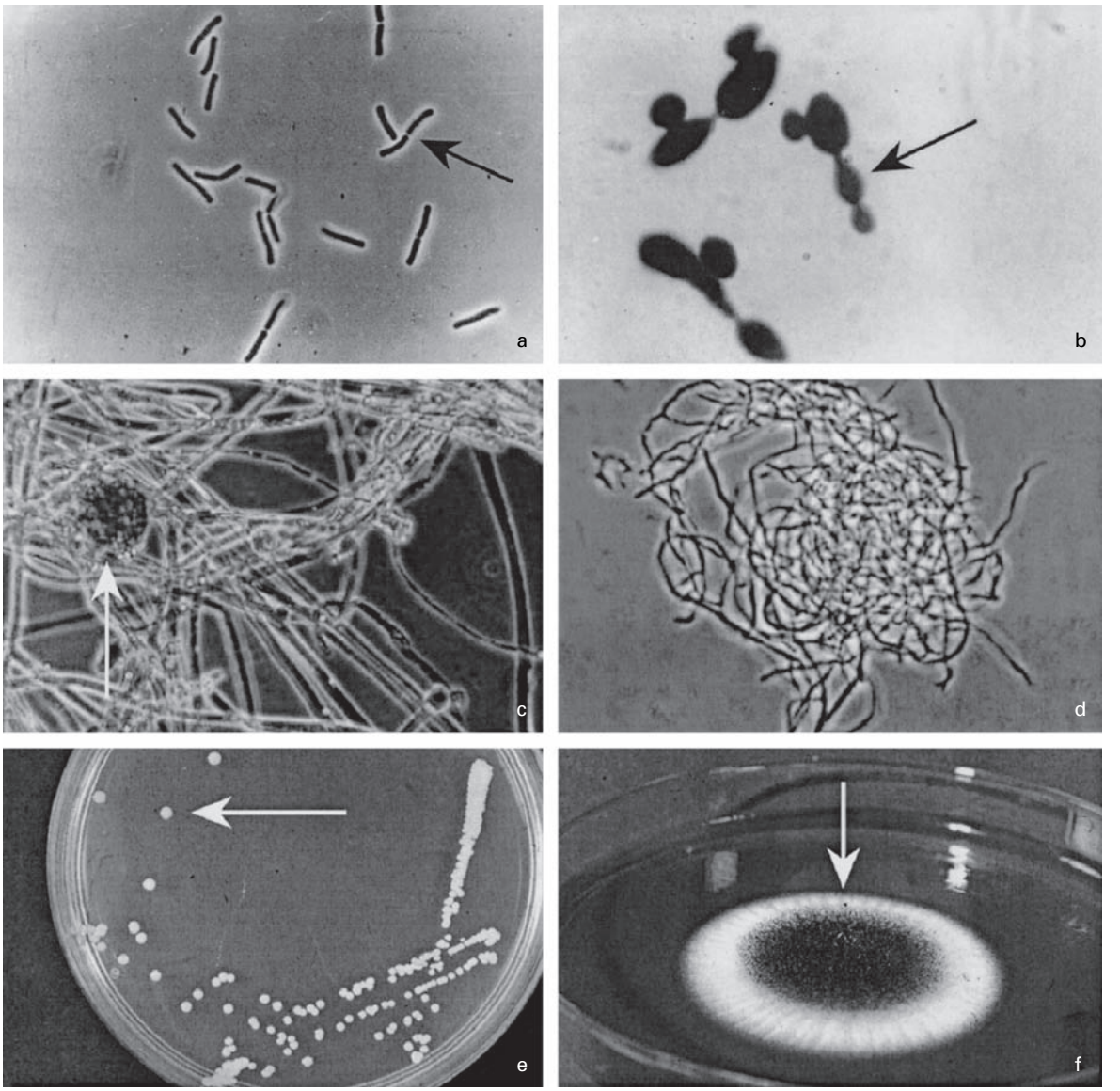

Figure 2.1 (a) A growing culture of *Bacillus megaterium* in which cells about to divide by binary fission display constrictions (arrowed) prior to separation. (b) A growing culture of the yeast *Saccharomyces cerevisiae* displaying budding (arrowed). (c) The mould *Mucor plumbeus* exhibiting the typical appearance of a mycelium in which masses of asexual zygospores (arrowed) are formed on specialized hyphae. (d) The bacterium *Streptomyces rimosus* displaying the branched network of filaments that superficially resembles a mould mycelium. (e) The typical appearance of an overnight agar culture of *Micrococcus luteus* inoculated to produce isolated colonies (arrowed). (f) A single colony of the mould *Aspergillus niger* in which the actively growing periphery of the colony (arrowed) contrasts with the mature central region where pigmented asexual spores have developed.

whose taxonomic status is uncertain. Many protozoa are free-living motile organisms that occur in water and soil, although some are parasites of plants and animals, including humans, e.g. the organisms responsible for malaria and amoebic dysentery. Protozoa are not normally found as contaminants of raw materials or manufactured medicines and the relatively few that are of pharmaceutical interest owe that status primarily to their potential to cause disease.

2 Naming of microorganisms

Microorganisms, just like other organisms, are normally known by two names: that of the genus (plural = genera) and that of the species. The former is normally written with an upper case initial letter and the latter with a lower case initial letter, e.g. *Staphylococcus aureus* or *Escherichia coli*. These may be abbreviated by shortening the name of the genus provided that the shortened form is unambiguous, e.g. *Staph. aureus, E. coli*. Both the full and the shortened names are printed in *italics* to designate their status as proper names (in old books, theses or manuscripts they might be in roman type but underlined). The species within a genus are sometimes referred to by a collective name, e.g. staphylococci or pseudomonads, and neither these names, nor names describing groups of organisms from different genera, e.g. coliforms, are italicized or spelt with an upper case initial letter.

3 Microbial metabolism

As in most other aspects of their physiology, microorganisms exhibit marked differences in their metabolism. While some species can obtain carbon from carbon dioxide and energy from sunlight or the oxidation of inorganic materials like sulphides, the vast majority of organisms of interest in pharmacy and medicine are described as chemoheterotrophs—they obtain carbon, nitrogen and energy by breaking down organic compounds. The chemical reactions by which energy is liberated by digestion of food materials are termed catabolic reactions, while those that use the liberated energy to make complex cellular polymers, proteins, carbohydrates and nucleic acids, are called anabolic reactions.

Food materials are oxidized in order to break them down and release energy from them. The term oxidation is defined as the removal or loss of electrons, but oxidation does not invariably involve oxygen, as a wide variety of other molecules can accept electrons and thus act as oxidizing agents. As the oxidizing molecule accepts the electrons, the other molecule in the reaction that provides them is simultaneously reduced. Consequently, oxidation and reduction are invariably linked and such reactions are often termed redox reactions. The term redox potential is also used, and this indicates whether oxidizing or reducing conditions prevail in a particular situation, e.g. in a body fluid or a culture medium. Anaerobic organisms prefer low redox potentials (typically zero to −200 mV or less) while aerobes thrive in high redox potential environments (e.g. zero to +200 mV or more).

There are marked similarities in the metabolic pathways used by pathogenic bacteria and by mammals. Many bacteria use the same process of glycolysis that is used by humans to begin the breakdown of glucose and the release of energy from it. Glycolysis describes the conversion of glucose, through a series of reactions, to pyruvic acid, and it is a process for which oxygen is not required, although glycolysis is undertaken by both aerobic and anaerobic organisms. The process releases only a relatively small amount of the energy stored in a sugar molecule, and aerobic microorganisms, in common with mammals, release much more of the energy by aerobic respiration. Oxygen is the molecule at the end of the sequence of respiratory reactions that finally accepts the electrons and allows the whole process to proceed, but it is worth noting that many organisms can also undertake *anaerobic* respiration, which uses other final electron acceptors, e.g. nitrate or fumarate.

As an alternative to respiration many microorganisms use fermentation as a means of releasing more energy from sugar; fermentation is, by definition, a process in which the final electron acceptor is an organic molecule. The term is widely understood to mean the production by yeast of ethanol and carbon dioxide from sugar, but in fact many organisms apart from yeasts can undertake fermentation and the process is not restricted to common sugar (sucrose) as a starting material or to ethanol and carbon dioxide as metabolic products. Many pathogenic bacteria are capable of fermenting several different sugars and other organic materials to give a range of metabolic products that includes acids (e.g. lactic, acetic and propionic), alcohols (e.g. ethanol, propanol, butanediol) and other commercially important materials like the solvents acetone and butanol. Fermentation is, like glycolysis, an anaerobic process, although the term is commonly used in the pharmaceutical and biotechnology

industries to describe the manufacture of a wide range of substances by microorganisms where the biochemical process is neither fermentative nor even anaerobic, e.g. many textbooks refer to antibiotic fermentation, but the production vessels are usually vigorously aerated.

Microorganisms are far more versatile than mammals with respect to the materials that they can use as foods and the means by which those foods are broken down. Some pathogenic organisms can grow on dilute solutions of mineral salts and sugar (or other simple molecules like glycerol, lactic or pyruvic acids), while others can obtain energy from rarely encountered carbohydrates or by the digestion of proteins or other non-carbohydrate foods. In addition to accepting a wide variety of food materials, many microorganisms can use alternative metabolic pathways to break the food down depending on the environmental conditions, e.g. facultative anaerobes can switch from respiration to fermentation if oxygen supplies are depleted. It is partly this ability to switch to different metabolic pathways that explains why none of the major antibiotics work by interfering with the chemical reactions microorganisms use to metabolize their food. It is a fundamental principle of antibiotic action that the drug must exploit a difference in metabolism between the organism to be killed and the human host; without such a difference the antibiotic would be very toxic to the patient too. However, not only do bacteria use metabolic pathways for food digestion that are similar to our own, many of them would have the ability to switch to an alternative energy-producing pathway if an antibiotic were developed that interfered with a reaction that is unique to bacteria.

The metabolic products that arise during the period when a microbial culture is actually growing are termed primary metabolites, while those that are produced after cell multiplication has slowed or stopped, i.e. in the 'stationary phase' (see Chapter 3), are termed secondary metabolites. Ethanol is a primary metabolite of major commercial importance although it is produced in large quantities only by some species of yeast. More common than ethanol as primary metabolites are organic acids, so it is a common observation that the pH of a culture progressively falls during growth, and many organisms further metabolize the acids so the pH often rises after cell growth has ceased. The metabolites that are found during secondary metabolism are diverse, and many of them have commercial or therapeutic importance. They include antibiotics, enzymes (e.g. amylases that digest starch and proteolytic enzymes used in biological washing powders), toxins (responsible for many of the symptoms of infection but some also of therapeutic value, e.g. botox—the toxin of *Clostridium botulinum*) and carbohydrates (e.g. dextran, used as a plasma expander and for molecular separations by gel filtration).

4 Microbial cultivation

The vast majority of microorganisms of interest in pharmacy and medicine can be cultivated in the laboratory and most of them require relatively simple techniques and facilities. Some organisms are parasites and so can only be grown inside the cells of a host species—which often necessitates mammalian cell culture facilities—and there are a few (e.g. the organism responsible for leprosy) that are not cultivated outside the living animal.

4.1 Culture media

A significant number of common microorganisms are capable of synthesizing all the materials they need for growth (e.g. amino acids, nucleotides and vitamins) from simple carbon and nitrogen sources and mineral salts. Such organisms can grow on truly synthetic (chemically defined) media, but many organisms do not have this capability and need a medium that already contains these biochemicals. Such media are far more commonly used than synthetic ones, and several terms have been used to describe them, e.g. routine laboratory media, general purpose media and complex media. They are complex in the sense that their precise chemical composition is unknown and likely to vary slightly from batch to batch. In general, they are aqueous solutions of animal or plant extracts that contain hydrolysed proteins, B-group vitamins and carbohydrates.

Readily available and relatively inexpensive sources of protein include meat extracts (from those parts of animal carcasses that are not used for human or domestic animal consumption), milk and soya. The protein is hydrolysed to varying degrees to give peptones (by definition not coagulable by heat or ammonium sulphate) or amino acids. Trypsin or other proteolytic enzymes are preferred to acids as a means of hydrolysis because acids cause more amino acid destruction; the term 'tryptic' denotes the use of the enzyme. Many microorganisms require B-group vitamins (but not the other water- or fat-soluble vitamins required by mammals) and this requirement is satisfied by yeast extract. Carbohydrates are used in the form of starch or sugars, but glucose (dextrose) is the only sugar regularly employed as a nutrient.

Microorganisms differ in terms of their ability to ferment various sugars, and their fermentation patterns may be used as an aid in identification. Thus, other sugars included in culture media are normally present for these diagnostic purposes rather than as carbon and energy sources. Sodium chloride may be incorporated in culture media to adjust osmotic pressure, and occasionally buffers are added to neutralize acids that result from sugar metabolism. Routine culture media may be enriched by the addition of materials like milk, blood or serum, and organisms that need such supplements in order to grow are described as 'exacting' in their nutritional requirements.

Culture media may be either liquid or solid; the latter term describes liquid media that have been gelled by the addition of agar, which is a carbohydrate extracted from certain seaweeds. Agar at a concentration of about 1–1.5% w/v will provide a firm gel that cannot be liquefied by the enzymes normally produced during bacterial growth (which is one reason it is used in preference to gelatin). Agar is unusual in that the melting and setting temperatures for its gels are quite dissimilar. Fluid agar solutions set at approximately 40 °C, but do not re-liquefy on heating until the temperature is in excess of 90 °C. Thus agar forms a firm gel at 37 °C which is the normal incubation temperature for many pathogenic organisms (whereas gelatin does not) and when used as a liquid at 45 °C is at a sufficiently low temperature to avoid killing microorganisms—this property is important in pour plate counting methods (see section 5).

In contrast to medium ingredients designed to support microbial growth, there are many materials commonly added to selective or diagnostic media whose function is to restrict the growth of certain types of microorganism while permitting or enhancing the growth of others. Examples include antibacterial antibiotics added to fungal media to suppress bacterial contaminants, and bile to suppress organisms from anatomical sites other than the gastrointestinal tract. Many such additives are used in media for organism identification purposes, and these are considered further in subsequent chapters. The term enrichment sometimes causes confusion in this context. It is occasionally used in the sense of making a medium nutritionally richer to achieve more rapid or profuse growth. Alternatively, and more commonly, an enrichment medium is one designed to permit a particular type of organism to grow while restricting others, so the one that grows increases in relative numbers and is 'enriched' in a mixed culture.

Solid media designed for the growth of anaerobic organisms usually contain non-toxic reducing agents, e.g. sodium thioglycollate or sulphur-containing amino acids; these compounds create redox potentials of −200 mV or less and so diminish or eliminate the inhibitory effects of oxygen or oxidizing molecules on anaerobic growth. The inclusion of such compounds is less important in liquid media where a sufficiently low redox potential may be achieved simply by boiling; this expels dissolved oxygen, which in unstirred liquids only slowly resaturates the upper few millimetres of liquid. Redox indicators like methylene blue or resazurin may be incorporated in anaerobic media to confirm that a sufficiently low redox potential has been achieved.

Media for yeasts and moulds often have a lower pH (5.5–6.0) than bacterial culture media (7.0–7.4). Lactic acid may be used to impart a low pH because it is not, itself, inhibitory to fungi at the concentrations used. Some fungal media that are intended for use with specimens that may also contain bacteria may be supplemented with antibacterial antibiotics, e.g. chloramphenicol or tetracyclines.

4.2 Cultivation methods

Most bacteria and some yeasts divide by a process of binary fission whereby the cell enlarges or elongates, then forms a cross-wall (septum) that separates the cell into two more or less equal compartments each containing a copy of the genetic material. Septum formation is often followed by constriction such that the connection between the two cell compartments is progressively reduced (see Figure 2.1a) until finally it is broken and the daughter cells separate. In bacteria this pattern of division may take place every 25–30 minutes under optimal conditions of laboratory cultivation, although growth at infection sites in the body is normally much slower owing to the effects of the immune system and scarcity of essential nutrients, particularly iron. Growth continues until one or more nutrients is exhausted, or toxic metabolites (often organic acids) accumulate and inhibit enzyme systems. Starting from a single cell many bacteria can achieve concentrations of the order of 10^9 cells ml^{-1} or more following overnight incubation in common liquid media. At concentrations below about 10^7 cells ml^{-1} culture media are clear, but the liquid becomes progressively more cloudy (turbid) as the concentration increases above this value; turbidity is, therefore, an indirect means of monitoring culture growth. Some bacteria produce chains of cells, and some produce elongated cells (filaments) that may exhibit branching to create a tangled

mass resembling a mould mycelium (Figure 2.1d). Many yeasts divide by budding (see section 1.2.3 and Figure 2.1b) but they, too, would normally grow in liquid media to produce a turbid culture. Moulds, however, grow by extension and branching of hyphae to produce a mycelium (Figure 2.1c) or, in agitated liquid cultures, pellet growth may arise.

When growing on solid media in Petri dishes (often referred to as 'plates') individual bacterial cells can give rise to colonies following overnight incubation under optimal conditions. A colony is simply a collection of cells arising by multiplication of a single original cell or a small cluster of them (called a colony-forming unit or CFU). The term 'colony' does not, strictly speaking, imply any particular number of cells, but it is usually taken to mean a number sufficiently large to be visible by eye. Thus, macroscopic bacterial colonies usually comprise hundreds of thousands, millions or tens of millions of cells in an area on a Petri dish that is typically 1–10 mm in diameter (Figure 2.1e). Colony size is limited by nutrient availability and/or waste product accumulation in just the same way as cell concentration in liquid media. Colonies vary between bacterial species, and their shapes, sizes, opacities, surface markings and pigmentation may all be characteristic of the species in question, so these properties may be an aid in identification procedures (see Chapter 3).

Anaerobic organisms may be grown on Petri dishes provided that they are incubated in an anaerobic jar. Such jars are usually made of rigid plastic with airtight lids, and Petri dishes are placed in them together with a low-temperature catalyst. The catalyst, consisting of palladium-coated pellets or wire, causes the oxygen inside the jar to be combined with hydrogen that is generated by the addition of water to sodium borohydride; this is usually contained in a foil sachet that is also placed in the jar; alternatively, oxygen may be removed by combination with ascorbic acid. After its removal, an anaerobic atmosphere is achieved and this is monitored by an oxidation–reduction (redox) indicator; resazurin is frequently used as a solution soaking a fabric strip.

Yeast colonies often look similar to those of bacteria, although they may be larger and more frequently coloured. The appearance of moulds growing on solid microbiological media is similar to their appearance when growing on common foods. The mould colony consists of a mycelium that may be loosely or densely entangled depending on the species, often with the central area (the oldest, most mature region of the colony) showing pigmentation associated with spore production (Figure 2.1f). The periphery of the colony is that part which is actively growing and it is usually non-pigmented.

4.3 Planktonic and sessile (biofilm) growth

Bacteria growing in liquid culture in the laboratory usually exist as individual cells or small aggregates of cells suspended in the culture medium; the term planktonic is used to describe such freely suspended cells. In recent years, however, it has become recognized that planktonic growth is not the normal situation for bacteria growing in their natural habitats. In fact, bacteria in their natural state far more commonly grow attached to a surface which, for many species, may be solid, e.g. soil particles, stone, metal or glass, or for pathogens, an epithelial surface in the body, e.g. lung or intestinal mucosa. Bacteria attached to a substrate in this way are described as sessile, and are said to exhibit the biofilm or microcolony mode of growth.

Planktonic cells are routinely used for almost all the testing procedures that have been designed to assess the activity of antimicrobial chemicals and processes, but the recognition that planktonic growth is not the natural state for many organisms prompted investigations of the relative susceptibilities of planktonic- and biofilm-grown cells to antibiotics, disinfectants and decontamination or sterilization procedures. In many cases it has been found that planktonic and sessile bacteria exhibit markedly different susceptibilities to these lethal agents, and this has prompted a reappraisal of the appropriateness of some of the procedures used (see Chapters 8, 13 and 18).

5 Enumeration of microorganisms

In a pharmaceutical context there are several situations where it is necessary to measure the number of microbial cells in a culture, sample or specimen:

- when measuring the levels of microbial contamination in a raw material or manufactured medicine
- when evaluating the effects of an antimicrobial chemical or decontamination process
- when using microorganisms in the manufacture of therapeutic agents
- when assessing the nutrient capability of a growth medium.

In some cases it is necessary to know the total number of microbial cells present, i.e. both living and dead: e.g. in vaccine manufacture dead and living cells may both

Table 2.2 Traditional and rapid methods of enumerating cells

Traditional methods		
Viable counts	Total counts	Rapid methods (indirect viable counts)
1 Pour plate (counting colonies *in* agar)	**1** Direct microscopic counting (using Helber or haemocytometer counting chambers)	**1** Epifluorescence (uses dyes that give characteristic fluorescence only in living cells) often coupled to image analysis
2 Surface spread or surface drop (Miles Misra) methods (counting colonies on agar surface)	**2** Turbidity methods (measures turbidity (opacity) in suspensions or cultures)	**2** ATP methods(measure ATP production in living cells using bioluminescence)
3 Membrane filter methods (colonies growing on membranes on agar surface)	**3** Dry weight determinations	**3** Impedance (measures changes in resistance, capacitance or impedance in growing cultures)
4 MPN (counts based on the proportion of liquid cultures growing after receiving low inocula)	**4** Nitrogen, protein or nucleic acid determinations	**4** Manometric methods (measure oxygen consumption or CO_2 production by growing cultures)

produce an immune response, and in pyrogen testing both dead and living cells induce fever when injected into the body. However, in many cases it is the number or concentration of *living* cells that is required. The terminology in microbial counting sometimes causes confusion. A *total count* is a counting procedure enumerating both living and dead cells, whereas a *viable count*, which is far more common, records the living cells alone. However, the term *total viable count* (*TVC*) is used in most pharmacopoeias and by many regulatory agencies to mean a viable count that records all the different species or types of microorganism that might be present in a sample (e.g. bacteria plus fungi).

Table 2.2 lists the more common counting methods available. The first three traditional methods of viable counting all operate on the basis that a living cell (or a CFU) will give rise to a visible colony when introduced into or onto the surface of a suitable medium and incubated. Thus, the procedure for pour plating (Figure 2.2A) usually involves the addition of a small volume (typically 1.0 ml) of sample (or a suitable dilution thereof) into molten agar at 45 °C which is then poured into empty sterile Petri dishes. After incubation the resultant colonies are counted and the total is multiplied by the dilution factor (if any) to give the concentration in the original sample. In a surface spread technique (Figure 2.2B) the sample (usually 0.1–0.25 ml) is spread over the surface of agar which has previously been dried to permit absorption of the added liquid. The Miles Misra (surface drop) method (Figure 2.2C) is similar in principle, but several individual drops of culture are allowed to spread over discrete areas of about 1 cm diameter on the agar surface. These procedures are suitable for samples that are expected to contain concentrations exceeding approximately 100 CFU ml^{-1} so that the number of colonies arising on the plate is sufficiently large to be statistically reliable. If there are no clear indications of the order of magnitude of the concentration in the sample, it is necessary to plate out the sample at each of two, three or more (decimal, i.e. 10-fold) dilutions so as to obtain Petri dishes with conveniently countable numbers of colonies (usually taken to be 30–300 colonies).

If 30 is accepted as the lowest reliable number to count and a pour plate method uses a 1.0 ml sample, it follows that the procedures described above are unsuitable for any sample that is expected to contain <30 CFU ml^{-1}, e.g. water samples where the count may be 1 CFU ml^{-1} or less. Here, membrane filter methods are used (Figure 2.2D) in which a large, known volume of sample is passed through the membrane which is placed, without inversion, on the agar surface. Nutrients then diffuse up through the membrane and allow the retained cells to grow into colonies on it just as they would on the agar

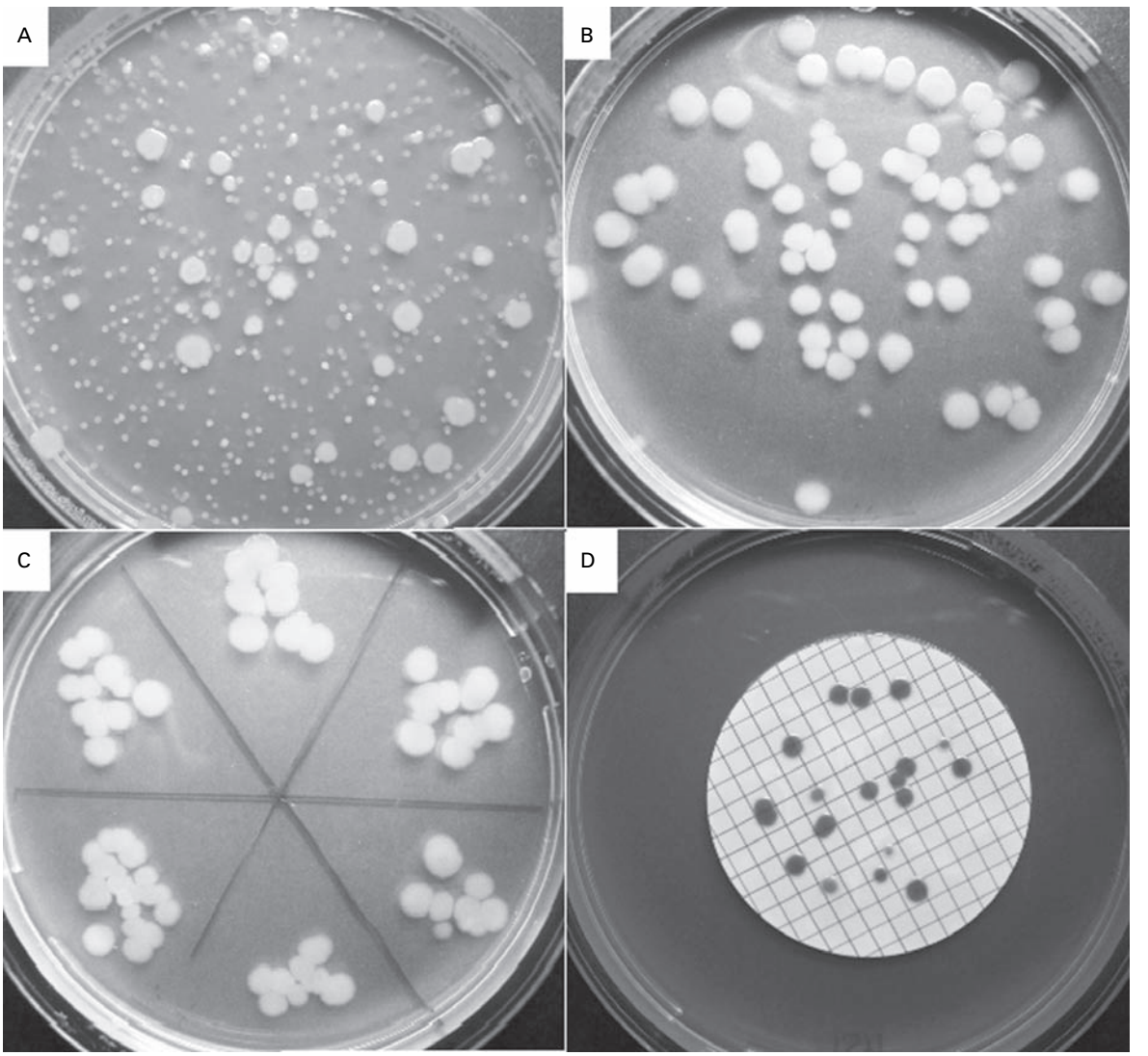

Figure 2.2 Viable counts of bacteria: (A) Pour plate method using *Bacillus subtilis*; the colonies on the surface of the agar are growing larger than those within the agar due to greater oxygen availability. (B) Surface spread, and (C) surface drop (Miles Misra) methods using *Bacillus subtilis*. (D) Membrane filtration method showing *Serratia marcescens* colonies growing on a 47 mm diameter membrane.

itself. Some of the relative merits of these procedures are described in Table 2.3.

Most probable number (MPN) counts may be used when the anticipated count is relatively low, i.e. from <1 up to 100 microorganisms ml^{-1}. The procedure involves inoculating multiple tubes of culture medium (usually three or five) with three different volumes of sample, e.g. three tubes each inoculated with 0.1 ml, three with 0.01 ml and three with 0.001 ml. If the concentration in the sample is in the range indicated above, there should be a proportion of the tubes receiving inocula in which no microorganisms are present; these will remain sterile after incubation, while others that received inocula actually containing one or more CFU show signs of growth. The proportions of positive tubes are recorded for each sample volume and the results are compared with stand-

Table 2.3 Relative merits of the common viable counting procedures

Counting method	Advantages	Disadvantages
Pour plate	Requires no predrying of the agar surface	Very small colonies of strict aerobes at the base of the agar may be missed
	Will detect lower concentrations than surface spread/surface drop methods	Colonies of different species within the agar appear similar—so it is difficult to detect contaminants
Surface spread and surface drop methods	Surface spread often gives larger colonies than pour plates—thus they are easier to count	Agar surface requires predrying to absorb sample
	Easier to identify contaminants by appearance of the colonies	Possibility of confluent growth, particularly with moulds, masking individual colonies
Membrane filtration	If necessary, will detect lower concentrations than other methods	Viscous samples will not go through the membrane and particulate samples may block the membrane thereby restricting filtration capacity
	Antimicrobial chemicals in the sample can be physically removed from the cells	

ard tables showing the MPN of organisms per ml (or per 100 ml) of original sample. The procedure is more commonly used in the water, food and dairy industries than in the pharmaceutical industry; nevertheless, it is a valid technique described in pharmacopoeias and appropriate for pharmaceutical materials, particularly water. The poor accuracy and precision associated with MPN counts usually means that the method is one of last resort—to be considered only when other counting methods are inappropriate.

Turbidity measurements are the most common means of estimating the total numbers of bacteria present in a sample. Measuring the turbidity using a spectrophotometer or colorimeter and reading the concentration from a calibration plot is a simple means of standardizing cell suspensions for use as inocula in antibiotic assays or other tests of antimicrobial chemicals. Fungi cannot readily be handled in this way because the suspension may not be uniform or may sediment in a spectrophotometer cuvette. Consequently, dry weight determinations on known volumes of culture are an alternative means of estimating fungal biomass. Direct microscopic counting may be an appropriate method for bacteria, yeasts and fungal spores but not for moulds, and indirect measures of biomass like assays of insoluble nitrogen, protein or nucleic acids are possible for all cell types, but rarely used outside the research laboratory.

The traditional methods of viable counting all suffer from the same limitations:

- relatively labour intensive
- not easy to automate
- slow, because they require an incubation period for colonies to develop or liquid cultures to become turbid
- may require relatively large volumes of culture media, many Petri dishes and a lot of incubator space.

For these reasons much interest and investigative effort has been invested in recent years in the use of so-called 'rapid' methods of detecting and counting microorganisms (see also Chapter 3). These methods enumerate viable organisms—usually bacteria and yeasts rather than moulds—in a matter of hours and eliminate the 24–48 hour (or longer) incubation periods that are typical of traditional procedures. The rapid methods employ various means of indirect detection of living cells, but the following operating principles are the most common:

• Epifluorescent techniques use fluorescent dyes that either exhibit different colours in living and dead cells (e.g. acridine orange) or appear colourless outside the cell but become fluorescent when absorbed and subjected to cellular metabolism (e.g. fluorescein diacetate).
• Living cells generate adenosine triphosphate (ATP) that can readily be detected by enzyme assays, e.g. luciferin emits light when exposed to firefly luciferase in the presence of ATP; light emission can be measured and related to bacterial concentration.
• The resistance, capacitance or impedance of a culture medium changes as a result of bacterial or yeast growth and metabolism, and these electrical properties vary in proportion to cell concentration.
• Manometric techniques are appropriate for monitoring the growth of organisms that consume or produce significant quantities of gas during their metabolism, e.g. yeasts or moulds producing carbon dioxide as a result of fermentation.

These methods are fast, readily automated and eliminate the need for numerous Petri dishes and incubators. On the other hand they require expensive equipment, have limitations in terms of detection limits and may be less readily adapted to certain types of sample than traditional methods. Furthermore, there are problems in some cases with reconciling the counts obtained by rapid methods and by traditional means. The newer techniques may detect organisms that are metabolizing but not capable of reproducing to give visible colonies (viable but non-culturable organisms), so may give values many times higher than traditional methods; this has contributed to the caution with which regulatory authorities have accepted the data generated by rapid methods. Nevertheless, they are becoming more widely accepted and are likely to become an integral part of enumeration procedures in pharmaceutical microbiology in the foreseeable future.

6 Microbial genetics

The nature of the genetic material possessed by a microbial cell and the manner in which that genetic material may be transferred to other cells depends largely upon whether the organism is a prokaryote or a eukaryote (see section 1.2).

6.1 Bacteria

The genes essential for growth and metabolism of bacteria are normally contained on a chromosome of double-stranded DNA, which is in the form of a covalently closed circle (and so designated ccc ds DNA). Additional genes that usually just confer upon the cell a survival advantage under certain circumstances may also be contained upon plasmids; these are usually similar in structure to chromosomes but much smaller and replicate independently (Chapters 3 and 13). The total complement of genes possessed by a cell, i.e. those in the chromosome, plasmid(s) and any received from other sources, e.g. bacteriophages (bacterial viruses), is referred to as the genome of the cell.

Typically bacterial chromosomes are 1 mm or more in length and contain about 1000–3000 genes. As many bacterial cells are approximately 1 μm long, it is clear that the chromosome has to be tightly coiled in order to fit in the available volume. Although all the genes are contained on a single chromosome (rather than being distributed over two or more), it is possible for a cell to contain several *copies* of that chromosome at any one time. Usually there are multiple copies during periods of rapid cell division, but some species seem to have many copies all the time. The mechanisms by which bacterial genes may be transferred from one organism to another are described in Chapter 3.

Plasmids usually resemble chromosomes except that they are approximately 0.1–1.0% of the size of a bacterial chromosome, and there are a few that are linear rather than circular. Plasmid genes are not essential for the normal functioning of the cell but may code for a property that affords a survival advantage in certain environmental conditions; bacteria possessing the plasmid in question would therefore be selected when such conditions exist. Properties which can be coded by plasmids include the ability to utilize unusual sugars or food sources, toxin production, production of pili that facilitate the attachment of a cell to a substrate (e.g. intestinal epithelium) and antibiotic resistance. A cell may contain multiple copies of any one plasmid and may contain two or more different plasmids. However, some plasmid combinations cannot coexist inside the same cell and are said to be incompatible; this phenomenon enables plasmids to be classified into incompatibility groups.

Plasmids replicate independently of the chromosome within the cell, so that both daughter cells contain a copy of the plasmid after binary fission. Plasmids may also be passed from one cell to another by various means (Chapter 3). Some plasmids exhibit a marked degree of host specificity and may only be transmitted between different strains of the same species, although others, particularly those commonly found in Gram-negative intestinal bacteria, may cross between different species within

a genus or between different genera. Conjugative (self-transmissible) plasmids code for genes that facilitate their own transmission from one cell to another by the production of pili. These sex pili initially establish contact between the two cells and then retract, drawing the donor and recipient cells together until membrane fusion occurs.

6.2 Eukaryotes

Eukaryotic microorganisms (yeasts, moulds, algae and protozoa) possess a nucleus that normally contains one or more pairs of linear chromosomes, in which the ds DNA is complexed with protein. The cells may divide asexually and the nucleus undergoes mitosis—a sequence of events by which the nucleus and the chromosomes within it are replicated to give copies identical to the originals. Most eukaryotes also have the potential for sexual reproduction during which the nucleus undergoes meiosis, i.e. a more specialized form of nuclear and chromosome division creating new gene combinations, so the offspring differ from the parents. Despite this potential, there are some eukaryotic cells, particularly fungi, in which a sexual stage in the life cycle has never been observed. Many eukaryotic microorganisms possess plasmids, and some fungal plasmids are based on RNA instead of DNA.

6.3 Genetic variation and gene expression

Microorganisms may adapt rapidly to new environments and devise strategies to avoid or negate stressful or potentially harmful circumstances. Their ability to survive adverse conditions may result from the organism using genes it already possesses, or by the acquisition of new genetic information. The term 'genotype' describes the genetic composition of an organism, i.e. it refers to the genes that the organism possesses, regardless of whether they are expressed or not. It is not uncommon for a microbial cell to possess a particular gene but not to express it, i.e. not to manufacture the protein or enzyme that is the product of that gene, unless or until the product is actually required; this is simply a mechanism to avoid wasting energy. For example, many bacteria possess the genes that code for β-lactamases; these enzymes hydrolyse and inactivate β-lactam antibiotics (e.g. penicillins). In many organisms β-lactamases are only produced in response to the presence of the antibiotic. This form of non-genetic adaptation is termed *phenotypic* adaptation, and there are many situations in which bacteria adopt a phenotypic change to counter environmental stress. But microorganisms may also use an alternative strategy of *genetic* adaptation, by which they acquire new genes either by mutation or by conjugation (Chapter 3); subsequently, a process of selection ensures that the mutant organisms that are better suited to the new environment become numerically dominant.

In bacteria, mutation is an important mechanism by which resistance to antibiotics and other antimicrobial chemicals is achieved, although the receipt of entirely new genes directly from other bacteria is also clinically very important. Spontaneous mutation rates (rates not influenced by mutagenic chemicals or ionizing radiation) vary substantially depending on the gene and the organism in question, but rates of 10^{-5}–10^{-7} are typical. These values mean that, on average, a mutant arises once in every 100 000 to every 10 million cell divisions. Although these figures might suggest that mutation is a relatively rare event, the speed with which microorganisms can multiply means, for example, that mutants exhibiting increased antibiotic resistance can arise quite quickly during the course of therapy.

7 Pharmaceutical importance of the major categories of microorganisms

Table 2.4 indicates the ways in which the different types of microorganism are considered relevant in pharmacy. The importance of viruses derives exclusively from their pathogenic potential, and because of their lack of intrinsic metabolism they are not susceptible to antibiotics. Partly for these reasons, viral infections are among the most dangerous and difficult to cure, and of all the categories of microorganism, only viruses appear in (the most serious) Hazard Category 4 as classified by the Advisory Committee on Dangerous Pathogens. Because they are not free-living, viruses are incapable of growing on manufactured medicines or raw materials, so they do not cause product spoilage, and they have no synthetic capabilities that can be exploited in medicines manufacture. Viruses are relatively easy to destroy by heat, radiation or toxic chemicals, so they do not represent a problem from this perspective. In this, they contrast with prions; although some authorities would question the categorization of these infectious agents as microorganisms, they are included here because of their undoubted ability to cause, as yet incurable, fatal disease, and their extreme resistance to lethal agents. Pharmacists and healthcare personnel in general should be aware of the ability of prions to easily withstand sterilizing conditions

Table 2.4 Pharmaceutical importance of the major categories of microorganisms

Type of organism	Pharmaceutical relevance				
	Contamination or spoilage of raw materials and medicines	Pathogens	Resistance to antibiotics and biocides	Resistance to sterilizing agents and processes	Used in the manufacture of therapeutic agents
Viruses	+	+			
Prions	+	+		+	
Bacteria					
Gram-negative	+	+	+		+
Gram-positive	+	+	+	+ (spores)	+
Mycobacteria		+	+		
Streptomycetes		+			+
Chlamydia		+			
Rickettsia		+			
Mycoplasma		+			
Fungi		+			
Yeasts	+	+	+		+
Moulds	+	+	+		+
Protozoa		+			

that would be satisfactory for the destruction of all other categories of infectious agent.

There are examples of bacteria that are important in each of the different ways indicated by the column headings of Table 2.4. Many of the medically and pharmaceutically important bacteria are pathogens, and some of these pathogens are of long-standing notoriety as a result of their ability to resist the activity of antibiotics and biocides (disinfectants, antiseptics and preservatives). In addition to these long-established resistant organisms, other bacteria have given more recent cause for concern including meticillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VSE) and multiply resistant *Mycobacterium tuberculosis* (Chapter 13). While penicillin and cephalosporin antibiotics are produced by fungal species, the majority of the other categories of clinically important antibiotics are produced by species of bacteria, notably streptomycetes. In addition, a variety of bacteria are exploited commercially in the manufacture of other medicines including steroids, enzymes and carbohydrates. The ability of bacteria to grow on diverse substrates ensures that their potential as agents of spoilage in manufactured medicines and raw materials is well recognized, and the ability of many species to survive drying means that they survive well in dust and so become important as contaminants of manufactured medicines. The ability to survive not only in dry conditions but in other adverse environments (heat, radiation, toxic chemicals) is well exemplified by bacterial spores, and their pre-eminence at or near the top of the 'league table' of resistance to lethal agents has resulted in spores acting as the indicator organisms that have to be eliminated in most sterilization processes (Chapter 21).

Like bacteria, fungi are able to form spores that survive drying, so they too arise commonly as contaminants of manufactured medicines. However, the degree of resistance presented by the spores is usually less than that exhibited by bacteria, and fungi do not represent a sterilization problem. Fungi do not generally create a significant infection hazard either; relatively few fungal species are considered major pathogens for animals that possess a fully functional immune system. There are, however, several fungi which, while representing little threat to immunocompetent individuals, are nevertheless capable

of initiating an infection in persons with impaired immune function; the term 'opportunist pathogens' is used to describe microorganisms (of all types) possessing this characteristic. In this context it is worth noting that the immunocomopromised represent an increasingly large group of patients, and this is not just because of HIV/AIDS. Several other conditions or drug treatments impair immune function, e.g. congenital immunodeficiency, cancer (particularly leukaemia), radiotherapy and chemotherapy, the use of systemic corticosteroids and immunosuppressive drugs (often following tissue or organ transplants), severe burns and malnutrition.

Protozoa are of significance largely owing to the pathogenic potential of a few species. Because protozoa do not possess cell walls they do not survive drying well (unless in the form of cysts), so they are not a problem in the manufacturing environment—and even the encysted forms do not display resistance to sterilizing processes to match that of bacterial spores. It should be noted that protozoal infections are not currently a major problem to human health in temperate climates, although they are more troublesome in veterinary medicine and in the tropics. There are concerns that the geographical ranges of protozoal infections such as malaria may extend substantially if current fears about global warming translate into reality.

8 Preservation of microorganisms

In addition to their uses in the manufacture of medicines, microorganisms are employed in a variety of tests and assays, particularly those to measure the activity of antimicrobial chemicals. Useful organisms, therefore, need to be correctly preserved in order to ensure that their desirable properties are not changed during storage or, worse, the culture dies completely and is irreplaceable. Many bacteria and fungi can conveniently be stored for a few days, or possibly weeks, in the form of liquid cultures in tubes, or as colonies on Petri dishes. Organisms that readily form spores—*Bacillus* and *Clostridium* species of bacteria and most fungi—can be stored for months or even years in this way provided that the culture medium does not evaporate to dryness, but non-sporing organisms vary substantially in their survival capacities. Gram-positive bacteria generally tend to survive better than Gram-negative ones: species like *Pseudomonas aeruginosa*, for example, may die in a few weeks, even at refrigeration temperatures, if maintained as colonies on unsealed Petri dishes. Even if a culture that is to be preserved does not die completely when stored in the refrigerator, there is a risk that the cells that *do* survive are not typical of the population as a whole; they may, for example, be mutants that have increased resistance to adverse conditions in general, and so fail to give the expected results when used in tests on antibiotic activity. The dual aims of a culture preservation procedure therefore are to maintain the viability of the highest possible percentage of cells and to minimize the risk of selecting atypical mutants.

The most common procedures for long-term storage are by freezing at −80 °C (or lower) in refrigerators, by storage in liquid nitrogen at −196 °C in special vessels, or by freeze-drying (also called lyophilization). In each case, cryoprotectant chemicals—compounds like glycerol or dimethylsulphoxide—are incorporated at a concentration of about 10% v/v in the liquid culture of the organism in order to minimize both the formation of damaging ice crystals and osmotic stresses that can accelerate cell death during freezing and thawing.

Reference cultures, those with well-defined biosynthetic capabilities or resistance properties, can be obtained in a freeze-dried form from internationally accessible culture collections like the American Type Culture Collection (cultures having the designation ATCC before a reference number) or the UK National Collection of Industrial and Marine Bacteria (NICMB). Increasingly. pharmacopoeias and regulatory agencies are requiring tests that employ microorganisms to be conducted with cultures or test suspensions of cells that are no more than five subcultures from the reference material obtained from the designated culture collection.

3 Bacteria

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1 Introduction

The smallest free-living microorganisms are the prokaryotes, comprising bacteria and archaea (see Chapter 2). Prokaryote is a term used to define cells that lack a true nuclear membrane; they contrast with eukaryotic cells (e.g. plants, animals and fungi) that possess a nuclear membrane and internal compartmentalization. Indeed, a major feature of eukaryotic cells, absent from prokaryotic cells, is the presence in the cytoplasm of membrane-enclosed organelles. These and other criteria differentiating eukaryotes and prokaryotes are shown in Table 2.1.

Bacteria and archaea share many traits and it was not until the early 1980s that differences first became evident from analyses of gene sequences. One major difference is

Hugo and Russell's Pharmaceutical Microbiology, Eighth Edition. Edited by Stephen P. Denyer, Norman Hodges, Sean P. Gorman, Brendan F. Gilmore.

the composition of cell walls. A more striking contrast is in the structure of the lipids that make up their cytoplasmic membranes. Differences also exist in their respective patterns of metabolism: most archaea are anaerobes, and are often found inhabiting extreme environments. It is possible that their unusual membrane structure gives archaeal cells greater stability under extreme conditions. Of notable interest is the observation that no disease-causing archaea have yet been identified; the vast majority of prokaryotes of medical and pharmaceutical significance are bacteria.

Bacteria represent a large and diverse group of micro-organisms that can exist as single cells or as cell clusters. Moreover, they are generally able to carry out their life processes of growth, energy generation and reproduction independently of other cells. In these respects they are very different from the cells of animals and plants, which are unable to live alone in nature and can exist only as part of a multicellular organism. They are capable of growing in a range of different environments and can not only cause contamination and spoilage of many pharmaceutical products but also a range of different diseases. For this reason, only bacteria are referred to throughout the remainder of this chapter.

1.1 Bacterial diversity and ubiquity

Bacterial diversity can be seen in terms of variation in cell size and shape (morphology), adaptation to environmental extremes, survival strategies and metabolic capabilities. Such diversity allows bacteria to grow in a multiplicity of environments ranging from hot sulphur springs (65 °C) to deep freezers (–20°C), from high (pH 1) to low (pH 13) acidity and high (0.7 m) to low osmolarity (water). In addition, they can grow in both nutritionally rich (compost) and nutritionally poor (distilled water) situations. Hence, although each organism is uniquely suited to its own particular environmental niche and rarely grows out of it, the presence of bacteria may be considered ubiquitous. Indeed, there is no natural environment that is free from bacteria. This ubiquity is often demonstrated by terms used to describe organisms that grow and/or survive in particular environments. An example of such descriptive terminology is shown in Table 3.1.

Table 3.1 Descriptive terms used to describe bacteria

Descriptive term	Adaptive feature
Psychrophile	Growth range –40 °C to +20 °C
Mesophile	Growth range +20 °C to +40 °C
Thermophile	Growth range +40 °C to +85 °C
Thermoduric	Endure high temperatures
Halophile	Salt-tolerant
Acidophile	Acid-tolerant
Aerobe	Air (oxygen) requiring
Obligate anaerobe	Air (oxygen) poisoned
Autotroph	Utilizes inorganic material
Heterotroph	Requires organic material

2 Bacterial ultrastructure

2.1 Cell size and shape

Bacteria are the smallest free-living organisms, their size being measured in micrometres (microns). Because of this small size a microscope affording a considerable degree of magnification (×400–1000) is necessary to observe them. Bacteria vary in size from a cell as small as 0.1–0.2 µm in diameter to those that are >5 µm in diameter. Bacteria this large, such as *Thiomargarita namibiensis*, are extremely rare: the majority of bacteria are 1–5 µm long and 1–2 µm in diameter. By comparison, eukaryotic cells may be 2 µm to >200 µm in diameter. The small size of bacteria has a number of implications with regard to their biological properties, most notably increased and more efficient transport rates. This advantage allows bacteria far more rapid growth rates than eukaryotic cells.

While the classification of bacteria is immensely complex, nowadays relying very much on 16S ribosomal DNA sequencing data, a more simplistic approach is to divide them into major groups on purely morphological grounds. The majority of bacteria are unicellular and possess simple shapes, e.g. round (cocci), cylindrical (rod, also called bacillus, spelt with a lower case initial letter to distinguish from *Bacillus*, the genus) or ovoid. Some rods are curved (vibrios), while longer rigid curved organisms with multiple spirals are known as spirochaetes. Rarer morphological forms include the actinomycetes which are rigid bacteria resembling fungi that may grow as lengthy branched filaments; the mycoplasmas which lack a conventional peptidoglycan (murein) cell wall and are highly pleomorphic organisms of indefinite shape; and some miscellaneous bacteria comprising stalked, sheathed, budded and slime-producing forms often associated with aquatic and soil environments.

Often bacteria remain together in specific arrangements after cell division. These arrangements are usually characteristic of different organisms and can be used as

part of a preliminary identification. Examples of such cellular arrangements include chains of rods or cocci, paired cells (diplococci), tetrads and clusters.

2.2 Cellular components

Compared with eukaryotic cells, bacteria possess a fairly simple base cell structure, comprising cell wall, cytoplasmic membrane, nucleoid, ribosomes and occasionally inclusion granules (Figure 3.1). Nevertheless it is important for several reasons to have a good knowledge of these structures and their functions. First, the study of bacteria provides an excellent route for probing the nature of biological processes, many of which are shared by multicellular organisms. Secondly, at an applied level, normal bacterial processes can be customized to benefit society on a mass scale. Here, an obvious example is the large-scale industrial production (fermentation) of antibiotics. Thirdly, from a pharmaceutical and healthcare perspective, it is important to be able to know how to kill bacterial contaminants and disease-causing organisms. To treat infections antimicrobial agents are used to inhibit the growth of bacteria, a process known as antimicrobial chemotherapy. The essence of antimicrobial chemotherapy is selective toxicity (Chapters 11, 12, 14 and 15), which is achieved by exploiting differences between the structure and metabolism of bacteria and host cells. Selective toxicity is, therefore, most efficient when a similar target does not exist in the host. Examples of such targets will be noted in the following sections.

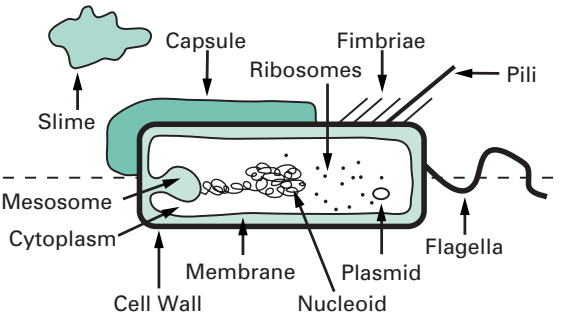

Figure 3.1 Diagram of a bacterial cell. Features represented above the dotted line are only found in some bacteria, whereas those below the line are common to all bacteria.

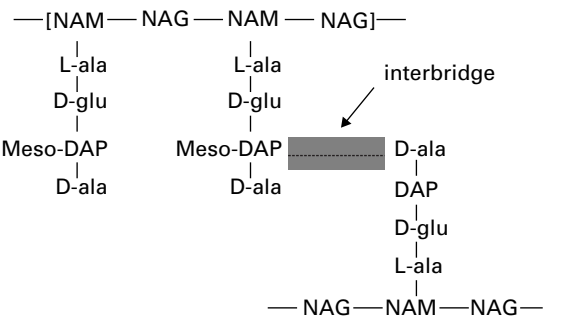

Figure 3.2 Structure of *Escherichia coli* peptidoglycan.

2.2.1 Cell wall

The bacterial cell wall is an extremely important structure, being essential for the maintenance of the shape and integrity of the bacterial cell. It is also chemically unlike any structure present in eukaryotic cells and is therefore an obvious target for antibiotics that can attack and kill bacteria without harm to the host (Chapter 12).

The primary function of the cell wall is to provide a strong, rigid structural component that can withstand the osmotic pressures caused by high chemical concentrations of inorganic ions in the cell. Most bacterial cell walls have in common a unique structural component called peptidoglycan (also called murein or glycopeptide); exceptions include the mycoplasmas, extreme halophiles and the archaea. Peptidoglycan is a large macromolecule containing glycan (polysaccharide) chains that are cross-linked by short peptide bridges. The glycan chain acts as a backbone to peptidoglycan, and is composed of alternating residues of *N*-acetyl muramic acid (NAM) and *N*-acetyl glucosamine (NAG). To each molecule of NAM is attached a tetrapeptide consisting of the amino acids L-alanine, D-alanine, D-glutamic acid and either lysine or diaminopimelic acid (DAP). This glycan tetrapeptide repeat unit is cross-linked to adjacent glycan chains, either through a direct peptide linkage or a peptide interbridge (Figure 3.2). The types and numbers of cross-linking amino acids vary from organism to organism. Other unusual features of the cell wall that provide potential antimicrobial targets are DAP and the presence of two amino acids that have the D-configuration.

Bacteria can be divided into two large groups, Gram-positive and Gram-negative, on the basis of a differential staining technique called the Gram stain. Essentially, the Gram stain consists of treating a film of bacteria dried on a microscope slide with a solution of crystal violet, followed by a solution of iodine; these are then washed with an alcohol solution. In Gram-negative organisms the cells lose the crystal violet–iodine complex and are rendered colourless, whereas Gram-positive cells retain the dye. Regardless, both cell types are counter-stained with a different coloured dye, e.g. carbolfuchsin, which is red.

Hence, under the light microscope Gram-negative cells appear red while Gram-positive cells are purple. These marked differences in response reflect differences in cell wall structure. The Gram-positive cell wall consists primarily of a single type of molecule whereas the Gram-negative cell wall is a multilayered structure and quite complex.

The cell walls of Gram-positive bacteria are quite thick (20–80 nm) and consist of between 60% and 80% peptidoglycan, which is extensively cross-linked in three dimensions to form a thick polymeric mesh (Figure 3.3). Gram-positive walls frequently contain acidic polysaccharides called teichoic acids; these are either ribitol phosphate or glycerol phosphate molecules that are connected by phosphodiester bridges. Because they are negatively charged, teichoic acids are partially responsible for the negative charge of the cell surface as a whole. Their function may be to effect passage of metal cations through the cell wall. In some Gram-positive bacteria glycerol–teichoic acids are bound to membrane lipids and are termed lipoteichoic acids. During an infection, lipoteichoic acid molecules released by killed bacteria trigger an inflammatory response. Cell wall proteins, if present, are generally found on the outer surface of the peptidoglycan.

The wall, or more correctly, envelope of Gram-negative cells is a far more complicated structure (Figure 3.4). Although it contains less peptidoglycan (10–20% of wall), a second membrane structure is found outside the peptidoglycan layer. This outer membrane is asymmetrical, composed of proteins, lipoproteins, phospholipids and a component unique to Gram-negative bacteria, lipopolysaccharide (LPS). Essentially, the outer

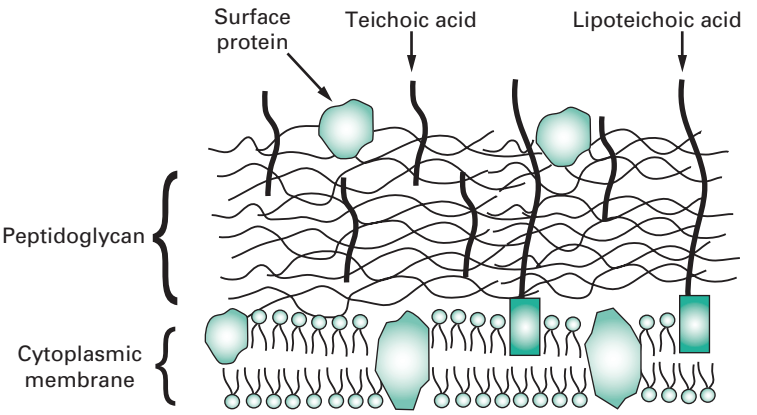

Figure 3.3 Structure of the Gram-positive cell wall.

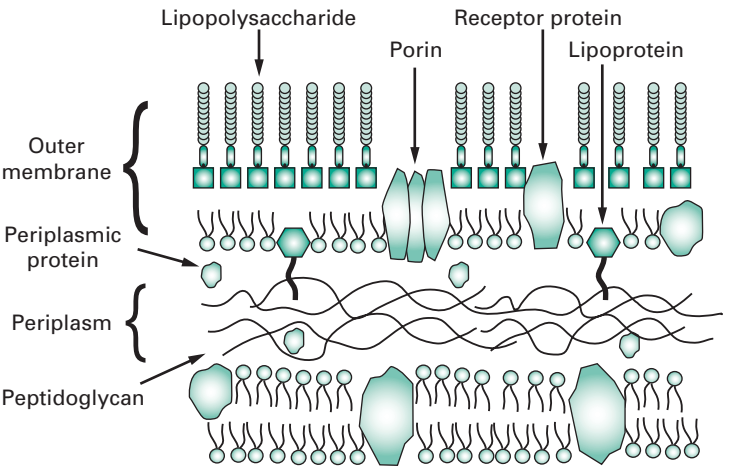

Figure 3.4 Structure of the Gram-negative cell envelope.

membrane is attached to the peptidoglycan by a lipoprotein, one end of which is covalently attached to peptidoglycan and the other end is embedded in the outer membrane. The outer membrane is not a phospholipid bilayer although it does contain phospholipids in the inner leaf, and its outer layer is composed of LPS, a polysaccharide–lipid molecule. Proteins are also found in the outer membrane, some of which form trimers that traverse the whole membrane and in so doing form water-filled channels or porins through which small molecules can pass. Other proteins are found at either the inner or outer face of the membrane.

The LPS (Figure 3.5) is an important molecule because it determines the antigenicity of the Gram-negative cell and it is extremely toxic to animal cells. The molecule consists of three regions, namely lipid A, core polysaccharide and O-specific polysaccharide. The lipid A portion is composed of a disaccharide of glucosamine phosphate bound to fatty acids and forms the outer leaflet of the membrane. It is the lipid A component that is responsible for the toxic and pyrogenic properties of Gram-negative bacteria. Lipid A is linked to the core polysaccharide by the unique molecule ketodeoxyoctonate (KDO), and at the other end of the core is the O-polysaccharide (O-antigen), which usually contains six-carbon sugars as well as one or more unusual deoxy sugars such as abequose.

Although the outer membrane is relatively permeable to small molecules, it is not permeable to enzymes or large molecules. Indeed, one of the major functions of the outer membrane may be to keep certain enzymes that are present outside the cytoplasmic membrane from diffusing away from the cell. Moreover, the outer membrane is not readily penetrated by hydrophobic compounds and is, therefore, resistant to dissolution by detergents.

The region between the outer surface of the cytoplasmic membrane and the inner surface of the outer membrane is called the periplasm. This occupies a distance of about 12–15 nm, is gel-like in consistency and, in addition to the peptidoglycan, contains sugars and an abundance of proteins including hydrolytic enzymes and transport proteins. Table 3.2 summarizes the major differences in wall composition between Gram-positive and Gram-negative cells.

2.2.2 Cytoplasmic membrane

Biochemically, the cytoplasmic membrane is a fragile, phospholipid bilayer with proteins distributed randomly throughout. These are involved in the various transport and enzyme functions associated with the membrane. A major difference in chemical composition between prokaryotic and eukaryotic cells is that eukaryotes have sterols in their membranes (e.g. cholesterol) whereas prokaryotes do not. The cytoplasmic membrane serves many functions, including transport of nutrients, energy generation and electron transport; it is the location for regulatory proteins and biosynthetic proteins, and it acts as a semipermeable selectivity barrier between the cytoplasm and the cell environment.

Invaginations of the cytoplasmic membrane are referred to as *mesosomes*. Those that form near the septum of Gram-positive cells serve as organs of attachment for the bacterial chromosome.

2.2.3 Cytoplasm

The cytoplasm consists of approximately 80% water and contains enzymes that generate ATP directly by oxidizing glucose and other carbon sources. The cytoplasm also

Table 3.2 Gram-positive and Gram-negative cell wall composition

Feature	Gram-positive cells	Gram-negative cells
Peptidoglycan	60–80%	10–20%
Teichoic acid	Present	Absent
Lipoteichoic acid	Present	Absent
Lipoprotein	Absent	Present
Lipopolysaccharide	Absent	Present
Protein	*c.*15%	*c.*60%
Lipid	*c.*2%	*c.*20%

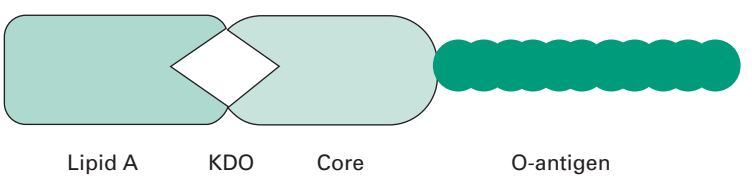

Figure 3.5 Schematic representation of lipopolysaccharide (LPS). KDO, ketodeoxyoctonate.

contains some of the enzymes involved in the synthesis of peptidoglycan subunits. Ribosomes, the DNA genome (nucleoid) and inclusion granules are also found in the cytoplasm.

2.2.4 Nucleoid

The bacterial chromosome exists as a singular, covalently closed circular molecule of double-stranded DNA comprising approximately 4600 kilobase pairs. It is complexed with small amounts of proteins and RNA, but unlike eukaryotic DNA, is not associated with histones. The DNA, if linearized, would be about 1 mm in length. In order to package this amount of material the cell requires that the DNA is supercoiled into a number of domains (*c.*50) and that the domains are associated with each other and stabilized by specific proteins into an aggregated mass or nucleoid. The enzymes, topoisomerases, that control topological changes in DNA architecture are different from their eukaryotic counterparts (which act on linear chromosomes) and therefore provide a unique biochemical target for antibiotic action.

2.2.5 Plasmids

Plasmids are relatively small, circular pieces of double-stranded extrachromosomal DNA. They are capable of autonomous replication and encode for many auxiliary functions that are not usually necessary for bacterial growth. One such function of great significance is that of antibiotic resistance (Chapter 13). Plasmids may also transfer readily from one organism to another, and between species, thereby increasing the spread of resistance.

2.2.6 Ribosomes

The cytoplasm is densely packed with ribosomes. Unlike eukaryotic cells these are not associated with a membranous structure; the endoplasmic reticulum is not a component of prokaryotic cells. Bacterial ribosomes are 70S in size, made up of two subunits of 30S and 50S. This is smaller than eukaryotic ribosomes, which are 80S in size (40S and 60S subunits). Differences will therefore exist in the size and geometry of RNA binding sites.

2.2.7 Inclusion granules

Bacteria occasionally contain inclusion granules within their cytoplasm. These consist of storage material composed of carbon, nitrogen, sulphur or phosphorus and are formed when these materials are replete in the environment to act as repositories of these nutrients when shortages occur. Examples include poly-β-hydroxybutyrate, glycogen and polyphosphate.

2.3 Cell surface components

The surface of the bacterial cell is the portion of the organism that interacts with the external environment most directly. As a consequence, many bacteria deploy components on their surfaces in a variety of ways that allow them to withstand and survive fluctuations in the growth environment. The following sections describe a few of these components that are commonly found, although not universally, that allow bacteria to move, sense their environment, attach to surfaces and provide protection from harsh conditions.

2.3.1 Flagella

Bacterial motility is commonly provided by flagella, long (*c.*12 μm) helical-shaped structures that project from the surface of the cell. The filament of the flagellum is built up from multiple copies of the protein flagellin. Where the filament enters the surface of the bacterium, there is a hook in the flagellum, which is attached to the cell surface by a series of complex proteins called the flagellar motor. This rotates the flagellum, causing the bacterium to move through the environment. The numbers and distribution of flagella vary with bacterial species. Some have a single, polar flagellum, whereas others are flagellate over their entire surface (peritrichous); intermediate forms also exist.

2.3.2 Fimbriae

Fimbriae are structurally similar to flagella, but are not involved in motility. Although they are straighter, more numerous and considerably thinner and shorter (3 μm) than flagella, they do consist of protein and project from the cell surface. There is strong evidence to suggest that fimbriae act primarily as adhesins, allowing organisms to attach to surfaces, including animal tissues in the case of some pathogenic bacteria, and to initiate biofilm formation. Fimbriae are also responsible for haemagglutination and cell clumping in bacteria. Among the best characterized fimbriae are the type I fimbriae of enteric (intestinal) bacteria.

2.3.3 Pili

Pili are morphologically and chemically similar to fimbriae, but they are present in much smaller numbers (<10) and are usually longer. They are involved in the genetic exchange process of conjugation (section 6.3).

2.3.4 Capsules and slime layers

Many bacteria secrete extracellular polysaccharides (EPS) that are associated with the exterior of the bacterial cell. The EPS is composed primarily of *c.*2% carbohydrate and 98% water, and provides a gummy exterior to the cell. Morphologically, two extreme forms exist: *capsules*, which form a tight, fairly rigid layer closely associated with the cell, and *slimes*, which are loosely associated with the cell. Both forms function similarly, to offer protection against desiccation, to provide a protective barrier against the penetration of biocides, disinfectants and positively charged antibiotics, to protect against engulfment by phagocytes and protozoa and to act as a cement binding cells to each other and to the substratum in biofilms (see below). One such polymer that performs all these functions is alginate, produced by *Pseudomonas aeruginosa*; dextran, produced by *Leuconostoc mesenteroides*, is another. Both polymers may be harvested and used variously as pharmaceutical aids, surgical dressings and drug delivery systems, although the preferred source of alginate is seaweed rather than bacteria.

2.3.5 S-layers

S-layers are the most common cell wall type amongst the archaea. These consist of a two-dimensional paracrystalline array of proteins or glycoproteins which show various ordered symmetries when viewed under the electron microscope. In many species of bacteria, S-layers are present on their outer surfaces in addition to other cell wall components such as polysaccharides. In such arrangements the S-layer is always the outermost layer. In addition to increasing the structural robustness of the cell, S-layers can act to a certain extent as an external permeability barrier.

3 Biofilms

Any surface, whether it is animate or inanimate, is of considerable importance as a microbial habitat owing to the adsorption of nutrients. A nutrient-rich microenvironment is thus produced in a nutrient-poor macroenvironment whenever a surface–liquid interface exists. Consequently, microbial numbers and activity are usually much greater on a surface than in suspension. Hence, in many natural, medical and industrial settings bacteria attach to surfaces and form multilayered communities called *biofilms*. These commonly contain more than one species of bacteria, which exist cooperatively together as a functional, dynamic consortium. Moreover, biofilms commonly possess unique properties that are distinct to unattached cells. Biofilm formation usually begins with pioneer cells attaching to a surface, either through the use of specific adhesins such as fimbriae, or non-specifically by EPS. Once established, these cells grow and divide to produce microcolonies, which with time, eventually coalesce to produce a biofilm. A key characteristic of biofilms is the enveloping of the attached cells in a matrix of EPS and other macromolecules. This helps to cement cells to the surface and to each other, and protects the bacteria from hazardous materials such as antibiotics and biocides, from desiccation and from engulfment by macrophages and phagocytes in much the same way as the capsules and slime layers mentioned above. In addition, strands of EPS hold the bacterial cells at a distance from one another, enabling small water channels to form in the biofilm. These channels act as a primitive circulatory system carrying trapped nutrients and oxygen to the enclosed cells and take waste products away.

Biofilms have a number of significant implications in medicine and industry. In the human body the resident cells within the biofilm are not exposed to attack by the immune system and in some instances can exacerbate the inflammatory response. An example of this is shown by the growth of *Ps. aeruginosa* as an alginate-enclosed biofilm in the lungs of cystic fibrosis patients. Bacterial biofilms are also profoundly less susceptible to antimicrobial agents than their free-living, planktonic counterparts. As a consequence, bacterial biofilms that form on contaminated medical implants and prosthetic devices, manufacturing surfaces or fluid conduit systems are virtually impossible to eliminate with antibiotics or biocides. In these situations antimicrobial resistance occurs as a population or community response. Biofilms are considered in more detail in Chapter 8.

4 Bacterial sporulation

In a few bacterial genera, notably *Bacillus* and *Clostridium*, a unique process takes place in which the vegetative cell undergoes a profound biochemical change to give rise to a specialized structure called an endospore or spore (Figure 3.6). This process of sporulation is not part of a reproductive cycle, but the spore is a highly resistant cell that enables the producing organism to survive in adverse environmental conditions such as lack of moisture or essential nutrients, or exposure to toxic chemicals, radiation or high temperatures. Because of their extreme resistance to radiation, ethylene oxide and heat, all steri-

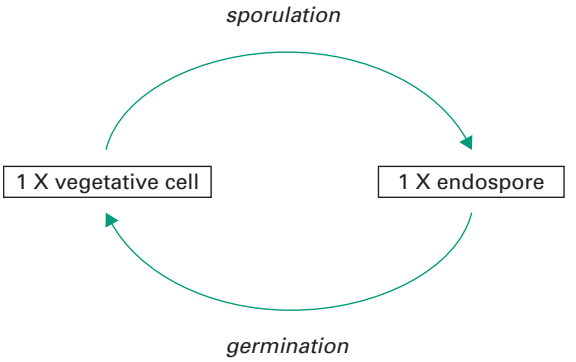

Figure 3.6 Bacterial sporulation and germination.

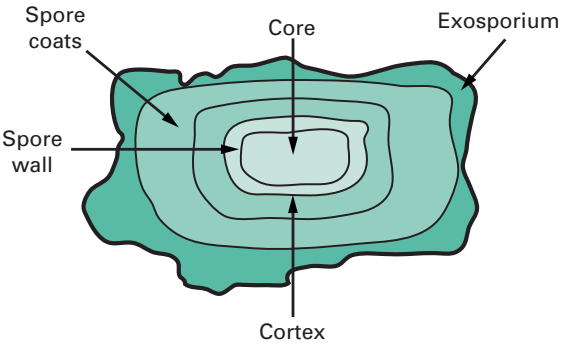

Figure 3.7 Diagram of endospore structure.

lization processes for pharmaceutical products have been designed to destroy the bacterial spore (Chapter 21). Removal of the environmental stress may lead to germination of the spore back to the vegetative cell form.

4.1 Endospore structure

Endospores are differentiated cells that possess a grossly different structure to that of the parent vegetative cell in which they are formed. The structure of the spore is much more complex than that of the vegetative cell in that it has many layers surrounding a central core (Figure 3.7). The outermost layer is the exosporium composed of protein; within this are the spore coats, which are also proteinaceous but with a high cysteine content, the cortex that consists of loosely cross-linked peptidoglycan and the central core that contains the genome. Characteristic of the spore is the presence of dipicolinic acid and high levels of calcium ions which complex together. The core is also partially dehydrated, containing only 10–30% of the water content of the vegetative cells. Dehydration has been shown to increase resistance to both heat and chemicals. In addition, the pH of the core is about 1 unit lower than the cytoplasm of the vegetative cell and contains high levels of core-specific proteins that bind tightly to the DNA and protect it from potential damage. These core-specific proteins also function as an energy source for the outgrowth or germination of a new vegetative cell from the endospore.

4.2 Endospore formation

During endospore formation the vegetative cell undergoes a complex series of biochemical events in cellular differentiation, and many genetically directed changes in the cell that underpin the conversion occur in a series of distinct stages. Sporulation requires that the synthesis of some proteins involved in vegetative cell function cease and that specific spore proteins are made. This is accomplished by activation of a variety of spore-specific genes such as *spo* and *ssp*. The proteins coded by these genes catalyse a series of events leading ultimately to the production of a dry, metabolically inert but extremely resistant endospore. The whole process can take only a matter of hours to complete under optimal conditions.

4.3 Endospore germination

Although endospores can lie dormant for decades, they can revert back to a vegetative cell very rapidly. Activation of the process may occur through removal of the stress inducer that initiated sporulation. During germination loss of resistance properties occurs along with a loss of calcium dipicolinate and cortex components, and degradation of the core-specific proteins. Outgrowth occurs, involving water uptake and synthesis of new RNA, proteins and DNA until eventually, after a matter of minutes, the vegetative cell emerges from the fractured spore coat and begins to divide again.

5 Bacterial toxins

Although bacteria are associated with disease, only a few species are disease-producing or pathogenic for healthy individuals (Chapter 7). Of greater concern are those organisms that, if presented with the correct set of conditions, can cause disease, i.e. opportunist pathogens. Examples include *Staphylococcus epidermidis*, a beneficial organism when present on the skin (its normal habitat) yet potentially fatal if attached to a synthetic heart valve,

and *Ps. aeruginosa*, a non-pathogenic environmental organism but again potentially lethal in immunocompromised patients.

The pathogens cause host damage in a number of ways. In most cases pathogens produce a variety of molecules or factors that promote pathogenesis, among which are the toxins: products of bacteria that produce immediate host cell damage. Toxins have been classified as either endotoxin, i.e. cell wall-related, or exotoxin, products released extracellularly as the organism grows.

Endotoxin is the lipid A component of LPS (see section 2.2.1). It possesses multiple biological properties including the ability to induce fever, initiate the complement and blood cascades, activate B lymphocytes and stimulate production of tumour necrosis factor. Endotoxin is generally released from lysed or damaged cells. Care must be taken to eliminate or exclude such heat-resistant material from parenteral products and their delivery systems through a process known as depyrogenation (Chapters 21 and 22).

Most exotoxins fall into one of three categories on the basis of their structure and activities. These are the A-B toxins, the cytolytic toxins and the superantigen toxins. The A-B toxins consist of a B subunit that binds to a host cell receptor and is also covalently bound to the A subunit that mediates the enzymic activity responsible for toxicity. Most exotoxins (e.g. diphtheria toxin, cholera toxin) are of the A-B category. The cytolytic toxins such as haemolysins and phospholipases do not have separable A and B portions but work by enzymatically attacking cell constituents, causing lysis. The superantigens also lack an A-B type structure and act by stimulating large numbers of immune response cells to release cytokines, resulting in a massive inflammatory reaction. An example of this type of reaction is *Staphylococcus aureus*-mediated toxic shock syndrome.

6 Bacterial reproduction and growth kinetics

6.1 Multiplication and division cycle

The majority of bacterial cells multiply in number by a process of binary fission. That is, each individual will increase in size until it is large enough to divide into two identical daughter cells. At the point of separation each daughter cell must be capable of growth and reproduction. While each daughter cell will automatically contain those materials that are dispersed throughout the mother cell (mRNA, rRNA, ribosomes, enzymes, cytochromes, etc.), each must also carry at least one copy of the chromosome. The bacterial chromosome is circular and attached to the cytoplasmic membrane where it is able to uncoil during DNA replication. The process of DNA replication proceeds at a fixed rate dependent on temperature, therefore the time taken to copy an entire chromosome depends on the number of base pairs within it and the growth temperature. For *Escherichia coli* growing at 37 °C, replication of the chromosome will take approximately 45 minutes. These copies of the chromosome must then segregate to opposite sides of the cell before cell division can proceed. Division occurs in different ways for Gram-positive and Gram-negative bacteria. Gram-negative cells do not have a rigid cell wall and divide by a process of constriction followed by membrane fusion. Gram-positive cells, on the other hand, having a rigid cell wall, must develop a cross-wall (see Figure 2.1a) that divides the cell into two equal halves. Constriction and cross-wall formation takes approximately 15 minutes to complete. DNA replication, chromosome segregation (C-phase) and cell division (D-phase) occur sequentially in slow-growing cells with generation times of greater than 1 hour and are the final events of the bacterial cell cycle. Cells are able to replicate faster than once every hour by initiating several rounds of DNA replication at a time. Thus partially replicated chromosomes become segregated into the newly formed daughter cells. In this fashion it is possible for some organisms growing under their optimal conditions to divide every 15–20 minutes. Rod-shaped organisms maintain their diameter during the cell cycle and increase their mass and volume by a process of elongation. When the length of the cell has approximately doubled then the division/constriction occurs centrally. Coccal forms increase in size by radial expansion, with the division plane going towards the geometric centre. In some genera the successive division planes are always parallel. Under such circumstances the cells appear to form chains (i.e. streptococci). In staphylococci successive division planes are randomized, giving dividing clusters of cells the appearance of a bunch of grapes. Certain genera, e.g. *Sarcina*, rotate successive division planes by 90° to form tetrads and cubical octets. The appearance of dividing cells under the microscope can therefore be a useful initial guide to identification.

6.2 Population growth

When placed in favourable conditions populations of bacteria can increase at remarkable rates, given that each division gives rise to two identical daughter cells, then

each has the potential to divide again. Thus cell numbers will increase exponentially as a function of time. For a microorganism growing with a generation time of 20 minutes, one cell will have divided three times within an hour to give a total of eight cells. After 20 hours of continued division at this rate then the accumulated mass of bacterial cells would be approximately 70 kg (the weight of an average man). Ten hours later the mass would be equivalent to the combined body weight of the entire population of the UK. Clearly this does not happen in nature; rather, the supply of nutrients becomes exhausted and the organisms grow considerably more slowly, if at all.

The time interval between one cell division and the next is called the *generation time*. When considering a growing culture containing thousands of cells, a mean generation time is usually calculated. As one cell doubles to become two cells, which then multiply to become four cells and so on, the number of bacteria n in any generation can be expressed as:

1st generation	$n = 1 \times 2 = 2^1$
2nd generation	$n = 1 \times 2 \times 2 = 2^2$
3rd generation	$n = 1 \times 2 \times 2 \times 2 = 2^3$
xth generation	$n = 1 \times 2^x = 2^x$

For an initial population of N_o cells, as distinct from one cell, at the xth generation the cell population will be:

$$N = N_o \times 2^x$$

where N is the final cell number, N_o the initial cell number and x the number of generations. To express this equation in terms of x, then:

$$\log N = \log N_o + x \log 2$$

$$\log N - \log N_o = x \log 2$$

$$x = (\log N - \log N_o)/\log 2 = (\log N - \log N_o)/0.301$$
$$= 3.3(\log N - \log N_o)$$

The actual generation time is calculated by dividing x into t where t represents the hours or minutes of exponential growth.

6.2.1 Growth on solid surfaces

If microorganisms are immobilized on a solid surface from which they can derive nutrients and remain moist, cell division will cause the daughter cells to form a localized colony. In spite of the small size of the individual organisms, colonies are easily visible to the naked eye. Indeed, microbial growth can often be seen on the tonsils of an infected individual or as colonies on discarded or badly stored foods. In the laboratory solidified growth media are deployed to separate different types of bacteria and also as an aid to enumerating viable cell numbers. These media comprise a nutrient soup (broth) that has been solidified by the addition of agar (see Chapter 2). Agar melts and dissolves in boiling water but will not resolidify until the temperature is below 45 °C. Agar media are used in the laboratory either poured as a thin layer into a covered dish (Petri dish or plate) or contained within a small, capped bottle (slant). If suspensions of different species of bacteria are spread on to the surface of a nutrient agar plate then each individual cell will produce a single visible colony. These may be counted to obtain an estimate of the original number of cells. Different species will produce colonies of slightly different appearance, enabling judgements to be made as to the population diversity. The colour, size, shape and texture of colonies of different species of bacteria vary considerably and form a useful diagnostic aid to identification. Transfer of single colonies from the plate to a slant enables pure cultures of each organism to be maintained, cultured and identified.

6.2.2 Growth in liquids

When growing on a solid surface the size of the resultant colony is governed by the local availability of nutrients. These must diffuse through the colony. Eventually growth ceases when the rate of consumption of nutrients exceeds the rate of supply. When grown in liquids the bacteria, being of colloidal dimensions and sometimes highly motile, are dispersed evenly through the fluid. Nutrients are therefore equally available to all cells. When considering growth of bacterial populations in liquids it is necessary to consider whether the environment is closed or open with respect to the acquisition of fresh nutrient. Closed systems are typified by batch culture in closed glass flasks. In these waste products of metabolism are retained and all the available nutrients are present at the beginning of growth. Open systems, on the other hand, have a continual supply of fresh nutrients and removal of waste products.

6.2.2.1 Liquid batch culture (closed)

Figure 3.8 shows the pattern of population growth obtained when a small sample of bacteria is placed within a suitable liquid growth medium held in a glass vessel. As the increase in cell numbers is exponential (1, 2, 4, 8, 16, etc.) then during active growth a logarithmic plot of

cell number against time gives a straight line (B). This period is often referred to as the *logarithmic growth phase*, during which the generation or doubling time may be calculated from the slope of the line. However, the exponential phase is preceded by a *lag period* (A), during which time the inoculum adapts its physiology to that required for growth on the available nutrients. As growth proceeds nutrients are consumed and waste materials accumulate. This has the effect of reducing the rate of growth (*late logarithmic phase*) towards an eventual halt (*stationary phase*, C). Starvation during the stationary phase will eventually lead to the death of some of the cells and adaptation to a dormant state in others (*decline phase*, D). Patterns of growth such as this occur within inadequately preserved pharmaceutical products, in water storage tanks and in industrial fermentations.

6.2.2.2 Growth in open culture

Except under circumstances of feast–famine, growth of bacteria in association with humans and in our environment is subject to a gradual but continuous provision of nutrients and a dilution of waste products. Under such circumstances the rate of growth of bacteria is governed by the rate of supply of nutrients and the population size. Accordingly, bacteria in our gastrointestinal tracts receive a more or less continuous supply of food and excess bacteria are voided with the faeces (indeed, bacteria make up >90% of the dry mass of faeces). In many situations the bacteria become immobilized, as a biofilm, upon a surface and extract nutrients from the bulk fluid phase.

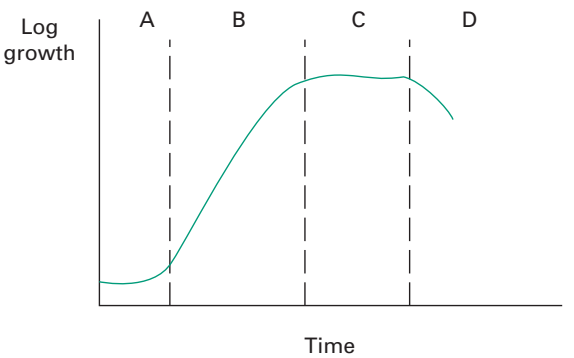

Figure 3.8 Typical bacterial growth curve in closed batch liquid culture: (A) lag or adaptive phase; (B) logarithmic or exponential phase; (C) stationary phase; (D) decline phase.

6.3 Growth and genetic exchange

For many years it was thought that bacteria, dividing by binary fission, had no opportunity for the exchange of genetic material and could only adapt and evolve through mutation of genes. This is not only untrue but masks the profound ability of bacteria to exchange and share DNA across diverse genera. This is of particular significance because it enables bacterial populations to adapt rapidly to changes in their environment, whether this is related to the appearance of a novel food or to the deployment of antibacterial chemicals and antibiotics. Three major processes of genetic exchange can be identified in bacteria—transformation, transduction and conjugation. Further details of these processes are given in Chapter 13 dealing with the development and spread of antibiotic resistance.

6.3.1 Transformation

In 1928 Griffith noticed that a culture of *Streptococcus pneumoniae* that had mutated to become deficient in capsule production could be restored to its normal capsulate form by incubation with a cell-free filtrate taken from a culture of the normal strain. While this discovery preceded the discovery of DNA as the genetic library and was only poorly understood at the time, it demonstrated the ability of certain types of bacteria to absorb small pieces of naked DNA from the environment that may recombine into the recipient chromosome. The process has become known as transformation and is likely to occur naturally in situations such as septic abscesses and in biofilms where high cell densities are associated with death and lysis of significant portions of the population. Transformation is also exploited in molecular biology as a means of transferring genes between different types of bacteria.

6.3.2 Transduction

Viruses are discussed more fully elsewhere (Chapter 5); however, there is a group of viruses, called bacteriophages, which have bacterial cells as their hosts. These bacteriophages inject viral DNA into the host cell. This viral DNA is then replicated and transcribed at the expense of the host and assembled into new viral particles. Under normal circumstances the host cell becomes lysed in order to release the viral progeny, but in exceptional circumstances, rather than enter a replication cycle the viral DNA becomes incorporated, by recombination, into the chromosome of the bacterium. This is known as a *temperate phage*. The viral DNA thus forms part of the bacterial chromosome and will be copied to all daughter cells.

Temperate phage will become active once again at a low frequency and phasing between temperate and lytic forms ensures the long-term survival of the virus. Occasionally during this transition back to the lytic form the excision of the viral DNA from the bacterial chromosome is inaccurate. The resultant virus may then either be defective, if viral DNA has been lost, or it may carry additional DNA of bacterial origin. Subsequent temperate infections caused by the latter virions will result in this bacterial DNA having moved between cells: a process of gene movement known as *transduction*. As the host range of some bacteriophages is broad then such processes can move DNA between diverse species.

6.3.3 Conjugation

Conjugation is thought to have evolved through transduction, and relates to the generation of defective viral DNA. This can be transcribed to produce singular viral elements, which cannot assemble or lyse the host cell. Such DNA strands are known as *plasmids*. They are circular and can either be integrated into the main chromosome, in which case they are replicated along with the chromosome and passed to daughter cells, or they are separate from it and can replicate independently. The simplest form of plasmid is the F-factor (fertility factor); this can be transcribed at the cell membrane to generate an F-pilus within the cell envelope and cells containing an F-factor are designated F^+. The F-pilus is a hollow appendage that is capable of transferring DNA from one cell to another, through a process that is very similar to the injection of viral DNA into a cell during infection. In its simplest form an unassociated F-factor will simply transfer a copy to a recipient cell, and such a transfer process is known as conjugation. Integration with, and dissociation of, the F-factor with the chromosome occurs randomly. When it is in the integrated form, designated Hfr (high frequency of recombination), then not only can a copy of the plasmid DNA be transferred across the F-pilus but so also can a partial or complete copy of the donor chromosome. Subsequent recombination events incorporate the new DNA into the recipient chromosome.

Just as the excision of temperate viral DNA from the host chromosome could be inaccurate, and lead to additions and deletions from the sequence, so too can the F-factor gather chromosomal DNA as the host cells change from Hfr to F^+. In such instances the plasmid that is formed will transfer not only itself but also this additional DNA into recipient cells. This is particularly significant because the unassociated plasmid can replicate autonomously from the chromosome to achieve a high copy number. It can also be transferred simultaneously to many recipient bacteria. If the transported DNA encoded a mechanism of antibiotic resistance (Chapter 13) it would not be difficult to imagine how whole populations could rapidly acquire the resistance characteristics.

7 Environmental factors that influence growth and survival

The rate of growth of a microbial population depends on the nature and availability of water and nutrients, temperature, pH, the partial pressure of oxygen and solute concentrations. In many laboratory experiments the microorganisms are provided with an excess of complex organic nutrients and are maintained at optimal pH and temperature. This enables growth to be very rapid and the results visualized within a relatively short time period. Such idealized conditions rarely exist in nature, where microorganisms not only compete with one another for nutrients but also grow under suboptimal conditions. Particular groups of organisms are adapted to survive under particular conditions; thus Gram-negative bacteria tend to be aquatic whereas Gram-positive bacteria tend to prefer more arid conditions such as the skin. The next two sections of this chapter will consider separately the physicochemical factors that affect growth and survival of bacteria, and the availability and nature of the available nutrients.

7.1 Physicochemical factors that affect growth and survival of bacteria

7.1.1 Temperature

Earlier in this chapter various classes of bacteria (thermophile, mesophile, etc.) were described according to the range of temperatures under which they could grow. The majority of bacteria that have medical or pharmaceutical significance are mesophiles and have optimal growth temperatures between ambient and body temperature (37 °C). Individual species of bacteria also have a range of temperatures under which they can actively grow and multiply (permissive temperatures). For every organism there is a minimum temperature below which no growth occurs, an optimum temperature at which growth is most rapid and a maximum temperature above which growth is not possible (Figure 3.9). As temperatures rise, chemical and enzymic reactions within the cell proceed more

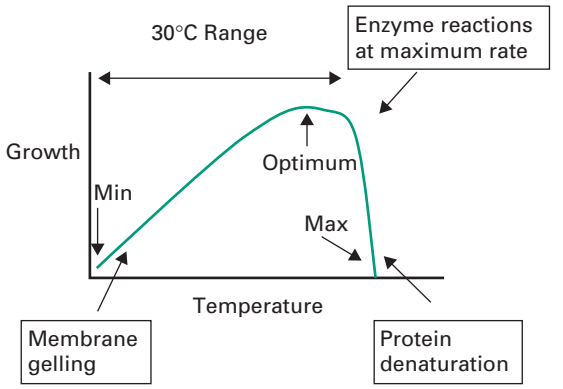

Figure 3.9 The effect of temperature on bacterial growth.

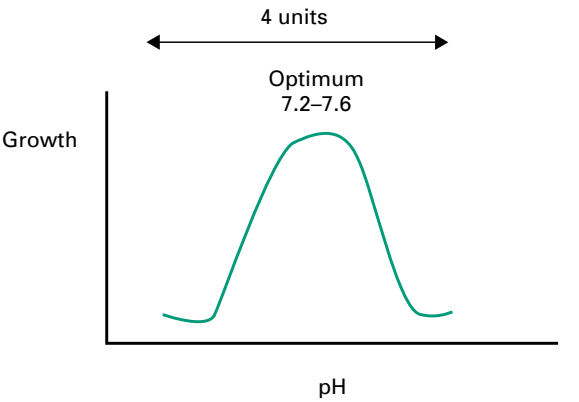

Figure 3.10 The effect of pH on bacterial growth.

rapidly, and growth becomes faster until an optimal rate is achieved. Beyond this temperature certain proteins may become irreversibly damaged through thermal lysis, resulting in a rapid loss of cell viability.

The optimum temperature for growth is much nearer the maximum value than the minimum, and the range of the permissive temperatures can be quite narrow (3–4 °C) for obligate pathogens yet broad (10–20 °C) for environmental isolates, reflecting the range of temperatures that they are likely to encounter in their specialized niches. If the temperature exceeds the permissive range then provided that lethal temperatures are not achieved (*c.*60 °C for most Gram-negative mesophiles) the organisms will survive but not grow. Temperatures of 105 °C and above are rapidly lethal and can be deployed to sterilize materials and products. Generally bacteria are able to survive temperatures beneath the permissive range provided that they are gradually acclimatized to them.

7.1.2 pH

As for temperature, each individual microorganism has an optimal pH for growth and a range about that optimum where growth can occur albeit at a slower pace. Unlike the response to temperature, pH effects on growth are bell-shaped (Figure 3.10), and extremes of pH can be lethal. Generally those microorganisms that have medical or pharmaceutical significance have pH growth optima of between 7.4 and 7.6 but may grow suboptimally at pH values of 5–8.5. Thus growth of lactobacilli within the vaginal vault reduces the pH to approximately 5.5 and prevents the growth of many opportunist pathogens. Accordingly, the pH of a pharmaceutical preparation may dictate the range of microorganisms that could potentially cause its spoilage.

7.1.3 Water activity/solutes

Water is essential for the growth of all known forms of life. Gram-negative bacteria are particularly adapted to an existence in, and are able to extract trace nutrients from, the most dilute environments. This adaptation has its limitations because the Gram-negative cell envelope cannot withstand the high internal osmotic pressures associated with rapid rehydration after desiccation and the organisms are unable to grow in the presence of high concentrations of solute. The availability of water is reflected in the water activity of a material or liquid. Water activity (A_w) is defined as the vapour pressure of water in the space above the material relative to the vapour pressure above pure water at the same temperature and pressure. Pure water by definition has an A_w of 1.00. Pharmaceutical creams might have A_w values of 0.8–0.98, whereas strawberry jam might have an A_w of *c.*0.7. Generally Gram-negative bacteria cannot grow if the A_w is below 0.97, whereas Gram-positive bacteria can grow in materials with A_w of 0.8–0.98 and can survive rehydration after periods of desiccation, hence their dominance in the soil. Yeasts and moulds can grow at low A_w values, hence their appearance on moist bathroom walls and on the surface of jam. The water activity of a pharmaceutical product can markedly affect its vulnerability to spoilage contaminants (see Chapter 17).

7.1.4 Availability of oxygen

For many aerobic microorganisms oxygen acts as the terminal electron acceptor in respiration and is essential for growth. Alternative terminal electron acceptors are organic molecules whose reduction leads to the generation of organic acids such as lactic acid. They can sometimes be utilized under conditions of low oxygen or where

carbon substrate is in excess (fermentation), and highly specialized groups of microorganisms can utilize inorganic materials such as iron as electron acceptors (e.g. iron–sulphur bacteria). Different groups of organisms therefore vary in their dependence on oxygen. Paradoxically, there are many bacteria for which oxygen is highly toxic (*obligate anaerobes*), so the presence or absence of oxygen within a nutrient environment can profoundly affect both the rate and nature of the microbial growth obtained. Strongly oxygen-dependent bacteria will tend to grow as a thin pellicle on the surfaces of liquid media where oxygen is most available. Special media and anaerobic chambers are required to grow obligate anaerobes within the laboratory, yet such organisms persist and actively grow within the general environment. This is because the close proximity of strongly aerobic cells and anaerobes will create an anoxic microenvironment in which the anaerobe can flourish. This is particularly the case for the mouth and gastrointestinal tract where obligate anaerobes such as *Bacteroides* and *Fusobacter* can be found in association with strongly aerobic streptococci.

The inability of oxygen to diffuse adequately into a liquid culture is often the factor that causes an onset of stationary phase, so culture density is limited by oxygen demand. The cell density at stationary phase can often be increased, therefore, by shaking the flask or providing baffles. Diffusion of oxygen may also be a factor limiting the size of bacterial colonies formed on an agar surface.

7.2 Nutrition and growth

Bacteria vary considerably in their requirements for nutrients and in their ability to synthesize for themselves various vitamins and growth factors. Clearly the major elemental requirements for growth will match closely the elemental composition of the bacteria themselves. In this fashion there is a need for the provision of carbon, nitrogen, water, phosphorus, potassium and sulphur with a minor requirement for trace elements such as magnesium, calcium, iron, etc. The most independent classes of bacteria are able to derive much of their nutrition from simple inorganic forms of these elements. These organisms are called *chemolithotrophs* and can even utilize atmospheric carbon dioxide and nitrogen as sources of carbon and nitrogen. Indeed, such bacteria are, in addition to the green plants and algae, a major source of organic molecules and so they are more beneficial than problematic to humans. The majority of bacteria require a fixed carbon source, usually in the form of a sugar, but this may also be obtained from complex organic molecules such as benzene, paraffin waxes and proteins. Nitrogen can generally be obtained from ammonium ions but is also available by deamination of amino acids, which can thus provide both carbon and nitrogen sources simultaneously. Many classes of bacteria are auxotrophic and can grow on simple sugars together with ammonium ions, a source of potassium and trace elements. Such bacteria can synthesize for themselves all the amino acids and ancillary factors required for growth and division. These bacteria, e.g. pseudomonads and *Achromobacter* species, are generally free-living environmental strains but they can sometimes cause infections in immunocompromised people. In the laboratory they can be grown in simple salts media with few, if any, complex supplements. The rate of growth of such organisms depends not only on temperature and pH but also on the nature of the carbon and nitrogen sources. Thus, a faster rate of growth is often obtained when glucose or succinate is the carbon source rather than lactose or glycerol, and when amino acids are provided as sources of nitrogen rather than ammonium salts. If faced with a choice of carbon and nitrogen sources then the bacteria will adapt their physiology to the preferred substrate and only when this is depleted will they turn their attention to the less preferred substrate. Growth in liquid cultures with dual provision of substrate such as this is often characterized by a second lag phase during the logarithmic growth period while this adaptation takes place. This is called *diauxic growth* (Figure 3.11).

As the association between bacteria and higher life forms becomes closer then more and more preformed biosynthetic building blocks become available without the need to synthesize them from their basic elements. Thus a pathogenic organism growing in soft tissues

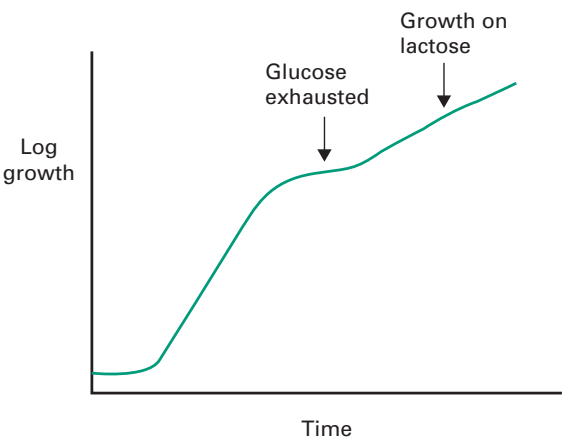

Figure 3.11 Diauxic growth on a mixture of glucose and lactose.

will have available to it glucose and metal ions from the blood and a whole plethora of amino acids, bases, vitamins, etc. from lysed tissue cells. While most bacteria will utilize these when they are available, a number of bacteria that have become specialized pathogens have lost their ability to synthesize many of these chemicals and so cannot grow in situations where the chemicals are not provided in the medium. Consequently many pathogens require complex growth media if they are to be cultured *in vitro*.

All that has been discussed earlier about the physicochemical and nutritional constraints on bacterial growth has been based on laboratory studies. By definition, the only bacteria that we can describe in this way are those that can be cultured artificially. It cannot be overstated that a majority of bacterial species and genera cannot be cultured in the laboratory. In the past the presence of such non-culturable bacteria has been attributed to moribund cells. With the advent of modern molecular tools, however, it has now been realized that these organisms are viable. By amplifying their DNA and sequence mapping, the genetic relationship of such bacteria to the culturable ones can be demonstrated and whole new families of hitherto unrecognized bacteria are being identified. It is possible that in the future many disease states currently thought to have no microbiological involvement could be identified as being of bacterial origin. A recent example of this has been the association of *Helicobacter pylori* with gastric ulcers and gastric cancers.

8 Detection, identification and characterization of organisms of pharmaceutical and medical significance

There are many situations in which microorganisms must not only be detected and enumerated but where they must also be identified either to make a specific diagnosis of infection or to ensure the absence of specified bacteria from certain types of product. In such circumstances various cultural approaches are available that deploy enrichment and selection media. Once a microorganism has been isolated in pure culture, usually from a single colony grown on an agar plate, then further characterization may be made by the application of microscopy together with some relatively simple biochemical tests. Over the last 20 years the biochemical characterization of individual organisms has become simplified by the introduction of rapid identification systems. In recent years molecular approaches have enabled identification of organisms without the need to culture them.

8.1 Culture techniques

Conventional approaches to microbiological examination of specimens require that they be cultured to assess the total numbers of specific groups of microorganisms or to determine the presence or absence of particular named species. The majority of samples taken for examination contain mixtures of different species, so simple plating onto an agar surface may fail to detect an organism that is present at <2% of the total viable population. Various enrichment culture techniques may therefore be deployed to detect trace numbers of particular pathogens, prior to confirmatory identification.

8.1.1 Enumeration

The simplest way in which to enumerate the microorganisms that contaminate an object or liquid sample is to dilute that sample to varying degrees and inoculate the surface of a predried nutrient agar with known volumes of those dilutions (see Chapter 2). Individual viable bacteria that are able to grow on the nutrients provided and under the conditions of incubation will produce visible colonies that can be counted and the numbers related back to the original sample. Such counting procedures are often lengthy and tedious; the number of colonies formed might not relate to the viable number of cells, as clumps of cells will only produce a single colony and they will only detect a particular subset of the viable bacteria present in the sample that can grow under the chosen conditions. Accordingly a variety of different media and cultural conditions are deployed to enumerate different categories of organism.

A number of techniques are currently being developed in order speed up the enumeration process, although some of these rapid enumeration techniques indirectly measure the most probable number of viable cells.

8.1.1.1 Enumeration media

Enumeration media will only ever culture a subset of cells towards which the medium and incubation conditions are directed. Thus, simple salts media with relatively simple sugars as carbon sources and trace levels of amino acids are often used to enumerate bacteria associated with water (e.g. R2A medium). Such plates may be incubated under aerobic or anaerobic conditions at a range of temperatures. Different temperatures will select for different subsets of cells, therefore any description of a viable bacterial count must specify the incubation condi-

tions. In medical microbiology temperatures akin to the human body are often deployed because only those bacteria able to grow at such temperatures are likely to cause infection. However, psychrophilic Gram-negative bacteria (growing in water at 10 °C) can be a major source of bacterial pyrogen, so a variety of incubation temperatures are often used in monitoring pharmaceutical waters and products. Highly nutritious media, e.g. blood agar, are also used as enumeration media. This is particularly the case when looking for microorganisms such as staphylococci that are usually found in association with animals and humans. Such agar plates may be deliberately exposed to air (settle plates) and the number of colonies formed related to the bacteria content of a room. In the pharmaceutical industry microbiological monitoring will generally report the total aerobic count and, less commonly, the total anaerobic counts obtained on a moderately rich medium such as tryptone soya agar. Sometimes inhibitors of bacterial growth (e.g. Rose Bengal) can be added to a medium in order to select for moulds.

8.1.1.2 Rapid enumeration techniques

The detection and quantification of components of bacterial cells is considerably faster than those approaches requiring the growth of colonies, and estimates of total viable cell number can thereby be obtained within minutes rather than hours and days.

Some of the rapid methods that have been used for bacteria and other microorganisms, e.g. bioluminescence, epifluorescence and impedance techniques, have been described in Chapter 2, but there are other rapid methods that have found more limited application; these will be considered here. In the examination of pharmaceutical waters and aqueous pharmaceutical products electronic particle counters, e.g. Coulter counters, can be used to determine bacterial concentration, although these instruments do not discriminate between living and dead cells. Similar counters are available that are able to analyse particles found in air. Other rapid techniques aim to detect microbial growth rather than to visualize individual cells and colonies. As bacteria grow in liquid culture they not only alter the conductivity of the culture (see Chapter 2), they also generate small quantities of heat. The time taken to detect this heat can be directly related to the numbers of viable cells present by means of microcalorimeters. Once again this is a considerable improvement over conventional culture, but unlike particle counting and bioluminescence can only detect those organisms that are able to grow in the chosen medium.

None of the rapid techniques are able to isolate individual organisms. They do not therefore aid in the characterization or identification of the contaminants.

8.1.2 Enrichment culture

Enrichment cultures are intended to increase the dominance of a numerically minor component of a mixed culture such that it can be readily detected on an agar plate. Enrichment media are always liquid and are intended to provide conditions that are favourable for the growth of the desired organism and unfavourable for the growth of other likely isolates. This can be achieved either through manipulation of the pH and tonicity of the medium or by the inclusion of chemicals that inhibit the growth of unwanted species. Thus, MacConkey broth contains bile salts that will inhibit the growth of non-enteric bacteria and may be used to enrich for Enterobacteriaceae. Several serial passages through enrichment broths may be made, and after enrichment it is not possible to relate the numbers of organisms detected back to that in the original sample.

8.1.3 Selective media

Selective media are solidified enrichment broths, so again they are intended to suppress the growth of particular groups of bacteria and to allow the growth of others. The methods of creating this situation are the same as for enrichment broths. Thus mannitol salts agar will favour the growth of micrococci and staphylococci, and cetrimide agar will favour the growth of pseudomonads. The use of selective media is an adjunct to characterizing the nature of contaminants. Counts of colonies obtained on selective solid media are often documented as presumptive counts, so for example, colonies formed on a MacConkey agar (containing bile salts) might be cited as a presumptive coliform count.

8.1.4 Identification media (diagnostic)

Identification media contain nutrients and reagents that indicate, usually through some form of colour formation, the presence of particular organisms. This enables them to be easily detected against a background of other species. In this fashion inclusion of lactose sugar and a pH indicator into MacConkey agar facilitates the identification of colonies of bacteria that can ferment lactose. Fermentation leads to a reduction in pH within these colonies and can be detected by an acid shift in the pH indicator, usually to red. Lactose-fermenting coliforms (*Escherichia* spp., *Klebsiella* spp.) can therefore be easily distinguished from non-fermentative coliforms

(*Salmonella* spp., *Shigella* spp.). Similarly, the inclusion of egg-yolk lecithin into an agar gives it a cloudy appearance that clears around colonies of organisms that produce lecithinase (a virulence factor in staphylococci). While there are numerous types of selective and diagnostic media available, they can only be used as a guide to identification, but microscopy and biochemical or genetic characterization are much more definitive.

8.2 Microscopy

Observation of stained and wet preparations of clinical specimens (blood, pus, sputum) and isolated pure cultures of bacteria from the manufacturing environment provides rapid and essential information to guide further identification. The application of simple stains such as the Gram stain can divide the various genera of bacteria into two convenient broad groups. The size and shapes of individual cells and their arrangement into clusters, chains and tetrads will also guide identification, as will specific stains for the presence of endospores, capsules, flagella and inclusion bodies. Examination of wet preparations can give an indication as to the motility status of the isolate, and these procedures all represent an important first stage in the identification process.

8.3 Biochemical testing and rapid identification

The differing ability of bacteria to ferment sugars, glycosides and polyhydric alcohols is widely used to differentiate the Enterobacteriaceae and in diagnostic bacteriology generally. Fermentation can be indicated by pH changes in the medium with or without gas production visualized by the collection of bubbles in inverted tubes. More specialized media examine the ability of certain strains to oxidize or reduce particular substrates. There are many hundreds of individual biochemical tests available that each separately seek the presence of a particular enzyme or physiological activity. Taxonomic studies have led to the recognition that certain of these tests in combination characterize particular species of bacteria. Various manuals such as *Bergey's Manual* (Holt, 1994) and *Cowan and Steel's Manual for the Identification of Medically Important Bacteria* (Barrow & Feltham, 2004) provide a logical and sequential framework for the conduct of such tests. Identification of particular species and genera by such processes is time-consuming, expensive and may require numerous media and reagents.

This process has become simplified in recent years by the development of rapid identification methods and kits. The latter often use multiwell microtitration plates that can be inoculated in a single operation either with an inoculated wire or with a suspension of a pure culture. Each individual well contains the medium and reagents for the conduct of a single biochemical test. Identification kits vary in their complexity and also in the precision of the identification made. Simple kits may perform only 8–15 tests, more complex ones are capable of performing 96 simultaneous biochemical evaluations. Scoring of each test and entry into a computer database then allows the pattern of test results to be compared with a large panel of organisms and a probability of identity calculated. As different sets of tests will be required for different classes of bacteria, guidance as to the initial choice of kit is given on the basis of the Gram stain reaction, and the results of oxidase and catalase tests performed directly on isolated colonies. In large diagnostic laboratories and in quality assurance laboratories automated systems are deployed that can inoculate, incubate and analyse hundreds of individual samples at a time.

8.4 Molecular approaches to identification

The need to identify microorganisms rapidly has led to the development of a number of molecular identification and characterization tools. These have not yet become routinely adopted in the analytical or diagnostic laboratory but probably will be in the future. One such technique (denaturing gradient gel electrophoresis; DGGE) isolates and amplifies 16S ribosomal DNA and, following sequencing of the bases, compares this with known sequences held in a reference library. This approach enables phylogenetic relationships to be derived even for those bacteria that have not previously been identified. Other systems examine the patterns of key constituents of the cells such as fatty acids and assign identities based on similarity matches to known reference cultures.

Molecular approaches can be of especially useful when attempting to detect a particular species. Thus, gene probes carrying fluorescent dyes can be used in hybridization procedures with the collected clinical material. Examination under the fluorescent microscope will show the targeted organism as fluorescent against a background of non-fluorescent organisms.

8.5 Pharmaceutically and medically relevant microorganisms

Microorganisms of medical and pharmaceutical relevance can be broadly classified into those organisms that

are harmful or problematic, and those that can be used to our advantage. Some microorganisms, depending on the situation, can fall into both categories. Microorganisms cause some of the most important diseases of humans and animals and they can also be found as major contaminants of pharmaceutical products. On the other hand, many large-scale industrial processes, e.g. antibiotic production, are based on microorganisms, and selected species can be used to test disinfectant efficacy and to monitor sterilization procedures. Tables 3.3 and 3.4 respectively, list examples of some of the more pharmaceutically relevant beneficial and problematic microorganisms. Specific texts should be referred to for more detailed descriptions.

Table 3.3 Examples of some pharmaceutically useful bacteria

Organism	Characteristics	Pharmaceutical relevance
Actinomyces spp.	Gram-positive, filamentous rods	Antibiotic production
Bacillus atrophaeus (formerly *Bacillus subtilis)*	Gram-positive rod, aerobic, spore-former	Used to validate and monitor dry heat and ethylene oxide sterilization processes
Bacillus pumilus	Gram-positive rod, aerobic, spore-former	Used to validate and monitor radiation sterilization processes
Bordetella pertussis	Gram-negative rod, aerobe	Vaccine against whooping cough
Brevundimonas (formerly *Pseudomonas*) *diminuta*	Gram-negative, microaerobic rod	0.22 µm filter challenge test
Clostridium sporogenes	Gram-positive rod, anaerobe, spore-former	Used to confirm anaerobic growth conditions
Clostridium tetani	Gram-positive rod, anaerobe, spore-former	Vaccine against tetanus
Corynebacterium diphtheriae	Gram-positive rod, aerobe	Vaccine against diphtheria
Escherichia coli	Gram-negative enteric rod, facultative anaerobe	Kelsey–Sykes disinfectant capacity test
		Preservative limit test
Geobacillus stearothermophilus (formerly *Bacillus stearothermophilus*)	Gram-positive rod, aerobic, spore-former	Used to validate and monitor moist heat sterilization processes
Haemophilus influenzae type b	Gram-negative rod, aerobe	Vaccine against Hib infections
Leuconostoc mesenteroides	Gram-positive rod	Dextran production
Neisseria meningitidis	Gram-negative cocci, aerobic	Vaccine against meningitis C
Pseudomonas aeruginosa	Gram-negative, microaerobic rod	Alginate production
		Kelsey–Sykes disinfectant capacity test
Proteus vulgaris	Gram-negative, aerobic rod	Kelsey–Sykes disinfectant capacity test
Salmonella enterica serovar Typhi	Gram-negative enteric rod, facultative anaerobe	Chick Martin/Rideal Walker disinfectant coefficient test
Staphylococcus aureus	Gram-positive, aerobic cocci	Kelsey-Sykes disinfectant capacity test
		Preservative limit test

Table 3.4 Examples of some pharmaceutically problematic bacteria

Organism	Characteristics	Pharmaceutical relevance
Bacteroides fragilis	Gram-negative enteric rod, anaerobe	Wound infections
Bordetella pertussis	Gram-negative rod, aerobe	Causative agent of whooping cough
Campylobacter jejuni	Gram-negative enteric spiral rod, microaerophilic	Severe enteritis
Clostridium tetani	Gram-positive rod, anaerobe, spore-former	Causative agent of tetanus
Corynebacterium diphtheriae	Gram-positive rod, aerobe	Causative agent of diphtheria
Escherichia coli	Gram-negative enteric rod, facultative anaerobe	Food poisoning, severe enteritis
Haemophilus influenzae	Gram-negative rod, aerobe	Causative agent of infantile meningitis and chronic bronchitis
Legionella pneumophila	Gram-negative rod, aerobic	Causative agent of Legionnaire's disease
Mycobacterium tuberculosis	Gram-positive, acid-fast rod, aerobe	Causative agent of tuberculosis
		Disinfectant resistance
		Intracellular pathogen
Pseudomonas aeruginosa	Gram-negative, microaerobic rod	General environmental contaminant
		Quintessential opportunist pathogen
		High resistance to antibiotics and biocides
		Biofilm-former
Salmonella spp.	Gram-negative enteric rods, facultative anaerobes	Varying degrees of food poisoning, typhoid fever
Staphylococcus aureus	Gram-positive, aerobic, catalase-positive cocci	Skin contaminant
		Food poisoning
		Toxic shock syndrome
		Pyogenic infections
Staphylococcus epidermidis	Gram-positive, aerobic, catalase-positive cocci	Implanted medical device/prosthetic device contaminant
		Biofilm-former
Streptococcus spp.	Gram-positive, aerobic, catalase-negative cocci	Causative agents of tonsilitis and scarlet fever

9 References and further reading

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4 Fungi

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1 What are fungi?

Yeast, such as brewers' yeast, and moulds, such as *Penicillium chrysogenum* which produces the antibiotic penicillin, are classified as fungi. Yeast cells tend to grow as single cells which reproduce asexually in a process known as *budding*, although a minority of species (e.g. *Schizosaccharomyces pombe*) reproduce by fission. Many yeast species are capable of sexual reproduction and the formation of spores. In contrast, moulds grow as masses of overlapping and interlinking hyphal filaments and reproduce by producing masses of spores in a variety of structures. This division between yeast and moulds based on growth morphology is not clear-cut since some yeast can produce hyphae under specific conditions (e.g. *Candida albicans*) while many normally filamentous fungi possess a yeast-like phase at some point in their life cycle. Fungi are eukaryotic organisms, i.e. their cells possess a nuclear membrane, consequently there are many similarities between the biochemistry of fungal cells and vertebrate (human) cells. Fungi are widely distributed in nature, occurring as part of the normal flora on the body of warm-blooded animals, as decomposers of organic matter and as animal and plant pathogens. Medically, fungi are an extremely important group of microbes, being responsible for a number of potentially fatal diseases in humans (Table 4.1), but a significant number of fungi are of great benefit to humanity in terms of the production of alcoholic beverages, bread, enzymes, antibiotics and recombinant proteins (Table 4.2). Fungi have also been utilized for a range of molecular biological applications.

Fungal taxonomy is extremely difficult and there is much discussion as to the number and inter-relatedness of fungal classes. Using one classification system, the kingdom Fungi can be subdivided into six classes. The class Oomycetes contains the mildews and water moulds, the class Ascomycetes contains the mildews, some moulds and most yeast species (including *Saccharomyces cerevisiae*), the class Basidiomycetes contains the mushrooms and bracket fungi, the class Teliomycetes contains the rust fungi (plant pathogens), the class Ustomycetes contains the smuts (plant pathogens) and the class Deuteromycetes contains species such as *Aspergillus*, *Fusarium* and *Penicillium* (see Figure 4.1). In contrast, phylogenetic

Hugo and Russell's Pharmaceutical Microbiology, Eighth Edition. Edited by Stephen P. Denyer, Norman Hodges, Sean P. Gorman, Brendan F. Gilmore.

Table 4.1 Examples of fungal diseases and selected causative agents

Type of mycosis	Disease	Species name
Superficial	Pityriasis versicolor	*Malassezia furfur*
	White piedra	*Trichosporon beigelii*
Cutaneous	Tinea pedis (athlete's foot)	*Trichophyton rubrum*
	Onychomycosis (nail infection)	*Trichophyton rubrum*
	Tinea capitis (scalp ringworm)	*Trichophyton tonsurans*
Subcutaneous	Chromoblastomycosis	*Fonsecaea pedrosoi*
	Mycetoma	*Acremonium* spp.
Systemic	Blastomycosis	*Blastomyces dermatitidis*
	Histoplasmosis	*Histoplasma capsulatum*
	Coccidioidomycosis	*Coccidioides immitis*
	Paracoccidioidomycosis	*Paracoccidioides brasiliensis*
Opportunistic	Candidosis (superficial/systemic)	*Candida albicans* *Candida glabrata* *Candida parapsilosis*
	Aspergillosis	*Aspergillus fumigatus*
	Pneumonia	*Pneumocystis jirovecii (carinii)*

analysis has revealed that there are four distinct phyla within the fungal kingdom; these are the Chytridiomycota, Zygomycota, Ascomycota and Basidiomycota.

2 Structure of the fungal cell

The typical yeast cell is oval in shape and is surrounded by a rigid cell wall which contains a number of structural polysaccharides and may account for up to 25% of the dry weight of the cell wall (see Figure 4.2). Glucan accounts for 50–60%, mannan for 15–23% and chitin for 1–9% of the dry weight of the wall, respectively, with protein and lipids also present in smaller amounts. The thickness of the cell wall may vary during the life of the cell but the average thickness in the yeast *C. albicans* varies from 100 to 300 nm. Glucan, the main structural component of the fungal cell wall, is a branched polymer of glucose which exists in three forms in the cell: β-1,6-glucan, β-1,3-glucan and β-1,3,-β-1,6-complexed with chitin. Mannan is a polymer of the sugar mannose and is found in the outer layers of the cell wall. The third principal structural component, chitin, is concentrated in bud scars that are areas of the cell from which a bud has detached. Proteins and lipids are also present in the cell wall and under some conditions may represent up to 30% of the cell wall contents. Mannoproteins form a fibrillar layer that radiates from an internal skeletal layer that is formed by the polysaccharide component of the cell wall. The innermost layer is rich in glucan and chitin which provides rigidity to the wall and is important in regulating cell division.

Enzymatic or mechanical removal of the cell wall leaves an osmotically fragile protoplast which will burst if not maintained in an osmotically stabilized environment. Incubation of protoplasts in an osmotically stabilized agar growth medium will allow the resynthesis of the wall and the resumption of normal cellular functions. The ability to generate fungal protoplasts opens the possibility of fusing these under defined conditions to generate strains with novel biotechnological applications.

The periplasmic space is a thin region that lies directly below the cell wall. It contains secreted proteins that do not penetrate the cell wall and is the location for a number of enzymes required for processing nutrients prior to entry into the cell. The cell membrane or plasmalemma is located directly below the periplasmic space and is a phopholipid bilayer which contains phospholipids, lipids, protein and sterols. The plasmalemma is

Table 4.2 Examples of economically important fungi

Fungal species	Application
Filamentous fungi	
Agaricus bisporus	Edible mushroom
Aspergillus, Penicillium spp.	Enzymes (catalase, lipase, amylase)
Aspergillus sp. + *Saccharomyces* sp.	Sake (rice wine)
Fusarium graminearum	Single cell protein
Penicillium chrysogenum	Penicillin production
Penicillium notatum	Enzyme (glucose oxidase)
Penicillium roqueforti	Cheese flavouring (Roqueforti 'blue' cheese)
Yeast	
Pichia sp.	Gene expression system
Saccharomyces cerevisiae	Bakers' yeast—bread
	Brewers' yeast—beer, wine, cider, etc.
	Enzyme (invertase)
	Gene expression system
	Dietary supplement

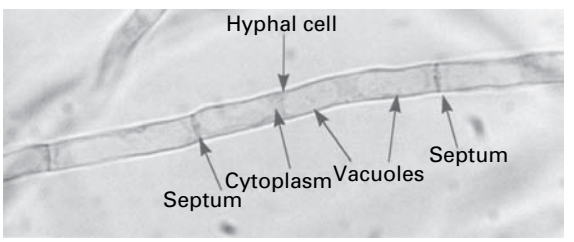


Figure 4.1 Septate hyphae of *Aspergillus fumigatus*.

approximately 10 nm thick and in addition to being composed of phospholipids also contains globular proteins. The dominant sterol in fungal cell membranes is ergosterol which is the target of the antifungal agent amphotericin B. Sterols are important components of the plasmalemma and represent regions of rigidity in the fluidity provided by the phospholipid bilayer.

Most of the cell's genome is concentrated in the nucleus which is surrounded by a nuclear membrane which contains pores to allow communication with the rest of the cell (see Figure 4.2). The nucleus is a discrete organelle and, in addition to being the repository of the DNA, also contains proteins in the form of histones. Yeast chromosomes vary in size from 0.2 to 6 Mb and the number per yeast is also variable with *S. cerevisiae* having as many as 16 while the fission yeast *Sch. pombe* has as few as 3. In addition to the genetic material in the nucleus the yeast cell often has extrachromosomal information in the form of plasmids. For example, the 2 µm plasmid is present in *S. cerevisiae,* although its function is unclear, and there are killer plasmids in the yeast *Kluyveromyces lactis* which encode a toxin.

Actively respiring fungal cells possess a distinct mitochondrion which has been described as the 'powerhouse' of the cell (Figure 4.2). The enzymes of the tricarboxylic acid cycle (Krebs' cycle) are located in the matrix of the mitochondrion while electron transport and oxidative phosphorylation occur in the mitochondrial inner membrane. The outer membrane contains enzymes involved in lipid biosynthesis. The mitochondrion is a semi-independent organelle as it possesses its own DNA and is capable of producing its own proteins on its own ribosomes which are referred to as mitoribosomes.

The fungal cell contains a vast number of ribosomes which are usually present in the form of polysomes—lines of ribosomes strung together by a strand of mRNA. Ribosomes are the site of protein biosynthesis. The system which mediates the export of proteins from the cell involves a number of membranous compartments including the Golgi apparatus, the endoplasmic reticulum and the plasmalemma. In addition, the vacuole is employed as a 'storage space' where nutrients, hydrolytic enzymes or metabolic intermediates are retained until required.

3 Medical significance of fungi

Fungi represent a significant group of pathogens capable of causing a range of diseases in humans under the right set of conditions. Although the majority of fungi appear to be harmless to humans it is worth bearing in mind that a normally non-pathogenic fungus can cause a clinically relevant problem if the immune system is suppressed as a result of therapy (e.g. for receipt of organ transplant) or disease (e.g. cancer). In a case of profound immuno-

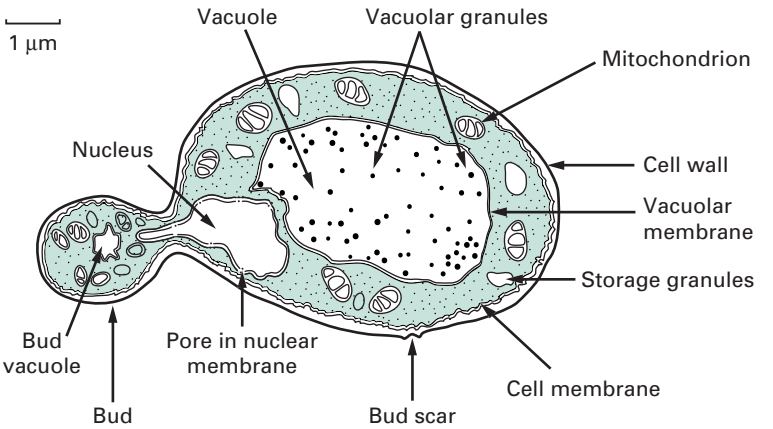


Figure 4.2 Diagrammatic representation of 'typical' fungal cell.

compromise a wide range of fungi can present as capable of inducing disease.

The most common fungal pathogens of humans can be divided into three broad classes: yeasts, moulds and dermatophytes. The yeast *C. albicans* is the most frequently encountered human fungal pathogen, being responsible for a wide range of superficial and systemic infections. The superficial infections include oropharyngeal and genital conditions, the former occurs predominantly in HIV-positive individuals, geriatric patients and premature infants and may arise when a weakened or immature immune system is present. Genital candidosis is very common and approximately 75% of women are affected by vulvovaginal candidosis (VVC) during their life with a further 5–12% suffering from recurring bouts of infection over a prolonged period of time.

The mould *Aspergillus fumigatus* is the dominant fungal pulmonary pathogen of humans and generally presents as a problem in those with pre-existing lung disease or damage. In addition to pulmonary infection other sites may be affected including the brain, kidneys and sinuses depending upon the level of immunocompromise of the individual. Groups particularly susceptible to colonization by *Aspergillus* species include those with cavities due to tuberculosis, patients affected with asthma or cystic fibrosis and those with profound immunosuppression due to leukaemia (neutropenia). Aspergillosis presents as a serious problem in patients immunosuppressed in advance of organ transplantation.

Dermatophyte is the term applied to a range of fungi capable of colonizing the skin, nails or hair. The principal dermatophytic fungi are *Trichophyton*, *Microsporum* and *Epidermophyton* species. The most commonly encountered dermatophytic infections are athlete's foot (infection of the foot) and ringworm (fungal infection of the scalp or skin).

4 Antifungal therapy

The choice and dose of an antifungal will depend upon the nature of the condition, whether there are any underlying diseases, the health of the patient and whether antifungal resistance has been identified as compromising therapy. Part of the difficulty in designing effective antifungal agents lies in the fact that fungi are eukaryotic organisms so agents that will kill fungi may also have a deleterious effect on human tissue. The ideal antifungal drug should target a pathway or process specific to the fungal cell, so reducing the possibility of damaging tissue and inducing unwanted side effects.

4.1 Polyene antifungals

Polyene antifungals are characterized by having a large macrolide ring of carbon atoms closed by the formation of an internal ester or lactone (Figure 4.3). In addition, polyenes have a large number of hydroxyl groups distributed along the macrolide ring on alternate carbon atoms. This combination of highly polar and non-polar regions within the molecule renders the polyenes amphiphatic, i.e. having hydrophobic and hydrophilic regions in the one molecule, which assists solubility in lipid membranes.

The principal polyenes are amphotericin B and nystatin. Amphotericin B is produced by the bacterium

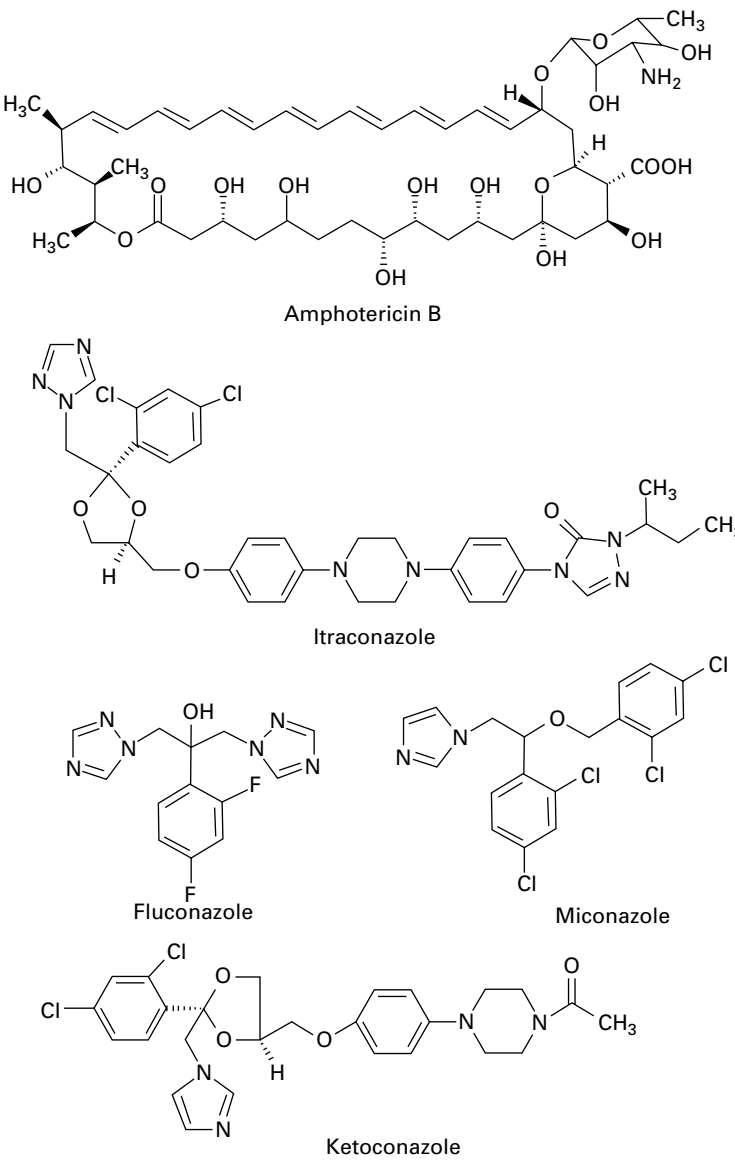


Figure 4.3 Structures of polyene (amphotericin B) and azole (itraconazole, fluconazole, miconazole and ketoconazole) antifungal agents.

Streptomyces nodosus and its activity is due to the ability to bind ergosterol, which is the dominant sterol in fungal cell membranes, and consequently increases membrane permeability by the formation of pores (Figure 4.4). The action of amphotericin B seems to rely on the formation of pores through which intracellular contents can escape from the cell. Amphotericin B can lead to renal damage during prolonged antifungal therapy. Amphotericin B is active against a broad range of fungal pathogens and is considered the 'gold standard' against which the activity of other antifungal agents is measured. Because of its renal toxicity amphotericin B tends to be reserved for severe cases of systemic fungal disease but recent formulations in which the drug is encapsulated within liposomes have been shown to have reduced toxicity.

Nystatin was discovered in 1950 and exhibits the same mode of action as amphotericin B but tends to have lower solubility, which has restricted its use to the treatment of topical infections. Although nystatin was effective for the

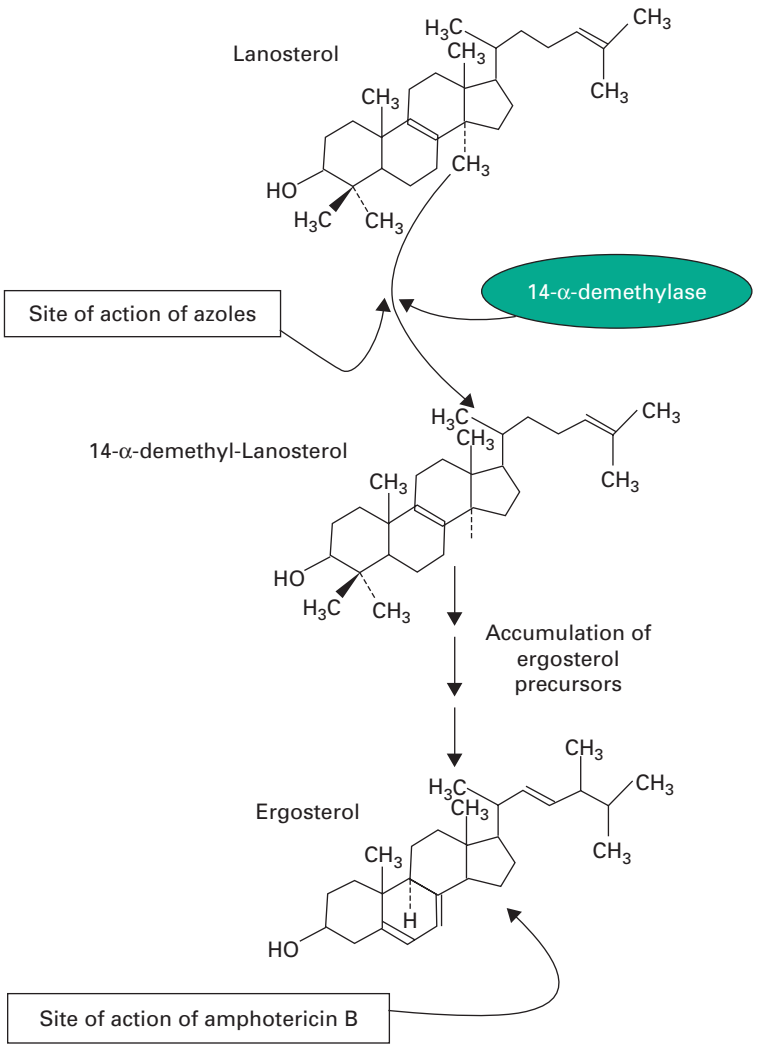


Figure 4.4 Modes of action of polyene (amphotericin B) and azole antifungal agents.

treatment of conditions such as oral and vaginal candidosis, its use has been overtaken by the introduction of azole antifungal drugs.

4.2 Azole antifungals

The first generation of azole antifungals revolutionized the treatment of mucosal and invasive fungal infections, and azoles are still the most widely used group of antifungal agents. The azole derivatives are classified as imidazoles or triazoles on the basis of whether they have two or three nitrogen atoms in the five-membered azole ring (Figure 4.3). The azoles in current clinical use are clotrimazole, miconazole, econazole and ketoconazole; newer drugs such as itraconazole, fluconazole and voriconazole have important applications in the treatment of systemic infections. Azoles function by interfering with ergosterol biosynthesis by binding to the cytochrome P-450 mediated enzyme known as 14-α-demethylase (P-450$_{DM}$). This blocks the formation of ergosterol by preventing the methylation of lanosterol (a precursor of ergosterol) (Figure 4.4). This result in a reduction in the amount of ergosterol in the fungal cell membrane which leads to membrane instability, growth inhibition and cell death. An additional consequence of the block in ergosterol biosynthesis is the build-up of toxic intermediates which can prove fatal to the fungal cell.

Azoles exhibit a broad spectrum of activity *in vitro*, being capable of inhibiting the growth of most *Candida*, *Cryptococcus* and *Aspergillus* species, and dermatophytes. Miconazole was the first azole used to treat systemic fungal infections but demonstrated a number of toxic side effects. Ketoconazole produced high serum concentrations upon oral administration but had poor activity against aspergillosis. In addition, ketoconazole was associated with a range of side effects which limited its applicability. Newer triazoles such as fluconazole and itraconazole have increased the options for dealing with fungal infections. Fluconazole was introduced for clinical use in 1990, is water soluble and shows good penetration and deposition into the pulmonary tissues; it also reaches high levels in the cerebrospinal fluid and the peritoneal fluids. Fluconazole has proved highly effective in the treatment of infections caused by *C. albicans* but shows limited activity against *Aspergillus*. Itraconazole became available for clinical use in the late 1980s and was the first azole with proven efficacy against *Aspergillus*. Itraconazole is effective in treating severe *Aspergillus* infections and exhibits both fungicidal and fungistatic effects. Upon ingestion itraconazole undergoes extensive hepatic metabolism which yields up to 30 metabolites, a number of which retain antifungal activity. Itraconazole is currently available as an intravenous formulation and is widely used for the treatment of severe *Aspergillus* infection in this form. Fluconazole and itraconazole demonstrate significantly reduced side effects compared to ketoconazole. Novel azole drugs with increased ability to inhibit the fungal 14-α demethylase are also becoming available. These agents, which include voriconazole, posaconazole and ravuconazole, have a wider spectrum of activity than fluconazole and it has been suggested that some of them show fungicidal effects to some species (e.g. *Aspergillus* spp.). Voriconazole is one of the newest second-generation triazole antifungal drugs and it shows good activity against pulmonary aspergillosis and cerebral aspergillosis.

4.3 Echinocandins

The echinocandins are a relatively new group of antifungal drug and are semisynthetic lipopeptides comprising a cyclic hexapeptide core connected to a lateral fatty acid chain. Three compounds of this group are currently in use: caspofungin, micafungin and anidulafungin (Figure 4.5). Unlike conventional antifungal therapy that targets ergosterol or its synthesis, the echinocandins target the synthesis of β-1,3-glucan, the major polymer of the fungal cell wall. The cell wall is essential to the fungus as

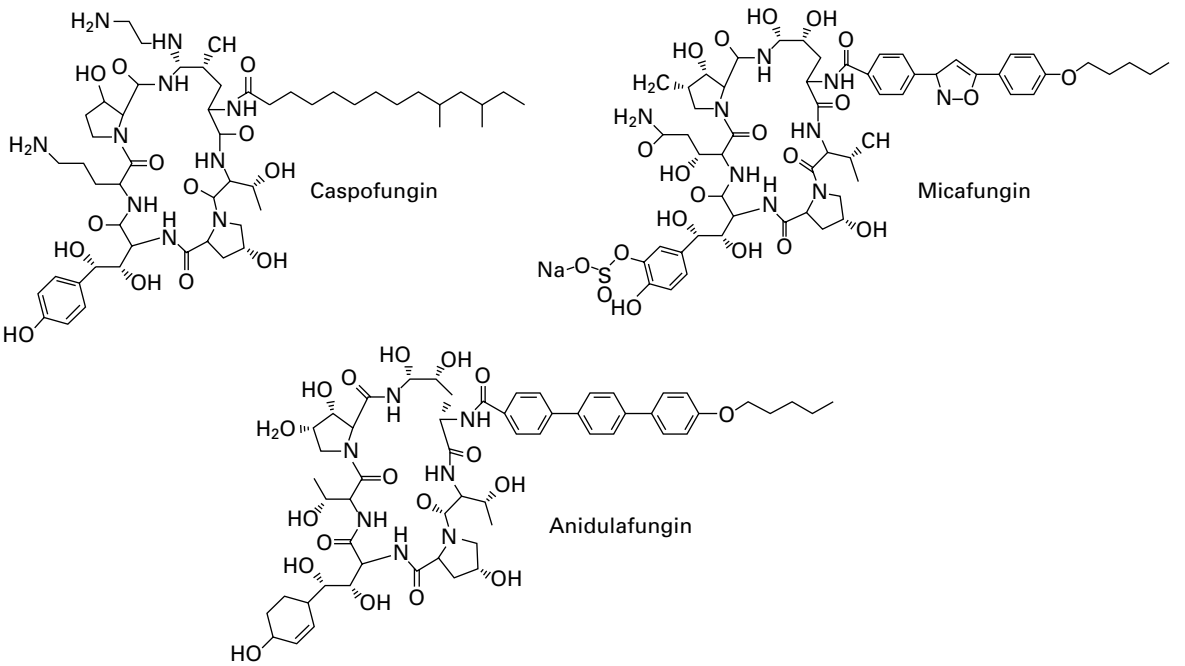


Figure 4.5 Structure of echinocandins.

it provides physical protection, maintains osmotic stability, regulates cell shape, acts as a scaffold for proteins, mediates cell–cell communication and is the site of a number of enzymatic reactions. Inhibition of β-1,3-glucan synthesis disrupts the structure of the growing cell wall, resulting in osmotic instability and ballooning out of the intracellular contents as a result of high osmotic pressure, and ultimately ends in cell lysis.

Caspofungin has demonstrated *in vitro* antifungal activity against various filamentous fungi and yeasts. It has activity against different *Aspergillus* species including *A. fumigatus*, *A. flavus*, *A. niger* and *A. terreus* but is considered to be more fungistatic than fungicidal. Conversely, caspofungin is particularly fungicidal against a range of *Candida* species including species that are resistant (e.g. *C. krusei*) or isolates that are less susceptible (e.g. C. *dubliniensis*, *C. glabrata*) to azoles, or resistant to amphotericin B.

The fungal cell wall represents an attractive target and the echinocandins have proven to offer a safer alternative to conventional antifungal therapies. (i.e. polyenes and azoles). Echinocandins display an unique mode of action which results in defects in cell wall morphology and osmotic instability. As the cell wall is an essential component for stability and ultimately virulence, the targeting of the wall by echinocandins results in the efficient destruction of the fungal cell. To avoid future problems with resistance, researches need to clarify the precise interactions of the echinocandins with the target enzyme, and fully examine the cell's complex response to this agent.

4.4 Synthetic antifungal agents

Flucytosine is a synthetic fluorinated pyrimidine which has been used as an oral antifungal agent and demonstrates good activity against a range of yeast species and moderate levels of activity against *Aspergillus* species. Two modes of action have been proposed for flucytosine. One involves the disruption of protein synthesis by the inhibition of DNA synthesis while the other possible mode of action is the depletion in the amino acid pools within the cell as a result of inhibition of protein synthesis. In general yeast cells increase in size when exposed to levels of flucytosine lower than the minimum inhibitory concentration (MIC) and display alterations in their surface morphology, both of which can be interpreted as a result of an imbalance in the control of cellular growth. Many fungi are inherently resistant to flucytosine or develop resistance after a relatively short exposure and resistance has been attributed to alteration in the enzyme (cytosine deaminase) required to process flucytosine once inside the cell or to an elevation in the amount of pyrimidine synthesis. The problem of resistance has limited the use of flucytosine so that now it is generally used in combination with an antifungal agent (e.g. amphotericin B) where it can potentiate the effect of the second agent.

5 Medically important fungal pathogens of humans

5.1 *Candida albicans*

The yeast *C. albicans* is an opportunistic fungal pathogen which can be present as a normal part of the body's microflora. *C. albicans* is also responsible for a range of systemic infections and many of these can start as superficial infections. Infection of the gastrointestinal tract is seen in diabetics, cancer patients and people with AIDS; the oesophagus is a common site of infection, rendering swallowing difficult. The urinary tract can also be the site of candidosis which may be due to renal infection, other underlying disease(s) or cystitis. The presence of an indwelling urinary catheter may also predispose to *Candida* infection.

A range of factors are capable of predisposing the individual to superficial or systemic candidal infections. Factors which impair the host's immune system such as the presence of underlying disease (AIDS, cancer, diabetes), the use of immunosuppressive therapy during organ transplantation and broad-spectrum antibiotic therapy can leave the individual susceptible to candidosis. Other factors that may predispose to *Candida* infection include the presence of indwelling catheters and skin damage as a result of burns or other trauma.

C. albicans displays a variety of virulence factors which aid colonization and persistence in the body. One of the most important of these factors is the ability to adhere to host tissue using a variety of mechanisms (Figure 4.6A). The importance of adherence may be illustrated by the ability of *C. albicans* to adhere to various mucosal surfaces and to withstand forces that may lead to its removal from the body, such as the bathing/washing action of body fluids. A hierarchy exists among *Candida* species indicating that the more common etiological agents of candidosis (*C. albicans* and C*andida tropicalis*) are more adherent to host tissue *in vitro* than relatively non-pathogenic species such as *C. krusei* and *Candida guilliermondii*.

C. albicans can exist in two morphologically distinct forms: budding blastospores or hyphae (Figure 4.6B).

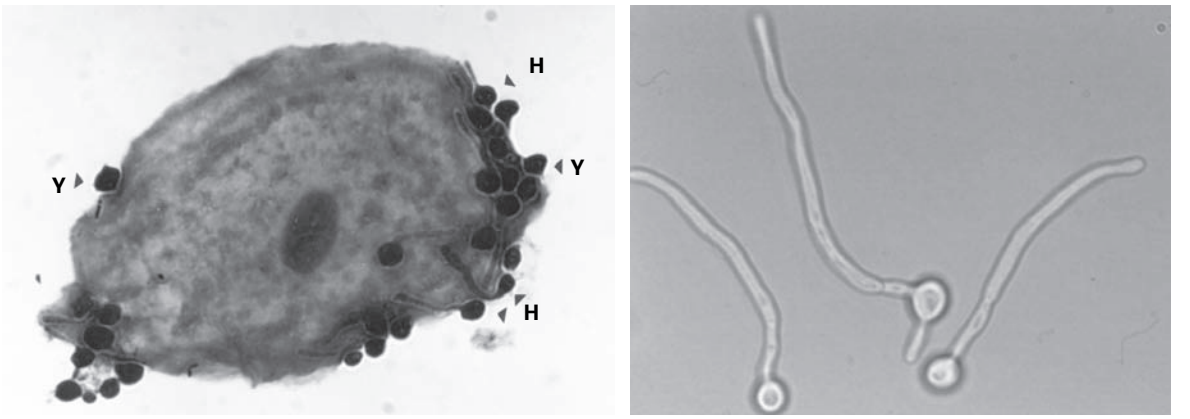


Figure 4.6 (A) Cells of *C. albicans* (arrows) adhering to a human buccal epithelial cell. (B) Germ tubes of *C. albicans*. Y, yeast cells; H, hyphal cells.

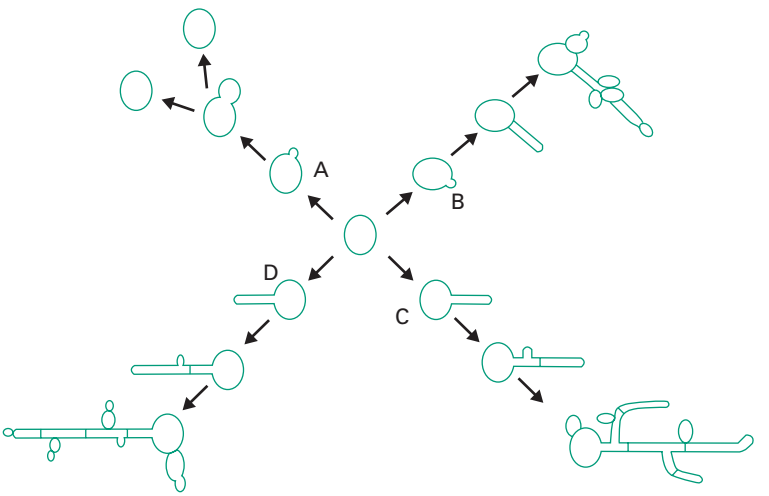


Figure 4.7 Growth morphologies of *Candida albicans*: A, budding morphology; B, hyphal formation; C, germ-tube formation leading to hyphal formation; D, pseudohyphal formation.

The yeast can switch between each form and is usually encountered in tissue samples in both morphological forms (Figure 4.7). The hyphae are capable of thigmotropism (contact sensing) which may aid in finding the line of least resistance between and through layers of cells in tissue. *C. albicans* produces a range of extracellular enzymes which facilitate adherence and/or tissue penetration. Phospholipases A, B, C and lysophospholipase may function to damage host cell membranes and facilitate invasion. *C. albicans* produces a range of acid proteinases which have been shown to aid adherence and invasion but which also play an important role in the degradation of the immunoglobulins IgG and IgA. There are now known to be 10 members of the secreted aspartic proteinase (SAP) family and these have a low pH optimum which may assist in the colonization of the vagina. Haemolysin production by *C. albicans* has also been documented and seems to be important in allowing the yeast access iron released from ruptured red blood cells. An important immune evasion tactic of *C. albicans* is the ability to bind to platelets via fibrinogen binding ligands which results in the fungal cell being surrounded by a cluster of platelets.

C. albicans is capable of giving rise to a variety of interconvertible phenotypes which can be considered as providing an extra dimension to the existing virulence factors associated with this yeast. A number of switching systems have been identified and phenotypic switching

may have evolved to compensate for the lack of variation achieved in other organisms that utilize sexual reproduction. Phenotypic switching allows the yeast to exploit microniches in the body and alters a variety of factors (e.g. antifungal drug resistance, adherence, extracellular enzyme production) in addition to the actual phenotype and so may be considered as the 'dominant' or 'controlling' virulence factor.

In terms of tissue colonization and invasion, adherence is the initial step in the process. Once the yeast has adhered, enzymes (phospholipase and proteinase) can facilitate adherence by damaging or degrading cell membranes and extracellular proteins. Hyphae may be produced and penetrate layers of cells using thigmotropism to find the line of least resistance. The passage through cells is undoubtedly aided by the production of extracellular enzymes. Once endothelial cells are reached enzymes may assist in the degradation of tissue and allow the yeast enter the host's blood stream where phenotypic switching or coating with platelets may be used to evade the immune system. While in the bloodstream the haemolysin may function to burst blood cells and release iron which is essential for growth. Escape from the bloodstream involves adherence to the walls of capillaries and passage across the wall.

5.2 *Aspergillus fumigatus*

Aspergillus fumigatus is a saprophytic fungus which is widely distributed in nature where it is frequently encountered growing on decaying vegetation and damp surfaces (Figure 4.8). *A. fumigatus* can present as an opportunistic pathogen of humans and is the commonest etiological agent of pulmonary aspergillosis, being responsible for 80–90% of cases. Although the incidence of disease due to *Aspergillus* species is less than that due to *Candida*, aspergillosis results in greater mortality rates.

A. fumigatus produces a number of extracellular enzymes which facilitate growth in the lung and dissemination through the body. Phospholipase production has been shown in clinical isolates of this fungus with optimal production occurring at 37°C. This enzyme plays a critical role in tissue degradation and may facilitate exit of the fungus from the lung into the bloodstream. Fungal proteases are responsible for tissue degradation and neutralization of the immune system and probably evolved to allow the fungus to degrade animal and plant material. Elastin constitutes almost 30% of lung tissue and many *A. fumigatus* isolates display elastinolytic activity while isolates incapable of elastinase production display reduced virulence in mice. *A. fumigatus* produces two elastinases: a serine protease and a metalloproteinase. Apart from their direct role in tissue degradation, *A. fumigatus* proteases (in particular the serine protease) may also function as allergens which may be important in the induction and persistence of allergic aspergillosis. Local inflammation due to the presence of proteases results in airway damage and *in vitro* studies have shown that proteases are capable of inducing epithelial cell detachment from basement membranes. Such desquamation may partly explain the extent of damage seen in the lungs of asthmatic and cystic fibrosis patients affected with allergic aspergillosis. In addition proteases induce the release of the proinflammatory IL-6 and IL-8 cytokines in cell lines derived from airway epithelial cells, which may induce mucosal inflammatory response and subsequent damage to the surrounding tissue.

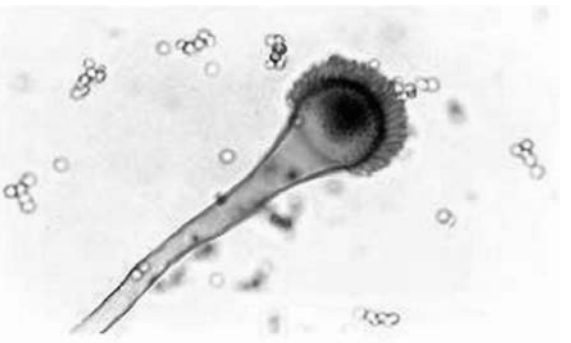


Figure 4.8 Conidiophore of *Aspergillus fumigatus*.

The production and secretion of toxins into surrounding tissues by the proliferating fungus is regarded as an important virulence attribute and may facilitate fungal growth in the lung. Gliotoxin is the main toxin produced by *A. fumigatus*, others include helvolic acid, fumigatin and fumagillin. It is believed that toxins play a significant role in facilitating the colonization of the lung by *A. fumigatus* since they can act to retard elements of the local immunity. Significantly, *Aspergillus* species that do not produce toxins to the same extent as *A. fumigatus* isolates are rarely seen in clinical cases.

5.3 *Histoplasma capsulatum*

Histoplasma capsulatum is a dimorphic fungus which is the cause of histoplasmosis, the most prevalent fungal

pulmonary infection in the USA. Histoplasmosis is common amongst AIDS patients but is also found among infants and elderly people living in endemic areas who may be susceptible to infection due to impaired immune function. The mortality rate among infants exposed to a large dose of *H. capsulatum* may be 40–50%. *H. capsulatum* grows in the mycelial form in soil while in tissue it is encountered as round, budding cells. Its natural habitat is soil that has been enriched with the droppings of bats or birds, and disturbance of such soil by natural (e.g. wind) or human (e.g. agriculture) activities releases large numbers of airborne spores that upon inhalation can establish pulmonary infection in individuals or in populations distant from the original source of spores.

Upon inhalation of *H. capsulatum* pulmonary macrophages engulf the yeast cells and provide a protected environment for the yeast to multiply and disseminate from the lungs to other tissues. The ability to survive within the hostile environment provided by the macrophage is the key to the success of *H. capsulatum* as a pathogen. Normal individuals who inhale a large number of *H. capsulatum* spores can develop a non-specific flu-like illness associated with fever, chills, headache and chest pains after a 3 week incubation period that resolves without treatment. Chronic pulmonary histoplasmosis is seen in men in the 45–55 year age group who may have been exposed to low levels of spores over a long period of time. The condition is progressive, leading to a loss of lung function and death if untreated.

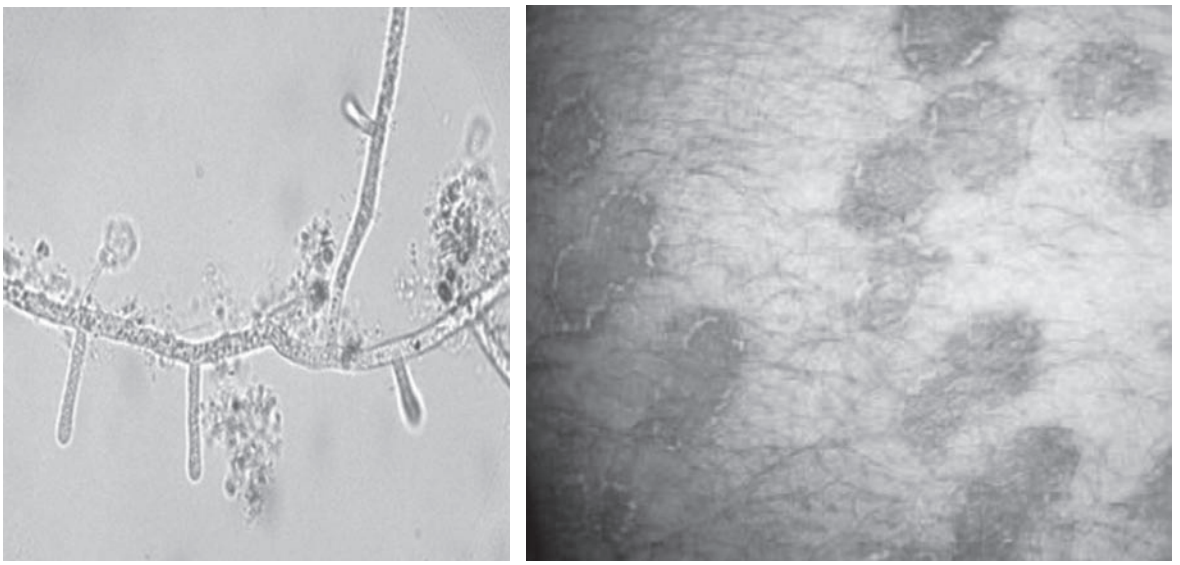
5.4 *Cryptococcus neoformans*

Crypotococcus neoformans is an encapsulated yeast that is most frequently associated with infection in immunocompromised patients, particularly those with AIDS, where meningitis is the most common clinical manifestation. *C. neoformans* is a facultative intracellular pathogen that is capable of surviving and replicating within macrophages and withstanding the lytic activity within these cells. In order to survive within macrophages *C. neoformans* appears to accumulate polysaccharides within cytoplasmic vesicle. As part of its survival strategy *C. neoformans* also produces melanin, which has the ability to bind and protect against microbicidal peptides. In addition, melaninization may allow the cell withstand the effects of harmful hydroxyl radicals and so survive in hostile environments . The capsule of *C. neoformans* is a virulence factor in that it protects the cell from the immune response, and capsular material (glucuronoxylomannans) induces the shedding of host cell adherence molecules required for the migration of host inflammatory cells to the site of infection, thus explaining the reduced inflammatory response to this yeast.

In immunocompromised patients pulmonary infection can lead to disseminated forms of the disease where the eyes, skin and bones become infected. Cryptococcal meningitis is particularly associated with AIDS patients where it is a major cause of death. While cryptococcosis may be controlled by antifungal therapy, in AIDS patients there is a danger of relapse unless antifungal therapy is constantly maintained.

5.5 Dermatophytes

The dermatophytes are a group of keratinophilic fungi which can metabolize keratin—the principal protein in skin, nails and hair (Figure 4.9). Tinea capitis is defined as the infection of the hair and scalp with a dermatophyte, usually *Microsporum canis* or *Trichophyton violaceum*. In Europe and North Africa *T. violaceum* may be responsible for approximately 60% of cases of tinea capitis. This condition is characterized by a mild scaling and loss of hair and in some cases it may be contagious. Tinea corporis is characterized by infection of the skin of the trunk, leg and arms with a dermatophyte and is usually caused by *Trichophyton* spp., *Microsporum* sp. or *Epidermophyton floccosum*. Lesions are itchy, dry and show scaling. Typically lesions retain a circular morphology and the condition is often referred to as 'ringworm'. Tinea cruris is seen where the skin of the groin is infected with a dermatophytic fungus, usually *Epidermophyton floccosum* or *Trichophyton rubrum*. This condition is highly contagious via fomites (towels, sheets, etc.) and up to 25% of patients show recurrence following antifungal therapy. Tinea pedis presents as infection of the feet with a dermatophyte; it is very common and easily contracted. The principal fungi responsible for this condition are *T. rubrum* and *E. floccosum* and the sites of infection may include the webs of the toes, the sides of the feet or the soles of the feet. Tinea manuum is a fungal infection of the hands and can be caused by a range of fungi most notably *Trichophyton mentagrophytes*. Infection is often seen in association with eczema and can result from transmission of fungi from another infected body site. Tinea unguium is defined as infection of the fingernails or toenails and is often described as onychomycosis, which includes infections due to a range of other fungi, bacteria and nail damage associated with certain disease states (e.g. psoriasis).



A B

Figure 4.9 Dermatophytic infections. Hyphae of *Microsporum canis* (A) and a dermatophyte infection of the skin (B).

6 Emerging fungal pathogens

In recent years a number of fungal species previously regarded as non-pathogenic have emerged as causes of disease in certain groups of patients (e.g. HIV-positive patients, cancer patients and transplant patients). While a number of these fungal species may have been causing disease previously, their emergence as significant pathogens is a cause of great concern. Undoubtedly the ability to keep immunocompromised patients alive for long periods is providing a set of novel niches for fungi, as is the introduction of novel drugs and therapies. This section focuses on a number of emerging fungal pathogens and demonstrate how the availability of novel niches has facilitated the appearance of this group of fungal pathogens.

6.1 *Saccharomyces cerevisiae*

The yeast *S. cerevisiae* is widely distributed in nature. It has been used for millennia in the production of bread and alcoholic beverages, and can be consumed directly as a dietary supplement. Traditionally it is better known as brewer's or baker's yeast. Because of the ease with which it can be genetically and biochemically manipulated it is probably the most studied and best-characterized organism on the planet. Recently a considerable number of reports have implicated this yeast as a cause of disease in immunocompromised patients but also in those with no apparent predisposing condition. As a result, *S. cerevisiae* is no longer regarded as a GRAS ('generally regarded as safe') organism but is now classified as a Biosafety Level 1 pathogen, indicating that it can cause superficial or mild systemic infections in certain instances. This change of status has serious implications for industries using this yeast and indicates that, in cases of impairment of the immune system, what was once regarded as a harmless yeast is capable of causing life-threatening illness.

S. cerevisiae has been implicated in a number of superficial and systemic cases of disease and often in patients who have no apparent predisposing illness. In the majority of cases it has been found in association with other microbes, but in a significant number of instances it was identified as the sole pathogen. It has been identified as a cause of vulvovaginitis in women, being responsible for possibly up to 5% of cases, and is responsible for pneumonia and widespread dissemination in AIDS patients. Other conditions attributed to this yeast include septicaemia, postoperative peritonitis and the induction of fever and coughs in transplant patients. In one instance a bone marrow transplant (BMT) patient taking a self-prescribed course of brewer's yeast tablets developed a fever, cough and chest pains. *S. cerevisiae* was isolated from the lung and identified as the source of the

infection. In relation to BMT patients the contamination of food with *S. cerevisiae* is now regarded as a potential source of infection.

Isolates of *S. cerevisiae* that have been associated with disease display a number of attributes that facilitate their persistence and dissemination in the host. Clinical isolates display the ability to grow at 42 °C, which is significantly greater than the upper temperature range for brewing yeast. This ability is regarded as important since febrile patients can attain this temperature and it is advantageous for a pathogenic microorganism to survive at this elevated temperature. Pathogenic isolates are capable of producing a number of extracellular enzymes such as acid proteinases and phospholipases which play a role in modulating the immune system's response to infection and allow the degradation of cell membranes, respectively. Isolates also demonstrate the ability to grow in a pseudohyphal form which may assist in the penetration of tissue. Brewing strains of *S. cerevisiae* are known to flocculate at the end of the fermentation and this phenomenon is also seen in pathogenic isolates where it plays a role in obstructing capillaries particularly in the brain with concomitant damage to surrounding tissue. Pathogenic isolates adhere to epithelial tissue via a proteinaceous adhesin which is critical to the survival of the yeast in areas where it could be washed away by the action of swallowing as in the mouth. Potentially the most important attribute of virulent isolates is their ability to alter their phenotype. In clinical isolates of *S. cerevisiae* this ability contributes to the yeast's persistence in the body where in complement factor 5 mice isolates were capable of persisting in the brain for up to 7 days without being cleared.

Conventional therapy for superficial or systemic *S. cerevisiae* infection relies on the use of azoles or polyenes. Interestingly, clinical isolates demonstrate a high level of resistance to fluconazole, which has been regarded as the first choice for the treatment of *C. albicans*-induced superficial candidosis in HIV-positive individuals. While the mechanism that confers resistance to fluconazole in *S. cerevisiae* is still poorly characterized it is thought to be mediated via a multidrug efflux pump, which would remove the drug from the cell before it can act.

6.2 Non-*albicans Candida* species

The rather cumbersome term 'non-*albicans Candida* species' covers a range of *Candida* species that have emerged as significant human pathogens in recent years. The principal species are *Candida dubliniensis*, *Candida krusei* and *Candida glabrata*, although it must be emphasized that other *Candida* species may be problematic in specific situations. All the emerging *Candida* species share a number of common characteristics i.e. they were either unknown or regarded as inconsequential pathogens until recently and their emergence as significant causes of disease has occurred by exploiting a series of novel niches produced either as a result of therapy or disease.

Candida dubliniensis was first identified in 1995 in samples taken from HIV-positive patients suffering from oropharyngeal candidosis. Upon streaking samples on CHROMagar plates *C. albicans* appeared green but the newly discovered *C. dubliniensis* produced colonies displaying a different shade of green. The yeast is similar to *C. albicans* in many ways but displays different carbohydrate assimilation and DNA restriction patterns. This yeast has been identified in HIV-positive and -negative populations from many parts of the world and is now the dominant cause of oral candidosis in the former group. The discovery of *C. dubliniensis* demonstrates that the provision of a novel niche allows previously unrecognized pathogens to emerge and out-compete the perceived dominant pathogen.

C. krusei was regarded as a harmless, transient commensal, being commonly found in the environment (on fruit) and on the human body, but now it is regarded as a significant cause of disease in HIV-postive patients, diabetics and cancer patients (both solid tumour and leukaemia where it is capable of colonizing the hastrointestinal, respiratory and urinary tracts). In terms of virulence attributes *C. krusei* demonstrates a reduced ability to adhere to epithelial cells compared to *C. albicans* although it does display a high cell surface hydrophobicity which allows it stick to and colonize catheters and implants. Its main virulence attribute is the high level of inherent resistance to fluconazole. Upon the introduction of this drug in 1990 to treat oropharyngeal candidosis in AIDS patients and systemic candidosis in transplant patients there was a reduction in the number of cases of disease caused by *C. albicans.* However the elimination of this yeast may have facilitated the emergence of *C. krusei* as the dominant fungal pathogen in certain classes of patient.

C. glabrata has emerged in recent years as a serious cause of disease in neutropenic cancer patients and has been responsible for mortality rates of 5–38% in some surveys. It appears to be a particular problem in the late stages of haematological malignancies, where mortality rates of 70–100% have been described. The incidence of *C. glabrata* infection has increased in recent years; it is

now the fourth most commonly isolated *Candida* species and this increase may be attributable, in part at least, to an alteration in local epidemiology. Since *C. glabrata* is common amongst leukaemia patients and bone marrow recipients, a range of other risk factors such as prolonged hospitalization, use of cytotoxic drugs and catheterization may also play a role in its prevalence. *C. glabrata* is recognized as being a yeast of relatively low virulence but its emergence as a serious pathogen has been attributed to it being partially resistant to fluconazole. In addition, other factors that may have contributed to its appearance as a serious pathogen have been identified as azole prophylaxis which eliminates *C. albicans* but not *C. glabrata* or *C. krusei* and local factors such as the use of broad-spectrum antibiotics or vascular catheters or the presence of neutropenia as part of the disease state.

6.3 *Penicillium marneffei*

Until recently *Penicillium marneffei* was regarded as a very rare and inconsequential cause of disease in humans. Now, however, it is the most frequent cause of fungal disease in AIDS patients who reside in, or have visited, South-East Asia. The principal areas of infection are Thailand and southern China although cases have been reported from Malaysia, Taiwan, Japan and Hong Kong. While the nature of the infection in humans is well characterized, the natural habitat or reservoir of this fungus is still unknown although it may be soil or decaying vegetation. *P. marneffei* is an asexual, dimorphic fungus growing as a mycelium at 37 °C in tissue and as single cells at 28 °C. It reproduces by a process known as fission. In the yeast form of growth it can be difficult to distinguish microscopically from *Histoplasma capsulatum* and *Cryptococcus neoformans*, so monoclonal antibody based assays specific for the detection of mannoproteins associated with *P. marneffei* have been developed. PCR (polymerase chain reaction) fingerprinting is also used to identify *P.marneffei* and to distinguish between infecting strains.

Infection in humans follows inhalation of fungal material, and in AIDS patients dissemination throughout the body can result. Pulmonary involvement is often seen particularly in AIDS patients but the conditions most associated with infection are fever, weight loss, anaemia, skin lesions, and liver and spleen inflammation. The condition is fatal if untreated with antifungal drugs. While the condition responds well to therapy if it is initiated at an early stage, relapse is common in AIDS patients and continuing antifungal therapy may be required. Because of the immunocompromised nature of AIDS patients other infections such as tuberculosis and pneumonia are often seen along with *P. marneffei* infection.

7 Antibiotic production by fungi

Perhaps one of the most important discoveries regarding the beneficial use of fungi for humans was the identification in 1929 by Sir Alexander Fleming that an isolate of *Penicillium notatum* produced a substance capable of killing Gram-positive bacteria. This compound was subsequently identified as penicillin and was the first member of the β-lactam class of antibiotics to be discovered. These compounds function by inhibiting peptidoglycan synthesis in bacteria and their use has reduced the importance of the Gram-positive bacteria as a cause of disease. Subsequent to the identification of penicillin production by *P. notatum*, a screen revealed that *Penicillium chrysogenum* was a superior producer. Following a series of mutagenic and selection procedures the strain used in conventional fermentations is capable of producing penicillin at a rate of 7000 mg/L compared to the 3 mg/L of Fleming's *P. notatum* isolate. A typical penicillin fermentation yields three types of penicillin, namely F, G and V. The latter can be used directly, however G is modified by the action of penicillin acylase to give a variety of semi-synthetic penicillins which show resistance to the action of bacterial penicillinases which are implicated in conferring antibacterial drug resistance.

The majority of antibiotics obtained from fungi are produced by fermentation and most are secondary metabolites, production of which occurs in the stationary phase and is linked to sporulation. Catabolite repression can inhibit antibiotic production and one way to avoid this is to use low levels of glucose in the fermentation medium or to obtain a mutant which is not catabolite repressed. The chemical content of the medium must be monitored since high levels of nitrogen or phosphate (PO_4) retard antibiotic production. One problem that seriously affects the productivity of antibiotic fermentations is feedback inhibition, where the antibiotic builds to high intracellular levels and retards production or kills the cell. One means of reversing this is to introduce low levels of the antifungal agent amphotericin B, which increases membrane permeability, leading to a decrease in intracellular antibiotic levels and a concomitant increase in production.

Antibiotic production can be maximized by optimizing production as a result of random mutagenesis and selection. Another approach has been to fuse or mate

high-producing strains with good secretors. Rational selection is a process where a chelating agent is introduced into the fermentation to complex all the metal ions present and consequently has a beneficial effect on antibiotic production. More recently genetic manipulation has been employed to express the genes for antibiotic production in another species which has the possibility of producing hybrid antibiotics with novel targets.

8 Further reading

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5 Viruses

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1 Introduction

Viruses were first discovered at the end of the 19th century, although the symptoms they cause were identified much earlier. One of the earliest pieces of evidence is the symptoms of poliomyelitis in an Egyptian priest depicted on a hieroglyph. Virus discovery came about when the cause of an infectious disease (rabies), could not be explained by the presence of bacteria. Unlike bacteria, the 'infectious materials' were not retained by filtration and thus viruses were then referred to as 'filterable agents' or 'filterable viruses'. Until the advent of electron microscopy in the 1940s, only the chemical nature of viruses (i.e. proteins and nucleic acid) could be identified and their infectivity was mainly studied in animal models. The observation of a virus by electron microscopy and the development of cell tissue culture started the golden era of virology. Since then a large number of viruses have been isolated, their structure identified and their replication understood, leading to the design of potent antiviral drugs and effective vaccines. Progress in virology over the last 50 years have been considerable, leading to the eradication of smallpox following a worldwide vaccination programme, and the likely future eradication of poliovirus. For the first time in human history an infectious disease has been vanquished. Despite such an achievement, much progress is still required to combat other viruses. For example, despite large financial investment and high profile studies, a cure or vaccine for HIV is still

Hugo and Russell's Pharmaceutical Microbiology, Eighth Edition. Edited by Stephen P. Denyer, Norman Hodges, Sean P. Gorman, Brendan F. Gilmore.

not available. The number of commercially available antivirals is also still limited, when compared to the number of antibiotics. Within the last few years, diseases caused by viruses in human and animals have reminded us that viral infections can easily spread and cause epidemics and pandemics. Recent examples are 'swine flu' and 'avian flu', both caused by influenza viruses, and also an outbreak of severe acute respiratory syndrome (SARS), the virus originating from Asia and spreading worldwide, and outbreaks of foot and mouth virus affecting cattle, sheep and pigs that had important economic consequences in the UK and mainland Europe.

2 General structure of viruses

Viruses are extremely diverse in size and in structure. The smallest virus is approximately 28 nm in size (poliovirus), while the largest is 750 nm (mimivirus). In simplistic terms, a virus consists of viral nucleic acid within a protein core, the *capsid* (also referred to as the coat), possibly surrounded by a lipidic *envelope* (Table 5.1). In reality, there are many differences between viruses in terms of nucleic acid, capsid structure, number of coats and envelope composition. Such differences account for the high diversity of viruses and the differences in their properties, notably their resistance to antiviral drugs and viricidal agents. Viral classification (Figure 5.1) is based on the physical and chemical properties of viruses, their structure and morphology (Figure 5.2).

2.1 Viral nucleic acid

The viral genome is composed of either DNA or RNA. It can be double stranded (ds) or single-stranded (ss), linear (e.g. poliovirus) or circular (e.g. hepatitis B virus), containing several segments (e.g. influenza—eight segments of minus-sense ss RNA) or one molecule (e.g. poliovirus). Six groups can be distinguished depending on their nucleic acid content (Table 5.2). Viruses that contain plus-sense ss RNA (e.g. poliovirus) can have their genome translated directly by the host ribosome. The nature of the viral nucleic acid is important for the effectiveness of antiviral treatments (see below). For example, retroviridae such as HIV require a specific virus-encoded enzyme, a reverse transcriptase, to convert their ss RNA into ss DNA, to be able to replicate within the host cell. This enzyme is a primary target site of many antiviral drugs.

On some occasions, the viral genome has been shown to be infectious, i.e. to cause an infection. Such an observation is important when one considers viricidal agents that damage the viral capsid or envelope but not the viral nucleic acid. Furthermore, in laboratory conditions, the phenomenon of *multiplicity reactivation* has been observed with poliovirus whereby random damage to viral capsid and nucleic acid following treatment with hypochlorite, a biocide intensively used for surface disinfection, resulted in complementary reconstruction of an infectious particle by hybridization of the gene pool of the inactivated virus. This again underlines the necessity of rendering the viral nucleic acid non-infectious following a viricidal treatment.

2.2 Viral capsid

The function of the capsid is to protect the viral nucleic acid from detrimental chemical and physical conditions (e.g. disinfection). The capsid is composed of a number of subunits named *capsomeres* genetically encoded by the viral genome. The nature and association of the capsomeres are fundamental for the virus, as they give the shape of the capsid, but also provide the virus with resistance to chemical and physical agents. The assembly of the capsomeres results in two different architectural styles—icosahedral and helical symmetries (Figure 5.3); in mammalian viruses, a more complex structure can be found, where several proteinaceous structures envelope the viral genome core (e.g. poxviruses, rhabdoviruses). Bacterial viruses (bacteriophages) also show a complex structure consisting of a capsid head, a tail and tail fibres (see section 8.1). The nature of the capsomeres in certain viruses allows for the self-assembly of the capsid within the host cell. The capsomeres are held together by non-covalent intermolecular forces. Such assembly also allows the release of the viral genome following dissociation of the non-covalently bonded subunits. These subunits offer considerable economy of genetic information within the viral genome since only a small number of different subunits contribute to the formation of the capsid. Viruses with an icosahedral capsid usually have capsomeres in the form of pentons and hexons. The number of these subunits varies considerably between viruses: for example, adenovirus is constructed from 240 hexons and 12 pentons, whereas the poliovirus is composed of 20 hexons and 12 pentons, forming a much smaller structure. Viruses with a helical capsid (e.g. influenza and mumps viruses) have their subunits symmetrically packed in a helical array, appearing like coils of wound rope under electron microscopy. Although the core of such a virus is hollow, the viral nucleic acid is embedded into ridges on the inside of each subunit and does not fill the hollow core. Such a close association between viral nucleic acid

Table 5.1 Structure and clinical importance of mammalian viruses

Group	Virus	Characteristics	Clinical importance
DNA viruses			
Poxviruses	Variola	Large particles 200–250 nm: complex symmetry	Variola is the smallpox virus, it produces a systemic infection with a characteristic vesicular rash affecting the face, arms and legs, and has a high mortality rate
	Vaccinia		Vaccinia has been derived from the cowpox virus and is used to immunize against smallpox
Adenoviruses	Adenovirus	Icosahedral particles 80 nm in diameter	Commonly cause upper respiratory tract infections; tend to produce latent infections in tonsils and adenoids; will produce tumours on injection into hamsters, rats or mice
Herpesviruses	Herpes simplex virus (HSV1 and HSV2)	Enveloped, icosahedral particles 150 nm in diameter	HSV1 infects oral membranes in children; >80% are infected by adolescence. Following the primary infection the individual retains the HSV1 DNA in the trigeminal nerve ganglion for life and has a 50% chance of developing 'cold sores' HSV2 is responsible for recurrent genital herpes
	Varicella zoster virus (VZV)	Enveloped, isocahedral particles 150 nm in diameter	Causes chickenpox in children; virus remains dormant in any dorsal root ganglion of the CNS; release of immune control in elderly people stimulates reactivation resulting in shingles
Cytomegalovirus (CMV)		Enveloped, icosahedral particles 150 nm in diameter	CMV is generally acquired in childhood as a subclinical infection. About 50% of adults carry the virus in a dormantstate in white blood cells. The virus can cause severe disease (pneumonia, hepatitis, encephalitis) in immunocompromised patients. Primary infections during pregnancy can induce serious congenital abnormalities in the fetus
Epstein–Barr virus (EBV)		Enveloped, icosahedral particles 150 nm in diameter	Infections occur by salivary exchange. In young children they are commonly asymptomatic but the virus persists in a latent form in lymphocytes. Infection delayed until adolescence often results in glandular fever. In tropical Africa, a severe EBV infection early in life predisposes the child to malignant facial tumours (Burkitt's lymphoma)

(*continued*)

Table 5.1 (*continued*)

Group	Virus	Characteristics	Clinical importance
Hepatitis viruses	Hepatitis B virus (HBV)	Spherical enveloped particle 42 nm in diameter enclosing an inner icosahedral 27 nm nucleocapsid	In areas such as South-East Asia and Africa, most children are infected by prenatal transmission. In the Western world the virus is spread through contact with contaminated blood or by sexual intercourse. There is strong evidence that chronic infections with HBV can progress to liver cancer
Papovaviruses	Papilloma virus	Naked icosahedra 50 nm in diameter	Multiply only in epithelial cells of skin and mucous membranes causing warts. There is evidence that some types are associated with cervical carcinoma
RNA viruses			
Myxoviruses	Influenza virus	Enveloped particles, 100 nm in diameter with a helically symmetric capsid; haemagglutinin and neuraminidase spikes project from the envelope	These viruses are capable of extensive antigenic variation, producing new types against which the human population does not have effective immunity. These new antigenic types can cause pandemics of influenza. In natural infections the virus only multiplies in the cells lining the upper respiratory tract. The constitutional symptoms of influenza are probably brought about by absorption of toxic breakdown products from the dying cells on the respiratory epithelium
Paramyxoviruses	Mumps virus	Enveloped particles variable in size, 110–170 nm in diameter, with helical capsids	Infection in children produces characteristic swelling of parotid and submaxillary salivary glands. The disease can have neurological complications, e.g. meningitis, especially in adults
	Measles virus	Enveloped particles variable in size, 120–250 nm in diameter, helical capsids	Very common childhood fever, immunity is lifelong and second attacks are very rare
Rhabdoviruses	Rabies virus	Bullet-shaped particles, 75–180 nm, enveloped, helical capsids	The virus has a very wide host range, infecting all mammals so far tested; dogs, cats and cattle are particularly susceptible. The incubation period of rabies is extremely varied, ranging from 6 days up to 1 year. The virus remains localized at the wound site of entry for a while before passing along nerve fibres to central nervous system, where it invariably produces a fatal encephalitis

Table 5.1 (*continued*)

Group	Virus	Characteristics	Clinical importance
Reoviruses	Rotavirus	An inner core is surrounded by two concentric icosahedral shells producing particles 70 nm in diameter	A very common cause of gastroenteritis in infants. It is spread through poor water supplies and when standards of general hygiene are low. In developing countries it is responsible for about a million deaths each year
Picornaviruses	Poliovirus	Naked icosahedral particles 28 nm in diameter	One of a group of enteroviruses common in the gut of humans. The primary site of multiplication is the lymphoid tissue of the alimentary tract. Only rarely do they cause systemic infections or serious neurological conditions like encephalitis or poliomyelitis
Rhinoviruses	Naked icosahedra 30 nm in diameter		The common cold viruses; there are over 100 antigenically distinct types, hence the difficulty in preparing effective vaccines. The virus is shed copiously in watery nasal secretions
	Hepatitis A virus (HAV)	Naked icosahedra 27 nm in diameter	Responsible for 'infectious hepatitis' spread by the oral–faecal route especially in children. Also associated with sewage contamination of food or water supplies
Togaviruses	Rubella	Spherical particles 70 nm in diameter; a tightly adherent envelope surrounds an icosahedral capsid	Causes German measles in children. An infection contracted in the early stages of pregnancy can induce severe multiple congenital abnormalities, e.g. deafness, blindness, heart disease and mental retardation
Flaviviruses	Yellow fever virus	Spherical particles 40 nm in diameter with an inner core surrounded by an adherent lipid envelope	The virus is spread to humans by mosquito bites; the liver is the main target; necrosis of hepatocytes leads to jaundice and fever
	Hepatitis C virus (HCV)	Spherical particles 40 nm in diameter consisting of an inner core surrounded by an adherent lipid envelope	The virus is spread through blood transfusions and blood products. Induces a hepatitis which is usually milder than that caused by HBV

(*continued*)

Table 5.1 (*continued*)

Group	Virus	Characteristics	Clinical importance
Filoviruses	Ebola virus	Long filamentous rods composed of a lipid envelope surrounding a helical nucleocapsid1000 nm long, 80 nm in diameter	The virus is widespread amongst populations of monkeys. It can be spread to humans by contact with body fluids from the primates. The resulting haemorrhagic fever has a 90% case fatality rate
Retroviruses	Human T-cell leukaemia virus (HTLV-1)	Spherical enveloped virus 100 nm in diameter; icosahedral cores contain two copies of linear RNA molecules and reverse transcriptase	HTLV is spread inside infected lymphocytes in blood, semen or breast milk. Most infections remain asymptomatic but after an incubation period of 10–40 years in about 2% of cases, adult T-cell leukaemia can result
	HIV	Differs from other retroviruses in that the core is cone-shaped rather than icosahedral	HIV is transmitted from person to person via blood or genital secretions. The principal target for the virus is the CD4+ T-lymphocyte cells. Depletion of these cells induces immunodeficiency
Hepatitis viruses	Hepatitis D virus (HDV)	An RNA-containing virus that can only replicate in cells co-infected with HBV. The spherical coat of HDV is composed of HBV capsid protein	The presence of the satellite HDV exacerbates the pathogenic effects of HBV producing severe hepatitis

CNS, central nervous system.

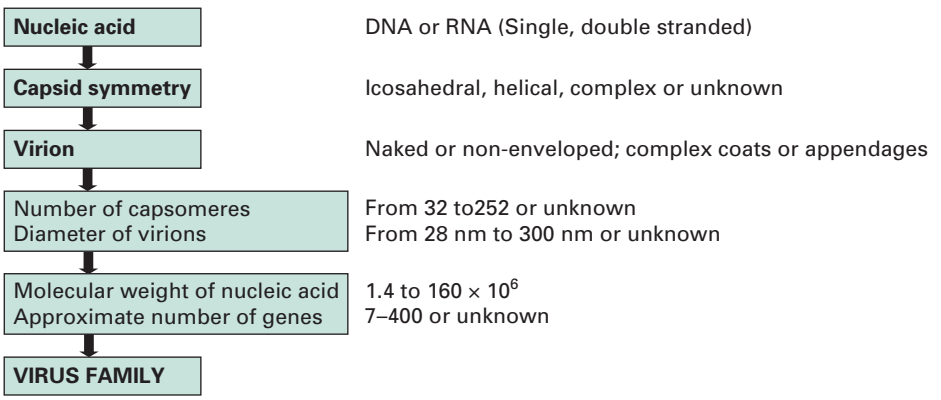

Figure 5.1 Classification of viruses.

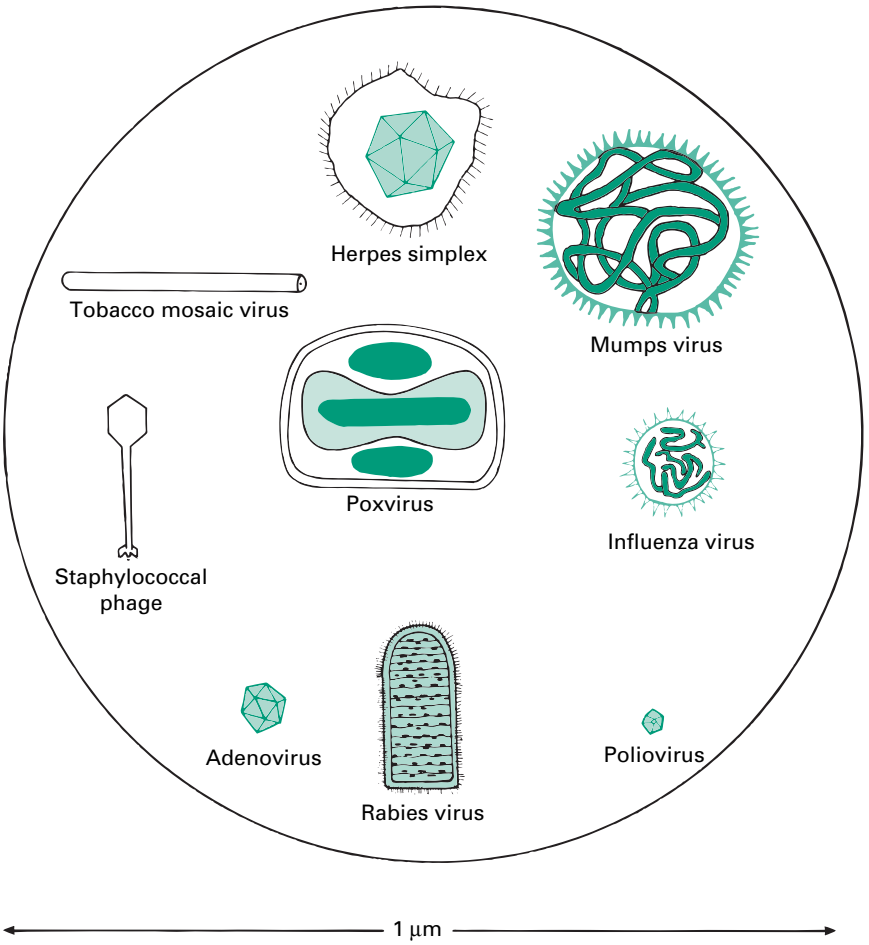

Figure 5.2 The morphology of a variety of virus particles. The large circle indicates the relative size of a *Staphylococcus* cell.

and capsid proteins can explain the damage caused to the nucleic acid following disaggregation of the capsid after chemical or physical treatments.

2.3 Viral envelope

The viral capsid can be surrounded by a lipidic envelope, which originates from the host cell. The envelope is added during the replication process or following excision of the viral progeny from the host cells. The envelope can come from the host cell nuclear membrane (e.g. herpes simplex virus) or the cytoplasmic membrane (e.g. influenza virus). One characteristic of the viral envelope is that host proteins are excluded, but proteins encoded by the viral genome are present. These viral proteins play an important serological role. Enveloped viruses are generally considered to be the most susceptible to chemical and physical conditions and do not survive well on their own outside the host cell (e.g. on surfaces), although they can persist longer in organic soil (e.g. blood, exudates, faeces). Lipids in viruses are generally phospholipids from the host envelope, although glycolipids, neutral fats, fatty acids, fatty aldehydes and cholesterol can be found.

2.4 Viral receptors

In addition to these structures, glycoproteins can be found usually protruding from the viral capsid or embedded in the envelope. These virus-encoded structures are important for viral infectivity as they recognize the host cell receptor site conveying viral specificity. In bacteriophages, these structures can take the shape of tail fibres.

Table 5.2 Examples of types of nucleic acid in human and bacterial viruses

Group	Nucleic acid	Family	Example
I	ds DNA	Herpesviridae, Poxviridae, Hepadnaviridae*, Papovaviridae, Adenoviridae	Herpes simplex virus, cytomegalovirus, poxvirus, HBV, papilloma virus, adenovirus
		Myxoviridae	Bacteriophage T and λ
II	ss DNA		Bacteriophage M13, X174, filamentous bacteriophages
III	ds RNA	Reoviridae	Reoviruses, rotaviruses
IV	ss RNA with mRNA identical in base sequence to virion RNA	Picornaviridae	Poliovirus, hepatitis A virus, rhinovirus
		Leviviridae	Bacteriophage MS2
V	ss RNA with mRNA complementary in base sequence to virion RNA	Paramyxoviridae, Orthomyxoviridae, Filoviridae	Influenza, paramyxovirus, measles, mumps, Ebola virus
VI	ss RNA with DNA intermediate in their growth	Retroviridae,	HIV

* Hepadnaviridae genome contains partially ds DNA and partially ss DNA.

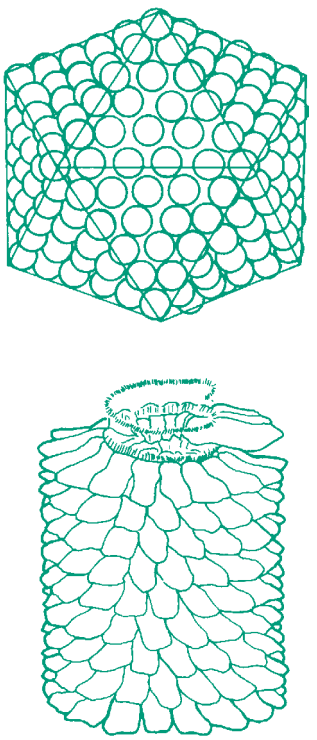

Figure 5.3 Icosahedral and helical symmetry in viruses.

3 Virus–host cell interactions

Viruses need to interact with a host cell as they cannot reproduce on their own. They have no metabolism and cannot synthesize their own proteins, lipids or nucleic acids. Thus viruses can be considered as true intracellular parasites that grow within living cells and use their energy and synthetic machinery to produce viral components. The production and excision of viruses from the host cell will result in cell death, although this might not be immediate. Following the replication of one virus within the host cell, hundreds of new viruses (virus progeny or *virions*) can be released and infect adjacent cells (within a tissue). The propagation from one infected cell to new cells, and the subsequent destruction of tissue or cells, provides signs of the viral disease.

On the basis of host specificity, three major viral groups can be distinguished: (1) viruses of bacteria and blue-green algae, (2) plant viruses and (3) animal (including insect) viruses. Viruses are usually very specific and rarely cross species barriers, although there are some exceptions, such as rabies and influenza that can cause diseases in both animals and humans. Viruses can also be asymptomatic in certain hosts where they do not cause an infection; the host becomes a reservoir and can trans-

mit the virus to a susceptible recipient (e.g. transmission of yellow fever to humans by mosquitoes).

Viruses can interact with the host cell in five different ways: (1) multiplication of the virus and destruction of the host cell upon release of the viral progeny, (2) multiplication of the virus and release of the virions without the immediate destruction of the host cell, (3) survival of the virus in a latent stage without noticeable changes to the infected cell, (4) survival of the infected cell in a dramatically altered or transformed state (e.g. transformation of a normal cell to one having the properties of a cancerous cell) and (5) incorporation of the viral nucleic acid in the host cell genome without noticeable changes to the infected cell. The interaction with the host cell will vary between viruses, but will generally follow one of these five routes.

There is a great diversity in viral infections and viral diseases. Many viral infections are asymptomatic or 'silent' whereby the virus replicates within the host but does not produce symptoms of a disease. Other common infections produce some mild symptoms, such as a low-grade fever and a 'runny nose'. This is the case of the common cold, caused by rhinoviruses, from which patients make a full recovery within a few days. At the other end of the spectrum, some viruses kill their host very quickly following infection, as in the case of haemorrhagic viruses such as the Ebola virus. On other occasions a range of symptoms can be observed in different hosts. This has been the case recently with 'swine flu', which produced a range of symptoms, from a slight fever to full influenza including a high fever, vomiting, and dizziness. Other problematic viruses might not cause immediate symptoms, but following the systematic destruction of host cells will lead to an incurable disease; e.g. HIV and oncogenic (tumour) viruses.

3.1 HIV

HIV is an enveloped virus with a cone-shaped nucleocapsid containing two copies of a positive-sense ss RNA molecule and the enzyme reverse transcriptase. The viral genome encodes for the following genes: *env*, envelope proteins; *gag*, capsid proteins; *pol*, enzymes involved in viral multiplication; and *tat,* enzymes regulating host metabolism. Seventy glycoprotein spikes (gp120) project from the envelope and interact specifically with the CD4 protein receptor on the T-lymphocyte (Chapter 9). The HIV core penetrates the cell cytoplasm following membrane fusion and is uncoated releasing the two RNA molecules and the reverse transcriptase into the cytoplasm. The RNA is copied by reverse transcriptase into ss DNA, which is then duplicated to form a ds DNA copy of the original viral RNA genome. This DNA moves into the host cell nucleus where it is integrated as a *provirus* into a host cell chromosome.

The provirus can lie dormant in the cell or can be expressed, producing viral mRNA and proteins and resuming the multiplication cycle producing virions. The viral mRNA is *polycistronic*, producing *polyproteins* that need to be cleaved by a specific virus-encoded protease. Following the assembly of viral proteins and viral RNA, the virions bud off the infected cells.

At present there is no prospect of any drugs that can be developed to eliminate proviruses from infected cells. Current therapy has evolved around maintaining a high count of T4 helper lymphocytes by regulating the viral load produced by infected cells (up to 10^{10} viral particles produced continuously per day). Indeed, the ultimate decrease in T4 helper cells (below 200/ml of blood) seriously compromises the host immune system and allows infection by a range of opportunist pathogens including fungi, protozoa, bacteria and other viruses. Currently, antiviral treatments (highly active antiretroviral therapy—HAART) combining a protease inhibitor and two reverse transcriptase inhibitors, reduce the number of HIV particles and slow the progression of the disease by restoring and maintaining the number of T4 helper lymphocytes. However, this triple therapy does not eliminate the virus completely. Patients who stop using the drugs experience a rapid rebound in levels of the virus in the blood and progression of the disease. The inability to eliminate the virus completely calls for lifelong therapy that is prohibitively expensive for countries with very limited health budgets.

3.2 Tumour viruses

There is a suggestion that 20% of human cancers might have a viral origin. Virus infected cells change dramatically, acquiring the characteristics of tumour cells exhibiting uncontrolled growth. For example, the Epstein–Barr virus (EBV) has been associated with the formation of lymphomas and nasopharyngeal carcinomas, the hepatitis B and C viruses with hepatocellular carcinoma, human papilloma viruses with cervical cancer, human T-cell lymphotrophic virus type 1 with adult T-cell leukaemia/lymphoma syndrome and HIV with Kaposi's sarcoma.

There are no identified single mechanisms by which viruses induce tumours. The acquisition of viral genes by the host must be followed by other events such as environmental or dietary exposures to chemical

carcinogens for cancer to occur. For example, there might be an association between EBV and malaria to trigger Burkitt's lymphoma in young children in Africa. EBV and consumption of smoked fish might trigger nasopharyngeal carcinoma in adults in China. The development of liver cancer following infection with hepatitis B virus (HVB) might be triggered by high alcohol consumption, smoking and exposure to fungal toxin (aflatoxins), events that damage the liver.

It is known that the viral genome of oncogenic viruses can integrate the host DNA. Indeed, viral DNA has been recovered from infected cells. Following integration of the provirus, the regulation of cell growth and division can be affected. There is no means of eradicating proviruses. However, progress in the prevention of papillomavirus has been made by designing an effective vaccine combined to a successful vaccination programme that is responsible for an important decrease in cervical cancer cases.

4 Multiplication of human viruses

The objective of the replication cycle is to ensure the multiplication of the virus with the formation of identical viral progeny. Viruses differ in their replication cycle and the time to produce and release new virions. The multiplication cycle of human viruses is generally slow, from 4 to more than 40 hours. Bacterial viruses are generally faster and can take as little as 20 minutes to replicate within the bacterial host. The replication cycle can be divided into six distinct phases (Figure 5.4) that are common to all viruses, although detail within each phase varies greatly between viruses. Understanding the viral multiplication process is crucial for the development of new antiviral drugs.

4.1 Attachment to the host cell

Viral attachment to the cell surface can be divided into three phases: (1) an initial contact mainly dependent

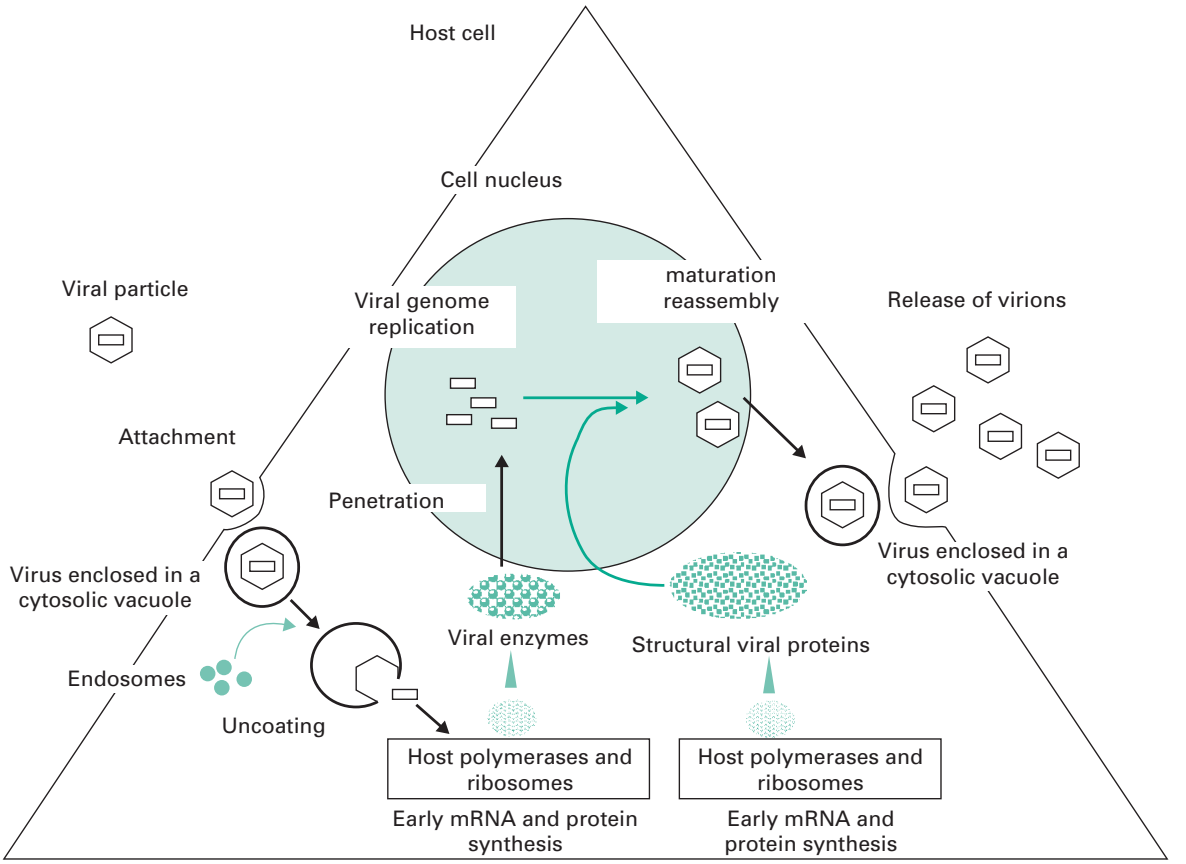

Figure 5.4 Diagrammatic representation of the production and release of viral particles from an infected cell.

on brownian motion, (2) a reversible phase during which electrostatic repulsion is reduced and (3) irreversible changes in virus-receptor–host-receptor configuration that initiates viral penetration through the cell membrane.

All viruses possess *receptors* on their surface, usually in the form of glycoproteins embedded in the viral envelope or protruding as spike from the viral capsid. These structures recognize and bind receptors on the host cell and provide the virus with its high specificity although different viruses might share the same receptor. The virus–cell recognition event is similar to any protein–protein interaction in that it occurs through a stereospecific network of hydrogen bonds and lipophilic associations. For example, the haemagglutinin receptor of influenza virus binds the terminal glycoside residues of gangliosides (cell surface glycolipids) of the target cell leading directly to the virus particle adhering to the cell. Similarly, the interaction between the HIV receptor (i.e. gp120) and the T-lymphocyte receptor (i.e. CD4) has been intensively studied.

4.2 Penetration of the viral particle

Following the irreversible attachment of the virus to the host cell, penetration of the virus through the cell membrane is initiated following two energy-dependent mechanisms, endocytosis or fusion. A third mechanism has been identified in some bacteriophages that can inject their nucleic acid inside the bacterium (see section 8.1). During endocytosis, the association between virus receptor and host receptor triggers a number of mechanisms that draw the cell membrane to engulf the virus particle forming a cytosolic vacuole. This process is widespread among non-enveloped viruses, but is also used with some enveloped viruses such as influenza (orthomyxoviruses). Certain enveloped viruses (e.g. herpes simplex virus, HIV) can penetrate following fusion of their envelope with the host cell membrane, liberating the viral capsid within the cell cytoplasm.

4.3 Uncoating of the viral particle

Following penetration of the virus in a vacuole or directly into the cell cytoplasm, the viral nucleic acid then needs to be released from the capsid/coat(s) to initiate viral replication. This is the *uncoating* process. For viruses that penetrate by endocytoses, the acidification of the cytosolic vacuoles following endosome fusion induces a conformational change in the capsid and the release of viral nucleocapsid (some helper proteins are associated with the viral nucleic acid) into the cytoplasm. For certain viruses, such as reovirus, only a partial uncoating is necessary for the expression of the viral genome. The release of the nucleocapsid from vacuoles can occur in the cytoplasm, close to the nucleus or within the cell nucleus.

4.4 Replication of viral nucleic acids and translation of the genome

This stage of viral replication ensures that (1) the host cell synthesis machinery is taken over by the virus, and (2) the viral genome is replicated. The structure, size and nature of the viral genome are extremely diverse and thus this stage of the viral multiplication cycle reflects this diversity. Three main mechanisms are, however, common to all viruses: the transcription of viral genes into viral mRNA, the translation of the viral genome into proteins, and the replication of the viral genome. Early transcription and translation usually occurring immediately after the release of the nucleocapsid in the cytoplasm is also common, and ensures the production of early proteins such as viral polymerases, and the hijacking of the cell synthesis machinery. In addition, some viruses can encode for genes the products of which regulate the host synthetic processes according to the needs of the virus (e.g. *tat* gene in HIV).

The replication of the viral genome depends on the type of nucleic acid carried by the virus. The positive strand RNA in viruses such as the poliovirus can be used directly as mRNA following the acquisition of a terminal sequence from the host cell. Negative strand RNA (e.g. in influenza virus) is transcribed into a positive RNA complementary in base sequence to the parent RNA using an RNA-dependent RNA polymerase carried by the virus. In ds DNA viruses (e.g. adenoviruses), the nucleic acid passes into the nucleus where it is usually transcribed by a host DNA-dependent RNA polymerase. In some viruses (e.g. poxvirus), this enzyme is contained within the virus and released during uncoating, allowing the viral genome to be replicated in the cell cytoplasm. In retroviruses (e.g. HIV), a single-stranded proviral DNA is produced from the viral ss RNA by a viral enzyme called reverse transcriptase. This unique enzyme acts both as an RNA- and DNA-directed DNA polymerase, and has associated RNAase activity. The proviral DNA can be transported to the cell nucleus where it can be integrated within the cell host genome by a viral integrase.

One important difference between the host cell and the virus is in the nature of their mRNA. Host cell mRNA encodes directly for functional proteins, whereas viral mRNA is *polycistronic*, which means several distinct proteins are encoded within a single piece of mRNA. This

implies that the virus needs to use a virus-specific protease to cut at the correct place the *polyprotein* produced by translation to restore the functionality of viral proteins.

Late protein synthesis during the replication cycle concerns the production of structural components (e.g. capsomeres) of the new virions.

4.5 Maturation or assembly of virions

Towards the end of the multiplication process, large amounts of viral materials accumulate within the host. Viral capsid starts to form from individual structural proteins. In certain viruses (e.g. poliovirus) the capsid self-assembles. The replicated viral genome and some viral proteins become packaged within the capsid. Most non-enveloped viruses accumulate within the cytoplasm or nucleus and are only released when the cell lyses. Packaging of viral components can occur within the cytoplasm or in the cell nucleus. For example, with influenza virus, the capsomeres are transported to the cell nucleus where they combined with the viral RNA and assemble into helical capsids. The envelope of enveloped viruses originates from the host. With the influenza virus, viral proteins such as neuraminidase and haemagglutinin migrate to the cell membrane, displacing cell protein. The assembled nucleocapsids pass out from the nucleus to the cytoplasm and as they impinge on the altered cytoplasmic membrane they cause it to bulge and bud off completed enveloped particles from the cell. In the herpesvirus, the envelope originates from the nucleus membrane. The nucleocapsid assembles into the nucleus and it acquires its envelope as it passes through the inner nuclear membrane. The complete virus is then incorporated into a vesicle which migrates to the cell surface.

The maturation of viruses and their assembly is not well understood at present. The presence of chaperone proteins may play an important role in the interaction between the viral nucleic acid and the structural proteins.

4.6 Release of virions into the surrounding environment

At the end of the multiplication process, the mature virions are released from the host cell. This can occur in a number of different ways. For most non-enveloped viruses, the virus progeny accumulates within the host cell cytoplasm and is released following cell lysis. Some viruses (e.g. bacteriophages) produce a lytic enzyme (peptide) or proteases to lyse the host enabling the release of infectious particles, although the host often self-disintegrates as it cannot maintain normal housekeeping functions during a viral infection. Enveloped viruses are usually released by a budding process over a period of hours. Ultimately the host cell will die following damage to its metabolism and housekeeping functions during viral replication.

5 Cultivation of human viruses

The study and identification of viruses (for diagnosis) depends on our ability to propagate them. In the early days of virology, viruses were propagated in a host. To some extent, this is still the case today with the use of the chick embryo (see below). However, progress in cell culture has revolutionized the cultivation of many viruses, enabling a better understanding of their replication properties, more rapid diagnosis and the easier production of vaccine (Table 5.3).

5.1 Cell culture

Cells to support the growth of human viruses are usually derived from humans or other primates, or from rodents. Cell cultures may be divided into three types according to their history: (1) primary, (2) secondary and (3) continuous cell culture. Primary and secondary cells are usually diploid cell lines. Primary cell lines are derived directly from an intact tissue such as human embryo kidney or monkey kidney. Secondary cell cultures are derived from primary cultures, usually those arising from embryonic tissue. These cells are more homogenous, better characterized, but might not be as susceptible to viral infection as primary cell lines. In addition a limited number of subcultures can be performed with these cells, generally up to a maximum of about 50 before the cells degenerate. Continuous cell lines are usually derived from malignant tissue (e.g. HeLa cells derived from a cervical carcinoma) and have the capacity to multiply indefinitely *in vitro*.

In principle, cell culture for propagating viruses relies on the growth of cells in a semiconfluent monolayer attached to a surface (e.g. the bottom of a flask). To subculture, the cells are separated from the monolayer or relevant tissue usually with trypsin to form a suspension of single cells. These suspended cells are then used to seed a new flask. Following growth at 37 °C, cells will multiply, attach to the surface and form a new monolayer within a few days. The media used to grow the cells consists of basic nutrients and salts (the composition of the medium varies depending on the type of cell) supplemented with

Table 5.3 Cultivation of viruses to produce vaccines

Virus	Cultivation
Hepatitis A virus	Human diploid cell
Influenza virus	Fertilized hen's eggs—membrane bounding the amniotic cavity
Smallpox	Fertilized hen's eggs—chorioallantoic membrane
Measles and mumps	Chick embryo cells
Rubella	Human diploid cells
Human herpesvirus 3 (shingles, herpes zoster)	Human diploid cells
Rabies virus	Chick embryo cells or in human diploid cells
Varicella zoster virus	Human diploid cells
Tick-borne encephalitis virus	Chick embryo cells
Yellow fever virus	Chick embryos
Poliovirus	Human diploid cell line, continuous cell line or primary, secondary or tertiary monkey kidney cells
Baculovirus (recombinant vaccine)	Insect vector

serum (usually bovine albumin) to provide growth factors; antibiotics and antifungals are included to prevent bacterial and fungal contamination in such a rich growth medium.

The established cell monolayer will support viral replication from which viruses can be harvested. Many types of viruses, upon inoculation of a cell culture, will produced a characteristic morphological change in the infected cells. This is called a *cytopathic* effect and usually indicates cell death. The cytopathic effect can take the form of cell shrinkage or ballooning, or the detachment of cells from the surface of the flask; in some instances cellular effects will be detected using various staining solutions. Cytopathic effects usually spread to adjacent cells and will result in the formation of a *plaque* that can easily be identified following staining. These plaques are used for the enumeration of viruses assuming one plaque results from infection by one virus.

To confirm the identity of a viral pathogen (e.g. herpes simplex virus, cytomegalovirus, influenza), viruses are grown in cell culture and following the appearance of a cytopathic effect, the identity of the virus is confirmed using an appropriate viral antisera labelled with a fluorescent dye.

Mammalian cells used for vaccine production are obtained from an approved cell bank. All such cells need to be checked for infectious agents and tumorigenicity (in the case of live vaccines). Such cells are also characterized biochemically (isoenzyme analysis), immunologically (histocompatibility antigens) and by cytogenic markers and are shown to be free from contaminating cells (nucleic acid fingerprint analysis). Depending on their origin, cells will be examined for the absence of specific infectious agents, for the absence of bacterial, fungal and mycoplasma contaminants, and for retroviruses—using product-enhanced reverse transcriptase (PERT) assay,

transmission electron microscopy and, if necessary, infectivity assays. The same controls apply to insect cells.

5.2 The chick embryo

Fertile chicken eggs, 9–11 days old, are used as a convenient cell system to grow a number of human pathogenic viruses. Their usefulness derives from the many types of different tissues found in the eggs, tissues that will support the growth of different viruses (Table 5.3). The use of chicken eggs is expensive and the use of cell culture is preferred wherever possible. The use of chicken eggs for the production of vaccine is necessarily subject to many controls. The eggs have to be free from specific pathogens and originate from healthy flocks. The processing of the fertilized eggs must be conducted under aseptic conditions in an area where no other infectious agents or cells are handled at the same time.

5.3 Animal inoculation

Some animals (e.g. rodents, primates) have to be used to culture certain viruses in order to study antiviral and vaccine effectiveness, and also as a source of cell lines for cell cultures. The use of animals follows strict ethical guidelines and is extremely expensive. A number of controls ensuring that the animals are free from diseases must be observed. Where animals are used to grow viruses or to test an antiviral or vaccine, growth of the virus is indicated by signs of disease or death.

6 Control of viruses

6.1 Antiviral chemotherapy

A number of antivirals are in use in the UK (*British National Formulary*, 2009) for a range of viral infections, including HIV, herpesvirus infections, viral hepatitis, influenza and respiratory syncytial virus. Antiviral treatments are particularly important for persons at high risk, notably immunocompromised patients. Most antivirals are prodrugs that need to be activated within the cell, usually by a kinase, and other cellular enzymes.

Antivirals can act at different stages of the viral replication cycle, with the most effective treatments targeting unique viral enzymes, such as proteases, polymerases and the reverse transcriptase (Table 5.4 and Chapter 11). A number of targets are being investigated to prevent viral attachment to the host cell: competition for CD4 receptors using a pentapeptide identical in sequence to the terminal amino acids of HIV gp120; inhibition of herpes simplex virus (HSV) ribonucleotide reductase; competition for the cell receptor using a hexapeptide fusion sequence at the N-terminus of the influenza haemagglutinin viral receptor. Proteases are particularly important for the uncoating process preventing the release of viral nucleocapsid and for the cleavage of viral polypeptide gene products (e.g. indinavir sulphate). The replication of viral DNA is also a well-exploited target with the use of nucleoside analogues (e.g. idoxiuridine is incorporated into viral and cellular DNA instead of thymidine), non-nucleoside analogues (e.g. nevirapine and foscarnet) and oligonucleotides (Table 5.5). These nucleic acid oligomers with base sequence complementary to conserved regions of proviral DNA have been successful use in the prevention of viral mRNA function. The inhibition of HIV reverse transcriptase has led to the synthesis of many successful antivirals (Table 5.4). The release of the mature virions after the multiplication process can also be blocked. This is the case of neuraminidase inhibitors (e.g. zanamivir and ozaltamivir) preventing the shedding of virions.

Unfortunately, antiviral chemotherapy is associated with a number of problems. Many viral diseases only become apparent after extensive viral multiplication and tissue damage have occurred, delaying treatments. Many antivirals are toxic (e.g. nucleoside analogues) since viral replication often depends on the use of host cell enzymes. There is also scope for improving the pharmacokinetic properties of antivirals, providing a better penetration and retarding drug degradation. The use of prodrugs has improved drug adsorption. Finally, antiviral monotherapy often leads to the development of virus resistance. Emerging HIV resistance has been well documented and current treatments are based on a triple therapy.

6.1.1 HIV

There is no cure for HIV infections as yet. The role of antivirals is to slow or halt disease progression. Since their discovery and use, these drugs (Table 5.4), called antiretrovirals, have considerably prolonged the life expectancy of patients, although not without some important side effects. Antiretroviral treatments aim to reduce HIV plasma levels for as much and as long as possible. Several antiretroviral drugs are usually given together to avoid emerging viral resistance. Initiation of HIV treatment (HAART) is therefore complex and involves two nucleoside reverse transcriptase inhibitors and two non-nucleoside reverse transcriptase inhibitors. Alternative regimens are possible following treatment failure and deterioration of a patient's condition. The use of antiretrovirals for prophylaxis after exposure is also possible,

Table 5.4 Current antiviral drugs in the UK

Type	Viral infection	Name
Nucleoside reverse transcriptase inhibitor	HIV	Zidovudine (AZT)
		Abacavir
		Didanosine
		Emtricitabine
		Lamivudine (3TC)
		Stavudine (d4T)
		Tenofovir
	HIV, hepatitis B	Adefovir dipivoxil
	Hepatitis C	Entecavir
Protease inhibitor	HIV	Atazanavir
		Darunavir
(Prodrug of amprenavir)		Fosemprenavir
		Indinavir
		Lopinavir
		Nelfinavir
		Ritonavir
		Saquinavir
		Tipranavir
Non-nucleoside reverse transcriptase inhibitor	HIV	Efavirenz
		Etravirine
		Nevirapine
Inhibition of HIV-host fusion	HIV	Enfuvirtide
Antagonist of CCR5 chemokine receptor	HIV	Maraviroc
Inhibitor of HIV integrase	HIV	Raltegravir
Inhibitor of DNA polymerase	Herpes viruses	Acyclovir
(Prodrug of penciclovir)	Herpes zoster and genital herpes	Famciclovir
(Prodrug of aciclovir)	Herpes simplex,	Valaciclovir
	Herpes zoster, cytomegalovirus	Ganciclovir

(*continued*)

Table 5.4 (*continued*)

Type	Viral infection	Name
(Ester of valaciclovir)	Cytomegalovirus	Valganciclovir
	Herpes simplex, cytomegalovirus	Cidofovir
	Herpes simplex, Herpes zoster, Epstein-Barr, vaccinia	Vidarabine
Stimulate cell mediated immune response—mode of action unknown	Herpes simplex	Inosine pranobex
Inactivation of virus-specific DNA polymerase and reverse transcriptase		Foscarnet
Nucleoside analogue; interference with viral nucleic acids synthesis	Hepatitis C, RSV, herpes virus, adenovirus, poxvirus, Lassa fever virus, influenza, measles, mumps, HIV	Ribavirin
Inhibition of viral neuraminidase	Influenza	Oseltamivir
		Zanamivir
Monoclonal antibody	RSV	Palivizumab

Data from BNF 2009.

Table 5.5 Oligonucleotide therapies

Methods	Principles	Effect
Antisense nucleotides	Production of an RNA sequence complementary to single-stranded viral RNA	Triggers the formation of double stranded duplex, inhibiting viral RNA replication
Antigen methods	Formation of triple helix of DNA	Inhibit transcription
Decoy methods	Production of synthetic decoys corresponding to a specific nucleic acid sequence which binds virally encoded regulatory proteins	Inhibit transcription
Ribozymes	Production of RNA molecules (oligo (ribo)nucleotides)	Cleave other RNA sequences at specific sites

where a patient has been exposed to HIV-contaminated materials (e.g. needle injury). Such use follows guidelines available locally (e.g. hospital) or nationally (e.g. Department of Health, British Association for Sexual Health and HIV).

The immune reconstitution syndrome and the lipodystrophy syndrome have been associated with antiretroviral treatments. The latter includes fat redistribution, insulin resistance, hyperglycaemia and dyslipidaemia. In addition, these antivirals can be damaging to liver function and have been associated with osteonecrosis following long-term combination treatments. A number of side effects are commonly associated with the use of antiretrovirals: gastrointestinal disturbance, anorexia, pancreatitis, liver damage, dyspnoea, cough, headache, insomnia, dizziness, fatigue, blood disorders, myalgia, arthralgia, rash, urticaria and fever. Protease inhibitors are metabolized by cytochrome P-450 and therefore have a significant potential for drug interactions. Non-nucleoside reverse transcriptase inhibitors have been shown to interact with a number of drugs metabolized in the liver. They have been associated with a number of side effects such as rash, psychiatric and central nervous system disturbances, and even fatal hepatitis.

6.1.2 Herpesvirus infections

Herpesviridae is a family of viruses that include the herpes simplex virus, chickenpox (varicella), shingles (herpes zoster) and cytomegalovirus. Mild herpes simplex virus infections in healthy individuals are treated with a topical antiviral drug (e.g. treatment of cold sores). However, for primary herpetic gingivostomatitis a change of diet and analgesics are recommended. For severe infections (e.g. neonatal herpes infection, infection in immunocompromised patients) a systemic antiviral drug is used (Table 5.4). Antiviral treatments for chickenpox are recommended in patients at risk and in neonates to reduce risks of severe diseases. In healthy adults, treatment taken with 24 hours of the appearance of a rash may decrease the duration and severity of symptoms. Systemic antivirals are used to decrease the severity and duration of shingles when taken within 72 hours of the onset of rash. Antivirals for herpes are also associated with a number of side effects which vary depending on the drug, but may include nausea, vomiting, stomach pain, headache, fatigue, rash, and increase in serum and urine uric acid. Antivirals for the treatment of cytomegalovirus are usually given to immunocompromised patients and they tend to be more toxic with notable nephrotoxicity (e.g. cidofovir) and a number of documented side effects (e.g. ganciclovir, foscarnet)

6.1.3 Viral hepatitis

Hepatitis B and C are major causes of viral chronic hepatitis. The initial treatment for acute hepatitis B is with interferons (peginterferon alfa-2a) which may reduce the risk of chronic infection. However, the use of interferon is limited by a poor response rate in patients and frequent relapse. A number of antivirals are licensed for the treatment of chronic hepatitis B (Table 5.4). The choice of antivirals depends upon the initial response to peginterferon-alfa, emerging viral resistance, and co-infection with HIV. For the treatment of chronic hepatitis C a combination of ribavarin and peginterferon-alfa is recommended, although the choice and duration of treatment depends upon the viral genotypes and viral load. These antivirals are also associated with a number of side effects including nausea, vomiting, abdominal pain and diarrhoea.

6.1.4 Influenza

Two antivirals are recommended for the treatment of influenza according to the National Institute of Health and Clinical Excellence (NICE) guidelines (Table 5.4). Oseltamivir was extensively used for the prevention and control of the swine flu outbreak in the UK in 2009. Following an intensive use, at least two major limitations in the usefulness of the drug have been identified. First, the drug needs to be taken within a few hours of the onset of symptoms, which proved very difficult with a number of symptoms from mild 'cold-like' to severe 'flu-like' symptoms reported. Second, the side effects, especially in young children and adolescents, have been very severe, prompting many parents to stop the medication, decreasing the willingness to give the antivirals to children who have been possibly exposed to the virus.

6.1.5 Respiratory syncytial virus

Respiratory syncytial virus (RSV) is responsible for severe bronchiolitis notably in infants. A monoclonal antibody (palivizumab) or an antiviral drug (ribavirin) is indicated for the treatment of RSV (Table 5.4). The antiviral is associated with a number of severe side effects.

6.2 Vaccination

Vaccination is undoubtedly the most successful measure against microbial infections, and particularly viral infections. Remarkably, human protection against smallpox

was achieved by Jenner in 1796 with the inoculation of cowpox, well before the 'germ theory' of disease was postulated. A worldwide vaccination program initiated in 1966 led to the eradication of smallpox in 1980. The poliovirus is almost completely eradicated following an intensive worldwide vaccination programme by the World Health Organization.

Vaccines are preparations containing antigens that elicit a specific and active immunity against an infecting agent; they can induce the innate and the adaptive (cellular, humoral) parts of the immune system (Chapter 9). There are currently a number of viral vaccines available against a diverse range of human viruses (Table 5.6). The success of vaccination relies on the prevention of a disease from occurring. Vaccination is particularly indicated to protect persons at risk (e.g. hepatitis B, varicella zoster vaccines for healthcare workers), prior to travelling (hepatitis A virus, Japanese encephalitis, yellow fever, tick-borne encephalitis virus) or to prevent cancer (human papillomavirus vaccine preventing cervival cancer). A vaccine can also be given to protect from a viral outbreak (e.g. measles, mumps and rubella vaccine—MMR) or following exposure to rabies.

Viral vaccines are prepared using different methods, the most common being the use of live attenuated viruses, inactivated viruses or the use of viral components. Viruses or their components can be prepared from animals, fertilized hen's eggs, in suitable cell cultures (see section 5) or in suitable tissues, or by culture of genetically engineered cells. MMR vaccine and live (oral) poliomyelitis vaccines are based on the use of live attenuated viruses, which are not as virulent as the original virus. The attenuated viruses will cause a strong immune response without causing the disease. Hepatitis A virus and influenza vaccines rely on chemically inactivated (formaldehyde, propiolactone) virus particles or components (surface antigens). Hepatitis B virus vaccine is a recombinant vaccine where the viral DNA encoding for a virus surface antigen (HBsAg) is expressed in yeast (*Saccharomyces cerevisiae*) or mammalian cells (Chinese hamster ovary cells or other suitable cell lines). The surface antigen is then purified. Human papillomavirus (HPV) vaccine contains virus-like particles and recombinant capsid protein expressed in yeast or using a baculovirus as an expression system.

Table 5.6 Viral vaccines

Vaccine	Type
Hepatitis A vaccine	Formaldehyde-inactivated hepatitis A virus
Hepatitis B vaccine	Inactivated HBV surface antigen (HBsAg)
HPV vaccine	Virus-like particle composed of the major capsid protein (L1) of HPV type 6, 11, 16 and 18
Influenza vaccine	Formaldehyde-inactivated influenza virus
Japanese encephalitis vaccine	Inactivated Japanese encephalitis virus
MMR vaccine	Live attenuated viruses
Poliomyelitis vaccines	Inactivated poliomyelitis vaccine (injection)
	Live poliomyelitis vaccine (oral)
Rabies vaccine	Inactivated rabies virus
Rotavirus vaccine	Live attenuated rotavirus
Tick-borne encephalitis vaccine	Formaldehyde-inactivated tick-borne encephalitis virus
Varicella zoster vaccine	Live attenuated varicella zoster virus
Yellow fever vaccine	Live attenuated yellow fever virus

HBsAg, hepatitis B virus surface antigen; HPV, human papillomavirus; MMR, measles, mumps and rubella.

It should be noted that most viral vaccines contain one or more adjuvants, e.g. aluminium salts (antigens are absorbed to the aluminium salts), monophosphoryl lipid A, to increase or modulate the host immune response to the antigens.

Immunoglobulin may play a role in the protection of patients with a compromised immunity against viral infections. Human normal immunoglobulin (HNIG) is prepared from a pool of donated human plasma that has been checked to be non-reactive for hepatitis B surface antigen, hepatitis C virus and HIV (types 1 and 2) but contains immunoglobulin G (IgG) and antibodies against viruses that are prevalent in the general population including hepatitis A, measles, mumps, rubella and varicella. Intramuscular normal immunoglobulin is thus used to protect against hepatitis A virus in immunocompromised patients visiting areas where the disease is highly endemic and to protect against or attenuate measles infection in immunocompromised patients. It can also be used for pregnant women against rubella virus, where the risk of termination of pregnancy is unacceptable.

Disease-specific immunoglobulins are prepared from a pool of plasma obtained from specific human donors who have high-levels of the specific antibody required. Disease-specific hepatitis B immunoglobulin is used following accidental inoculation by a risk material (e.g. needlestick injury) or for infants born from mothers infected with the virus. Disease-specific rabies immunoglobulin is available following the bite of an animal suspected of carrying the disease or originating from an area where the disease is endemic. Disease-specific varicella zoster immunoglobulin is indicated for individuals who are at a high risk such as neonates whose mothers develop chickenpox, or for those exposed to the virus while requiring intensive care or prolonged special care, and for immunocompromised individuals.

6.3 Viricidal effects of chemical and physical agents on viruses

Viruses are generally transmitted via surfaces and therefore the use of viricidal disinfectants on hard surfaces and viricidal antiseptics on skin is important. In addition, viruses are often associated with organic materials, such as secretions from the host, blood, faeces etc., which enable them to persist on surfaces for longer periods of time (weeks, months, and rarely years) and to survive better viricidal challenges.

In general, viruses are not particularly resistant to chemical or physical agents, although some exceptions exist. In terms of susceptibility to viricidal agents, small non-enveloped viruses (e.g. poliovirus) are more resistant than large enveloped viruses (e.g. HIV, influenza), the lipid-rich envelope being damaged easily by chemical and physical agents. The susceptibility of large non-enveloped viruses varies, but it is generally considered to be between that of the small naked and large enveloped viruses, however some rotaviruses are proving particularly difficult to destroy.

The viricidal activity of biocides (antiseptics, disinfectants) varies and not all biocides show a strong viricidal activity against non-enveloped viruses (e.g. cationic biocides, phenolics and alcohols). In addition, biocidal activity depends upon a number of factors, such as concentration, contact time, presence of soiling, and formulation (Chapters 18 and 19). Soiling is particularly an issue with water- and food-borne viruses. Indeed, even enveloped viruses can survive for many days on a soiled surface even when exposed to biocides. The interactions between biocides and viruses have been poorly studied. In general, viruses present only a few target sites to biocides, mainly the envelope (when present), glycoproteins, the capsid and viral nucleic acid (Chapter 20). Some biocides (e.g. cationic) are likely to interact with the envelope and glycoproteins, inhibiting viral infectivity, without altering the viral capsid and genome. The main target site is most probably the capsid which has been shown to be severely damaged in the presence of highly reactive biocides such as aldehydes (e.g. glutaraldehyde) and oxidizing agents (e.g. peracetic acid, hydrogen peroxide). Less reactive biocides, such as quaternary ammonium compounds (QACs) and biguanides (e.g. chlorhexidine) have been shown to damage viral capsid to a lesser extent, explaining the limited activity of these agents against non-enveloped viruses. The viral genome is the infectious part of the virus and is the ideal target for biocides. The destruction/alteration of the viral nucleic acid would ensure complete viral inactivation. However, only a limited number of reactive biocides, mainly oxidizing agents (e.g. hydrogen peroxide, chlorine dioxide) have been shown to penetrate within the capsid and damage viral nucleic acid, or to damage viral nucleic acid released from a damaged capsid. The nature of the association of the viral nucleic acid with the capsid also plays a role in the susceptibility of the virus to chemical and physical agents. Viruses with a helical capsid structure might be more susceptible since the destruction/alteration of capsid is more likely to cause damage to the viral nucleic acid which is closely associated to this type of structure.

Physical agents such as heat and irradiation are viricidal and play an important role in the control of viral contaminants in pharmaceutical products. Most viruses are susceptible to exposure to temperatures above 60 °C for 30 minutes. Such susceptibility is used for the inactivation of viral contaminants, such as HIV, in blood products. However, other viruses such as the hepatitis B virus are less susceptible and appropriate assurance of the absence of such a virus is needed. Viruses survive well at low temperatures and they can be routinely stored at −40 °C to −70 °C. In addition to thermal processes, UV irradiation and ionizing radiation (γ-ray and accelerated electrons) are viricidal mainly following the destruction of the viral nucleic acid. Ionizing irradiation and thermal processes are used for terminal sterilization processes applied to medical and pharmaceutical products (Chapter 21).

6.4 Control of viruses in pharmaceutical products

The presence of certain viruses needs to be controlled in pharmaceutical products derived from human and animal origin. This includes blood products such as human plasma for fractionation intended for the manufacture of human antithrombin III, human coagulation factor VII, VIII, IX, XI, dried prothrombin complex, dried fibrinogen, normal immunoglobulin, human α_1-proteinase inhibitor, and human von Willebrand factor, for which tests for the presence of antibodies against HIV-1 and HIV-2, hepatitis B surface antigen (HBsAg), and antibodies against hepatitis C virus are required. For the urine-derived urofollitropin (obtained from postmenopausal women), tests for hepatitis virus antigens and HIV antigen are needed.

The risk of a pharmaceutical product being contaminated by viruses depends mainly on the origin of the product component (e.g. species, organ, tissue), the history of the donor, the amount of material used, the manufacturing process and its capacity to remove/destroy any contaminants. In addition, the infectivity and pathogenicity of possible viral contaminants must be taken into consideration, notably when considering the route of administration of the medicinal product (i.e. transdermal delivery would carry less risk than an injection). A risk assessment is generally carried out for these products containing a component from human or animal origin, which takes into consideration factors affecting the potential level of infectious particles and those related to the use of the product.

Thus stringent controls are applied to the raw materials, in process samples and to the final product. In addition, one or more validated procedures to remove or destroy viruses can be applied. The type of inactivation measures used must be validated against a range of representative viruses (i.e. enveloped, non-enveloped, DNA, and RNA viruses) with different degrees of resistance to that type of treatment. Furthermore, early inactivation limits the extent of contamination. For the preparation of vaccines, the inactivation process must ensure that it does not affect the antigenicity while killing the virus and other potential contaminants such as mycoplasmas; for example for the preparation of influenza vaccine, the inactivation process chosen must cause minimum alteration of the haemagglutinin and neuraminidase antigens.

7 Viruses and gene therapy

Certain viruses or virus components are now used as vectors for the delivery of genes to targeted cells. A number of viruses are being used in gene transfer medicinal products (GTMP) and these include adenoviruses (AAV), poxviruses, retroviruses, lentiviruses, adeno-associated viruses and herpesviruses. Viral vectors for human use are freeze-dried or liquid preparations of recombinant viruses, genetically modified to transfer genetic material to human somatic cells *in vivo* (i.e. injected directly into the patient's body) or *ex vivo* (i.e. transferred into host cells before administration).

There are different approaches for the design and construction of viral vectors. The chosen approach depends upon the type of virus used. The procedure aims to minimize the risk of generating replicating viruses or to eliminate helper viruses when used during production. In addition, a number of stringent controls are performed ensuring the complete genetic and phenotypic characterization of the viral vector is carried out. These include the complete sequence of the genome of the viral vector, verification of genomic integrity of the vector, determination of the concentration of the infectious vector, residual host-cell protein and DNA, and residual reagents including antibiotics.

For retroviridae-derived vectors, genetic modification aims to ensure that the recombinant retroviruses are rendered replication-incompetent. Adeno-associated virus vectors (rAAV) are deficient adenovirus in which certain genes (i.e. *cap* and *rep*) necessary for viral replication have been replaced. A helper virus is thus needed during production of the rAAV and needs to be eliminated from the final GTMP. As with other viral vectors, the sequence integrity of the viral genes and expression cassette as well

as genetic stability need to be controlled, and the absence of wild-type virus (e.g. AAV) verified.

8 Viruses as antimicrobials

8.1 Bacteriophages

Bacteriophages (phages) are viruses that infect only bacteria. They were first described at the end of the 19th century. They are typically 20–200 nm in size and are highly diverse in their structure and host range and it is likely that all bacterial species can be infected by a phage. Phages are extremely specific in their host range and some will only infect a specific bacterial strain. Such a high specificity is used for bacterial typing as discussed below. The most studied phages are complex ones (e.g. T-phage) sometimes referred to as 'tadpole-shaped' consisting of a head (often icosahedral) that contains the viral genome and a tail which function is to recognize the host receptor, attach and subsequently serve as a nucleic acid injection device (Figure 5.5). Indeed, one of the main differences between such phages and common mammalian viruses is that these phages inject their viral genome inside the host cell.

Phages have proved to be very useful genetic tools over the years, since they are easy to propagate to high concentration and easy to study. Because of their similarity to mammalian viruses, it is not surprising that phages have been used to elucidate the viral multiplication cycle and their study has led to many discoveries such as mRNA, the understanding of the genetic code and the control of genes, contributing to important advances in molecular biology.

From the study of phage replication cycles, two scenarios have emerged, one resulting in the lysis of the bacterial host—the *lytic cycle*—and the other resulting in the viral nucleic acid being integrated into the host genome—the *lysogenic cycle* (Figure 5.6). Infection with a lytic phage, also called *virulent* phage, results in the replication of the phage within the susceptible bacteria and the release of infectious phage progeny from the host cell following cell lysis. Such a lytic property is used to enumerate phages. Phages inoculated on to a lawn of a susceptible host bacterium form clear 'holes' (plaques) which result from phage infection and lysis of a bacterial host and the release of phage progeny, subsequently infecting, replicating in, and lysing adjacent cells, ultimately forming these *plaques* which are easily identifiable with the naked eye (Figure 5.7). Since each of these plaques is assumed to result from the infection from a single phage, the number of plaques counted is used to represent the number of phages.

In the lysogenic cycle, the viral nucleic acid which has integrated the host genome is called *prophage*, and the host cell that contains the viral genome *lysogenic.* Following infection with lysogenic phages, both a lytic and lysogenic responses are observed. The integration of the prophage ensures that the viral genome is passed on to the daughter cells following bacterial cell replication. Lysogeny is an extremely common phenomenon and through evolution most bacteria will host several prophages. Indeed, sequencing of the whole bacterial genome often indicates the presence of prophages (or their remnants), that have become disabled with time. On occasions, a prophage dormant in its host can be reactivated and resume a lytic cycle. Upon excision from the

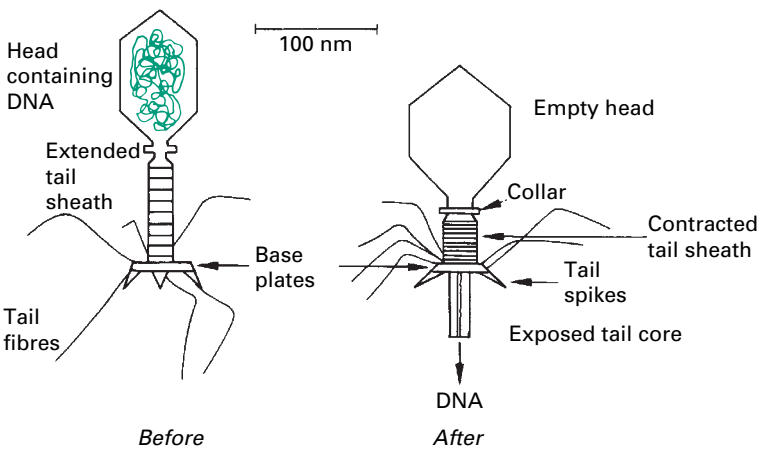

Figure 5.5 T-even phage structure before and after tail contraction.

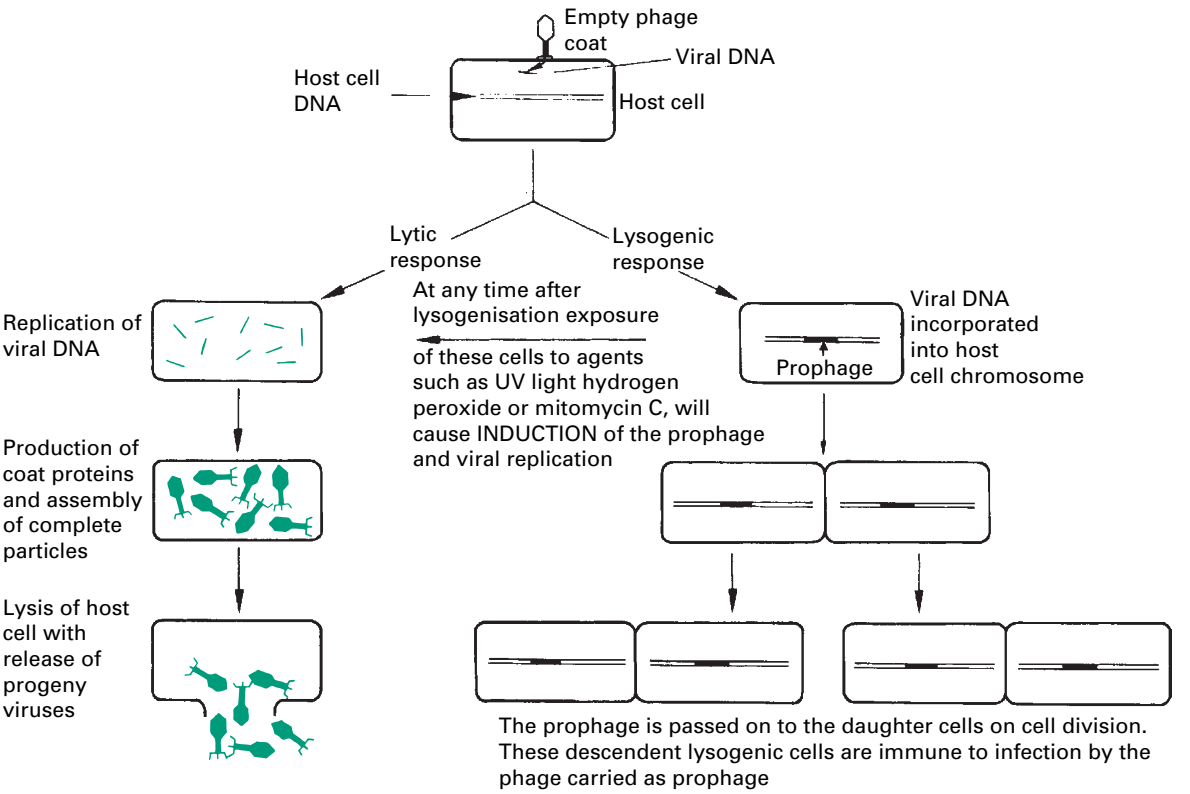

Figure 5.6 Scheme to illustrate the lytic and lysogenic responses of bacteriophages.

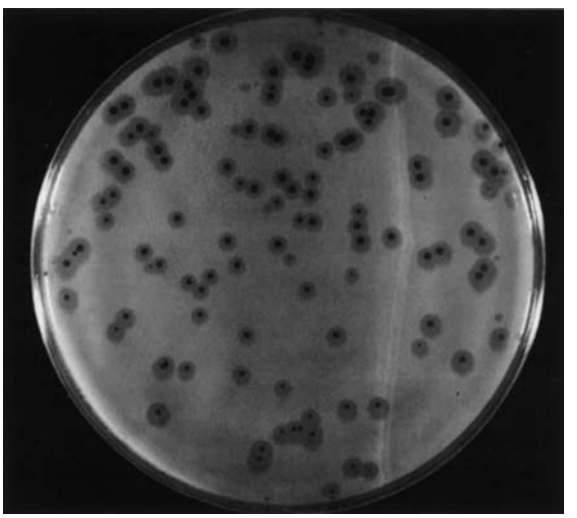

Figure 5.7 Plaques formed by phage on a plate seeded with *Bacillus subtilis*.

host genome, the prophage can take adjacent bacterial genes that become incorporated in the virion and transmitted to a new susceptible host cell. Genes carried on the prophage can then be expressed in the new host. This process is called *transduction* and is responsible for gene transfer between bacteria. Sometimes, genetic factors transferred by transduction encode for antibiotic resistant determinants and/or virulence factors such as toxins. The induction of prophages to a lytic cycle can be artificially triggered by exposure to chemical and physical agents such as mitomycin C and ultraviolet light. If the use of lytic phages might be appropriate to kill bacteria, the use of lysogenic phages is best confined to the genetic engineering of bacteria.

8.2 Use of bacteriophages to treat bacterial infection

The discovery of the lytic property of phages quickly resulted in their use as a potential bactericidal agent at the beginning of the 20th century, to combat bacteria responsible for dysentery outbreaks, first against *Vibrio*

cholera (work of Hankin) and then *Shigella shiga* (work of d'Herelle). Phages targeting bacteria causing a number of diseases such as anthrax, scarlet fever, cholera and diphtheria were quickly isolated, but with the exception of 'cholera' phages these did not result in useful treatments. The introduction of antibiotics in the early 1940s resulted in the end of phage therapy in the West, although it continues in the former Soviet Union, where it was used to treat a variety of bacterial infections in both the First and Second World Wars and is still used nowadays in clinical practice (Chapter 27).

With the threat of bacterial resistance to antibiotics, there has been a renewed interest in the use of phages to control bacterial infection and product contamination and this has led to the licensing of phage products for a number of applications; the use of phages to combat *Listeria monocytogenes* in ready-to-eat meat and poultry products was authorized by the US Food and Drug Administration (FDA) in 2006 and in cheeses by the EU commission in 2009. In the UK, a phage-based product to combat *Pseudomonas aeruginosa* in ear infection is now in phase III clinical trials. Phage preparations are also successfully employed in aquaculture, notably against *Lactococcus garvieae*, the cause of a serious fish disease.

There are a number of ways to prepare a phage product. 'Natural' phages from the environment can be selected on the basis of their activity and incorporated into a product on the basis that they are lytic and do not contain any detrimental genes encoding for example antibiotic resistance or bacterial virulence factors. Often several phages (cocktail) attacking the same species or strain are added to the product to minimize the risk of emerging bacterial resistance. Non-replicating phages have been used with some degree of success, but in this case the lytic effect is short lived. Genetically modified phages have also been used, whereby detrimental genes can be removed and phage virulence genes added. Another advantage of using genetically modified phages is commercial where patents might be easier to file. Finally, lysogenic phages can be used following treatment to remove their lysogenic property.

Phage-based products can be developed using phage components, mainly phage lytic enzymes which are employed during phage penetration and during virion excision from the host cell. Phage lytic enzymes (e.g. peptides) can be harvested and use on their own to lyse/kill bacteria (Chapter 27).

The use of phages for surface disinfection and antisepsis or for the treatment of a bacterial infection (phage therapy) is at an early stage and further work is needed to develop appropriate phage-based products, notably the effect of the different routes of administration on phage viability and effectiveness. Animal studies have shown that the route of administration is crucial for phage efficacy. Phage therapy is unlikely to supplant antibiotic therapy in the future; however, it is highly probable that commercially available phage products will increase as the advantages of phage therapy outweigh its disadvantages (Table 5.7 and Chapter 28).

8.3 Epidemiological uses and diagnosis

The high specificity of phage against their host leads to other applications. Phage typing is a method that differentiates distinct strains of the same bacterial species on the basis of their susceptibility to phages. This has been particularly relevant with bacteria such as *Salmonella enterica* serovar Typhi and to some extent *Staphylococcus aureus* that can be 'typed' during an outbreak, and thus provides some important epidemiological information. Molecular techniques based on genetic differences are now preferred for *S. aureus*.

A further application for phage is in developing rapid diagnostics, notably against slow-growing bacteria such as *Mycobacterium tuberculosis*. One specific kit relies on the protection of TB phages from an externally applied viricide when inside the targeted host, *M. tuberculosis* if present; if the bacterium is not present in the sputum or other biological fluid sample then the phages will be unprotected and destroyed. Phages that are protected and able to propagate are then harvested and inoculated to a lawn of rapidly growing susceptible mycobacteria. The presence of plaques will indicate that the phages were able to replicate first within *M. tuberculosis*, i.e. a positive sample.

9 Prions

The causative agents of the neurodegenerative diseases bovine spongiform encephalopathy (BSE), scrapie in sheep and Creutzfeldt–Jakob disease (CJD) in humans used to be referred to as slow viruses. However, it is now clear that they are caused by a distinct class of infectious agents termed *prions* that have unique and disturbing properties. They can be recovered from the brains of infected individuals as rod-like structures which are oligomers of a 30 kDa glycoprotein. They are devoid of nucleic acid and are extremely resistant to heating and ultraviolet irradiation. They also fail to produce an immune response in the host. Just how such proteins can

Table 5.7 Some advantages and disadvantages of phage therapy

Advantages	Disadvantages
Highly specific	High specificity requires pathogen identification Need for 'custom' treatments based on organisms present
Replicate at site of infection Less frequent dosing required Active against bacterial biofilms	Bacterial resistance can emerge—the use of a cocktail containing several distinct phages is often proposed
No reported side effects	Phage therapy could result in toxic shock from bacterial lysis; i.e. release of bacterial components such as lipopolysaccharide wall material Potentially immunogenic
Easy to isolate and propagate Phages can be developed against a wide range of bacterial hosts Believed that every bacterium will possess at least one phage due to natural selection	The genomes of selected phages need to be sequenced to ensure they do not contain unwanted genes
Can be easily manipulated; e.g. incorporated into capsules, etc. for ease of delivery	Quality assurance controls are complex and will need to address biological variation, phage yield, and endotoxin limits
Development and production costs may be less than a new antibiotic	

replicate and be infectious has only recently been understood. It seems that a glycoprotein (designated PrPc) with the same amino acid sequence as the prion (PrPsc) but with a different tertiary structure, is present in the membranes of normal neurons of the host. The evidence suggests that the prion form of the protein combines with the normal form and alters its configuration to that of the prion. The newly formed prion can then in turn modify the folding of other PrPc molecules. In this way the prion protein is capable of autocatalytic replication. As the prions slowly accumulate in the brain, the neurons progressively vacuolate. Holes eventually appear in the grey matter and the brain takes on a sponge-like appearance. The clinical symptoms take a long time to develop, up to 20 years in humans, but the disease has an inevitable progression to paralysis, dementia and death.

It is now clear that the large-scale outbreak of BSE that began in the UK during the 1980s resulted from feeding cattle with supplements prepared from sheep and cattle offal. The recognition of this fact led to changes in animal feed policies and eventually to the imposition of a ban on the human consumption of bovine brain, spinal cord and lymphoid tissues that were considered to be potentially infectious. Unfortunately people had been consuming potentially contaminated meat for a number of years. Concerns that the agent had already been disseminated to humans in the food chain were realized in 1996 with the advent of a novel human disease that was called *variant* or vCJD. This condition was unusual as it attacked young adults with an average age of 30 rather than the 60-year-olds who typically succumb to classical sporadic CJD. Studies on the experimental transmission of prions to mice provided evidence that vCJD represents infection by the BSE agent. The pathology in the mouse brain induced by the vCJD agent and the incubation time of the disease are different from that of classical CJD and very similar to that of BSE. Gel electrophoresis of the polypeptides from the brains of infected mice revealed that the different transmissible spongiform encephalitis agents have characteristic molecular signatures. These signatures are based on the lengths of protease-resistant fragments and the glycosylation patterns on the prion molecules. The patterns from vCJD agent were very different patterns from those of the classical CJD but remarkably similar to those formed by BSE.

Since 1996 there has been a slow but gradual increase in the numbers of confirmed cases of vCJD. By November 2010 the number of deaths in the UK from vCJD had reached 170. As the average incubation time for vCJD is not yet known, it is difficult to estimate how many more cases will develop. The measures taken to protect the public will hopefully have prevented any further human infections, but sadly no effective treatment is available for those who have already contracted the disease.

10 Further reading

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6 Protozoa

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1 Introduction

1.1 Parasitism

Parasitism is a specific type of interaction between two organisms that has many features in common with other infectious processes, but host–parasite interactions often operate over a longer timescale than those seen with other pathogens. This extended process results in significant host–parasite interaction at the cellular and organismal level. It is known, for example, that some parasites alter the behaviour of the host, while others, such as *Giardia lamblia,* induce biochemical change in the host cells at the site of infection (the duodenal epithelium). Most parasites have a life cycle that often involves several hosts; this means that survival and transmission between different hosts requires the parasite to exhibit more than one physiologically distinct form.

1.2 Habitats

Parasites inhabit a wide range of habitats within their hosts. Some parasites will inhabit only one site throughout their life cycle, but many move to various sites within the body. Such movement may require the formation of motile cellular forms, and it will produce a significant change in the physiology and morphology of the parasite as a result of environmental change. Parasites moving from the gut to other tissues, for example, will encounter

Hugo and Russell's Pharmaceutical Microbiology, Eighth Edition. Edited by Stephen P. Denyer, Norman Hodges, Sean P. Gorman, Brendan F. Gilmore.

higher levels of oxygen, changes in pH and significant exposure to the host immune response. When life cycles involve more than one host organism these changes are greater. The reasons why parasites move to various sites in with the host is driven by evasion of host immune attack and to aid transmission.

1.3 Physiology of parasitic protozoa

Parasitic protozoa, like their free-living counterparts, are single-celled eukaryotic organisms that utilize flagella, cilia or amoeboid movement for motility. The complexity of some parasite life cycles means that some species may exhibit, at different times, more than one form of motility. All pathogenic protozoa are heterotrophs, using carbohydrates or amino acids as their major source of carbon and energy. Some parasitic protozoa utilize oxygen to generate energy through oxidative phosphorylation, but many protozoan parasites lack functional or 'typical' mitochondria, or have mitochondria that do not function like those in mammalian cells. As a result of this adaptation many parasites exhibit a fermentative metabolism that functions even in the presence of oxygen. The reason for the utilization of less efficient fermentative pathways is not clear, but it is presumably due in part to the fact that such parasites survive in environments where oxygen is only present occasionally or at low levels. For some parasites oxygen is toxic, and they appear to utilize it possibly in an effort to remove it and thus maintain an anaerobic metabolism.

The metabolism of parasites is highly adapted, with many possessing unique organelles such as kienetoplasts and hydrogenosomes. Many synthetic pathways that are found in other eukaryotes are absent because many metabolic intermediates or precursors such as lipids, amino acids and nucleotides are actively scavenged from their environment. This minimizes energy expenditure, which is finely balanced in parasites and means that the membrane of parasitic protozoa is rich in transporters. Secretion of haemolysins, cytolysins, proteolytic enzymes, toxins, antigenic and immunomodulatory molecules that reduce host immune response also occurs in pathogenic protozoa.

Survival of parasites is partly due to their high rate of reproduction, which may be either sexual or asexual; some organisms such as *Plasmodium* exhibit both forms of reproduction in their life cycle. Simple fission is characteristic of many amoeba, but some species also undergo nuclear division in the cystic state (cysts are forms required for survival outside the host) with each nucleus giving rise to new trophozoites (the growing, motile and pathogenic form).

2 Blood and tissue parasites

This section considers the life cycles, disease and pathology of some blood and tissue parasites; this is not an exhaustive list but covers some of the most important species. These diseases are commonly associated with travel to tropical and subtropical countries, but diseases such as leishmaniasis are frequently seen in southern Spain and France. It should also be noted that climate change is altering the geographical distribution of many parasitic diseases.

2.1 Malaria

Malaria has been a major disease of humankind for thousands of years. Despite the availability of drugs for treatment, malaria is still one of the most important infectious diseases of humans, with approximately 200–500 million new cases and 1–2.5 million deaths each year. Protozoa of the genus *Plasmodium* cause malaria and four species are responsible for the disease in humans: *P. falciparum, P. vivax, P. ovale* and *P. malariae*. *P. falciparum* and *P. vivax* account for the vast majority of cases, although *P. falciparum* causes the most severe disease. Other species of plasmodia infect reptiles, birds and other mammals. Malaria is spread to humans by the bite of female mosquitoes of the genus *Anopheles* but transmission by inoculation of infected blood and through congenital routes is also seen. These mosquitoes feed at night and their breeding sites are primarily in rural areas.

2.1.1 Disease

The most common symptom of malaria is fever, although chills, headache, myalgia and nausea are frequently seen and other symptoms such as vomiting, diarrhoea, abdominal pain and cough occasionally appear. In all types of malaria, the periodic febrile response (fever) is caused by rupture of mature schizonts (one of the cell forms arising as part of the life cycle). In *P. vivax* and *P. ovale* malaria fever occurs every 24–48 hours, whereas in *P. malariae,* maturation occurs every 72 hours. In falciparum malaria fever may occur every 48 hours, but is usually irregular, showing no distinct periodicity. Apart from anaemia, most physical findings in malaria are often non-specific and offer little aid in diagnosis, although enlargement of some organs may be seen after prolonged infection. If the diagnosis of malaria is missed or delayed, especially with *P. falciparum* infection, potentially fatal complicated malaria may develop. The most frequent and serious complications of malaria are cerebral malaria and severe anaemia.

2.1.2 Life cycle

Plasmodia have a complex life cycle (Figure 6.1) involving a number of life cycle stages and two hosts. The human infective stage comprises the sporozoites (*c.*1–7 μm), which are produced by sexual reproduction in the midgut of the mosquito (vector) and migrate to its salivary gland. When an infected *Anopheles* mosquito bites a human, sporozoites are injected into the bloodstream and are thought to enter liver parenchymal cells within 30 minutes of inoculation. In these cells the parasite differentiates into a spherical, multinucleate schizont which may contain 2000–40 000 uninucleate merozoites. This process of growth and development is termed *exoerythrocytic schizogony*. This exoerythrocytic phase usually takes between 5 and 21 days, depending on the species of *Plasmodium*; however, in *P. vivax* and *P. ovale* the maturation of schizonts may be delayed for up to 1–2 years. These 'quiescent' parasites are called *hypnozoites*. Clinical illness is caused by the erythrocytic stage of the parasite life cycle; no disease is associated with sporozoites, the developing liver stage of the parasite, the merozoites released from the liver, or gametocytes.

The common symptoms of malaria are due to the rupture of erythrocytes when erythrocytic schizonts mature (Figure 6.2a). This release of parasite material triggers a host immune response, which in turn induces the formation of inflammatory cytokines, reactive oxygen intermediates and other cellular products. These proinflammatory molecules play a prominent role in pathogenesis, and are probably responsible for the fever, chills, sweats, weakness and other systemic symptoms associated with malaria. In *P. falciparum* malaria, infected erythrocytes adhere to the endothelium of capillaries and postcapillary venules, leading to obstruction of the microcirculation and localized anoxia. The pathogenesis of anaemia appears to involve haemolysis or phagocytosis of parasitized erythrocytes and ineffective erythropoiesis.

2.2 Trypanosomatids

The family Trypanosomatidae consists of two genera, *Trypanosoma* and *Leishmania*. These are important pathogens of humans and domestic animals and the diseases they cause constitute serious medical and economic problems. Because these protozoans have a requirement for haematin obtained from blood, they are called *haemoflagellates*. The life cycles of both genera involve insect and vertebrate hosts and have up to eight life cycle stages, which differ in the placement and origin of the flagellum. Trypanosomatids have a unique organelle called the kinetoplast. This appears to be a special part of the mitochondrion and is rich in DNA. Two types of DNA have been found in the kinetoplast: *maxicircles* that encode many mitochondrial enzymes, and *minicircles*, which serve a function in the process of RNA editing. Replication of trypanosomatids occurs by single or multiple fission, involving first the kinetoplast, then the nucleus, and finally the cytoplasm. There are four major diseases associated with this group: Chagas disease is caused by *Trypanosoma cruzi*; sleeping sickness (African trypanosomiasis) is associated with *T. brucei*; cutaneous and mucocutaneous leishmaniasis are caused by a range of species including *Leishmania tropica*, *L. major*, *L. mexicana, L. amazonensis* and *L. braziliensis*; and visceral leishmaniasis, which is also known as kala-azar, is typically caused by *L. donovani.*

2.2.1 American trypanosomiasis (Chagas disease)

Chagas disease begins as a localized infection that is followed by parasitaemia and colonization of internal organs and tissues. Infection may first be evidenced by a small tumour (chagoma) on the skin. Symptoms of the disease include fever, oedema and myocarditis (infection of the heart muscle) with or without heart enlargement, and meningoencephalitis in children. The acute disease is frequently subclinical and patients may become asymptomatic carriers; this chronic phase may result, after 10–20 years, in cardiopathy. Chagas disease is transmitted by several genera of triatomine bugs (*Triatoma, Rhodnius* and *Panstrongylus*) and in nature the disease exists among wild mammals and their associated triatomines. Human trypanosomiasis is seen in almost all countries of the Americas, including the southern USA, but the main foci are in poor rural areas of Latin America.

T. cruzi exhibits two cell types in vertebrate hosts, a blood form termed a trypomastigote, and in the tissues (mainly heart, skeletal and smooth muscle, and reticuloendothelial cells) the parasite occurs as an amastigote (Figure 6.3). Trypomastigotes ingested when the insect takes a blood meal from an infected host transform into epimastigotes in the intestine. Active reproduction occurs and in 8–10 days metacyclic trypomastigote forms appear which are flushed out of the gut with the faeces of the insect. These organisms are able to penetrate the vertebrate host only through the mucosa or abrasions of the skin; hence, transmission does not necessarily occur at every blood meal. Within the vertebrate the trypomastigotes transform into amastigotes, which, after a period of intracellular multiplication at the portal of entry, are

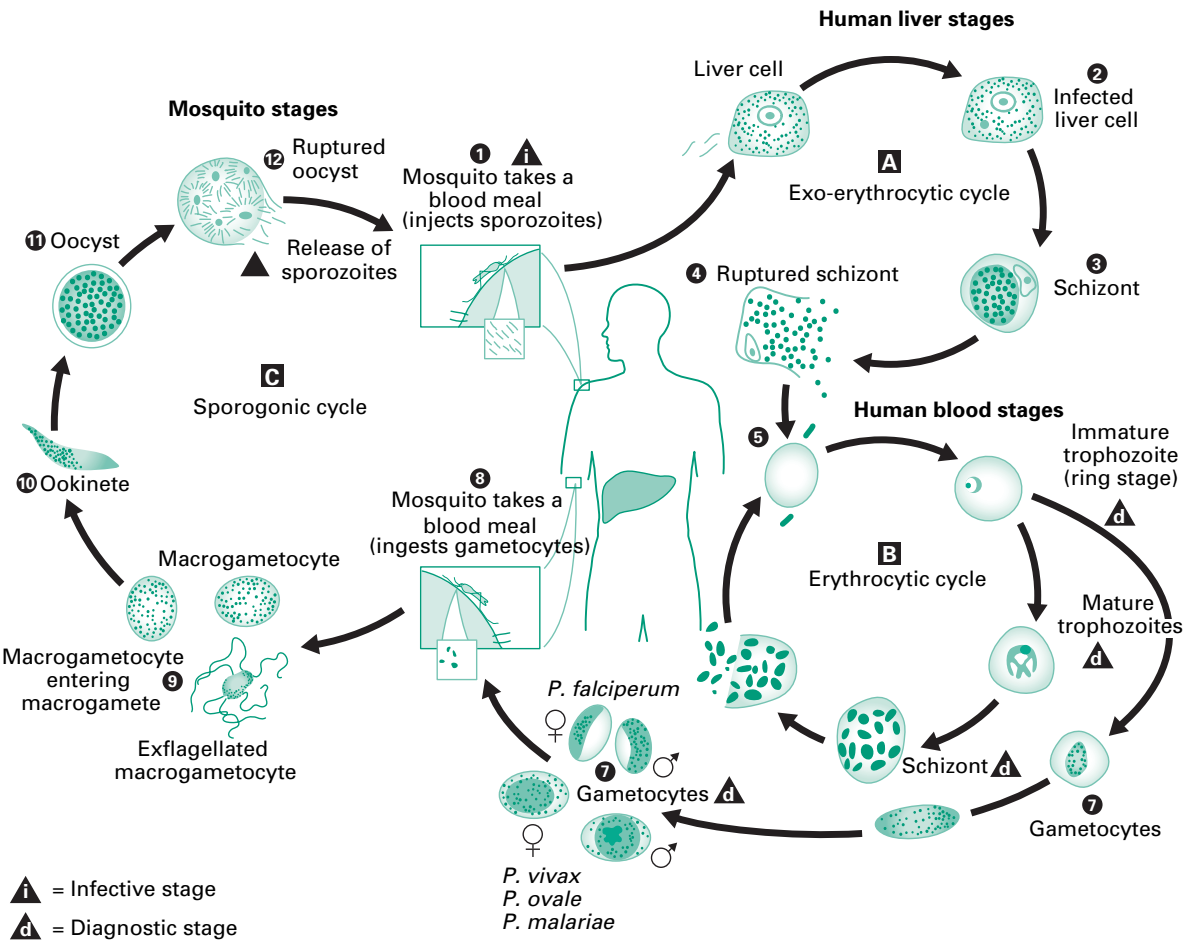

Figure 6.1 The malaria parasite life cycle involves two hosts. During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host (1). Sporozoites infect liver cells (2) and mature into schizonts (3), which rupture and release merozoites (4). (Of note, in *P. vivax* and *P. ovale* a dormant stage (hypnozoites) can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.) After this initial replication in the liver (exo-erythrocytic schizogony, A), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony, B). Merozoites infect red blood cells (5). The ring stage trophozoites mature into schizonts, which rupture releasing merozoites (6). Some parasites differentiate into sexual erythrocytic stages (gametocytes) (7). Blood-stage parasites are responsible for the clinical manifestations of the disease. The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal (8). The parasite's multiplication in the mosquito is known as the sporogonic cycle (C). While in the mosquito's stomach, the microgametes penetrate the macrogametes, generating zygotes (9). The zygotes in turn become motile and elongated (ookinetes) (10), which invade the midgut wall of the mosquito where they develop into oocysts (11). The oocysts grow, rupture and release sporozoites (12), which make their way to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle (1).

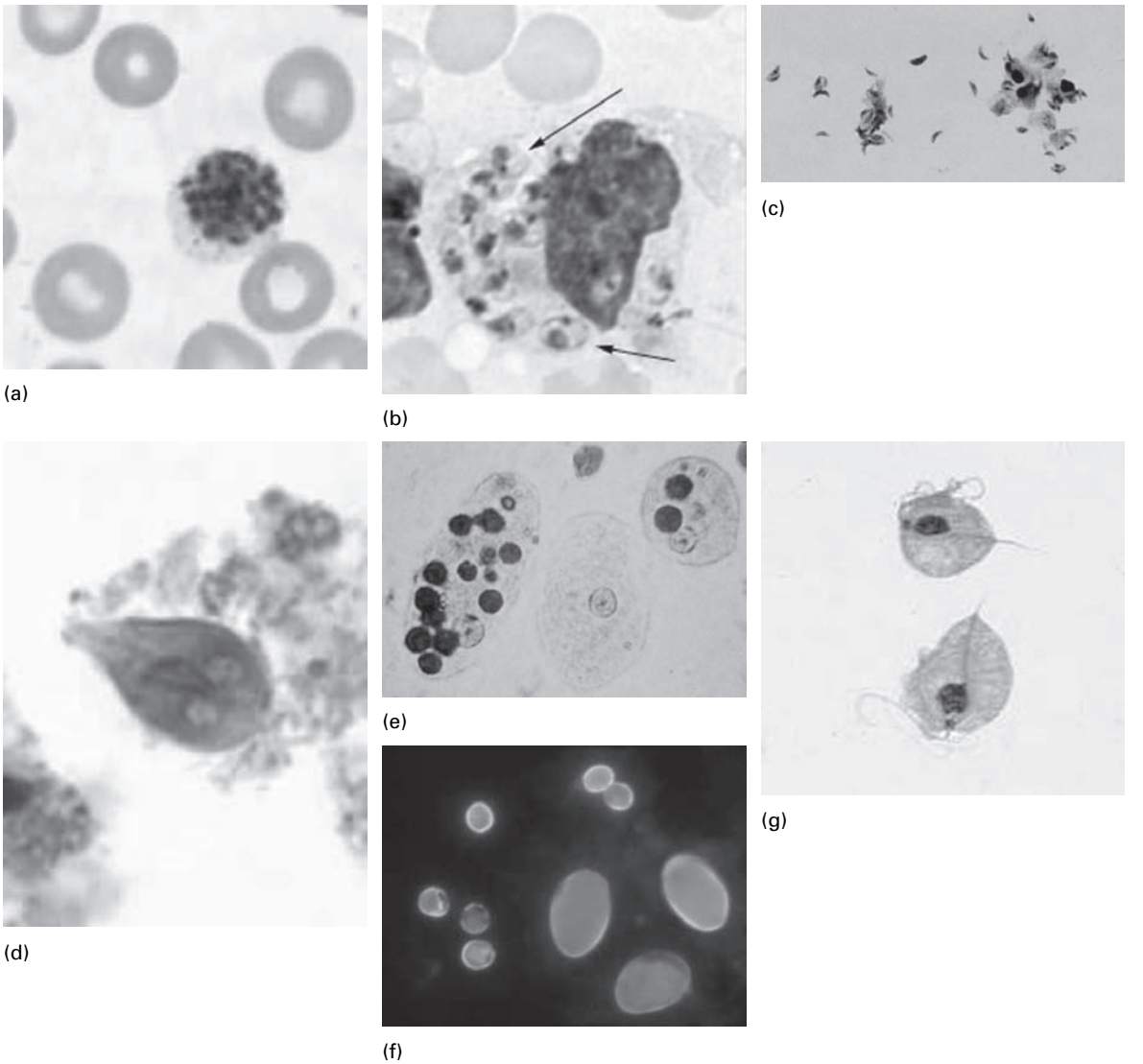

Figure 6.2 (a) Mature schizonts of *Plasmodium vivax. P. vivax* schizonts are large, have 12–24 merozoites, and may fill the red blood cell (RBC). RBCs are enlarged 1.5–2 times and may be distorted. Under optimal conditions Schüffner's dots may be seen. (b) *Leishmania tropica* amastigotes within an intact macrophage. (c) *Toxoplasma gondii* trophozoites in the bronchial secretions from an HIV-infected patient. (d) Trophozoites of *Giardia intestinalis*. Each cell has two nuclei and is 10–20 mm in length. (e) Trophozoites of *Entamoeba histolytica* with ingested erythrocytes, which appear as dark inclusions. (f) Oocysts of *Cryptosporidium parvum* (upper left) and cysts of *Giardia intestinalis* (lower right) labelled with immunofluorescent antibodies. (g) Trophozoites of *Trichomonas vaginalis.*

released into the blood as trypanosomes; these invade other cells or tissues, becoming amastigotes again.

The pathology of the infection is associated with inflammatory reactions in infected tissues. These can lead to destruction of the infected tissue and if it involves heart tissue this can cause acute myocarditis. Parasite enzymes may also cause cell and tissue damage. In the absence of parasites, an autoimmune pathological process seems to be mediated by T lymphocytes (CD4+) (see Chapter 9) and by the production of certain cytokines;

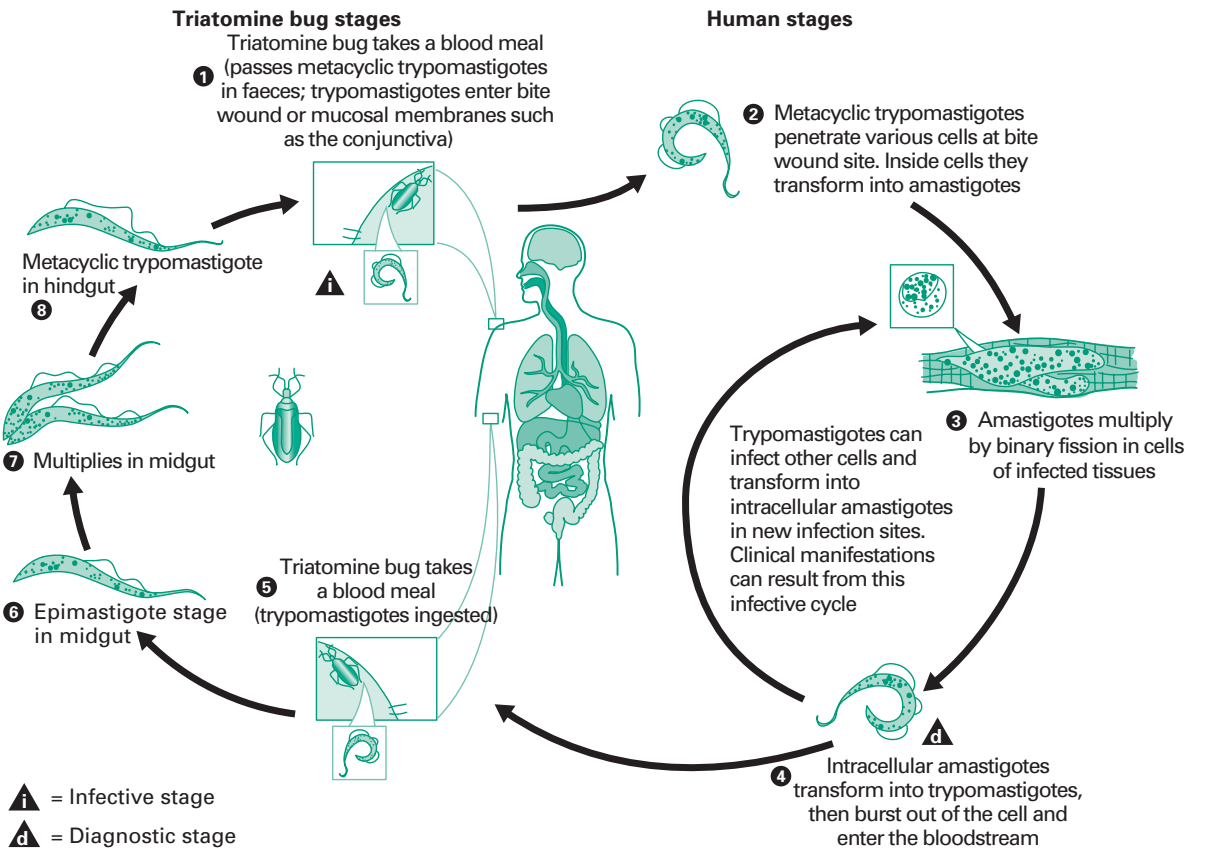

Figure 6.3 An infected triatomine insect vector (or 'kissing' bug) takes a blood meal and releases trypomastigotes in its faeces near the site of the bite wound. Trypomastigotes enter the host through the wound or through intact mucosal membranes, such as the conjunctiva (1). Common triatomine vector species for trypanosomiasis belong to the genera *Triatoma*, *Rhodinius* and *Panstrongylus*. Inside the host, the trypomastigotes invade cells, where they differentiate into intracellular amastigotes (2). The amastigotes multiply by binary fission (3) and differentiate into trypomastigotes, and then are released into the circulation as bloodstream trypomastigotes (4). Trypomastigotes infect cells from a variety of tissues and transform into intracellular amastigotes in new infection sites. Clinical manifestations can result from this infective cycle. The bloodstream trypomastigotes do not replicate (different from the African trypanosomes). Replication resumes only when the parasites enter another cell or are ingested by another vector. The 'kissing' bug becomes infected by feeding on human or animal blood that contains circulating parasites (5). The ingested trypomastigotes transform into epimastigotes in the vector's midgut (6). The parasites multiply and differentiate in the midgut (7) and differentiate into infective metacyclic trypomastigotes in the hindgut (8). *Trypanosoma cruzi* can also be transmitted through blood transfusions, organ transplantation, transplacentally, and in laboratory accidents.

these induce a polyclonal activation of B-lymphocytes and the secretion of large quantities of autoantibodies.

2.2.2 African trypanosomiasis (sleeping sickness)

Sleeping sickness (African trypanosomiasis) is caused by *Trypanosoma brucei*, of which there are two morphologically indistinguishable subspecies: *T. brucei rhodesiense* and *T. brucei gambiense*. After infection the parasite undergoes a period of local multiplication then enters the general circulation via the lymphatics. Recurrent fever, headache, lymphadenopathy and splenomegaly may occur. Later, signs of meningoencephalitis appear, followed by somnolence (sleeping sickness), coma and death.

T. brucei, unlike *T. cruzi,* multiplies in the blood or cerebrospinal fluid. Trypanosomes ingested by a feeding

fly must reach the salivary glands within a few days, where they reproduce actively as epimastigotes attached to the microvilli of the salivary gland where they transform into metacyclic trypomastigotes, which are found free in the lumen. Around 15–35 days after infection the fly becomes infective through its bite.

The pathology of the infection is due to inflammatory changes associated with an induced autoimmune demyelination of nerve cells. Interestingly, the immunosuppressive action of components of the parasite's membrane is probably responsible for frequent secondary infections such as pneumonia. Liberation of common surface antigens (the mechanism involved in immune evasion) in every trypanolytic crisis (episode of trypanosome lysis) leads to antibody and cell-mediated hypersensitivity reactions. It is believed that some cytotoxic and pathological processes are the result of biochemical and immune mechanisms.

2.2.3 Cutaneous and mucocutaneous leishmaniasis

Leishmaniasis is the term used for diseases caused by species of the genus *Leishmania* that are transmitted by the bite of infected sand flies. The lesions of cutaneous and mucocutaneous leishmaniasis are localized to the skin and mucous membranes. Visceral leishmaniasis is a much more severe disease, which involves the entire reticuloendothelial system, and is discussed in section 2.2.4 of this chapter. Cutaneous leishmaniasis appears 2–3 weeks after the bite of an infected sand fly as a small cutaneous papule; this slowly develops and often becomes ulcerated and develops secondary infections. Secondary or diffuse lesions may develop. The disease is usually chronic but may occasionally be self-limiting. Leishmaniasis from a primary skin lesion may involve the oral and nasopharyngeal mucosa. *Leishmania* species that infect humans are all morphologically similar and only exhibit one form, the intracellular amastigotes (3–6 μm long and 1.5–3 μm in diameter). Promastigotes (Figure 6.2b) are found in the sand fly.

In mammalian hosts amastigotes are phagocytosed by macrophages, but resist digestion and divide actively in the phagolysosome (Figure 6.4). The female sand fly ingests parasites in the blood meal from an infected person or animal and these pass into the stomach where they transform into promastigotes, and multiply actively. The parasites attach to the walls of the oesophagus, midgut and hindgut of the fly, and some eventually reach the proboscis and are inoculated into a new host.

The obvious symptoms of this infection are caused by the uptake of parasites by local macrophages. Host response to infection produces tubercle-like structures designed to limit the spread of infected cells. Some lesions may resolve spontaneously after a few months but other types of lesion may become chronic, sometimes with lymphatic and bloodstream dissemination. In infections due to *L. braziliensis* there is a highly destructive spread of infected macrophages to the oral or nasal mucosa. In *L. mexicana*, *L. amazonensis* and *L. aethiopica* infections the disease becomes more disseminated. The immunological response of the host plays an important factor in determining the precise pathology of the disease and this is apparent from the more severe type of infection seen in individuals with HIV. In Europe and Africa several rodents may act as reservoirs of the disease, but in countries such as India, transmission can occur in a human–sand fly–human cycle without rodent intervention. In rural semi-arid zones of Latin America, both wild and domestic dogs enter the epidemiological chain and the vector is a common sand fly, *Lutzomyia longipalpis*, abundant in and around houses. The disease is more common in children in both Latin America and the Mediterranean area.

2.2.4 Visceral leishmaniasis (kala-azar)

Like cutaneous leishmaniasis, visceral leishmaniasis begins with the formation of a nodule at the site of inoculation but this lesion rarely ulcerates and usually disappears in a few weeks. However, symptoms and signs of systemic disease such as undulating fever, malaise, diarrhoea, organ enlargement and anaemia subsequently develop. In more serious cases of visceral leishmaniasis the parasites, which can resist the internal body temperature, invade internal organs (liver, spleen, bone marrow and lymph nodes), where they occupy the reticuloendothelial cells. The pathogenic mechanisms of the disease are not fully understood, but enlargement occurs in those organs that exhibit marked cellular alteration such as hyperplasia. Parasitized macrophages replace tissue in the bone marrow. Patients with advanced disease are prone to superinfection with other organisms which are predominantly bacterial.

2.3 Toxoplasma gondii

The term *coccidia* describes a group of protozoa that contains the genus *Cryptosporidium* (see intestinal parasites) as well as a number of important veterinary parasites. *Toxoplasma gondii* is an intestinal coccidian but the major pathology of infection is associated with other tissues and organs. *T. gondii* infects members of the cat family as definitive hosts and has a wide range of intermediate hosts. Infection is common in many warm-blooded

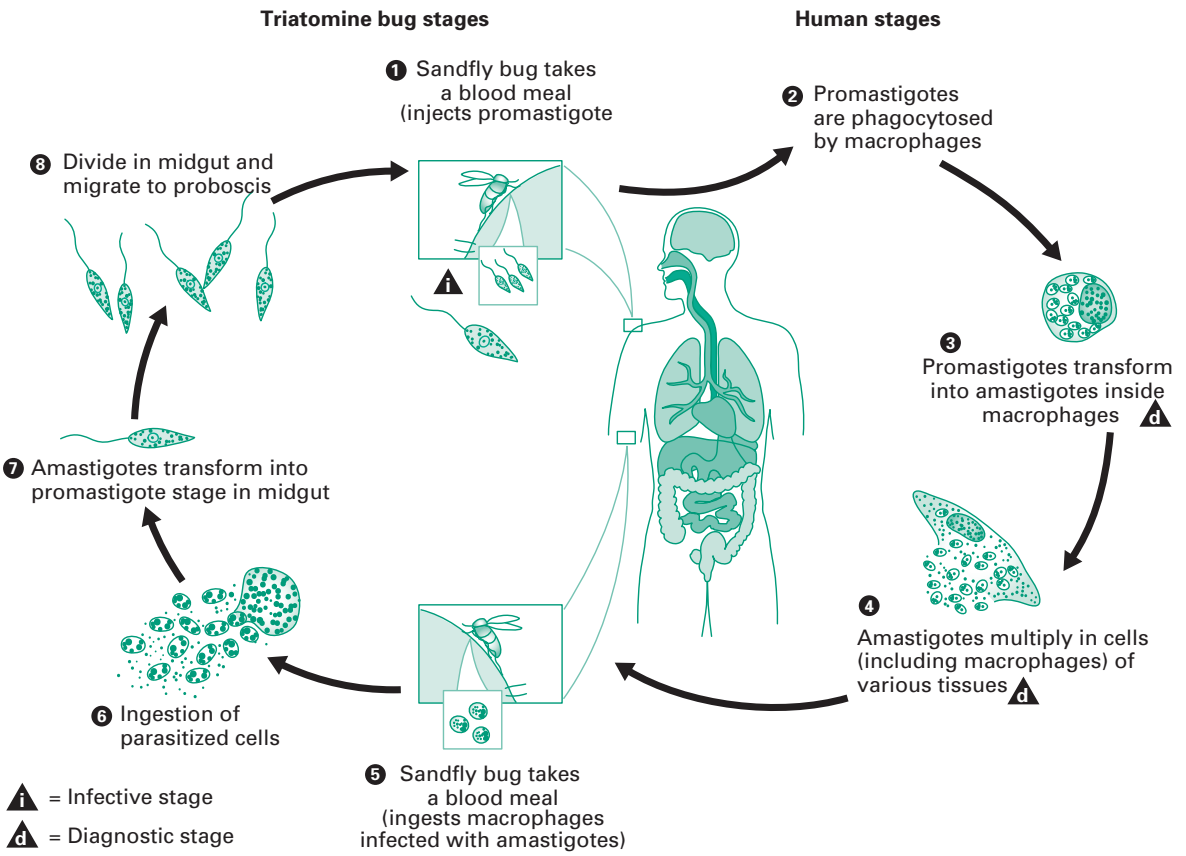

Figure 6.4 Leishmaniasis is transmitted by the bite of female phlebotomine sandflies. The sandflies inject the infective stage, promastigotes, during blood meals (1). Promastigotes that reach the puncture wound are phagocytosed by macrophages (2) and transform into amastigotes (3). Amastigotes multiply in infected cells and affect different tissues, depending in part on the *Leishmania* species (4). This originates the clinical manifestations of leishmaniasis. Sandflies become infected during blood meals on an infected host when they ingest macrophages infected with amastigotes (5, 6). In the sandfly's midgut, the parasites differentiate into promastigotes (7), which multiply and migrate to the proboscis (8).

animals, including humans. In most cases infection is asymptomatic, but devastating disease can occur congenitally in children as a result of infection during pregnancy. *T. gondii* infection in humans is a worldwide problem, although the rates of human infection vary from country to country. The reasons for these variations include environmental factors, cultural habits and the presence of domestic and native animal species. The frequency of postnatal toxoplasmosis acquired by eating raw meat and by ingesting food contaminated by oocysts from cat faeces (oocyst formation is greatest in the domestic cat) is not well established but is thought to be significant. Widespread natural infection is possible because infected animals may excrete millions of resistant oocysts, which can survive in the environment for prolonged periods (months–years). Mature oocysts are approximately 12 µm in diameter and contain eight infective sporozoites.

T. gondii infection in most animals including humans is asymptomatic. Severe disease in humans is observed only in congenitally infected children and in immunosuppressed individuals. The most common symptom associated with postnatal infection in humans is lymphadenitis which may be accompanied by fever, malaise, fatigue, muscle pains, sore throat and headache (flu-like symptoms). Typically infection resolves spontaneously in weeks or months, but in immunosuppressed individuals, a fatal encephalitis may occur producing symptoms such as headache, disorientation, drowsiness, hemiparesis, reflex changes and convulsions. Prenatal *T. gondii* infections often target the brain and retina and can cause a

wide spectrum of clinical disease. Mild disease may consist of impaired vision, whereas severely diseased children may exhibit a 'classic tetrad' of signs: retinochoroiditis, hydrocephalus, convulsions and intracerebral calcifications. Hydrocephalus is the least common but most dramatic lesion of congenital toxoplasmosis.

The life cycle of *T. gondii* was only fully described in the early 1970s when felines including domestic cats were identified as the definitive host and various warm-blooded animals were identified as intermediate hosts. *T. gondii* is transmitted by three mechanisms: congenitally, through the consumption of uncooked infected meat and via faecal matter contamination. Figure 6.5 shows the life cycle of *T. gondii*. Cats acquire *Toxoplasma* by ingesting any of three infectious stages of the organism: the rapidly multiplying forms, tachyzoites, the dormant bradyzoites (cysts) in infected tissue and the oocysts shed in faeces. The probability of infection and the time between infection and the shedding of oocysts varies with the stage of *T. gondii* ingested. Fewer than 50% of cats shed oocysts after ingesting tachyzoites or oocysts, whereas nearly all cats shed oocysts after ingesting bradyzoites. When a cat ingests tissue cysts, the cyst wall is dissolved by intestinal and gut proteolytic enzymes, which causes the release of bradyzoites. These enter the epithelial cells of the small intestine and initiate the formation of numerous asexual generations before the sexual cycle begins. At the same time that some bradyzoites invade the surface epithelia, other bradyzoites penetrate the lamina propria and begin to multiply as tachyzoites (trophozoites) (Figure 6.2c). Within a few hours, tachyzoites may disseminate to other tissues through the lymph and blood. Tachyzoites can enter almost any type of host cell and multiply until the cell becomes packed with parasites. The host cell then lyses and releases more tachyzoites to enter new host cells. The host usually controls this phase of infection, and as a result the parasite enters the 'resting' stage in which bradyzoites are isolated in tissue cysts. Tissue cysts are formed most commonly in the brain, liver and muscles. These cysts usually cause no host reaction and may remain dormant for the life of the host. In intermediate hosts, such as humans, the extraintestinal cycle of *T. gondii* is similar to the cycle in cats except that there is no sexual stage.

Most cases of toxoplasmosis in humans are probably acquired by the ingestion of either tissue cysts in infected meat or oocysts in food contaminated with cat faeces. Bradyzoites from the tissue cysts or sporozoites released from oocysts invade intestinal epithelia and multiply. *T. gondii* may spread both locally to mesenteric lymph nodes and to distant organs by invading the lymphatic and blood systems. Focal areas of necrosis (caused by localized cell lysis) may develop in many organs. The extent of the disease is usually determined by the extent of injury to infected organs, especially to vital and vulnerable organs such as the eye, heart and adrenals.

Opportunist toxoplasmosis in immunosuppressed patients usually represents reactivation of chronic infection. The predominant lesion of toxoplasmosis—encephalitis in these patients—is necrosis, which often results in multiple abscesses, some as large as a tennis ball.

3 Intestinal parasites

Gut protozoan parasites include *Entamoeba histolytica, Giardia lamblia, Dientamoeba fragilis, Balantidium* sp., *Isospora* sp. and *Cryptosporidium parvum*. All these organisms are transmitted by the faecal–oral route and most of them are cosmopolitan in their distribution. A good example of this is *Giardia*, which is found in nearly all countries of the world. In many developed countries, including the UK and USA, it is one of the most commonly identified waterborne infectious organisms. *Cryptosporidium,* like *Toxoplasma,* has a complex life cycle utilizing both sexual and asexual reproduction. In contrast, *Giardia* and *Entamoeba* have simple life cycles utilizing only asexual reproduction. These latter organisms are members of a small group of eukaryotes that do not have mitochondria. It had long been assumed that they never had mitochondria, but recent studies showing the presence of mitochondrial-like enzymes and structural proteins suggest that it is more likely that this organelle was lost as a result of metabolic/physiological adaptation.

3.1 Giardia lamblia (syn. intestinalis, duodenalis)

*Giardia duodenalis (*syn *lamblia* and *intestinalis)* is the causative agent of giardiasis, a severe diarrhoeal disease. The incidence of *Giardia* infection worldwide ranges from 1.5% to 20% but is probably significantly higher in countries where standards of hygiene are poor. The most common route of spread is via the faecal–oral route, although spread can also occur through ingestion of contaminated water and these modes of transmission are particularly prevalent in institutions, nurseries and daycare centres. Recent outbreaks and epidemics in the UK, USA and eastern Europe have been caused by drinking contaminated water from community water supplies

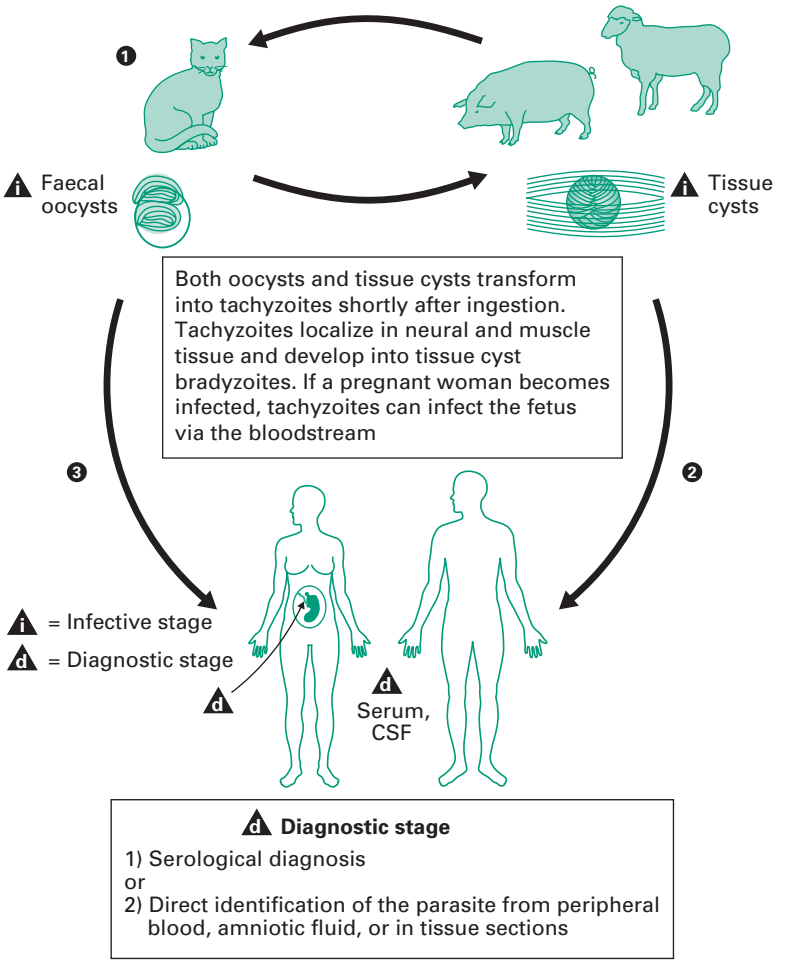

Figure 6.5 Members of the cat family (Felidae) are the only known definitive hosts for the sexual stages of *Toxoplasma gondii* and thus are the main reservoirs of infection. Cats become infected with *T. gondii* by carnivorism (1). After tissue cysts or oocysts are ingested by the cat, viable organisms are released and invade epithelial cells of the small intestine where they undergo an asexual cycle followed by a sexual cycle and then form oocysts, which are then excreted. The unsporulated oocyst takes 1–5 days after excretion to sporulate (become infective). Although cats shed oocysts for only 1–2 weeks, large numbers may be shed. Oocysts can survive in the environment for several months and are remarkably resistant to disinfectants, freezing and drying, but are killed by heating to 70 °C for 10 minutes. Human infection may be acquired in several ways: (A) ingestion of undercooked infected meat containing *Toxoplasma* cysts (2); (B) ingestion of the oocyst from faecally contaminated hands or food (3); (C) organ transplantation or blood transfusion; (D) transplacental transmission; (E) accidental inoculation of tachyzoites. The parasites form tissue cysts, most commonly in skeletal muscle, myocardium and brain; these cysts may remain throughout the life of the host.

or directly from rivers and streams. Many animals harbour *Giardia* species that are indistinguishable from the human infective types. There is now clear evidence from genotyping studies (see section 6.1.2) that the *G. duodenalis* species is made up of a number of genetically distinct groups which may represent species. This has raised the question of the existence of animal reservoirs of *Giardia*. Recent findings of *Giardia*-infected animals in watersheds from which humans acquired giardiasis, and the successful interspecies transfer of these organisms, suggests that human giardiasis can be acquired by zoonotic transfer, however it is not clear if the major route of transfer is from animal to human or from human to animal. More recently, it has been recognized that

Giardia infection may be transmitted by sexual activity, particularly among homosexual men.

This organism exhibits only two life cycle forms: the vegetative binucleate trophozoite (10–20 µm long × 2–3 µm wide) (Figure 6.2d) and the transmissible quadranucleate cyst (10–12 µm long × 1–3 µm wide). Trophozoites have four pairs of flagella and an adhesive disc, which is thought to help adhesion to the intestinal epithelium. Division in trophozoites is by longitudinal fission.

It was long believed that *Giardia* was a non-pathogenic commensal. However, we now know that *Giardia* can produce disease ranging from a self-limiting diarrhoea to a severe chronic syndrome. Immune-competent individuals with giardiasis may exhibit some or all of the following signs and symptoms: diarrhoea or loose, foul-smelling stools; steatorrhoea (fatty diarrhoea); malaise; abdominal cramps; excessive flatulence; fatigue and weight loss. Infected individuals with an immune deficiency or protein-calorie malnutrition may develop a more severe disease and will exhibit symptoms such as interference with the absorption of fat and fat-soluble vitamins, retarded growth, weight loss, or a coeliac disease-like syndrome.

Giardia infection is initiated by ingestion of viable cysts (Figure 6.6), the infective dose of which can be as low as one cyst, although infection initiated by 10–100 viable cysts is more likely. As the cysts pass through the stomach the low pH and elevated CO_2 induce excystation (cyst–trophozoite transformation). From each cyst two complete trophozoites emerge and these rapidly undergo division then attach to the duodenal and jejunal epithelium. Once attached, they will undergo division, and 4–7 days later they will detach and begin to round up and form cysts (encystment). This process is thought to be induced in response to bile. The first cysts are found in faeces after 7–10 days.

The underlying pathology of giardiasis is not fully understood. The trophozoites do not invade the mucosa, and although their presence may have some physical effects on the surface it is more likely that some of the pathology is caused by inflammation of the mucosal cells of the small intestine causing an increased turnover rate of intestinal mucosal epithelium. The immature replacement cells have less functional surface area and less digestive and absorptive ability. This would account for the microscopic changes seen in infected epithelia. It has been suggested that other mechanisms may exist, e.g. toxin production, but to date no such molecule has been observed.

3.2 Entamoeba histolytica

Entamoeba histolytica is the causative agent of amoebic dysentery, another infection transmitted via the faecal–oral route. The severity of this and related pathologies caused by this organism can vary from diarrhoea associated with the intestinal infection to extraintestinal amoebiasis producing hepatic and often lung infection. The prevalence of amoebiasis in developing countries reflects the lack of adequate sanitary systems. It had long been known that most infections associated with *E. histolytica* are asymptomatic or exhibit minimal symptomology, but in the late 1980s a separate, but morphologically and biochemically similar species, *E. dispar,* was identified. This organism exhibits limited pathogenicity and in many cases produces no symptoms but is commonly misidentified as *E. histolytica*. This species is the most likely cause of 'asymptomatic *Entamoeba* infection'.

E. histolytica has a relatively simple life cycle and, like *Giardia*, exhibits only two morphological forms: the trophozoite and cyst stages. Trophozoites (Figure 6.2e) vary in size from 10 to 60 µm and are actively motile. The cyst is spherical, 10–20 µm in diameter, with a thin transparent wall. Fully mature cysts contain four nuclei.

Symptoms of amoebic dysentery are associated with mucosal invasion and ulceration. Mucosal erosion causes diarrhoea, the severity of which increases with the level of invasion and colonization. Symptoms can also be affected by the site of the infection. Peritonitis as a result of perforation has been reported in connection with severe amoebic infection. Extraintestinal amoebiasis is usually associated with liver infection, causing abscesses and/or enlargement. The abscess appears as a slowly enlarging liver mass and will cause noticeable pain. Jaundice may also occur due to blockage of the bile. Pleural, pulmonary, and pericardial infection results from metastatic spread from the liver, but can also manifest in other parts of the viscera or give rise to a brain abscess. However, these complications are uncommon.

The life cycle of *E. histolytica* (Figure 6.7) is simple, but the ability of trophozoites to infect sites other than the intestine make it more complex than that of *Giardia.* Infection is initiated by ingestion of mature cysts, and again, excystation occurs during transit through the gut. After this, trophozoites rapidly divide by simple fission to produce four amoebic cells which undergo a second division; thus each cyst yields eight trophozoites. Survival outside the host depends on the resistant cyst form.

The pathology of the disease is only partially understood. The process of tissue invasion has been well studied

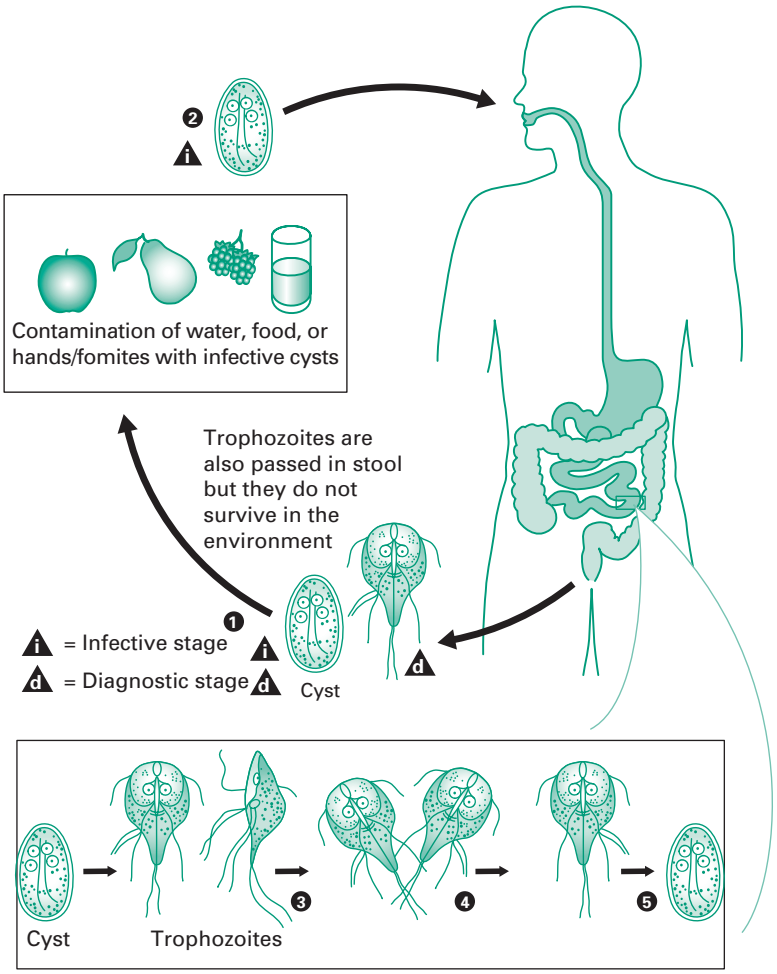

Figure 6.6 Cysts are resistant forms and are responsible for transmission of giardiasis. Both cysts and trophozoites can be found in the faeces (diagnostic stages) (1). The cysts are hardy, and can survive several months in cold water. Infection occurs by the ingestion of cysts in contaminated water, food or by the faecal–oral route (hands or fomites) (2). In the small intestine, excystation releases trophozoites (each cyst produces two trophozoites) (3). Trophozoites multiply by longitudinal binary fission, remaining in the lumen of the proximal small bowel where they can be free or attached to the mucosa by a ventral sucking disc (4). Encystation occurs as the parasites transit toward the colon. The cyst is the stage found most commonly in non-diarrhoeal faeces (5). Because the cysts are infectious when passed in the stool or shortly afterwards, person-to-person transmission is possible. Although animals are infected with *Giardia*, their importance as a reservoir is unclear.

and involves binding and killing of the host cells by specific adhesin molecules and the action of a pore-forming protein, amoebapore. The initial superficial ulcer may deepen into the submucosa and become chronic. Spread may occur by direct extension, by undermining of the surrounding mucosa until it sloughs, or by penetration that can lead to perforation. If the trophozoites gain access to the vascular or lymphatic circulation, metastases may occur first to the liver and then by direct extension or further metastasis to other organs, including the brain.

3.3 Cryptosporidium parvum

Cryptosporidium parvum is a ubiquitous coccidian parasite that causes cryptosporidiosis in humans; however, other species are known to cause infection in immunocompromised patients and in total this genus comprises

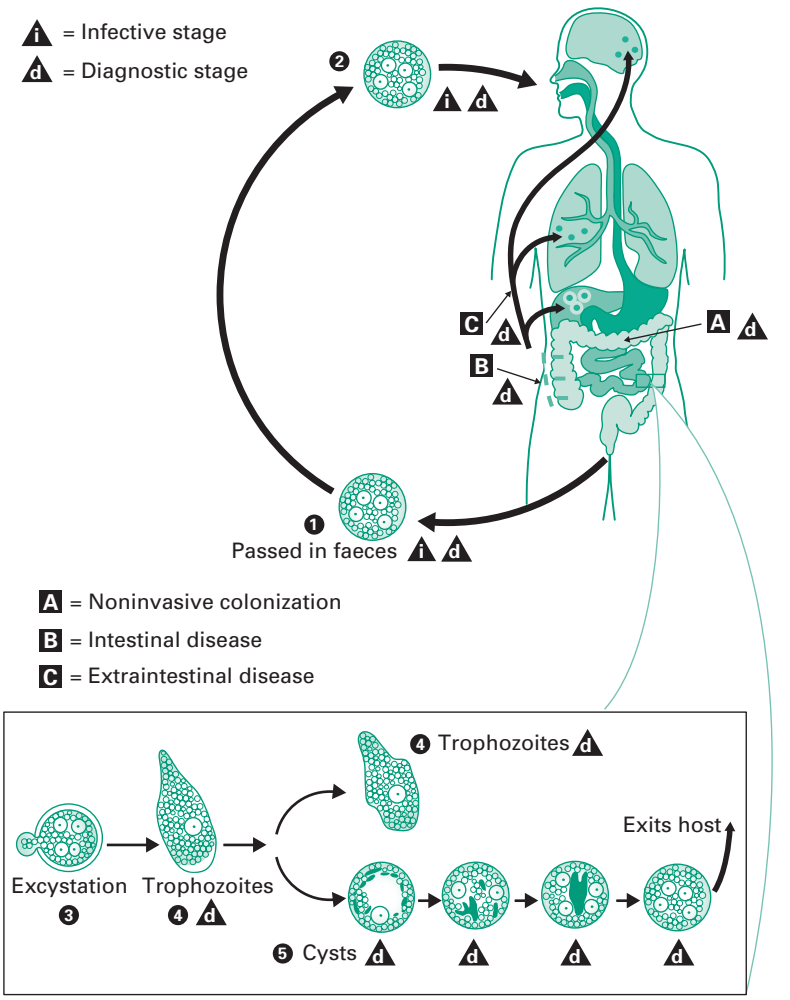

Figure 6.7 Cysts are passed in faeces (1). Infection by *Entamoeba histolytica* occurs by ingestion of mature cysts (2) in faecally contaminated food, water or hands. Excystation (3) occurs in the small intestine and trophozoites (4) are released, which migrate to the large intestine. The trophozoites multiply by binary fission and produce cysts (5), which are passed in the faeces (1). Because of the protection conferred by their walls, the cysts can survive days to weeks in the external environment and are responsible for transmission. (Trophozoites can also be passed in diarrhoeal stools, but are rapidly destroyed once outside the body, and if ingested would not survive exposure to the gastric environment.) In many cases, the trophozoites remain confined to the intestinal lumen (A, non-invasive infection) of individuals who are asymptomatic carriers, passing cysts in their stool. In some patients the trophozoites invade the intestinal mucosa (B, intestinal disease), or through the bloodstream, extraintestinal sites such as the liver, brain and lungs (C, extraintestinal disease), with resultant pathological manifestations. It has been established that the invasive and non-invasive forms represent two separate species, respectively, *E. histolytica* and *E. dispar*. However, not all persons infected with *E. histolytica* will have invasive disease. These two species are morphologically indistinguishable. Transmission can also occur through faecal exposure during sexual contact (in which case not only cysts, but also trophozoites could prove infective).

19–20 distinct species. Recently there has been a re-naming of the major species in this group to reflect the host specificity of the particular species or genotype. The life cycle of the parasite is complex but is completed in a single host. Infection follows the ingestion of oocysts associated with contaminated water or food. According to the World Health Organization (WHO) the health significance of *C. parvum* is high due to the persistence of the organism in the environment. Cattle represent the most important reservoir of *C. parvum* but other mammals,

domestic and wild, can be infected and act as carriers of the disease, even if asymptomatic. It is now known that a number of genetically distinct subspecies exist that can be divided into two groups. Group I organisms infect humans only (soon to be reclassified as *C. hominis*); group II organisms infect a wider range of hosts. There have been several large outbreaks of this infection in the UK and USA in which water was identified as the initial vehicle for transmission. Members of this genus are intracellular parasites infecting the intestinal mucosal epithelium. The two major life cycle forms are the oval oocyst and the sporozoite.

C. parvum infections are often asymptomatic, but symptoms such as profuse watery diarrhoea, stomach cramps, nausea, vomiting and fever are typical. The symptoms can last from several days to a few weeks in immunocompetent individuals, but in immunocompromised patients infection can become chronic, lasting months or even years. The mean infective dose for immunocompetent people is dependent on the strain of *C. parvum* but it is considered to be approximately 100 cells, and infants are more vulnerable to infection. Diarrhoea is a major cause of childhood mortality and morbidity as well as malnutrition in developing countries. *Cryptosporidium* is the third most common cause of infective diarrhoea in children in such countries, and consequently it plays a role in the incidence of childhood malnutrition.

Cryptosporidium infection has a higher nutritional impact in boys than girls, becausee of the need for micronutrients in boys to build up larger muscle mass. However, breast-feeding does offer some protection against infection. In immunocompromised individuals *Cryptosporidium* infection causes a severe gastroenteritis, and often the parasites infect other epithelial tissues causing pneumonia; the mortality rate due to *C. parvum* in AIDS patients is between 50% and 70%. Infection occurs when oocysts (Figure 6.2f) excyst following environmental stimuli (typical intestinal conditions) and parasitize the epithelial cells which line the intestine wall (Figure 6.8). After several further stages of the cycle, two forms of oocyst are produced; soft-walled oocysts reinitiate infection of neighbouring enterocytes while hard-walled cysts are expelled in the faeces.

Little is known about the mechanism by which these organisms cause disease. They are known to invade cells but this process is atypical in that the parasites form a vacuole just below the epithelial cell membrane. During infection a variety of changes are seen such as partial villous atrophy, crypt lengthening and inflammation; these responses are probably due in part to cell damage that occurs during the growth of the intracellular forms. It has also been proposed that parasite enzymes and/or immune-mediated mechanisms may also be involved. It should be remembered, however, that cryptosporidiosis is resolved by the immune system in healthy patients normally within 3 weeks.

4 *Trichomonas* and free-living amoebas

4.1 Trichomonas vaginalis

Trichomonas vaginalis is a common sexually transmitted parasite. Infection rates vary from 10% to 50%, with the highest reported rates found in the USA. Infections are usually asymptomatic or mild although symptomatic infection is most common in women. Trichomonads are all anaerobes and contain hydrogenosomes. This organelle is found in very few other anaerobic eukaryotes and is often termed the 'anerobic mitochondrion'. A number of functions have been assigned to it, and it has been shown to function in the generation of ATP. This organism does not exhibit a life cycle as only the motile (flagellate/amoeboid) trophozoite (Figure 6.2g) has been seen and division is by binary fission. Trichomonads have a pear-shaped body 7–15 μm long, a single nucleus, three to five forward-directed flagella, and a single posterior flagellum that forms the outer border of an undulating membrane.

Trichomoniasis in women is frequently chronic and is characterized by vaginal discharge and dysuria. The inflammation of the vagina is usually diffuse and is characterized by reddening of the vaginal wall and migration of polymorphonuclear leucocytes into the vaginal lumen (these form part of the vaginal discharge).

Because there is no resistant cyst, transmission from host to host must be direct. The inflammatory response in trichomoniasis is the major pathology associated with this organism; however, the mechanisms of induction are not known. It is likely that mechanical irritation resulting from contact between the parasite and vaginal epithelium is a major cause of this response but the organism produces high concentrations of acidic end-products and polyamines, both of which would also irritate local tissues.

4.2 Free-living opportunist amoebas

The free-living opportunist amoebas are an often-forgotten group of protozoans. The two major groups, *Naegleria* and *Acanthamoeba*, infect humans and both can cause fatal encephalitis. Both types of infections are rare, with less than 200 cases of *Naegleria fowleri* infection recorded worldwide and approximately 100–200

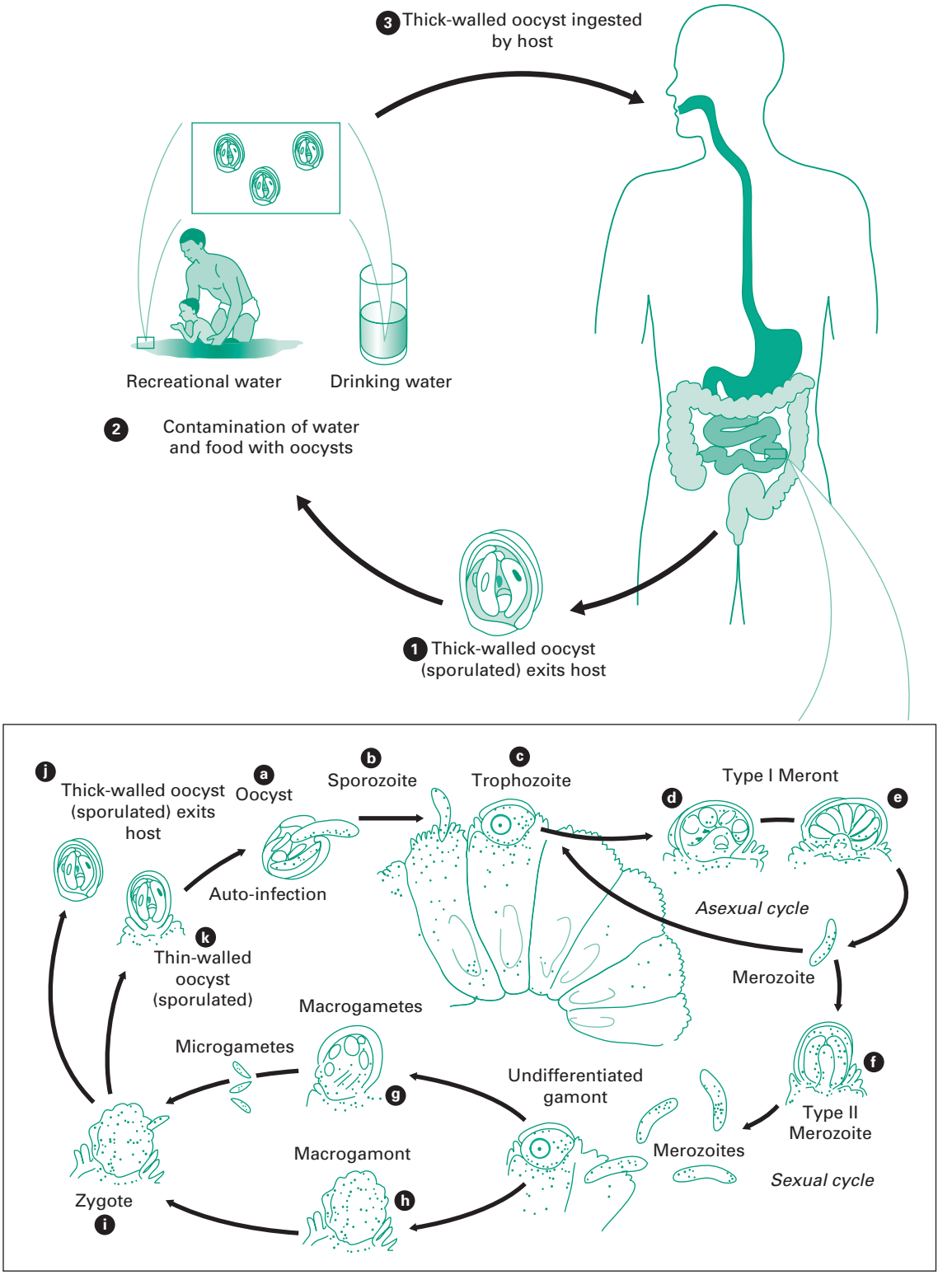
3 Thick-walled oocyst ingested by host
Recreational water
Drinking water
2 Contamination of water and food with oocysts
1 Thick-walled oocyst (sporulated) exits host
j
Thick-walled oocyst (sporulated) exits host
a
Oocyst
b
Sporozoite
c
Trophozoite
Type I Meront
d
e
Auto-infection
Asexual cycle
k
Thin-walled oocyst (sporulated)
Merozoite
Macrogametes
Microgametes
g
f
Type II Merozoite
Undifferentiated gamont
Merozoites
Sexual cycle
Macrogamont
Zygote
i
h

Figure 6.8 Life cycle of *Cryptosporidium*. Sporulated oocysts, containing four sporozoites, are excreted by the infected host through faeces and possibly other routes such as respiratory secretions (1). Transmission of *Cryptosporidium parvum* occurs mainly through contact with contaminated water (e.g. drinking or recreational water). Occasionally food sources, such as chicken salad, may serve as vehicles for transmission. Many outbreaks in the USA have occurred in water parks, community swimming pools and daycare centres. Zoonotic transmission of *C. parvum* occurs through exposure to infected animals or exposure to water contaminated by faeces of infected animals (2). Following ingestion (and possibly inhalation) by a suitable host (3), excystation (a) occurs. The sporozoites are released and parasitize epithelial cells (b, c) of the gastrointestinal tract or other tissues such as the respiratory tract. In these cells, the parasites undergo asexual multiplication (schizogony or merogony) (d, e, f) and then sexual multiplication (gametogony) producing microgamonts (male, g) and macrogamonts (female, h). Upon fertilization of the macrogamonts by the microgametes (i), oocysts (j, k) develop that sporulate in the infected host. Two different types of oocysts are produced, the thick-walled oocyst, which is commonly excreted from the host (j) and the thin-walled oocyst (k), which is primarily involved in autoinfection. Oocysts are infective upon excretion, thus permitting direct and immediate faecal–oral transmission. (From Juranek, D.D. Cryptosporidiosis. In: *Hunter's Tropical Medicine*, 8th edn, ed. G.T. Strickland.)

cases of *Acanthamoeba* ulcerative keratitis per year. This disease is commonly associated with contact lens use and it is thought that infection is caused by a combination of corneal trauma and dirty contact lenses. Both types of amoeba produce resistant cysts and *Naegleria* also exhibits a flagellate form. Both *Acanthamoeba* and *Naegleria* are free-living inhabitants of fresh water and soil, but *Naegleria fowleri* (the human pathogen) reproduces faster in warm waters up to 46 °C. Treatment of water by chlorination or ozonolysis does not entirely eliminate cysts and both amoebae have been isolated from air-conditioning units.

Naegleria fowleri is the causative agent of primary amoebic meningoencephalitis, a rapidly fatal disease that usually affects children and young adults. In all cases, contact with amoebae occurs as a result of swimming in infected fresh water. The organisms enter the brain via the olfactory tract after amoebae are inhaled or splashed into the olfactory epithelium. The incubation period ranges from 2 to 15 days and depends both on the size of the inoculum and the virulence of the strain. The disease appears with the sudden onset of severe frontal headache, fever, nausea, vomiting and stiff neck. Symptoms develop rapidly to lethargy, confusion and coma and in all cases to date the patient died within 48–72 hours.

Acanthamoeba castellanii, A. culbertsoni and other pathogenic *Acanthamoeba* species can cause opportunist lung and skin infections in immunocompromised individuals. Where amoebae spread from such lesions to the brain, they can cause a slowly progressive and usually fatal encephalitis. In addition, *Acanthamoeba* can cause an ulcerating keratitis in healthy individuals, usually in association with improperly sterilized contact lenses. The presence of cysts and trophozoites in alveoli or in multiple nodules or ulcerations of the skin characterizes acanthamoebic pneumonitis and dermatitis. Spread of amoebae to the brain produces an encephalitis, characterized by neurological changes, drowsiness, personality changes and seizures in the early stages of infection, which progress to altered mental status, lethargy and cerebellar ataxia. The end point of infection is usually coma followed by death of the patient. *Acanthamoeba* keratitis is characterized by painful corneal ulcerations that fail to respond to the usual anti-infective treatments. The infected and damaged corneal tissue may show a characteristic annular infiltrate and congested conjunctivae. If not successfully treated, the disease progresses to corneal perforation and loss of the eye or to a vascularized scar over thinned cornea, with impaired vision.

5 Host response to infection

Mechanisms to control parasitic protozoa are similar to those utilized for other infectious agents; they can be divided into non-specific mechanism(s) and specific mechanism(s) involving the immune system. The best-studied non-specific mechanisms include those that affect the entry of parasites into the red blood cell. The sickle cell haemoglobin trait and lack of the Duffy factor on the erythrocyte surface make the red cell more resistant to invasion by *Plasmodium*. These traits are commonly found in populations from malaria-endemic regions. A second example of a non-specific factor is the presence of trypanolytic factors in the serum of humans which confer resistance to *T. brucei*, although non-specific factors can play a key role in resistance, usually they work in conjunction with the host's immune system.

5.1 Immune response

Unlike most other types of infection, protozoan diseases are often chronic, lasting for months to years. When associated with a strong host immune response, this type of long-term infection is apt to result in a high incidence of immunopathology. Until recently the importance of host immune response in controlling many parasite infections was not fully appreciated, but the impact of HIV infection on many parasitic diseases has highlighted this relationship.

Different parasites elicit different humoral and/or cellular immune responses (Chapter 9). In malaria and trypanosome infections, antibody appears to play a major role in immunity, although it would seem that for many organisms both humoral and cellular immunity are required for killing of parasites. Cellular immunity is believed to be the most important mechanism in the killing of *Leishmania* and *Toxoplasma*. Cytokines are involved in the control of both the immune response and also the pathology of many parasitic diseases. Helper (h) and cytotoxic (c) T cells play major roles in the induction/control of the response. The various subsets of these produce different profiles of cytokines. For example, the Th1 subset produces gamma interferon (IFN-γ) and interleukin-2 (IL-2) and is involved in cell-mediated immunity. In contrast, the Th2 subset produces IL-4 and IL-6, and is responsible for antibody-mediated immunity. The induction of the correct T-cell response is key to recovery. The Th1 subset and increased IFN-γ are important for the control of *Leishmania*, *T. cruzi* and *Toxoplasma* infections, whereas the Th2 response is more important in parasitic infections in which antibody is a major factor. It is important to recognize that the cytokines produced by one T-cell subset can up- or down-regulate the response of other T-cell subsets; IL-4 will down-regulate Th1 cells for example. The cytokines produced by T and other cell types do not act directly on the parasites but induce changes in the metabolism of glucose, fatty acid and protein in other host cells. Cytokines can also stimulate cell division and, therefore, clonal expansion of T- and B-cell subsets. This can lead to increased antibody production and/or cytotoxic T-cell numbers. The list of cytokines and their functions is growing rapidly, and it would appear that these chemical messages influence all phases of the immune response. They are also clearly involved in the multitude of physiological responses (fever, decreased food intake, etc.) observed in an animal's response to a pathogen, and in the pathology that results.

5.2 Immune pathology

The protozoa can elicit humoral responses in which antigen–antibody complexes are formed and these can trigger coagulation and complement systems. Immune complexes have been found circulating in serum and deposited in the kidneys where they may contribute to conditions such as glomerulonephritis. In other tissues these complexes can also induce localized hypersensitivities. It is thought that this type of immediate hypersensitivity is responsible for various clinical syndromes including blood hyperviscosity, oedema and hypotension.

Another important form of antibody-mediated pathology is autoimmunity. Autoantibodies to a number of different host antigens (e.g. red blood cells, laminin, collagen and DNA) have been demonstrated. These autoantibodies may play a role in the pathology of parasitic diseases by exerting a direct cytotoxic effect on the host cells, e.g. autoantibodies that coat red blood cells produce haemolytic anaemia; they may also cause damage through a build-up of antigen–antibody complexes.

Many parasites can elicit the symptoms of disease through the action of their surface molecules such as the pore-forming proteins of *E. histolytica* that induce contact-dependent cell lysis, and trypanosome glycoproteins that can fix and activate complement resulting in the production of biologically active and toxic complement fragments. A range of parasite-derived enzymes such as proteases and phospholipases can cause cell destruction, inflammatory responses and gross tissue pathology.

5.3 Immune evasion

Parasites exhibit a number of mechanisms that allow them to evade host immune response. Two such mechanisms are displayed by trypanosomes, which are able to exhibit both antigenic masking and antigenic variation. Masking means the parasite becomes coated with host components and therefore is not recognized as foreign. In addition parasites can undergo antigenic variation which results in surface antigens being changed during the course of an infection. The ultimate goal of these is that the hosts immune response is evaded. Parasites can also suppress the host's immune response either to the parasite specifically or to foreign antigens in general. This, however, can cause a number of problems, as general immune suppression may make the individual more susceptible to secondary infection.

6 Detection of parasites

The detection of parasites in the host and environment (including foods) is vital for proper treatment and also for understanding mechanisms of transmission. In addition the use of molecular typing is of major benefit in studying epidemiology and for some of the zoonotic potential of parasites.

6.1 Methods of detection

It is not always easy or possible to culture parasites, so the detection of these organisms in samples requires the use of methods such as microscopy and DNA amplification. The most commonly used method involves microscopy and this can be applied to clinical as well as environmental samples. For some organisms this approach remains the 'gold standard' for detection and identification. The advantages of microscopy are speed, cost and availability. In addition, fluorescent-labelled antibodies raised to species-specific antigens can be utilized to help identify organisms to species or subspecies level, and other stains can be used to help determine the viability of cells. Examples of such stains include fluorescein diacetate which is cleaved by esterases in viable cells, releasing fluorescein (gives a green fluorescence) and propidium iodide which is excluded from viable cells but taken up by cells with damaged membranes (gives red fluorescence). However, there are a number of obvious limitations including the requirement for well-trained staff to perform the microscopy, limits of sensitivity of the method and, for some parasites, difficulty in differentiating species based on morphology. In addition, the parasite may not be present in easily available samples. Thus a variety of other approaches are used to help in detection.

6.1.1 Antibody-based technologies

For the major protozoan parasites a number of immunology-based methods exist to detect the presence of organisms in clinical samples. These include the use of agglutination, complement fixation and enzyme-linked immunosorbent assay (ELISA). The most commonly used method is the ELISA and this can be used to detect the presence of antigens in samples (direct assay) or antigen-specific antibodies in patients' serum (indirect). The ELISA method has a number of advantages in that it can be automated and has good specificity and sensitivity. Although a number of ELISA-based detection kits are available their cost can be a limitation to use especially in those developing countries where parasitic infections are endemic. The use of indirect ELISA methods for determining the infection status of the patient can also be difficult because previous exposure to the parasite can cause problems and the immune status of the patients can also impact on antibody production.

6.1.2 DNA-based technologies

Nucleic acids, DNA or RNA, are found in all types of microorganism and the sequences of these molecules can be used to help in the identification of species and individuals. The robust nature of DNA has allowed researchers to use these sequence in a variety applications including forensics, archaeology (sequences from animal tissues over 100 years old have routinely been determined) and epidemiology. The advent of highly sensitive DNA amplification technologies such as the polymerase chain reaction (PCR) has allowed the development of molecular tools for the identification and detection of parasites. Initial work on these techniques focused on their ability to help define species and many of these studies utilized the ribosomal DNA genes and were very successful. Using these genes it was possible to discriminate many genera and species, but the genes lacked the level of sequence variation required for separation below the level of species (important for some pathogenic organisms and for epidemiological studies). In addition ribosomal DNA sequencing was also not always appropriate for identifying species that are highly diverse. Other DNA markers that have been used included genes that encode for metabolic enzymes and structural proteins. For example *Cryptosporidium* can be speciated by using a combination of target genes including the *Cryptosporidium* oocyst wall protein (COWP), heat shock protein (hsp70), dihydrofolate reductase (DHFR) and 18S ribosomal DNA. Complete genomes are now available for the major protozoan parasites and this has helped in the development of improved methods for species identification. These genomes can be accessed at http://eupathdb.org/eupathdb/ and this website links to many others resources including the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Other DNA-based techniques are now available such as the multiplex PCR (amplification of more than one gene at a time) and quantitative real-time PCR (qPCR), and these have improved specificity and sensitivity in detection and increased the usefulness of DNA technology for the detection of parasites in environmental as well as clinical samples. It is also possible to assess the viability of protozoan parasites

using reverse transcription PCR (RT-PCR). This is due to the fact that mRNAs degrade quickly once parasites are killed. However, it should be noted that increasing evidence exists to show that mRNA can survive in some cells for up to 24 hours

A recent method, DNA microarray, is a fast developing technology. This is a 'chip' based system that measures the binding of target DNA sequences (parasite) from samples (fluorescently labelled using PCR) to complementary sequences bound to the chip surface. The binding of parasite sequence DNA and the amount that is bound can be detected using fluorescence (Figure 6.9). In theory this technique allows the detection of multiple parasite species at the same time in one sample.

6.1.3 Alternative methods

Alternative methods are being developed for the detection of parasites; these include mass spectrometry (MS) and biosensors. MS can be used by identifying known surface characteristics of appropriate life cycle forms. This type of MS uses low-energy electrons to generate a fingerprint of the cells. Other mass spectrometry methods can generate fingerprints based on parasite-derived peptides and metabolites. These can be detected easily with a high level of sensitivity and can be used in complex mixtures that would be present in clinical and environmental samples. Over the past 10 years the production of kits for near-patient testing has driven the development of detection technology and this has generated interest in the use of biosensors. These sensors detect the presence of 'marker molecules' that are specific to a particular pathogen and utilize mechanical, electrochemical or piezoelectric methods to generate a signal that can be detected. Such sensors can be combined onto 'chips' to detect a range of pathogens.

6.2 Analysis of samples

6.2.1 Clinical samples

Diagnosis of parasitic infection is dependent on the demonstration of the parasite in appropriate samples. The type of samples can vary from blood where preparation can be minimal (e.g. sample of smears for microscopy) to faeces or intestinal aspirates. Faecal samples require more processing: for example, fresh or preserved stools can be concentrated to increase the yield of the parasites by sedimentation using the formol–ether or formol–ethyl acetate techniques or by faecal parasite flotation method using copper sulphate. These concentrates can be stained for microscopy. For the extraction of DNA from samples a number of methods exist; however, there are various resin-based kits that will separate DNA from complex biological samples.

6.2.2 Environmental samples

Many parasites have life cycle forms that can survive in the environment. These act to initiate infections in susceptible hosts; for some parasites this only requires inges-

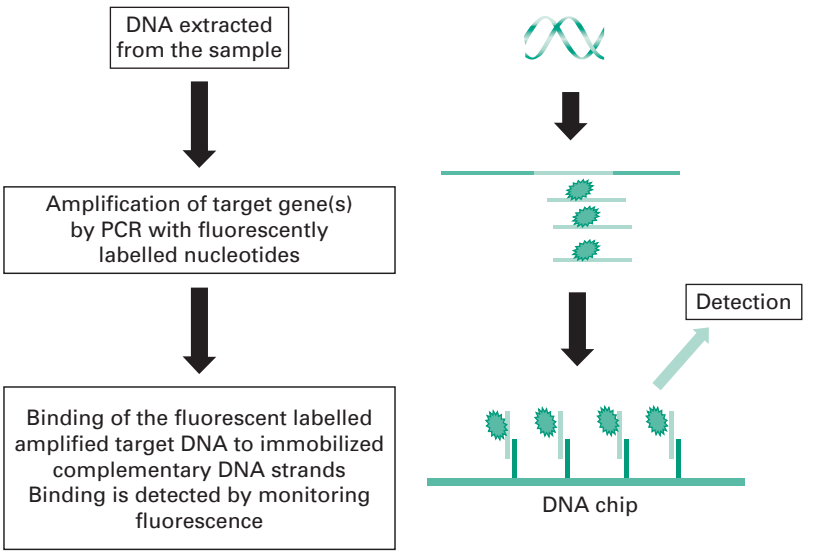

Figure 6.9 Diagram showing the steps involved in detecting parasite DNA using a microarray 'gene chip' system.

tion of no more than 10 cells or cysts, thus the ability to detect them in the environment is vital. This section focuses on water and foods as they are often major routes of transmission for parasites such as *Giardia* and *Cryptosporidium* which are included in the monitoring standards for potable waters (drinking-waters) by regulatory bodies in both the European Union and the USA. In addition, food and pharmaceutical companies that utilize water in their processes are required to test for a number of pathogens, including waterborne parasites. Foods can also be a source of infection and methods for the detection of pathogens in foods are as important as those for water samples.

As outlined in section 6.1, a number of methods can be used to detect and to identify parasites but the performance of these techniques will often be impaired when used on real samples. This is due to a number of factors that include:

- the levels of parasites present
- volume of samples required for analysis
- the presence of other microorganisms
- the presence of compounds that interfere with detection
- turbidity of samples (water)
- high levels of protein, lipids and carbohydrates in samples (foods).

Often these factors combine to make detection difficult. For example, if levels of a parasite in water are low (<1/100 ml) several litres must be collected and analysed. This is possible, but would require the sample volume to be reduced for analysis. This can be achieved either by filtration or centrifugation. If the sample contains sediment, both of these approaches are difficult; however, methods such as tangential flow filtration (fluid passed parallel to the filter) can be used. Waters can also contain organic acids that can inhibit the PCR process so these must be removed from the sample before analysis. DNA-based technology may also fail to detect the presence of a parasite if the DNA present in the sample is degraded, e.g. by other organisms. In foods, the process can be even more complex and separation of parasites from the foodstuff is often the factor that impacts most on the limits (sensitivity) of the method.

7 Control of protozoan parasites

It is now clear that the best approach for the successful control of parasites requires the integration of a number of methods which draw upon our increasing understanding of the parasites' life cycle, epidemiology and host response to infection.

7.1 Chemotherapy

The origins of chemotherapy are closely linked to the development of antiparasitic agents, but there has been slow progress in the development of new and novel antiprotozoal agents over the past 30 years. Recently, with the support of the WHO and government-sponsored research, new antiparasitic drugs are slowly coming into the market. Interestingly there are still a number of protozoan parasite infections such as cryptosporidiosis for which there is no effective treatment.

7.1.1 Mechanisms of action and selective toxicity

For many of the commonly used antiprotozoal drugs the modes of action and mechanisms of selective toxicity are well understood, although for some the precise mechanism remains unclear. The most common antiprotozoal drugs and their modes of action are shown in Table 6.1.

Considering the drugs in relation to modes of action, dapsone and the sulphonamides block the biosynthesis of tetrahydrofolate by inhibiting dihydropteroate synthetase, while the 2,4-diamino-pyrimidines (proguanil and pyrimethamine) block the same pathway but at a later step catalysed by dihydrofolate reductase.

The drugs that interfere with nucleic acid synthesis include those that bind to the DNA and intercalate with it such as chloroquine, mefloquine and quinine, and also pentamidine, which is unable to intercalate but probably interacts ionically. Other compounds such as benznidazole and metronidazole may alkylate DNA through activation of nitro groups via a one-electron reduction step. Several of these compounds, however, including chloroquine, mefloquine, quinine and metronidazole, have more than one potential mode of action. Chloroquine, for example, inhibits the enzyme haem polymerase, which functions to detoxify the cytotoxic molecule haem that is generated during the degradation of haemoglobin. Metronidazole is reduced in the parasite cell and forms a number of cytotoxic intermediates, which can cause damage not only to DNA but also to membranes and proteins.

Tetracycline targets protein synthesis in *Plasmodium* via a similar mechanism to that seen in bacteria: inhibition of chain elongation and peptide bond formation. Eflornithine interferes with the metabolism of the amino acid ornithine in *T. brucei gambiense* by acting as a suicide substrate for the enzyme ornithine decarboxylase.

Table 6.1 Common antiprotozoal drugs and their modes of action

Drug	Mode of action (if known)	Mechanism of selectivity	Target organism(s)
Dapsone	Cofactor synthesis	Unique target	*Plasmodium* spp.
Proguanil	Cofactor synthesis	Differences in the target	*Plasmodium* spp.
Pyrimethamine	Cofactor synthesis	Differences in the target	*Plasmodium* spp.
Sulphonamides	Cofactor synthesis	Differences in the target	*Plasmodium* spp.
Benznidazole	Nucleic acid synthesis	Activation in the parasite	*Trypanosoma* spp.
Chloroquine	Nucleic acid synthesis	Differential uptake	*Plasmodium* spp.
Mefloquine	Nucleic acid synthesis	Differential uptake	*Plasmodium* spp.
Metronidazole	Nucleic acid synthesis	Activation in the parasite	*Giardia*, *Trichomonas*, *Entamoeba*
Pentamidine	Nucleic acid synthesis	Differential uptake	*Leishmania* spp.
Quinine	Nucleic acid synthesis	Differential uptake	*Plasmodium* spp.
Eflornithine	Protein function	Differences in the target	*T. brucei gambiense*
Tetracycline	Protein function	Differential uptake	*Plasmodium* spp.
Benzimadazoles	Microtubule function	Differences in the target	*Giardia*, *Trichomonas*
Amphotericin B	Membrane function	Differences in the target	*Leishmania* spp.
Atovaquone	Energy metabolism	Differences in the target	*Plasmodium* spp.
Melarasaprol	Energy metabolism in the parasite	Target pathway more important	*T. brucei gambiense*
Primaquine	Energy metabolism	Differences in the target	*Trypanosoma* spp.

Albendazole has recently been shown to have significant antigiardial activity, although its mode of action is unclear. In *Leishmania*, amphotericin B binds to ergosterol in the membrane making it leaky to ions and small molecules (e.g. amino acids), while the anti-protozoal drugs atovaquone and primaquine bind to the cytochrome bc_1 complex and inhibit electron flow. The anti-trypanosomal drug melarsaprol is most likely to act by blocking glycolytic kinases, especially the cytoplasmic pyruvate kinase, although it may also disrupt the reduction of trypanothione.

7.1.2 Drug resistance

As with bacteria, drug resistance in some parasites such as *Plasmodium* is a major problem and tends to appear where chemotherapy has been used extensively. This problem is exacerbated by the fact there are so few drugs available for the control of some parasites, which utilize the same five basic resistance mechanisms that are displayed by bacteria: (1) metabolic inactivation of the drug; (2) use of efflux pumps; (3) use of alternative metabolic pathways; (4) alteration of the target; (5) elevation of the amount of target enzyme.

7.2 Other approaches to control

The early success in malaria control can be attributed to the use of professional spray teams who treated the inside of huts with DDT, without any direct involvement of the infected population. However, the problem with pesticides like DDT is that they lack specificity, and as

application is not always well directed there is often destruction of a wide range of insects, which may have undesirable side effects. Further problems include the accumulation of pesticide residues in the food chain and pesticide resistance in the target organism. Window screens and bed nets do prevent mosquito bites, however, and there has been a lot of interest in using bed nets impregnated with insecticide. Environmental control was a major strategy used before the development of modern insecticides. A good example of this is mosquito control through the removal of breeding sites by drainage, land reclamation projects, removal of vegetation overhanging water, speeding up water flow in canals, and periodic drainage and drying out of canals. Life cycle forms that enter the water system, such as cysts and oocysts of *Giardia* and *Cryptosporidium*, can present a major public health problem. These forms are often resistant to common disinfection methods and require physical removal from waters. Cysts and oocysts can be destroyed by use of proper sewage treatments such as anaerobic digestion, but these systems require regular maintenance in order to remain effective.

7.2.1 Biological control

Biological control is an active but developing area. Genetic control of insect vectors, particularly the use of irradiated sterile males, has been widely publicized, and the release of chemically sterile males has been attempted to control anopheline mosquitoes. Other similar methods include the release of closely related species within the environment in order to produce sterile hybrids. Genetically modified mosquitoes are currently being developed that are resistant to *Plasmodium* infection, and larvivorous fish have also been employed for mosquito control; other organisms considered for the same purpose include bacteria, fungi, nematodes and predatory insects. One of the best-studied agents is the bacterium *Bacillus thuringiensis*; the spore or the isolated toxin from this species can be used as a very effective and specific insecticide.

7.2.2 Vaccination

Where exposure to infection is likely to occur, killing the parasite as it enters the host is a sensible approach to control. There are two options available, chemoprophylaxis or vaccination. Unfortunately long-term chemotherapy can have adverse side effects, and in the absence of symptoms members of the at-risk population may fail to take the treatment. Vaccination would seem to be the ideal method of parasite control, as lifelong resistance may result from just a single treatment. Despite a huge amount of effort, the only successful parasite vaccines are those for the control of veterinary parasites. However, there has been significant success with the development of recombinant vaccines for the control of malaria. The recent development of DNA vaccines may be of use in the control of parasites. In this method the DNA encoding an important parasite protein is injected into host cells and the foreign 'vaccinating' protein is synthesized in or on the surface of the cell. This intracellular foreign protein enters the cell's major histocompatibility complex (MHC) class 1 pathway resulting in a cell-mediated immune response. In contrast, a protein that is extracellular enters the MHC class 2 pathway, which results primarily in an antibody or humoral response.

Despite advances in technology, few commercially available parasite vaccines exist and there are none for use in humans. Several vaccines are being evaluated in clinical trials and most of these are against malaria. Information about these can be found at the Malaria Vaccine Initiative website (www.malariavaccine.org). In addition there are vaccines being evaluated against other parasites, for example a *Leishmania* vaccine co-administered with BCG (bacillus of Calmette and Guérin) is under trial. Recently, DNA and viral vector-based vaccines have been tested in clinical trials including one utilizing an adenovirus human serotype 35-based vector encoding the malarial circumsporozoite protein.

8 Acknowledgement

The figures for this chapter originate in the copyright-free DPDx website maintained by the United States Centre for Disease Control Division of Parasitic Diseases; this source is gratefully acknowledged.

9 Further reading

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Part 2 Pathogens and host responses

7 Principles of microbial pathogenicity and epidemiology

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1 Introduction

Microorganisms are ubiquitous, and most of them are free-living and derive their nutrition from inert organic and inorganic materials. The association of humans with such microorganisms is generally harmonious, as the majority of those encountered are benign and, indeed, are often vital to commerce, health and a balanced ecosystem. The ability of bacteria and fungi to establish infections of plants, animals and humans varies considerably. Some are rarely, if ever, isolated from infected tissues, while opportunist pathogens (e.g. *Pseudomonas aeruginosa* or *Staphylococcus epidermidis*) can establish themselves only in compromised individuals. Only a few species of bacteria may be regarded as obligate pathogens, for which animals or plants are the only reservoirs for their existence (e.g. *Neisseria gonorrhoeae, Mycobacterium tuberculosis* and *Treponema pallidum*). Viruses (Chapter 5), on the other hand, must parasitize host cells in order to replicate and are therefore inevitably associated with disease. Even among the viruses and obligate bacterial

Hugo and Russell's Pharmaceutical Microbiology, Eighth Edition. Edited by Stephen P. Denyer, Norman Hodges, Sean P. Gorman, Brendan F. Gilmore.

pathogens the degree of virulence varies, in that some (particularly the bacteria) are able to coexist with the host without causing overt disease (e.g. *Staph. aureus*), while others will always cause some detriment to the host (e.g. rabies virus). Organisms such as these invariably produce their effects, directly or indirectly, by actively growing on or in the host tissues.

Other groups of microorganisms may cause disease through ingestion of substances (toxins) produced during microbial growth on foods (e.g. *Clostridium botulinum,* botulism; *Bacillus cereus,* vomiting). In this case, the organisms themselves do not have to proliferate in the host for the effects of the toxin to be manifested.

Animals and plants constantly interact with bacteria present within their environment. For an infection to develop, such microorganisms must remain associated with host tissues and increase their numbers more rapidly than they can be either eliminated or killed. This balance relates to the ability of the bacterium to mobilize nutrients and multiply in the face of innate defences and a developing immune response by the now compromised host.

The greater the number of bacterial cells associated with the initial challenge to the host, the greater will be the chance of disease. If the pathogen does not arrive at its 'portal of entry' to the body or directly at its target tissues in sufficient number, then an infection will not ensue. The minimum number of viable microorganisms that is required to cause infection and thereby disease is called the *minimum infective number* (MIN). The MIN varies markedly between the various pathogens and is also affected by the general health and immune status of the individual host. The course of an infection can be considered as a sequence of separate events that includes initial contact with the pathogen, its consolidation and spread between and within organs and its eventual elimination (Figure 7.1). Growth and consolidation of the microorganisms at the portal of entry commonly involves the formation of a microcolony (biofilm, see Chapters 3 and 8). Biofilms and microcolonies are collections of microorganisms that are attached to surfaces and enveloped within exopolymers (biofilm matrix) composed of polysaccharides, glycoproteins and/or proteins. Growth within the matrix not only protects the pathogens against opsonization and phagocytosis by the host but also modulates their microenvironment and reduces the effectiveness of many antibiotics. The localized high cell densities present within the biofilm communities also initiate production, by the colonizing organism, of extracellular virulence factors such as toxins, proteases and siderophores

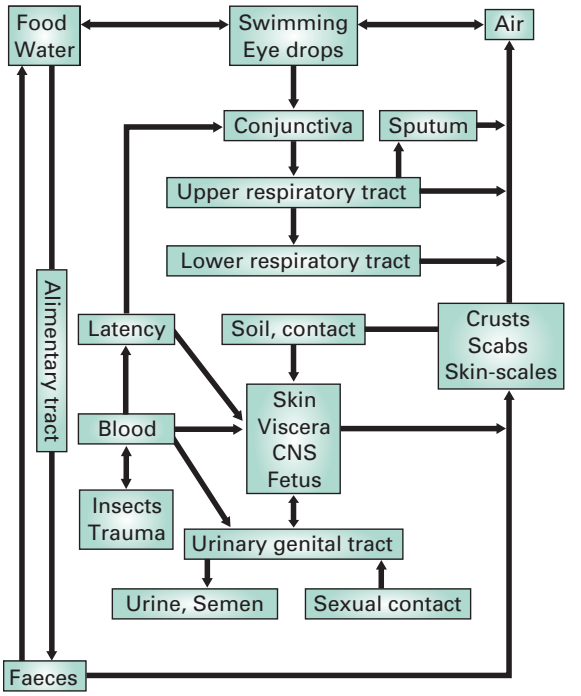

Figure 7.1 Routes of infection, spread and transmission of disease.

(low molecular weight ligands responsible for the solubilization and transport of iron(III) in microbial cells). These are associated with a phenomenon termed *quorum sensing* (see section 4.2) and help the pathogen to combat the host's innate defences and also promote the acquisition of nutrients.

Viruses are incapable of growing extracellularly and must therefore rapidly gain entry to cells (normally epithelial) at their initial site of entry. Once internalized in the non-immune host, they are to a large extent protected against the non-specific host defences. Following these initial consolidation events, the organisms may expand into surrounding tissues and/or disperse, via the blood, plasma, lymph or nerves, to distant tissues in order to establish secondary sites of infection or to consolidate further. In some instances microorganisms are able to colonize the host indefinitely and remain viable for many years (e.g. the herpesvirus varicella zoster which causes chickenpox, and herpes simplex virus 1 responsible for cold sores), but more generally they succumb to the heightened defences of the host, and in order to survive must either infect other individuals or survive in the general environment.

2 The human microbiome

Even though some parts of the human body are free from microorganisms (*axenic* state), the body harbours millions of mutualistic and commensal symbionts. Each of us unwittingly carries a population of bacterial cells on our skin and in our mouth and digestive tract that outnumbers cells carrying our own genome by approximately ten to one. *Mutualistic* relationships occur when both organisms (microbial and host cell) benefit from the interactions, whereas *commensalism* is a state in which one member of the relationship benefits without significantly affecting the other. Microorganisms that colonize body surfaces (internal and external) without normally causing disease constitute the *microbiome*. The microbiome comprises a number of distinct microbiotas which are characteristic of the region of the body colonized.

The microbiome is present throughout life and may comprise predominantly bacteria, with some fungi and protozoa, the majority of which are commensal. The microbiome begins to develop when the amniotic membrane surrounding the unborn child ruptures, allowing contact with vaginal, faecal and skin-associated microorganisms from the mother during childbirth. Microorganisms enter the infant's mouth and nose during passage through the birth canal, colonization of the upper respiratory tract occurs with the first breath of air, and the beginning of the colon microbiota occurs during feeding. The development of the resident microbiota is therefore initiated during the first few months of life. By comparison, transient microbiotas remain in the body for only a few hours, days or months. They are found in the same locations as the resident flora, but cannot persist because of their inability to compete with the microbiome, to resist elimination by the body's defence mechanisms or to tolerate the chemical and physical changes encountered.

Changes in relative abundance of normal microbiota, for whatever reason (e.g. major changes to diet, antibiotic treatment, hormonal changes, chemo- or radiotherapy), may allow one or more members of the microbiome to become opportunistic pathogens. For example, reductions in numbers of protective lactobacilli within the vagina brought about through antibiotic use can allow *Candida albicans*, a minority member of the normal microbiota, to grow more prolifically, resulting in an opportunistic vaginal yeast infection such as vaginal candidiasis. Following appropriate treatment, the normal flora is typically re-established in adults. In some areas of the body, such as the gastrointestinal tract, it is claimed that the recolonization by desired species can be encouraged by administration of probiotics. These are live cultures of intestinal bacteria that are marketed as conferring a health benefit and preventing digestive problems. Prebiotics, (normally fibre-like carbohydrates) that represent preferred or selective growth substrates for probiotic bacteria already present within the digestive tract are also of demonstrable benefit.

3 Portals of entry

3.1 Skin

The part of the body that is most widely exposed to microorganisms is the skin. Intact skin is usually impervious to microorganisms, and its surface is of acid pH and contains relatively few nutrients that are favourable for microbial growth. The vast majority of organisms falling on to the skin surface will die, while the survivors must compete with the commensal microflora for nutrients in order to grow. These commensals, which include coryneform bacteria, staphylococci and yeasts, derive nutrients from compounds like urea, hormones (e.g. testosterone) and fatty acids found in the apocrine and epocrine secretions. Such organisms are highly adapted to growth in this environment and will normally prevent the establishment of chance contaminants of the skin. Infections of the skin itself, such as ringworm (*Trichophyton mentagrophytes*) and warts (human papillomavirus, HPV), rarely, if ever, involve penetration of the epidermis. Infection can, however, occur through the skin following trauma such as burns, cuts and abrasions and, in some instances, through insect or animal bites or the injection of contaminated medicines. In recent years extensive use of intravascular and extravascular medical devices and implants has led to an increase in the occurrence of hospital-acquired infection. Commonly, these infections involve the growth of skin commensals such as *Staph. epidermidis* when associated with devices that penetrate the skin barrier. The organism grows as an adhesive biofilm on the surfaces of the device, and infection arises either from contamination of the device during its implantation or by growth along it of the organism from the skin. In such instances the biofilm sheds bacterial cells to the body and may give rise to a bacteraemia (the presence of bacteria in the blood).

The weak spots, or Achilles heels, of the body occur where the skin ends and mucous epithelial tissues begin (mouth, anus, eyes, ears, nose and urinogenital tract).

These mucous membranes present a much more favourable environment for microbial growth than the skin, in that they are warm, moist and rich in nutrients. Such membranes, nevertheless, possess certain characteristics that allow them to resist infection. Most of them, for example, possess their own highly adapted microbiotas (see section 2) which reduce the chances of infection by any invading organisms through a process termed *colonization resistance*. The resident flora varies greatly between different sites of the body, but is usually common to particular host species. Each site can be additionally protected by physicochemical barriers such as extreme acid pH in the stomach and bile salts in the large bowel, the presence of freely circulating non-specific antibodies and/or opsonins, and/or by macrophages and phagocytes (see Chapter 9). All infections start from contact between these tissues and the potential pathogen. Contact may be direct, from an infected individual to a healthy one; or indirect, and may involve inanimate vectors such as soil, food, drink, air and airborne particles. These may directly contact the body or be ingested or inhaled or enter wounds via infected bed linen and clothing. Indirect contact may also involve animal vector intermediates (carriers).

3.2 Respiratory tract

Air contains a large amount of suspended organic matter and, in enclosed occupied spaces, may hold up to 1000 microorganisms/m^3. Almost all of these airborne organisms are non-pathogenic bacteria and fungi, of which the average person inhales approximately 10 000/day. The respiratory tract is protected against this assault by a mucociliary blanket that envelops the upper respiratory tract and nasal cavity. Both present a tortuous path down which microbial particles travel and impact on these surfaces to become trapped within an enveloping blanket of mucus. Beating cilia move the mucus coating to the back of the throat where it, together with adherent particles, is swallowed. The alveolar regions of the lower respiratory tract have additional protection in the form of alveolar macrophages. To be successful, a pathogen must avoid being trapped in the mucus and swallowed; if deposited in the alveolar sacs it must avoid phagocytosis by macrophages, or if phagocytosed it must resist subsequent digestion by them. The possession of surface adhesins, specific for epithelial receptors, aids attachment of the invading microorganism and avoidance of removal by the mucociliary blanket. Other strategies include the export of ciliostatic toxins (i.e. *Corynebacterium diphtheriae*) that paralyse the cilial bed. As the primary defence of the respiratory tract is the mucociliary blanket, it is easy to envisage how infection with respiratory viruses (i.e. influenza virus, which kills respiratory epithelia) or the chronic inhalation of tobacco smoke, which increases mucin production and decreases the proportion of ciliated epithelial cells, increases the susceptibility of individuals to infection.

3.3 Intestinal tract

The intestinal tract must contend with whatever it is given in terms of food and drink. The extreme acidity and presence of digestive enzymes in the stomach will kill many of the bacteria challenging it, and the gastrointestinal tract carries its own commensal flora of yeast and lactobacilli that afford protection by, for example, competing with potential pathogens like *Helicobacter pylori*. Bile salts are mixed with the semidigested solids exiting from the stomach into the small intestine. These salts not only neutralize the stomach acids but also represent biological detergents or surfactants that are able to solubilize the outer membrane of many Gram-negative bacteria. Consequently, the small intestine is normally colonized by lower numbers of bacteria than the colon. The lower gut on the other hand is highly populated by commensal microorganisms ($10^{12}\,g^{-1}$ gut tissue) that are often associated with the intestinal wall, either embedded in layers of protective mucus or attached directly to the epithelial cells or attached to particulate food residues. The pathogenicity of incoming bacteria and viruses depends on their ability to survive passage through the stomach and duodenum and their capacity for attachment to, or penetration of, the gut wall, despite competition from the commensal flora and the presence of secretory antibodies (Chapter 9).

3.4 Urinogenital tract

In healthy individuals, the bladder, ureters and urethra are sterile, and sterile urine constantly flushes the urinary tract. Organisms invading the urinary tract must avoid being detached from the epithelial surfaces and washed out during urination. Because the male urethra is long (*c.*20 cm), bacteria must be introduced directly into the bladder, possibly through catheterization, in order to initiate an infection. The female urethra is much shorter (*c.*5 cm) and is more readily traversed by microorganisms that are normally resident within the vaginal vault. Bladder infections are therefore much more common in the female. Spread of the infection from the bladder to the kidneys can easily occur through reflux of urine into the ureter. As for the implantation of devices across

the skin barrier (above), long-term catheterization of the bladder will promote the occurrence of bacteriuria (the presence of bacteria in the urine) with all of the associated complications.

Lactic acid in the vagina gives it an acidic pH (5.0); this, together with other products of metabolism, inhibits colonization by most bacteria, except some lactobacilli that constitute the commensal flora. Other types of bacteria are unable to establish themselves in the vagina unless they have become extremely specialized. These species of microorganism tend to be associated with sexually transmitted infections.

3.5 Conjunctiva

The conjunctiva is usually free of microorganisms and protected by the continuous flow of secretions from lachrymal and other glands, and by frequent mechanical cleansing of its surface by the eyelid periphery during blinking. Lachrymal fluids contain a number of inhibitory compounds, together with lysozyme, that can enzymically degrade the peptidoglycan of Gram-positive bacteria such as staphylococci. Damage to the conjunctiva, caused through mechanical abrasion or reductions in tear flow, will increase microbial adhesion and allow colonization by opportunist pathogens. The likelihood of infection is thus promoted by the use of soft and hard contact lenses, physical damage, exposure to chemicals, or damage and infection of the eyelid border (blepharitis).

4 Consolidation

To be successful, a pathogen must be able to survive at its initial portal of entry, frequently in competition with the commensal flora and generally while subject to the attention of macrophages and wandering white blood cells. Such survival invariably requires the organism to attach itself firmly to the epithelial surface, eventually forming a biofilm. The initial attachment must be highly specific in order to displace the commensal microflora, and subsequently governs the course of an infection. Attachment can be mediated through provision, on the bacterial surface, of adhesive substances such as mucopeptide and mucopolysaccharide slime layers, fimbriae (Chapter 3), pili (Chapter 3) and agglutinins (Chapter 9). These are often highly specific in their binding characteristics, differentiating, for example, between the tips and bases of villi in the large bowel and the epithelial cells of the upper, mid and lower gut. Secretory antibodies, which are directed against such adhesions, block the initial attachment of the organism and thereby confer resistance to infection.

The outcome of the encounter between the tissues and potential pathogens is governed by the ability of the microorganism to multiply at a faster rate than it is removed from those tissues. Factors that influence this are the organism's rate of growth, the initial number arriving at the site and their ability to resist the efforts of the host tissues at removing/starving/killing them. The outcome of an encounter between a microorganism and a host can therefore be described as a balance between the accumulation of the pathogen and its elimination by the host.

The definition of *virulence* (i.e. the degree of pathogenicity caused by a microorganism) for pathogenic microorganisms must include the MIN. This will vary between individuals, but will invariably be lower in compromised hosts such as those who are catheterized, diabetics, smokers and cystic fibrosis patients, and those suffering trauma such as malnutrition, chronic infection or physical damage. A number of the individual factors that contribute towards virulence are discussed below.

4.1 Nutrient acquisition

Because the initial inoculum of a pathogen is usually too small to cause immediate damage to the host, it must acquire sufficient nutrients to allow it to multiply and increase in number. Not all nutrients, vitamins or growth factors are soluble and present in adequate quantities to allow pathogens to multiply. Moreover, trace elements may also be in short supply and can influence establishment of the pathogen. One such example is that of iron (Fe^{3+}) which is essential for microbial growth and function. Normally this is complexed with host iron-binding proteins such as lactoferrin or transferrin, resulting in insufficient iron being made available to the pathogen. In order to survive and multiply, pathogens need to be able to compete with the host (and normal microbiota) for iron. Some bacteria can do this through production of iron-chelating compounds called *siderophores* which have a greater affinity for iron than the host's iron-binding proteins, while others secrete hydrolytic enzymes that release iron from the host. Some organisms, such as *Ps. aeruginosa*, possess three or more different mechanisms of iron capture, giving them great versatility.

4.2 Biofilms

Cell attachment, and subsequent biofilm formation, is a means by which pathogens can remain in a favourable environment (i.e. one where there are plenty of nutrients)

without getting washed away. As a consequence, bacterial cell numbers and activities can become quite high. Biofilms can form on any surface (e.g. soft tissue, bone, medical implants) and may contain only one or two species (e.g. *Staph. aureus* mediated osteomyelitis) or more commonly several species of bacteria (e.g. dental plaque). Hence, biofilms may be considered as a functional microbial community. Within a biofilm intracellular signalling molecules (e.g. *N*-acyl homoserine lactones) are produced that when sufficient (threshold) concentrations are reached, up-regulate biofilm-specific genes. This process is known as quorum sensing, and is responsible for the formation and maintenance of the biofilm.

Once formed, biofilms can be extremely difficult, if not impossible, to remove. Their size and morphology can help protect the underlying cells, resisting physical forces or removal, phagocytosis and penetration of toxic molecules such as antibiotics. In addition, biofilms allow cells to live in close proximity to each other, thereby facilitating intercellular communication and genetic exchange. Finally, due to their profound resistance, biofilms provide foci of infection which often can only be removed by surgery.

4.3 Resistance to host defences

Most bacterial infections confine themselves to the surface of epithelial tissue (e.g. *Bordetella pertussis*, *Corynebacterium diphtheriae*, *Vibrio cholerae*). This is, to a large extent, a reflection of their inability to combat that host's deeper defences. Survival at these sites is largely due to firm attachment to the epithelial cells. Such organisms manifest disease through the production and release of toxins (see below).

Other groups of organisms regularly establish systemic infections after traversing the epithelial surfaces (e.g. *Brucella abortus*, *Salmonella enterica* serovar Typhi, *Streptococcus pyogenes*). This property is associated with their abilities either to gain entry into susceptible cells and thereby enjoy protection from the body's defences, or to be phagocytosed by macrophages or polymorphs and yet resist their lethal action and multiply within them. Other organisms are able to multiply and grow freely in the body's extracellular fluids. Microorganisms have evolved a number of different strategies that allow them to suppress the host's normal defences and thereby survive in the tissues.

4.3.1 Modulation of the inflammatory response

Growth of microorganisms releases cellular products into their surrounding medium, many of which cause non-specific inflammation associated with dilation of blood vessels. This increases capillary flow and access of phagocytes to the infected site. Increased lymphatic flow from the inflamed tissues carries the organisms to lymph nodes where further antimicrobial and immune forces come into play (Chapter 9). Many of the substances that are released by microorganisms in this fashion are chemotactic towards polymorphs that tend, therefore, to become concentrated at the site of infection: this is in addition to the inflammation and white blood cell accumulation that is associated with antibody binding and complement fixation (Chapter 9). Many organisms have adapted mechanisms that allow them to overcome these initial defences. Thus, virulent strains of *Staph. aureus* produce a mucopeptide (peptidoglycan), which suppresses early inflammatory oedema, and related factors that suppress the chemotaxis of polymorphs.

4.3.2 Avoidance of phagocytosis

Resistance to phagocytosis is sometimes associated with specific components of the cell wall and/or with the presence of capsules surrounding the cell wall. Classic examples of these are the M-proteins of the streptococci, the polysaccharide capsules of the pneumococci and the alginate-like slime associated with *Ps. aeruginosa* infections of the cystic-fibrotic lung. The acidic polysaccharide K-antigens of *Escherichia coli* and *S. enterica* serovar Typhi behave similarly, in that (1) they can mediate attachment to the intestinal epithelial cells, and (2) they render phagocytosis more difficult. Generally, possession of an extracellular capsule and/or slime will reduce the likelihood of phagocytosis.

Microorganisms are more readily phagocytosed when coated with antibody (opsonized). This is due to the presence on the white blood cells of receptors for the Fc fragment of IgM and IgG (discussed in Chapter 9). Avoidance of opsonization will clearly enhance the chances of survival of a particular pathogen. A substance called protein A is released from actively growing strains of *Staph. aureus*, which acts by non-specific binding to IgG at the Fc region (see also Chapter 9), at sites both close to, and remote from, the bacterial surface. This blocks the Fc region of bound antibody masking it from phagocytes. Protein A–IgG complexes remote from the infection site will also bind complement, thereby depleting it from the plasma and negating its actions near to the infection site.

4.3.3 Survival following phagocytosis

Death of microorganisms following phagocytosis can be avoided if the microorganisms are not exposed to the

killing and digestion processes within the phagocyte. This is possible if fusion of the lysosomes with phagocytic vacuoles can be prevented. Such a strategy is employed by virulent *Mycobacterium tuberculosis*, although the precise mechanism is unknown. Other bacteria seem able to grow within the vacuoles despite lysosomal fusion (*Listeria monocytogenes, S. enterica* serovar Typhi). This can be attributed to cell wall components that prevent access of the lysosomal substances to the bacterial membranes (e.g. *Brucella abortus,* mycobacteria) or to the production of extracellular catalase which neutralizes the hydrogen peroxide liberated in the vacuole (e.g. staphylococci).

If microorganisms are able to survive and grow within phagocytes, they will escape many of the other body defences such as the lymph nodes, and be distributed around the body. As the lifespan of phagocytes is relatively short, such bacteria will eventually be delivered to the liver and gastrointestinal tract where they are 'recycled'.

4.3.4 Killing of phagocytes

An alternative strategy is for the microorganism to kill the phagocyte. This can be achieved by the production of leucocidins (e.g. staphylococci, streptococci) which promote the discharge of lysosomal substances into the cytoplasm of the phagocyte rather than into the vacuole, thus directing the phagocyte's lethal activity towards itself.

5 Manifestation of disease

Once established, the course of a bacterial infection can proceed in a number of ways. These can be related to the relative ability of the organism to penetrate and invade surrounding tissues and organs. The vast majority of pathogens, being unable to combat the defences of the deeper tissues, consolidate further on the epithelial surface. Others, including most viruses, penetrate the epithelial layers, but no further, and can be regarded as partially invasive. A small group of pathogens are fully invasive. These permeate the subepithelial tissues and are circulated around the body to initiate secondary sites of infection remote from the initial portal of entry (Figure 7.1).

Other groups of organisms may cause disease through ingestion by the victim of substances produced during microbial growth on foods. Such diseases may be regarded as intoxications rather than as infections and are considered later (section 6.1.1). Treatment in these cases is usually an alleviation of the harmful effects of the toxin rather than elimination of the pathogen from the body.

5.1 Non-invasive pathogens

Bordetella pertussis (the aetiological agent of whooping cough) is probably the best described of these pathogens. This organism is inhaled and rapidly localizes on the mucociliary blanket of the lower respiratory tract. This localization is very selective and is thought to involve agglutinins on the organism's surface. Toxins produced by the organism inhibit ciliary movement of the epithelial surface and thereby prevent removal of the bacterial cells to the gut. A high molecular weight exotoxin is also produced during the growth of the organism which, being of limited diffusibility, pervades the subepithelial tissues to produce inflammation and necrosis. Corynebacterium *diphtheriae* (the causal organism of diphtheria) behaves similarly, attaching itself to the epithelial cells of the respiratory tract. This organism produces a diffusible toxin of low molecular weight, which enters the blood circulation and brings about a generalized toxaemia.

In the gut, many pathogens adhere to the gut wall and produce their effect via toxins that pervade the surrounding gut wall or enter the systemic circulation. *V. cholerae* and some enteropathic *E. coli* strains localize on the gut wall and produce toxins that increase vascular permeability. The end result is a hypersecretion of isotonic fluids into the gut lumen, acute diarrhoea and, as a consequence, dehydration that may be fatal in young or elderly people. In all these instances, binding to epithelial cells is not essential but increases permeation of the toxin and prolongs the presence of the pathogen.

5.2 Partially invasive pathogens

Some bacteria, and most viruses, are able to attach to the mucosal epithelia and then penetrate rapidly into the epithelial cells. These organisms multiply within the protective environment of the host cell, eventually killing it and inducing disease through erosion and ulceration of the mucosal epithelium. Typically, members of the genera *Shigella* and *Salmonella* utilize such mechanisms in infections of the gastrointestinal tract. These bacteria attach to the epithelial cells of the large and small intestines, respectively, and, following their entry into these cells by induced pinocytosis, multiply rapidly and penetrate laterally into adjacent epithelial cells. The mechanisms for such attachment and movement are unknown but involve a transition from a non-motile to motile phenotype. Some species of salmonellae produce, in addition, exotoxins that induce diarrhoea (section 6.1.4). There are innumerable serotypes of *Salmonella,* which

are primarily parasites of animals but are important to humans in that they colonize farm animals such as pigs and poultry and ultimately infect foods derived from them. *Salmonella* food poisoning (salmonellosis), therefore, is commonly associated with inadequately cooked meats, eggs and also with cold meat products that have been incorrectly stored following contact with the uncooked product. Dependent upon the severity of the lesions induced in the gut wall by enteric pathogens, red blood cells and phagocytes pass into the gut lumen, along with plasma, and cause the classic 'bloody flux' of bacillary dysentery. Similar erosive lesions are produced by some enteropathic strains of *E. coli.*

Viral infections such as influenza and the 'common cold' (in reality 300–400 different strains of rhinovirus) infect epithelial cells of the respiratory tract and nasopharynx, respectively. Release of the virus, after lysis of the host cells, is to the void rather than to subepithelial tissues. The residual uninfected epithelial cells are rapidly infected, resulting in general degeneration of the tracts. Such damage not only predisposes the respiratory tract to infection with opportunist pathogens such as *N. meningitidis* and *Haemophilus influenzae* but also causes the associated fever.

5.3 Fully invasive pathogens

Invasive pathogens either aggressively invade the tissues surrounding the primary site of infection (active spread) or are passively transported around the body in the blood, lymph, cerebrospinal, axonal or pleural fluids (passive spread). Some, especially aggressive organisms, move both passively and actively, setting up multiple, expansive secondary sites of infection in various organs.

5.3.1 Active spread

Active spread of microorganisms through normal subepithelial tissues is difficult in that the gel-like nature of the intercellular materials physically inhibits bacterial movement. Induced death and lysis of the tissue cells produces, in addition, a highly viscous fluid, partly due to undenatured DNA. Physical damage, such as wounds, is rapidly sealed with fibrin clots, thereby reducing the effective routes for spread of opportunist pathogens. Organisms such as *Strep. pyogenes, Cl. perfringens* and, to some extent, the staphylococci, are able to establish themselves in tissues by virtue of their ability to produce a wide range of extracellular enzyme toxins. These are associated with killing of tissue cells, degradation of intracellular materials and mobilization of nutrients. A selection of such toxins will be considered briefly.

- *Haemolysins* are produced by most of the pathogenic staphylococci and streptococci. They have a lytic effect on red blood cells, releasing iron-containing nutrients.
- *Fibrinolysins* are produced by both staphylococci (staphylokinase) and streptococci (streptokinase). These toxins indirectly activate plasminogen and so dissolve fibrin clots that the host forms around wounds and lesions to seal them. The production of fibrinolysins therefore increases the likelihood of the infection spreading. Streptokinase may be employed clinically in conjunction with streptodornase (Chapter 26) in the treatment of thrombosis.
- *Collagenases* and *hyaluronidases* are produced by most of the aggressive invaders of tissues. These are able to dissolve collagen fibres and the hyaluronic acids that function as intercellular cements; this causes the tissues to break up and produce oedematous lesions.
- *Phospholipases* are produced by organisms such as *Cl. perfringens* (α-toxin). These toxins kill tissue cells by hydrolysing the phospholipids that are present in cell membranes.
- *Amylases, peptidases* and *deoxyribonucleases* mobilize many nutrients that are released from lysed cells. They also decrease the viscosity of fluids present at the lesion by depolymerization of their biopolymer substrates.

Organisms possessing the above toxins, particularly those also possessing *leucocidins*, are likely to cause expanding oedematous lesions at the primary site of infection. In the case of *Cl. perfringens,* a soil microorganism that has become adapted to a saprophytic mode of life, infection arises from an accidental contamination of deep wounds when a process similar to that seen during the decomposition of a carcass ensues (gangrene). This organism is most likely to spread through tissues when blood circulation, and therefore oxygen tension, in the affected areas is minimal.

Abscesses formed by streptococci and staphylococci can be deep-seated in soft tissues or associated with infected wounds or skin lesions; they become localized through the deposition of fibrin capsules around the infection site. Fibrin deposition is partly a response of the host tissues, but is also partly a function of enzyme toxins such as *coagulase*. Phagocytic white blood cells can migrate into these abscesses in large numbers to produce significant quantities of pus. Such pus, often carrying the infective pathogen, might be digested by other phagocytes in the late stages of the infection or discharged to the exterior or to the capillary and lymphatic network. In the latter case, blocked capillaries might serve as sites for

secondary lesions. Toxins liberated from the microorganisms during their growth in such abscesses can freely diffuse to the rest of the body to set up a generalized toxaemia.

S. enterica serovar Typhi, *S. enterica* serovar Paratyphi and *S. enterica* serovar Typhimurium are serotypes of *Salmonella* (section 4.2) that are not only able to penetrate into intestinal epithelial cells and produce exotoxins, but are also able to penetrate beyond into subepithelial tissues. These organisms therefore produce a characteristic systemic disease (typhoid and enteric fever), in addition to the usual symptoms of salmonellosis. Following recovery from such infection the organism is commonly found associated with the gallbladder. In this state, the recovered person will excrete the organism and become a reservoir for the infection of others.

5.3.2 Passive spread

When invading microorganisms have crossed the epithelial barriers they will almost certainly be taken up with lymph in the lymphatic ducts and be delivered to filtration and immune systems at the local lymph nodes. Sometimes this serves to spread infections further around the body. Eventually, spread may occur from local to regional lymph nodes and thence to the bloodstream. Direct entry to the bloodstream from the primary portal of entry is rare and will only occur when the organism damages the blood vessels or if it is injected directly into them. This might be the case following an insect bite or surgery. Bacteraemia such as this will often lead to secondary infections remote from the original portal of entry.

6 Damage to tissues

Damage caused to the host organism through infection can be direct and related to the destructive presence of microorganisms (or to their production of toxins) in particular target organs; or it can be indirect and related to interactions of the antigenic components of the pathogen with the host's immune system. Effects can therefore be closely related to, or remote from, the infected organ.

Symptoms of the infection can in some instances be highly specific, relating to a single, precise pharmacological response to a particular toxin; or they might be non-specific and relate to the usual response of the body to particular types of trauma. Damage induced by infection will therefore be considered in these categories.

6.1 Direct damage

6.1.1 Specific effects

For the host, the consequences of infection depend to a large extent upon the tissue or organ involved. Soft tissue infections of skeletal muscle are likely to be less damaging than, for instance, infections of the heart muscle and central nervous system. Infections associated with the epithelial cells that make up small blood vessels can block or rupture them to produce anoxia or necrosis in the tissues that they supply. Cell and tissue damage is generally the result of a direct local action by the microorganisms, usually concerning action at the cytoplasmic membranes. The target cells are usually phagocytes and are generally killed (e.g. by *Brucella, Listeria, Mycobacterium*). Interference with membrane function through the action of enzymes such as phospholipase causes the affected cells to leak. When lysosomal membranes are affected, the lysosomal enzymes disperse into the cells and tissues causing them, in turn, to autolyse. This is mediated through the vast battery of enzyme toxins available to these organisms (section 4). If these toxins are produced in sufficient concentration they may enter the circulatory systems to produce a generalized toxaemia. During their growth, other pathogens liberate toxins that possess very precise, singular pharmacological actions. Diseases mediated in this manner include diphtheria, tetanus and scarlet fever.

In diphtheria, the organism *C. diphtheriae* confines itself to epithelial surfaces of the nose and throat and produces a powerful toxin which affects an elongation factor involved in eukaryotic protein biosynthesis. The heart and peripheral nerves are particularly affected, resulting in myocarditis (inflammation of the myocardium) and neuritis (inflammation of a nerve). Little damage is produced at the infection site.

Tetanus occurs when *Cl. tetani,* ubiquitous in the soil and the faeces of herbivores, contaminates wounds, especially deep puncture-type lesions. These might be the result of a minor trauma such as a splinter, or a major one such as a motor vehicle accident. At these sites, tissue necrosis ,and possibly also microbial growth, reduce the oxygen tension to allow this anaerobe to multiply. Its growth is accompanied by the production of a highly potent toxin that passes up peripheral nerves and diffuses locally within the central nervous system. The toxin has a strychnine-like action and affects normal function at the synapses. As the motor nerves of the brainstem are the shortest, the cranial nerves are the first affected, with twitches of the eyes and spasms of the jaw (lockjaw).

A related organism, *Cl. botulinum*, produces a similar toxin that may contaminate food if the organism has grown in it and if conditions are favourable for anaerobic growth. Meat pastes and pâtés are likely sources. This toxin interferes with acetylcholine release at cholinergic synapses and also acts at neuromuscular junctions. Death from this toxin eventually results from respiratory failure.

Many other organisms are capable of producing intoxication following their growth on foods. Most common among these are the staphylococci and strains of *B. cereus*. Some strains of *Staph. aureus* produce an enterotoxin which acts on the vomiting centres of the brain. Nausea and vomiting therefore follow ingestion of contaminated foods and the delay between eating and vomiting varies between 1 and 6 hours depending on the amount of toxin ingested. *B. cereus* also produces an emetic toxin but its actions are delayed and vomiting can follow up to 20 hours after ingestion. The latter organism is often associated with rice products and will propagate when the rice is cooked (spore activation) and subsequently reheated after a period of storage.

Scarlet fever is produced following infection with certain strains of *Strep. pyogenes*. These organisms produce a potent toxin that causes an erythrogenic skin rash that then accompanies the more usual effects of a streptococcal infection.

6.1.2 Non-specific effects

If the infective agent damages an organ and affects its functioning, this can manifest itself as a series of secondary disease features that reflect the loss of that function to the host. Thus, diabetes may result from an infection of the islets of Langerhans, paralysis or coma from infections of the central nervous system, and kidney malfunction from loss of tissue fluids and its associated hyperglycaemia. In this respect virus infections almost inevitably result in the death and lysis of the host cells. This will result in some loss of function by the target organ. Similarly, exotoxins and endotoxins can also be implicated in non-specific symptoms, even when they have well-defined pharmacological actions. Thus, a number of intestinal pathogens (e.g. *V. cholerae, E. coli*) produce potent exotoxins that affect vascular permeability. These generally act through adenylate cyclase, raising the intracellular levels of cyclic AMP (adenosine monophosphate). As a result of this the cells lose water and electrolytes to the surrounding medium, the gut lumen. A common consequence of these related, yet distinct, toxins is acute diarrhoea and haemoconcentration. Kidney malfunction might well follow and in severe cases lead to death. Symptomologically there is little difference between these conditions and the food poisoning induced by ingestion of staphylococcal enterotoxin (above).

6.2 Indirect damage

Inflammatory materials are released not only from necrotic cells but also directly from the infective agent. Endotoxins are derived from constituents of the bacterial cell rather than being deliberately exported cellular products. Thus, during the growth and autolysis of Gram-negative bacteria components of their cell envelopes, such as lipopolysaccharide (see Chapter 3) are shed to the environment. Endotoxins tend to be less toxic than exotoxins and have much less precise pharmacological actions. Indeed, it is not always clear to what extent these can be related to actions by the host or by the pathogen. Reactions include local inflammation, elevations in body temperature, aching joints, and head and kidney pain. Inflammation causes swelling, pain and reddening of the tissues, and sometimes loss of function of the organs affected. These reactions may sometimes be the major sign and symptom of the disease.

While various toxic effects have been attributed to these endotoxins, their role in the establishment of the infection, if any, remains unclear. The most notable effect of these materials is their ability to induce a high body temperature (pyrogenicity) (Chapters 3 and 22). The pyrogenic effect of lipopolysaccharide relates to the action of the lipid A component directly upon the hypothalamus and also to its direct action on macrophages and phagocytes. Elevation of body temperature follows within 1–2 hours. In infections such as meningitis the administration of antibiotics may cause such a release of pyrogen that the resultant inflammation and fever is fatal. In such instances antibiotics are co-administered with steroids to counter this effect. The pyrogenic effects of lipid A are unaffected by moist heat treatment (autoclave). Growth of Gram-negative organisms such as *Pseudomonas aeruginosa* in stored water destined for use in terminally sterilized products will cause the final product to be pyrogenic. Processes for the destruction of pyrogen associated with glassware, and tests for the absence of pyrogen in water and product, therefore form an important part of parenteral drug manufacture (Chapter 22).

Many microorganisms minimize the effects of the host's defence system against them by mimicking the antigenic structure of the host tissue. The eventual immunological response of the host to infection then leads to its autoimmune self-destruction. Thus, infec-

tions with *Mycoplasma pneumoniae* can lead to production of antibody against normal group O erythrocytes, with concomitant haemolytic anaemia.

If antigen released from the infective agent is soluble, antigen–antibody complexes are produced. When antibody is present at a concentration equal to or greater than the antigen, such as in the case of an immune host, these complexes precipitate and are removed by macrophages present in the lymph nodes. When antigen is present in excess, the complexes, being small, continue to circulate in the blood and are eventually filtered off by the kidneys, becoming lodged in kidney glomeruli and in the joints. Localized inflammatory responses in the kidneys are sometimes then initiated by the complement system (Chapter 9). Eventually the filtering function of the kidneys becomes impaired, producing symptoms of chronic glomerulonephritis.

7 Recovery from infection: exit of microorganisms

The primary requirement for recovery is that multiplication of the infective agent is brought under control, so that it ceases to spread around the body and that the damaging consequences of its presence are arrested and repaired. Such control is brought about by the combined functions of the phagocytic, immune and complement systems. A successful pathogen will not seriously debilitate its host; rather, the continued existence of the host must be ensured in order to maximize the dissemination of the pathogen within the host population. From the microorganism's perspective the ideal situation is where it can persist permanently within the host and be constantly released to the environment. Although this is the case for a number of virus infections (chickenpox, herpes) and for some bacterial ones, it is not common. Generally, recovery from infection is accompanied by complete destruction of the organism and restoration of a sterile tissue. Alternatively, the organism might return to a commensal relationship with the host on the epithelial and skin surface.

Where the infective agent is an obligate pathogen, a means must exist for it to infect other individuals before its eradication from the host organism. The route of exit is commonly related to the original portal of entry (Figure 7.1). Thus, pathogens of the intestinal tract are liberated in the faeces and might easily contaminate food and drinking water. Infective agents of the respiratory tract might be exhaled during coughing, sneezing or talking, survive in the associated water droplets, and infect nearby individuals through inhalation. Infective agents transmitted by insect and animal vectors may be spread through those same vectors, the insects/animals having been themselves infected by the diseased host. For some 'fragile' organisms (e.g. *N. gonorrhoeae, T. pallidum*), direct contact transmission is the only means of spread between individual hosts. In these cases, intimate contact between epithelial membranes, such as occurs during sexual contact, is required for transfer to occur. For opportunist pathogens, such as those associated with wound infections, transfer is less important because the pathogenic role is minor. Rather, the natural habitat of the organism serves as a constant reservoir for infection.

8 Epidemiology of infectious disease

Spread of a microbial disease through a population of individuals can be considered as *vertical* (transferred from one generation to another) or *horizontal* (transfer occurring within genetically unrelated groups). The latter can be divided into *common source outbreaks*, relating to infection of a number of susceptible individuals from a single reservoir of the infective agent (i.e. infected foods), or *propagated source outbreaks*, where each individual provides a new source for the infection of others.

Common source outbreaks are characterized by a sharp onset of reported cases over the course of a single incubation period (Figure 7.2) and relate to a common experience of the infected individuals (e.g. a contaminated food product). The number of cases will persist until the source of the infection is removed. If the source remains (i.e. a reservoir of insect vectors) then the disease becomes endemic to the exposed population, with a constant rate of infection. Propagated source outbreaks, on the other hand, are brought about by person-to-person spread, and show a gradual increase in reported cases over a number of incubation periods and eventually decline when most of the susceptible individuals in the population have been affected (Figure 7.2). Factors that contribute to propagated outbreaks of infectious disease are the infectivity of the agent (I), the population density (P) and the numbers of susceptible individuals in it (F). The likelihood of an epidemic occurring is given by the product of these three factors (i.e. FIP). An increase in any one of them might initiate an outbreak of the disease in epidemic proportions. Thus, reported cases of particular diseases show periodicity, with outbreaks of epidemic

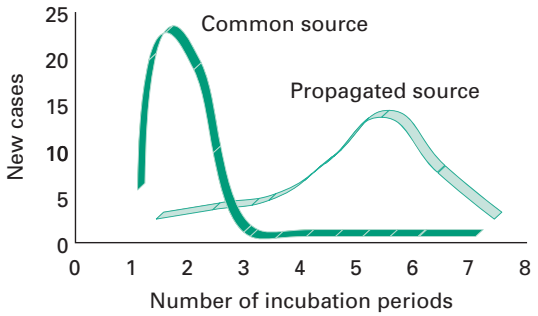

Figure 7.2 Comparison of timescale for common source outbreak and propagated source outbreak infections.

proportion occurring only when FIP exceeds certain critical threshold values, related to the infectivity of the agent. Outbreaks of measles and chickenpox therefore tend to occur annually in the late summer among children attending school for the first time. This has the effect of concentrating all susceptible individuals in one, often confined, space at the same time. The proportion of susceptible individuals can be reduced through rigorous vaccination programmes (Chapter 9). Provided that the susceptible population does not exceed the threshold FIP value, then herd immunity against epidemic spread of the disease will be maintained.

Certain types of infectious agent (e.g. influenza virus) are able to combat herd immunity such as this through undergoing major antigenic changes. These render the majority of the population susceptible, and their occurrence is often accompanied by spread of the disease across the entire globe (pandemics).

9 Further reading

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8 Microbial biofilms: consequences for health

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1 Introduction

Traditionally, microbiologists have grown bacteria as suspension cultures in rich media, in order to optimize cell yield. This planktonic mode of growth also became part of the standard assay on which all existing antimicrobials were selected and developed, and continues to be the basis for the selection of antimicrobials for specific patient treatment. We now recognize that in most environments, including our bodies, bacteria typically exist as adherent microcolonies termed *biofilms*, which afford bacteria a number of growth advantages including an inherent lack of susceptibility to antimicrobials. This antimicrobial tolerance differs from classical genetic resistance in that this reduced susceptibility disappears when the biofilm is returned to planktonic growth. Biofilm tolerance is multifactorial, which includes the spatial and structural parameters of the biofilm as well as the increased phenotypic diversity within the biofilm population. Biofilms are believed to be associated with approximately 60% of human infections including chronic, recurrent and device-related infections, therefore treatment of biofilm infections has become an important focus in modern medicine. As planktonic susceptibility testing, via the minimal inhibitory concentration (MIC) test, provides little guidance in the selection of antimicrobials to treat biofilms, a change in paradigm is required to determine appropriate treatment of biofilms and for the discovery of next-generation antimicrobials.

2 Biofilms

Biofilms are microcolonies of one or more species of bacteria or fungi typically growing adherent to a biotic

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or abiotic surface. Biofilms form to allow bacteria to maintain themselves in a niche of their choosing rather than being washed away by the shear force of running water in the natural environment or the movement of body fluids and mucins in the body. Biofilms provide a more energy-efficient means of growth, capturing nutrients as they flow past and easily expelling waste. They also provide a more secure environment for sustainability, making it difficult for phagocytes, found both in nature and as part of the immune system, to eradicate the biofilm. Also, as a biofilm, bacteria and fungi are less susceptible to antimicrobials, allowing them to be more tolerant than their planktonic brethren to antibiotics found in nature and those used clinically. A cartoon of the biofilm life cycle of bacteria is shown in Figure 8.1. In the centre of the figure bacteria exist in a mature biofilm that may be formed from many species, as in a consortium formed on the face of a rock in a stream or those found in the mouth, as part of our dental plaque. Chemical signals regulate the interactions between members of the biofilm just as hormones regulate the cells of our body. For example, under specific stress conditions appropriate signalling may lead to an increase in phenotypic diversity within the biofilm to accommodate the stress or these signals may cause bacteria to revert to their more motile planktonic phenotype and leave the biofilm to establish new microcolonies that will give rise to a mature biofilm.

2.1 Biofilms in nature and the consequences to health

Bacteria exist often as multispecies biofilms in nature, which may allow pathogens to survive in nature away from their natural host where they may serve as a nidus for reinfection. Examples of this are enteric organisms that form biofilms in drinking-water pipes or in wells. Following an original contamination event, biofilms allow for sustainability of these populations, which then serve to shed further organisms into potable water supplies, even after the apparent clearance of the original contamination event. Contamination of our groundwater sources pose concerns for the future of our potable water supplies; this threat is accentuated by the stability that biofilms of these contaminants bring into the equation.

2.2 Biofilms in the food industry

Food-borne outbreaks of infections are often associated with biofilms formed on the hard surfaces of food-

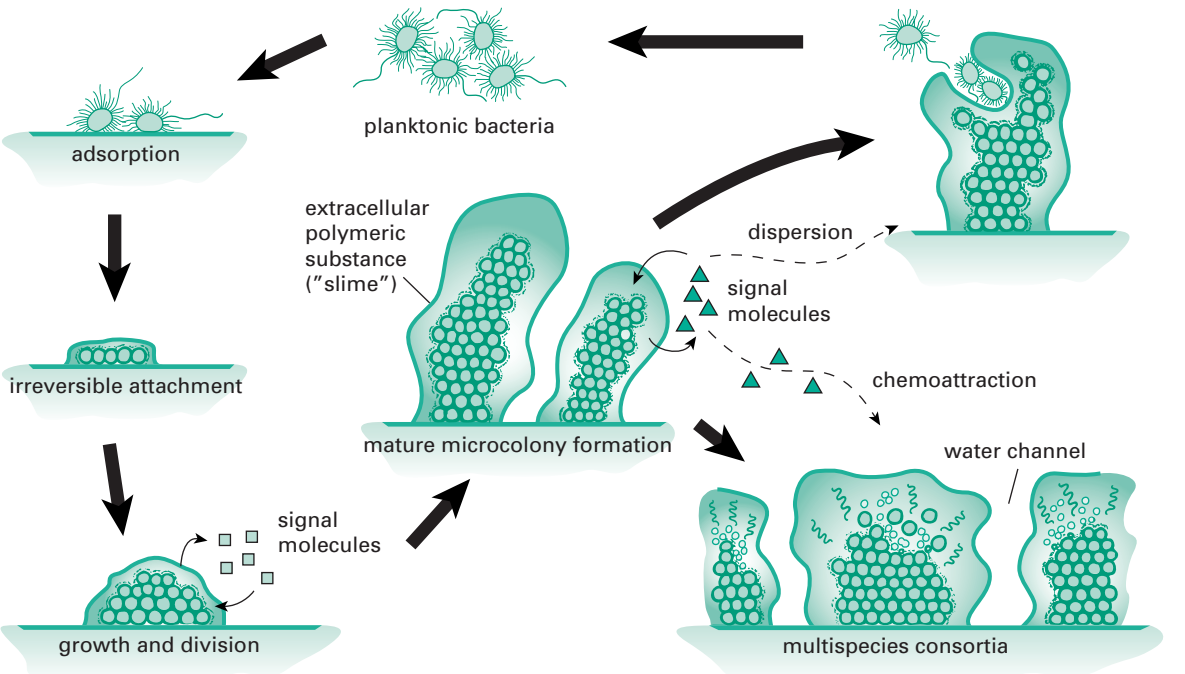

Figure 8.1 The main stages in microbial surface attachment and biofilm formation (From Harrison, J.J. *et al.* (2005) Biofilms. *Am Sci*, **93**, 508–515, with permission from *American Scientist.*)

processing plants, such as tables, knives and processing equipment. The inherent resistance of biofilms to many biocides used in the cleaning process allows these bacteria to multiply and contaminate food products.

2.3 Biofilms in hospitals

Nosocomial infections within healthcare facilities often result from pathogens surviving in the environment as biofilms. Biofilms may facilitate carriage by patients, hospital staff, or visitors, as for example in colonization of nasal passages by meticillin-resistant *Staphylococcus aureus* (MRSA). Biofilms may also become associated with hard surfaces, drains or waterpipes within the facility. Infections may also be associated with instruments or devices used in the hospital, such as the contamination of endoscopes by biofilms, where they are responsible for the passage of infectious organisms from one patient to another. Whether biofilms form on animate or inanimate surfaces, their recalcitrant nature makes it difficult to remove them from the environment.

2.4 Biofilms and medical devices

Medical devices have become an essential aspect of patient care, with tens of millions of implantable or indwelling medical devices (such as catheters, endotracheal tubes, artificial joint protheses and so on) used each year in patients worldwide. However, despite the evolution of medical devices and the biomaterials from which they are manufactured, their use *in vivo* is significantly compromised by their seemingly ubiquitous propensity to succumb to microbial colonization and biofilm formation, otherwise known as *medical device-associated infection*. Immediately after implantation, the device surface becomes modified by the adsorption of host-derived proteins, extracellular matrix proteins, coagulation products, etc., depending on the site. This 'conditioning film' renders the surface of the device favourable for microbial adhesion and is often followed by rapid primary attachment of microorganisms to the material surface and biofilm formation.

The microorganisms responsible for causing medical device-associated infections may be either from exogenous (carers, visitors, healthcare environment) or endogenous sources (via the migration of microorganisms from normally colonized body sites). Although site-dependent, the main causative organisms of medical device-associated nosocomial infections are frequently normal skin biota including *Staph. aureus* and coagulase-negative staphylococci, predominantly *Staph. epidermidis*, which is the most common causative organism of infections related to intravascular catheters and other implanted medical devices. A number of other key microorganisms have been shown to be significant causative organisms of medical device-related nosocomial infections, including *Pseudomonas aeruginosa* (ventilator-associated pneumonia, VAP), enterococci, *Escherichia coli* (urinary tract infection (UTI), septicaemia) and *Proteus* species such as *Proteus mirabilis* (UTI, device encrustation).

At least half of all cases of healthcare-associated infections are estimated to be due to biofilm-mediated, medical device-associated infections, with medical device use now regarded as the greatest external predictor of healthcare-associated infections. The development of medical device-associated infections generally necessitates the complete removal and replacement of the device, with the level of clinical intervention depending on the nature and site of the device implantation. Systemic antibiotics (often a combination therapy of two or more antimicrobial agents) represent the conventional approach to the treatment of device-associated infections; however, given the high degree of tolerance to antimicrobial challenge that is a feature of biofilm populations, eradication proves extremely difficult and infection relapses frequently occur. This has led to the development of a range of anti-infective and antimicrobial biomaterials for use in device manufacture, though the long-term efficacy of these devices in the reduction of medical device-associated infection is an area of considerable debate.

Healthcare-associated infections typically occur at four main body sites (urinary tract, respiratory tract, surgical sites and bloodstream infections), three of which (UTI, pneumonia, bloodstream infections) are commonly associated with the use of indwelling devices. Indeed, around 95% of nosocomial UTIs reported are linked to the use of urological devices (mainly urinary catheters), and more than 85% of nosocomial respiratory infections (mainly VAPs) are device-related. Central venous catheters pose the greatest risk of mortality due to catheter-related bloodstream infections, with incidences in the USA ranging from 100 000 to 500 000 cases annually, resulting in more than 25 000 deaths per year. Although these represent the most common device-associated infections, it is worth noting that all types of implantable medical device are susceptible to infection: for example, peritoneal catheter infections in peritoneal dialysis, orthopaedic implant infections, and biofilm formation on prosthetic heart valves. In addition to patient morbidity and mortality, device-associated infections

impose significant financial burdens on healthcare providers, related primarily to increased hospitalization time and associated care costs. Despite this, the use of and dependence on implantable, indwelling medical devices increases annually, correlated to an increasing ageing population in industrialized nations.

3 Tolerance of biofilms to antimicrobials

With the discovery of antibiotics the world changed. Acute infectious diseases, the leading cause of morbidity and mortality in humans, became treatable, resulting in an increase in life expectancy and quality of life. While infections continue to be a leading contributor to mortality, they are often associated with pre-existing conditions that compromise the patient. However, what became known as the 'antibiotic era' is now being compromised itself by the emergence of more and more antibiotic-resistant strains of bacteria that have caused modern medicine to question if we are not now entering the 'postantibiotic era'.

What is often ignored in these discussions of antimicrobial resistance and our reduced ability to treat infection is the fact that even in the halcyon times of antimicrobial therapy chronic or recurrent infections were poorly resolved with antibiotics. In fact, the designation of these infections as chronic was presumably derived due to their lack of responsiveness to antimicrobial therapy allowing them to become chronic or to recur in the face of therapy. Diseases such as recurrent ear infections in children, recurrent UTIs in women, and medical device-associated infections were and still remain a challenge to antimicrobial therapy, even when the isolates of these infections have been shown *in vitro* to be susceptible to antibiotics used in their treatment.

It is now recognized that these chronic infections involve bacteria associated within biofilms. In fact the US Food and Drug Administration (FDA) and Centers for Disease Control (CDC) both state that more than 60% of North American infections involve biofilms. We also now recognize that the confounding issue in the treatment of chronic infections is the inherent tolerance of biofilms to antibiotics predicted to have efficacy against the organism on the basis of planktonic susceptibility testing. In fact, Ceri and colleagues demonstrated that for an antibiotic to be effective in biofilms can require a concentration over 1000 times that needed to treat the same planktonic population (Ceri *et al.* 1999); concentrations that cannot be achieved or used safely in patient treatment. This altered tolerance to drugs is an adaptation of the biofilm, as bacteria derived from the biofilm show the same susceptibility profile as planktonics when the organisms are returned to the planktonic growth phase.

This reduced susceptibility to antibiotics differs from antibiotic resistance in a number of fundamental ways. First, this tolerance is only demonstrated when the isolate is in the biofilm mode of growth and is lost when the culture is returned to planktonic growth, hence it is not a permanent genetic change. Secondly, tolerance implies that the biofilm is not killed by the antimicrobial but it may not necessarily be able to grow in the presence of the drug, whereas in resistance the organism can grow in the presence of the antimicrobial. The tolerance of biofilm populations to antimicrobials would imply that in many instances of chronic infections microbes are in fact exposed to sublethal concentrations of drug, which may be an important implication in the development of classical antimicrobial resistance.

4 Mechanisms of biofilm tolerance

It is now believed that the tolerance to antimicrobials displayed by biofilms is a multifactorial process involving, to some degree or another, a number of different mechanisms contributing to the survival of the population, if not the individual cell. A model for this multifactorial tolerance is shown in Figure 8.2. Contributing to the tolerance of biofilms are factors ranging from the structural components of the biofilm, the physiological potential of cells spread throughout the biofilm and the expression of the genetic phenotypes of disparate populations of cells, all derived from the original clonal population(s) that made up the biofilm.

4.1 Biofilm structure

The hypothesis that the extracellular matrix acts as the gatekeeper for the penetration of antimicrobials into the biofilm, as shown in point 3 in Figure 8.2, has engendered many studies and a great deal of controversy. When biofilms were first visualized using both transmission and scanning electron microscopy, the dehydrated matrix seen in these original micrographs led to the belief that biofilms were very flat and dense structures where the compact and highly charged matrix around the biofilm would prevent penetration of antibiotics into the biofilm; hence this diffusion barrier would render them resistant to antimicrobial treatment. Stabilization of the matrix

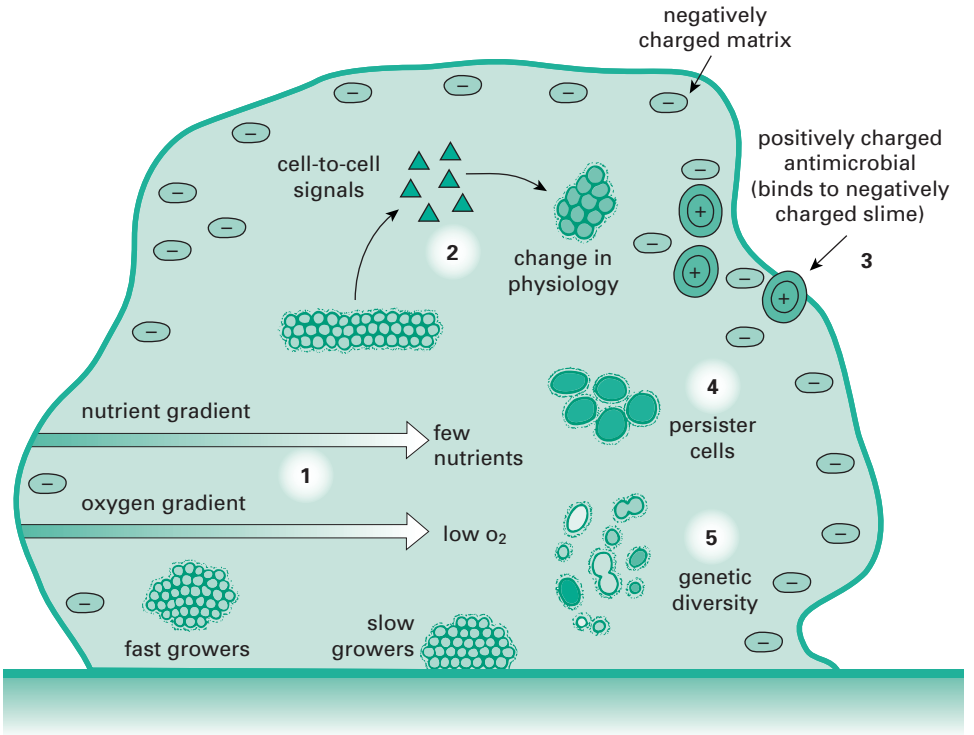

Figure 8.2 Factors contributing to antimicrobial tolerance of biofilms. (1) Low O_2 and decreased nutrient levels near the centre of the microcolony result in slow microbial bacterial growth and hence tolerance to antibiotic drugs which are more effective against fast-growing cells. (2) Intercellular signals can alter biofilm physiology, resulting in expression of molecular efflux pumps which expel antibiotics from the cells allowing the community to grow even in the presence of antimicrobial drug. (3) Negatively charged biofilm matrix may bind positively charged antimicrobials retarding their penetration into the biofilms. (4) Persister cells, a specialized population of cells which do not grow but do not die in the presence of antibiotic. Persister cells can, upon removal of the antimicrobial challenge, give rise to a normal bacterial colony. (5) Genetic and physiological diversity within the population act as an 'insurance policy', improving the chances that some cells survive any challenge. (From Harrison, J.J. *et al.* (2005) Biofilms. *Am Sci*, **93**, 508–515, with permission from *American Scientist.*)

and cross-sections through the biofilm revealed a very different picture of the biofilm, where cells were seen to exist within a very hydrated matrix containing channels to allow for nutrient transfer into the biofilm and the diffusion of waste out. The matrix is now believed to be composed of bacterially derived carbohydrate, the composition of which is dependent upon the bacterial species, nutrient availability and the growth conditions of the biofilm. Recently it has been established that DNA is an important component of the matrix, which may be specifically transported into the matrix. The role of DNA in the matrix is only now being deciphered. It has been shown to play a role in the conformation of the carbohydrate and hypothesized to serve as a gene pool for the diversity seen within the biofilm. The highly anionic charge of this matrix could be hypothesized to still play an important role in preventing charged antibiotics from effectively entering the biofilm and thereby still act as a primary inhibitor of antibiotic killing, as was originally proposed. Several studies of antibiotic penetration into biofilms demonstrated that the charge of the antibiotic could affect its penetration. For example, fluoroquinolones (ciprofloxacin, ofloxacin) that are not highly charged easily penetrate the matrix, while the penetration of charged aminoglycosides (tobramycin, gentamicin) is delayed. These studies have not, however, resolved the issue of the importance of the matrix in the resistance of biofilms. The rapid entry of fluoroquinolones, for

example, may be only into water channels of the biofilm and not into areas where cells of the biofilm are found, while the delay in entry of aminoglycosides may affect the rate of entry but may not affect final concentration significantly enough to alter susceptibility of the biofilm. Further, penetration of antimicrobials alone may not be as key an issue as the physiological state of the cells, which is also affected by the structure and organization of the biofilm. The diffusion into the biofilm of multiple factors, not limited to just the antibiotics themselves, may impact the biofilm's physiological status, thereby affecting the efficacy of antibiotics against the biofilm.

4.2 Biofilm physiology

The physiological state of the biofilm is also affected by its organizational structure, as diffusion of oxygen, nutrients and waste will ultimately affect all properties associated with growth and sustainability of the biofilm (shown in part 1 of Figure 8.2). Sophisticated experiments based on microelectrode probing of the biofilm and confirmed by dye distribution confocal microscopy assays have established the presence of oxygen and pH gradients within the biofilm. Gradients of nutrients and end products are also implicated in defining the different growth properties throughout the biofilm, which, as described above, has been linked to antibiotic susceptibility. The hypothesis predicts that antibiotics dependent on cell growth for activity will be less effective against biofilms because of the variability of growth within the biofilm. However biofilms, prove to be as recalcitrant to antibiotics that are not dependent upon cell growth as to those that do. Further, live dead staining of biofilms does not always correlate with cell death occurring most rapidly at the outer edges of the biofilm, where nutrient and oxygen levels are at their highest and hence where growth should be most rapid. In mixed species biofilms, where each component of the population may exist within its own niche for optimal growth, this model becomes even more complex to understand. The complexity of these interactions will make dissecting the process of antimicrobial tolerance more challenging. One can, however, argue that targeting one member of the mixed population within the biofilm may alter the susceptibility patterns of other species that are dependent upon the symbiotic interactions within the biofilm.

Another focus of study in biofilm resistance related to spatial orientation and physiology has been to look at how biofilms deal with oxidative stress. The mechanism of killing of many antimicrobials, including antibiotics, biocides and metals, is often associated with redox reactions involving various cellular components. These reactions may oxidize sensitive cellular thiol (RSH) groups or result in the production of reactive oxygen species (ROS), such as superoxides, hydroxy radicals, or hydrogen peroxide. The regulation of antioxidant pathways within the cell, such as glutathione (GSH) and thioredoxin pathways that manage thiol-disulfide homeostasis, and the *oxyRS, soxRS* and *marR* regulons that render cells resistant to ROS, play an important role in the susceptibility of biofilms to antimicrobials. The difference in oxygen tension in the biofilm and of the cells' response to oxygen stress may prove vital in survival of biofilms to naturally occurring antibiotics or those used in patient treatment. The role of the redox potential of *E. coli* to metals has demonstrated a very complex picture of these interactions and has shown a difference in mechanisms between planktonic and biofilm populations.

4.3 Cellular signalling and biofilm resistance

Our perspective of the microbial lifestyle has changed from one where bacteria exist mainly as solitary independent planktonic populations to one where bacteria form adherent communal populations of bacteria organized into microcolonies called biofilms. This shift in lifestyle suggests the presence of specific signalling between cells to allow them to organize these complex structures. Many different genes have been identified that can alter biofilm formation or antimicrobial susceptibility, but two global signalling pathways have come to the forefront as biofilm regulators in many different species of bacteria (see point 2 in Figure 8.2). Although models of biofilm formation have been proposed that do not require cell signal molecules, the importance of the following molecules in biofilm formation and antimicrobial resistance is well established and has even led to attempts to develop signal antagonists for treatment of biofilm disease or to create greater efficacy of existing antimicrobials by returning biofilms to a planktonic-like level of susceptibility.

4.3.1 Quorum sensing

Quorum sensing (QS) has been recognized as a key regulatory process associated with biofilm formation and antibiotic susceptibility. Well studied in *Vibrio fischeri*, QS involves an enzyme LuxI that produces a small signalling molecule, or *autoinducer*, that diffuses out of the cell. Upon reaching a threshold concentration the autoinducer will diffuse back into the cell, where cellular transcription is altered when the autoinducer binds the

transcription regulator LuxR and initiates QS-specific gene expression. In Gram-negative organisms the autoinducer is typically an acyl-homoserine lactone (AHL), but in some organisms multiple QS systems exist. For example, in *Ps. aeruginosa* signalling involves interactions of two distinct AHL compounds, produced by the Luxl homologues Lasl and Rhll respectively, that interact with their cognate receptors LasR and RhlR. Yet a third signal system, PQS, is also active in *Ps. aeruginosa*. In Gram-positive bacteria QS is typically carried out by autoinducing peptides. As QS is an integral step in biofilm formation and antibiotic tolerance, it has become a target for new therapeutics. Inhibitors of the QS signal pathway, assayed for their ability to either block biofilm formation or the expression of QS-dependent genes, may provide new approaches to treatment of biofilm disease.

4.3.2 Cyclic diguanylate

The universal use of nucleotides as signalling molecules is well recognized in biology. The importance of cyclic diguanylate (c-di-GMP) as a switch to move bacteria from a motile planktonic lifestyle to that of an adherent biofilm is now just being systematically explored. As with other nucleotide regulation systems, two components are involved in the regulatory pathway. The first is responsible for the synthesis of the signal, in this case a diguanylate cyclase (DGC) defined by proteins expressing GGDEF domains. The second component, a phosphodiesterase (PDE), degrades the active signal and is associated with two distinct domains, EAL and HD-GYP. The high level of redundancy of these domains makes understanding the mechanisms by which c-di-GMP regulates biofilm formation and virulence a complex issue, where patterns of temporal and spatial separation remain to be resolved.

4.4 Plasticity of biofilms

The final mechanism by which biofilms become less susceptible to antimicrobials is sketched in parts 4 and 5 of Figure 8.2. Biofilms are able to give rise to unique subpopulations; in some cases these may be part of the normal diverse metabolic activity found within the nutrient and oxygen gradients of the biofilm, an example being persister cell populations, or they may be subpopulations that are derived in response to stress but which are not classically resistant populations according to definitions discussed previously. Being part of a population, as opposed to being a single cell, allows for the adaption of subpopulations within the biofilm through phenotypic expression of unique gene sets, which can ensure the survival of the population as a whole, but often at the expense of individuals within the biofilm. This mechanism can be considered either altruism of a subset of the population or simply adaptation to the gradients of growth conditions present in the biofilms discussed in section 2 that result in the expression of different phenotypes. There have been many proposed models for phenotypic plasticity of a bacterial population but we will focus on two prominent hypotheses demonstrated in Figure 8.2. These are persister cell populations and the genetic diversity associated with the insurance hypothesis.

4.4.1 Persister cells

Clonal populations of cells, grown either as a planktonic or biofilm culture, give rise to persistent subpopulations of cells that resist killing by high concentrations of antimicrobials. Persistence is not the same as resistance, as persister populations possess no resistance mechanisms carried on transposable elements. They survive but do not grow in the presence of the selective agent, and when regrown the persister population recapitulates the killing curve of the original population when challenged again with the same antimicrobial. These persister populations typically represent about 0.1% of a logarithmic planktonic culture and up to about 10% of the initial population in a biofilm and they may therefore account for the higher level of antimicrobial tolerance seen in biofilms. Although persister cells were first reported in the 1940s the molecular mechanisms responsible for their properties are still a subject of debate. Persister cells are not produced in response to a challenge but pre-exist in the population and can be selected from any homogenous population of cells for being a slow growing, physiological distinct subpopulations of small cells capable of tolerance to environmental stress. The mechanism by which persistence is manifested remains a focus of many studies. Persistence in *E. coli* has been mapped to the high persistence operon (*hip*), containing the *hipA* gene that encodes a toxin and the *hipB* gene that encodes an antitoxin that functions as a DNA-binding protein that both binds HipA and also autoregulates the expression of the *hip* operon itself. Homologues of the *hip* operon are found across many bacterial genera, suggesting this is a common mechanism of resistance. Interestingly, in *E. coli* and other species redundant toxin–antitoxin genes have been identified, suggesting the possible specialization of sets of genes to deal with specific stress factors. Recently a toxin–antitoxin pair, *yafQ-dinJ* has been shown to act on biofilm but not planktonic populations to protect them against

very specific antimicrobials, which may provide a possible rationale for the redundancy seen in these systems.

4.4.2 Subpopulations of cells—the 'insurance hypothesis'

Biofilms are made up of cells that have adapted to a wide range of physiological states associated with the nutritional gradients within the biofilm, and hence even if the biofilm is initiated from a clonal population it will display heterogeneity at both the phenotypic and genotypic level. This diversity is enhanced when the biofilm is placed under antimicrobial stress. Persister cell populations, discussed above, represent only one adaptive state that contributes to the increased tolerance of biofilms to antimicrobials. The combination of diverse populations found in the biofilm that contribute to tolerance has been referred to as the *insurance hypothesis*, where diversity is increased in an attempt to ensure a population will survive the stress of antimicrobial challenge or increased metal or toxin concentrations in nature. This is an area of immense interest and a field of study that is just in its infancy. At this point numerous variants, separated on morphological criteria from challenged populations, have been identified, but only now with the advent of new-generation sequencing can these populations be screened for possible mutations that lead to their tolerant phenotype. This is an area worth keeping an eye on in the future.

5 Treatment of chronic biofilm infections

As already stated, chronic, recurrent and device-related infections are now recognized to be associated with biofilm formation. Biofilms, as discussed above, possess multiple mechanisms that render them less susceptible to antibiotic treatment than the same isolate in the planktonic mode of growth. The antibiotics we have today were all selected for efficacy against planktonic cultures and all our diagnostics are based on planktonic assays. Finally, we now recognize that MIC values provide us with little relevant information on how to treat biofilm infections. Clearly, a new paradigm for treatment of biofilms is needed.

5.1 A new biofilm assay

As part of the development of the next generation of antimicrobials, techniques must be developed and set in place to determine efficacy of these 'new' antimicrobials against cells grown in biofilms. Antimicrobial assays described in Chapter 18 include those targeted at identifying antimicrobials effective against biofilms. It is tempting to speculate that, since screening of all combinatorial libraries of antibiotics was based on efficacy against planktonic targets, potential new antibiotics with efficacy against chronic disease may have been overlooked. The potential of identifying 'next-generation' antibiotics in existing libraries by appropriate biofilm screens is a distinct possibility. The ability to now produce multiple reproducible biofilms of defined target size provides the potential for high-throughput screens for antibiotic efficacy against biofilms.

5.2 Better use of existing antimicrobials

Although all existing antimicrobials were selected for their efficacy against planktonic cultures and this efficacy has not always translated to activity against biofilms, it may still be possible to make better use of existing antimicrobials to treat biofilm disease. A marked trend in treating chronic infections of many kinds is the use of antibiotic combinations. The possible existence of synergies between existing antimicrobials represents one area of progress in treating biofilm infections. Rational approaches to the application of combinations of antimicrobials, based on the differences of their targets, have not provided clear use predictions. Most combinations are still empirically derived from past experience. The bioFILM PA assay used to select combinations of antibiotics for the treatment of *Ps. aeruginosa* lung infections in cystic fibrosis patients is one of the first diagnostics to receive regulatory approval (in Canada) and has had a measure of success in the treatment of seriously ill patients. This test is now in clinical trials as a standard diagnostic in cystic fibrosis. Based on the Calgary Biofilm Device (see Chapter 18), which is the only high-throughput device for selecting antimicrobials with efficacy against biofilms, this represents one of the first attempts to address the differences in susceptibility of organisms in the biofilm growth mode.

5.3 Next-generation antimicrobials

It is clear that biofilms represent an important challenge in the treatment of infectious diseases. Biofilm are involved in more than 60% of infections, typically associated with chronic, recurring and device-related infections. Furthermore, we now know that that existing antimicrobials have been selected for efficacy against planktonic organisms and are often much less effective against biofilms. What is clearly needed is a paradigm shift for the selection and development of antimicrobials, which will ensure efficacy against biofilms.

5.3.1 Conceptual evaluation of antibiotics

There is a great deal of discussion about the development of future antibiotics and whether this is the correct direction to follow. It is noteworthy that every antibiotic on the market has been derived or modified from natural products produced by microbial life forms. It is also interesting to note that these life forms continue to synthesize antibiotics. If they had lost efficacy in nature one would expect that these organisms would have evolved to stop wasting energy on their synthesis, suggesting that in nature antibiotics continue to have efficacy. The question is then, what disconnect is there between how antibiotics function in nature and how they are used in medicine?

For one thing, in the production of antibiotics an active compound is purified from the fermentation liquor and tested as an antibiotic against a planktonic target population of bacteria. This approach assumes that the planktonic population is a relevant target for antibiotic selection. It also assumes that other components of the liquor are not important components in the activity of the antibiotic. It is very possible that these discarded components may define activity against the biofilms found in nature. It is more difficult, if not impossible, to bring complex mixtures of active ingredients through regulatory process; however, this may be the active form of antibiotics in nature that are able to function against biofilms.

5.3.2 Biofilm assays

It should now be obvious that to find antibiotics capable of treating biofilm infections we require a biofilm assay of antibiotic efficacy. The lack of such an assay and the inability to deal with biofilm infections has in part slowed the adoption of the importance of biofilms in modern medicine. High-throughput screening of antibiotics against biofilms must be achieved for this field to move forward. More diagnostics like the bioFILM PA assay must be developed, and our understanding of the composition of natural antibiotic fermentations must be better defined for us to understand the synergies that might exist between existing antibiotics and to formulate the composition of ingredients of future antibiotics yet to be defined.

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9 Immunology

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1 Introduction

1.1 Historical perspective and scope of immunology

Progress in immunological science has been driven by the need to understand and exploit the generation of immune states exemplified now by the use of modern vaccines. From almost the first recorded observations, it was recognized that persons who had contracted and recovered from certain infectious diseases were not susceptible (i.e. were immune) to the effects of the same disease when re-exposed to the infection. Thucydides, over 2500 years ago, described in detail an epidemic in Athens (which could have been typhus or plague) and noted that sufferers were 'touched by the pitying care of those who had recovered because they were themselves free of apprehension, for no one was ever attacked a second time or with a fatal result'.

Since that time many attempts have been made to induce this immune state. In ancient times the process of variolation (the inoculation of live organisms of smallpox obtained from the diseased pustules of patients who were recovering from the disease) was practised extensively in India and China. The success rate was very variable and often depended on the skill of the variolator. In the late

Hugo and Russell's Pharmaceutical Microbiology, Eighth Edition. Edited by Stephen P. Denyer, Norman Hodges, Sean P. Gorman, Brendan F. Gilmore.

18th century Edward Jenner, an English country doctor, observed the similarity between the pustules of smallpox and those of cowpox, a disease that affected the udders of cows. He also observed that milkmaids who had contracted cowpox by the handling of diseased udders were immune to smallpox. Jenner deliberately inoculated a young boy with cowpox, and after the boy's recovery, inoculated him again with the contents of a pustule taken from a patient suffering from smallpox; the boy did not succumb to infection from this first, or any subsequent challenges, with the smallpox virus. Even though the mechanisms by which this protected against smallpox were not understood, Jenner's work had shown proof of principle that the harmless stimulation of our adaptive immune system (see section 1.2) was capable of generating an immune state against a specific disease, and thereby provided the basis for the process we now understand as vaccination. The cowpox virus is otherwise known as the vaccinia virus and the term vaccine was introduced by Pasteur to commemorate Jenner's work.

In 1801 Jenner prophesied the eradication of smallpox by the practice of vaccination. In 1967 smallpox infected 10 million people worldwide. The World Health Organization (WHO) initiated a programme of confinement and vaccination with the aim of eradicating the disease. In Somalia in 1977 the last case of naturally acquired smallpox occurred, and in 1979 WHO announced the total eradication of smallpox, thus fulfilling Jenner's prophecy. Many vaccine products are now available designed to provide protection against a range of infectious diseases. Their value has been proven in national vaccination programmes leading to dramatic reductions in morbidity and mortality of such diseases as diphtheria, pertussis, mumps, measles, rubella, and hepatitis A and B.

Further progress in the understanding of the complex nature and functioning of the immune system has been gained through the recognition that many varied forms of pathology, beyond that of infectious disease *per se*, have an underlying immunological basis, including such diseases as asthma, diabetes, rheumatoid arthritis and many forms of cancer. A basic knowledge of how the immune system functions is essential for health professionals involved in understanding the nature of disease and rationalizing therapeutic strategies. This chapter aims to provide a sound overview of the structure and functioning of the immune system and impart the reader with knowledge which will serve as a platform for the study of more complex specialized texts if and when required.

1.2 Definitions and outline structure of the immune system

The primary function of the immune system is to defend against and eliminate 'foreign' material, and to minimize any damage that may be caused as a result of the presence of such material. The term 'foreign' includes not only potentially pathogenic microorganisms but also cells recognized as 'non-self' and therefore foreign such as the human body's own virally infected or otherwise transformed (e.g. cancerous) host cells. Foreign material would also include *allogeneic* (within species) or *xenogeneic* (between species) transplant tissue and therapeutic proteins administered as medicines if they arose from a different species or were of human origin but had undergone inappropriate post-translational modifications during manufacture or contained impurities. It is also possible that small organic-based drugs may form adducts with endogenous proteins leading to the generation of an immunogen. A good example of such adduct formation is that between the serum protein albumin and the glucuronide metabolite of some non-steroidal anti-inflammatory drugs. This adduct is proposed as the basis of some hypersensitivity reactions.

For clarity there are a number of terms that should be defined at this point. An organism which has the ability to cause disease is termed a *pathogen*. The term *virulence* is used to indicate the degree of *pathogenicity* of a given strain of microorganism. Reduction in the virulence of a pathogen is termed *attenuation*; this can eventually result in an organism losing its virulence completely and it is then said to be *avirulent*.

An *antigen* is a component of the 'foreign' material that gives rise to the primary interaction with the body's immune system. If the antigen elicits an immune response it may then be termed an *immunogen*. Within a given antigen, e.g. a protein, there will be *antigenic determinants* or *epitopes*, which actually represent the antigen recognition sites for our *adaptive immune system* (see below). For example, within a protein antigen, an epitope for an antibody response will consist of 5–20 amino acids that form either part of a linear chain or a cluster of amino acids brought together conformationally by the folding of the protein. Antibodies (otherwise known as immunoglobulins—Ig) are produced and secreted into biological fluids by our adaptive immune system, are widely used in *in vitro* diagnostics and have been investigated in therapeutics as a means of targeting drugs to specific sites in the body. A *monoclonal antibody* is an antibody nominally recognizing only a single antigen (e.g. a single protein) and within which only a single

common epitope (e.g. clusters comprising a common single specific amino acid sequence or pattern) is recognized. In contrast, a *polyclonal antibody* is an antibody nominally recognizing only a single antigen but within which a number of different epitopes (e.g. clusters comprising different amino acid sequences or patterns) are recognized.

The immune system is broadly considered to exhibit two forms of response:

- The *innate immune response*, which is non-specific, displays no time lag in responsiveness, and is not intrinsically affected by prior contact with infectious agent.
- The *adaptive immune response*, which displays a time lag in response, involves highly specific recognition of antigen and affords the generation of immunological memory. An example of immunological memory is that provided by the generation of specific lymphocyte memory cell populations following vaccination with an antigen (e.g. diphtheria toxoid). These memory cells reside over a long term in our lymphoid tissue and permit a more rapid and pronounced protective immunological response on future exposure to the same antigen. The adaptive immune system is further subdivided into:
  - *Humoral immunity*, within which the effector cells are *B-lymphocytes* and where antigen recognition occurs through interactions with antibodies.
  - *Cell-mediated immunity*, within which the effector cells are *T-lymphocytes* and where antigen recognition occurs through interactions of peptide antigen (presented on the surface of other cell types) with *T-cell receptors* (*TCR*) on the plasma membrane of T-lymphocytes. In cell-mediated immunity the peptide antigen must be presented to T-lymphocytes by other cell types in association with a class of plasma membrane molecules termed *major histocompatibility complex (MHC) proteins.*

1.3 Cells of the immune system

A schematic overview of the cells involved in both the innate and adaptive components of the immune response is shown in Figure 9.1. Most of the cells involved in the immune system arise from progenitor cell populations within the bone marrow. The differentiation of these progenitor cells is under the control of a variety of growth factors, e.g. granulocyte- or macrophage-colony stimulating factors (G-CSF and M-CSF, respectively) released by monocyte and macrophage cells as well as by fibroblasts and activated endothelial cells. These growth factors promote the growth and maturation of monocyte and granulocyte populations within the bone marrow before their release into the lymphoid and blood circulations.

The principal cells of the innate immune system include the following:

- *Mononuclear phagocytic* cells, which are short-lived (<8 hours) monocytes in the blood circulation that migrate into tissues and undergo further differentiation to give rise to the long-lived and key effector cell—the macrophage.
- The *granulocyte cell* populations which include the neutrophil, basophil and eosinophil.
- The *mast cell* which is a tissue-resident cell that is triggered by tissue damage or infection to release numerous initiating factors leading to an inflammatory response. Such initiating factors include histamine, leukotrienes B4, C4 and D4, proinflammatory cytokines (signal proteins released by leucocytes—white blood cells), such as tumour necrosis factor-α (TNF-α), and chemotactic substances such as interleukin-8 (IL-8). The sudden degranulation of the contents of mast cells is also responsible for the acute anaphylactic reactions to bee stings, penicillins, nuts, etc.
- The *natural killer* (NK) cell which has a phenotype similar to that of lymphocytes but lacks their specific recognition receptors. The NK cell exploits non-specific recognition to elicit cytotoxic actions against host cells infected with virus and those host cells that have acquired tumour cell characteristics.

The lymphocyte populations also arise from bone marrow progenitor cells. The B-lymphocytes mature or differentiate in the bone marrow before leaving to circulate in the blood and lymph, while T-lymphocytes undergo maturation in the thymus. Antibodies mediating the effector functions of the humoral immune system are produced and secreted from a differentiated B-lymphocyte cell population termed *plasma cells*.

2 The innate immune system

2.1 Innate barriers at epidermal and mucosal surfaces

Innate defence against the passage of potentially pathogenic microorganisms across epidermal and mucosal barriers involves a range of non-specific mechanisms. Commensal microorganisms living on mucosal surfaces and on membranes such as skin and conjunctiva constitute one such mechanism (see Chapter 8). These commensals are, under normal circumstances, non-pathogenic, and help prevent colonization by pathogenic

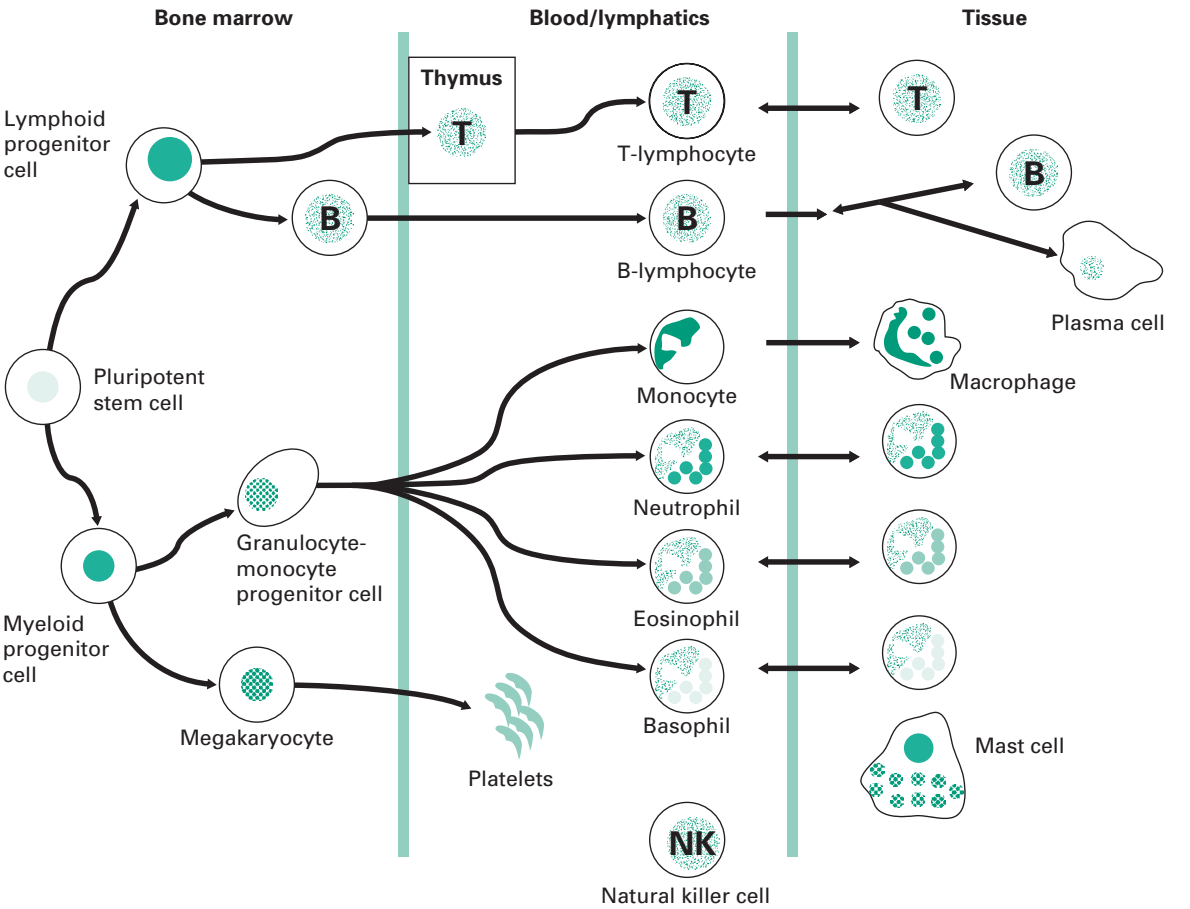

Figure 9.1 An overview of the cells involved in the immune response: both innate and adaptive components. The cells arise from a pluripotent progenitor cell within the bone marrow, with their growth and differentiation controlled by numerous growth factors. The T-lymphocytes differentiate in the thymus gland.

strains. There are also a number of physical and chemical barriers against microbial entry, including the flow of fluid secretions from tear ducts, the urogenital tract and the skin. Many of these secretions possess bacteristatic or bactericidal activity due to their low pH or the presence of hydrolytic enzymes such as lysozyme (a peptidoglycan hydrolase). Similarly, the mucus barrier covering mucosal surfaces such as the epithelium of the lung serves as a false binding platform for microorganisms, preventing them from interacting with the underlying host cells. In the normal state the hydrated mucus barrier is efficiently cleared under the driving force of beating cilia. The serious lung infections seen in cystic fibrosis arise because patients are unable to clear the bacteria-laden dehydrated mucus effectively.

2.2 Innate defence once epidermal or mucosal barriers have been compromised

The function of the innate defence system against microorganisms that have penetrated into interstitial tissues and the vascular compartment relies largely on the processes of phagocytosis (see section 2.2.3) and of activation of the alternative complement pathway (see section 2.2.4). However, the functions of the innate system when exposed to microbial infection are also critical in the recruitment and activation of cells of the adaptive immune response (see later).

The main cells mediating phagocytosis are the mononuclear phagocytic cells and granulocyte cell populations; of the latter, neutrophils are particularly important. For such cells to function, they must possess receptors to

sense signals from their environment. In executing their effector functions they need to secrete a range of molecules that will recruit or activate other immune cells to a site of infection.

Before consideration of the process of phagocytosis, an overview of the mononuclear phagocytic cell and granulocyte cell populations is useful.

2.2.1 Mononuclear phagocytic cells

The mononuclear phagocytic cells include monocytes and macrophages. Monocytes make up approximately 5% of the circulating blood leucocyte population and are short-lived cells (circulating in blood for ≤8 hours), but migrate into tissue to give rise to tissue macrophages. The macrophages constitute a long-lived, widely distributed heterogeneous population of cell types which bear different names within different tissues, such as the migrating Kupffer cell within the liver or the fixed mesangial cell within the kidney glomerulus.

The mononuclear phagocytic cells secrete a wide range of molecules too numerous to list in full here. However, these secretions include:

- Molecules which can break down or permeabilize microbial membranes and thereby mediate extracellular killing of microorganisms, e.g. enzymes (lysozyme or cathepsin G), bactericidal reactive oxygen species and cationic proteins.
- *Cytokines* which can provide innate protective antiviral (e.g. interferon (IFN)-α or -β) and antitumour (e.g. TNF-α) activity against other host cells. A group of cytokines termed *chemokines* can also serve to chemoattract other leucocytes into an area of ongoing infection or inflammation, for example IL-8 which attracts neutrophils. Yet another group of cytokines has proinflammatory actions (e.g. IL-1 and TNF-α) which, among other outcomes, leads to activation of endothelial and leucocyte cells promoting increased leucocyte extravasation into tissues and, in the case of IL-1, activation of T-lymphocyte populations.
- *Bioactive lipids* (e.g. thromboxanes, prostaglandins and leukotrienes), which further promote the inflammatory response through actions to increase capillary vasodilation and permeability.

The mononuclear phagocytic cells also possess numerous receptors that interact with their environment. These cells possess, among others:

- Receptors for chemotaxis toward microorganisms, e.g. receptors for secreted bacterial peptides such as formylmethionyl peptide.
- Receptors for complement proteins that serve as leucocyte activators (e.g. C3a and C5a; see section 2.2.4) or complement proteins that serve to coat (opsonize) microorganisms (e.g. C3b). An opsonized microbial surface more readily adheres to a phagocyte membrane, with the opsonin triggering enhanced activity of the phagocyte itself.
- Receptors for promoting adherence, such as lectin receptors interacting with carbohydrate moieties on the surface of the microorganism, or receptors for Fc domains (non-antigen-recognition domains) of antibodies which opsonize microorganisms (e.g. the receptor for the Fc domain of IgG is Fcg), or integrin receptors for cell–cell adhesion (e.g. promoting interaction between a macrophage and T-lymphocyte).
- Receptors for cytokines including those involved in macrophage activation (e.g.IFN.-γ) or limiting macrophage mobility (e.g. macrophage inhibitory factor, MIF) and hence increasing cell retention at a site of infection.

2.2.2 Granulocyte cell populations

The granulocyte cell populations include the neutrophils, basophils and eosinophils. The short-lived (2–3 days) neutrophil is the most abundant granulocyte (comprising >90% of all circulating blood granulocytes) and is the most important in terms of phagocytosis; indeed, this is the main function of the neutrophil. The receptors and secretions of the neutrophil are similar to those of the macrophage, although notably the neutrophil does not present antigen via MHC class II proteins (see later). The neutrophil is recruited to sites of tissue infection or inflammation by a neutrophil-specific chemotactic factor (IL-8) and is also chemoattracted and activated by some of the same factors described for mononuclear phagocytic cells, including complement protein C3a, bacterial formylmethionyl peptides and leukotrienes. Like macrophages, neutrophils undergo a respiratory burst and are very effective generators of reactive oxygen species.

Eosinophils are poor phagocytic cells and have a specialized role in the extracellular killing of parasites such as helminths, which cannot be physically phagocytosed. Basophils are non-phagocytic cells.

2.2.3 Phagocytosis

Macrophages and neutrophils in particular demonstrate a high capacity for the physical engulfment of particles such as microorganisms or microbial fragments from their immediate extracellular environment. This process (Figure 9.2) is made up of a number of steps:

- Chemotaxis of the phagocyte toward the microorganism through signals arising from the microorganism itself (e.g. formylmethionyl peptide), signals arising from complement proteins (e.g. C3a and C5a) generated as part of the activation of the alternative complement

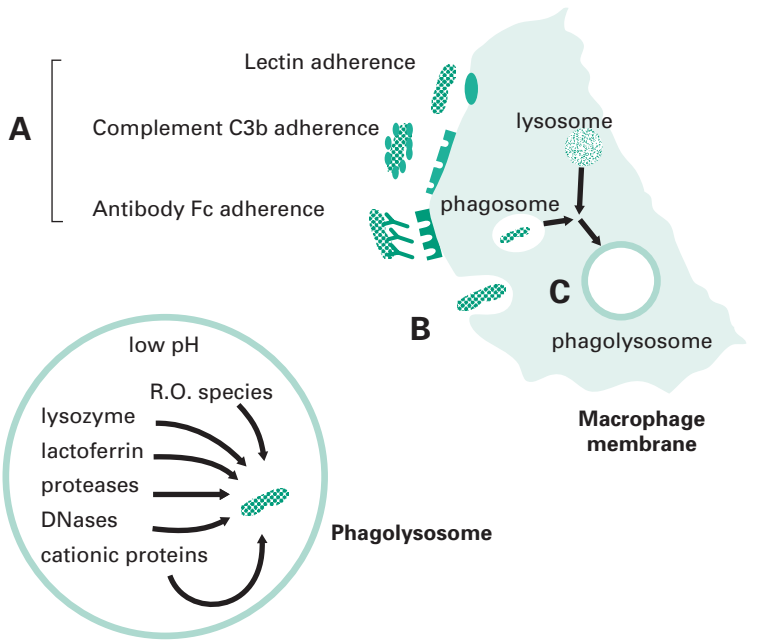

Figure 9.2 Schematic of phagocytosis showing: (step A) adherence of the microorganism to the surface of the phagocyte; (step B) membrane activation of the phagocyte; (step C) enclosure of phagocytosed material within the phagosome and subsequently the phagolysosome. The mediators of the phagolysosome degradation of the microorganism are shown in the enlarged phagolysosome insert.

pathway (see section 2.2.4), or signals due to release of inflammatory factors (e.g. leukotrienes) secreted by other leucocyte cells situated at the site of an infection.

- Adherence of the microorganism to the surface of the phagocyte (step A in Figure 9.2), involving adhesion through lectin receptors present on the surface of the phagocyte which interact with carbohydrate moieties on the surface of the microorganism; adhesion through complement C3b receptors present on the surface of the phagocyte interacting with C3b molecules that have opsonized the surface of the microorganism; and adhesion through Fc receptors which interact with the Fc domain of antibodies that have opsonized the surface of a microorganism.
- Membrane activation of the phagocyte actin–myosin contractile network to extend pseudopodia around the attached microorganism (step B in Figure 9.2). Membrane activation will also lead to the generation of a 'respiratory burst' by the phagocyte which involves an increase in the activity of the phagocyte membrane NADPH oxidase which converts molecular oxygen into bactericidal reactive oxygen species such as superoxide anion ($\bullet O^-$), hydrogen peroxide (H_2O_2), and in particular hydroxyl radicals ($\bullet OH$) and halogenated oxygen metabolites ($HOCl^-$).
- The enclosure of phagocytosed material, initially within a membranous vesicle termed a *phagosome*. Here, cationic proteins such as defensins and reactive oxygen species begin microbial membrane degradation. This is followed within minutes by fusion of the phagosome with a lysosome to form a phagolysosome, whose contents are at an acidic pH of about 5 which is optimal for the continued active breakdown of microbial structural components (step C in Figure 9.2).

2.2.4 Alternative complement pathway

The alternative complement pathway fulfils a critical role in innate immune defence. The complement system comprises at least 20 different serum proteins; many are known by the letter C and a number, e.g. C3. Many of the complement proteins are *zymogens*, i.e. proenzymes requiring proteolytic cleavage to be enzymically active themselves; some are regulatory in function. The cleavage products of complement proteins are distinguished from their precursor by the suffix 'a' or 'b', e.g. C3a and C3b, with the suffix 'b' generally denoting the larger fragment that stays associated with a microbial membrane, and the suffix 'a' generally denoting the smaller fragment that diffuses away. The activation of the complement pathway occurs in a cascade sequence, with amplification

occurring at each stage, such that each individual enzyme molecule activated at one stage generates multiple activated molecules at the next. In the 'resting' state, in the absence of infection, the complement proteins are inactive or have only a low level of spontaneous activation. The cascade is tightly regulated by both soluble and membrane-bound associated proteins. The regulation of the complement pathway prevents inappropriate activation of the cascade (i.e. when no infection is present) and also minimizes damage to host cells during an appropriate complement response to a microbial infection. Complement activation is normally localized to the site(s) of infection.

There are three main biological functions of the alternative complement pathway.

- *Opsonization of microbial membranes.* This involves the covalent binding of complement proteins to the surface of microbial membranes. This opsonization or coating by complement proteins promotes adherence of the opsonized microbial component(s) to the cell membranes of phagocytic cells. The complement protein C3b is a potent opsonin.
- *Activation of leucocytes.* This involves complement proteins acting on leucocytes, either at the site of infection or at some distance away, with the result of raising the level of functioning of the leucocytes in immune defence. For example, C3a is a potent leucocyte chemoattractant and also an activator of the respiratory burst.
- *Lysis of the target cell membrane.* This involves a collection of complement proteins associating on the surface of a microbial membrane to form a membrane attack complex (MAC), which leads to the formation of membrane pores and, ultimately, microbial cell lysis.

Figure 9.3 shows a highly schematized view of the activation cascade for the alternative complement pathway on a microbial membrane surface. The activation steps in the alternative pathway are also shown in Figure 9.7, which contrasts with the activation steps in the classical complement pathway involving antibody.

The pivotal protein in the alternative pathway is C3 (195 kDa). Under normal circumstances (in the absence of infection) C3 is cleaved very slowly through reaction with water or trace amounts of proteolytic enzyme to give C3b and C3a. The C3b formed is susceptible to nucleophilic attack by water and is rapidly inactivated to give iC3b. The C3a is not generated in sufficient amounts to lead to leucocyte activation and is rapidly inactivated. This normal low-level cleavage of the C3 molecule is termed 'C3 tickover' and it provides low levels of starting material, i.e. C3b, which will be required for full activation of the alternative complement pathway in the case of a microbial infection.

In the presence of a microbial membrane the C3b formed by C3 tickover will be susceptible to nucleophilic attack by hydroxyl or amine groups on the membrane surface, leading to the covalent attachment of C3b to the membrane (step A in Figure 9.3). Once C3b has attached to the membrane, factor B can bind to form a molecule termed C3bB. This complex is stabilized by a soluble protein called properdin. Factor D then enzymically cleaves the bound factor B to generate a molecule termed C3bBb which is the C3 convertase of the alternative pathway (Figure 9.3 inset).

This newly generated stable C3 convertase enzymically cleaves C3 to generate further C3b and C3a molecules, leading to leucocyte activation (by C3a) and greater deposition of C3b on the microbial membrane and hence further generation of C3 convertase molecules. In effect the microbial membrane has activated a positive feedback loop with cleavage of C3 to generate high amounts of C3b and C3a molecules.

The deposited C3b not only leads to the formation of the C3 convertase but also coats the microbial membrane as an opsonin and so promotes binding to phagocyte cell membranes. Some of the deposited C3b associates with the newly formed C3 convertase to generate a complex termed C3bBb3b, which is the C5 convertase of the alternative pathway (step B in Figure 9.3). This C5 convertase binds the complement protein C5 and cleaves it into C5a (a leucocyte activator) and C5b (an opsonin). The C5b remains associated with the membrane and acts as a platform for the sequential binding of complement proteins C6, C7, C8 (step C in Figure 9.3). The α-chain of the C8 molecule penetrates into the microbial membrane and mediates conformational changes in the incoming C9 molecules such that C9 becomes *amphipathic* (simultaneously containing hydrophilic and hydrophobic groups). In this form it is capable of insertion through the microbial membrane where it mediates a polymerization process that gives the MAC. The MAC generates transmembrane channels within the microbial membrane with the osmotic pressure of the cell leading to an influx of water and eventual microbial cell lysis.

Differences between host cell membranes and microbial cell membranes mean that the cascade is only activated in the presence of microorganisms, so C3 tickover cannot give rise to full activation of the alternative pathway in the absence of microbial membrane. Stable deposition of a functional C3 convertase only occurs on

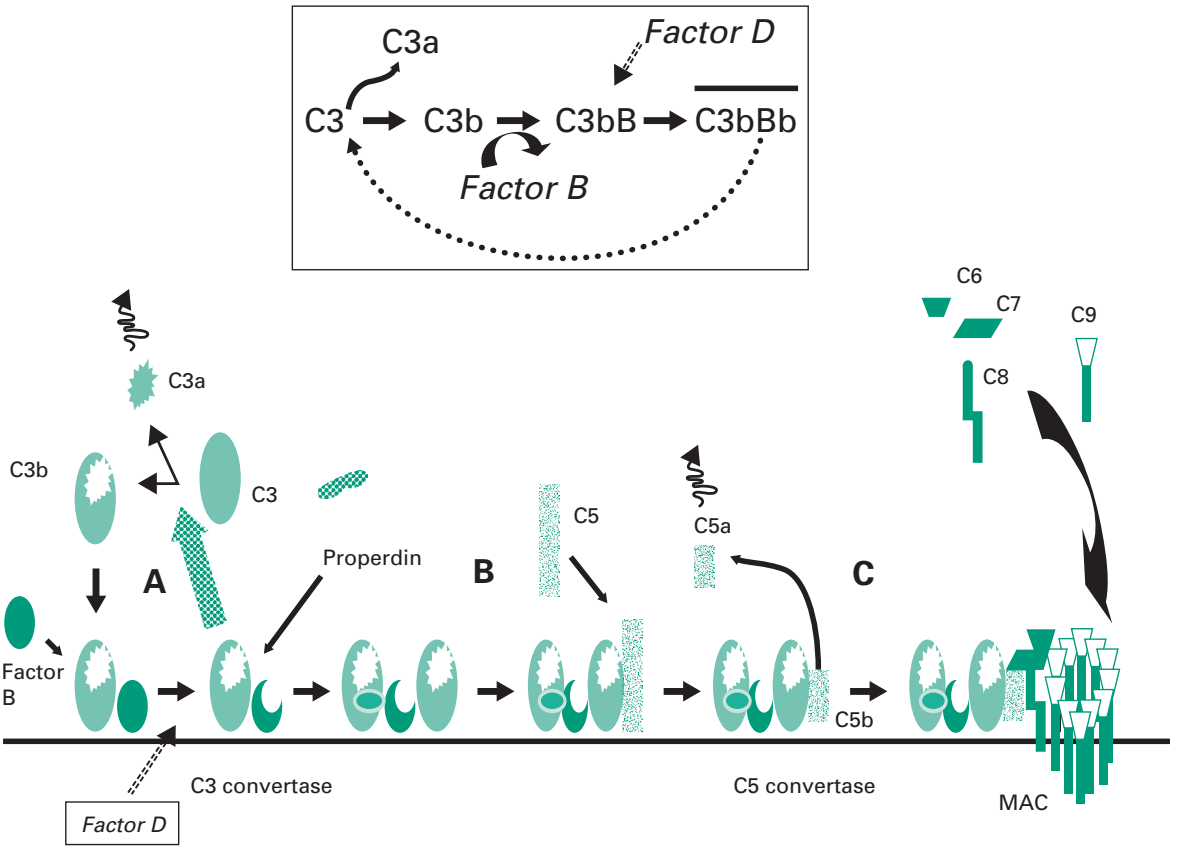

Figure 9.3 A highly schematized overview of the activation cascade for the alternative complement pathway on a microbial membrane surface. In the presence of a microbial membrane the C3b formed by C3 tickover deposits on the microbial membrane (step A). C3a diffuses away, leading to leucocyte activation. The deposited C3b leads to the generation of a stabilized C3 convertase (step B) which, through a positive feedback loop, leads to the amplified cleavage of more C3. Some C3b associates with the C3 convertase to generate a C5 convertase (step C) which will eventually lead to the generation of an MAC.

the microbial cell surface. The differences that exist include, for example:

- lipopolysaccharide or peptidoglycan on microbial membranes that promote the binding of C3b
- the high sialic acid content of host cell membranes that promotes the dissociation of any C3 convertase formed on host surfaces
- the presence of specific host cell membrane proteins that also serve a key regulatory function.

Decay activating factor (DAF) or complement receptor type 1 (CR1) are host cell membrane proteins that serve to competitively block the binding of factor B with C3b and hence inhibit formation of a C3 convertase; they also promote disassembly of any C3 convertase formed. Membrane cofactor protein (MCP) and CR1 are further host cell membrane proteins that promote the displacement of factor B from its binding with C3b. Host cell membranes also possess a protein, CD59, which prevents the unfolding of C9—a required step for membrane insertion to form an effective MAC.

3 The humoral adaptive immune system

The humoral immune response is mediated through antibody–antigen interactions. B-lymphocytes in their naive state, unstimulated by antigen, possess antibody molecules on their cell membrane, which serve a surveil-

lance function to recognize any invading antigen. A B-lymphocyte that has bound antigen is capable of differentiating into a plasma cell which, under the influence of signals from helper T-cells, produces a fuller repertoire of antibody molecules (i.e. a fuller range of antibody classes), which are then secreted from the plasma cell into the extracellular environment to bring about a range of humoral effector functions.

3.1 B-lymphocyte antigens

As briefly discussed in section 1.2, a B-cell antigen is a substance or molecule specifically interacting with an antibody, and which may lead to the further production of antibody and an immunological response. Typically B-lymphocyte antigens are proteins within which the epitopes each consist of clusters of 5–20 amino acid residues. B-lymphocyte epitopes arise most commonly from the three-dimensional folding of proteins (i.e. conformational epitopes), although they may also consist of a sequential linear sequence of amino acids within the polypeptide chain (linear epitopes). As a general rule, there is a gradient of increasing immunogenicity with increasing molecular weight of protein. Further, the higher the structural complexity of the protein or polypeptide antigen, the higher the level of immunogenicity it is likely to exhibit. Thus, a polypeptide comprising a single amino acid such as polylysine will be expected to be a weaker immunogen than a protein of equivalent molecular weight made up of a diverse range of amino acids.

Polysaccharides tend not to be good immunogens for B-lymphocytes. When a polysaccharide serves as the sole immunogen, the humoral response obtained is termed 'T-cell-independent' because the polysaccharide does not elicit helper T-lymphocyte cooperation (see section 4). The consequences of a T-cell-independent humoral response include the lack of production of memory B-cell populations and the lack of synthesis by the plasma cell of the full range of antibody subclasses, i.e. T-cell-independent humoral responses mainly involve the production of IgM antibody. For improved immunogenicity carbohydrate antigens are conjugated to proteins which allow a more effective 'T-cell dependent' humoral response, i.e. one that affords the generation of memory B-cell populations and of the synthesis of the full range of antibody subclasses. This strategy is used in a number of current vaccine products, e.g. meningococcal group C conjugate vaccine contains the capsular polysaccharide antigen of *Neisseria meningitidis* group C conjugated to *Corynebacterium diphtheriae* protein. Pure nucleic acid and lipid serve as very poor antigens.

3.2 Basic structure of antibody molecule

Figure 9.4 shows an antibody monomer with a four-polypeptide subunit structure, where the subunits are linked through disulphide bonding. The basic monomer structure can be considered the same for all the different classes of antibody (see below) even though some may form higher-order structures, e.g. IgM is a pentamer made up of five antibody monomer units.

The subunits of the antibody monomer comprise two identical 'heavy' polypeptide chains and two identical 'light' polypeptide chains, with each of these containing a 'constant' region and a 'variable' region. The light chain variable regions (V_L) and the heavy chain variable regions (V_H) are the parts of the antibody molecule involved in antigen recognition. Specifically, antibodies produced by different B-lymphocytes or plasma cells will have variable regions possessing different amino acid sequences leading to differences in antibody variable region surface conformation. At the extreme tips of the variable regions are hypervariable domains that serve the specific antigen recognition function discriminating between, for example, diphtheria toxin and tetanus toxin. The structural differences in the variable and hypervariable domains enable different antibodies to recognize different structural epitopes; this meets the needs of the immune system to combat a large and diverse range of antigens.

A horizontal line of symmetry can be drawn through the antibody structure in Figure 9.4, bisecting the molecule into two equivalent halves each containing a single heavy chain and a single light chain and clearly showing the antibody monomer to possess bivalency in its ability to interact with antigen, i.e. each antibody monomer can bind two epitopes, although the epitopes bound by a single antibody must be identical. The antigen recognition domain of an antibody monomer is termed the Fab domain. The structure of the constant region of the heavy chain (C_H) does not influence the antigen recognition function of the molecule but defines the different classes of antibody that are produced and hence the effector functions arising from antigen–antibody interaction; this heavy chain constant region is termed the Fc domain.

An analogy that may assist visualization of the function of an antibody molecule is one that views it as a hand (Fab domain) attached to the arm (Fc domain) (Figure 9.4). The palm of the hand (variable region) can take up different shapes to allow the fingertips (hypervariable regions) to gain a very precise interaction with

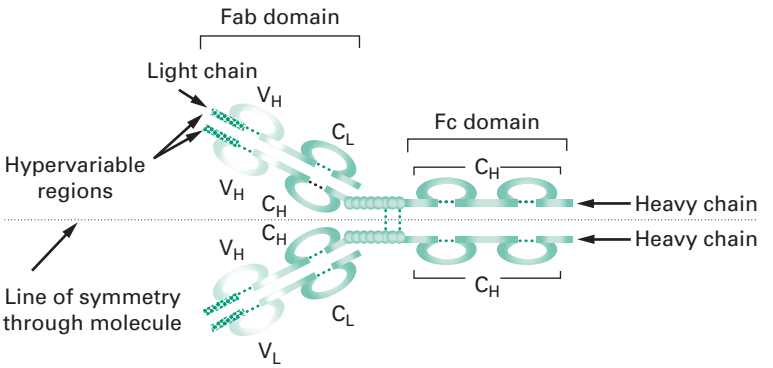

Figure 9.4 An antibody monomer consisting of a four-polypeptide subunit structure, where the subunits are linked through disulphide bonding. The Fab fragment is concerned with antigen recognition, while the Fc region determines the various effector functions of antibodies. A horizontal line of symmetry can be drawn through the antibody structure bisecting the molecule into a single heavy chain and a single light chain and clearly showing the bivalency in antigen recognition.

an object (antigen). At the wrist (hinge region) the hand is highly flexible relative to the arm (Fc domain) to allow the hand and fingertips (Fab domain) maximum flexibility to orientate an interaction with objects (antigen). The structure of the arm (Fc domain) does not influence interaction with an object (antigen). Once the object (antigen) has interacted with the fingertips (hypervariable regions) of the hand then the arm (Fc domain) can mediate a variety of effector functions.

A B-lymphocyte and plasma cell can produce different classes of antibody depending on the stage of immune activation and on the intercellular signals that the B-lymphocyte and plasma cell receive from other effector cells within the immune system. As stated above, the class of antibody is determined by the structure of the Fc domain and the different classes of antibodies possess different effector functions. The basic classes of antibodies are: IgM (heavy chain constant region defined as μ); IgA (heavy chain constant region defined as α); IgD (heavy chain constant region defined as δ); IgG (heavy chain constant region defined as γ) and IgE (heavy chain constant region defined as ε). The different classes of antibody can be remembered using the acronym MADGE. In addition to the heavy chain constant region classes, there are two light chain constant region classes, κ and λ; however, these do not mediate different antibody effector functions.

Each B-lymphocyte and the plasma cell that derives from it is capable of producing all the different antibody classes. However, all the antibody classes produced by a single B-lymphocyte and its derived plasma cell will recognize only a single epitope, i.e. only a single specific set of chemical features within a sequence or pattern of amino acid residues. In other words, all antibodies produced by a single B-lymphocyte, and its derived plasma cell, possess the same Fab domain recognizing the same antigenic determinant but clearly may possess different Fc domains capable of mediating different effector functions. Thus the same epitope can stimulate various different forms mediated via the IgM, IgA, IgD, IgG, IgE classes of humoral immune attack.

Within the antibody pool it is estimated that there are approximately 10^9 different epitope recognition specificities, sufficient to cover the range of pathogens likely to be encountered in life. This enormous diversity in antigen recognition is due to the amino acid sequence diversity in the variable and hypervariable domains of the antibody molecule. However, this large diversity cannot result from the presence of an equivalent number of separate protein-coding genes; the human genome project has estimated there to be only approximately 30 000 protein-coding genes. Rather, the clonal diversity in antigen recognition is due in the main to a process termed *gene rearrangement*, which occurs in each B-lymphocyte during maturation in the bone marrow. For example, the DNA coding for a single heavy chain molecule will result from the splicing together of genes from four separate regions termed a variable region gene (V), a diversity region gene (D), a joining region gene (J) and a constant region gene (C). There are approximately 100 V genes, 25 D genes and 50 J genes. Gene rearrangement will allow combinatorial freedom for any V, D and J genes to splice together, providing a large number of VDJ combined gene product permutations and hence diversity in antigen recognition. Inaccurate splicing together of the regional genes at the V–D and D–J junctions further increases diversity, as does the process of random nucleotide insertion. The C genes dictate the

different classes of antibody and not the antigen recognition specificity. An additional process which occurs in a B-lymphocyte memory cell population while it resides within the lymphoid tissue is that of somatic mutation, in which only very slight changes in antibody Fab domains occur through single base mutations. Sometimes these mutations prove advantageous by increasing the affinity of an antibody to the same original epitope. Under these circumstances the antibody clone with the highest binding affinity to the original target epitope will proliferate and dominate. The light chain gene also has V, J and C regions and the V and J genes undergo a similar rearrangement to that described for the heavy chain, and hence further add to diversity. The heavy chain and light chain polypeptides are joined together via disulphide bond formation following protein synthesis of the individual heavy and light chains. In summary, all antibodies produced by a single B-lymphocyte and its derived plasma cell are 'programmed' to recognize only a single antigen recognition feature determined by the recombination pattern of the V, D and J genes (heavy chain) and the V and J genes (light chain). The class of antibody is determined by further excisions within the DNA to allow the same VDJ gene combination to lie next to a different C gene, which codes for the structure of the antibody constant region and therefore determines antibody class. The five C gene classes are m, a, d, g and e, although various subclasses also exist. Antibody class switching is not a random process but one that is regulated by helper T-lymphocyte cytokine secretions.

3.3 Clonal selection and expansion

Within the body there may exist at any one time only a handful of naive B-lymphocytes capable of recognizing the same epitope. The meeting of an antigen and a naive B-lymphocyte capable of recognizing an epitope within the antigen occurs through the delivery of antigen to lymphoid tissues of the spleen, lymph nodes and local lymphoid tissue within mucosal surfaces (mucosal associated lymphoid tissue, MALT) and skin (SALT). This lymphoid tissue is rich in lymphocytes. Further, a proportion of B-lymphocytes will always be recirculating from the lymphoid tissue through the lymph and blood circulations and so able to encounter circulating antigen.

Antigen will be specifically recognized by IgM molecules present on the surface of the naive B-lymphocyte. Following this antigen-driven selection of a specific B-lymphocyte clone, the clone will undergo repeated cell divisions. Some of the daughter cells will differentiate into short-lived (2–3 days) plasma cells able to secrete antibody of different classes to combat the initial primary antigen exposure. Other clonal daughter cells will become long-lived B-lymphocyte memory cells populating the lymphoid tissue and spreading around the body through the lymph and blood circulations. These cells will provide 'immunological memory' able to generate a more rapid and pronounced secondary response on subsequent exposure to the original antigen (Figure 9.5).

This process of clonal selection and expansion to form memory cell populations is the basis of vaccination. The initial introduction of antigen gives rise to a primary response (Figure 9.6) in which there is a significant latent period before increased serum antibody levels are observed; the main antibody response is IgM production, although some IgG is also synthesized and secreted. On re-exposure to the same antigen a secondary response is elicited. The features of the secondary response include:

- a reduced latent period between antigen challenge and increases in serum antibody (e.g. latent period of 5–7 days for the secondary response vs 7–20 days for the primary response)
- an antibody response dominated by IgG which is more pronounced with higher serum levels achieved.

In the absence of helper T-lymphocyte involvement (T-cell-independent humoral responses, e.g. where antigen is carbohydrate alone) B-lymphocyte memory cell populations are not produced, and antibody class switching is restricted. Hence, under these circumstances, the primary and secondary antibody responses to antigen

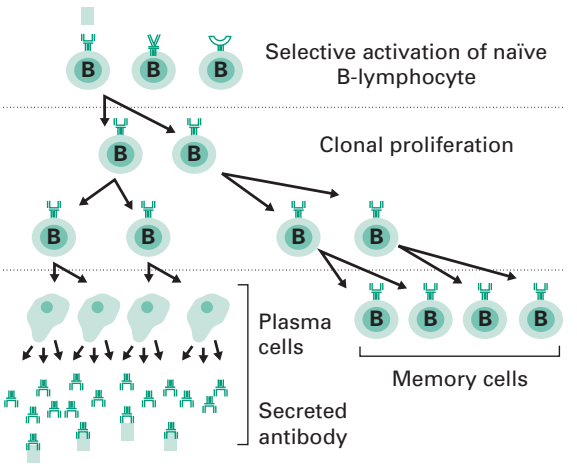

Figure 9.5 Clonal proliferation of B-lymphocytes. Following antigen-driven selection of a specific B-lymphocyte clone, it will undergo repeated cell divisions to give effector cell populations and memory cell populations.

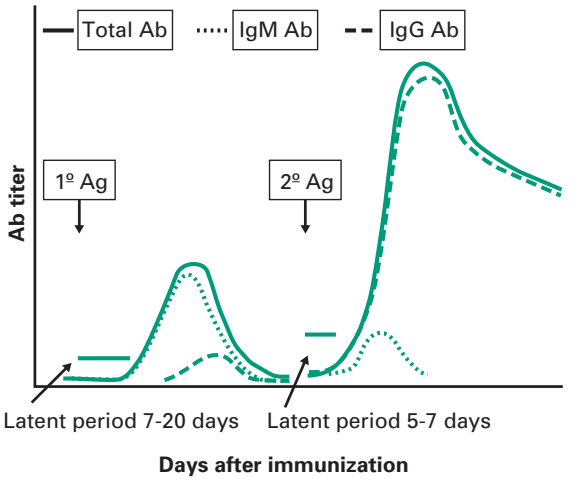

Figure 9.6 Primary and secondary responses to antigen. A primary response of the humoral system involves a significant latent period before elevated serum antibody levels are seen; the major serum antibody generated is IgM. Memory cell populations provide the basis of the secondary response, which displays a significant reduction in the latency period to achieve elevated serum antibody. The antibody serum levels are greater than in the primary response and involve mainly IgG.

challenge are essentially indistinguishable and exhibit a prolonged latent period, relatively low levels of serum antibody produced, and IgM as the main serum antibody.

3.4 Humoral immune effector functions

The humoral immune response is mediated by the initial antibody–antigen interaction, but with the different antibody classes offering a range of effector functions. The effector functions of antibodies include those described below.

3.4.1 Cognitive function on B-lymphocyte cell surface

Antibody on the surface of naive or memory B-lymphocytes serves to recognize and bind specific antigen; IgM serves this main cognitive function. It exists as a pentamer of five monomer units with an antigen valency of 10 and is extremely efficient at binding antigen. IgD appears to function mainly on the surface of B-lymphocytes and may also contribute to cognition in some way.

3.4.2 Neutralization of antigen by secreted antibody

Secreted antibody, in particular IgG, IgA and IgM, can bind antigen and sterically hinder the interaction of toxins, viruses, bacteria, etc. with host cell surfaces. In the circulatory and interstitial fluids IgG (which exists as a monomer with an antigen valency of 2) is the main antibody that fulfils this role in the secondary response, while IgM is the main antibody produced in the primary response. IgA has specific roles in mucosal immunity.

3.4.3 Opsonization of antigen

Secreted antibody, in particular IgG, opsonizes antigenic material and in doing so promotes association (e.g. through Fcg receptors) of the antigenic material with phagocyte membranes. Occupancy of the Fc receptor by the antibody also serves to activate a phagocyte's killing mechanisms.

3.4.4 Mucosal immunity

Mucosal immunity involves the interaction of antibody with antigen at mucosal surfaces such as those of the gastrointestinal tract, lung or urogenital tract. The major antibody of the mucosal lining fluid is IgA, which exists as a dimer of two monomer units (antigen valency of 4). IgA is actively secreted across mucosal epithelium into the lining fluid; it will neutralize antigen and may also serve as an opsonin. IgA is also present in secretions such as tears, saliva, etc. but it has a limited role in systemic immunity.

3.4.5 Antibody-dependent cell cytotoxicity (ADCC)

Through specific binding to antigen on the surface of membranes perceived as 'foreign', e.g. microbial cells or host cells virally infected or otherwise transformed, antibody can direct (through its Fc domains) the close association of 'killing' cells, such as neutrophils, eosinophils, NK cells and even cytotoxic T-lymphocytes, with the 'foreign' membrane. This close association depends on the antibody's Fc domain binding to the respective Fc receptor present on the surface membrane of the 'killing' cell. Such close proximity to the 'foreign' cell enables the efficient and targeted release of cytotoxic molecules into the extracellular environment. IgG is the main antibody of systemic body fluids and is an important mediator of ADCC, although IgE and IgA may undertake this role in certain circumstances, e.g. against certain parasites IgE directs ADCC mediated by eosinophils.

3.4.6 Immediate hypersensitivity

Mast cells express high-affinity receptors (Fce) that bind the Fc domain of IgE antibodies. In the absence of antigen these receptors are occupied by the IgE monomer (antigen valency of 2) secreted previously from plasma cells. In this circumstance the IgE molecules are serving a cognitive function which, on appropriate antigen binding, results in aggregation of the membrane-bound IgE and causes immediate mast cell degranulation and release of inflammatory mediators. Mast cells possess in their membranes IgE monomers able to recognize different antigenic epitopes. This contrasts with each single B-lymphocyte, which possesses IgM antibody on its surface membrane that performs a cognitive function but is capable of recognizing only a single epitope specificity.

3.4.7 Neonatal immunity

The neonate lacks the ability to mount a full immunological response; accordingly, maternal IgG is transported across the placenta late in pregnancy and is also absorbed across the gastrointestinal tract from breast milk. Maternal IgA secreted into breast milk will also provide mucosal protection for the neonate.

3.4.8 Activation of the classical complement pathway

A complement cascade similar to that of the alternative pathway can be activated through specific antibody–antigen interactions. The antibodies that activate the classical complement pathway are IgM and IgG.

Key steps in the activation of the classical pathway are shown in Figure 9.7, where this pathway is also compared to the alternative pathway. In the classical pathway the initiating step is the specific binding of IgG or IgM to antigen. Once this occurs, a complement protein termed C1 (which comprises a single C1q subunit, two C1r subunits and two C1s subunits) binds to adjacent Fc domains in the antibody–antigen complex. This binding of C1 activates the catalytic activity of the C1r subunits, and in turn the C1s subunits. The activated C1s subunits cleave C4 into C4b and C4a; the latter can diffuse away and serve as a leucocyte activator. The C4b covalently associates with the antibody–antigen complex on the surface of a microbial membrane and can serve as an opsonin. A further complement protein, C2, binds to this membrane complex to give C4b2. The C1s subunit then enzymically cleaves the bound C2a to generate on the membrane a new complex termed C4b2b, which is the C3 convertase of the classical pathway. (In some texts the C2a is referred to as the larger subunit remaining with the membrane while C2b is the smaller subunit that diffuses away.)

This C3 convertase molecule is distinct from that within the alternative pathway, but it is from this point onwards that parallels can be drawn between the two cascades.

The host proteins that serve key regulatory functions within the alternative pathway (DAF, CR1 factor I, CD59)

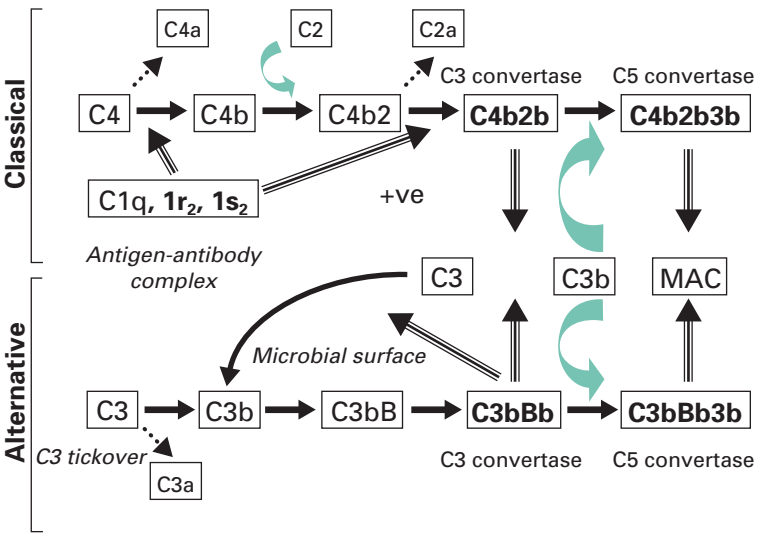

Figure 9.7 The classical and alternative complement pathways.

also serve similar functions within the classical pathway. However, in contrast to the alternative pathway the activation step in the classical pathway requires specific antibody–antigen interactions. In this context the C1 protein can only become catalytically active when it is bound to at least two adjacent Fc domains. In the case of the IgG and IgM molecules the Fc domains will only align adjacent to each other when the corresponding Fab domains bind antigen. Further, when C1 is free in the circulation it is bound to a protein termed C1 inhibitor (C1-INH) which prevents any possible activation of C1 in the absence of antibody. Once C1 binds to adjacent Fc domains within an antibody–antigen complex C1-INH is displaced.

The functions of the classical complement pathway are similar to those described for the alternative pathway, i.e. opsonization, leucocyte activation and membrane lysis of target cells. The classical pathway can additionally lead to complement protein deposition on insoluble antibody–antigen immune complexes circulating within blood, and in doing so promote the clearance of such potentially harmful complexes by Kupffer cells of the liver. The presence of two complement pathways provides for rapid (alternative) and specific (classical) activation of a key defence mechanism, and offers greater protection against the development of microbial resistance mechanisms.

4 Cell-mediated adaptive immune system

Cell-mediated adaptive immune responses are mediated by T-lymphocytes which arise from bone marrow progenitor cells and undergo maturation in the thymus before release into the systemic blood and lymph circulations (Figure 9.1). A number of parallels can be drawn between the B-lymphocyte-mediated immune response and the T-lymphocyte-mediated response. First, membrane-bound antibodies serve the cognitive function for B-lymphocytes, while the cognitive function for T-lymphocytes is served by T-lymphocyte receptor (TCR) present on the cell's plasma membrane surface. Second, in response to antigen T-lymphocytes, like B-lymphocytes, will undergo clonal proliferation and form a population clone of memory T-cells specific for a single epitope. Third, each single B-lymphocyte and plasma cell that derives from it is capable of producing antibodies that will only recognize a single epitope. In the same manner each single T-cell is programmed to make TCR of only a single specificity able to recognize only a single specific set of chemical features within a T-cell epitope. This is achieved for the TCR in a similar manner to that for antibodies in that different gene segments termed V-D-J are brought together by the process of gene rearrangement into single RNA products. The recombined RNA will code for the polypeptide chains that make up the TCR. When all the possible recombinations are considered, the number of different TCR molecules that an individual can make is in excess of 10^9, a number similar to the primary antigen recognition repertoire of T-cells.

There are two general classes of T-lymphocytes: helper T-lymphocytes and cytotoxic T-lymphocytes. The latter function to kill host cells that have undergone a transformation such as a viral infection or cancer and they recognize specific antigens on the surface of host cells that have arisen as a result of such cell transformation. Through this specific antigen recognition, the cytotoxic T-cell becomes closely apposed to the target host cell and is activated to synthesize and release cytotoxic secretory products (e.g. pore-forming molecules such as perforins) leading to lysis of the affected host cell. In contrast, the helper T-cell can be viewed as the coordinator of the adaptive immune system, providing appropriate activation signals, in the form of secreted cytokines, to promote the functioning of both the cytotoxic T-cell populations and that of the antibody-producing B-lymphocyte and plasma cell populations. The actions of the helper T-cell populations also promote the function of the innate immune system, for example IFN-γ released by helper T-cells increases the phagocytic activity of macrophages. The helper T-cells are further divided into T_H1 or T_H2 subpopulations depending on the nature of cytokines they secrete and, as a consequence, the arms of the immune system they predominantly influence. The T_H1 helper T-cells mainly regulate cell-mediated immunity while T_H2 helper T-cells regulate humoral immunity.

4.1 T-lymphocyte antigen recognition and MHC proteins

Epitopes for T-lymphocytes comprise exclusively linear peptide sequences. T-lymphocytes are unable to respond to carbohydrate, lipid or nucleic acid material and they only respond to peptide antigen when it is presented to the T-lymphocyte by surface proteins on the plasma membrane of host cells. These surface proteins are termed MHC proteins and can be subdivided into two main classes:

- *MHC class I proteins* are expressed on the surface of all nucleated host cell membranes and present peptide antigen to cytotoxic T-lymphocytes.

• *MHC class II* proteins are expressed only on a more specialized group of cells termed antigen-presenting cells (APCs), and present peptide antigen to helper T-lymphocytes.

Such a distinct cellular distribution of MHC proteins and restriction in presentation to discrete T-lymphocyte subpopulations may be remembered by considering the different T-lymphocyte functions. That is, all cells of the body have the potential to become infected with virus and undergo a cancerous change, and hence all cells must have the capacity to be destroyed by the actions of cytotoxic T-cells. As such, all cells of the body must possess MHC I molecules to afford antigen presentation to cytotoxic T-cells. In contrast, as a coordinator cell of the immune system, the helper T-cell must be able to respond to its environment in order to give appropriate signals or 'help' to other immune cells. Specialized APCs with the capacity to phagocytose interstitial proteinaceous material therefore undertake the function of 'environmental sampling'. MHC class II proteins expressed on the surface of APCs will present peptide antigen to helper T-lymphocytes.

APCs include the macrophage tissue cell population, specialized APCs such as dendritic cells within the lymphatic system or Langerhans cells within the skin. B-lymphocytes also serve the function of an APC because they interact with protein antigen through high-affinity surface IgM molecules. Subsequently, they internalize the protein antigen for processing to generate peptides that will be presented by MHC II molecules expressed on the B-lymphocyte cell surface. Another cell type that can serve the function of an APC is the endothelial cell, which can be induced to express MHC II molecules by the action of the cytokine IFN-γ. It should not be overlooked that the APC can itself become infected with virus and undergo cancerous transformation, and therefore the APC, in addition to MHC II molecules, will also express the full complement of MHC I molecules on its surface.

This process of MHC presentation of peptide and interaction with T-lymphocyte receptor is shown in Figure 9.8. A foreign peptide presented by a MHC molecule will be recognized by a TCR expressed on the surface of an appropriate T-lymphocyte. Once a particular TCR recognizes a peptide sequence as foreign, intracellular signals to activate the T-cell are sent via the CD3 complex present within the T-cell membrane. The recognition of peptide as foreign will lead to an immune response. Beyond antigen presentation, the interaction between MHC molecule and T-lymphocyte also serves to identify that the T-lymphocyte and host cell membrane arise from the same embryonic tissue. Tremendous inter-individual differences, or more specifically polymorphisms, exist in the MHC proteins within a population. The T-lymphocyte undertakes this MHC surveillance through the possession of accessory molecules. Cytotoxic T-lymphocytes possess CD8+ molecules which interact with MHC I (Figure 9.8a), while helper T-lymphocytes possess $CD4^+$ molecules which interact with MHC II (Figure 9.8b); hence the use of the terms $CD8^+$ lymphocytes to refer to cytotoxic T-cells and $CD4^+$ lymphocytes to refer to helper T-cells.

4.2 Processing of proteins to allow peptide presentation by MHC molecules

Peptide epitope presented by MHC I is derived from the processing of proteins (e.g. a viral protein) synthesized within the actual cell that eventually will present the peptide to cytotoxic T-lymphocytes. The MHC I molecule is composed of two polypeptide chains, an α-chain which has α1, α2 and α3 domains, and a second

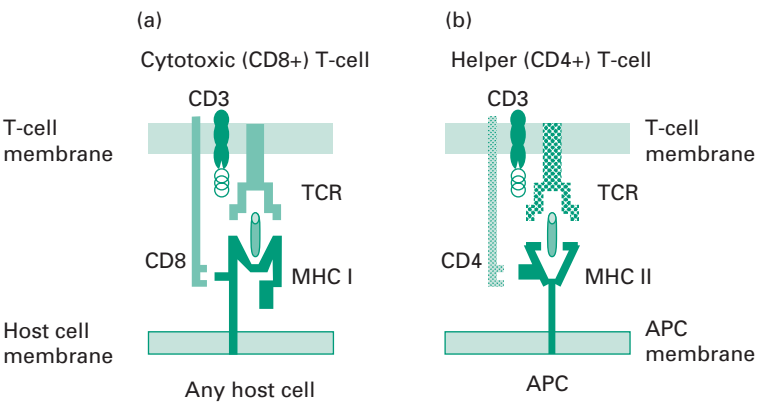

Figure 9.8 The process of MHC presentation of peptide and interaction with T-lymphocyte receptor (TCR). Cytotoxic T-lymphocytes ($CD8^+$) interact with MHC I (a), while helper T-lymphocytes ($CD4^+$) interact with MHC II (b).

polypeptide termed β_2-microglobulin. The $\alpha 1$ and $\beta 2$ domains form a peptide-binding cleft which can accommodate peptides up to 11 amino acids in length. Figure 9.9a shows the processing of a protein into peptide fragments for presentation by MHC I. The synthesized protein (indicated by an asterisk) is present in the cytoplasm of the cell and is degraded by a subcellular organelle termed a proteasome. The derived peptide fragments are actively transported, via a TAP peptide transporter, into the lumen of the endoplasmic reticulum (ER) where they fit within the binding clefts of MHC I molecules. From the ER the MHC I with bound peptide is transported to the trans-Golgi network (TGN), from which it is transported via endosomes to the plasma membrane where the MHC I molecule with bound peptide is accessible to surveillance by cytotoxic $CD8^+$ T-lymphocytes.

Peptide epitopes presented by MHC II are derived from proteins present within the extracellular fluid and are presented to helper T-lymphocytes by APCs. The MHC II molecule is composed of two polypeptide chains, an α-chain which has $\alpha 1$ and $\alpha 2$ domains, and a β-chain which has $\beta 1$ and $\beta 2$ domains. The $\alpha 1$ and $\beta 1$ domains form a peptide-binding cleft that can accommodate peptides up to 20 amino acids in length. Figure 9.9b shows the processing of a protein into peptide fragments for presentation by MHC II. In this case the protein (indicated by an asterisk) is internalized from the extracellular fluid by the APC and restricted to an endosomal compartment without access to the APC's cytoplasm. The endosome delivers the protein to a lysosome compartment which degrades the protein into peptide fragments, after which the peptide fragments are returned to an

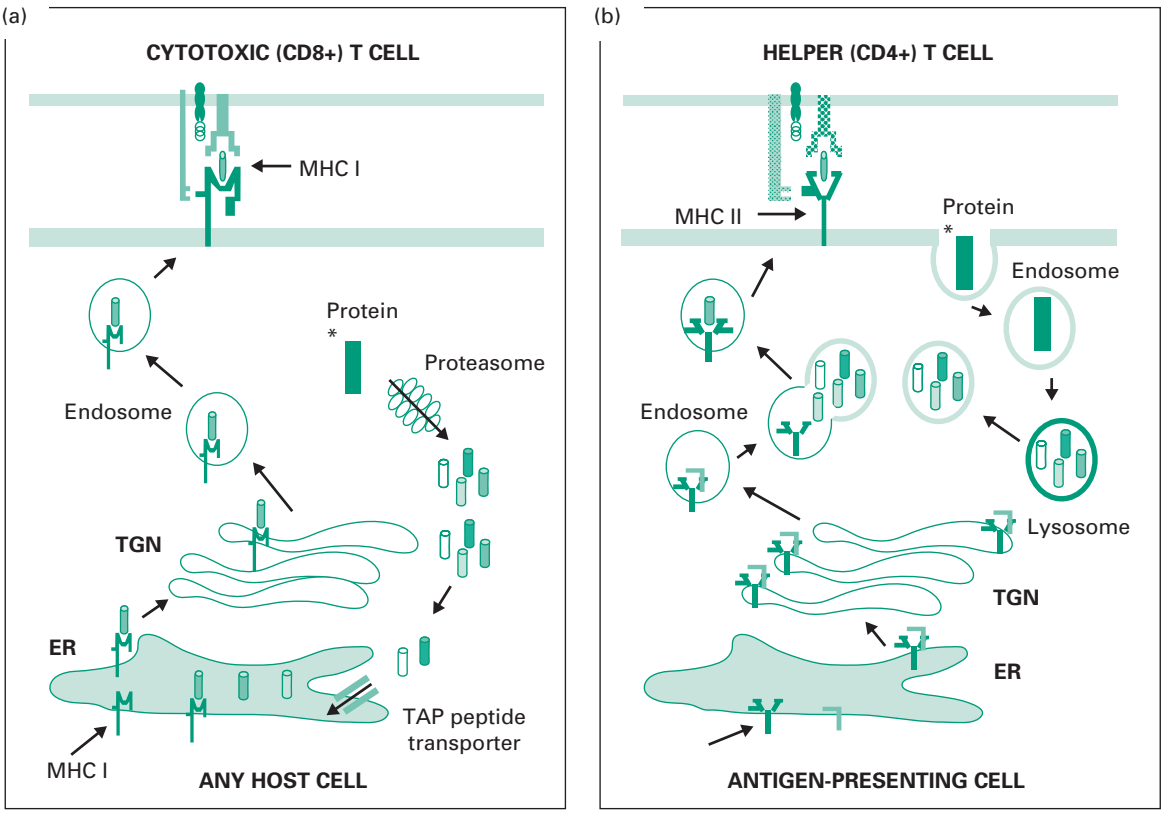

Figure 9.9 Schematic of the processing of a protein into peptide fragments for presentation by MHC. (a) The synthesized protein (*) is present in the cytoplasm of the cell and is degraded into peptide by the proteasome. The derived peptide fragments are presented by MHC I to cytotoxic $CD8^+$ T-lymphocytes. (b) Protein (*) is endocytosed by an APC and processed into peptide within lysosomes. The derived peptide fragments are presented by MHC II to helper $CD4^+$ T-lymphocytes.

endosome compartment. In the lumen of the ER the MHC II molecule becomes associated with another protein termed an invariant chain which blocks access of peptides to the binding cleft of the MHC II molecule; the MHC II–invariant chain complex is transferred to the TGN and then to an endosomal compartment. At this point the endosomes that contain the processed peptide and the MHC II molecules merge, and the invariant chain disintegrates, allowing peptides access to the MHC II-binding cleft. The MHC II molecules with bound peptide are transported to the plasma membrane where they are accessible to surveillance by helper $CD4^+$ T-lymphocytes.

4.3 More on T-lymphocyte subpopulations

4.3.1 Effector T-helper cell subtypes

Following maturation in the thymus, mature but naive $CD4^+$ helper T-cells access the systemic blood and lymphatic circulations. In this naive state they have yet to be stimulated by antigen. The antigen-driven activation signals involve initially TCR interactions with MHC-presented peptide and subsequent CD3 activation. This is followed by the interaction of a variety of costimulatory molecules on the surface of the APC (e.g. CD80, CD86) with surface receptors (e.g. CD28) on the helper T-cell. Once the helper T-cell is activated it can proliferate in an autocrine or paracrine fashion driven by secreted IL-2. These proliferating helper T-cells will then differentiate depending on their cytokine environment; for example, IFN-γ and IL-12 drive the differentiation to a T_H1 subpopulation of cells while IL-4 drives the differentiation to a T_H2 subpopulation of cells.

Apart from IL-2 the main cytokines produced by T_H1 cells are IFNγ and TNFβ, and the main cell partner for T_H1 cells are the APCs. The T_H1 cells classically promote cell-mediated immune responses maximizing the effectiveness of APCs and the proliferation of cytotoxic $CD8^+$ T-cells. Apart from IL-2 the main cytokines produced by T_H2 cells are IL-4, 5,6,10 and 13, while the main cell partner for T_H2 cells is the B-cell. The T_H2 cells classically promote the humoral immune responses stimulating B-cells to proliferate to undergo Ig class switching and increase Ig production and secretion.

The above model of helper T-cell subpopulations and how various cytokines can serve to promote the differentiation pathway to either T_H1 or T_H2 phenotype is recognized to be an oversimplification. However, the basic model serves to emphasize that distinct populations of helper T-cells exist that fulfil many different and varied functions.

4.3.2 T-regulatory cells

T-regulatory cells (T_{regs}) are a subset of T lymphocytes that serve an immune suppressor function leading to peripheral tolerance to self- or foreign antigens. T_{regs} are characterized by a $CD4^+/CD8^-$ phenotype, but among a number of other identity markers that these cells display the expression of the Foxp3 transcription factor is the main distinguishing feature. T_{regs} are distinct from those effector T-cells which are induced to switch to secrete immunosuppressive cytokines as a typical immune response progresses with time from an immunostimulatory to immunoinhibitory character. The T_{regs} represent approximately 10% of all $CD4^+$ T-cells and can acquire the immunosuppressive phenotype in the thymus or via induction in the periphery. In the thymus this subset of $CD4^+$ T-cells are positively induced by interactions with MHC molecules and the recognition of agonist peptide. This contrasts to the situation for effector T-cells where TCR recognition in the thymus of MHC-presented peptide normally triggers the positive deletion of the affected T-cell. In particular TGFβ appears particularly important for the expression of the T_{regs} phenotype while IL-2 as the key T-cell mitogen is also important.

T_{regs} essentially serve to suppress immune responses of effector T-cells, effector B-cells and APCs leading to peripheral immune tolerance. Direct cell–cell contact as well as cytokine signalling are mechanisms important in mediating their actions. These cells play an important role in self-limiting immune responses. T_{regs} display a number of disease associations, with decreased numbers of T_{regs} or reduced function in a range of autoimmune diseases. T_{regs} also appear to fulfil a role in the active immune evasion of tumours, with the experimental depletion of T_{regs} improving natural antitumour immunity and effectiveness of active immunotherapy.

4.3.3 γδ T-cells

The vast majority of T-cells possess a TCR comprised of two polypeptide chains, a single α-chain and a single β-chain. γδ T-cells possess a TCR made of a single γ-chain and a single δ-chain. The antigenic molecules or ligands that activate γδ T-cells remain essentially unknown, although they appear not to require antigen processing or MHC presentation. These cells have characteristics of both innate and adaptive immune cells possessing a TCR, but also undergoing early activation capable of

phagocytosis and rapid production of cytokines that regulate inflammation and pathogen removal.

5 Some clinical perspectives

This section is intended to provide a brief overview of some clinical issues that exemplify the basic aspects of immune system functioning discussed previously.

5.1 Transplantation rejection

Transplantation is the process of transferring cells, tissues or organs—termed a graft—from one location to another. An *autologous* graft is a transplant between two sites within the same individual, e.g. skin graft from the thigh to the hand. An *allogeneic* graft is a transplant between two genetically different individuals of the same species, e.g. kidney transplant from a donor to a recipient individual. A *xenogenic* graft is a transplant across different species, e.g. pig to human.

The tempo of clinical rejection—in kidney transplantation, for example—is often categorized by the following stages.

- *Hyperacute rejection* occurs within minutes to hours following revascularization of a graft. The cause is due to the presence of preformed circulating antibody (IgG) that reacts with the blood cell antigens (the ABO system), or MHC I molecules or other poorly defined antigens. This should now be a rare event clinically as recipients are tested (cross-matched) before transplantation for the presence of antibodies reactive with cells of the donor.
- *Acute rejection* occurs within weeks to months following transplantation and involves humoral (antibody) and cell-mediated induced cytotoxicity. Damage may be reversed with early diagnosis and more aggressive immunosuppressive therapy.
- *Chronic rejection* occurs many months or even years following transplantation. The pathology is characterized by fibrosis and may require differential diagnosis to distinguish between a chronic rejection event and the recurrence of the original disease that necessitated transplantation in the first place.

The major alloantigens (i.e. antigens responsible for rejection of allogeneic grafts) are the MHC proteins. Although there are two distinct classes of MHC protein (described in section 4.1), the MHC molecules actually have a number of subclasses which vary further in the general nature of peptides that they will accept within their binding clefts. The MHC I molecules are composed of three subclasses, MHC IA, MHC IB and MHC IC, on each nucleated cell of the body; all three subclasses are simultaneously expressed. The MHC II molecules are also made up of three subclasses, MHC II DR, MHC II DP and MHC II DQ, and again on each APC all three subclasses of MHC II molecule are simultaneously expressed. APCs, like other cells in the body, will also express MHC I molecules on their surface in addition to MHC II.

As indicated previously, the major cause of allogeneic tissue transplantation rejection is the polymorphic nature of the MHC phenotype between individuals. Polymorphism in MHC arises within the population because the genes for each of the MHC subclasses can exist in multiple different forms or alleles. For example, in humans there are at least 52 different forms of the MHC IB gene and at least 24 different forms of the MHC IA gene. It follows that individuals in a population can possess any one of the 52 different forms of MHC IB gene and any one of the 24 different forms of MHC IA gene, so the number of different combinations for the six classes of MHC proteins is many millions. The situation is further complicated by the fact that each individual inherits and coexpresses a set of MHC I and II genes from each parent. This means that on each nucleated cell of the body there will be coexpressed paternally derived and maternally derived versions of the MHC IA, MHC IB and MHC IC molecules. The same principle will apply for coexpression on APCs of paternal and maternal MHC II protein subclasses.

This tremendous polymorphism is important in immune defence because it allows the broadest possible scope of peptide antigen presentation, and thus the best chance of survival of a population as a whole, but it also confers the very high probability of MHC mismatch during allogeneic transplantation. As a result of the mode of MHC inheritance, the highest probability of a MHC tissue match between individuals who are not genetically identical twins will be that obtained between siblings, where there is a 1 in 4 chance of a sibling possessing an exact match for all the MHC I and MHC II subclasses. The MHC proteins are also termed human leucocyte antigens (HLA), and HLA tissue typing is undertaken routinely before transplantation to gain improved matches between donor and recipient. In kidney transplantation it has been found that matching the MHC IA, IB and IIDR genes in particular appears to improve short- and long-term graft survival.

The main target for the modern immunosuppressants such as ciclosporin and tacrolimus is inhibition of cytokine gene transcription in a highly selective manner

in the helper T-lymphocyte populations. The consequence of this is to inhibit helper T-cell autoactivation and helper T-cell coactivation of cytotoxic T-lymphocytes and of B-lymphocytes, and thus considerably 'damp down' cell-mediated and humoral immune responses to the graft.

5.2 Hypersensitivity

Hypersensitivity can be defined as an exaggerated response of the immune system leading to host tissue damage. However, some of the immune responses described in the hypersensitivity classification below are, in some circumstances, appropriate responses to invading antigen. For example, a component in what is an appropriate immune response to tissue transplant rejection can be defined as a type II hypersensitivity reaction.

The highly influential Gell and Coombs classification scheme defines four categories of hypersensitivity:

• *Type I—immediate hypersensitivity*. This is also called anaphylactic or acute hypersensitivity. It involves IgE antibody and is mediated via degranulation of mast cells leading to release of preformed factors which promote an influx of immune cells to the site of mast cell activation and initiation of a rapid inflammatory reaction. In the extreme case the inflammatory response extends beyond the localized site of initiation and affects systemic tissues leading to life-threatening anaphylactic reactions such as those documented to penicillin, to peanut antigen, or to bee-sting antigen. Examples of localized type I hypersensitivity include hay fever. The term 'allergy' has become synonymous with type I hypersensitivity.

• *Type II hypersensitivity—antibody-mediated cytotoxicity*. This is caused by antibodies that are directed against cell surface antigens. IgG and IgM are the key antibodies involved that direct cytotoxic events against the cell surface with which they interact. The cytotoxic events include activation of the classical complement pathway leading to the formation of a MAC, and the attraction and activation of killing cells such as NK cells or phagocytes which can bind to the antigen–antibody complex via receptors for antibody Fc domains or complement C3b. Type II hypersensitivity disorders include blood transfusion reactions arising from mismatch of the blood ABO antigens between donor and recipient, or haemolytic disease of the newborn. Autoimmune disorders such as myasthenia gravis, Goodpasture's syndrome and autoimmune haemolytic anaemias are initiated by autoantibodies reacting against 'self' tissue.

• *Type III hypersensitivity—complex-mediated*. This involves the formation of large antigen–antibody complexes that circulate in the blood, are usually coated by complement proteins and are removed by phagocytosis. If this process is compromised for any reason then the antigen–antibody complexes will be deposited in tissue capillary beds, with kidney deposition being clinically the most important site. This deposition of high molecular weight antigen–antibody complexes in the glomerular capillaries of the kidney can lead to a condition termed glomerulonephritis which involves disruption of the glomerular basement membrane, destruction of glomeruli and ultimately renal failure which may necessitate organ transplantation. Systemic lupus erythematosus is a condition where autoantibodies are directed against the host's DNA and RNA with subsequent complement-coated immune complexes deposited throughout systemic tissues such as in the kidney, skin, joints and brain.

• *Type IV hypersensitivity—cell-mediated*. This results from inappropriate accumulation of macrophages at a localized site, and may or may not involve the presence of antigen. Under conditions of ongoing localized infection or inflammation, macrophages release proteases, which destroy infected or otherwise damaged tissue. However, with the inappropriate recruitment and/or activation of excessive numbers of macrophages, continuing damage to normal tissue may result, leading to chronic inflammation. The recruitment and activation of macrophages in type IV hypersensitivity is augmented by the activity of helper T-lymphocytes (specifically the T_H1 subpopulation). Examples of type IV hypersensitivity include granuloma formation and contact dermatitis. Granulomas are initiated and maintained by the recruitment of macrophages into the site of a persistent source of antigen or toxic material. A granuloma is a fibrotic core of tissue composed of tissue cells and macrophages surrounded by lymphocytes and then further surrounded by layers of calcified collagenous material. Sarcoidosis is a granulomatous disease of unknown cause but characterized by granuloma nodule formation in the lung and skin, among other sites.

6 Summary

The immune system is a complex body system whose various functions display a high level of inter-regulation. As such, any attempt to describe the functioning of the immune system within a single chapter will inevitably represent an oversimplification. However, the authors consider this chapter to be a comprehensive, but nevertheless basic, overview of the immune system that will

serve as a sound foundation for further reading on the clinical immunological basis of disease or for the consultation of more specialized texts on immunological function.

The discussion in this chapter is structured by delineating the immune system into innate and adaptive responses. The innate system, responding immediately but non-specifically to antigen, is complementary to the adaptive immune system which reacts in a highly specific manner to antigen but which displays a delay in its response. It should not be forgotten, however, that the functioning of the two systems are intimately related, showing dependency on each other for the optimal maintenance of health.

7 Acknowledgement

The authors acknowledge Dr Jim Furr, an inspirational educator.

8 Further reading

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10 Vaccination and immunization

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1 Introduction

People rarely suffer from the same infectious disease twice. Reinfections normally occur, primarily (1) when the infectious agent exhibits antigenic plasticity such as with the common cold and influenza; (2) if the patient is immunocompromised, due for example to immunosuppressive therapy or immunological disorders; or (3) when a significant amount of time has passed after the first infection. Alternatively, the patient may have failed to eliminate the primary infection which remained latent and emerged later in a modified of similar form as for example with herpes simplex (oral and genital herpes), herpes zoster, (chickenpox) and HIV/AIDS.

Immunity against reinfection was recognized long before the discovery of the causal agents of infectious disease. Consequently, efforts were made towards developing treatment strategies that could generate immunity to infection without the individual suffering the infection. An early development was the attempted prevention of smallpox (variola major) through the dermal inoculation of healthy individuals with material taken from active smallpox lesions. Such treatments often produced

Hugo and Russell's Pharmaceutical Microbiology, Eighth Edition. Edited by Stephen P. Denyer, Norman Hodges, Sean P. Gorman, Brendan F. Gilmore.

single localized lesions and commonly, but not always, protected the recipient from contracting full-blown smallpox. The process became known as *variolation* and, unknown to its practitioners, protected against the disease by changing the route of infection of the causal organism from respiratory transmission to cutaneous. Unfortunately, occasional cases of smallpox resulted from such practices and variolated individuals could also (rarely) infect others, resulting in infection. Further developments recognized that immunity developed towards one pathogen may be associated with cross-immunity towards related infectious agents. Cowpox is a disease of cattle that can be transmitted to humans. The symptoms are similar to those of smallpox, but considerably less severe. Following the observation that individuals exposed to cowpox were conferred protection against smallpox, Edward Jenner substituted material taken from active cowpox (vaccinia) into the variolation procedures. This conferred much of the protection against smallpox that had become associated with variolation but without the associated risks. This discovery, made over two centuries ago, became known as *vaccination* and heralded a new era in disease control. The term vaccination was originally used to refer to prophylactic measures that use living microorganisms or their products to induce immunity, but the term is now used to refer to all immunization procedures.

Vaccination is used to protect individuals against infection and also to protect communities against epidemic disease. Such public health measures have met with spectacular success and in instances where there is no reservoir of the pathogen other than in infected individuals and survival of the pathogen outside the host is therefore limited, vaccination has the potential to eradicate the disease permanently. This has already been achieved for smallpox where the coordinated deployment of an effective vaccine over many decades led to the eradication of this disease. The global eradication of smallpox was endorsed by the World Health Assembly on 8 May 1980. Another candidate disease for global eradiation by vaccination is poliomyelitis, where effective vaccination programmes have reduced the annual incidence to fewer than 2000 cases. The virus persists, however, in India, Pakistan, Afghanistan and Nigeria. The Global Polio Eradication Initiative, spearheaded by the World Health Organization (WHO), Rotary International, the U.S. Centers for Disease Control (CDC) and the United Nations Children's Fund (UNICEF) is now actively working towards the eradication of this virus. It has been calculated that eradication is a more cost-effective option than containing the disease, as well as reducing morbidity associated with the residual cases. The effectiveness of poliomyelitis vaccines is clearly indicated by data shown in Figure 10.1.

2 Spread of infection

Infectious diseases may either be spread from a common reservoir (common source) of the infectious agent that is distinct from the diseased individuals, or through a population by serial transfer from diseased to healthy, susceptible individuals (propagated source; Figure 10.2).

2.1 Common-source infections

In common source infections, potential reservoirs of infection include infected drinking-water, contaminated water vapour from a cooling tower, or contaminated food. In the simplest of cases the source of the infection is transient (i.e. food sourced to a single retail outlet, or to an isolated event such as a dinner party). In such instances, the onset of new cases will be phased over a timescale approximately equivalent to one incubation period, and the decline in new cases closely follows the elimination of the source (Figure 10.2). This leads to an acute outbreak of infection, limited to those linked with the source. Such incidents are epitomized by the 1996 outbreak of *Escherichia coli* O157 infections in Lanarkshire, Scotland, that resulted in 5 deaths and left 280 people ill. Similarly, in Clemenstone, South Wales, an outbreak resulted in 157 cases of *E. coli* including the death of a 5-year-old boy in 2005. Both of these outbreaks were linked to a single retail outlet.

If the source of the infection persists beyond the onset, then the incidence of new cases may be maintained at a level that is commensurate with the infectivity of the pathogen and the frequency of exposure of individuals. For those infectious diseases that are transmitted to humans via insect vectors that may act as reservoirs of infection, onset and decline phases of epidemics are rarely observed, other than as reflections of the seasonal variation in the prevalence of the insect. Diseases such as these are generally controlled by public health measures and environmental control of the insect vector with vaccination and immunization being deployed to protect individuals (e.g. yellow fever vaccination).

2.2 Propagated-source infections

Propagated outbreaks of infection relate to the direct transmission of an infective agent from a diseased

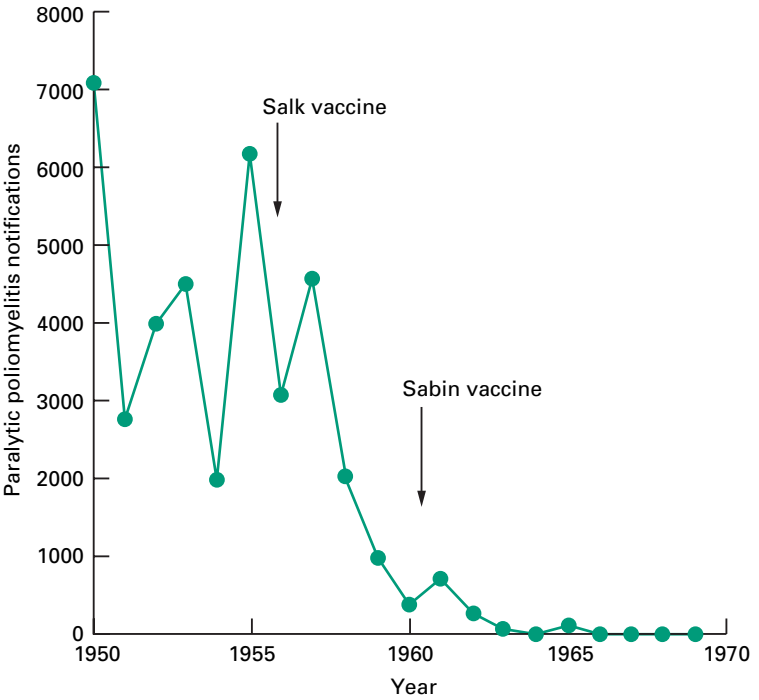

Figure 10.1 Reported incidence of paralytic poliomyelitis in England and Wales during the 1950s and 1960s. After the introduction of vaccination programmes the incidence of this disease dropped from an endemic incidence of around 5000 cases per year to fewer than 10.

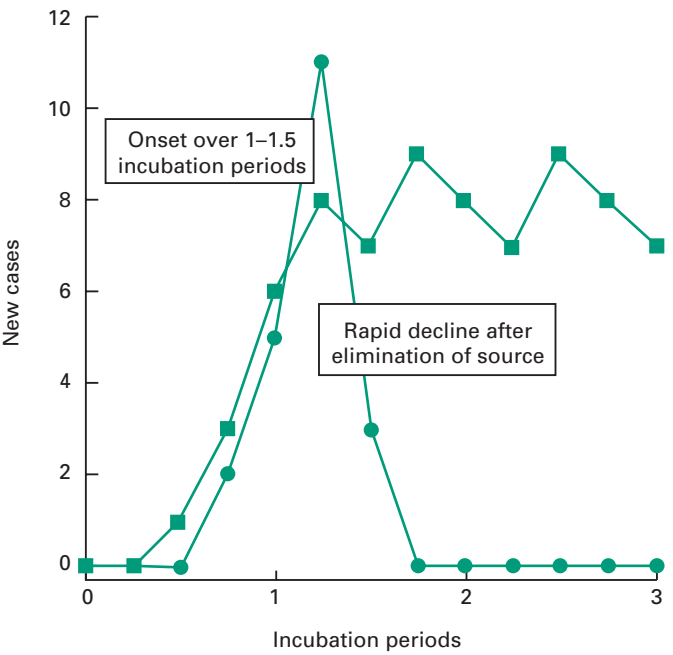

Figure 10.2 Incidence pattern for common-source outbreaks of infection where the source persists (■) and where it is short-lived (●).

individual to a healthy, susceptible one. Mechanisms of such transmission have been described in Chapter 7 and include inhalation of infective aerosols such as with measles, mumps and diphtheria; direct physical contact such as may occur with syphilis, herpesvirus and human papillomavirus; and, where sanitation standards are poor, through the introduction of infected faecal material into drinking-water (i.e. cholera and typhoid fever) or onto food (e.g. *Salmonella* and *Campylobacter*). The ease of transmission, and hence the rate of onset of an epidemic, relates to the susceptibility status and general state of health of the individuals concerned, the virulence properties of the organism, the route of transmission, the duration of the infective period associated with the disease, behavioural patterns, age of the population group and the population density (i.e. urban versus rural).

Each infectious individual will be capable of transmitting the disease to the susceptible individuals that they encounter during their infectious period. The number of persons to which a single infectious individual might transmit the disease and hence the rate of occurrence of the infection within the population will depend upon the population densities of susceptible and infective individuals, the degree and nature of their social interaction and the duration and timing of the infective period. If infectivity precedes the manifestation of disease, then spread of the infection may be greater than if these were concurrent. As each infected individual will in turn, become a source of infection, this leads to a near exponential increase in the incidence of disease. Figure 10.3 shows the incidence of disease within a population group. This group is perfectly mixed and all individuals are susceptible to the infection. The model infection has an incubation period of 1 day and an infective period of 2 days commencing at the onset of symptoms with recovery occurring 1 day later. For the sake of this illustration it has been assumed that each infective individual will infect two others per day until the entire population group has contracted the disease (solid lines). In reality, the rate of transmission will decrease as the epidemic progresses, because recovered individuals may become immune to further infection, reducing the population density of susceptible individuals, and thereby the likelihood of onward transmission. Epidemics therefore often cease before all members of the community have been infected (Figure 10.3, dotted line). If the proportion of immune individuals within a population group can be maintained above this threshold level then the likelihood of an epidemic arising from a single isolated infection incident is small (this is referred to as *herd immunity*). The threshold level itself is a function of the infectivity

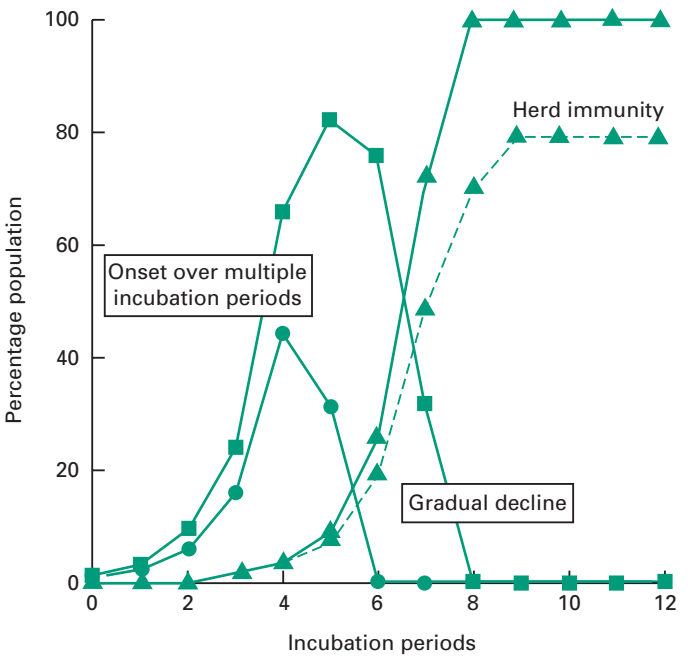

Figure 10.3 Propagated outbreaks of infection showing the incidence of new cases (●), diseased individuals (■) and recovered immune individuals (▲). The dotted line indicates the incidence pattern for an incompletely mixed population group.

of the agent and the population density. Outbreaks of measles and chickenpox therefore tend to occur annually in the late summer among children attending school for the first time. This has the effect of concentrating all susceptible individuals in one, space and thereby reducing the proportion of immune subjects to a value below the threshold for propagated transmission. An effective vaccination programme that maintains the proportion of individuals who are immune to a given infectious disease above the critical threshold level for herd immunity. Such a programme will not prevent isolated cases of infection but will prevent these from becoming epidemic.

3 Objectives of a vaccine/immunization programme

There is the potential to develop a protective vaccine/immunization programme for all infectious diseases, although some pathogens are considerably more challenging candidates than others. Whether or not such vaccines are developed and deployed is related to the severity and economic impact of the disease on the community as well as the effects upon the individual. Various factors governing the likelihood of an immunization programme being adopted are discussed below, while the principles of immunity, and of the production and quality control of immunological products are discussed in Chapters 9 and 24 respectively.

3.1 Disease severity

The severity of the disease in terms of its morbidity and mortality, the probability of permanent injury to its survivors and the likelihood of infection must be sufficient to warrant the costly development of a vaccine and its subsequent use. Thus, although influenza vaccines are constantly reviewed and stocks maintained, the control of influenza epidemics through vaccination is not recommended. Rather, those groups of individuals, such as elderly people, who are at special risk from the infection, are protected.

Vaccines to be included within national immunization and vaccination programmes should be chosen to reflect the infection risks within that country. Additional immunizations, appropriate for persons travelling abroad, are intended to protect the at-risk individual, but also to prevent importation of the disease into an unprotected home community.

3.2 Vaccine effectivness

Vaccination and immunization programmes seldom confer 100% protection against the target disease. More commonly the degree of protection is 60–95%. In such instances, although individuals receiving treatment have a high probability of becoming immune, virtually all members of a community must be treated in order to reduce the actual proportion of susceptible individuals to below the threshold for epidemic spread of the disease. Antidiphtheria and antitetanus prophylaxes, which utilize toxoids, are among the most efficient immunization programmes, whereas the performance of BCG is highly variable.

3.3 Safety

No medical or therapeutic procedure comes without some risk to the patient, but all possible steps are taken to ensure safety, quality and efficacy of vaccines and immunological products (see Chapter 24). The risks associated with immunization procedures are constantly reviewed and balanced against the risks associated with contracting the disease. In this respect, the incidence of paralytic poliomyelitis in the USA and UK in the late 1990s was low, with the majority of cases being related to vaccine use (vaccine-associated paralytic polio or VAPP). As the worldwide elimination of poliomyelitis approaches, there is debate as to the value of the live (Sabin) vaccine outside endemic areas, and the inactivated polio vaccine (IPV) is now the vaccine of choice in the UK for prophylaxis against paralytic polio.

3.4 Public perceptions

Public confidence in the safety of vaccines and immunization procedures is essential if compliance is to match the needs of the community. The correlation between actual risk and perception of risk is not always reliable, however. In this respect, public concern and anxiety in the mid 1970s over the perceived safety of pertussis vaccine led to a reduction in coverage of the target group from about 80% to 30%. Major epidemics of whooping cough, with over 100 000 notified cases, followed in the late 1970s and early 1980s. By 1992, public confidence had returned and coverage had increased to 92%, with a considerable associated decrease in disease incidence. Similarly, links have been claimed between the incidence of autism in children and the change in the UK from single measles and German measles vaccines to the combined measles, mumps and rubella (MMR) vaccine. Such claims have been proved to be unfounded beyond

reasonable doubt but have nevertheless decreased the uptake of the MMR vaccine and thereby increased the likelihood and magnitude of measles epidemics.

3.5 Cost

Cheap, effective vaccines are an essential component of the global battle against infectious disease. It was estimated that the 1996 costs of the USA childhood vaccination programme, directed against polio, diphtheria, pertussis, tetanus, measles and tuberculosis, was $1 for the vaccines and $14 for the programme costs. The newer vaccines, particularly those that have been genetically engineered, are considerably more expensive, putting the costs beyond the budgets of many developing countries.

3.6 Longevity of immunity

The ideal of any vaccine is to provide lifelong protection of the individual against disease. Immunological memory (Chapter 9) depends on the survival of cloned populations of B- and T-lymphocytes (memory cells). Although these lymphocytes can persist in the body for many decades, the duration of protection varies from one individual to another and depends on the vaccine; commonly ranging between 10 and 20 years. Thus, if the immune system is not boosted, either by natural exposure to the organism or by reimmunization, protective immunity gained in childhood may be lost by the age of 30. Those vaccines that provide only poor protection against disease may have proportionately reduced timespans of effectiveness. Equally, vaccines may be less effective and have a shorter duration when administered to neonates. Yellow fever vaccination, which is highly effective, must therefore be repeated at 10-year intervals, while the typhoid vaccine is only effective for up to 3 years. Whether or not immunization in childhood is boosted at adolescence or in adulthood depends on the relative risks associated with the infection as a function of age and the longevity of immunity conferred by the vaccine.

4 Classes of immunity

The theoretical background that underlies immunity to infection has been discussed in detail in Chapter 9. Immunity to infection may be passively acquired through the receipt of preformed, protective antibodies or it may be actively acquired through an immune response following deliberate or accidental exposure to microorganisms or their component parts. Active, acquired immunity might involve either or both of the humoral and cell-mediated responses.

4.1 Passive (artificially acquired) immunity

Humoral antibodies of the IgG class are able to cross the placenta from mother to fetus. These antibodies will provide passive protection of the newborn against those diseases which involve humoral immunity and to which the mother is immune. In this manner, most newborn infants in the UK will have passive protection against tetanus, but not against tuberculosis. Protection against the latter relies to a large extent on cell-mediated immunity. Secreted (IgA) antibodies are also passed to the gut of newborn, together with the first deliveries of breast milk (colostrum). Such antibodies provide some passive protection against infections of the gastrointestinal tract. Maternally acquired antibodies will react with antigens associated with an infection but also with antigens introduced to the body as part of an immunization programme. Premature immunization, i.e. before degradation of the maternal antibodies, may reduce the potency of an administered vaccine. This aspect of the timing of a course of vaccinations is discussed later.

Administration of preformed antibodies taken from animals, pooled human serum, or human cell lines is often used to treat existing infections (e.g. tetanus, diphtheria) or condition (e.g. venomous snake bite). Pooled serum may also be administered prophylactically, within a slow-release vehicle, for individuals travelling to parts of the world where diseases such as hepatitis A are endemic. Such administrations confer no long-term immunity and may interfere with concurrent vaccination procedures.

4.2 Active (artificially acquired) immunity

Active immunity (Chapter 9) relates to exposure of the immune system to antigenic materials and the subsequent response. Such exposure might be related to an infection or to the multiplication of an attenuated vaccine strain, or it might be associated with the direct introduction of non-viable antigenic material into the body e.g. a non-living or inactivated vaccine. The route of exposure to antigen will influence the nature of the subsequent immune response. Thus, injection of antigen will lead primarily to humoral (IgG, IgM) production, while exposure of epithelial tissues (gut, respiratory tract) will lead to the production of secretory antibodies (IgA, IgE) and to the stimulation of humoral antibody production.

The magnitude and specificity of an immune response depends upon the duration of the exposure to antigen and on its time–concentration profile. During a naturally occurring infection (or the administration of a live, attenuated vaccine), the levels of antigen in the host may be low at the onset and localized to the portal of entry to the host. As the amounts of antigen are small, they will react only with a small, highly defined subgroup of small lymphocytes. These may undergo transformation to produce various antibody classes specific to the antigen and undergo clonal expansion. These immune responses and the progress of the infection may progress simultaneously. With time, microorganisms will produce greater amounts of antigenic materials that will, in turn, react with an increasing number of cloned lymphocytes to produce yet more antibodies. Eventually the antibody levels may be sufficient to eliminate the infecting organism from the host. Antibody levels will then decline, with the net result of this encounter being the clonal expansion of particular small lymphocytes relating to a highly specific 'immunological memory' of the encounter.

This situation should be contrasted with the injection of a killed or non-living vaccine where the amount of antigen introduced is relatively high when compared with the levels present during the initial stages of an infection. In a non-immune animal, the antigens may react not only with those lymphocytes that are capable of producing antibody of high specificity but also with those of a lower specificity. Antibody of both high and lower specificity may react with and remove the residual antigen. The immune response will cease after this initial (primary) challenge. On a subsequent (secondary) challenge (during a course of vaccinations), the antigen will react with residual preformed antibody relating to the first challenge, together with a more specific subgroup of the original cloned lymphocytes. As the number of challenges is increased, the proportion of stimulated lymphocytes that are specific to the antigen rises. After a sufficient number of consecutive challenges the magnitude and specificity of the immune response matches that which would occur during a natural infection with an organism bearing the antigen. This pattern of exposure brings with it certain problems. Firstly, as the introduced immunogen will react preferentially with preformed antibody rather than lymphocytes then sufficient time must elapse between exposures to allow the natural loss of antibody to occur. Secondly, immunity to infection will only be complete after the final challenge with immunogen. Thirdly, low specificity antibody produced during the early exposures to antigen might be capable of cross-reaction with host tissues to produce an adverse response to the vaccine.

5 Types of vaccine

Vaccines may comprise living, attenuated microorganisms, killed microorganisms, or purified bacterial and viral components (component vaccines). Recent innovations include the development of DNA vaccines that encode for the transcription of antigen when introduced directly into host tissues or vaccines that might be delivered nasally by non-pathogenic bacteria (e.g. *Lactococcus lactis*) or otherwise by viral vectors (e.g. adenovirus). Some aspects of these vaccine classes are discussed below.

5.1 Live vaccines

Live, infective microorganisms, attenuated with respect to their pathogenicity but retaining their ability to infect, can be used to confer protective immunity. Two major advantages stem from the use of live vaccines. First, the immunization mimics the course of a natural infection such that only a single exposure is required to render an individual immune. Secondly, the exposure may be mediated through the natural route of infection (e.g. oral), thereby stimulating an immune response that is appropriate to a particular disease (e.g. secretory antibody as a primary defence against poliomyelitis virus in the gut with oral polio vaccine (OPV); see below). Disadvantages associated with the use of live vaccines are also apparent. Live, attenuated vaccines, administered through the natural route of infection, will replicate in the patient and could be transmitted to others. If attenuation is reduced during the replication process, infections might result (see OPV below). A second, major disadvantage, of live vaccines is that the course of their action, and possible side effects, might be affected by the infection and immunological status of the patient.

5.2 Killed and component vaccines

Since these vaccines are unable to evoke a natural infection profile with respect to the release of antigen, they must be administered on a number of occasions. Immunity may not reach optimal levels until the course of immunization is complete and, with the exception of toxin-dominated diseases such as diphtheria and tetanus where the immunogen is a toxoid, are unlikely to match the performance of a live vaccine. The specificity of the immune response generated in the patient may initially be low. This is particularly the case when the vaccine is

composed of a relatively crude cocktail of killed cells, where the immune response is directed only partially towards antigenic components of the pathogen. This increases the possibility of adverse reactions in the patient. Release profiles of these immunogens can be improved through their formulation with adjuvants (Chapters 9 and 24), and the immunogenicity of certain purified bacterial components such as polysaccharides can be improved by their conjugation to a carrier.

5.3 DNA vaccines

A development associated with research into gene therapy has been the use of DNA encoding specific virulence factors of defined pathogens to evoke an immune response. The DNA is introduced directly into tissue cells by means of a transdermal 'gene gun' and is transcribed by the recipient cells. Accordingly, the host responds to the antigenic material produced as though it were an infection. The course of release of the antigen reflects that of a natural infection and, therefore, a highly specific response is invoked. Eventually the introduced DNA is lost from the recipient cells and antigen release ceases. To date, few experimental trials have demonstrated convincing protection in humans, but this remains a promising approach. Protective immunity has been reported in a trial of a human vaccine for bird flu and a West Nile virus vaccine for horses has been approved.

6 Routine immunization against infectious disease

6.1 Poliomyelitis vaccination

Poliovirus, a picornavirus, has three immunologically distinct serotypes (I, II and III) and there may be three phases of the disease. The first is an acute infection of lymphoid tissues associated with the gastrointestinal tract (Peyer's patches), during which time the virus can be found in the throat and in faeces. The second phase is characterized by an invasion of the bloodstream, and in the third phase the virus migrates from the bloodstream into the meninges. Infections range in severity from asymptomatic (the majority of cases) to paralytic poliomyelitis which may cause permanent neurological damage and muscle paralysis. Paralytic poliomyelitis is a major illness, but occurs in only 0.1–2% of cases. It is characterized by the destruction of large nerve cells in the anterior horn of the brain, resulting in varying degrees of paralysis, and unvaccinated adults are at greater risk of paralytic infection than children. The infection is transmitted by the faecal–oral route.

Polio is the only disease, at present, for which both live and killed vaccines compete. Since the introduction of the killed virus (Salk) in 1956 and the live, attenuated virus (Sabin) in 1962 there has been a remarkable decline in the incidence of poliomyelitis (Figure 10.1). The inactivated polio (Salk) vaccine (IPV) contains formalin-killed poliovirus of all three serotypes. On injection, the vaccine stimulates the production of antibodies of the IgM and IgG class that neutralize the virus in the second stage of infection. A course of three injections at monthly intervals produces long-lasting immunity to all three poliovirus types. The live, oral polio (Sabin) vaccine (OPV) is now less commonly used. Advantages over the IPV vaccine include lower costs and easier administration. OPV contains attenuated poliovirus of each of the three types and is administered, as a liquid, onto the tongue. The vaccine strains infect the gastrointestinal mucosa and oropharynx, promoting the common immune response, and involving both humoral and secretory antibodies. IgA, secreted within the gut epithelium, provides local resistance to the first stages of poliomyelitis infection. OPV therefore provides protection at an earlier stage of the infection than does IPV. Infection of epithelial cells with one strain of enterovirus, however, may inhibit simultaneous infection by related strains. At least three administrations of OPV are therefore given, with each dose conferring immunity to one of the vaccine serotypes. These doses must be separated by a period of at least 1 month in order to allow the previous infection to lapse. Booster vaccinations are also provided to cover the eventuality that some other enterovirus infection, present at the time of vaccination, had reduced the response to the vaccine strains. Faecal excretion of vaccine virus will occur and may last for up to 6 weeks after administration. Such released virus may spread to close contacts and infect or (re)immunize them. Vaccine-associated poliomyelitis may occur through reversion of the attenuated strains to the virulent wild type, particularly with types II and III and has been estimated to occur once per 4 million doses. Since the introduction of OPV, notifications of paralytic poliomyelitis in the UK have markedly dropped. However, from 1985 to 1995, 19 of the 28 notified cases of paralytic poliomyelitis were associated with reverted vaccine strains (14 recipients, 5 contacts). As the risk of natural infections with poliomyelitis within developed countries has now diminished markedly, the live vaccine strains present the greatest risk. Consequently, OPV has now

been replaced with IPV as the polio vaccine of choice in the UK and USA.

6.2 Measles, mumps and rubella vaccination (MMR)

Measles, mumps and rubella (German measles) are infectious diseases with respiratory routes of transmission and infection and each is caused by distinct members of the paramyxovirus group. Each virus only has one serotype. Although the primary multiplication sites of these viruses are within the respiratory tract, the diseases are associated with viral multiplication elsewhere in the host.

6.2.1 Measles

Measles is a severe, acute, highly contagious infection that frequently occurs in epidemic form. After multiplication within the respiratory tract, the virus is transported throughout the body, particularly to the skin where a characteristic maculopapular rash develops. Complications of the disease can occur, including measles encephalitis, which can cause permanent neurological injury and death, and subacute, sclerosing panencephalitis (SSPE), which is a rare form of progressive encephalitis associated with persistence of the measles virus that primarily affects children and young adults. It is incurable, although early treatment can slow progression or improve the remission rate.

A live, attenuated vaccine strain of measles was introduced in the USA in 1962 and to the UK in 1968. A single injection produces high-level immunity in over 95% of recipients. Moreover, as the vaccine induces immunity more rapidly than the natural infection, it may be used to control the impact of measles outbreaks. The measles virus cannot survive outside an infected host. Widespread use of the vaccine therefore has the potential, as with smallpox, of eliminating the disease worldwide. Mass immunization has markedly reduced the incidence of measles, although a 15-fold increase in the incidence was noted in the USA between 1989 and 1991 because of poor compliance with the vaccine.

6.2.2 Mumps

Mumps virus infects the parotid glands to cause swelling and a general viraemia. Complications include pancreatitis, meningitis and orchitis, the last occasionally leading to male sterility. Infections can also cause permanent unilateral deafness. In the absence of vaccination, infection occurs in more than 90% of individuals by age 15 years. A live, attenuated mumps vaccine has been available since 1967 and has been part of the childhood vaccination programme in the UK since 1988 when it was included as part of the MMR triple vaccine (see below).

6.2.3 Rubella

Rubella is a mild, often subclinical infection that is common among children aged between 4 and 9 years. Infection during the first trimester of pregnancy brings with it a major risk of abortion or congenital deformity in the fetus (congenital rubella syndrome, CRS). Rubella immunization was introduced to the UK in 1970 for prepubertal females and non-immune women intending to start families. The vaccine utilizes a live, cold-adapted strain of the virus. The major disadvantage of the vaccine is that, as with the wild type, the fetus can be infected. While there have been no reports of CRS associated with use of the vaccine, the possible risk makes it imperative that women do not become pregnant within 1 month of vaccination. Prepubertal females were immunized to extend the period of immunity through the childbearing years. Until 1988 boys were not routinely protected against rubella. Their susceptibility to the virus was thought to maintain the natural prevalence of the disease in the community and thereby reinforce the vaccine-induced immunity in vaccinated, adult females. This proved not to be the case and in fact cases of CRS could be related to incidence of the disease in younger children within the family. Rubella vaccine is now given to both sexes at the age of about 13 months as part of the MMR programme (below).

6.2.4 MMR vaccine

MMR vaccine was introduced to the UK in 1988 for young children of both sexes, replacing the single measles vaccine. It consists of a single dose of a lyophilized preparation of live attenuated strains of the measles, mumps and rubella viruses. The MMR vaccine had previously been deployed in the USA and Scandinavia for a significant number of years without any indication of increased adverse reaction or of decreased seroconversion over separate administration of the component parts. Immunization results in seroconversion to all three viruses in >95% of recipients. For maximum effect, MMR vaccine is recommended for children of both sexes aged 12–15 months but can also be given to non-immune adults. From October 1996 a second dose of MMR was recommended for children aged approximately 4 years in order to prevent the reaccumulation of sufficient susceptible children to sustain future epidemics.

In 1998 a research paper attempted to associate an increase in autism to the introduction of the triple

vaccine. This led to a decreased public confidence in the vaccine. Detailed examination of the data and also the results of several clinical studies have indicated that there is no association between use of the triple vaccine and autism. This is backed up by over 20 years of successful deployment of the vaccine outside of the UK. Currently, much effort is being made to restore confidence in the vaccine in order to avoid the lack of compliance leading to the occurrence of measles epidemics.

6.3 Tuberculosis

Tuberculosis (TB) is a major cause of death and morbidity worldwide, particularly where poverty, malnutrition and poor housing prevail. Human infection is acquired by inhalation of *Mycobacterium tuberculosis* or *M. bovis.* Tuberculosis is primarily a disease of the lungs, causing chronic infection of the lower respiratory tract, but may spread to other sites or proceed to a generalized infection (miliary tuberculosis). Active disease can result either from a primary infection or from a subsequent reactivation of a quiescent infection. Following inhalation, the mycobacteria are taken up by alveolar macrophages where they survive and multiply. Circulating macrophages and lymphocytes, attracted to the site, carry the organism to local lymph nodes where a cell-mediated immune response is triggered. The host, unable to eliminate the pathogen, contains the bacteria within small granulomas or tubercles. If high numbers of mycobacteria are present then the cellular responses can result in tissue necrosis. The tubercles contain viable pathogens that may persist for the remaining life of the host. Reactivation of the healed primary lesions is thought to account for over two-thirds of all newly reported cases of the disease.

The incidence of TB in the UK declined 10-fold between 1948 and 1992, to just over 5000 new cases being notified each year. Those most at-risk include pubescent children, health service staff and individuals intending to stay for more than 1 month in countries where TB is endemic. However, since 1994 there has been a gradual increase in the number of notified cases in England and Wales to approximately 7000 new cases per year which can be associated with age group (20–45-year-olds more susceptible), sex (males slightly more susceptible), geographical region and ethnicity. The rise in notifications can be attributed to a number of different factors, including increased immigration from countries where TB is more prevalent, crowded conditions (e.g. prisons, hostels, wards, etc.), a greater number of susceptible individuals now in society (e.g. substance abusers, immunocompromised individuals) and limited training for GPs in recognizing the symptoms of TB. Fortunately this latter issue has now been addressed, along with the re-emergence of TB research programmes.

A live vaccine is required to elicit protection against TB and both antibody and cell-mediated immunity are required for protective immunity. Vaccination with BCG (bacille Calmette–Guérin), derived from an attenuated *M. bovis* strain, is commonly used in countries where TB is endemic. The vaccine was introduced in the UK in 1953. Efficacy in the UK has been shown to be over 70%, with protection lasting at least 15 years. In other countries, where the general state of health and well-being of the population is less than in the developed world, the efficacy of the vaccine has been shown to be markedly lower than this.

Because of the risks of adverse reaction to the vaccine by individuals who have already been exposed to the disease, a sensitivity test must be carried out before immunization with BCG. A Mantoux skin test assesses an individual's sensitivity to a purified protein derivative (PPD) prepared from heat-treated antigens (tuberculin) extracted from *M. tuberculosis.* A positive test implies past infection or past, successful immunization. Those with strongly positive tests may have active disease and should be referred to a chest clinic. However, many people with active TB, especially disseminated TB, seroconvert from a positive to a negative skin test. Results of the skin test must therefore be interpreted with care.

Much debate surrounds the use of BCG vaccine, a matter of some importance, considering that TB kills around 3 million people annually and that drug-resistant strains have emerged. Although the vaccine has demonstrated some efficacy in preventing childhood TB, it has little prophylactic effect against postprimary TB in those already infected. One solution is to bring forward the BCG immunization to include neonates. Immunization at 2–4 weeks of age will ensure that immunization precedes infection, and also negates the requirement for a skin test. Passive, acquired maternal antibody to TB is unlikely to interfere with the effectiveness of the immunization as immunity relates primarily to a cell-mediated response. Alternative strategies involve improvement of the vaccine, possibly through the introduction into the BCG strain of genes that encode protective antigens of *M. tuberculosis*.

The current UK policy (introduced in September 2005) is to reserve the BCG vaccine for those considered to be at highest risk based on location or familial links to TB endemic areas. As such, this targeted approach will

seek to immunize all infants in areas where the incidence of TB is 40 or more per 100 000; infants with at least one parent or grandparent who was born in a country with a TB incidence of 40 or more per 100 000; previously unvaccinated new immigrants from high-prevalence countries for TB; those who have lived in the same household or had prolonged, close contact with someone with TB; or at-risk workers (doctors, nurses, social workers, etc.).

6.4 Diphtheria, tetanus and acellular pertussis (DTaP) immunization

Immunization against these three unrelated diseases is considered together because the vaccines are non-living and are often co-administered as a triple vaccine as part of the childhood vaccination programme.

6.4.1 Diphtheria

This is an acute, non-invasive infectious disease associated with the upper respiratory tract (Chapter 7). The incubation period is 2–5 days although the disease remains communicable for up to 4 weeks. A low molecular weight toxin is produced which affects the myocardium, nervous and adrenal tissues. Death results in 3–5% of infected children. Diphtheria immunization stimulates the production of an antitoxin which protects against the disease but not against infection/colonization of the respiratory tract. The immunogen is a toxoid, prepared by formaldehyde treatment of the purified toxin (Chapter 24) and administered while adsorbed to an adjuvant, usually aluminium phosphate or aluminium hydroxide. The primary course of diphtheria prophylaxis consists of three doses starting at 2 months of age and separated by an interval of at least 1 month. The immune status of adults may be determined by administration of Schick test toxin, which is essentially a diluted form of the vaccine.

6.4.2 Tetanus

Tetanus results from the production of a toxin by germinating spores and vegetative cells of *Clostridium tetani* that can potentially infect deep wounds. The organism, which may be introduced into the wound, grows anaerobically at such sites. The toxin is adsorbed into nerve cells and has a profound effect on nerve synapses resulting in spastic paralysis in affected individuals (Chapter 7). Mortality rates are highest in individuals over 60 years of age and the unvaccinated. Tetanus immunization employs a toxoid and protects by stimulating the production of antitoxin. This antitoxin will neutralize the toxin as the organisms release it and before it can be adsorbed into nerves. As the toxin is produced only slowly after infection, the vaccine, which acts rapidly, may be used prophylactically in non-immunized individuals who have recently suffered a high-risk injury. The toxoid, as with diphtheria toxoid, is formed by reaction with formaldehyde and is adsorbed onto an inorganic adjuvant. The primary course of tetanus vaccination consists of three doses starting at 2 months of age and is separated by an interval of at least 1 month.

6.4.3 Pertussis (whooping cough)

Whooping cough is caused by the non-invasive respiratory pathogen *Bordetella pertussis* (Chapter 7). This disease may be complicated by bronchopneumonia, or by repeated post-tussis vomiting leading to weight loss and to cerebral hypoxia associated with a risk of brain damage. Until the mid 1970s the mortality from whooping cough was about 1 per 1000 notified cases, with a higher rate for infants under 1 year of age. A full course of vaccine now consists of highly purified selected components of the *Bordetella pertussis* organism (i.e. acellular pertussis) which are treated with formaldehyde or glutaraldehyde and absorbed on to aluminium phosphate or aluminium hydroxide adjuvants. This vaccine gives considerably lower incidence of local and systemic reactions in comparison to the whole-cell pertussis vaccine that preceded it and gives comparable protection (>80%). The primary course of pertussis prophylaxis consists of three doses starting at 2 months of age and separated by an interval of at least 1 month.

6.4.4 DTaP vaccine combinations and administration

The primary course of DTaP protection consists of three doses of a combined vaccine, each dose separated by at least 1 month and commencing not earlier than 2 months of age. In such combinations, the pertussis component of the vaccine acts as an additional adjuvant for the toxoid elements. Tetanus and diphtheria vaccines are also available in a combined tetanus/diphtheria/inactivated polio vaccine (Td/IPV) vaccine.

The primary course of pertussis vaccination is considered sufficient to confer lifelong protection, especially as the mortality associated with disease declines markedly after infancy. The risks associated with tetanus and diphtheria infection however persist throughout life. The Td/IPV vaccination is therefore repeated before school entry, at 4–5 years of age, and once again at puberty.

6.5 Immunization against bacteria associated with meningitis

6.5.1 Meningococcal immunization

Meningococcus *(Neisseria meningitidis)* is a bacterium that exclusively colonizes and/or infects humans; there is no animal reservoir. It is present as part of the normal microbiota of the pharynx in approximately 10% of individuals but can rarely spread through the bloodstream and to the brain through poorly understood mechanisms, causing meningitis and septicaemia. These are life-threatening, systemic infections; overall mortality from meningococcal disease is approximately 10%, assuming symptoms are recognized and treatment is commenced without delay, but rises considerably if there are delays. Diagnosis is therefore considered a medical emergency. At least 12 subtypes or serogroups of meningococcus have been identified, but groups B and C account for the majority of cases in Europe and the Americas. In the UK, group B accounts for approximately two-thirds of reported cases with group C accounting for the remaining third. *Neisseria meningitidis* group A does not normally cause disease in the UK but is an endemic cause of meningitis in other parts of the world, particularly sub-Saharan Africa in an area between Senegal (West Africa) to Ethiopia (East) that has been termed the 'meningitis belt'. It is believed that reasons for the hyperendemic incidence of group A meningococcal meningitis in this part of the world include high incidence of upper respiratory tract infections during the dry (dusty) season, combined with overcrowding, migration and pilgrimages.

Whilst group C meningitis is most common in the under 1-year-old group in the UK, mortality is highest in adolescents. Although meningitis accounts for the majority of invasive meningococcal disease, in 15–20% of cases septicaemia predominates and is associated with significantly higher mortality. There is currently no vaccine available for group B meningococcus but vaccines are available for groups A and C. As with the Hib vaccine (see below), the preparations are intended to invoke protective immunity towards the polysaccharide component of the bacterium. Early vaccines, composed of purified polysaccharide, worked in adults but had poor efficacy in infants—the most at-risk group. The new MenC conjugate vaccine comprises capsular polysaccharide components conjugated to a carrier protein (usually diphtheria or tetanus toxoid). This vaccine is effective in the very young and is therefore suitable for protecting infants. The vaccine is normally administered along with DTaP and Hib at 3, 4 and 12 months and a single dose is sufficient to immunize individuals over 12 months of age; it has also been used to provide prophylaxis for teenagers, adolescents and young adults. Group A vaccine is available for those travelling to areas of the world where the infection is epidemic.

6.5.2 *Haemophilus influenzae* type b (Hib) immunization

Haemophilus influenzae can cause infections ranging from bronchitis and otitis media to life-threatening, invasive disease (meningitis and bacteraemia). Invasive infections, which are most common in young children, are normally caused by encapsulated strains of the bacterium that can be serologically differentiated into six typeable capsular serotypes (a–f). Before the introduction of vaccination, *H. influenzae* type b (Hib) was the most prevalent of these in invasive disease. Non-invasive haemophilus disease is most often caused by non-encapsulated strains that are not amenable to typing based on capsular serology. Although the most common form of invasive Hib disease is meningitis, accounting for 60% of cases, Hib can also cause other infections, including pneumonia and pericarditis.

The fatality rate for treated Hib meningitis infections is approximately 5% and complications include deafness and intellectual impairment (in *c.*10% of cases). Hib often forms part of the normal microbiota of the nasopharynx in healthy individuals and the frequency of carriage before the Hib vaccine was introduced was approximately 4 in every 100 for preschool children. Carriage of this bacterium is now rare because of the effectiveness of the Hib vaccination. Hib meningitis is rare in children under 3 months and peaks in its incidence at around 10–11 months of age; infection is uncommon after 4 years of age. Before the introduction of Hib vaccination the incidence of the disease in the UK was estimated at 34 per 100 000.

The vaccine utilizes purified preparations of the polysaccharide capsule of the major serotypes of the bacterium associated with disease. Polysaccharides are poorly immunogenic and must be conjugated onto a protein carrier (diphtheria or tetanus toxoids) to enhance their efficacy. The Hib vaccine is given as part of a combined product of *H. influenzae* type b, diphtheria, tetanus, acellular pertussis and inactivated polio vaccine (DTaP/IPV/Hib), or as the Hib/MenC conjugate vaccine.

6.5.3 Pneumococcal vaccination

Streptococcus pneumoniae (pneumococcus) is an encapsulated Gram-positive coccus. As with meningococcus

and Hib, the capsule is an important virulence factor for this bacterium (non-capsulated strains are normally avirulent). Many different capsular types have been characterized but approximately 66% of the serious infections in adults and 80% of invasive pneumococcal infections in children are caused by about 10 capsular types. Pneumococci are often part of the normal microbiota of the nasal cavity and associated tissues but commensal strains are often avirulent. Conversely, invasive infections (i.e. meningitis and septicaemia) are often caused by strains not considered to be normal commensals. *S. pneumoniae* is a versatile pathogen which can cause sinusitis or otitis media (infections of the sinuses or middle ear). The bacterium may also cause deep lung infections (pneumonia), which accounts for its species name, and is also capable of causing systemic infections including bacteraemic pneumonia, bacteraemia and meningitis. The incidence of infection by pneumococci is highest in the winter, and transmission by aerosols or direct contact with respiratory secretions is believed to require either frequent or prolonged close contact. Two distinct vaccines have been developed to provide protection against this bacterium: the pneumococcal polysaccharide vaccine (PPV) which contains purified capsular polysaccharide from 23 capsular types of bacterium and the pneumococcal conjugate vaccine (PCV) which comprises capsular polysaccharides from 7 common capsular types. Importantly, PCV is conjugated to protein in a manner similar to the *Haemophilus influenzae* type b (Hib) and MenC vaccines. While PPV is an effective vaccine in adults, the effectiveness of pneumococcal prophylaxis is considerably improved in children by protein conjugation; PCV is immunogenic in children and the childhood vaccination schedule recommends that doses be given at 2 and 4 months of age with a booster at 13 months. The PPV vaccine is recommended for adults over 65 years and at-risk groups aged 2 years or over. It will provide additional prophylaxis to individuals who have already received the PCV vaccine because it protects from additional serotypes.

6.6 Human papillomavirus (HPV) vaccination

Human papillomaviruses (HPV) are a group of viruses that infect squamous epithelia, including the skin and mucosal surfaces of the upper respiratory and anogenital tracts. Approximately 100 types of HPV have been identified, of which around 40 infect the genital tract. The majority of genital infections are asymptomatic but in some cases may be associated with genital warts and (the reason for the development of the HPV vaccine), cervical cancer. The time between infection and development of cancers may range from 12 months to over 10 years. It is important to note that HPV is associated with genital and anal cancers in both men and women and also with cancers of the mouth and throat. Importantly, not all types of HPV are carcinogenic; the risk of cancer development associated with different strains is variable and HPV viruses that cause warts (a common sexually transmitted infection) may be low risk for carcinogenicity and in some cases strains are not considered carcinogenic. Genital HPV infections are transmitted primarily through sexual intercourse with infected individuals and the use of condoms reduces the risk of sexual transmission. HPV may also be transmitted vertically from mother to child. Persistent HPV infection with HPV 16 and HPV 18 (high-risk strains) is associated with the majority of cervical cancers. There are however, strains other than HPV 16 and HPV 18 that are carcinogenic.

The HPV vaccine comprises recombinant subunits expressed in yeast or in cells of insect origin such that the vaccine contains non-infectious, virus-like particles. The current UK vaccine, Cervarix, affords protection against HPV 16 and HPV 18 and is very effective at preventing precancerous lesions associated with these virus strains for at least 6 years, probably longer. Since the main aim of the HPV vaccine programme is to reduce the incidence of cervical cancer, the vaccine is currently administered to females (not males) between the age of 12 and 13 with a catch-up programme for older females up to the age of 18.

7 The UK routine childhood immunization programme

The timing of the various components of the childhood vaccination programme is subject to continual review. In the 1960s, the primary course of DTP vaccination consisted of three doses given at 3, 6 and 12 months of age, together with OPV. This separation gave adequate time for the levels of induced antibody to decline between successive doses of the vaccines. Current recommendations (Table 10.1) accelerate the vaccination programme with no reductions in its efficacy. Thus, the MMR vaccination has replaced separate measles and rubella prophylaxis and BCG vaccination may now be given at birth, but only for infants living in areas of the UK where the annual incidence of TB is 40 per 100 000 or greater and those with familial or other links to high-risk countries.

Table 10.1 Schedule for the UK's routine childhood immunizations (2009)

Vaccines given	Administration	Timing
Diphtheria, tetanus, pertussis, polio and Hib (DTaP/IPV/Hib)	One injection	2 months old
Pneumococcal conjugate vaccine (PCV)	One injection	
Diphtheria, tetanus, pertussis, polio and Hib (DTaP/IPV/Hib)	One injection	3 months old
Meningococcal MenC	One injection	
Diphtheria, tetanus, pertussis, polio and Hib (DTaP/IPV/Hib)	One injection	4 months old
Meningococcal (MenC)	One injection	
Pneumococcal conjugate vaccine (PCV)	One injection	
Hib/MenC	One injection	12 months old
Measles, mumps and rubella (MMR)	One injection	*c.*13 months old
Pneumococcal conjugate vaccine (PCV)	One injection	
Diphtheria, tetanus, pertussis and polio (DTaP/IPV or (Td/IPV)	One injection	3 years 4 months to 5 years old
Measles, mumps and rubella (MMR)	One injection	
Human papilloma virus (HPV)	Three injections; over a *c.*6 month period	12–13 years old (females only)
Tetanus, diphtheria (Td/IPV)	One injection	13–18 years old

Adapted from Salisbury *et al.* (2006).
The BCG vaccine (for TB prophylaxis) is now administered only to children considered to be at increased risk of developing severe disease and/or of exposure to TB infection.
The table is intended as a general guide to the vaccines used. **Policies can and do change. For definitive information, see the UK Department of Health website:** (http://www.dh.gov.uk/prod_consum_dh/groups/dh_digitalassets/@dh/@en/documents/digitalasset/dh_120973.pdf) Accessed 5 November 2010.

DTaP vaccination occurs at 2, 3 and 4 months to coincide with administration of Hib and IPV. It is imperative that as many individuals as possible benefit from the vaccination programme. Fewer visits to the doctor's surgery translate into improved patient compliance and less likelihood of epidemic spread of the diseases in question. The current recommendations minimize the number of separate visits to the clinic while attempting to maximize the protection generated.

8 Immunization of special risk groups

While not recommended for routine administration, vaccines additional to those represented in the childhood programme are available for individuals in special risk categories. These categories relate to occupational risks or risks associated with travel abroad. Such immunization protocols include those directed against cholera, typhoid, meningitis (group A), anthrax, hepatitis A and B, influenza, Japanese encephalitis, rabies, tick-borne encephalitis and yellow fever.

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(2006 and earlier versions); an authoritative source of information about the composition and schedules for current UK vaccines and vaccination policies.

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Part 3 Prescribing therapeutics

11 Antibiotics and synthetic antimicrobial agents: their properties and uses

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1 Antibiotic development, past and present

An antibiotic was originally defined in the 1940s as a substance produced by one microorganism which, in low concentrations, inhibited the growth of other microorganisms. This definition necessarily meant that an antibiotic was a naturally occurring substance, a microbial metabolite, so any antimicrobial agent that was manufactured by chemical synthesis fell outside the definition. The use of the term has changed over the years, both because an increasing number of synthetic analogues or derivatives of 'true' antibiotics have come on to the market, and because several agents that bear little or no resemblance to natural microbial metabolites have also been developed which, for all practical purposes, mimic traditional antibiotics in their potency, low toxicity and, crucially, systemic action. Consequently, drugs like trimethoprim, metronidazole, imidazole derivatives and

Hugo and Russell's Pharmaceutical Microbiology, Eighth Edition. Edited by Stephen P. Denyer, Norman Hodges, Sean P. Gorman, Brendan F. Gilmore.

fluoroquinolones are commonly referred to as antibiotics, and the term 'antibiotic' will be used in this chapter to include these and other agents with systemic antimicrobial activity.

By this broader definition, sulphonamides, introduced into therapy in the 1930s, were the first significant antibiotics and predated penicillin by about 10 years. Although Fleming received the credit for discovering penicillin in 1929, much of the development work was undertaken at Oxford University over the next 10 years, and the onset of the Second World War was the impetus for the American pharmaceutical industry to convert the discovery into a medicine that could be manufactured on a large scale. Benzylpenicillin was the original antibiotic in this class, but in the late 1940s and early 1950s it was joined both by other penicillins and by several other classes of antibiotic that are still in widespread use today, such as tetracyclines, macrolides and aminoglycosides. All of these were 'true' antibiotics in the traditional sense, i.e. they were extracted from large-volume cultures of *Streptomyces* bacteria or fungi (in the case of penicillins), but the 1960s saw the advent of semisynthetic antibiotics—penicillins particularly—in which the naturally occurring substance was extracted from the microbial culture, purified and then structurally modified by conventional chemical means. Since then, an increasing number of antibiotics have been totally synthesized.

Although bacterial resistance was a problem that was recognized from the start of the antibiotic era, the international pharmaceutical industry developed new antibiotics steadily throughout the period 1950–1970, so that new drugs regularly became available to replace those to which resistance developed; this ability to keep ahead of the problem lead to a degree of complacency and a belief that the industry would maintain its supremacy indefinitely. The naivety of this assumption became steadily apparent during the remaining years of the century as antibiotic resistance became a major problem and hospital 'superbugs' like MRSA (meticillin-resistant *Staphylococcus aureus*) and 'C. diff' (*Clostridium difficile*) rose to prominence, whilst at the same time the industry diverted research resources away from antibiotics.

Most of the antibiotics developed during the period 1970–2000 were structural modifications of existing ones, and it was not until the new millennium that genuinely new antibiotics like linezolid and quinupristin/dalfopristin (Synercid) came into use. Unfortunately, though, despite the need for new drugs to treat the superbugs mentioned above and others that have more recently come into prominence, such as multiply resistant tuberculosis (MRTB) and vancomycin-resistant enterococci (VRE), the short duration of therapy and the likelihood of eventual resistance and falling sales mean that the commercial incentive to develop new antibiotics is still much lower than that for drugs treating chronic diseases like diabetes or hypertension, or 'lifestyle' problems like obesity. Consequently, the supply of new antibiotics is likely to remain limited, and the use and distribution of those that are developed will be carefully managed to avoid their indiscriminate use which predisposes to resistance development (see Chapter 15 on antimicrobial stewardship). The effects of short durations of therapy (typically 5–10 days for many infections), antimicrobial stewardship policies limiting their prescribing, relatively short patent life, and the likelihood of some degree of resistance eventually developing anyway, all combine to make modern antibiotics very expensive drugs. This trend of escalating cost is starkly illustrated by comparing the UK price differential of more than 700-fold between trimethoprim (from the 1960s) and linezolid (marketed in 2000) (based on a single day's treatment using current *British National Formulary* prices).

1.1 Antibiotic usage

Despite the fact that few new classes of antibiotics have been developed in recent years, there are, nevertheless, a large number of different antibiotics in current use. Some of these are only available in particular countries; and even within a single country the list of available antibiotics changes year by year as new products replace old ones that are withdrawn for commercial reasons or as a result of toxicity concerns. Currently in the UK there are approximately 70 antibacterial antibiotics, 20 antifungals and 40 antiviral agents on the market, and numbers are similar in other countries, so it is not the intention in this chapter to describe, or necessarily even to mention, all of these drugs. Instead, the approach will be to consider the important constituents of the major antibiotic classes and refer the reader to more detailed sources for information about some of the less frequently used agents.

The various classes of antibacterial antibiotics will be considered in their order of importance in terms of usage, which will be taken to mean the frequency with which they are prescribed. Surprisingly perhaps, this does not necessarily coincide with their rank order in terms of the amount manufactured by the pharmaceutical industry

Table 11.1 Relative frequency of prescribing of antibiotics in the UK

β-Lactams (total)	7.582
Penicillins	5.854
Penicillins with β-lactamase inhibitors	0.967
Cephalosporins	0.761
Monobactams and carbapenems	<0.001
Tetracyclines	3.352
Macrolides	2.226
Trimethoprim	1.042
Quinolones	0.477
Sulphonamides	0.034
Aminoglycosides	0.005
Glycopeptides	0.001
Chloramphenicol	<0.001
Others	0.246

UK antibiotic use in defined daily doses per 1000 inhabitants per day based on ambulatory outpatients. Based on data from the European Surveillance of Antimicrobial Consumption project: http://www.esac.ua.ac.be/esac_service/applet/eidb.html

each year, because a significant fraction of the annual production is used in animal feed supplements. According to one estimate, the amount used in this way in the USA has been as much as eight times that used to treat human infections.

Table 11.1 shows the relative frequency of prescribing of the major antibiotic classes as determined by recent data from an ongoing Europe-wide survey of hospital outpatient prescriptions. The relative numbers would be likely to change somewhat if the survey were to be conducted on inpatient prescriptions or on those issued by community medical practitioners, but some general trends are clear. β-Lactam antibiotics are, by far, the most frequently prescribed class, and of these, prescriptions for penicillins substantially outnumber those for cephalosporins. Trimethoprim features strongly, and as an individual drug would rank very highly even in comparison to penicillins given that the value of 5.854 represents total prescriptions for nearly 10 different penicillins (see section 2 below). Antibiotics that are available primarily as parenteral products, e.g. aminoglycosides and glycopeptides, are much less likely to be prescribed for outpatients than for inpatients because of the problems of administration, so they appear low in the rank order on this particular survey. It is worth emphasizing that this is part of a European survey, and the popularity of particular antibiotics and, consequently, the rank order of the table, is likely to vary from one country to another.

2 β-Lactam antibiotics

For 20 years after their introduction, the penicillins were the only category of β-lactam antibiotics. They were joined, in the mid 1960s, by the cephalosporins, and towards the end of the 20th century by carbapenems and monobactams. These antibiotics all have the same mechanism of antibacterial action and all possess the β-lactam ring as an integral part of their structure, but they differ widely in other characteristics.

2.1 Penicillins

The penicillins (general structure shown in Figure 11.1A) may be considered as being of the following types:

- *Naturally occurring.* For example, those produced by fermentation of moulds such as *Penicillium notatum* and *P. chrysogenum*. The most important examples are benzylpenicillin (penicillin G) and phenoxymethylpenicillin (penicillin V).
- *Semisynthetic.* In 1959, scientists at Beecham Research Laboratories succeeded in isolating the penicillin 'nucleus', 6-aminopenicillanic acid (6-APA; Figure 11.1A: R represents H). During the commercial production of benzylpenicillin, phenylacetic (phenylethanoic) acid ($C_6H_5.CH_2.COOH$) is added to the medium in which the *Penicillium* mould is growing. This substance is a precursor of the side chain (R; see Figure 11.2) in benzylpenicillin. Growth of the organism in the absence of phenylacetic acid led to the isolation of 6-APA; this has a different R_F value from benzylpenicillin, which allowed it to be detected chromatographically.

A second method of producing 6-APA came with the discovery that certain microorganisms produce enzymes, penicillin amylases (amylases), which catalyse the removal of the side chain from benzylpenicillin (Figure 11.1B). Acylation of 6-APA with appropriate substances results in new penicillins being produced which differ only in the nature of the side chain (Table 11.2; Figure 11.2). Some of these penicillins have considerable activity against Gram-negative as well as Gram-positive bacteria,

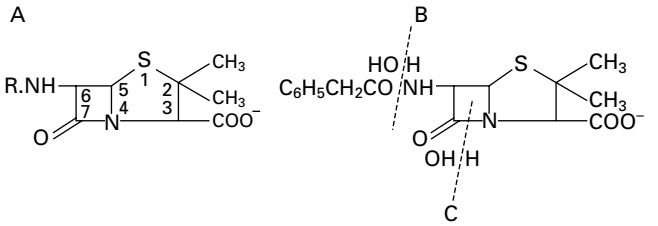


Figure 11.1 (A) General structure of penicillins. (B) Removal of the side chain from benzylpenicillin. (C) Site of action of β-lactamases.

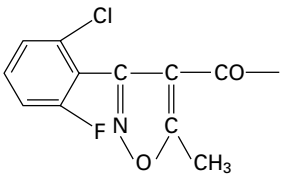
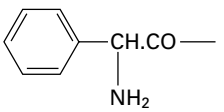
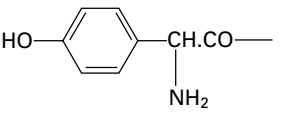
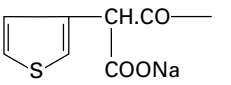
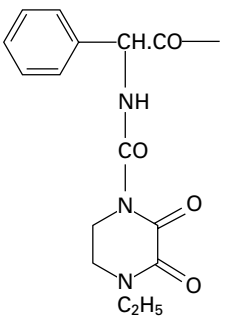
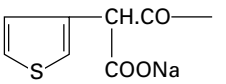
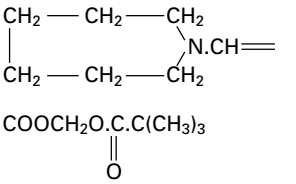
Drug	R	Drug	R	Drug	R
1	$C_6H_5CH_2CO$—	2	$C_6H_5OCH_2CO$—	3	
4		5		6	
7		8		9	

Figure 11.2 Examples of the side chain R in various penicillins (the numbers 1–9 correspond to those in Table 11.2). Number 8 (temocillin) has a methoxy (-OCH3) group at position 6α. Number 9 (pivmecillinam) is a 6-β-amidinopenicillanic acid.

and are thus broad-spectrum antibiotics. Pharmacokinetic properties may also be altered.

The sodium and potassium salts are very soluble in water, but they are hydrolysed in solution at a temperature-dependent rate to the corresponding penicilloic acid (Figure 11.3A) which is not antibacterial. Penicilloic acid is produced at alkaline pH or (via penicillenic acid; Figure 11.3B) at neutral pH, but at acid pH a molecular rearrangement occurs, giving penillic acid (Figure 11.3C). Susceptibility to hydrolysis means that penicillins cannot be formulated as aqueous products, so oral syrups and mixtures must be manufactured as dry granules for resuspension in water, and injections freeze dried in vials or ampoules. Typically, aqueous solutions of penicillins lose 10% or more of their activity in 24 hours at room temperature. Instability in acid medium logically precludes oral administration, since the antibiotic may be destroyed in the stomach; for example at pH 1.3 and 35 °C benzylpenicillin has a half-life of less than 5 minutes and is therefore not administered orally, whereas ampicillin, with a half-life of 600 minutes, is obviously suitable for oral use. Benzylpenicillin is also rapidly excreted, but this can be overcome by the use of sparingly soluble salts (benzathine, benethamine and procaine) which slowly release penicillin into the circulation over a period of time, thus giving a continuous high concentration in the blood.

Table 11.2 Properties of the common penicillins

Penicillin	Oral activity	Activity against			Stability towards β-lactamases from		Doses/day	Combined with β-lactamase inhibitor
		Gram +ve	Gram –ve[a]	*Ps. aeruginosa*	*Staph. aureus*	Gram –ve		
1 Benzylpenicillin	–	+	–	–	–	–	4	
2 Phenoxymethylpenicillin	+	+	–	–	–	–	4	
3 Flucloxacillin	+	+	–	–	+	+	4	
4 Ampicillin	+	+	+	–	–	–	4	Sulbactam (not UK)
5 Amoxicillin	+	+	+	–	–	–	3	Clavulanic acid
6 Ticarcillin	–	±	+	+	–	±	3–4	Clavulanic acid
7 Piperacillin	–	+	+	+	–	–	4	Tazobactam
8 Temocillin	–	–	+	–	+	+	2	
9 Pivmecillinam	+	–	+	–	NR	±	3–4	

+, applicable; –, inapplicable; NR, not relevant: pivmecillinam has no effect on Gram-positive bacteria; ±, variable result depending on species and strain.
[a]Except *Ps. aeruginosa*. All penicillins show some degree of activity against Gram-negative cocci.
Notes
1 For additional information on β-lactamase-mediated resistance, see Chapter 13.
2 Most penicillins are active against Gram-positive bacteria, although in the case of *Staph. aureus* this may depend upon the resistance of the antibiotic to β-lactamase.
3 The table is not intended to list every penicillin available worldwide; for encyclopaedic data, see the books edited by Bryskier (2005) and Finch *et al.* (2003).

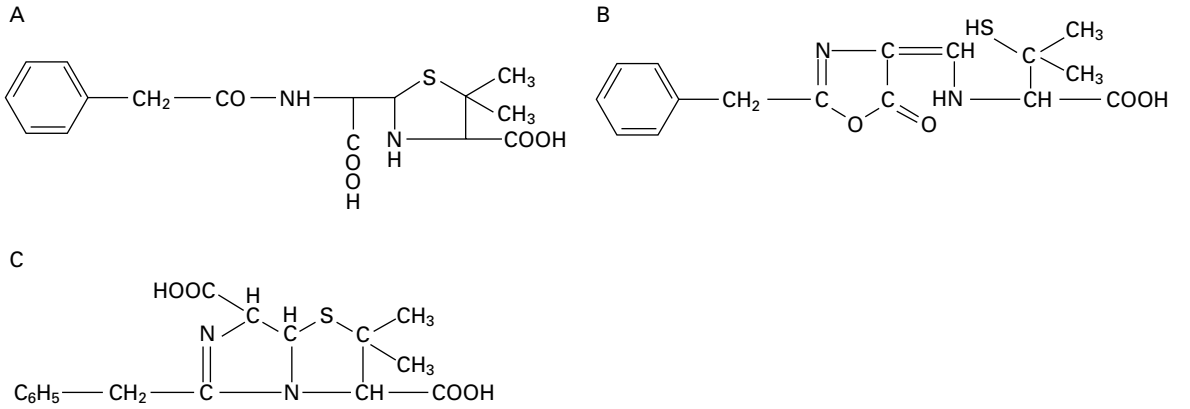


Figure 11.3 Degradation products of benzylpenicillin in solution: (A) penicilloic acid; (B) penicillenic acid; (C) penillic acid.

Many bacteria produce enzymes, β-lactamases (formerly called penicillinase; see Chapter 13), which may inactivate a penicillin by opening the β-lactam ring, as in Figure 11.1C. The only clinically significant β-lactamase produced by a Gram-positive species is that of *Staph. aureus*, but most, if not all, Gram-negative bacteria have the potential to produce the enzymes, albeit in such small amounts in some cases that they are of no clinical importance. The Gram-negative β-lactamases exhibit small interspecies, and even interstrain, differences in chemical structure which can have profound effects on their ability to hydrolyse the various β-lactam antibiotics. Some penicillins (Table 11.2) are considerably more enzyme-resistant than others, and consequently may be extremely valuable in the treatment of infections caused by β-lactamase-producing bacteria.

The older penicillins, benzylpenicillin and phenoxymethylpenicillin, are primarily active against Gram-positive bacteria; they are also β-lactamase sensitive. These shortcomings were overcome to varying degrees by the semisynthetic penicillins developed in the 1960s. Ampicillin remained β-lactamase sensitive, but its possession of an amino group on the benzyl side chain gave the molecule a much broader spectrum of activity than its parent, benzylpenicillin. The oral absorption of ampicillin was found to be adequate rather than good; a figure of less than 50% is commonly quoted. This was significantly improved by the inclusion of a *p*-hydroxyl group on the benzene ring of the ampicillin side chain, thus creating amoxicillin, which has largely superseded the older antibiotic. Both ampicillin and amoxicillin are effective against many Gram-negative bacteria including *Haemophilus influenzae, Escherichia coli, Salmonella, Shigella* and *Proteus* species, though not *Pseudomonas aeruginosa*. This last organism has represented a problem in antibiotic therapy for many years, and carbenicillin, also developed in the early 1960s, was the first penicillin showing antipseudomonal activity, although it has now been largely replaced by ticarcillin. Piperacillin, an acyl derivative of ampicillin, also possesses activity against *Pseudomonas*, and, like ticarcillin, is moderately susceptible to β-lactamases, so both antibiotics are normally used as combination products with β-lactamase inhibitors (see section 2.3).The property of resistance to Gram-negative β-lactamases, strongly exhibited by temocillin, is conferred by the possession of a 6α-methoxy group, although this causes the molecule to exhibit an antibacterial spectrum confined almost exclusively to Gram-negative species. A similar spectrum arises with pivmecillinam, an amidinopenicillin, but in this case the enzyme resistance is much weaker.

Penicillins possess a carboxylic acid group on C3 which can be esterified to create lipophilic prodrugs with enhanced absorption from the gastrointestinal tract, after which tissue esterases hydrolyse the ester to release the active antibiotic. This strategy has been particularly successful in remedying the poor oral absorption of ampicillin and resulted in the development of bacampicillin, pivampicillin and talampicillin; the first two of these, in particular, are widely available elsewhere, but not currently in the UK.

The problem of sensitivity to staphylococcal β-lactamase was overcome by the development of meticillin in which the bulky substituent groups of the side chain largely prevented enzyme binding. Meticillin, which was only available as an injection, has largely been replaced

with other β-lactamase-stable penicillins, particularly the orally active flucloxacillin, although there are several related drugs whose availability varies from country to country. The great majority of *Staph. aureus* strains remained sensitive to meticillin for about 15 years after its introduction in 1960, but the emergence of MRSA gathered momentum from the mid 1970s; the incidence in the USA was 2.4% in 1975 but rose to 35% by 1996.

Penicillins generally are of low toxicity, with allergic reactions as the only serious problem; these arise more commonly with benzylpenicillin and ampicillin than the rest. All penicillins, but particularly those administered orally, can cause diarrhoea, and, rarely, pseudomembranous colitis; this is more of a problem with ampicillin because a higher proportion of an oral dose remains in the colon to disturb the natural flora. Penicillins are excreted primarily in the urine, in which they achieve much higher levels than in the blood, and accumulation of sodium and potassium may arise with high-dose injections in patients with poor kidney function.

2.2 Cephalosporins

There are quite a number of similarities between cephalosporins and penicillins, including their early history. In both cases, the academic research was conducted at Oxford University and the American pharmaceutical companies converted that early work into a marketed product. The discovery of the first cephalosporin was made in 1948 in Sardinia, the research was conducted at the William Dunn School of Pathology during the 1950s and the first antibiotics in the class, cephalothin and cephaloridine, became available in the mid 1960s. Since then many cephalosporins have been synthesized, and more than 60 have been marketed in various countries over the last 40 years, although not all of them are still available. As with the penicillins, the policy in this chapter is to consider the general properties of this class of antibiotics and to provide more detail about selected important cephalosporins, rather than provide a comprehensive listing.

Cephalosporins consist of a six-membered dihydrothiazine ring fused to a β-lactam ring (top structure in Figure 11.4). The position of the double bond in Δ^3-cephalosporins is important, since Δ^2-cephalosporins (double bond between 2 and 3) are not antibacterial, irrespective of the composition of the side chains. The similarity to the basic penicillin structure is immediately apparent, but the crucial difference is that there is much greater scope for structural modification of the cephalosporins because of the presence of two side chains (on carbons 3 and 7) in contrast to the single side chain of penicillins. Again, the fundamental properties of acid stability (and hence oral availability), antimicrobial spectrum, resistance to β-lactamases and pharmacokinetics can all be substantially varied by side-chain modifications. Several of the cephalosporins act as good inducers of β-lactamases.

The many cephalosporins have been classified into 'generations', although the usefulness of such a classification has been questioned. There is general acceptance of four generations, but ceftobiprole has been claimed as the first member of the fifth. The assignment of cephalosporins into the first four generations has broadly followed the time course of their introduction, but there has been some overlap, so certain drugs that have been classified into one particular generation were marketed in some countries after the earliest ones of the next. The situation is further complicated by the fact that there is not universal agreement on the generation to which some cephalosporins belong; cefaclor, for example, is considered a first-generation antibiotic in Japan, but is regarded as second-generation in most other countries.

The general trends have been for an increase in activity towards Gram-negative species (usually with a corresponding loss of antistaphylococcal action), and increased resistance to β-lactamase as the development of cephalosporins has progressed through the generations. First-generation drugs are those that have moderate antimicrobial activity and resistance to staphylococcal, but not Gram-negative, β-lactamases. These were originally used primarily as alternative antibiotics for the treatment of staphylococcal infections, and are rarely first-choice therapy. Like the oral penicillins, the oral cephalosporins may disturb the gut flora and give rise to diarrhoea. The possession of good resistance to both staphylococcal and Gram-negative β-lactamases is the principal characteristic distinguishing the second- from the first-generation antibiotics, although improved potency, particularly towards *H. influenzae* and enterobacteria, is also a feature. Yet higher activity towards Gram-negative bacteria is displayed by third-generation drugs, to an extent that some of them have little or no value in the treatment of staphylococcal infections. The parenterally administered third-generation cephalosporins, e.g. cefotaxime and ceftazidime, are sometimes used in combination with gentamicin or other aminoglycosides with the intention of achieving synergy. The usefulness of the third-generation drugs has diminished somewhat since their introduction as a consequence of the spread of strains capable of producing extended-spectrum

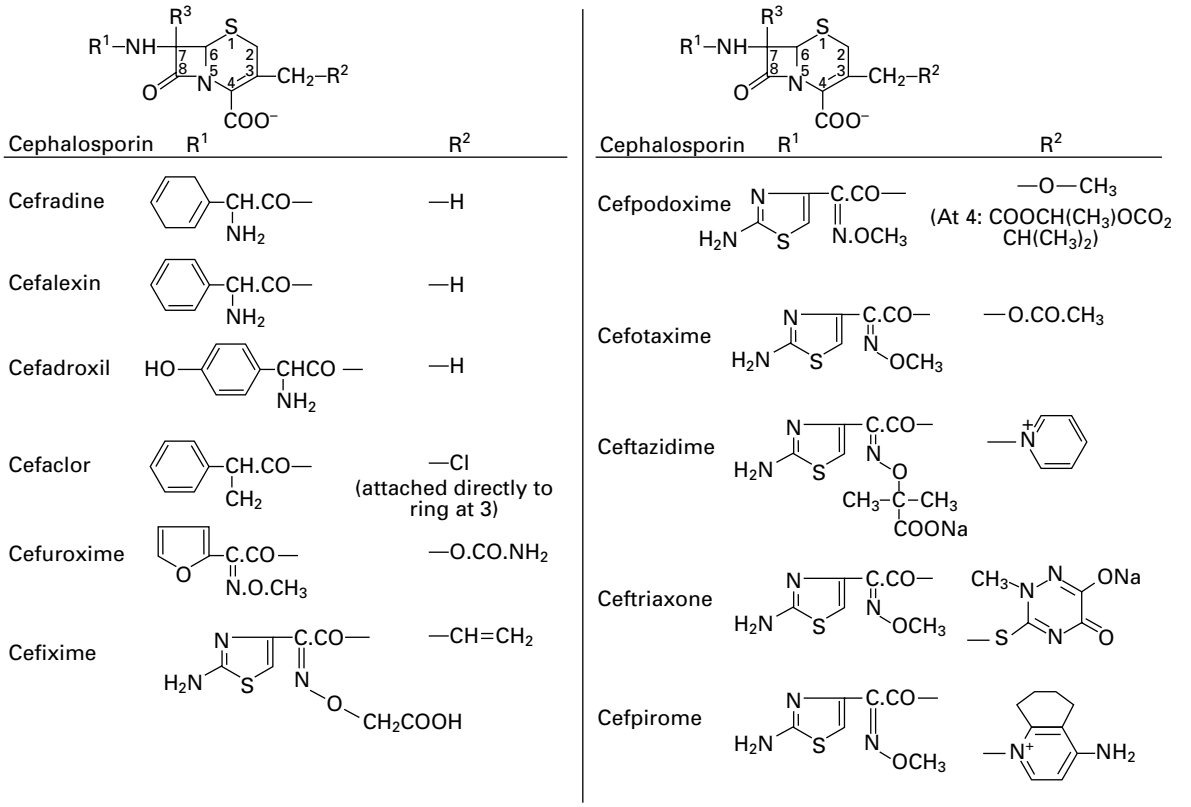


Figure 11.4 General structure of cephalosporins and examples of side chains R^1 and R^2 (R^3 is H in all examples in this figure).

β-lactamases (see Chapter 13) and it was this deficiency that the fourth-generation cephalosporins were intended to remedy. Cefpirome and cefepime exhibit extremely good enzyme resistance, but otherwise have much the same antibacterial spectrum as ceftazidime and other third-generation molecules.

2.2.1 Structure–activity relationships

The activity of cephalosporins (and other β-lactams) against Gram-positive bacteria depends on antibiotic affinity for penicillin-sensitive enzymes (PSEs) also known as penicillin binding proteins (PBPs). Resistance results from altered PBPs or, more commonly, from β-lactamases. Activity against Gram-negative bacteria depends on penetration of β-lactams through the outer membrane, resistance to β-lactamases found in the periplasmic space and binding to PBPs. (For further information on mechanisms of action and bacterial resistance, see Chapters 12 and 13). Modification of the cephalosporin nucleus (Figure 11.4) at 7α (i.e. R^3) by addition of a methoxy group increases β-lactamase stability but decreases activity against Gram-positive bacteria because of reduced affinity for PBPs; molecules possessing a 7α-methoxy group, e.g. cefoxitin, are termed cephamycins.

Side chains containing a 2-aminothiazolyl group at R^1, e.g. cefotaxime, ceftriaxone and ceftazidime, yield cephalosporins with enhanced affinity for PBPs of Gram-negative bacteria and streptococci. An iminomethoxy group (-C=N.OCH_3) in, for example, cefuroxime, provides β-lactamase stability against common plasmid-mediated β-lactamases. A propylcarboxy group ($(CH_3)_2$-C-COOH) as in ceftazidime increases β-lactamase resistance and also provides activity against *Ps. aeruginosa*, while at the same time reducing β-lactamase induction capabilities. In cephalosporins susceptible to β-lactamases, opening of the β-lactam ring occurs with concomitant loss of the substituent at R^2 (except in cefalexin, where R^2 represents H; see Figure 11.4). This is followed by fragmentation of the molecule.

The nature of the R^2 substituent influences both the pharmacokinetic properties of the molecule and its ability to enter bacterial cells—particularly to cross the outer membrane of Gram-negative bacteria via porins. For good oral absorption: (1) the R^2 substituent must be small, non-polar and stable; a methyl group is considered desirable but might decrease antibacterial activity; and (2) the 7-acyl group (R^1) must be based on phenylglycine and the amino group must remain unsubstituted. Esterification of the carboxylic acid group at C4 can, as with the penicillins, result in enhanced oral absorption provided that the ester is rapidly hydrolysed by tissue esterases; this is exemplified in both cefuroxime axetil and cefpodoxime proxetil. The possession of a quaternary nitrogen on the side chain at position 3 has two benefits: it reduces the affinity of the cephalosporin for Gram-negative β-lactamases, in other words, makes it more resistant to enzyme attack, and it makes the molecule zwitterionic which increases the rate at which it can pass through the porin channels into the Gram-negative cell.

Side chains of the various cephalosporins, including those most recently developed, are presented in Figure 11.4 and a summary of the properties of these antibiotics in Table 11.3.

2.3 β-Lactamase inhibitors

The strategy of protecting vulnerable penicillins against enzyme-mediated hydrolysis by combining them with a β-lactamase inhibitor resulted in the first such combination product, co-amoxiclav, in 1981. Table 11.1 shows that penicillins with β-lactamase inhibitors represent approximately 6.5% of the antibiotics prescribed to UK outpatients in the European hospital survey, and co-amoxiclav, which consists of amoxicillin plus clavulanic acid, is by far the most important combination available. Clavulanic acid has also been used to protect ticarcillin from β-lactamase attack, and two penicillanic acid sulphones, sulbactam and tazobactam, have been used to protect ampicillin and piperacillin respectively. In each case the protecting molecule is itself a β-lactam antibiotic, but one possessing little antimicrobial activity in its own right. The benefit afforded is an extension of the antimicrobial spectrum of the antibiotic receiving protection; this is achieved by negating the effects of β-lactamases produced by staphylococci and some Gram-negative species, which would otherwise be resistant. In the case of co-amoxiclav for example, the combination exhibits activity not only against many strains of *Staph. aureus*, but also against strains of *E. coli*, *H. influenzae* and *Klebsiella* species, against which amoxicillin alone would be ineffective. This means that co-amoxiclav should, in theory, be reserved for infections known, or likely, to be due to amoxicillin-resistant β-lactamase-producing strains, but unfortunately it is not always used in this prudent manner.

Clavulanic acid was isolated from *Streptomyces clavuligerus* and belongs to a class of β-lactams termed clavems, which differ from penicillins in two respects: namely the replacement of sulphur in the penicillin thiazolidine ring (Figure 11.1) with oxygen in the clavam oxazolidine ring (Figure 11.5A), and the absence of the side chain at position 6. Clavulanic acid also affords some protection against enzymes that are primarily active against cephalosporins rather than penicillins, but this protection is quite modest compared with its activity against 'penicillinases'.

Sulbactam and tazobactam (Figure 11.5 B and C) can be regarded as β-lactam molecules that resemble penicillins except that the sulphur atom of the thiazolidine ring is converted to a sulphone, and again, there is no side chain at position 6. Sulbactam is effective against a similar range of β-lactamases to clavulanic acid, although it is not quite as potent. In both cases, the range of enzymes does not normally include those manufactured by *Ps. aeruginosa* and other problem Gram-negative organisms. Sulbactam has been combined with both ampicillin and cefoperazone, but in both products the two ingredients were separate entities; this poses the potential problem that their pharmacokinetics might not perfectly match, so that the two agents might not appear at the infection site in the optimal concentration ratio at the same time. In the case of sulbactam and ampicillin this problem was partly overcome by covalently linking the two molecules to create sultamicillin which is well absorbed following oral administration and then hydrolysed to liberate equimolar proportions of the individual components.

2.4 Carbapenems and aztreonam

The spread of organisms that had developed various forms of resistance to the early penicillins and cephalosporins lead to a search for other β-lactam antibiotics that avoided these resistance problems. Many naturally occurring and synthetic compounds were examined but only a few were developed to become marketed products, and the most useful of these were the carbapenems. This group may be considered as penicillin or cephalosporin derivatives in which the sulphur atom has been replaced with a carbon. The nomenclature is confusing because some reference sources used terms like carbapenems,

Table 11.3 Properties of selected cephalosporins

Cephalosporin	Generation	Dose forms	Antibacterial spectrum							Doses/ day	Comments
			Staphylococci	Enterococci	Streptococci	Enterobacteria	*Neisseria*	*Haemophilus*	*Ps aeruginosa*		
Cefradine	1	O + I	+	+	R	+	+	R	R	4	Least active of first-generation cephalosporins
Cefalexin	1	O	+	+	R	+	+	R	R	2–4[a]	
Cefadroxil	1	O	+	+	R	+	+	R	R	2	Similar to cefalexin in most respects
Cefaclor	2	O	+	+	R	+	+	+	R	3	More active than first-generation cephalosporins
Cefuroxime	2	O[b]+ I	+	+	R	+	+	+	R	2	Generally more active than cefaclor
Cefixime	3	O	V	+	R	+	+	+	R	1–2	Longer duration of action than other cephalosporins
Cefpodoxime	3	O	+	+	R	+	+	+	R	2	Good activity against respiratory pathogens
Cefotaxime	3	I	+	+	R	+	+	+	R	2–4[a]	Broad-spectrum; particularly good against Gram –ves
Ceftazidime	3	I	V	+	R	+	+	+	+	2–3[a]	Good anti-*Pseudomonas* activity
Ceftriaxone	3	I	+	+	R	+	+	+	R	1	Long plasma half-life means less frequent dosing
Cefpirome	4	I	+	+	V	+	+	+	+	2	Particularly stable against β-lactamases

+, active; I, injection; O, oral; R, resistant; V, varies between strains, or weak activity.
[a]Depending on dose.
[b]As axetil ester.

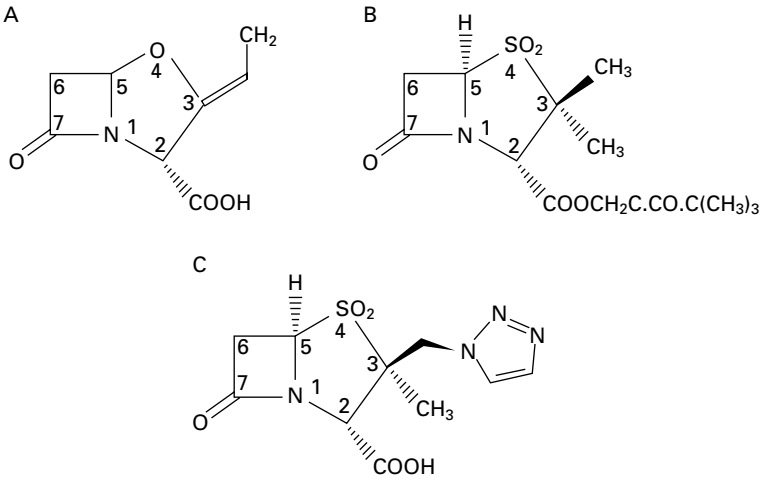


Figure 11.5 β-Lactamase inhibitors. (A) clavulanic acid; (B) sulbactam; (C) tazobactam.

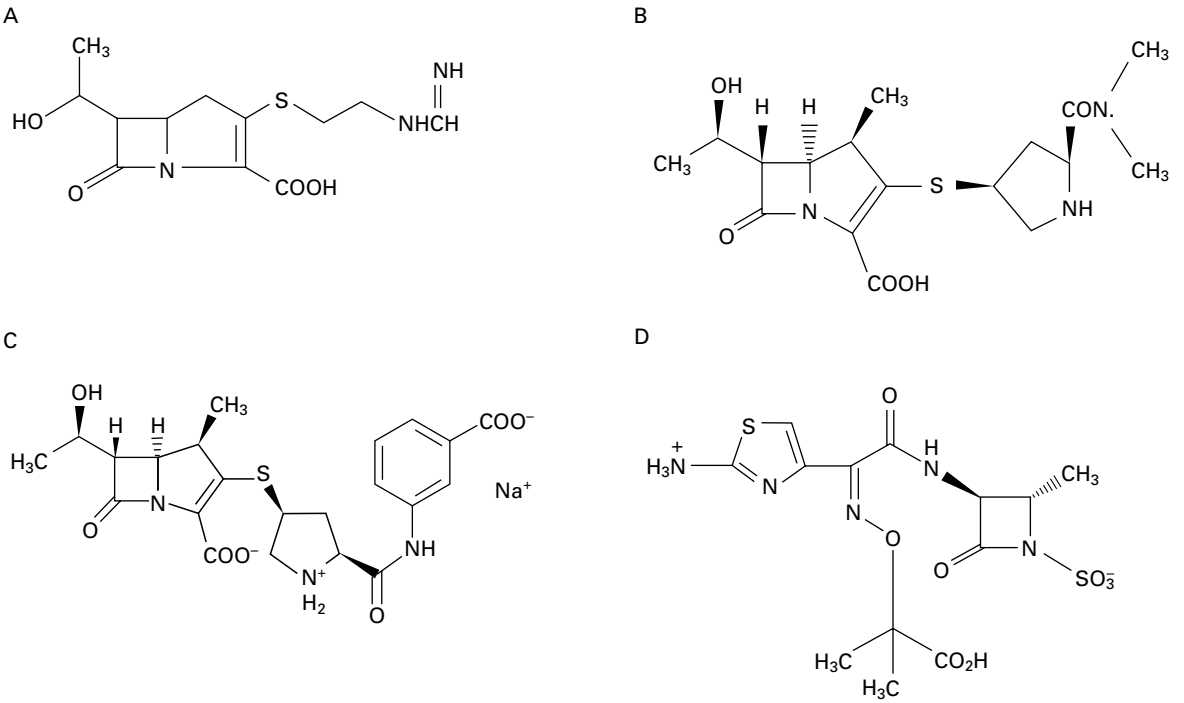


Figure 11.6 Carbapenems and aztreonam: (A), imipenem; (B), meropenem; (C), ertapenem; (D), aztreonam.

olivanic acids and thienamycins as if they were synonymous. In fact, carbapenems is the generic term for the group which includes olivanic acids (of which there are no products in therapeutic use) and the thienamycins. The earliest thienamycins were discovered in the 1970s but proved difficult to develop because of their poor stability. The *N*-formimidoyl derivative of thienamycin, which was named imipenem (Figure 11.6A), proved to combine the desirable properties of *in vitro* stability, a broad spectrum of antimicrobial activity and good resistance to almost all of the then-known β-lactamases. Its only shortcoming was poor *in vivo* stability because it was

vulnerable to hydrolysis by mammalian renal dipeptidase, but this was solved by the development of a renal dipeptidase inhibitor, cilastatin, with which imipenem was marketed. Meropenem, marketed more recently, is more stable than imipenem to dipeptidase and may thus be administered without cilastatin; its chemical structure is depicted in Figure 11.6B. Ertapenem (Figure 11.6C) has properties similar to those of meropenem but affords the additional advantage of once daily dosing.

Examination of the structure–activity relationships of the early β-lactam antibiotics led to an expectation that molecules possessing only the β-lactam ring without a second ring fused to it would have no antimicrobial activity. This proved not to be so when such naturally occurring antibiotics, termed monobactams, were discovered, and found not only to possess activity, but to exhibit good resistance to β-lactamases. The naturally occurring monobactams were not developed for clinical use, but an analogue, aztreonam, produced totally by conventional chemical synthesis, was marketed in 1986. It is highly active against most Gram-negative bacteria and stable to most types of β-lactamases, although its resistance to staphylococcal β-lactamases is irrelevant because it is inactive against all strains of *Staph. aureus* as well as other Gram-positive species and anaerobes. Like the carbapenems, it is formulated as an intravenous injection and, as such, it tends to be limited to use in hospitals for the treatment of serious Gram-negative infections, including those due to *Ps. aeruginosa*. As with the carbapenems and third-generation cephalosporins, it exhibits synergy with aminoglycosides like gentamicin and tobramycin, and these combinations are employed for the treatment of *Pseudomonas* lung infections in cystic fibrosis.

2.5 Hypersensitivity

Although penicillins are among the safest antimicrobial agents available, allergic reactions, particularly skin allergies, may occur in 1–10% of patients receiving them, and the much more serious, and potentially fatal, anaphylactic reactions occur in 0.005–0.05% of cases. Patients sensitive to one penicillin will be sensitive to all others because it is the basic penicillin structure that is responsible for the hypersensitivity, but benzylpenicillin and ampicillin are the ones most likely to cause anaphylaxis and skin allergy respectively. Although hypersensitivity reactions can occur after administration via any route, the more severe reactions are most likely to arise after intravenous injection. About 10% of patients allergic to penicillins will also be sensitive to cephalosporins.

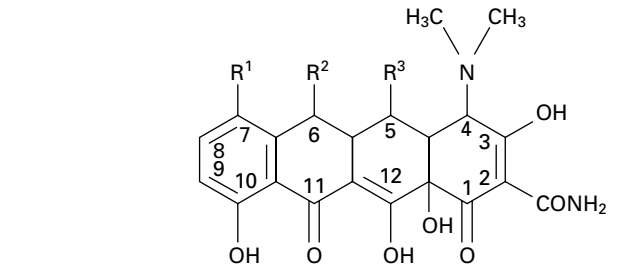
3 Tetracyclines

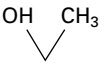
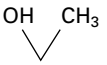
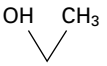
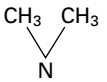
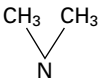
The tetracyclines are a group of broad-spectrum antibiotics that are declining in use as a result of increasing bacterial resistance. Despite that, they remain important antibiotics for several dangerous, but relatively rare, infections due to chlamydia (e.g. trachoma), rickettsia (e.g. typhus and Q-fever) and spirochaetes (e.g. Lyme disease) as well as those caused by 'typical' bacteria (e.g. brucellosis and bubonic plague). They also represent useful alternatives to macrolides (see section 4 of this chapter) and to β-lactams (particularly in cases of allergy) for the treatment of more common infections, including those of the respiratory tract.

The tetracyclines (Figure 11.7) were first developed during the 1940s and 1950s and several that are still in use date from that time, e.g. tetracycline itself, oxytetracycline and chlortetracycline. Doxycycline and minocycline are more potent semisynthetic analogues discovered in 1966 and 1972 respectively, after which there were no significant developments until the introduction, in 2005, of tigecycline (a glycylglycine derivative), which is generally more potent again than other tetracyclines and maintains activity against some organisms that have become resistant to earlier members of the group.

Although the tetracyclines can exhibit bactericidal activity by inhibiting ribosome function at concentrations that might be used in the laboratory, they are bacteristatic at concentrations that can safely be achieved in the body. They are active against Gram-positive bacteria, although many strains of *Staph. aureus* have become resistant to all but tigecycline which, as a consequence, is of value in the treatment of MRSA infections. Many Gram-negative species are also sensitive to tetracyclines, and although the proportion of strains responding to treatment has significantly diminished in recent years there are substantial geographical variations in resistance patterns; *Ps. aeruginosa* and *Proteus* species are normally resistant. In addition to the infections mentioned above, tetracyclines are prescribed for the treatment of acne, various genital infections, the eradication of *Helicobacter pylori* in gastric and peptic ulcer disease (as part of a multidrug regimen) and, in the case of doxycycline particularly, for the prophylaxis of drug-resistant *Plasmodium falciparum* malaria.

Resistance to the tetracyclines (see also Chapter 13) develops relatively slowly, but there is cross-resistance, i.e. an organism resistant to one member is usually resistant to all other members of this group, but there are excep-



Drug	R^1	R^2	R^3
1	H		OH
2	H		H
3	Cl		H
4	H		OH
5	H	CH_3	OH
6		H_2	H
7		H_2	H

with an N-lysinomethyl group at C2

with a t-butylglycylamido group at C9

Figure 11.7 Tetracycline antibiotics: (1) oxytetracycline; (2) tetracycline; (3) demeclocycline; (4) lymecycline; (5) doxycycline; (6) minocycline; (7) tigecycline.

tions: tigecycline (mentioned above), and tetracycline-resistant *Staph. aureus* strains may still be sensitive to minocycline. Superinfection ('overgrowth') with naturally tetracycline-resistant organisms, for example *Candida albicans* and other yeasts, affecting the mouth, upper respiratory tract or gastrointestinal tract, may occur as a result of the suppression of tetracycline-susceptible microorganisms.

Tetracyclines are absorbed from the gastrointestinal tract and oral products are the only form in which they are currently available in the UK, although ophthalmic, topical and injectable products have been used in the past and some are still available in other countries. Absorption of tetracyclines is inhibited by food, antacids, milk or products containing di- or trivalent cations, so tetracycline, oxytetracycline and chlortetracycline have to be administered four times a day in order to maintain adequate tissue concentrations. Such impaired absorption is much less evident in the more recently developed drugs, and partly as a consequence of both this improvement and of extended half-lives, lymecycline, minocycline and tigecycline are administered twice daily, and doxycycline just once. The newer tetracyclines are also more lipophilic than the early ones, so doxycycline, and to an even greater extent minocycline, exhibit good tissue distribution and achieve concentrations in the biliary tract, liver, kidneys and other organs which may substantially exceed those in the blood.

Dose-dependent nausea and vomiting are the most common side effects, and diarrhoea may arise as a consequence of alterations in the bacterial flora of the colon. The ability to chelate with calcium results in tetracyclines being deposited in bones and teeth, and precludes their administration to children younger than 12 years or to women in late pregnancy. Their use in patients with poor kidney function is also contraindicated because most of the tetracyclines accumulate in this situation; again, doxycycline and minocycline are exceptions.

4 Macrolides

The macrolide antibiotics are large molecules comprising 12–16-membered lactone rings linked through glycosidic bonds with amino sugars. Erythromycin was the first member of the group to be discovered in 1952 and it is still an important antibiotic today. It was quickly followed 2 years later by spiramycin and oleandomycin but, although still available in certain countries, these last two are now little used. Erythromycin suffers from several disadvantages: its antimicrobial spectrum is largely restricted to Gram-positive species, it has poor acid stability so its absorption is erratic, it commonly exhibits gastrointestinal side effects and bacteria acquire resistance to it relatively easily. These shortcomings prompted the search for new macrolides, and several semisynthetic derivatives were forthcoming: roxithromycin was marketed in 1987, clarithromycin and azithromycin in 1991 and the most recent, telithromycin, in 2001. Erythromycin (Figure 11.8) and roxithromycin are chemically similar in possessing a 14-membered ring structure. A distinction is sometimes drawn between them and both azithromycin which, strictly speaking, is an azalide (a 15-membered ring containing an additional nitrogen atom) and telithromycin which is a ketolide (a 14-membered ring with an additional keto group). The term macrolide, however, is commonly used to describe all five antibiotics, and that terminology will be used here.

The macrolides are active against most Gram-positive bacteria, *Neisseria* and *H. influenzae* but, with the exception of azithromycin, not against the Enterobacteriaceae. Because their antibacterial spectrum is similar to those of the early penicillins, the macrolides were, and still are, considered alternatives for patients with penicillin allergy. They are commonly used for respiratory, skin and soft tissue infections, but one of the factors that stimulated the development of the more recent macrolides was their activity against emerging pathogens like species of *Legionella*, *Campylobacter*, *Helicobacter* and *Chlamydia*, as well as some mycoplasmas and rickettsias and the *Mycobacterium avium* complex to which AIDS/HIV patients are susceptible. The semisynthetic macrolides do not afford a significant advantage over erythromycin in terms of their activity against staphylococci, streptococci and enterococci, but they represent an advantage in several other respects: they are generally more active against the other organisms mentioned above; they exhibit better stability and pharmacokinetics, thus permitting less frequent dosage and better tissue penetration; they generally have fewer side effects; and, particularly in the case of telithromycin and other ketolides, they may be active against some strains that have acquired resistance to erythromycin, and they are, themselves, less vulnerable to resistance development.

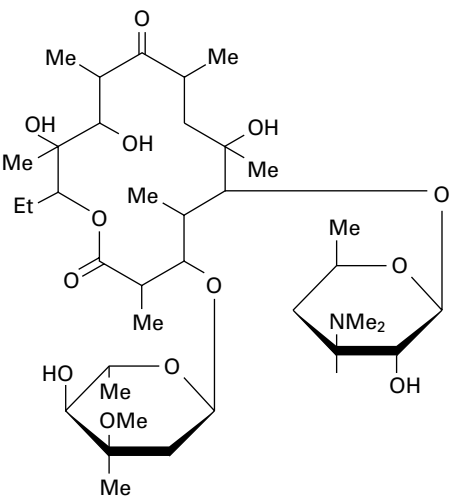


Figure 11.8 Erythromycin.

The macrolides all act by inhibiting protein synthesis in bacteria and they are regarded as bacteristatic drugs, although bactericidal activity may be achieved at high concentrations. The antimicrobial activity of erythromycin is pH-dependent, increasing with pH up to about 8.5, and the same effect occurs to varying degrees with other members of the group. The macrolides are extremely bitter and their tablets are often coated, both to disguise the taste and to protect the antibiotic from stomach acid. Erythromycin exhibits particularly poor acid stability and erratic oral absorption, and a variety of esters have been used to minimize these problems which, although present, are much less evident in the semisynthetic molecules.

All the macrolides are orally active and they are concentrated intracellularly, particularly into neutrophils by which they are transported to infection sites. The longer elimination half-lives of the newer drugs permit less frequent dosing than that required for erythromycin. The group as a whole are regarded as relatively safe antibiotics which do not exhibit severe adverse reactions, although gastrointestinal disturbances (nausea, vomiting, abdominal pain and, infrequently, diarrhoea) are relatively common with erythromycin and much reduced or absent in the others. These and other characteristics are summarized in Table 11.4.

Table 11.4 Properties of macrolides

Macrolide	Route	Doses/day	Distinguishing features
Erythromycin	O, IV	2–4	Especially poor acid stability, erratic oral absorption and gastrointestinal side effects
Roxithromycin	O	1–2	Slightly less potent but substantially better oral absorption than erythromycin
Clarithromycin	O, IV	2	Very good acid stability and oral absorption. Tissue concentrations very much higher than those in the plasma
Dirithromycin	O	1	Slightly less active than erythromycin, but long half-life and good tissue concentration
Azithromycin	O	1	More active against Gram-negative bacteria, including Enterobacteriaceae, than erythromycin; very high concentration in neutrophils
Telithromycin	O	1	Active against bacteria resistant to penicillins and other macrolides, with recommendations that its use be restricted for that purpose

IV, intravenous; O, oral.

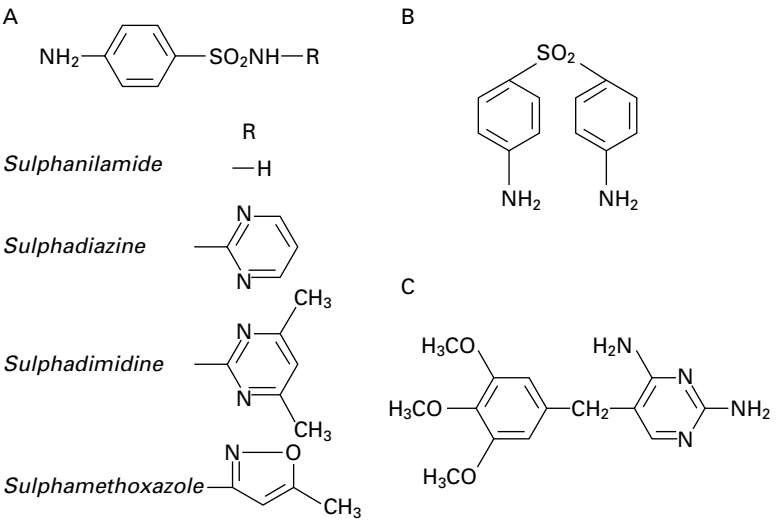


Figure 11.9 (A) Some sulphonamides; (B) dapsone; (C) trimethoprim.

5 Sulphonamides, trimethoprim and related drugs

Sulphonamides were discovered by Domagk in 1935. It had been shown that a red azo dye, prontosil, had a curative effect on mice infected with β-haemolytic streptococci; it was subsequently found that *in vivo*, prontosil was converted into sulphanilamide (Figure 11.9A). The basis of the antimicrobial activity of the sulphonamides is their structural similarity to *p*-aminobenzoic acid (PABA) which is an integral part of the B vitamin, folic acid. In sensitive bacteria, sulphonamides compete with PABA with the result that folic acid synthesis is reduced. Because the vitamin is essential for the manufacture of nucleic acids (and other important biochemicals), this leads to a reduction in, or cessation of, bacterial growth.

Chemical modifications of sulphanilamide (see Figure 11.9A) gave compounds with higher antibacterial activity or special properties like prolonged activity. The drugs were extensively used from the 1930s to the 1970s, after which their popularity declined due to resistance development and the introduction of safer and more effective antibiotics. A few sulphonamides are still used in topical therapies(notably silver sulphadiazine for burns) and in veterinary medicine, while sulphadiazine itself remains available for the prevention of rheumatic fever. Dapsone (Figure 11.9B) is a sulphonamide derivative that has been used extensively in the past for the treatment of leprosy, but again, although still available and currently employed as part of a multidrug treatment for leprosy, its use has declined as a result of resistance development and the introduction of better antibiotics.

In the body, folic acid must be reduced to dihydrofolic acid and then tetrahydrofolic acid in order to become active, and it was discovered that a group of synthetic drugs called diaminopyrimidines could inhibit the enzymes responsible for this reduction. These dihydrofolate reductase inhibitors, of which trimethoprim (11.9C) is the most important, were found to act synergistically with sulphonamides because they blocked successive steps in the synthesis of reduced folic acid. Consequently trimethoprim was introduced in 1969 as a combination product with sulphamethoxazole (co-trimoxazole) for the treatment of urinary tract and, less commonly, respiratory infections. Unfortunately, the advantages of using the combination product were not as great as anticipated partly because pharmacokinetic differences between the two drugs resulted in relative concentrations in the body which were far from optimal for synergy. This, together with increasing evidence that the antibacterial activity of co-trimoxazole was due largely to the trimethoprim component with the more toxic sulphamethoxazole contributing little, lead to the use of trimethoprim alone from the mid 1970s. Co-trimoxazole is currently recommended only for treatment of pneumocystis pneumonia, toxoplasmosis and nocardiasis.

Trimethoprim remains one of the least expensive orally active agents available for the treatment of urinary tract infections, for which it is still widely prescribed, although it, too, is suffering from increasing resistance development and the trend is towards its replacement with fluoroquinolones (see section 6). Other trimethoprim analogues, notably tetroxoprim, have been introduced as antibacterial agents, but have not demonstrated significant advantages over trimethoprim itself. The other important diaminopyrimidine possessing antimicrobial activity is pyrimethamine. This is used in combination with sulphadoxine or other drugs for the treatment (but not any longer for the prophylaxis) of malaria. It is used on its own for the treatment of toxoplasmosis and pneumocystis pneumonia.

6 Quinolones

The quinolones and the cephalosporins have much in common in terms of their history and development: both classes of antibiotic were first introduced in the early 1960s and the first members of each class, nalidixic acid and cephalothin respectively, had limited applications. However, both groups were extensively developed over the next 50 years with four generations of antibiotics being produced in both classes, each an improvement on its predecessor, so that the quinolones and the cephalosporins are currently amongst the most widely used and valuable antibiotics available.

Nalidixic acid (Figure 11.10B) was developed as a treatment for urinary tract infections, and it, together with other first-generation quinolones produced in the 1960s and 1970s (e.g. cinoxacin, acrosoxacin and pipemidic acid), had an antibacterial spectrum that was largely restricted to *E. coli* and other Enterobacteriaceae. As with the cephalosporins, there is no universal acceptance of the definitions of each generation of the antibiotics, so particular quinolones have been assigned to different generations in various textbooks. There is agreement, however, that the distinguishing feature of the first generation is the absence of a fluorine atom at position 6, so they are simply designated 'quinolones', whereas the second, third and fourth generations are termed fluoroquinolones. The first-generation drugs are now little used, but those that followed in the 1980s, e.g. ciprofloxacin, norfloxacin, ofloxacin and levofloxacin (the L-isomer of ofloxacin) (Figure 11.10C–E), are still very much in use today and owe their popularity both to their greater activity against *E. coli* and other Gram-negative urinary pathogens and their wider antibacterial spectrum which includes Gram-positive cocci and *Ps. aeruginosa*. The third-generation quinolones, e.g. moxifloxacin (Figure 11.10F), possess the attributes of their predecessors but exhibit greater activity against *Strep. pneumoniae*. The fourth generation, e.g. trovafloxacin (not available in Europe), are also active against anaerobes.

All the quinolones are bactericidal, and act by inhibiting the bacterial enzymes responsible for coiling of DNA (see Chapter 12); they are all orally active, and several are

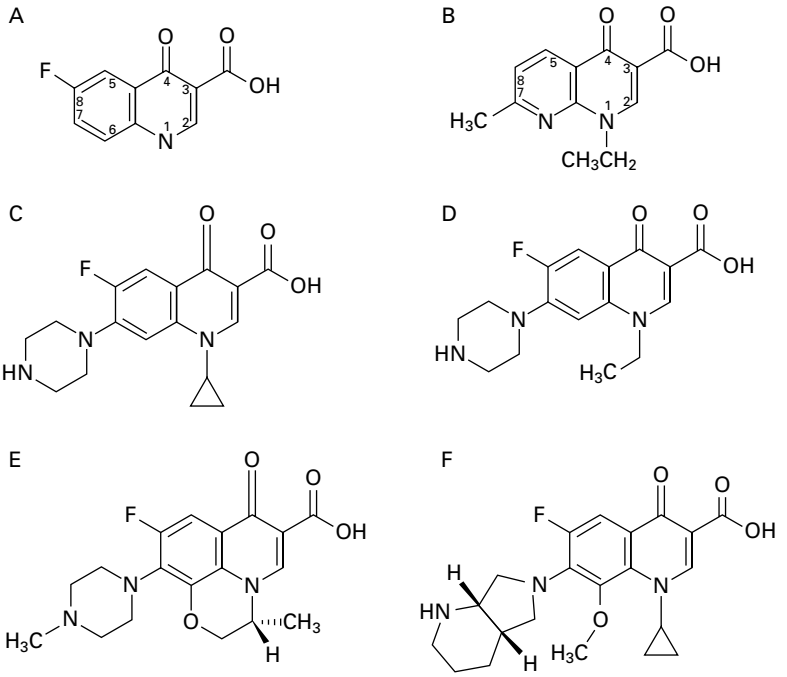


Figure 11.10 (A) Fluoroquinolone; (B) nalidixic acid; (C) ciprofloxacin; (D) norfloxacin; (E) levofloxacin and ofloxacin; (F) moxifloxacin.

available as intravenous injections. The first-generation drugs were only used for the treatment of urinary infections, but the much broader spectra of those that followed permitted their use for respiratory, soft tissue, bone and joint, gastrointestinal and sexually transmitted infections as well. Quinolones have been shown to cause rare cases of tendon damage, including rupture, and for this reason are not generally prescribed for children, but their most common minor side effect is gastrointestinal disturbances, which are reported in about 5% of patients. They are relatively susceptible to resistance development, and Gram-positive species tend to mutate to resistance at higher frequencies than Gram-negatives. Some degree of cross-resistance usually occurs, so that bacteria that become resistant to one quinolone are often less susceptible to others. They are now the most widely prescribed antibiotics for adults in the USA, and the growing resistance problem has been attributed in part to their indiscriminate use in human medicine and to cross-resistance between the drugs used for human infections and the several quinolones licensed exclusively for veterinary medicine which have been overused, particularly in Europe, for prophylaxis of bacterial infections in poultry flocks and other intensively reared animals.

The characteristics of some of the important quinolones are shown in Table 11.5.

7 Aminoglycosides

The aminoglycosides are a large group of broad-spectrum antibiotics possessing significant activity against many Gram-negative bacteria and a more limited range of Gram-positive organisms. The antibiotics are all bactericidal and administered by injection because they are poorly absorbed from the gastrointestinal tract. They are cationic, water-soluble drugs that interfere with protein synthesis in bacteria by binding to the 30S ribosome subunit (which is not possessed by mammals).

The earliest aminoglycoside was streptomycin, discovered in 1944, and at least 10 more were discovered over the next 30 years although not all were marketed. However, their susceptibility to resistance development by inactivating enzymes and other mechanisms (see Chapter 13), and their potential to cause damage to the kidneys and the eighth cranial nerve (to the ear) limited the use of the naturally occurring aminoglycosides and

Table 11.5 Properties of quinolones

Quinolone	Generation	Routes	Doses/day	Uses
Nalidixic acid	1	O	4	Restricted to UTIs; still available but little used
Ciprofloxacin	2	O, IV	2	Has good activity against Gram-negative species but only moderate activity against Gram-positives. Used primarily for UTIs, respiratory (but not pneumococcal pneumonia), GI and bone and joint infections
Ofloxacin	2	O, IV	1 or, more commonly, 2	Primarily for UTIs and lower respiratory infections, as well as gonorrhoea and genital infections
Levofloxacin	2	O, IV	1–2	As for ciprofloxacin, and as a second-line treatment for community-acquired pneumonia
Norfloxacin	2	O	2	UTIs and chronic prostatitis
Moxifloxacin	3	O	1	Sinusitis, community-acquired pneumonia or exacerbations of chronic bronchitis

GI, gastrointestinal; IV, intravenous; O, oral; UTI, urinary tract infection.

lead to the development of several semisynthetic members of the group.

Streptomycin was the first effective antibiotic for the treatment of tuberculosis and it is still used as a second-line drug for that purpose, although it now has few other applications. Neomycin was also discovered in the 1940s, but its high toxicity curtailed its use as a systemic drug and it is now largely restricted to ophthalmic and topical products, although in this limited field its use is widespread, and it is often encountered with other antibiotics or with steroids in antibiotic/anti-inflammatory creams. Oral preparations of neomycin are employed, again often in combination with other antibiotics, to reduce the bacterial population of the colon prior to surgery.

Three of the most important aminoglycosides currently available are gentamicin, tobramycin and amikacin. The first two of these are naturally occurring drugs discovered in the 1960s, whilst amikacin is a semisynthetic derivative of kanamycin, which it has superseded. Gentamicin, the drug of choice in the UK, is used alone or in combination with β-lactam antibiotics (with which it exhibits synergy) both for 'blind' therapy of infections prior to identification of the infecting organism, and for the treatment of bacterial endocarditis and serious Gram-negative infections; in common with other members of the group, it has no activity against anaerobes. Amikacin has similar applications, but is more stable to inactivation by bacterial enzymes, though rather less potent, than gentamicin. All three antibiotics possess useful activity against *Ps. aeruginosa* and are particularly valuable, again with β-lactams, for the eradication or suppression of this organism in the lungs of cystic fibrosis patients; tobramycin is slightly more active than gentamicin against *Ps. aeruginosa* and for this reason it is more frequently used for this purpose than any other.

Aminoglycosides have in the past often been administered twice or three times per day, but the more recent trend has been towards a single, higher, daily dose. Although not suitable in all situations, once-daily dosing is undoubtedly more convenient and considered to be at least as safe and efficacious. Regardless of the dosing schedule however, monitoring of serum levels during treatment to avoid potential toxic high concentrations or ineffective low ones is still an integral part of aminoglycoside therapy.

8 Glycopeptides

The only two important glycopeptide antibiotics currently available are vancomycin and teicoplanin. Like many of the other antibiotics in current use, vancomycin is a relatively old drug, having been introduced in 1958,

but its activity against MRSA resulted in it becoming progressively more valuable as MRSA became more prevalent. It has a complex tricyclic glycopeptide structure and its large molecular size means that it cannot penetrate through the outer membrane of most Gram-negative bacteria, so its use is effectively restricted to the treatment of infections by aerobic or anaerobic Gram-positive species. In addition to *Staph. aureus*, it is active against *Staph. epidermidis*, streptococci, *Cl. difficile* and *Ent. faecalis,* although resistant enterococci are posing an increasing clinical problem.

Vancomycin is bactericidal to most susceptible bacteria at concentrations near its minimum inhibitory concentration (MIC) and is an inhibitor of bacterial cell wall peptidoglycan synthesis, although at a site different from that of β-lactam antibiotics. Employed as the hydrochloride and administered by dilute intravenous injection, vancomycin is indicated in potentially life-threatening infections that cannot be treated with other, less toxic, antibiotics. Oral vancomycin, which is not absorbed from the gastrointestinal tract, is the drug of choice in the treatment of antibiotic-induced pseudomembranous colitis associated with the administration of antibiotics such as clindamycin and lincomycin (section 11). Although not chemically related to the aminoglycosides, vancomycin suffers the same toxicity problems and has the potential to damage both the kidney and the ears, and it too is normally subject to blood-level monitoring during therapy.

Because of its potential toxicity, its poor penetration of bile and cerebrospinal fluid, the requirement for twice-daily dosing, and pain on intramuscular administration that effectively limits vancomycin to the intravenous route, there was scope for the introduction of a second glycopeptide antibiotic in which these deficiencies were eliminated or reduced; accordingly, teicoplanin was marketed in Europe in the early 1990s. Teicoplanin has the same mode of action and antimicrobial spectrum as vancomycin, as well as a similar chemical structure, but, crucially, teicoplanin possesses more fatty acid side chains which (1) make the molecule more acidic, thereby permitting the formulation of a sodium salt that can be given both by intravenous and intramuscular injection, and (2) make teicoplanin more lipophilic, which affords better tissue penetration and a longer half-life; as a consequence, teicoplanin is normally administered once daily rather than twice. Other advantages over vancomycin are a slightly higher potency against most target organisms and a better toxicity profile, thereby eliminating the need for *routine* blood monitoring.

9 Antitubercular antibiotics

The antibiotics used for the treatment of tuberculosis belong to a variety of chemical classes, but it is convenient to consider them together in the same section for two reasons: first, most of them are used exclusively for tuberculosis, and second, therapy extends over several months, during which time resistance development is a significant possibility. This risk is minimized by the use of two or three antibiotics in combination, so it is appropriate to consider them together because that represents their normal pattern of use.

Streptomycin (Section 7), introduced in the late 1940s, was the first effective treatment for tuberculosis, but its use in isolation was short lived because of the ease with which the bacteria became resistant to a single antibiotic. Isoniazid, which became available about 5 years later, was used first in combination with streptomycin then with rifampicin after the latter was introduced in 1967. The combination of isoniazid and rifampicin has been the mainstay of tuberculosis therapy from that time until the present day, although other drugs like pyrizinamide and ethambutol have been added to the combination, and multidrug-resistant tuberculosis has become an increasing problem in recent years; this is, by definition, simultaneous resistance to isoniazid and rifampicin.

Tuberculosis may be caused by any one of three *Mycobacterium* species, but all three characteristically grow slowly in the body and may persist for long periods in a near dormant state. Because the course of therapy is normally 4–6 months, antibiotics that are orally effective are much preferred; this is because unsupervised patients are more likely to take oral medicines, and in many countries where patients may have difficulty travelling to clinics the daily administration of injectable antibiotics is not feasible. The ideal antitubercular drug should also have the potential to kill rather than merely inhibit the growth of the infecting organism. Because mycobacteria can survive, and even reproduce, within macrophages, relying on the immune system to eradicate an infection following the use of a bacteristatic drug is unlikely to be an effective strategy. Rifampicin kills dormant bacteria, while pyrizinamide is active against bacteria that are slowly reproducing in acidic environments; isoniazid is most useful against more rapidly growing cells.

The current approach is to treat tuberculosis in two phases: an initial phase of 2 months using isoniazid, rifampicin and pyrizinamide (with or without

ethambutol), and a 4 month continuation phase with isoniazid and rifampicin. If, however, the infecting organism is resistant to any of the above, second-line drugs may be used and the duration of treatment possibly extended. The status of streptomycin is equivocal; it is now rarely used in the UK and is not a first-choice treatment recommendation of the European Respiratory Society either, but it is more commonly used in front-line therapy in the USA. Second-line drugs available for infections caused by resistant organisms, or when first-line drugs cause unacceptable side effects, include amikacin, capreomycin, cycloserine, newer macrolides (e.g. azithromycin and clarithromycin) and moxifloxacin. Drug regimens are often indicated using a shorthand notation with single letters to indicate the drugs employed, initial numbers indicating months of therapy and following numbers (which may be in parentheses or subscripts) indicating days per week. For example, 2RHZ(E)/4HR(3) or 2RHZ(E)/4HR$_3$ would mean 2 months of rifampicin, isoniazid and pyrizinamide (with the possible addition of ethambutol) followed by 4 months of isoniazid and rifampicin three times per week. Several of the antitubercular drugs have specific contraindications or require monitoring during use; some of these requirements and cautions are shown in Table 11.6.

Rifampicin is the only one of the common antitubercular drugs that is used in the treatment of non-mycobacterial infections. It is active against Gram-positive bacteria and some Gram-negative species, but not Enterobacteriaceae or pseudomonads. Rifampicin possesses significant bactericidal activity at very low concentrations against staphylococci. Unfortunately, resistant mutants may arise very rapidly, both *in vitro* and *in vivo*, so it has been recommended that rifampicin should be combined with another antibiotic, e.g. vancomycin, in the treatment of staphylococcal infections. Rifabutin, a semisynthetic rifamycin, may be used in the prophylaxis of *M. avium* complex infections in immunocompromised patients and in the treatment, with other drugs, of pulmonary tuberculosis and non-tuberculous mycobacterial infections.

10 Newer antibiotics for MRSA and other Gram-positive cocci infections

It is not only MRSA that has become a major problem organism in recent years; VRE and penicillin-resistant *Strep. pneumoniae* (pneumococci) have also become much more prevalent, prompting fears of serious treatment problems due to the small number of antibiotics that remained effective. The emergence of *Staph. aureus* strains which showed intermediate sensitivity to vancomycin or to glycopeptides in general (designated VISA or GISA respectively) caused particular concern, and accelerated the introduction of three new antibiotics intended primarily for the treatment of Gram-positive cocci infections.

Table 11.6 Properties of selected antitubercular antibiotics

Antibiotic	Status	Route	Properties
Isoniazid	1	O, IM or IV	S/E of peripheral neuropathy, preventable with pyridoxine
Rifampicin	1	O or IV	Colours the urine red; induces liver enzymes so oral contraception is less effective
Pyrizinamide	1	O	Used with caution in patients with poor liver function
Ethambutol	1	O	Visual acuity check required before therapy and plasma drug monitoring required during use
Streptomycin	2	IV	Plasma drug monitoring required during use
Capreomycin	2	IM	Hearing and balance function and serum potassium checks recommended before and during treatment
Cycloserine	2	O	Avoided in patients with a history of epilepsy, neurological and psychiatric problems

1, first-choice therapy; 2, second-choice therapy; IM, intramuscular; IV, intravenous; O, oral.

The first of these, approved in 1999, is a fixed-ratio combination of the two streptogramin antibiotics, dalfopristin and quinupristin (30:70). This bactericidal intravenous product is recommended for the treatment of serious infections with vancomycin-resistant *Ent. faecium* and multiresistant strains of staphylococci and pneumococci. The second, linezolid (2001), is an orally active, synthetic antimicrobial possessing an essentially bacteristatic action against Gram-positive organisms, with no useful activity against Gram-negatives. It is used for the treatment of MRSA, VRE and pneumococcal infections, and although isolated early reports of resistance did arise, the development of resistance during treatment has been rare. Daptomycin (2003) is a lipopeptide with a novel mode of bactericidal action destabilizing the bacterial cell membrane, which means that cross-resistance with existing drugs is unlikely to arise. It is administered intravenously and used for skin and soft tissue infections by Gram-positive organisms and for *Staph. aureus* endocarditis; it is particularly useful for infections by VISA and GISA strains of this species.

11 Miscellaneous antibacterial antibiotics

11.1 Clindamycin

Clindamycin (Figure 11.11A) is another antibiotic possessing significant bacteristatic activity towards Gram-positive cocci (including MRSA, but not *Ent. faecalis*), although rather less activity is shown towards Gram-negative cocci and none at all against enterobacteria. Clindamycin is not related structurally to the macrolides (section 4) but has a similar mechanism of action, so cross-resistance may occur between them. The streptogramins also have a similar mechanism of action, but it is claimed that the quinupristin-dalfopristin combination does not demonstrate cross-resistance to clindamycin or the macrolide antibiotics.

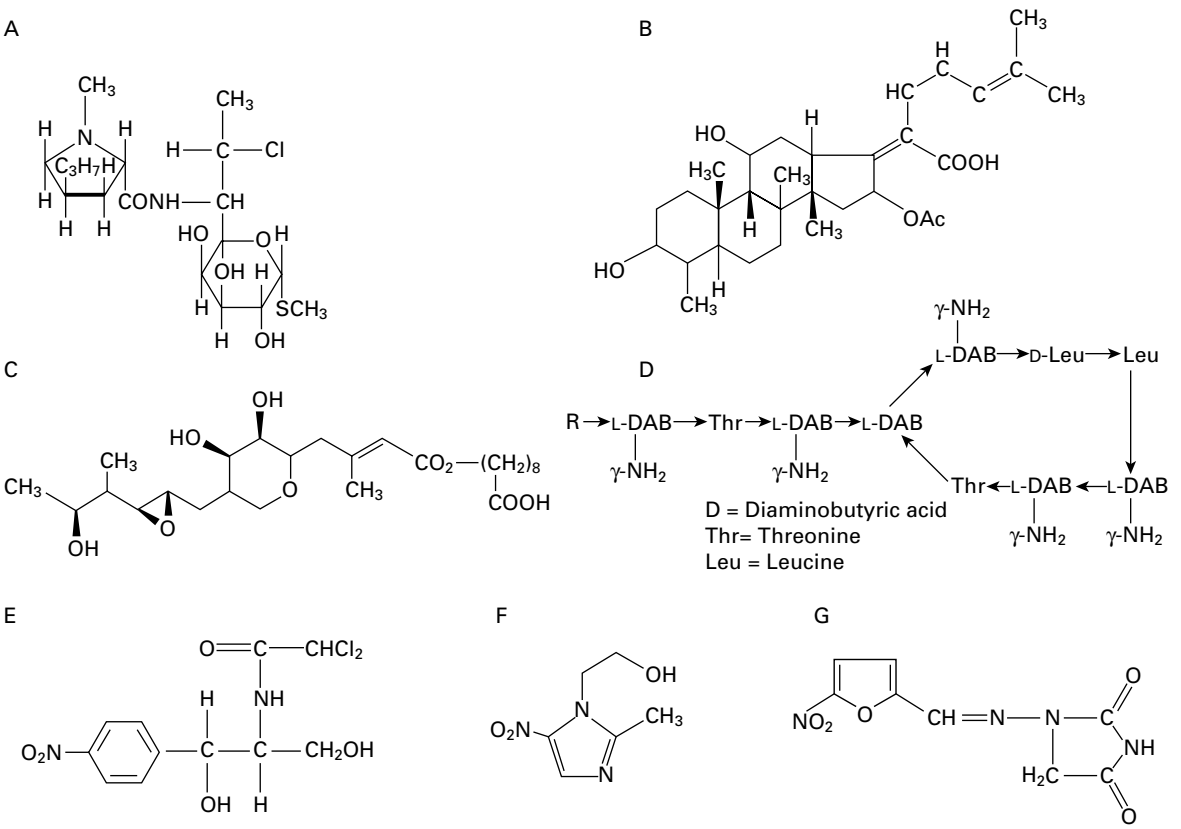


Figure 11.11 Miscellaneous antibiotics: (A) clindamycin; (B) fusidic acid; (C) mupirocin; (D) colistin; (E) chloramphenicol; (F) metronidazole; (G) nitrofurantoin.

Clindamycin is currently recommended for the oral treatment of staphylococcal bone and joint infections, acne, peritonitis, infections by anaerobic bacteria and falciparum malaria. Together with oral cephalosporins, it is one of the antibiotics most firmly associated with pseudomembranous colitis caused by *Cl. difficile*, and this reputation limits its use.

11.2 Fusidic acid

Fusidic acid (Figure 11.11B) is a steroid-like, bactericidal antibiotic used primarily for its activity against staphylococci. It does possess activity against other Gram-positive species, although streptococci are relatively resistant and Gram-negative bacilli completely so. It is active against penicillin-resistant strains of *Staph. aureus*, including MRSA, and may be administered in combination with erythromycin or clindamycin for severe staphylococcal infections. Fusidic acid is available as a paediatric oral suspension, cream and ointment, and, in the form of its water-soluble sodium salt, as tablets or an intravenous injection. Resistance arises with relative ease *in vitro*, but despite this, resistance is relatively uncommon in clinical isolates.

11.3 Mupirocin

Mupirocin (Figure 11.11C) is an antibiotic active against staphylococci, streptococci and a limited range of Gram-negative species. Its use is largely confined to topical treatment of *Staph. aureus* infections, particularly the eradication of MRSA from the nose; for this purpose it is claimed normally to be more effective than chlorhexidine or fusidic acid.

11.4 Colistin

Colistin (Figure 11.11D) is the only member of the polymyxin group of peptide antibiotics that is still in use. It is active against many types of Gram-negative bacteria, but not against cocci, *Serratia marcescens* and *Proteus* spp.; it is inactive against Gram-positive organisms. Its use is largely restricted to the treatment of *Ps. aeruginosa* lung infections in cystic fibrosis sufferers, in which case it is nebulized or given by intravenous injection, and in the treatment of infections, particularly in burns, caused by *Acinetobacter* species.

11.5 Chloramphenicol

Chloramphenicol (Figure 11.11E) is a true antibiotic but is manufactured totally by chemical synthesis. It has a broad spectrum of activity including some rickettsias and larger viruses, but aplastic anaemia, which is dose-related, may result from treatment in a proportion of patients and this has largely restricted its use as a systemic antibiotic to life-threatening infections with *H. influenzae*. It is, however, still used significantly in the treatment of ophthalmic infections and in veterinary medicine.

11.6 Metronidazole and other nitroimidazoles

The nitroimidazoles are a group of synthetic antimicrobials that are unusual in possessing activity against a wide range of organisms including bacteria, protozoa, and some helminths. They are similar in terms of their structure, mode of action, uses and toxicity and the principal factor distinguishing them is their pharmacokinetics. Metronidazole (Figure 11.11F) is by far the most commonly used, and is the only one considered in this section, although nimorazole (no longer available in the UK) and tinidazole are alternative drugs that may afford the advantage of less frequent dosing. They may all be regarded as prodrugs in the sense that they become active only after reduction of the nitro group in low redox environments, so they are able to kill cells growing anaerobically by damaging their DNA. This damage could, in theory, occur in any type of cell regardless of its taxonomic status, and the fact that sufficiently reducing conditions do not arise in mammalian cells is the reason for the drugs' lack of toxicity for humans.

Metronidazole was introduced in 1960 for the treatment of vaginitis caused by the protozoan *Trichmonas vaginalis*, and its wider application for the treatment of bacterial anaerobic infections was recognized later. Currently metronidazole is used alone, or quite commonly in combination with other antibiotics, for the treatment of a wide variety of bacterial infections shown in Table 11.7. For the treatment of amoebiasis, giardiasis or trichomonal vaginitis, metronidazole is often used alone. Metronidazole is available in more dosage forms (oral, topical, injectable or suppositories) than most other antibiotics, and by the oral route is often given two or three times daily. Toxicity and side effects are relatively uncommon, although it does exhibit a disulphiram-like reaction with alcohol in some patients.

11.7 Nitrofurantoin

Nitrofurantoin (Figure 11.11G) is the one remaining member of the nitrofuran group of drugs that is still in common use. Like metronidazole, nitrofurantoin requires its nitro group to be reduced in order to exhibit antimicrobial activity, and it too exhibits bactericidal activity by damaging DNA. It has a wide spectrum of activity which

Table 11.7 Some common bacterial infections for which metronidazole may be used

Bacterial infection	Examples of causative organism(s)	Drugs with which metronidazole may be combined
Vaginosis	*Gardnerella vaginalis*	Often used alone
Gastroduodenal ulcers	*Helicobacter pylori*	Clarithromycin and amoxicillin
Dental infections (gingivitis)	*Streptococcus* and *Fusobacterium* species	Used alone or with amoxicillin
Gut flora reduction prior to surgery	*Bacteroides* spp. and other colon bacteria	Gentamicin or cefuroxime
Infected bedsores or tumours	Various anaerobes	Quinolones
Pelvic inflammatory disease	*Neisseria gonorrhoea* and *Chlamydia trachomatis*	Doxycycline + ceftriaxone, or ofloxacin

includes Gram-positive cocci and many Gram-negative enteric bacteria, but after oral administration the blood levels achieved are very low and a significant fraction of the dose is rapidly excreted in the urine, so its use is restricted to the treatment of cystitis. The size of the drug crystals used in tablet manufacture has an effect on the dissolution of the drug, and it is claimed that the macrocrystalline form affords steadier release. Its antimicrobial activity is substantially greater in acid urine, which unfortunately conflicts with the common symptomatic treatment of cystitis by alkalizing the urine with potassium citrate or similar compounds. Nitrofurantoin is unusual in being one of the few antimicrobial drugs to which resistance has not significantly increased since its introduction, and for this reason there is a degree of renewed interest in it.

12 Antifungal antibiotics

There has been a significant increase in the number of both systemically and topically acting antifungal agents in recent years; this has been prompted in part by the increase in patients with impaired immunity who are particularly vulnerable to such infections. For much of the second half of the 20th century nystatin, amphotericin and griseofulvin were the principal antifungal antibiotics available, and these were supplemented with a range of synthetic imidazoles which were used primarily for superficial rather than systemic fungal infections. The introduction of the triazole antifungals in the 1980s was a major advance and, more recently, the echinocandins have further increased the range of drugs available for severe infections.

Lack of toxicity is, as always, of paramount importance, but the differences between bacterial and fungal cells in both structure and biosynthetic processes mean that the low-toxicity antibacterial antibiotics are usually inactive against fungi. This limitation is further complicated by the fact that both fungal and human cells are eukaryotic in structure, which means that there are few differences that can be exploited in order to achieve selective toxicity towards fungi whilst leaving the human cells unharmed. Fungal infections are normally less virulent in nature than are bacterial or viral ones but may, nevertheless, pose major treatment problems in individuals with a depressed immune system, particularly in the case of systemic infection.

12.1 Azoles

The azoles may be considered as two subgroups: the older imidazole drugs, the majority of which were introduced as topical products or pessaries for the treatment of superficial infections by dermatophytes (skin pathogens), *Pityriasis* species (causing flaky skin and dandruff) and *C. albicans*, and the more recently developed, more versatile and, in some cases, much more expensive, triazoles.

12.1.1 Imidazoles

The imidazoles are a large and diverse group of compounds with activity against bacteria and protozoa (metronidazole and tinidazole), helminths (mebendazole) and fungi (clotrimazole, miconazole, ketoconazole, econazole, sulconazole and tioconazole). Table 11.8 lists infections

Table 11.8 Antifungal imidazoles

Drug	Common formulations	Uses
Clotrimazole	T, P, VC, Pdr, S; Soln	Dermatophytes, pityriasis or *Candida* in the skin, vagina or ear
Miconazole	T, P, VC, Soln, Pdr	Oral, intestinal, skin or vaginal *Candida* infections, dermatophytes and pityriasis
Econazole	T, P	Dermatophytes , pityriasis and skin or vaginal *Candida*
Sulconazole	T	Dermatophytes , pityriasis and skin or vaginal *Candida*
Ketoconazole	T, Sham, Tab	Oral systemic treatment of dermatophytes or *Candida* resistant to other drugs or patients intolerant to them
Tioconazole	NS	Fungal infections of finger and toe nails

NS, nail solution; P, pessary; Pdr, powder; S, spray; Sham, shampoo; Soln, solution or gel for external or oral use; T, topical cream or ointment; Tab, tablets; VC, vaginal cream.

for which the common antifungal drugs are employed; other imidazoles are available in various countries.

As Table 11.8 indicates, the imidazoles are available in a wide variety of dosage forms, but most of them have the same uses. They exhibit modest activity against some Gram-positive bacteria, but they are all essentially fungistatic drugs which might exert fungicidal action during prolonged exposure to high concentrations. Miconazole may be given orally for the treatment of intestinal fungal infections, and in this situation may show some absorption into the systemic circulation, although this may be more of a problem from the perspective of potential drug interactions than a benefit. Ketoconazole, however, achieves greater absorption from the gastrointestinal tract, but it has been largely superseded by newer and less toxic drugs so its use tends to be restricted to situations where resistance or patient intolerance to those drugs is a problem.

12.1.2 Triazoles

Fluconazole (Figure 11.12A) and itraconazole were introduced in the 1980s and posaconazole and voriconazole much more recently. The two earlier drugs are more widely used, whilst the recent ones tend to be reserved for severe, possibly life-threatening infections, in which other antibiotics have failed or are inappropriate. All four of the triazole drugs are orally active and all but posaconazole are available in injection form.

Fluconazole is better absorbed from the gastrointestinal tract than itraconazole and, in addition to the treatment of dermatophytes, pityriasis and *Candida* infections, it is valuable in cases of cryptococcosis which, although uncommon, is very dangerous in immunocompromised patients. Itraconazole is used for similar infections to fluconazole but, in addition, it is more commonly selected as an alternative to amphotericin (see below) in cases of systemic *Aspergillus* infection (again, more common in the immunocompromised) and other rare systemic mycoses. It is more frequently associated with liver toxicity than fluconazole.

12.2 Polyenes

Polyene antibiotics are characterized by possessing a large ring containing a lactone group and a hydrophobic region consisting of a sequence of four to seven conjugated double bonds. The only important polyenes are amphotericin B and nystatin.

Amphotericin B (Figure 11.12C) is active against most fungal pathogens and is used for systemic mycoses as a potentially more toxic, but possibly more effective, alternative to itraconazole. It is poorly absorbed from the gastrointestinal tract and is thus usually administered by intravenous injection under strict medical supervision. Lipid-based and liposomal formulations of amphotericin are available which exhibit lower toxicity than conventional aqueous formulations; they may therefore be given in higher doses. Nystatin is administered orally in the treatment of *C. albicans* infections in the intestine or the mouth (often referred to as thrush), and by cream or pessary for skin or vaginal infections by that organism. It is rarely used for the treatment of other infections and is too toxic to be given by injection.

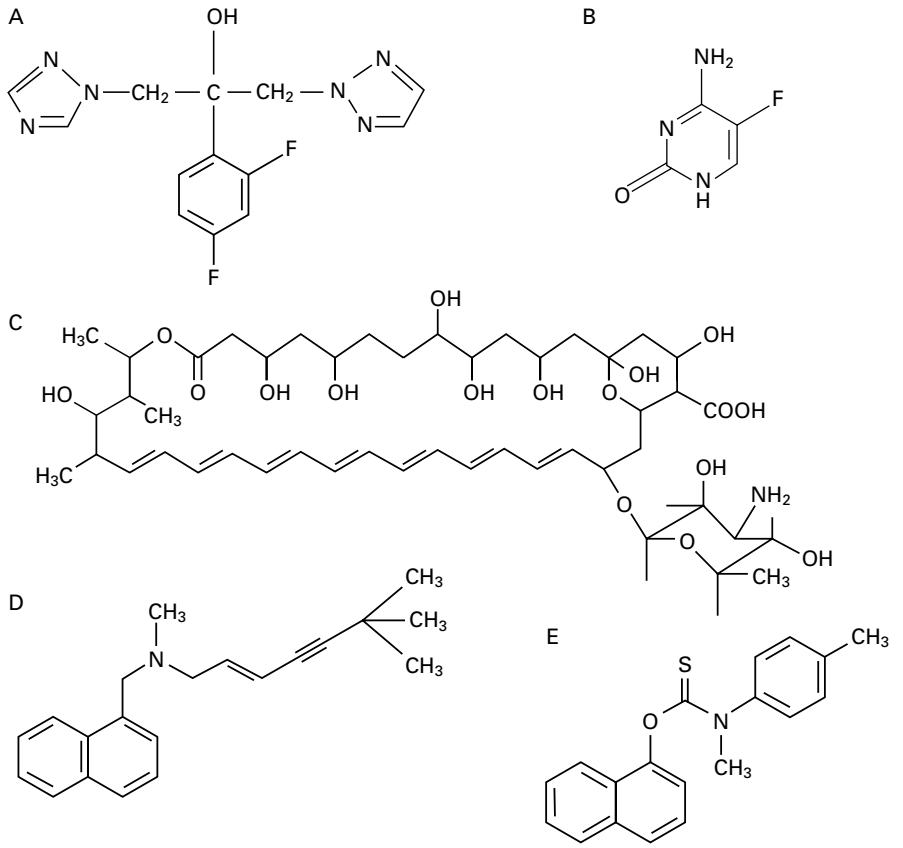


Figure 11.12 Antifungal agents: (A) fluconazole; (B) flucyctosine; (C) amphotericin; (D) terbinafine; (E) tolnaftate.

12.3 Echinocandins

The echinocandins are a new class of semisynthetic lipopeptide antibiotics that are fungicidal towards *Aspergillus* spp., *Candida* spp. and *Pneumocystis jirovecii* (previously known as *P. carinii*). Caspofungin, the first member of the class, is given as an intravenous infusion recommended for invasive aspergillosis or candidiasis that is unresponsive to treatment with amphotericin or itraconazole. Anidulafungin and micafungin are similarly injectable products licensed for the treatment of invasive candidiasis, and they, like caspofungin, tend to be used as reserve drugs, both to minimize the risk of resistance development and due to cost considerations.

12.4 Other antifungal agents

Flucytosine (5-fluorocytosine, Figure 11.12B) is a narrow-spectrum antifungal agent with greatest activity against yeasts such as *Candida*, *Cryptococcus* and *Torulopsis*. It is normally used in combination with fluconazole or as a synergistic combination with amphotericin which permits amphotericin dose reduction and a lower risk of toxicity. Terbinafine (Figure 11.12D), a member of the allylamine class of antimycotics, is orally active, fungicidal, effective against a broad range of dermatophytes and yeasts and is the drug of choice for fungal nail infections. Griseofulvin too is employed for the treatment of dermatophyte infections of hair, skin and nails, but usually only when topical therapy has failed, and with the exception of *Trichophyton* infections in children, it is no longer regarded as a drug of choice. It is orally active, although the particle size of the powder used to manufacture the tablets has a significant effect on bioavailability. Tolnaftate (Figure 11.12E) is a synthetic thiocarbamate which is used topically in the treatment or prophylaxis of tinea (commonly referred to as ringworm, although not due to worms at all) and amorolfine is used as a cream or nail lacquer for the same purpose.

13 Antiviral drugs

Most of the antibacterial and antifungal agents described earlier in this chapter have little or no activity against viruses because they target structures or enzyme systems that are only found in bacterial and fungal cells. In contrast to other microorganisms, viruses do not possess the enzymes necessary for their own replication. After entry into the host cell, the virus uses the enzymes already present, or induces the formation of new ones, in order to synthesize the individual components of the virus particle which are then assembled and released from the host cell. Because viruses literally 'take over' the machinery of an infected human cell, there are very few unique features of viral replication that can be exploited for the purposes of achieving selective toxicity, i.e. creating antiviral agents that inhibit or kill the virus without harming the human host.

Prior to the identification of the HIV virus in 1983 there was a very limited range of effective synthetic antiviral agents; the fourth edition of this book, published in 1987, described only nine. The HIV/AIDS pandemic provided a major stimulus for fundamental research into the structure and reproduction of viruses in general and retroviruses in particular, and this, together with (1) better understanding of the role played by some viruses in the development of specific cancers, (2) more sophisticated diagnostic methods, and (3) elucidation of the genomes of several viruses, has led to a wealth of new antiviral drugs; Table 11.9 lists 21, and a further 20 that are used largely or exclusively for the treatment of HIV are shown in Table 11.10. Although these new drugs may be categorized on the basis of their chemical structure (Figure 11.14) or mode of action, most of them are licensed for use against a limited number of viruses, and often just a single one, so the most convenient and useful way of considering antivirals is on the basis of the infections they are intended to treat.

13.1 HIV

There is a large and progressively increasing variety of antiretroviral agents available to treat HIV, and their use requires specialist knowledge. A detailed account of the characteristics of each individual drug is beyond the scope of this chapter, but it is possible to gain a

Table 11.9 Antiviral drugs used in the treatment of selected viral infections

Herpes	Cytomegalovirus	Viral hepatitis	Influenza	Respiratory syncytial virus
Aciclovir	Cidofovir	Adefovir	Amantadine	Palivizumab
Famciclovir	Ganciclovir	Entecavir	Oseltamivir	Ribavirin
Penciclovir	Foscarnet	Lamivudine	Zanamivir	
Valaciclovir	Valganciclovir	Telbivudine		
Inosine Pranobex		Tenofovir		
Idoxuridine		Interferon-α		

Table 11.10 Mechanisms of action of common antiretroviral drugs

Mechanism of action	Examples
Nucleoside reverse transcriptase inhibitors (nucleoside analogues)	Abacavir, didanosine, emtricitabine, lamivudine, stavudine, tenofovir, zidovudine
Protease inhibitors	Atazanavir, darunavir, fosamprenavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir, tipranavir
Non-nucleoside reverse transcriptase inhibitors	Efavirenz, etravirine, nevirapine
Miscellaneous agents with unique mechanisms of action	Enfuvirtide, maraviroc, raltegravir

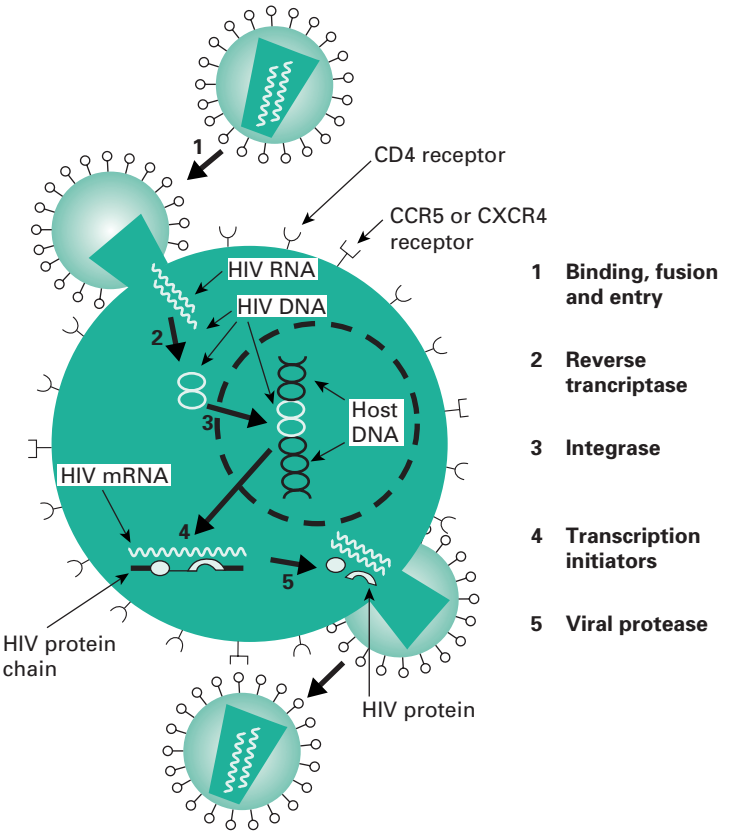


Figure 11.13 HIV life cycle showing stages vulnerable to antiretroviral drugs.

good understanding of the principles of HIV chemotherapy by considering the life cycle of the virus (Figure 11.13) in relation to the modes of action of the drugs in current use.

The virus particle initially binds to a CD4 protein receptor and one of two coreceptors on the surface of a T-lymphocyte. Maraviroc, an antagonist of the CCR5 coreceptor, is licensed in the UK for the treatment of patients exclusively infected with CCR5-tropic HIV. The bound virus then fuses with the host cell membrane and the viral RNA is released into the cell. This step is targeted by enfuvirtide, a fusion inhibitor, that is used for managing infection that has failed to respond to a regimen of other antiretroviral drugs. The single-stranded viral RNA is used as a template from which a complementary DNA strand is manufactured by viral reverse transcriptase; there are several nucleoside analogue inhibitors of this enzyme (Table 11.10), together with a smaller number of non-nucleoside inhibitors (e.g. efavirenz, etravirine, and nevirapine). The DNA is duplicated, and in its double-stranded form it enters the cell nucleus where an HIV enzyme integrates it into the host DNA to create what is termed a provirus. The integrase enzyme can be inhibited by raltegravir, a drug which again is largely reserved for the treatment of HIV infection resistant to multiple antiretrovirals.

The provirus may remain latent (dormant) within the cell nucleus for a period of time varying from 2 weeks to 20 years; it is then activated by regulatory proteins termed transcription initiators which cause the viral DNA to be transcribed into viral mRNA and several long protein molecules. It is the latent provirus that represents the major hurdle to complete eradication of HIV, and recent research interest has focused on the transcription initiators as alternative potential targets for antiretroviral drug action. The long proteins only become functional after being split into smaller molecules by viral protease enzymes and there are now many protease inhibitor drugs available for the treatment of HIV (Table 11.10).

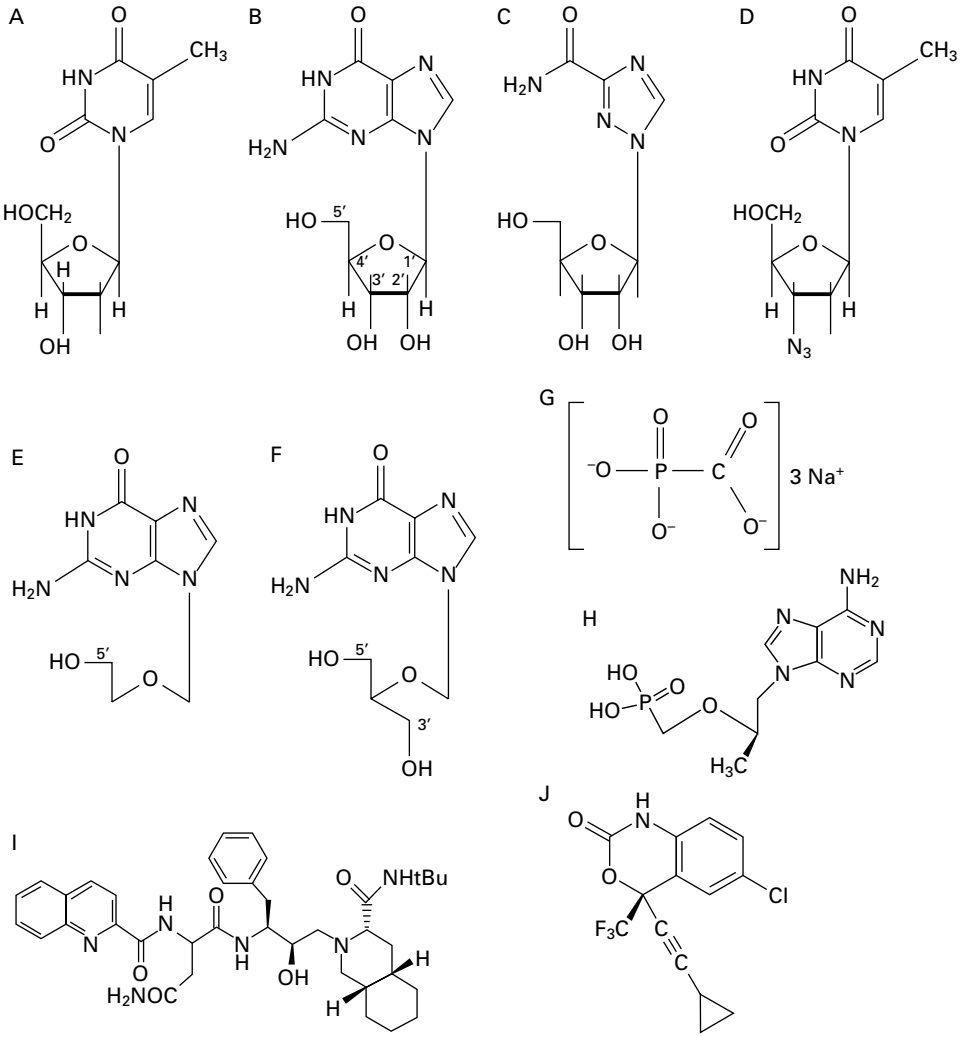


Figure 11.14 Structures of nucleosides and selected antiviral agents: (A) thymidine; (B) guanosine; (C) ribavirin; (D) zidovudine; (E) aciclovir; (F) ganciclovir; (G) foscarnet; (H) tenofovir; (I) saquinavir; (J) efavirenz.

HIV infection cannot be cured, but strict adherence to a regimen of antiretroviral drugs can substantially extend survival. However, there are several problems that may arise during therapy, of which one of the most significant is the risk of the virus becoming resistant. This risk may be minimized by using combinations of three or more drugs with different mechanisms of action in regimens that have become known as *highly active antiretroviral therapy* (HAART). Treatment is normally initiated with two nucleoside reverse transcriptase inhibitors and a non-nucleoside reverse transcriptase inhibitor; the regimens currently recommended in the UK contain *either* tenofovir, emtricitabine and efavirenz, *or* abacavir, lamivudine, and efavirenz. Retinovir, used in low concentrations at which it has no intrinsic antiviral activity, has been shown to increase the duration of effective blood concentrations of almost all the other protease inhibitors listed in Table 11.10, and these synergistic combinations have given rise to the term *boosted protease inhibitor*; a combination product of retinovir and lopinavir is commercially available. Regimens containing two nucleoside reverse transcriptase inhibitors and a boosted protease inhibitor are reserved for patients with resistance to first-line treatment. Synergy is often observed between antiret-

roviral drugs, both between agents having the same, and different, modes of action.

Other problems of HIV therapy are drug toxicity and patient adherence to their prescribed medication, and these two are often linked. The variety and severity of the side effects, particularly those relating to redistribution of body fat, make it more difficult for patients to achieve the adherence and persistence required for effective treatment. All the common drugs used in therapy are orally active because any drug that required lifelong daily injections would so predispose to non-adherence as to prejudice its commercial viability. Several of the antiretroviral drugs listed in Table 11.10, particularly those with unique mechanisms of action, are used in such restricted circumstances that they are extremely expensive.

13.2 Herpes and cytomegalovirus infections

There are eight herpesviruses capable of causing human infection, but of these the most important are:

- the two *herpes simplex viruses*, HSV-1 and HSV-2, which, respectively, cause cold sores on the face and lips, and genital herpes
- the *varicella zoster virus* causing chickenpox and shingles
- the *Epstein–Barr virus* responsible for infectious mononucleosis (glandular fever)
- the *cytomegalovirus* (CMV) which may cause retinitis (inflammation of the retina) and, infrequently, similar symptoms to infectious mononucleosis.

The first four of the drugs listed in Table 11.9 for the treatment of herpes infections are the most important. Inosine pranobex is an orally active immunomodulator the effectiveness of which has not been proven, and idoxuridine, a pyrimidine analogue used as a solution in dimethylsulphoxide for the treatment of cutaneous herpes, has largely been superseded by aciclovir.

Many antiviral drugs are nucleoside analogues, and aciclovir together with its prodrug valaciclovir, and penicyclovir and *its* prodrug famciclovir, are important examples of this group. Aciclovir and peniciclovir are structurally very similar and they act in the same way both to inhibit viral DNA polymerase and to cause premature termination of DNA synthesis. Both drugs are only effective when they have been phosphorylated in the cell, and selective toxicity arises because phosphorylation is achieved much more efficiently by virus-encoded thymidine kinase than by the corresponding mammalian enzyme, so human cell DNA synthesis is little affected. All four drugs are used primarily to treat herpes simplex and varicella zoster infections; CMV is normally resistant to them and Epstein–Barr virus shows intermediate sensitivity. Aciclovir is available as an intravenous injection, tablets and a cream, but the last two of these need to be administered five times daily in order to maintain effective levels. Its prodrug valaciclovir has a much longer half-life and the dose frequency is correspondingly reduced to 2–3 times a day. Peniciclovir has little oral activity and is used topically, primarily for cold sores, but it is applied every 2 hours during waking hours. The orally active famciclovir is taken between one and three times daily for the treatment of genital herpes and shingles.

Because CMV does not produce thymidine kinase it is not normally susceptible to aciclovir and peniciclovir, so a different group of drugs is used to treat it. Ganciclovir has a similar structure to aciclovir but it is phosphorylated more effectively in infected cells than healthy ones, albeit by a host cell enzyme. It is injected intravenously for the treatment of life-threatening or sight-threatening CMV infections in immunocompromised patients only, or for the prevention of CMV infection following organ transplants. Valganciclovir is an expensive, orally active, valine ester prodrug of ganciclovir which is used in similar circumstances. Both drugs are toxic, and the latter carries a specific warning in the UK British National Formulary about potential teratogenic and carcinogenic activity. Cidofovir is a cytosine analogue that is active against most herpesviruses, and is used by injection in AIDS patients to treat CMV retinitis that is unresponsive to other drugs. Foscarnet, too, is given for CMV retinitis when ganciclovir cannot be used; it has a relatively simple phosphonoformic acid structure capable of chelating metal ions, which is thought to be the basis of its inhibitory action on polymerase enzymes.

13.3 Viral hepatitis

Hepatitis, inflammation of the liver, can be caused by various drugs and toxins, but hepatitis due to viral infection is more common. The viruses most frequently responsible are the hepatitis viruses A–E (which are not all related), but about 5% of cases of viral hepatitis are due to other viruses, e.g. herpesvirus, CMV, Epstein–Barr virus, etc. Hepatitis virus A (formerly known as infectious hepatitis) is a self-limiting, rarely fatal, food-borne infection that does not result in permanent liver damage and is not normally treated with antiviral drugs. Hepatitis E is similarly self-limiting and relatively uncommon, and hepatitis D can only arise as a co-infection with the

hepatitis B virus, so it is hepatitis viruses B and C (HBV and HCV respectively) that are the most problematic and which require antiviral therapy.

It has been estimated that approximately one third of the world's population are infected with HBV and just over one tenth of that number with HCV. The two infections have several features in common, although the viruses are not of the same family. In both cases the disease may be acute or chronic and in the latter case there may be progression to liver damage and a higher risk of contracting liver cancer. Acute HBV cases do not normally receive antiviral drugs, and that was formerly the case for HCV also, but recent evidence suggests that early treatment of HCV has a higher success rate and shorter treatment time than that required for chronic disease, so this practice is now more common.

HBV is unusual in that it is not a retrovirus like HIV but it does use reverse transcriptase in its replication process. For this reason two of the drugs used to treat HIV infection, lamivudine and tenofovir, are also effective for HBV; in addition, adefovirdipivoxil, entecavir and telbivudine are also used, typically for 6 months or more for all five drugs. In patients with decompensated liver disease (liver cirrhosis with fluid accumulation in the abdomen) lamivudine or adefovir are recommended. Adefovir is effective in lamivudine-resistant HBV infection, but telbivudine should not be used because cross-resistance may arise. Entecavir is effective in patients not previously treated with nucleoside analogues,but resistance can occur in patients who have received lamivudine. Again, all of these antivirals are available as relatively expensive, oral products.

There are several genotypes of HCV which exhibit different drug sensitivities, so the genotype should be determined before drug selection. Interferon-α is used together with ribovirin for the treatment of chronic infection by both HCV and HBV. Interferons are low molecular weight proteins produced by virus-infected cells that themselves induce the formation of a second protein inhibiting the transcription of viral mRNA. Interferon-α needs to be injected on a daily basis or at least three times weekly, but in its polyethylene glycol-derivatized form (peginterferon-α2a) it is much longer acting and requires less frequent dosing. The combination of ribavirin and interferon-α is less effective against HCV than the combination of peginterferon-α2a and ribavirin. Peginterferon-α2a alone should be used if ribavirin is contraindicated or not tolerated, but ribavirin alone is ineffective.

13.4 Influenza and respiratory syncytial virus

There are three related influenza viruses, A, B and C, of which C is relatively rare and causes only mild infections. All three reproduce in the same way and possess the enzyme neuraminidase which is responsible for liberating the newly formed virus particles from the host cell. There are two neuraminidase inhibitors available, oseltamivir which is formulated as an ethyl ester prodrug for oral administration, and zanamivir which is administered by inhalation. Neither drug has activity against other viruses.

Both oseltamivir and zanamivir are most effective if taken within 48 hours of the onset of symptoms, when they may reduce the duration of the symptoms by about 1–1.5 days; they may also reduce the risk of complications in elderly patients or those with chronic diseases. They are useful too for postexposure prophylaxis of influenza, but again need to be administered within a few hours of exposure. Mutant strains of influenza viruses have developed resistance to oseltamivir but currently these are uncommon, and no such resistance has so far been observed with zanamivir.

Amantadine is a drug that inhibits an ion-channel protein in influenza A but not influenza B virus (which does not possess the target molecule). Again, it is effective if given soon after the onset of symptoms, but resistance has now become widespread so the drug, although still available, is no longer recommended in the UK.

Respiratory syncytial virus (RSV) is a commonly occurring virus related to those causing measles and mumps. It infects most infants by the age of 2 years, but unfortunately there is no long-lasting immunity following infection and no vaccine available. Ribavirin (Section 13.3) is one of the few antiviral agents used in the treatment of RSV, but its value is very much in doubt. Palivizumab is a monoclonal antibody used for preventing serious lower respiratory tract disease caused by RSV in children at high risk of the disease.

14 References and further reading

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12 Mechanisms of action of antibiotics and synthetic anti-infective agents

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Hugo and Russell's Pharmaceutical Microbiology, Eighth Edition. Edited by Stephen P. Denyer, Norman Hodges, Sean P. Gorman, Brendan F. Gilmore.

1 Introduction

The antibiotics and synthetic anti-infective agents described in Chapter 11 are used to treat infections caused by bacteria, fungi and protozoa. Most exert a highly selective toxic action on their target microbial cells but have little or no toxicity towards mammalian cells. They can therefore be administered at concentrations sufficient to kill or inhibit the growth of infecting organisms without damaging mammalian cells. By comparison, the disinfectants, antiseptics and preservatives described in Chapter 17 are too toxic for systemic treatment of infections. Figure 12.1 illustrates the five broad target areas of the major groups of antibiotics and synthetic agents used to treat microbial infections. Note that most of the agents are selective antibacterials; relatively few agents are available for treatment of fungal or protozoal infections. Study of the mechanism of action reveals the basis for the selective toxicity.

2 The microbial cell wall

2.1 Peptidoglycan biosynthesis in bacteria and its inhibition

Peptidoglycan is a vital component of the cell wall of virtually all bacteria. About 50% of the weight of the wall of Gram-positive bacteria is peptidoglycan; smaller amounts occur in mycobacterial walls (30%) and Gram-negative bacterial cell walls (10–20%). It is a macromolecule composed of sugar (glycan) chains cross-linked by short peptide chains (Figure 12.2). The glycan chains contain alternating units of *N*-acetylmuramic acid and *N*-acetylglucosamine. Each *N*-acetylmuramic acid contains a short peptide substituent made up of four amino acids (the stem peptides). A key feature of peptidoglycan is the occurrence of the D-isomers of some amino acids in the stem peptides (particularly D-alanine and D-glutamic acid) and unusual amino acids such as *meso*-diaminopimelic acid which are not found in proteins. In some organisms (e.g. *Escherichia coli*) cross-linking of the stem peptides involves a direct peptide bond between the fourth amino acid of the stem peptide on one chain and the third amino acid in the stem peptide on an adjacent chain. In other organisms (e.g. *Staphylococcus aureus*) the linkage is made by a short peptide bridge (e.g. five glycines) between the stem peptides. The precise composition of peptidoglycan varies between different organisms but the overall structure is the same.

In all cases the peptidoglycan plays a vital role: it is responsible for maintaining the shape and mechanical strength of the bacterial cell. If it is damaged in any way, and particularly if its synthesis is inhibited, then the shape of the cells becomes distorted, they swell and will eventually burst (lyse) as a result of the high internal osmotic pressure. Mammalian cells do not possess a cell wall and contain no other macromolecules resembling peptidoglycan. Consequently, antibiotics which interfere with the synthesis and assembly of peptidoglycan show excellent selective toxicity.

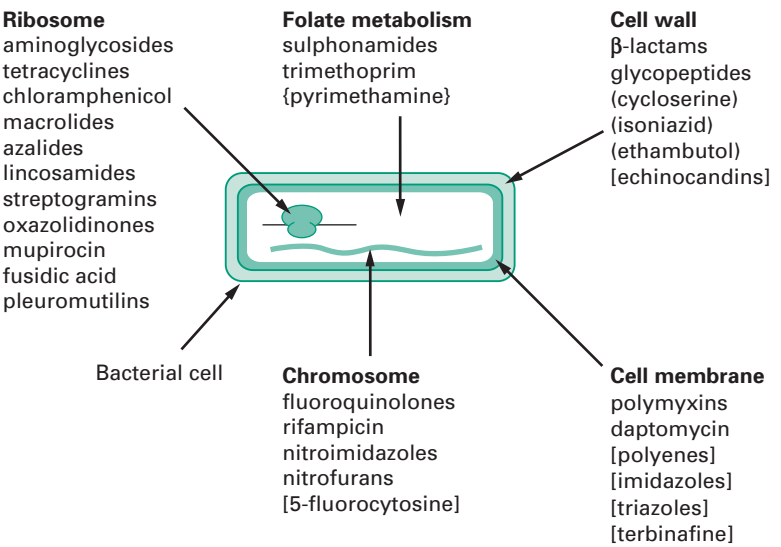


Figure 12.1 Schematic diagram of a typical bacterial cell showing the sites of action of the major classes of antibiotics and antimicrobial agents used to treat infections. Agents listed without brackets are used to treat bacterial infections. Agents used against mycobacterial, fungal or protozoal infections are indicated by the use of (), [] and {} brackets, respectively.

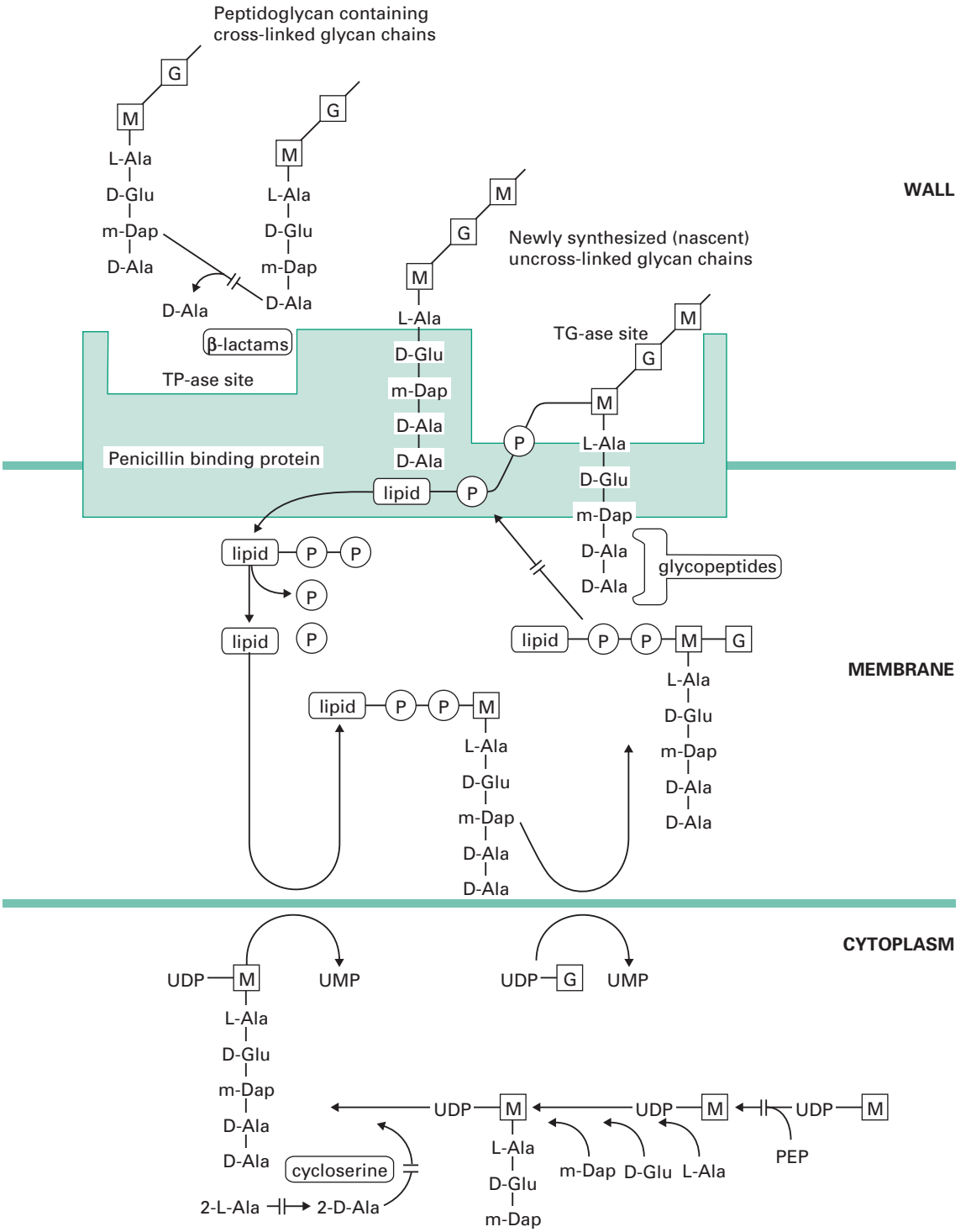


Figure 12.2 Pathway for the biosynthesis of peptidoglycan in bacterial cells showing the sites of action of cycloserine, glycopeptide and β-lactam antibiotics.

2.1.1 D-Cycloserine

D-Cycloserine interferes with the early stage of synthesis of peptidoglycan involving the assembly of the dipeptide D-alanyl-D-alanine. This occurs inside the cytoplasm and involves a racemase enzyme which converts L-alanine to D-alanine and a ligase which couples two D-alanines together (Figure 12.2). Both of these enzymes are inhibited by D-cycloserine, which bears some structural similarities to D-alanine. The antibiotic binds to the pyridoxal phosphate cofactor of the enzymes, effectively preventing them from forming D-alanyl-D-alanine. Subsequent stages of peptidoglycan synthesis, involving coupling of the dipeptide to three other amino acids forming the stem peptide on UDP-*N*-acetylmuramic acid, are blocked. Note that initially the peptide contains five amino acids, terminating in D-alanyl-D-alanine. The terminal D-alanine is removed on insertion into the cell wall during the final step in which the cross-links are formed between stem peptides on adjacent glycan strands.

2.1.2 Glycopeptides—vancomycin and teicoplanin

The peptidoglycan macromolecule is assembled in the cell wall by the sequential action of two enzymes (transglycosylases and transpeptidases) which are located on the outer face of the cytoplasmic membrane. To reach the assembly site (i.e. a region of cell wall growth) the precursors, which are assembled in the cytoplasm, must cross the cell membrane. They do this linked to a lipid, undecaprenylphosphate, which acts as a carrier molecule, cycling between the inner and outer faces of the membrane. The biochemical details of this process are outlined in Figure 12.2. Antibiotics interfering with this stage of peptidoglycan synthesis have been identified, e.g. bacitracin, but they have not found major applications in the treatment of infections.

The glycopeptides vancomycin and teicoplanin act at the stage where the peptidoglycan precursors are inserted into the cell wall by the transglycosylase enzyme on the outer face of the cell membrane. This enzyme assembles linear glycan chains that are not initially cross-linked to the existing peptidoglycan in the cell wall. The linear glycan chains are assembled by the transglycosylase by transfer of the growing glycan chain to the disaccharide peptidoglycan precursor on the lipid carrier as it crosses the cell membrane. Glycopeptides block this process by binding, not to the enzyme itself, but to the disaccharide peptidoglycan precursor, specifically to the D-alanyl-D-alanine portion on the stem peptide. The presence of the bulky glycopeptides tightly bound to each D-alanyl-D-alanine residue prevents the transglycosylase from carrying out the transfer reaction. Binding involves formation of a network of five hydrogen bonds between amino acid residues on the glycopeptide antibiotics and D-alanyl-D-alanine. Resistance to this unusual mechanism of enzyme inhibition can result from alteration in the D-alanyl-D-alanine substrate to D-alanyl-D-lactate, which occurs in glycopeptide (e.g. vancomycin)-resistant enterococci (VRE). Vancomycin does not penetrate the cell membrane of bacteria and is thought to bind to the disaccharide-pentapeptides on the outer face of the cytoplasmic membrane. It has been suggested that two vancomycin molecules form a back-to-back dimer which bridges between pentapeptides on separate glycan chains, thus preventing further peptidoglycan assembly. Teicoplanin also binds tightly to the D-alanyl-D-alanine region of the peptidoglycan precursor. However, as a lipoglycopeptide it may act slightly differently from vancomycin, by locating itself in the outer face of the cytoplasmic membrane and binding the pentapeptide as the precursors are transferred through the membrane. Glycopeptides must cross the cell wall to reach the outer face of the cell membrane where transglycosylation takes place. They are too large to penetrate the outer membrane of most Gram-negative bacteria and are consequently used for treatment of serious Gram-positive infections.

2.1.3 β-Lactams—penicillins, cephalosporins, carbapenems and monobactams

The final stage of peptidoglycan assembly is the cross-linking of the linear glycan strands assembled by transglycosylation to the existing peptidoglycan in the cell wall. This reaction is catalysed by transpeptidase enzymes, which are also located on the outer face of the cell membrane. They first remove the terminal D-alanine residue from each stem peptide on the newly synthesized glycan chain. The energy released from breaking the peptide bond between the two alanines is used in the formation of a new peptide bond between the remaining D-alanine on the stem peptide and a free amino group present on the third amino acid of the stem peptides in the existing cross-linked peptidoglycan. In many organisms, including *E. coli*, this acceptor amino group is supplied by the amino acid diaminopimelic acid. In other organisms, e.g. *Staph. aureus*, the acceptor amino group is supplied by the amino acid L-lysine. Although there is considerable variation in the composition of the peptide cross-link among different species of bacteria, the essential transpeptidation mechanism is the same. Therefore, virtually all bacteria can be inhibited by interference with this group of enzymes.

The β-lactam antibiotics inhibit transpeptidases by acting as alternative substrates. They mimic the D-alanyl-D-alanine residues and react covalently with the transpeptidases (Figure 12.3). The β-lactam bond (common to all members of the β-lactam antibiotics) is broken but the remaining portion of the antibiotic is not released immediately. The half-life for the transpeptidase–antibiotic complex is of the order of 10 minutes; during this time the enzyme cannot participate in further rounds of peptidoglycan assembly by reaction with its true substrate. The vital cross-linking of the peptidoglycan is therefore blocked while other aspects of cell growth continue. The cells become deformed in shape and eventually burst through the combined action of a weakened cell wall, high internal osmotic pressure and the uncontrolled activity of autolytic enzymes in the cell wall. Penicillins, cephalosporins, carbapenems and monobactams all inhibit peptidoglycan cross-linking through interaction

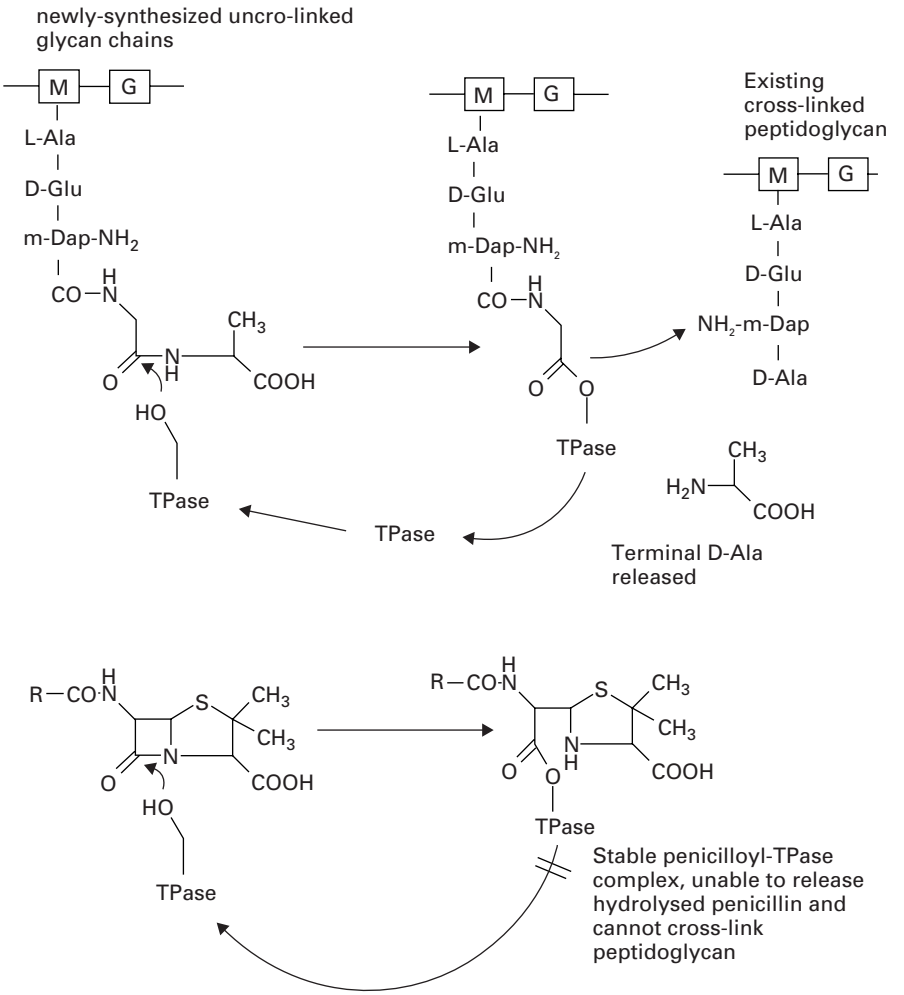


Figure 12.3 The action of transpeptidase (TPase) with its natural substrate (upper) and penicillin G (lower). The -OH group on a serine residue at the active site of the TPase attacks the peptide bond between the terminal D-alanyl-D-alanine residues of the stem peptide in the non-cross-linked glycan chain. The terminal D-alanine is released and a new peptide cross-link is formed with existing peptidoglycan in the cell wall. Penicillin G blocks this process by forming a stable penicilloyl-TPase complex which cannot release the penicillin and therefore cannot form the peptide cross-link in the peptidoglycan.

of the common β-lactam ring with the transpeptidase enzymes. However, there is considerable variation in the morphological effects of different β-lactams owing to the existence of several types of transpeptidase. The transpeptidase enzymes are usually referred to as penicillin-binding proteins (PBPs) because they can be separated and studied after reaction with ^{14}C-labelled penicillin. This step is necessary because there are very few copies of each enzyme present in a cell. They are usually separated according to their size by electrophoresis and are numbered PBP1, PBP2, etc., starting from the highest molecular weight species. In Gram-negative bacteria most of the high molecular weight transpeptidases also possess transglycosylase activity, i.e. they have a dual function in the final stages of peptidoglycan synthesis with the transglycosylase and transpeptidase activities located in separate regions of the protein structures. Furthermore, the different transpeptidases have specialized functions in the cell; all cross-link peptidoglycan but some are involved with maintenance of cell integrity, some regulate cell shape and others produce new cross wall between elongating cells, securing chromosome segregation prior to cell division. The varying sensitivity of the PBPs towards different β-lactams helps to explain the range of morphological effects observed in treated bacteria. For example, penicillin G (benzylpenicillin), ampicillin and cephaloridine are particularly effective in causing rapid lysis of Gram-negative bacteria such as *E. coli*. These antibiotics act primarily upon PBP1B, the major transpeptidase of the organism. Other β-lactams have little activity against this PBP, e.g. mecillinam binds preferentially to PBP2 and it produces a pronounced change in the cells from a rod shape to an oval form. Many of the cephalosporins, e.g. cephalexin, cefotaxime and ceftazidime, bind to PBP3 resulting in the formation of elongated, filamentous cells. The lower molecular weight PBPs, 4, 5 and 6, do not possess transpeptidase activity. These are carboxypeptidases, which remove the terminal D-alanine from the pentapeptides on the linear glycans in the cell wall but do not catalyse the cross-linkage. Their role in the cells is to regulate the degree of cross-linking by denying the D-alanyl-D-alanine substrate to the transpeptidases but they are not essential for cell growth. Up to 90% of the amount of antibiotic reacting with the cells may be consumed in inhibiting the carboxypeptidases, with no lethal consequences to the cells.

Gram-positive bacteria also have multiple transpeptidases, but fewer than Gram-negatives. Shape changes are less evident than with Gram-negative rod-shaped organisms. Cell death follows lysis of the cells mediated by the action of endogenous autolytic enzymes (autolysins) present in the cell wall which are activated following β-lactam action. Autolytic enzymes able to hydrolyse peptidoglycan are present in most bacterial walls; they are needed to re-shape the wall during growth and to aid cell separation during division. Their activity is regulated by binding to wall components such as the wall and membrane teichoic acids. When peptidoglycan assembly is disrupted through β-lactam action, some of the teichoic acids are released from the cells, which are then susceptible to attack by their own autolysins.

2.1.4 β-Lactamase inhibitors—clavulanic acid, sulbactam and tazobactam

Expression of β-lactamase enzymes is the most important mechanism through which organisms become resistant to β-lactams. Over 300 different β-lactamase enzymes have been described and they can be classified either by amino acid sequence or by their biochemical properties. The majority of the enzymes have a serine residue at their active site and bear structural and mechanistic similarities to the carboxypeptidases from which they are thought to have evolved. Unlike the transpeptidases and carboxypeptidases, the β-lactamases hydrolyse β-lactam antibiotics very efficiently, releasing fragments of the antibiotics rapidly instead of remaining bound to the ring-opened forms for several minutes. A number of successful inhibitors, including clavulanic acid, sulbactam and tazobactam have been developed for use in combination with susceptible β-lactams (amoxicillin, ampicillin and piperacillin, respectively), protecting them from inactivation by the β-lactamases. The inhibitors are hydrolysed by the β-lactamases in the same manner as susceptible β-lactam antibiotics, the β-lactam ring being broken by attack by a serine residue in the active site of the enzyme. Instead of undergoing rapid release from the active site serine, the inhibitors remain bound and undergo one of several different fates. It is thought that the hydrolysed inhibitors can interact with a second enzyme residue in the active site of the β-lactamase, forming a covalently cross-linked, irreversibly inhibited complex. Other categories of β-lactamase enzymes have zinc atoms at their active sites and hydrolyse the β-lactam ring by a different mechanism to the serine-based enzymes. These metallo-β-lactamases are not inhibited by clavulanic acid, sulbactam and tazobactam.

2.2 Mycolic acid and arabinogalactan biosynthesis in mycobacteria

The cell walls of mycobacteria contain an arabinogalactan polysaccharide in addition to the peptidoglycan, plus a variety of high molecular weight lipids, including the mycolic acids, glycolipids, phospholipids and waxes. The lipid-rich nature of the mycobacterial wall is responsible for the characteristic acid-fastness on staining and serves as a penetration barrier to many antibiotics. Isoniazid and ethambutol have long been known as specific antimycobacterial agents, exerting no activity towards other bacteria, but their mechanisms of action have only recently been established.

2.2.1 Isoniazid

Isoniazid interferes with mycolic acid synthesis by inhibiting an enoyl reductase (InhA) which forms part of the fatty acid synthase system in mycobacteria. Mycolic acids are produced by a diversion of the normal fatty acid synthetic pathway in which short-chain (16-carbon) and long-chain (24-carbon) fatty acids are produced by addition of 7 or 11 malonate extension units from malonyl coenzyme A to acetyl coenzyme A. InhA inserts a double bond into the extending fatty acid chain at the 24-carbon stage. The long-chain fatty acids are further extended and condensed to produce the 60–90-carbon β-hydroxymycolic acids which are important components of the mycobacterial cell wall. Isoniazid is converted inside the mycobacteria to a free radical species by a catalase peroxidase enzyme, KatG. The active free radicals then attack and inhibit the enoyl reductase, InhA, by covalent attachment to the active site.

2.2.2 Ethambutol

Ethambutol blocks assembly of the arabinogalactan polysaccharide by inhibition of an arabinotransferase enzyme. Cells treated with ethambutol accumulate the isoprenoid intermediate decaprenylarabinose, which supplies arabinose units for assembly in the arabinogalactan polymer.

2.3 Echinocandins—caspofungin, anidulafungin and micafungin

These members of the echinocandin group of antifungal agents have been developed for treatment of serious fungal infections. They interfere with the synthesis of the β-1,3-D-glucan polymer in the fungal cell wall. Without the glucan polymer, the integrity of the fungal cell wall is compromised, yeast cells lose their rigidity and become like protoplasts; the effect is especially pronounced in *Candida* and *Aspergillus* species.

3 Protein synthesis

3.1 Protein synthesis and its selective inhibition

Figure 12.4 outlines the process of protein synthesis involving the ribosome, mRNA, a series of amino-acyl transfer RNA (tRNA) molecules (at least one for each amino acid) and accessory protein factors involved in initiation, elongation and termination. As the process is essentially the same in prokaryotic (bacterial) and eukaryotic cells (i.e. higher organisms and mammalian cells) it is surprising that there are so many selective agents which act in this area (see Figure 12.1).

Bacterial ribosomes are smaller than their mammalian counterparts. They consist of one 30S and one 50S subunit (the S suffix denotes the size, which is derived from the rate of sedimentation in an ultracentrifuge). The 30S subunit comprises a single strand of 16S rRNA and over 20 different proteins that are bound to it. The larger 50S subunit contains two single strands of rRNA (23S and 5S) together with over 30 different proteins. The subunits pack together to form an intact 70S ribosome. The equivalent subunits for mammalian ribosomes are 40S and 60S, making an 80S ribosome. Some agents exploit subtle differences in structure between the bacterial and mammalian ribosomes. The macrolides, azalides and chloramphenicol act on the 50S subunits in bacteria but not the 60S subunits of mammalian cells. By contrast, the tetracyclines derive their selective action through active uptake and concentration within microbial cells but only limited penetration of mammalian cells.

3.2 Aminoglycoside–aminocyclitol antibiotics

Most of the information on the mechanisms of action of aminoglycoside–aminocyclitol (AGAC) antibiotics comes from studies with streptomycin. One effect of AGACs is to interfere with the initiation and assembly of the bacterial ribosome (Figure 12.4). During assembly of the initiation complex, *N*-formylmethionyl-tRNA (fmet-tRNA) binds initially to the ribosome binding site on the untranslated 5′ end of the mRNA together with the 30S ribosomal subunit. Three protein initiation factors (designated IF-1, 2 and 3) and a molecule of guanosine triphosphate (GTP) are involved in positioning the fmet-tRNA on the AUG start codon of mRNA. IF-1 and IF-3 are then released from the complex, GTP is hydrolysed to guanosine diphosphate (GDP) and released with IF-2 as the 50S subunit joins the 30S subunit and mRNA

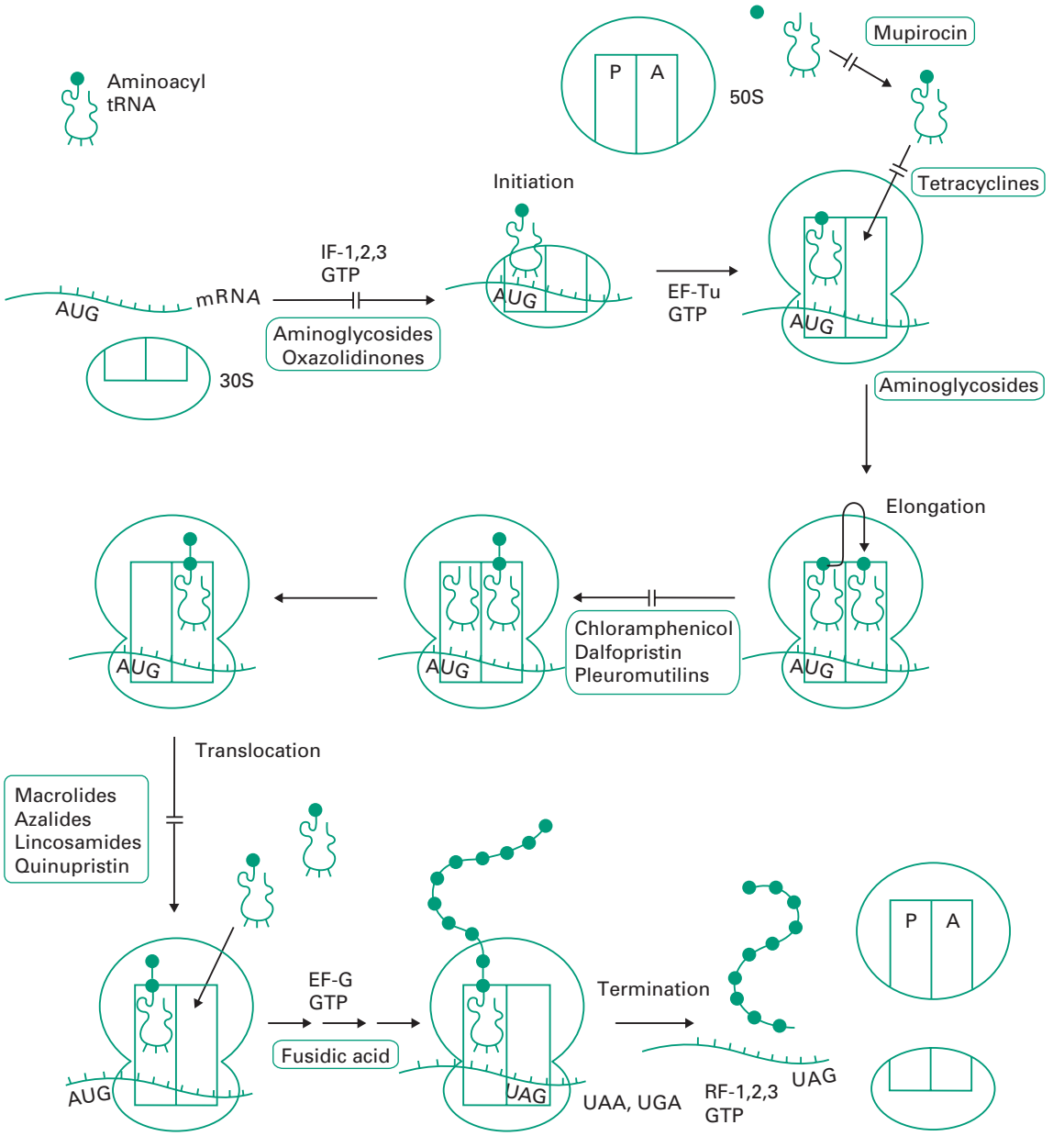


Figure 12.4 Outline of the process of protein synthesis (translation of messenger RNA) in bacterial cells. The four stages of synthesis are shown: initiation, elongation, translocation and termination with the sites of action of antibiotics. AUG is the start codon on messenger RNA (mRNA) specifying the first amino acid in bacterial proteins, *N*-formylmethionine. UAG, UAA and UGA are termination codons specifying no amino acid. 30S and 50S are the subunits of the ribosome. Other protein factors involved in protein synthesis are initiation factors (IF-1, 2, 3), elongation factors (EF-Tu and EF-G) and release factors (RF-1, 2, 3).

to form a functional ribosome. The fmet-tRNA occupies the peptidyl site (P site) leaving a vacant acceptor site (A site) to receive the next aminoacyl-tRNA specified by the next codon on the mRNA. Streptomycin binds tightly to one of the protein components of the 30S subunit. Binding of the antibiotic to the protein, which is the receptor for IF-3, prevents initiation and assembly of the ribosome.

Streptomycin binding to the 30S subunit also distorts the shape of the A site on the ribosome and interferes with the positioning of the aminoacyl-tRNA molecules during peptide chain elongation. Streptomycin therefore exerts two effects: inhibition of protein synthesis by freezing the initiation complex, and misreading of the codons through distortion of the 30S subunit. Simple blockage of protein synthesis would be bacteriostatic rather than bactericidal. As streptomycin and the other AGACs exert a potent lethal action, it seems that the formation of toxic, non-functional proteins through misreading of the codons on mRNA is a more likely mechanism of action. This can be demonstrated with cell-free translation systems in which isolated bacterial ribosomes are supplied with artificial mRNA template such as poly(U) or poly(C) and all the other factors, including aminoacyl-tRNAs needed for protein synthesis.

In the absence of an AGAC the ribosomes will produce artificial polypeptides, polyphenylalanine (as specified by the codon UUU) or polyproline (as specified by the codon CCC). However, when streptomycin is added, the ribosomes produce a mixture of polythreonine (codon ACU) and polyserine (codon UCU). The misreading of the codons does not appear to be random: U is read as A or C, and C is read as A or U. If such misreading occurs in whole cells, the accumulation of non-functional or toxic proteins would eventually prove fatal to the cells. There is some evidence that the bacterial cell membrane is damaged when the cells attempt to excrete the faulty proteins.

The effectiveness of the AGACs is enhanced by their active uptake by bacteria, which proceeds in three phases. First, a rapid uptake occurs within a few seconds of contact, which represents binding of the positively charged AGAC molecules to the negatively charged surface of the bacteria. This phase is referred to as the energy-independent phase (EIP) of uptake. In the case of Gram-negative bacteria the AGACs damage the outer membrane causing release of some lipopolysaccharide, phospholipid and proteins but this is not directly lethal to the cells. Second, there follows an energy-dependent phase of uptake (EDP I) lasting about 10 minutes, in which the AGAC is actively transported across the cytoplasmic membrane. A second energy-dependent phase (EDP II) which leads to further intracellular accumulation follows after some AGAC has bound to the ribosomes in the cytoplasm. Although the precise details of uptake by EDP I and EDP II are not clear, both require organisms to be growing aerobically. Anaerobes do not take up AGACs by EDP I or EDP II and are consequently resistant to their action.

3.3 Tetracyclines

This group of antibiotics is actively transported into bacterial cells, possibly as the magnesium complex, achieving a 50-fold concentration inside the cells. Mammalian cells do not actively take up the tetracyclines (small amounts enter by diffusion alone) and it is this difference in uptake that determines the selective toxicity. Resistance to the tetracyclines occurs through failure of the active uptake system or the action of active efflux pumps, which remove the drug from the cells before it can interfere with ribosome function. Other resistance mechanisms involve ribosomal protection and modification. Protein synthesis by both bacterial and mammalian ribosomes is inhibited by the tetracyclines in cell-free systems. The action is on the smaller subunit. Binding of just one molecule of tetracycline to the bacterial 30S subunit occurs at a site involving the 3′ end of the 16S rRNA, a number of associated ribosomal proteins and magnesium ions. The effect is to block the binding of aminoacyl-tRNA to the A site of the ribosome and halt protein synthesis. Tetracyclines are bacteriostatic rather than bactericidal, consequently they should not be used in combination with β-lactams, which require cells to be growing and dividing to exert their lethal action.

3.4 Chloramphenicol

Of the four possible optical isomers of chloramphenicol, only the D-*threo* form is active. This antibiotic selectively inhibits protein synthesis in bacterial ribosomes by binding to the 50S subunit in the region of the A site involving the 23S rRNA. The normal binding of the aminocyl-tRNA in the A site is affected by chloramphenicol in such a way that the peptidyl transferase cannot form a new peptide bond with the growing peptide chain on the tRNA in the P site. Studies with aminocyl-tRNA fragments containing truncated tRNA chains suggest that the shape of the region of tRNA closest to the amino acid is distorted by chloramphenicol. The altered orientation of this region of the aminoacyl-tRNA in the A site is sufficient to prevent peptide bond forma-

tion. Chloramphenicol has a broad spectrum of activity, which covers Gram-positive and Gram-negative bacteria, mycoplasmas, rickettsia and chlamydia. It has the valuable property of penetrating into mammalian cells and is therefore the drug of choice for treatment of intracellular pathogens, including *Salmonella enterica* serovar Typhi, the causative organism of typhoid. Although it does not inhibit 80S ribosomes, the 70S ribosomes of mammalian mitochondria are sensitive and therefore some inhibition occurs in rapidly growing mammalian cells with high mitochondrial activity.

3.5 Macrolides and azalides

Erythromycin is a member of the macrolide group of antibiotics; it selectively inhibits protein synthesis in a broad range of bacteria by binding to the 50S subunit. The site at which it binds is close to that of chloramphenicol and involves the 23S rRNA. Resistance to chloramphenicol and erythromycin can occur by methylation of different bases within the same region of the 23S rRNA. The sites are therefore not identical, but binding of one antibiotic prevents binding of the other. Unlike chloramphenicol, erythromycin blocks translocation. This is the process by which the ribosome moves along the mRNA by one codon after the peptidyl transferase reaction has joined the peptide chain to the aminoacyl-tRNA in the A site. The peptidyl-tRNA is moved (translocated) to the P site, vacating the A site for the next aminocyl-tRNA. Energy is derived by hydrolysis of GTP to GDP by an associated protein elongation factor, EF-G. By blocking the translocation process, erythromycin causes release of incomplete polypeptides from the ribosome. It is assumed that the azalides, such as azithromycin, have a similar action to the macrolides. The azalides have improved intracellular penetration over the macrolides and are resistant to the metabolic conversion which reduces the serum half-life of erythromycin.

3.6 Clindamycin

This agent binds selectively to a region of the 50S ribosomal subunit close to that of chloramphenicol and erythromycin. It blocks elongation of the peptide chain by inhibition of peptidyl transferase.

3.7 Streptogramins—quinupristin and dalfopristin

The two unrelated streptogramins, quinupristin and dalfopristin, have been used in combination (in a 30:70 ratio) to treat infections caused by staphylococci and enterococci, particularly methicillin-resistant *Staph. aureus* (MRSA) and VRE. Their action is synergistic, and is generally bactericidal compared with either agent used alone or compared with antibiotics in the macrolide group. The main target is the bacterial 50S ribosome, with the formulation acting to inhibit protein synthesis. The agents bind sequentially to the 50S subunit; dalfopristin alters the shape of the subunit so that more quinupristin can bind. Dalfopristin blocks an early step in protein synthesis by forming a bond with the ribosome, preventing elongation of the peptide chain by the peptidyl transferase. Quinupristin blocks a later step by preventing the extension of peptide chains and causing incomplete chains to be released. The overall effect is to block elongation. Use of streptogramins is limited by vasculitis, causing pain on intravenous administration.

3.8 Oxazolidinones—linezolid

Oxazolidinones such as linezolid act at the early stage of protein synthesis, preventing the formation of the initiation complex between the 30S subunit, mRNA and fmet-tRNA.

3.9 Mupirocin

The target of mupirocin is one of a group of enzymes which couple amino acids to their respective tRNAs for delivery to the ribosome and incorporation into protein. The particular enzyme inhibited by mupirocin is involved in producing isoleucyl-tRNA. The basis for the inhibition is a structural similarity between one end of the mupirocin molecule and isoleucine. Protein synthesis is halted when the ribosome encounters the isoleucine codon through depletion of the pool of isoleucyl-tRNA.

3.10 Fusidic acid

This steroidal antibiotic does not act on the ribosome itself, but on one of the associated elongation factors, EF-G. This factor supplies energy for translocation by hydrolysis of GTP and GDP. Another elongation factor, EF-Tu, promotes binding of aminoacyl-tRNA molecules to the A site through binding and hydrolysis of GTP. Both EF-G and EF-Tu have overlapping binding sites on the ribosome. Fusidic acid binds the EF-G:GDP complex to the ribosome after one round of translocation has taken place. This prevents further incorporation of aminoacyl-tRNA by blocking the binding of EF-Tu:GTP. Fusidic acid owes its selective antimicrobial action to active uptake by bacteria and exclusion from mammalian cells. The equivalent elongation factor in mammalian cells, EF-2, is susceptible to fusidic acid in cell-free systems.

3.11 Pleuromutilins—retapamulin

These agents bind to the 23S rRNA component of the 50S bacterial ribosome and block peptide bond formation by interfering with the binding of the peptidyl transferase region with the aminoacyl-tRNA substrates in the A and P sites on the ribosome. This mechanism is different to that of other peptidyl transferase inhibitors (chloramphenicol and clindamycin) so cross-resistance to these agents does not occur.

4 Chromosome function and replication

4.1 Basis for the selective inhibition of chromosome replication and function

As with protein synthesis, the mechanisms of chromosome replication and function are essentially the same in prokaryotes and eukaryotes. There are, however, important differences in the detailed functioning and properties of the enzymes involved and these differences are exploited by a number of agents as the basis of selective inhibition. The microbial chromosome is large in comparison with the cell that contains it (approximately 500 times the length of *E. coli*). It is therefore wound into a compact, supercoiled form inside the cell. During replication the circular double helix must be unwound to allow the DNA polymerase enzymes to synthesize new complementary strands. The shape of the chromosome is manipulated by the cell by the formation of regions of supercoiling. Positive supercoiling (coiling in the same sense as the turns of the double helix) makes the chromosome more compact. Negative supercoiling (generated by twisting the chromosome in the opposite sense to the helix) produces localized strand separation which is required both for replication and transcription. In a bacterium such as *E. coli* four different topoisomerase enzymes are responsible for maintaining the shape of DNA during cell division. They act by cutting one or both of the DNA strands; they remove and generate supercoiling, then reseal the strands. Their activity is essential for the microbial cell to relieve the complex tangling of the chromosome (both knotting and chain link formation) which results from progression of the replication fork around the circular chromosome. Type I topoisomerases cut one strand of DNA and pass the other strand through the gap before resealing. Type II enzymes cut both strands and pass another double helical section of the DNA through the gap before resealing. In *E. coli* topoisomerases I and III are both type I enzymes while topoisomerases II and IV are type II enzymes. Topoisomerase II (also known as DNA gyrase) and topoisomerase IV are essential enzymes which are inhibited by the fluoroquinolone group of antimicrobials. Topoisomerase II is responsible for introducing negative supercoils into DNA and for relieving torsional stress, which accumulates ahead of sites of transcription and replication. Topoiosomerase IV provides a potent decatenating (unlinking) activity that removes links and knots generated behind the replication fork.

The basic sequence of events for microbial chromosome replication is described below.

4.1.1 Synthesis of precursors

Purines, pyrimidines and their nucleosides and nucleoside triphosphates are synthesized in the cytoplasm. At this stage the antifolate drugs (sulphonamides and dihydrofolate reductase inhibitors) act by interfering with the synthesis and recycling of the cofactor dihydrofolic acid (DHF). Thymidylic acid (2-deoxythymidine monophosphate, dTMP) is an essential nucleotide precursor of DNA synthesis. It is produced by the enzyme thymidylate synthetase by transfer of a methyl group from tetrahydrofolic acid (THF) to the uracil base on uridylic acid (2-deoxyuridine monophosphate, dUMP) (Figure 12.5). THF is converted to DHF in this process and must be reverted to THF by the enzyme dihydrofolate reductase (DHFR) before the cycle can be repeated. By inhibiting DHFR, the antifolates effectively block the production of dTMP and hence DNA synthesis.

The antifungal agent 5-fluorocytosine also interferes with these early stages of DNA synthesis. Through conversion to the nucleoside triphosphate it subsequently blocks thymidylic acid production through inhibition of the enzyme thymidylate synthetase (Figure 12.6).

The antiviral nucleosides aciclovir and ganciclovir are also converted to their respective nucleoside triphosphates in the cytoplasm of infected cells. They proceed to inhibit viral DNA replication either by inhibition of the DNA polymerase or by incorporation into DNA with subsequent termination of chain extension. Finally, the anti-HIV drug AZT acts in an analogous manner, being converted to the corresponding triphosphate and inhibiting viral RNA synthesis by the HIV reverse transcriptase.

4.1.2 Unwinding of the chromosome

As described in section 4.1, the DNA double helix must unwind to allow access of the polymerase enzymes to produce two new strands of DNA. This is facilitated by topoisomerase II (DNA gyrase) which is the target of the

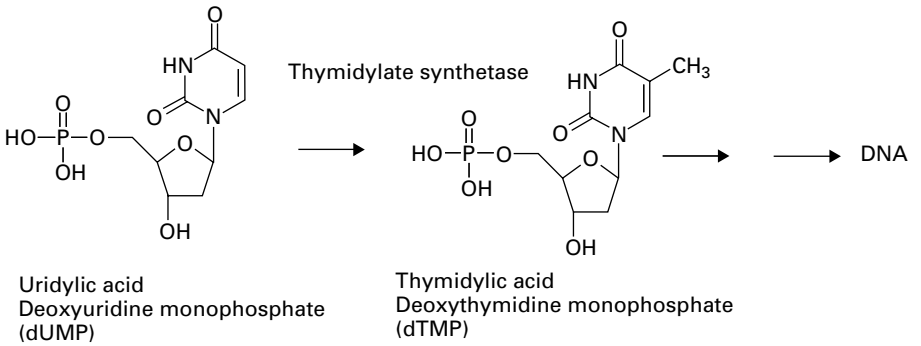


Figure 12.5 Conversion of uridylic acid to thymidylic acid by the enzyme thymidylate synthetase, a vital early stage in the synthesis of DNA.

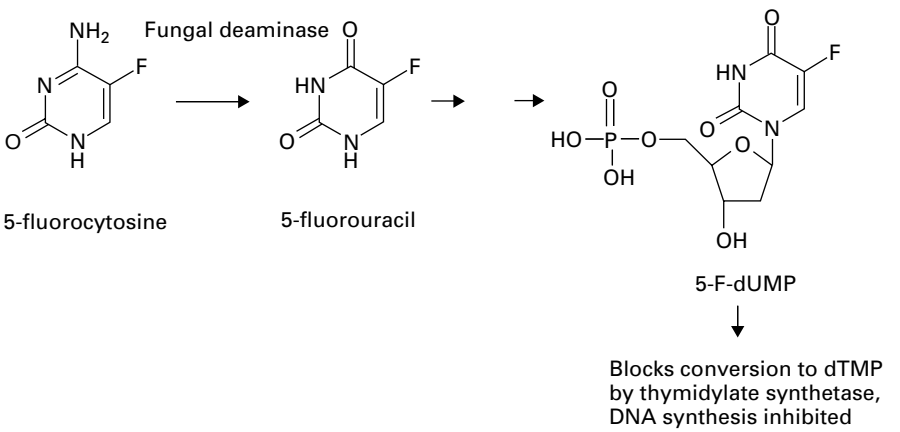


Figure 12.6 Conversion of the antifungal agent 5-fluorocytosine (5-FC) to 5-fluorouracil by a deaminase enzyme inside fungal cells and subsequent inhibition of fungal DNA synthesis through inhibition of thymidylate synthetase.

fluoroquinolones. Some agents interfere with the unwinding of the chromosome by physical obstruction. These include the acridine dyes, of which the topical antiseptic proflavine is the most familiar, and the antimalarial acridine mepacrine. They prevent strand separation by insertion (intercalation) between base pairs from each strand, but exhibit very poor selective toxicity.

4.1.3 Replication of DNA strands

The unwound DNA strands are kept unfolded during replication by binding a protein called Albert's protein. A series of enzymes produce new strands of DNA using each of the separated strands as templates. One strand is produced continuously. The other is produced in a series of short strands called Okazaki fragments that are joined by a DNA ligase. The entire process is carefully regulated, with proofreading stages to check that each nucleotide is correctly incorporated as specified by the template sequence. There are no therapeutic agents yet known which interfere directly with the DNA polymerases.

4.1.4 Transcription

The process of transcription, the copying of a single strand of mRNA sequence using one strand of the chromosome as a template, is carried out by RNA polymerase. This is a complex of four proteins (2 α, 1 β and 1 β' subunits) which make up the core enzyme. Another small protein, the σ factor, joins the core enzyme, which binds to the promoter region of the DNA preceding the gene that is to be transcribed. The correct positioning and

orientation of the polymerase is obtained by recognition of specific marker sites on the DNA at positions −10 and −35 nucleotide bases before the initiation site for transcription. The σ factor is responsible for recognition of the initiation signal for transcription and the core enzyme possesses the activity to join the nucleotides in the sequence specified by the gene. Mammalian genes possess an analogous RNA polymerase but there are sufficient differences in structure to permit selective inhibition of the microbial enzyme by the semisynthetic rifamycin antibiotics rifampicin and rifabutin.

4.2 Fluoroquinolones

The fluoroquinolones selectively inhibit topoisomerases II and IV, which are not found in mammalian cells. The enzymes, both tetramers comprising two A and two B subunits, are capable of catalysing a variety of changes in DNA topology. The topoisomerases bind to the chromosome at points where two separate double-stranded regions cross. This can be at a supercoiled region, a knotted or a linked (catenane) region. The A subunits (gyrA for topoisomerase II and parC for topoisomerase IV) cut both DNA strands on one chain with a 4 base pair stagger; the other chain is passed through the break which is then resealed. The B subunits (gyrB for topoisomerase II and parE for topoisomerase IV) derive energy for the reaction by hydrolysis of ATP. The precise details of the interaction are not clear but it appears that the fluoroquinolones do not simply eliminate enzyme function, they actively poison the cells by trapping the topoisomerases as drug–enzyme–DNA complexes in which double-stranded DNA breaks are held together by the enzyme protein alone. The enzymes are unable to reseal the DNA, with the result that the chromosome in treated cells becomes fragmented. The number of fragments (approximately 100 per cell) is comparable to the number of supercoils in the chromosome. The action of the fluoroquinolones probably triggers secondary responses in the cells which are responsible for death. One notable morphological effect of fluoroquinolone treatment of Gram-negative rod-shaped organisms is the formation of filaments. In Gram-positive cocci topoisomerase IV may be the more important target for fluoroquinolone action.

4.3 Nitroimidazoles (metronidazole, tinidazole) and nitrofurans (nitrofurantoin)

These agents also cause DNA strand breakage, but by a direct chemical action rather than by inhibition of a topoisomerase. Metronidazole is active only against anaerobic organisms. The nitro group of metronidazole is converted to a nitronate radical by the low redox potential within cells. The pyruvate:ferredoxin oxidoreductase (POR) is a major metabolic pathway in anaerobic bacteria and protozoa used for generation of ATP. This system converts metronidazole to its active form which then attacks the DNA, producing strand breakage. Another nitroimidazole, tinidazole, and the nitrofuran nitrofurantoin are thought to act in a similar manner.

4.4 Rifampicin and rifabutin

Rifampicin acts on the β subunit of RNA polymerase. Binding of just one molecule of rifampicin inhibits the initiation stage of transcription in which the first nucleotide is incorporated in the RNA chain. Once started, transcription itself is not inhibited. It has been suggested that the structure of rifampicin resembles that of two adenosine nucleotides in RNA; this may form the basis of the binding of the antibiotic to the β subunit. One problem is the rapid development of resistance in organisms due to alterations in the amino acids comprising one particular region of the β subunit. These changes do not affect the activity of the polymerase but render it insensitive to rifampicin. The action of rifampicin is specific for the microbial RNA polymerase, the mammalian version being unaffected. Rifabutin, which has enhanced activity against *Mycobacterium avium* complex, is thought to act in the same way as rifampicin.

4.5 5-Fluorocytosine

This antifungal agent inhibits DNA synthesis at the early stages involving production of the nucleotide thymidylic acid (TMP). 5-Fluorocytosine (5-FC) is converted by a deaminase inside fungi to 5-fluorouracil, then to the corresponding nucleoside triphosphate, 5-fluorodeoxyuridine monophosphate (5-F-dUMP) which then acts as an inhibitor of thymidylate synthetase (Figure 12.6). This enzyme normally produces thymidylic acid (TMP) from deoxyuridine monophosphate (dUMP) by addition of a methyl group (supplied by a folate cofactor, section 4.1.1) to the 5 position of the uracil ring. As this position is blocked by the fluoro group in 5-FC, the phosphate acts as an inhibitor of the enzyme. 5-FC can be considered as a prodrug; it has the value of being taken up by fungi as the nucleoside, whereas the active triphosphate produced inside the cells would not be taken up because of its negative charge. Although 5-FC is an important antifungal agent in the treatment of life-threatening infections, resistance can occur due to active efflux of the drug from the cells before it can inhibit DNA synthesis.

5 Folate antagonists

5.1 Folate metabolism in microbial and mammalian cells

Folic acid is an important cofactor in all living cells. In the reduced form, tetrahydrofolate (THF), it functions as a carrier of single-carbon fragments, which are used in the synthesis of adenine, guanine, thymine and methionine (Figure 12.7). One important folate-dependent enzyme is thymidylate synthetase, which produces TMP by transfer of the methyl group from THF to UMP. In this and other folate-dependent reactions THF is converted to dihydrofolic acid (DHF), which must be reduced back to THF before it can participate again as a carbon fragment carrier. The enzyme responsible for the reduction of DHF to THF is dihydrofolate reductase (DHFR) which uses the nucleotide $NADPH_2$ as a cofactor. Bacteria, protozoa and mammalian cells all possess DHFR but there are sufficient differences in the enzyme structure for inhibitors such as trimethoprim and pyrimethamine to inhibit the bacterial and protozoal enzymes selectively without damaging the mammalian form. In the case of protozoa such as the *Plasmodium* species responsible for malaria, the DHFR is a double enzyme which also contains the thymidylate synthetase activity.

There is another fundamental difference between folate utilization in microbial and mammalian cells (Figure 12.7). Bacteria and protozoa are unable to take up exogenous folate and must synthesize it themselves. This is carried out in a series of reactions involving first the synthesis of dihydropteroic acid from one molecule each of pteridine and *p*-aminobenzoic acid (PABA). Glutamic acid is then added to form DHF, which is reduced by DHFR to THF. Mammalian cells do not make their own DHF, instead they take it up from dietary nutrients and convert it to THF using DHFR.

5.2 Sulphonamides

Sulphonamides (e.g. sulphamethoxazole and dapsone) are structural analogues of PABA (Figure 12.8). They competitively inhibit the incorporation of PABA into dihydropteroic acid and there is some evidence for their incorporation into false folate analogues, which inhibit subsequent metabolism. The presence of excess PABA will reverse the inhibitory action of sulphonamides, as will thymine, adenine, guanine and methionine. However these nutrients are not normally available at the site of infections for which the sulphonamides are used.

5.3 DHFR inhibitors—trimethoprim and pyrimethamine

Trimethoprim is a selective inhibitor of bacterial DHFR. The bacterial enzyme is several thousand times more sensitive than the mammalian enzyme. Pyrimethamine, likewise, is a selective inhibitor of plasmodial DHFR. Both

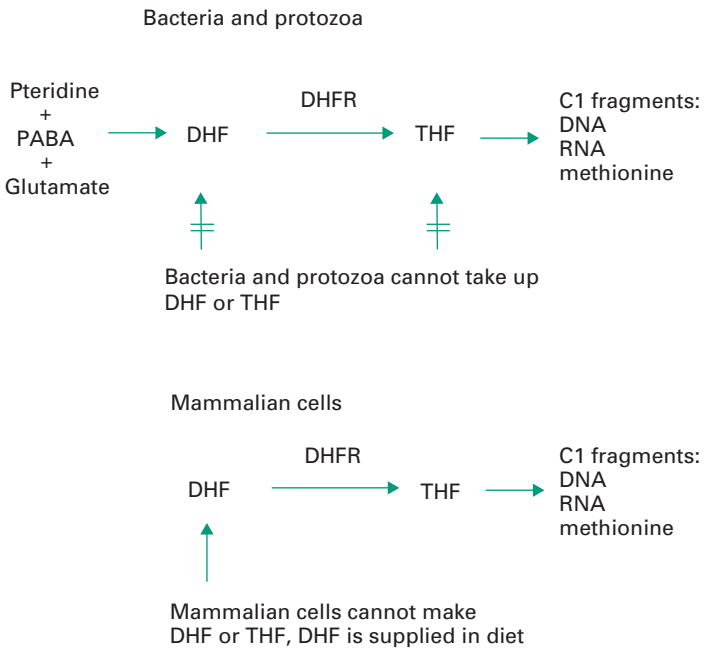
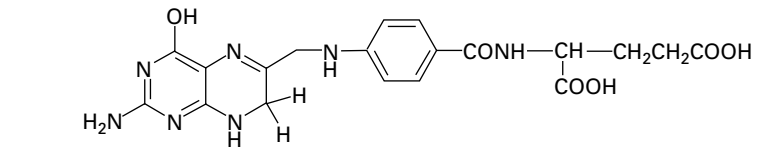
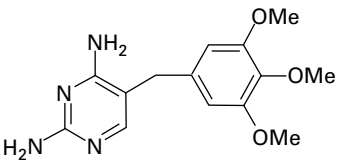


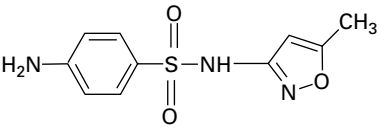
Figure 12.7 Pathways of folate metabolism and use in microbial cells (upper) and mammalian cells (lower). Bacterial and protozoal cells must synthesize dihydrofolic acid (DHF) from pteridine, *p*-aminobenzoic acid (PABA) and glutamate. DHF is converted to tetrahydrofolic acid (THF) by the enzyme dihydrofolate reductase (DHFR). THF supplies single carbon units for various pathways including DNA, RNA and methionine synthesis. Mammalian cells do not make DHF, it is supplied from the diet, conversion to THF occurs via a DHFR enzyme as in microbial cells.



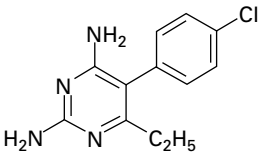
Dihydrofolic acid (DHF)



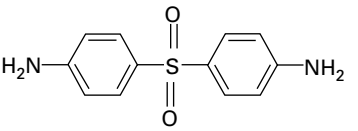
Trimethoprim (antibacterial)



Sulphamethoxazole (antibacterial)



Pyrimethamine (antimalarial)



Dapsone (antimalarial, antileprotic)

Figure 12.8 Structural relationships between dihydrofolate reductase inhibitors (trimethoprim and pyrimethamine), sulphonamides (e.g. sulphamethoxazole, sulphadiazine and dapsone) and dihydrofolic acid.

are structural analogues of the dihydropteroic acid portion of the DHF substrate (Figure 12.8). Crystal structures of the bacterial, plasmodial and mammalian DHFRs, each containing either bound substrate or the inhibitors, have been determined by X-ray diffraction studies. These show how inhibitors fit tightly into the active site normally occupied by the DHF substrate, forming a pattern of strong hydrogen bonds with amino acid residues and water molecules lining the site. Another DHFR is proguanil, a guanidine-containing prodrug which is metabolized in the liver to cycloguanil, an active selective inhibitor of plasmodial DHFR. Methotrexate is a potent DHFR inhibitor that has an analogous structure to the whole DHF molecule, including the glutamate residue. It has no selectivity towards microbial DHFR and therefore cannot be used to treat infections; however, it is widely used as an anticancer agent. A derivative of methotrexate that is used for treatment of *Pneumocystis jirovecii* infections in AIDS patients is trimetrexate. Although it is very toxic to mammalian cells, simultaneous administration of leucovorin (formyl-THF or folinic acid) as an alternative source of folate which cannot be taken up by the organism protects host tissues. DHFR inhibitors can be used in combination with a sulphonamide to achieve a double interference with folate metabolism. Suitable combinations with matching pharmacokinetic properties are sulphamethoxazole with trimethoprim (the antibacterial co-trimoxazole) and dapsone with pyrimethamine (the antimalarial Maloprim).

6 The cytoplasmic membrane

6.1 Composition and susceptibility of membranes to selective disruption

The integrity of the cytoplasmic membrane is vital for the normal functioning of all cells. Bacterial membranes do not contain sterols and in this respect differ from membranes of fungi and mammalian cells. Fungal membranes contain predominantly ergosterol as the sterol component whereas mammalian cells contain cholesterol. Gram-negative bacteria contain an additional outer-membrane structure that provides a protective penetration barrier to potentially harmful substances, including many antibiotics. The outer membrane has an unusual asymmetric structure in which phospholipids occupy the inner face and the lipopolysaccharide (LPS) occupies the outer face. The outer membrane is attached to the peptidoglycan by proteins and lipoproteins. The stability of all membranes is maintained by a combination of non-covalent interactions between the constituents involving ionic, hydrophobic and hydrogen bonding. The balance of these interactions can be disturbed by the

intrusion of molecules (membrane-active agents) which destroy the integrity of the membrane, thereby causing leakage of cytoplasmic contents or impairment of metabolic functions associated with the membrane. Most membrane-active agents that function in this way, e.g. the alcohols, quaternary ammonium compounds and bisbiguanides (considered in Chapters 17 and 18) have very poor selectivity. They cannot be used systemically because of their damaging effects upon mammalian cells; instead they are used as skin antiseptics, disinfectants and preservatives. A few agents can be used therapeutically: the polymyxins (colistin), which act principally upon the outer membrane of Gram-negative bacteria, and the antifungal polyenes, which act upon fungal membranes. Other antifungal agents, the imidazoles, triazoles and terbinafine act by blocking the synthesis of ergosterol, the major sterol present in fungal membranes (see also Chapter 4).

6.2 Polymyxins

Polymyxin E (colistin) is used in the treatment of serious Gram-negative bacterial infections, particularly those caused by *Pseudomonas aeruginosa*. It binds tightly to the lipid A component of LPS in the outer membrane of Gram-negative bacteria. The outer leaflet of the membrane structure is distorted, segments of which are released and the permeability barrier is destroyed. The polymyxin molecules can then penetrate to the cytoplasmic membrane where they bind to phospholipids, disrupt membrane integrity, and cause irreversible leakage of cytoplasmic components. Their detergent-like properties are a key feature of this membrane-damaging action, which is similar to that of quaternary ammonium compounds. With increasing resistance to the major groups of antibiotics, some multiresistant organisms (e.g. *Acinetobacter* species) remain sensitive only to membrane-active agents such as colistin. However, some Gram-negative bacteria produce LPS that does not bind polymyxins (e.g. *Bacteroides* species and *Burkholderia cenocepacia*) while resistance can occur in some normally sensitive organisms such as *E. coli* and *Pseudomonas aeruginosa* through modification of their LPS structure (e.g. by addition of aminoarabinose or aminoethanol substituents to the lipid A regions of their LPS).

6.3 Daptomycin

This negatively charged bactericidal cyclic lipopetide binds to the surface of the Gram-positive bacterial cell membrane. The binding is dependent on calcium ions. The acyl tail portion of the compound inserts itself into the cytoplasmic membrane and drug molecules aggregate together forming channels. The leakage of potassium ions from the cells results in inhibition of macromolecular synthesis and cell death.

6.4 Polyenes

Amphotericin B and nystatin are the most commonly used members of this group of antifungal agents. They derive their action from their strong affinity towards sterols, particularly ergosterol. The hydrophobic polyene region binds to the hydrophobic sterol ring system within fungal membranes. In so doing, the hydroxylated portion of the polyene is pulled into the membrane interior, destabilizing the structure and causing leakage of cytoplasmic constituents. It is possible that polyene molecules associate together in the membrane to form aqueous channels. The pattern of leakage is progressive, with small metal ions such as K^+ leaking first, followed by larger amino acids and nucleotides. The internal pH of the cells falls as K^+ ions are released, macromolecules are degraded and the cells are killed. The selective antifungal activity of the polyenes is poor, depending on the higher affinity for ergosterol than cholesterol. Kidney damage is a major problem when polyenes are used systemically to treat severe fungal infections. The problem can be reduced, but not eliminated by administration of amphotericin as a lipid complex or liposome.

6.5 Imidazoles and triazoles

The azole antifungal drugs act by inhibiting the synthesis of the sterol components of the fungal membrane (see also Chapter 4). They are inhibitors of one step in the complex pathway of ergosterol synthesis involving the removal of a methyl group from lanosterol (Figure 12.9). The 14-α-demethylase enzyme responsible is dependent on cytochrome P-450. The imidazoles and triazoles cause rapid defects in fungal membrane integrity due to reduced levels of ergosterol, with loss of cytoplasmic constituents leading to similar effects to the polyenes. The azoles are not entirely specific for fungal ergosterol synthesis and have some action on mammalian sterol metabolism; for example, they reduce testosterone synthesis.

6.6 Terbinafine

This synthetic antifungal agent inhibits the enzyme squalene epoxidase at an early stage in fungal sterol biosynthesis. Acting as a structural analogue of squalene, terbinafine causes the accumulation of this unsaturated hydrocarbon, and a decrease in ergosterol in the fungal cell membrane (Figure 12.9).

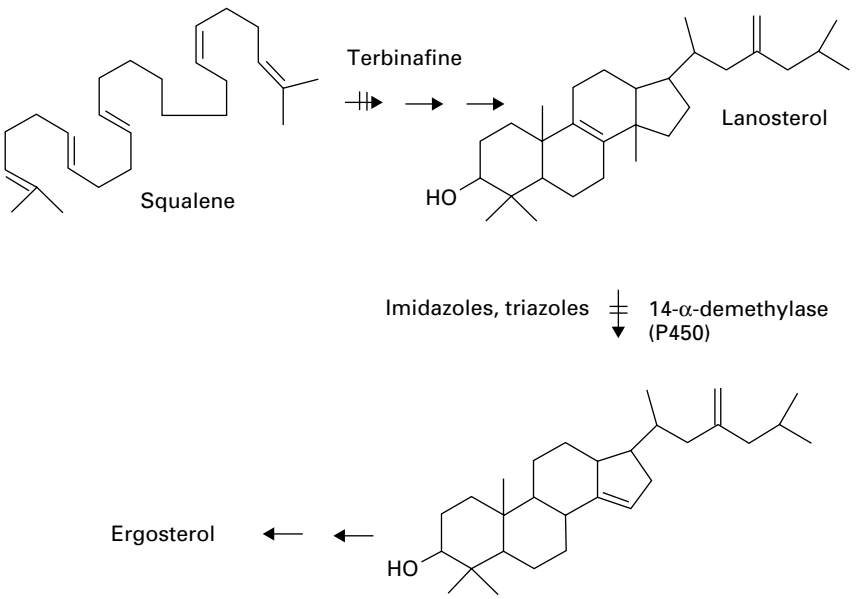


Figure 12.9 Pathway for synthesis of the essential fungal sterol ergosterol and the sites of inhibition by the antifungal agents terbinafine, imidazoles and triazoles.

7 Further reading

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13 Bacterial resistance to antibiotics

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1 Introduction

It is both a cliché and a truism to state that antibiotic resistance has been around for as long as antibiotics have been used to treat infection. Indeed, the origin of antibiotic resistance extends much further back in evolutionary terms and reflects the attack and counter-attack of complex microbial flora in order to establish ecological niches and survive. It is true to say that early treatment failures with antibiotics did not represent a significant clinical problem because other classes of agents, with different cellular targets, were available. It is the emergence of multiple resistance, i.e. resistance to several types of antibiotic agent, that is causing major problems in the clinic today. Several factors drove this situation in the 1970s and 1980s, including the introduction of extended-spectrum agents and advances in medical techniques, such as organ transplantation and cancer chemotherapy. The net result has been a huge selective pressure in favour of multiply resistant species. Coupled with this, there has been a sharp decline in the introduction of agents acting on new cellular targets over the last 30 years compared with the 20-year period following the Second World War. There are a number of resistant organisms causing concern at present. Notable Gram-positive organisms include meticillin-resistant *Staphylococcus aureus* (MRSA) and coagulase-negative staphylococci,

Hugo and Russell's Pharmaceutical Microbiology, Eighth Edition. Edited by Stephen P. Denyer, Norman Hodges, Sean P. Gorman, Brendan F. Gilmore.

glycopeptide-intermediate sensitivity *Staphylococcus aureus* (GISA), vancomycin-resistant *Enterococcus* (VRE) species and penicillin-resistant *Streptococcus pneumoniae*. Concerns among the Gram-negative organisms include multidrug-resistant *Pseudomonas aeruginosa, Stenotrophomonas maltophilia* and *Acinetobacter baumannii* and members of the Enterobacteriaceae with extended-spectrum β-lactamases. Multidrug resistance in the acid-fast bacilli *Mycobacterium tuberculosis* and *M. avium* complex pose major health threats worldwide.

2 Origins of resistance

Some bacteria are said to have *innate* resistance against antibiotics and this typically reflects variations in the structure of their cell envelope. These will be identified in subsequent sections on resistance mechanisms. Resistance or reduced susceptibility may also be *phenotypic*, resulting from adaptation to growth within a specific environment. A characteristic of such phenotypic resistance is reversion to antibiotic susceptibility upon subculture in conventional laboratory media and failure to isolate genotypic resistant mutants (section 17). The origins of antibiotic resistance genes are unclear; however, studies using clinical isolates collected before the introduction of antibiotics demonstrated susceptibility, although conjugative plasmids (section 16.1) were present. Resistance can be achieved by horizontal acquisition of resistance genes, mobilized via insertion sequences, transposons and conjugative plasmids, by recombination of foreign DNA into the chromosome, or by mutations in different chromosomal loci. Given that it is only 60 years since the introduction of antibiotics, mutation of common ancestral genes could not be the only resistance mechanism. Many resistance genes will have derived from the diverse gene pool present in environmental microorganisms, most likely produced as protective mechanisms by antibiotic-producing organisms. Genetic exchange is likely to arise in soil and the general environment as well as in the gut of humans and animals. Rapid mutation can occur and there is clearly a heavy selective pressure resulting from the overuse of antibiotics in medical practice. Agricultural and veterinary use of antibiotics also makes an important and unhelpful contribution. The mutation process is not a static event and a complex network of factors influences the rate and type of mutants that can be selected under antibiotic selective pressure. Antibiotic concentration, physiological conditions such as nutrient availability and stress can each regulate mutation rates. The structure of a gene is relevant to mutability. Size is not the main factor, as not every mutation in a gene that encodes an antibiotic target leads to resistance. Resistance only occurs by mutations which are both permissive (i.e. not lethal or leading to an unacceptable reduction in 'fitness' or ability to cause infection) and able to produce a resistance phenotype. The probability that such a mutation arises will be proportional to the number of target sites within the gene. In *Escherichia coli*, mutations in the *gyrA* gene, encoding the GyrA subunit of topoisomerase II and leading to fluoroquinolone resistance (section 8) have been identified in at least seven locations, whereas mutational changes in only three positions in the *parC* gene, encoding a subunit of topoisomerase IV, have been observed. As a consequence, the prediction that the mutation rate would be higher in *gyrA* than *parC* is correct. Such observations and predictions cannot be extrapolated to other organisms. Indeed, the opposite is true for fluoroquinolone resistance in *Strep. pneumoniae*.

3 Mechanisms of resistance

Resistance to antimicrobial agents typically occurs by one or more of the following mechanisms:

- Inactivation of the drug
- Alteration of the target
- Reduced cellular uptake
- Increased efflux.

In this chapter, resistance will be examined by agent, but attention will be drawn to mechanisms which can permit resistance to multiple, chemically different agents. This is most commonly associated with efflux and this will be described in section 16.4.

4 Resistance to β-lactam antibiotics

β-Lactam antibiotics act by inhibiting the carboxy/transpeptidase or penicillin-binding proteins (PBPs) involved in the late stages of peptidoglycan biosynthesis. Although introduced nearly 60 years ago, β-lactam antibiotics still represent the most widely used class of agents in the clinic today. Resistance to many β-lactam agents is common and is most often caused by β-lactamases or by mutation in the PBPs resulting in reduced affinity. Reduced uptake and efflux are also seen, but they are less significant.

4.1 β-Lactamases

A number of different β-lactamases have been described, but all share the feature of catalysing the ring-opening of the β-lactam moiety (Figure 13.1). Thus, the structural homology with the terminal D-Ala-D-Ala of maturing peptidoglycan, shared by all β-lactam antibiotics, is lost.

β-Lactamases may be chromosomal or plasmid-borne, inducible or constitutive, and for this reason their terminology can be confusing. A number of classification systems have been proposed, including classes A–D based on peptide sequence. Classes A, C and D have a serine at the active site, whereas class B enzymes have four zinc atoms at their active site and these are also called metallo-β-lactamases. Class A enzymes are highly active against benzylpenicillin; class B β-lactamases are effective against penicillins and cephalosporins. Class C enzymes are usually inducible, but mutation can lead to overexpression. Class D consists of the OXA-type enzymes, which can hydrolyse oxacillin. Increasing resistance to β-lactam agents, mainly by β-lactamase, prompted the discovery and introduction of agents with greater β-lactam stability such as cephalosporins, carbapenems and monobactams. Resistance first appeared in organisms such as *Enterobacter cloacae* and *Pseudomonas aeruginosa*, due to mutations causing overproduction of the class C chromosomal AmpC β-lactamase. Subsequently, in the late 1980s, resistance occurred in organisms such as *Klebsiella pneumoniae* and *E. coli* that lack an inducible AmpC enzyme. Resistance was found to be mediated by plasmids encoding extended-spectrum β-lactamases (ESBLs). These arose from mutational development of more limited-spectrum β-lactamases such as TEM and SHV that either increased the size of the active-site pocket or altered its binding characteristics to allow the larger cephalosporins to enter and be broken down. TEM derivatives predominate, possibly favoured by the use of ceftazidime and other slowly penetrating cephalosporins. These mutations also increase the binding of clavulanic acid and so these ESBLs remain susceptible to inhibition by this and other β-lactamase inhibitors such as sulbactam and tazobactam, which are generally ineffective against class C β-lactamases.

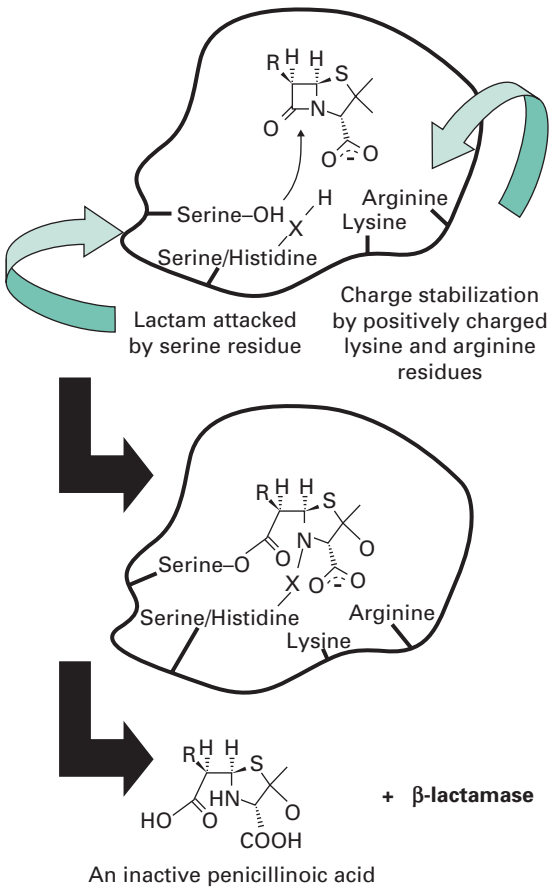


Figure 13.1 General scheme for inhibition of β-lactam-type antibiotics by β-lactamase.

Continuing use of the third-generation cephalosporins and the introduction of β-lactamase inhibitor combinations (clavulanate with amoxycillin or ticarcillin, sulbactam with ampicillin, and tazobactam with piperacillin; see section 4.2) resulted in the appearance of plasmids encoding class C β-lactamases. After several unconfirmed reports, the first proof that a class C β-lactamase had been captured on a plasmid came in 1990 when transmissible resistance to α-methoxy and oxyimino-β-lactams was shown to be mediated by an enzyme whose gene was 90% identical to the *ampC* gene of *Ent. cloacae.* They have subsequently been found worldwide. Strains with plasmid-mediated AmpC enzymes are typically resistant to aminopenicillins (ampicillin or amoxycillin), carboxypenicillins (carbenicillin or ticarcillin) and ureidopenicillins (piperacillin). The enzymes also provide resistance to the oxyimino cephalosporins (ceftazidime, cefotaxime, ceftriaxone) and the 7-α-methoxy group (cefoxitin, cefmetazole and moxalactam) as well as the monobactam aztreonam.

In December 2009, the first report of a carbapenemase β-lactamase, referred to as New Delhi metallo-β-lactamase (NDM-1), was recorded. It was discovered in a carbapenem-resistant *K. pneumoniae* strain isolated in Sweden from a Swedish national who acquired the

infection in India. The enzyme is one of the class of B metallo-β-lactamases and is conferred by the gene bla_{NDM-1}. This is considered a serious threat to the carbapenem family of antibiotics.

4.2 β-Lactamase inhibitors

In addition to introducing agents with increased stability to β-lactamase inhibition, β-lactamase inhibitors including clavulanic acid, sulbactam and tazobactam have been developed (Figure 13.2). Clavulanic acid is produced by a streptomyces and is a suicide inhibitor of β-lactamases from a number of Gram-negative and Gram-positive organisms. These β-lactamase inhibitors do not have any significant antimicrobial activity against bacterial transpeptidases, but their combination with a β-lactam antibiotic (see above) has extended the clinical usefulness of the latter.

4.3 Altered penicillin-binding proteins and meticillin-resistant *Staphylococcus aureus*

Altered PBPs are responsible for reduced sensitivity to β-lactam agents by *Strep. pneumoniae* (PBP1a, PBP2b and PBP2x) and *Haemophilus influenzae* (PBP3a and PBP3b), but by far the most clinically significant example is MRSA. By the early 1950s, the acquisition and spread of plasmid-encoded β-lactamases had blunted the effectiveness of penicillin for treating *Staph. aureus* infections such as boils, carbuncles, pneumonia, endocarditis and osteomyelitis. The β-lactamase-stable agent meticillin was introduced in 1959, but by 1960, meticillin-resistant strains were identified. This was the result of *Staph. aureus* acquiring the *mecA* gene, which encodes an altered PBP gene, *PBP2a*. The *mecA* gene is chromosomal and expression is either constitutive or inducible, but not by meticillin. PBP2a has low affinity for most β-lactam antibiotics.

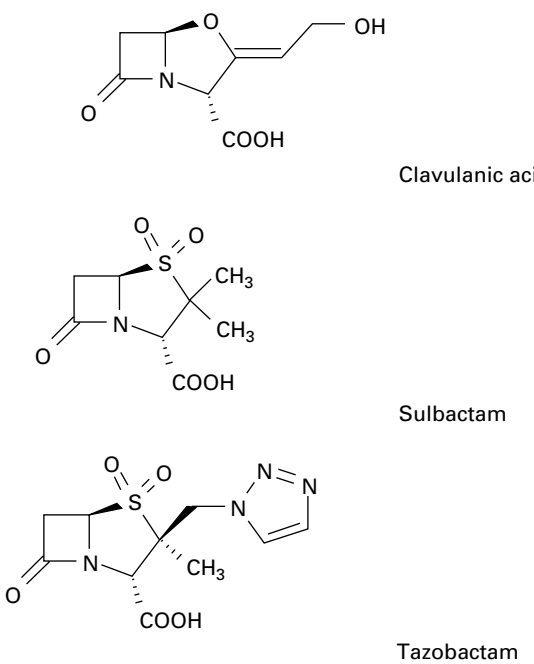


Figure 13.2 Structures of β-lactamase inhibitors.

5 Resistance to glycopeptide antibiotics

Vancomycin and teicoplanin are the two glycopeptides used clinically. They bind the terminal D-alanyl-D-alanine side chains of peptidoglycan and prevent cross-linking in a number of Gram-positive organisms. They are not active against Gram-negative organisms because of the presence of the outer membrane. Vancomycin use increased dramatically in response to the increasing incidence of MRSA and resistance was first reported in the enterococci in 1988. VRE now account for more than 20% of all enterococcal infections. Resistance is greatest amongst *Ent. faecium* strains, but significant numbers of the more clinically significant *Ent. faecalis* are also resistant. Five types of resistance, VanA–VanE, have now been reported. Phenotypic VanA resistance is the most common and confers high-level resistance to vancomycin and teicoplanin. VanA resistance is mediated by a seven-gene cluster on the transposable genetic element Tn*1546* (Figure 13.3).

Resistance to vancomycin is via a sensor histidine kinase (VanS) and a response regulator (VanR). VanH encodes a D-lactate dehydrogenase/α-keto acid reductase and generates D-lactate, which is the substrate for VanA, a D-Ala-D-Lac ligase. The result is cell wall precursors terminating in D-Ala-D-Lac to which vancomycin binds with very low affinity. This change in affinity is mediated by one hydrogen bond. The complex formed between vancomycin and D-Ala-D-Ala is stabilized by five hydro-

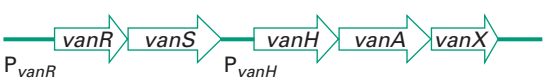


Figure 13.3 Organization of vancomycin resistance gene cluster.

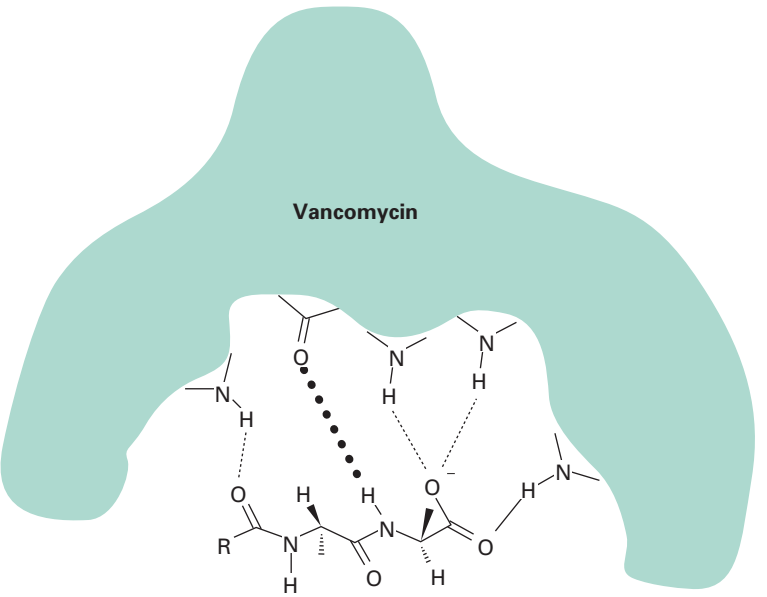
gen bonds, whereas only four hydrogen bonds can form between vancomycin and D-Ala-D-Lac and the complex is unstable (Figure 13.4). Further, VanX encodes a D-Ala-D-Ala dipeptidase which can modify endogenous D-Ala-D-Ala precursors. Recent genetic analysis has identified close homology between this cluster and genes present in the vancomycin-producing organism *Amycolatopsis orientalis*, suggesting that selective pressure has forced genes originally present to protect antibiotic-producing organisms to jump to other species. VanB resistance is also acquired and the peptidoglycan precursor is again D-Ala-D-Lac, but isolates often remain susceptible to teicoplanin. VanC resistance is intrinsic and chromosomally encoded in some enterococcal species such as *Ent. gallinarum* and the peptidoglycan precursor is D-Ala-D-Ser. Less is known of VanD and VanE resistance, but both are acquired. VanD uses D-Ala-D-Lac and VanE uses D-Ala-D-Ser.

5.1 MRSA and reduced glycopeptide susceptibility

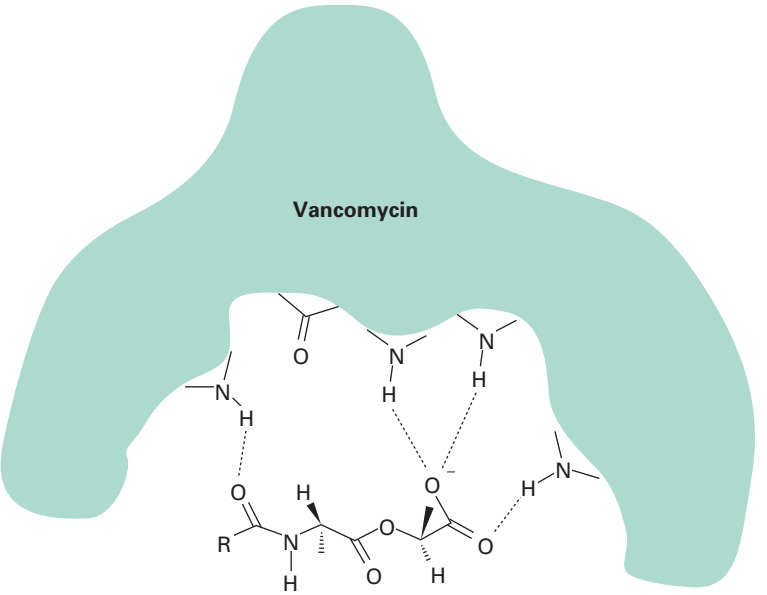
There is major concern that high-level, VanA-type resistance could transfer to staphylococci, particularly MRSA. Experimental transfer of the enterococcal VanA system to *Staph. aureus* on the skin of mice has been reported, but other mechanisms resulting in intermediate-level resistance occur in clinical isolates. In the 1960s and 1970s MRSA was not feared because several other treatment options existed, including use of tetracyclines, macrolides and aminoglycosides. But multiple resistance was accumulating and by the 1980s empirical therapy of staphylococcal infections, particularly nosocomial sepsis, was changed to the glycopeptide antibiotic vancomycin. MRSA levels were rising and the early 1990s saw a major increase in vancomycin use. The inevitable consequence of the selective pressure was the isolation in 1997 of the first *Staph. aureus* strain with reduced susceptibility to vancomycin and teicoplanin (vancomycin MIC = 8 μg/ml). At the beginning of the 21st century, MRSA is responsible for up to 25% of nosocomial infections in the USA and reports of community-acquired MRSA infections are increasing. While reports of 'superbugs' resistant to all known antibiotics abound, it is important to distinguish between reduced susceptibility and resistance, recognizing that there are conflicting definitions of resistance and resistance breakpoints. Strains with MIC values <4 μg/ml are considered sensitive, 8–16 μg/ml intermediate and >32 μg/ml resistant. Thus the acronyms VISA (vancomycin-intermediate *Staph. aureus*) and GISA (glycopeptide-insensitive *Staph. aureus*) are used to denote strains with vancomycin or teicoplanin MICs of 8 μg/ml, whereas VRSA (vancomyin-resistant *Staph. aureus*) is reserved for strains with MIC values >32 μg/ml. The mechanism of glycopeptide resistance is poorly understood, but strains show longer doubling times and decreased susceptibility to lysostaphin. Increased quantities of PBP2 and PBP2′ and cell wall precursors are presumed to trap vancomycin, while amidation of glutamine residues in cell wall muropeptides reduces the cross-linking and consequently the number of vancomycin target molecules.

6 Resistance to aminoglycoside antibiotics

The aminoglycosides are hydrophilic sugars possessing a number of amino and hydroxy substituents. The amine groups are protonated at biological pH and it is the polycationic nature of the molecules that affords them their affinity for nucleic acids, particularly the acceptor (A) site of 16S ribosomal RNA. Aminoglycoside binding to the A site interferes with the accurate recognition of cognate tRNA by rRNA during translation and may also perturb translocation of the tRNA from the A site to the peptidyl-tRNA site (P site). While high-level resistance in aminoglycoside-producing microorganisms is by methylation of the rRNA, this is *not* the mechanism of resistance in previously susceptible strains. The most common mechanism for clinical aminoglycoside resistance is their structural modification by enzymes expressed in resistant organisms, which compromises their ability to interact with rRNA. There are three classes of these enzymes: aminoglycoside phosphatases (APHs), aminoglycoside nucleotidyltransferases (ANTs) and aminoglycoside acetyltransferases (AACs). Within each class, there are enzymes with differing specificities around the sugars. There are four ANTs (ANT(6), ANT(4′), ANT(3″) and ANT(2″)), seven APHs (APH(3′), APH(2″), APH(3″), APH(6), APH(9), APH(4) and APH(7″)) and four AACs (AAC(2′), AAC(6′), AAC(1) and AAC(3)). There is also a bifunctional enzyme, AAC(6′)–AAC(2″). Aminoglycosides are typically susceptible to attack by multiple enzymes (Figure 13.5). Attempts to circumvent these modifying enzymes have centred on structural modifications. Examples include tobramycin which lacks the 3′-hydroxyl group and is thus not a substrate for APH(3′), and amikacin which has an acylated N-1 group and is not a substrate for several modifying enzymes. Other strategies are exemplified by experimental compounds such



Stable complex between vancomycin and D-Ala-D-Ala



Unstable complex between vancomycin and D-Ala-D-Lac

Figure 13.4 Mechanism of high-level vancomycin resistance. Hydrogen bonds are denoted by dotted lines. The key hydrogen bond present in the stable complex with D-Ala-D-Ala, but missing in the unstable complex with D-Ala-D-Lac, is shown in bold type.

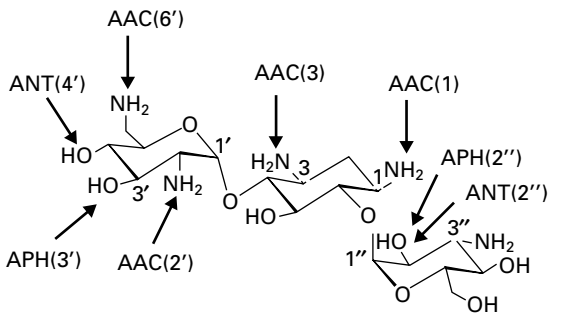


Figure 13.5 Structure of kanamycin B showing system of ring numbering and sites of action of some aminoglycoside-modifying enzymes. AAC, aminoglycoside acetyltransferase; ANT, aminoglycoside nucleotidyltransferase; APH, aminoglycoside phosphotransferase.

as 3′-oxo-kanamycin. This molecule is a substrate for APH(3′), but the phosphorylation product is unstable and regenerates the original antibiotic.

7 Resistance to tetracycline antibiotics

Chlortetracycline and oxytetracycline were discovered in the late 1940s and studies of representative populations before their widespread use suggests that emergence of resistance is a relatively modern event. More than 60% of *Shigella flexneri* isolates are resistant to tetracycline; resistant isolates of *Salmonella enterica* serovar Typhimurium are becoming more common and among Gram-positive species, approximately 90% of MRSA strains and 60% of multiply resistant *Strep. pneumoniae* are now tetracycline-resistant. The major mechanisms of resistance are efflux and ribosomal protection. One exception is the *tet(X)* gene that encodes an enzyme which modifies and inactivates the tetracycline molecule, although this does not appear to be clinically significant. The Tet efflux proteins belong to the major facilitator superfamily (MFS). These proteins exchange a proton for a tetracycline–cation (usually Mg^{2+}) complex, reducing the intracellular drug concentration and protecting the target ribosomes in the cell. In Gram-negative bacteria, the efflux determinants comprise divergently oriented efflux and repressor proteins that share overlapping promoter and operator regions. In the absence of a tetracycline–Mg^{2+} complex, the repressor protein binds and blocks transcription of both genes. Drug binding alters the conformation of the repressor so that it can no longer bind the DNA operator region and block transcription. This method of regulation probably applies to all of the Gram-negative efflux systems including *tet*(A), *tet*(C), *tet*(D), *tet*(E), *tet*(G) and *tet*(H).

No repressor proteins have been identified in the Gram-positive *tet*(K) or *tet*(L) genes and regulation of plasmid-borne tetracycline resistance appears to be by translational attenuation, involving stem-loop mRNA structures and tetracycline-induced unmasking of the ribosome binding site permitting translation of the efflux protein. Regulation of chromosomal *tet*(L) expression involves tetracycline-promoted stalling of the ribosomes during translation of early codons of the leader peptide, which allows reinitiation of translation at the ribosome binding site for the structural gene. Ribosomal protection is mediated by cytoplasmic proteins that inhibit tetracycline and also confer resistance to doxycycline and minocycline. These proteins share homology with the elongation factors EF-Tu and EF-G, and expression of Tet(M) and Tet(O) proteins appears to be regulated. A 400-bp region upstream from the coding region for *tet*(O) is needed for full expression, but the mechanism(s) has not been characterized. The widespread emergence of efflux- and ribosome protection-based resistance to first- and second-generation tetracyclines has prompted the development of the 9-glycinyltetracyclines (9-glycylcyclines). 9-Amino-acylamido derivatives of minocycline have similar activity to earlier compounds; however, when the acyl group is modified to include an *N*,*N*-dialkylamine or 9-*t*-butyl-glycylamido moiety (Figure 13.6), antimicrobial activity is retained and the compounds are active against strains containing *tet* genes responsible for resistance by efflux and ribosomal protection.

8 Resistance to fluoroquinolone antibiotics

Fluoroquinolones bind and inhibit two bacterial topoisomerase enzymes: DNA gyrase (topoisomerase II) which is required for DNA supercoiling, and topoisomerase IV which is required for strand separation during cell division. DNA gyrase tends to be the major target in Gram-negative bacteria, whereas both topoisomerases are inhibited in Gram-positive bacteria. Each topoisomerase is termed a heterotetramer, being composed of two copies of two different subunits designated A and B. The A and B subunits of DNA gyrases are encoded by *gyrA* and *gyrB*, respectively, whilst topoisomerase IV is encoded by *parC* and *parE* (*grlA* and *grlB* in *Staph. aureus*). Mutations in

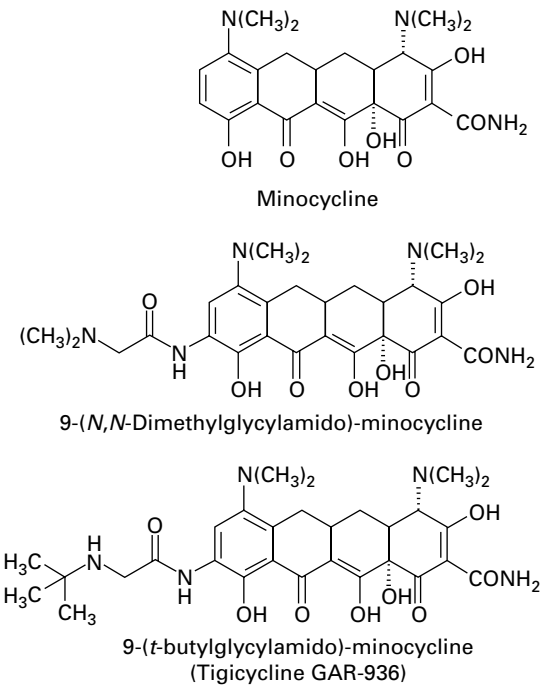


Figure 13.6 Structural modifications at the 9 position of the tetracycline antibiotic minocycline conferring increased stability against resistance mechanisms.

gyrA, particularly involving substitution of a hydroxyl group with a bulky hydrophobic group, induce conformational changes such that the fluoroquinolone can no longer bind. Mutations have also been detected in the B subunit, but these are probably less important. Alterations involving Ser80 and Glu84 of *Staph. aureus grlA* and Ser79 and Asp83 of *Strep. pneumoniae parC* have led to quinolone resistance. Like GyrB, mutations in ParE leading to resistance are not common. While changes in GyrA and ParC give resistance to the older fluoroquinolones, MIC values do not always rise above clinically defined breakpoints for newer agents such as gemifloxacin and moxifloxacin.

Topoisomerases are located in the cytoplasm and thus fluoroquinolones must cross the cell envelope to reach their target. Changes in outer-membrane permeability have been associated with resistance in Gram-negative bacteria, but permeability does not appear to be an issue with Gram-positive species. Efflux, however, does make a contribution to resistance, mainly low level, in both Gram-positive and Gram-negative bacteria. The NorA-mediated efflux system in *Staph. aureus* was characterized in 1990. It is expressed weakly in wild-type strains and resistance is thought to occur via mutations leading to increased expression of *norA*. NorA is a member of the MFS and homologues are also present in *Streptococcus pneumoniae* and *Bacillus* sp. There is a tendency for it to be more effective for hydrophilic fluoroquinolones, but there is no strict correlation. Fluoroquinolones are now being used for treating *M. avium* and multidrug-resistant *M. tuberculosis* and efflux-mediated resistance has been identified. A number of efflux pumps have been identified among Gram-negative bacteria, including AcrA in *E. coli*, which is regulated in part by the multiple-antibiotic resistance (Mar) operon (section 16.3).

9 Resistance to macrolide, lincosamide and streptogramin antibiotics

Although chemically distinct, members of the macrolide, lincosamide and streptogramin (MLS) group of antibiotics all inhibit bacterial protein synthesis by binding to a target site on the ribosome. Gram-negative bacteria are intrinsically resistant due to the permeability barrier of the outer membrane, and three resistance mechanisms have been described in Gram-positive bacteria. Target modification, involving adenine methylation of domain V of the 23S ribosomal RNA, is the most common mechanism. The adenine-N^6-methyltransferase, encoded by the *erm* gene, results in resistance to erythromycin and other macrolides (including the azalides), as well as the lincosamides and group B streptogramins. Streptogramin A-type antibiotics are unaffected and streptogramin A/B combinations remain effective. Expression of the *erm* gene may be constitutive or inducible. When expression is inducible, resistance is seen only against 14- and 15-membered macrolides; lincosamide and streptogramin antibiotics remain active. Telithromycin (Figure 13.7), the first of a new class of ketolide agents in the MLS family, does not induce MLS resistance and also retains activity against domain V-modified ribosomes and inhibition of protein synthesis through strong interaction with domain II. The second resistance mechanism is efflux. Expression of the *mef* gene confers resistance to macrolides only, whereas *msr* expression results in resistance to macrolides and streptogramins. Efflux-mediated resistance of *Staph. aureus* to streptogramin A antibiotics is also conferred by *vga* and *vgaB* gene products. A third resistance mechanism, involving ribosomal mutation, has been reported in a small number of clinical isolates of *Strep. pneumoniae*.

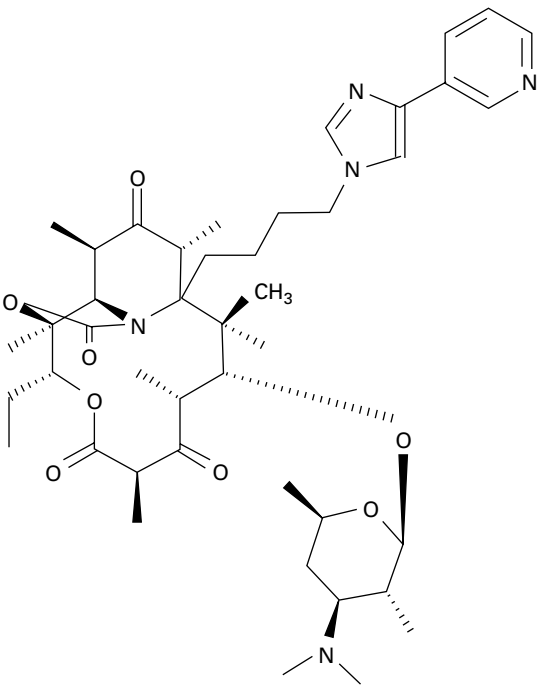


Figure 13.7 Structure of telithromycin, a ketolide macrolide antibiotic that retains activity against 23S domain V modified ribosomal RNA.

10 Resistance to chloramphenicol

Chloramphenicol inhibits protein synthesis by binding the 50S ribosomal subunit and preventing the peptidyltransferase step. Decreased outer-membrane permeability and active efflux have been identified in Gram-negative bacteria; however, the major resistance mechanism is drug inactivation by chloramphenicol acetyltransferase. This occurs in both Gram-positive and Gram-negative species, but the *cat* genes, typically found on plasmids, share little homology.

11 Resistance to the oxazolidinone antibiotics

Linezolid is the first of a new class of oxazolidinone antimicrobials with a novel target in protein synthesis. Linezolid does not interfere with translation initiation at the stage of mRNA binding or formation of 30S preinitiation complexes; rather, it involves binding the 50S rRNA. Its affinity for 50S rRNA from Gram-positive bacteria is twice that for the corresponding molecule in Gram-negative bacteria and as such linezolid has been approved for treating various Gram-positive infections, including MRSA. Resistance is appearing, although rare at present. Mutation in the central loop of domain V of the component 23S rRNA subunit appears to be the main mechanism, including a G2576T mutation in three isolates of linezolid-resistant MRSA.

12 Resistance to trimethoprim

Trimethoprim competitively inhibits dihydrofolate reductase (DHFR) and resistance can be caused by overproduction of host DHFR, mutation in the structural gene for DHFR and acquisition of the *dfr* gene encoding a resistant form. There are at least 15 DHFR enzyme types based on sequence homology and acquisition of *dfr* genes encoding alternative DHFR of type I, II or V is the most common mechanism of trimethoprim resistance among the Enterobacteriaceae.

13 Resistance to mupirocin

Nasal carriage of MRSA strains has been identified as an important target for infection control protocols aimed at reducing spread and acquisition. Mupirocin (pseudomonic acid A) is an effective topical antimicrobial used in MRSA eradication. It is an analogue of isoleucine that competitively binds isoleucyl-tRNA synthetase (IRS) and inhibits protein synthesis. Low-level resistance (MIC 4–256 μg/ml) is usually due to mutation of the host IRS, whereas high-level resistance (MIC >512 μg/ml) is due to acquisition of a distinct IRS that is less sensitive to inhibition. The *mupA* gene, typically carried on transferable plasmids, is found in *Staph. aureus* and coagulase-negative staphylococci, and encodes an IRS with only 30% homology to the mupirocin-sensitive form.

14 Resistance to peptide antibiotics—polymyxin

Many peptide antibiotics have been described and can be broadly classified as non-ribosomally synthesized peptides; they include the polymyxins, bacitracins and gramicidins as well as the glycopeptides (section 5) and the ribosomally synthesized peptides such as the antimicrobial peptides of the innate immune system. Polymyxins and other cationic antimicrobial peptides

have a self-promoted uptake across the cell envelope and perturb the cytoplasmic membrane barrier. Addition of a 4-amino-4-deoxy-L-arabinose (L-Ara4N) moiety to the phosphate groups on the lipid A component of Gram-negative lipopolysaccharide has been implicated in resistance to polymyxin. Details of the pathway for L-Ara4N biosynthesis from UDP glucuronic acid, encoded by the *pmr* operon, are emerging.

15 Resistance to antimycobacterial therapy

The nature of mycobacterial infections, particularly tuberculosis, means that chemotherapy differs from other infections. Organisms tend to grow slowly (long generation time) in a near dormant state with little metabolic activity. Hence, a number of the conventional antimicrobial targets are not suitable. Isoniazid is bactericidal, reducing the count of aerobically growing organisms. Pyrazinamide is active only at low pH, making it well suited to killing organisms within necrotic foci early in infection, but less useful later on when these foci have reduced in number. Rifampicin targets slow-growing organisms. Resistance mechanisms have now been described and multiple resistance poses a serious threat to health. Current treatment regimens result in a high cure rate and the combination of agents makes it highly unlikely that there will be a spontaneous resistant isolate to all the components. Problems most commonly occur in patients who receive inadequate therapy, which provides a serious selection advantage. Resistance can occur to single agents and subsequently to multiple agents. Resistance to rifampicin arises from mutation in the β subunit of RNA polymerase encoded by *rpoB* and resistant isolates show decreased growth rates. Modification of the catalase gene *katG* results in resistance to isoniazid, mainly by reduced or absent catalase activity. Catalase activity is absolutely required to convert isoniazid to the active hydrazine derivative. Interestingly, animal model studies suggest that *M. tuberculosis* strains in which the *katG* gene is inactivated are attenuated compared with wild-type strains. Low-level rifampicin resistance can be obtained by point mutations in *inhA* leading to its overexpression. Pyrazinamide is a prodrug requiring pyrazinamidase to produce the active pyrazinoic acid. Most cases of resistance are due to mutations in the pyrazinamidase gene (*pncA*), but gene inactivation by the insertion sequence IS*6110* has been reported. Streptomycin resistance can arise through mutations in *rrs* and *rpsL* which affect streptomycin binding. However, these account for only half of the resistant isolates, so further resistance mechanisms await definition. Ethambutol resistance has been noted in *M. tuberculosis* and other species such as *M. smegmatis*. Ethambutol inhibits the polymerization of arabinan in the arabinogalactan and lipoarabinomannan of the mycobacterial cell wall and one of its likely targets is the family of arabinosyltransferases encoded by the *emb* locus. Missense mutations in the *embB* gene in this locus confer resistance to ethambutol.

16 Multiple drug resistance

16.1 R-factors

Several issues of multiple drug resistance have already been raised in this chapter. Notable examples are MRSA, which can harbour both small cryptic plasmids and larger plasmids encoding resistance to antiseptics, disinfectants, trimethoprim, penicillin, gentamicin, tobramycin and kanamycin, and multidrug-resistant *M. tuberculosis*. Of equal concern are instances where isolates can become resistant to multiple, chemically distinct agents in a single biological event. One of the earliest examples was in Japan in 1959. Previously sensitive *E. coli* became resistant to multiple antibiotics through acquisition of a conjugative plasmid (R-factor) from resistant *Salmonella* and *Shigella* isolates. A number of R-factors have now been characterized including RP4, encoding resistance to ampicillin, kanamycin, tetracycline and neomycin, found in *Ps. aeruginosa* and other Gram-negative bacteria; R1, encoding resistance to ampicillin, kanamycin, sulphonamides, chloramphenicol and streptomycin, found in Gram-negative bacteria and pSH6, encoding resistance to gentamicin, trimethoprim and kanamycin, found in *Staph. aureus*.

16.2 Mobile gene cassettes and integrons

Many Gram-negative resistance genes are located in gene cassettes. One or more of these cassettes can be integrated into a specific position on the chromosome termed an *integron*. More than 60 cassettes have been identified, each comprising only a promotor-less single gene (usually antibiotic resistance) and a 59-base element forming a specific recombination site. This recombination site confers mobility because it is recognized by specific recombinases encoded by integrons that catalyse integration of the cassette into a specific site within the integron. Thus, integrons are genetic elements that recognize and

capture multiple mobile gene cassettes. As the gene typically lacks a promoter, expression is dependent on correct orientation into the integron to supply the upstream promoter. Four classes of integron have been identified, although only one member of class 3 has been described and class 4 integrons are limited to *Vibrio cholerae*. Analysis of the resistant *Shigella* strains isolated in Japan has shown that some of the conjugative plasmids included an integron with one or two integrated cassettes.

16.3 Chromosomal multiple-antibiotic resistance (*mar*) locus

The multiple-antibiotic resistance (*mar*) locus was first described in *E. coli* by Stuart Levy and colleagues at Tufts University and has since been recognized in other enteric bacteria. The locus consists of two divergently transcribed units, *marC* and *marRAB*. Little is known of *marC* and *marB*; however, *marR* encodes a repressor of the operon, and *marA* encodes a transcriptional activator affecting expression of more than 60 genes. Increased expression of the *MarRAB* operon resulting from mutations in *marO* or *marR*, or from inactivation of MarR following exposure to inducing agents such as salicylate, leads to the Mar phenotype. This phenotype is characterized by resistance to structurally unrelated antibiotics, organic solvents, oxidative stress and chemical disinfectants. A number of effector mechanisms have been identified, including increased expression of the *acrAB-tolC* multidrug efflux system (section 16.4) and the *soxRS* regulon.

16.4 Multidrug efflux pumps

Whereas some efflux pumps excrete only one drug or class of drugs, a multidrug efflux pump can excrete a wide range of compounds where there is often little or no chemical similarity between the substrates. One common characteristic may be agents with a significant hydrophobic domain. For this reason, hydrophilic compounds such as the aminoglycoside antibiotics are not exported by these systems. A distinction needs to be drawn between those efflux systems, typically in Gram-positive bacteria, that pump their substrate across the cytoplasmic membrane, such as the QacA and Smr pumps which both export quaternary ammonium compounds and basic dyes, and those which efflux across the cytoplasmic and outer membranes of Gram-negative bacteria. There are some examples of single membrane systems in Gram-negative bacteria, such as the EmrE protein in *E. coli*, but they are not of great clinical significance. The majority of Gram-negative pumps span both membranes and

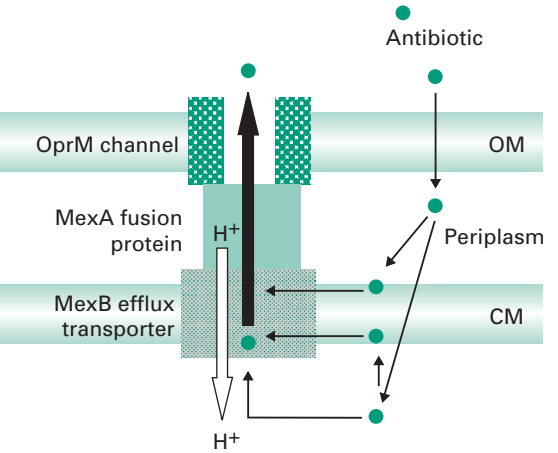


Figure 13.8 Schematic diagram of the MexAB-OprM efflux pump from *Pseudomonas aeruginosa*. CM, cytoplasmic membrane; OM, outer membrane. (Kindly supplied by K. Poole.)

include the AcrAB-TolC system in *E. coli* and the MexAB-OprM system in *Ps. aeruginosa*. Genomic analyses are revealing numerous homologues. Using the MexAB-OprM system as the prototypic example (Figure 13.8), MexA is the linker protein and MexB is in the cytoplasmic membrane. MexB is a resistance-nodulation-division (RND) family member and is predicted to be a proton antiporter with 12 membrane-spanning α-helices. OprM shows homology with outer-membrane channels of systems thought to export such diverse molecules as nodulation signals and alkaline proteases. Mutations in regulatory genes such as *nalB* cause overexpression of MexAB-OprM and consequently multidrug resistance. MexB is a proton antiporter and efflux by this and other members of the RND family is energized by the proton motive force. This contrasts with mammalian multidrug efflux pumps (MDR) that are powered by ATP hydrolysis.

17 Clinical resistance—MIC values, breakpoints, phenotype and outcome

The resistance mechanisms described in this chapter typically lead to an increase in MIC value, although it should be remembered that this does not always equate to clinical failure. If the MIC value remains below the breakpoint value, which can itself be difficult to determine, then the antibiotic will remain effective in the

clinic. But such arguments assume that MIC values, which are typically determined when the isolate is growing in complex, sensitivity-test broth, equate with the sensitivity of the organism when growing in the many subtly different environments encountered during infection *in vivo*. Unfortunately, the antibiotic literature contains numerous examples of treatment failures despite apparent sensitivity in the test tube and this resistance is referred to as being *phenotypic*. In other words, resistance is a consequence of the adaptation of the organism to grow and survive within the *in vivo* environment, and subculture into conventional laboratory growth medium rarely shows the existence of resistant mutants. There are several key factors at play here, particularly slow/no growth, nutrient depletion and mode of growth. There are numerous papers showing a tendency for nutrient depletion, i.e. restricted or non-availability of an important nutrient, and slow/no growth to be associated with reduced susceptibility to antibiotics and biocides.

There is now increasing concern over the role played by microbial biofilms in infection (see Chapter 8). These include the well-known examples of medical device-related infections, such as those associated with artificial joints, prosthetic heart valves and catheters. Many chronic infections, not related to medical devices, are also due to bacteria either not growing and relatively dormant or growing slowly as biomasses or adherent biofilms on mucosal surfaces. A bacterial biofilm is typically defined as a population of cells growing as a consortium on a surface and enclosed in a complex exopolymer matrix. Commonly in the wider environment but less so in infections, the population is mixed and also of heterogeneous physiologies. Growth as a biofilm almost always leads to a large increase in resistance to antimicrobial agents, including antibiotics, biocides and preservatives, compared with cultures grown in suspension (planktonic) in conventional liquid media, but there is no generally agreed mechanism to account for this resistance. Although there is general acceptance that there are numerous planktonic phenotypes, many papers refer to 'the biofilm phenotype', implicitly assuming (wrongly) that there is only one. Those same parameters known to influence planktonic physiology and antibiotic susceptibility, including growth rate and/or specific nutrient limitation, also apply to biofilm physiology and antibiotic susceptibility. The general resistance of biofilms is clearly phenotypic. The well-characterized resistance mechanisms described above—lack of antibiotic penetration, inactivation, efflux and repair—make contributions in some circumstances. However, compelling evidence that they are uniquely responsible for biofilm resistance is lacking. Reduced growth rate probably has an involvement, particularly in that it is associated with responses to stress. During stress responses, key structures are protected and cellular processes close down to a state of dormancy, and it has been proposed that exceptional vegetative cell dormancy is the basic explanation of biofilm resistance.

18 Concluding comments

It has been said that in the early years of the 21st century we are in an interim between the first antibiotic era, exemplified by the β-lactams, macrolides, tetracyclines, aminoglycosides and fluoroquinolones, and a second era of new agents directed against targets waiting to be revealed by genomics and proteomics research. In this interim, the goals must be to reduce antibiotic usage and encourage the return of a susceptible human commensal flora. Finally, it must be remembered that antibiotics are not a human invention. Microorganisms have used them in attack and counter-attack against each other for billions of years. Our efforts over the last 60 years seem trivial in comparison.

19 Further reading

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14 Clinical uses of antimicrobial drugs

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1 Introduction

The worldwide use of antimicrobial drugs continues to rise; in 2005 these agents accounted for an expenditure of approximately £26 billion. In the UK, prescribing in general practice accounts for approximately 90% of all antibiotics and largely involves oral and topical agents. Hospital use accounts for the remaining 10% of antibiotic prescribing, with a much heavier use of injectable agents. Although this chapter is concerned with the clinical use of antimicrobial drugs, it should be remembered that these agents are also extensively used in veterinary practice and, to a diminishing extent, in animal husbandry as growth promoters. In humans the therapeutic use of anti-infectives has revolutionized the management of most bacterial infections, many parasitic and fungal diseases and, with the availability of aciclovir and a growing number of antiretroviral agents (see Chapters 5 and 11), selected herpesvirus infections and HIV infection, respectively. Although originally used for the treatment of established bacterial infections, antibiotics have proved useful in the prevention of infection in various high-risk circumstances; this applies especially to patients undergoing various surgical procedures where perioperative antibiotics

Hugo and Russell's Pharmaceutical Microbiology, Eighth Edition. Edited by Stephen P. Denyer, Norman Hodges, Sean P. Gorman, Brendan F. Gilmore.

have significantly reduced postoperative infectious complications.

The advantages of effective antimicrobial chemotherapy are self-evident, but this has led to a significant problem in ensuring that they are always appropriately used. Prescribers face a dilemma: initial antimicrobial therapy must be effective against all likely infective organisms for the individual presentation, but excessive use of broad-spectrum agents contributes to the development and selection of drug-resistant organisms. Hence, antiinfectives are the only class of drug where inappropriate use in one patient can jeopardize the efficacy of treatment in other individuals.

Examples of inappropriate antimicrobial use include prescribing in situations where antibiotics are either ineffective, such as viral infections, or where the selected agent, its dose, route of administration or duration of use are inappropriate. Of particular concern is the unnecessarily prolonged use of antibiotics for surgical prophylaxis. Apart from encouraging superinfection by drug-resistant organisms, prolonged use is wasteful of health resources and unnecessarily increases the risk of adverse drug reactions. Thus, it is essential that the clinical use of these agents be based on a clear understanding of the principles that have evolved to ensure safe, yet effective, prescribing.

Further information about the properties of antimicrobial agents described in this chapter can be found in Chapter 11.

2 Principles of use of antimicrobial drugs

2.1 Susceptibility of infecting organisms

Drug selection should be based on knowledge of its activity against infecting microorganisms. Selected organisms may be predictably susceptible to a particular agent, and laboratory testing is therefore rarely performed. For example, *Streptococcus pyogenes* is uniformly sensitive to penicillin. In contrast, the susceptibility of many Gram-negative enteric bacteria is less predictable and laboratory guidance is essential for safe prescribing. The susceptibility of common bacterial pathogens and widely prescribed antibiotics is summarized in Table 14.1. It can be seen that, although certain bacteria are susceptible *in vitro* to a particular agent, use of that drug may be inappropriate, either on pharmacological grounds or because other less toxic agents are preferred.

2.2 Host factors

In vitro susceptibility testing does not always predict clinical outcome. Host factors play an important part in determining outcome and this applies particularly to circulating and tissue phagocytic activity. Infections can progress rapidly in patients suffering from either an absolute or functional deficiency of phagocytic cells. This applies particularly to those suffering from various haematological malignancies, such as the acute leukaemias, where phagocyte function is impaired both by the disease and also by the use of potent cytotoxic drugs which destroy healthy, as well as malignant, white cells. Under these circumstances it is essential to select agents that are bactericidal, as bacteriostatic drugs, such as the tetracyclines or sulphonamides, rely on host phagocytic activity to clear bacteria. Widely used bactericidal agents include the aminoglycosides, broad-spectrum penicillins, the cephalosporins and quinolones (see Chapter 11).

In some infections the pathogenic organisms are located intracellularly within phagocytic cells and therefore remain relatively protected from drugs that penetrate cells poorly, such as the penicillins and cephalosporins. In contrast, erythromycin, rifampicin and the fluoroquinolones readily penetrate phagocytic cells. Legionnaires' disease is an example of an intracellular infection and is treated with erythromycin with or without rifampicin.

2.3 Pharmacological factors

Clinical efficacy is also dependent on achieving satisfactory drug concentrations at the site of the infection; this is influenced by the standard pharmacological factors of absorption, distribution, metabolism and excretion. If an oral agent is selected, gastrointestinal absorption should be satisfactory. However, it may be impaired by factors such as the presence of food, drug interactions (including chelation), or impaired gastrointestinal function either as a result of surgical resection or malabsorptive states. Although effective, oral absorption may be inappropriate in patients who are vomiting or have undergone recent surgery; under these circumstances a parenteral agent will be required and has the advantage of providing rapidly effective drug concentrations.

Antibiotic selection also varies according to the anatomical site of infection. Lipid solubility is of importance in relation to drug distribution. For example, the aminoglycosides are poorly lipid-soluble and although achieving therapeutic concentrations within the extracellular fluid compartment, penetrate the cerebrospinal fluid (CSF) poorly. Likewise the presence of inflammation may affect drug penetration into the tissues. In the

Table 14.1 Sensitivity of selected bacteria to common antibacterial agents

	Staphylococcus aureus(pen. sensitive)	*Staphylococcus aureus*(pen. resistant)	*Streptococcus pyogenes* and *Streptococcus pneumoniae*	*Enterococcus*	*Cl. perfringens*	*Neisseria gonorrhoeae*	*Neisseria meningitidis*
Penicillin V/G	+	R	+*	+	+	+*	+
Methicillin, flucloxacillin	+	+*	+	R	(+)	(±)	(±)
Ampicillin, amoxicillin	+	R	+*	+	+	+*	+
Ticarcillin	(+)	R	(+)	R	+	(+)	(+)
Cefazolin	+	+*	+	R	(±)	(+)	(+)
Cefamandole, cefuroxime	+	+	+	R	+	+	+
Cefoxitin	+	+	+	R	+	(+)	(+)
Cefotaxime, ceftriaxone	+	+	+	R	+	+	+
Ceftazidime	+	+	+	R		+	+
Erythromycin	+	+	+	R	±	+	(+)
Clindamycin	+*	+*	+*	R	+*	R	R
Tetracyclines	+*	+*	±	+	+	+	(+)
Chloramphenicol	+	+	+	+	+	+	+
Ciprofloxacin	±	±	+	±	R	+	+
Gentamicin, tobramycin, amikacin, netilmicin	+	+	R	R	R	R	R
Sulphonamides	+	+	±	±	(±)	±	±
Trimethoprim–sulphamethoxazole	+	+	+	+	R	+	+

	Haemophilus influenzae	*Escherichia coli*	*Klebsiella* spp.	*P roteus* spp. (indole-negative)	*Proteus* spp. (indole-positive)	*Serratia* spp.	*Salmonella* spp.
Penicillin V/G	±	R	R	R	R	R	R
Methicillin, flucloxacillin	R	R	R	R	R	R	R
Ampicillin, amoxicillin	±	±	R	+	R	R	±
Ticarcillin	(+)	±	±	+	±	±	(+)
Cefazolin	±	+	±	+	R	R	(+)
Cefamandole, cefuroxime	+	+	+	+	+	R	(+)
Cefoxitin	+	+	+	+	+	±	(+)
Cefotaxime, ceftriaxone	+	+	+	+	+	+	(+)
Ceftazidime	+	+	+	+	+	+	(+)
Erythromycin	±	R	R	R	R	R	R
Clindamycin	R	R	R	R	R	R	R
Tetracyclines	+	+	±	±	R	R	(+)
Chloramphenicol	+*	+	±	+	±	+	+*
Ciprofloxacin	+	+	+	+	+	+	+
Gentamicin, tobramycin, amikacin, netilmicin	+	+	+*	+	+	+*	(+)
Sulphonamides	±	±	±	±	±	R	±
Trimethoprim–sulphamethoxazole	+	+	+	+	+	R	+

(*continued*)

Table 14.1 (*continued*)

	Shigella spp.	*Pseudomonas* spp.	*Bacteroides fragilis*	Other *Bacteroides* spp.	*Chlamydia* spp.	*Mycoplasma pneumoniae*	*Rickettsia* spp.
Penicillin V/G	R	R	R	+	R	R	R
Methicillin, flucloxacillin	R	R	R	(±)	R	R	R
Ampicillin, amoxicillin	±	R	R	+	R	R	R
Ticarcillin	(+)	+*	±	±	R	R	R
Cefazolin	(+)	R	R	±	R	R	R
Cefamandole, cefuroxime	(+)	R	R	R	R	R	R
Cefoxitin	(+)	R	+	+	R	R	R
Cefotaxime, ceftriaxone	(+)	±	R/±	R/+	R	R	R
Ceftazidime	(+)	+	R	±	R	R	R
Erythromycin	R	R	±	±	+	+	R
Clindamycin	R	R	+	+	R	R	R
Tetracyclines	(±)	R	±	±	+	+	+
Chloramphenicol	+	R	+	+	+	+	+
Ciprofloxacin	+	(+)	R	R	R	R	R
Gentamicin, tobramycin, amikacin, netilmicin	(+)	+*	R	R	R	R	R
Sulphonamides	±	R	R	R	+	R	R
Trimethoprim–sulphamethoxazole	+	R	R	R	+	R	R

+, Sensitive; R, resistant; ±, some strains resistant; ( ), not appropriate therapy; *, rare strains resistant.

presence of meningeal inflammation, β-lactam agents achieve satisfactory concentrations within the CSF, but as the inflammatory response subsides drug concentrations fall. Hence it is essential to maintain sufficient dosaging throughout the treatment of bacterial meningitis. Other agents such as chloramphenicol are little affected by the presence or absence of meningeal inflammation.

Therapeutic drug concentrations within the bile duct and gallbladder are dependent on biliary excretion. In the presence of biliary disease, such as gallstones or chronic inflammation, the drug concentration may fail to reach therapeutic levels. In contrast, drugs that are excreted primarily via the liver or kidneys may require reduced dosaging in the presence of impaired renal or hepatic function. The malfunction of excretory organs may not only risk toxicity from drug accumulation, but will also reduce urinary concentration of drugs excreted primarily by glomerular filtration. This applies to the aminoglycosides and the urinary antiseptics nalidixic acid and nitrofurantoin, where therapeutic failure of urinary tract infections may complicate severe renal failure.

2.4 Drug resistance

Drug resistance may be a natural or an acquired characteristic of a microorganism. This may result from impaired cell wall or cell envelope penetration, enzymatic inactivation, altered binding sites or active extrusion from the cell as a result of efflux mechanisms (Chapter 13). Acquired drug resistance may result from mutation, adaptation or gene transfer. Spontaneous mutations occur at low frequency, as in the case of *Mycobacterium tuberculosis* where a minority population of organisms is resistant to isoniazid. In this situation the use of isoniazid alone will eventually result in overgrowth by this subpopulation of resistant organisms.

Genetic resistance may be chromosomal or transferable on transposons or plasmids. Plasmid-mediated resistance has been increasingly recognized among Gram-negative enteric pathogens. By the process of conjugation (Chapter 13), resistance plasmids may be transferred between bacteria of the same and different species and also different genera. Such resistance can code for multiple antibiotic resistance. For example, the penicillins, cephalosporins, chloramphenicol and the aminoglycosides are all subject to enzymatic inactivation, which may be plasmid-mediated. Knowledge of the local epidemiology of resistant pathogens within a hospital, and especially within high-dependency areas such as intensive care and haemodialysis units, is invaluable in guiding appropriate drug selection.

2.4.1 Multidrug resistance

In recent years multidrug resistance has increased among certain pathogens. These include *Staphylococcus aureus*, enterococci and *M. tuberculosis*. *Staph. aureus* resistant to meticillin is known as meticillin-resistant *Staph. aureus* (MRSA). These strains are resistant to many antibiotics and have been responsible for major epidemics worldwide, usually in hospitals where they affect patients in high-dependency units such as intensive care units, burns units and cardiothoracic units. MRSA have the ability to colonize staff and patients and to spread readily among them. Several epidemic strains are currently circulating in the UK. The glycopeptides vancomycin or teicoplanin and the oxazolidinone linezolid are the currently recommended agents for treating patients infected with these organisms. Newer agents such as daptomycin and tigecycline are also active against MRSA and are increasingly used in the hospital setting; meanwhile, some strains retain sensitivity to older agents such as tetracyclines, rifampicin and clindamycin, and combinations of these agents are sometimes used, but only following full analysis of microbiological sensitivities.

Another serious resistance problem is that of drug-resistant enterococci. These include *Enterococcus faecalis* and, in particular, *E. faecium*. Resistance to the glycopeptides has again been a problem among patients in high-dependency units. Four different phenotypes are recognized (VanA, VanB, VanC and VanD). The VanA phenotype is resistant to both glycopeptides, while the others are sensitive to teicoplanin but demonstrate high (VanB) or intermediate (VanC) resistance to vancomycin; VanD resistance has only recently been described and remains uncommon. Those fully resistant to the glycopeptides are increasing in frequency and causing great concern as they are essentially resistant to almost all antibiotics.

Extended-spectrum β-lactamase (ESBL) producing Gram-negative organisms are an increasing problem in hospitals, and occasionally seen as a cause of urinary tract infection in primary care; ESBLs can hydrolyse most cephalosporins and penicillins, limiting therapeutic options to carbapenems or aminoglycosides. ESBLs can be chromosomally mediated (e.g. *Pseudomonas* spp., *Citrobacter* spp.) or plasmid-mediated (e.g. *Klebsiella* spp.), the latter often being implicated in hospital outbreaks. Furthermore, carbapenem resistance, via metallo-β-lactamase, has been seen in outbreak strains of *Pseudomonas aeruginosa* and *Acinetobacter baumannii*; in such situations, potentially toxic agents such as colistin are often the only effective choice.

Tuberculosis is on the increase after decades in which the incidence had been steadily falling. Drug-resistant strains have emerged largely among inadequately treated or non-compliant patients. These include the homeless, alcoholic, intravenous drug misusing, HIV-positive and immigrant populations. Resistance patterns vary but increasingly include rifampicin and isoniazid. Furthermore, outbreaks of multidrug-resistant tuberculosis have been increasingly reported from a number of hospital centres in the USA and more recently Europe, including the UK. These infections have occasionally spread to healthcare workers and are giving rise to considerable concern.

The underlying mechanisms of resistance are considered in Chapter 13.

2.5 Drug combinations

Antibiotics are generally used alone, but may on occasion be prescribed in combination. Combining two antibiotics may result in synergism, indifference or antagonism. In the case of synergism, microbial inhibition is achieved at concentrations below that for each agent alone and may prove advantageous in treating relatively insusceptible infections such as enterococcal endocarditis, where a combination of penicillin and gentamicin is synergistically active. Another advantage of synergistic combinations is that it may enable the use of toxic agents where dose reductions are possible. For example, meningitis caused by the fungus *Cryptococcus neoformans* responds to an abbreviated course of amphotericin B when it is combined with 5-flucytosine, thereby reducing the risk of toxicity from amphotericin B.

Combined drug use is occasionally recommended to prevent resistance emerging during treatment. For example, treatment may fail when fusidic acid is used alone to treat *Staph. aureus* infections, because resistant strains develop rapidly; this is prevented by combining fusidic acid with flucloxacillin. Likewise, tuberculosis is initially treated with a minimum of three agents, such as rifampicin, isoniazid and pyrazinamide; again drug resistance is prevented, which may result if either agent is used alone.

The most common reason for using combined therapy is in the treatment of confirmed or suspected mixed infections where a single agent alone will fail to cover all pathogenic organisms. This is the case in serious abdominal sepsis where mixed aerobic and anaerobic infections are common and the use of metronidazole in combination with either an aminoglycoside or a broad-spectrum cephalosporin is essential. Finally, drugs are used in combination in patients who are seriously ill and about whom uncertainty exists concerning the microbiological nature of their infection. This initial 'blind therapy' frequently includes a broad-spectrum penicillin or cephalosporin in combination with an aminoglycoside. The regimen should be modified in the light of subsequent microbiological information.

2.6 Adverse reactions

Regrettably, all chemotherapeutic agents have the potential to produce adverse reactions with varying degrees of frequency and severity, and these include hypersensitivity reactions and toxic effects. These may be dose-related and predictable in a patient with a history of hypersensitivity or a previous toxic reaction to a drug or its chemical analogues. However, many adverse events are idiosyncratic and therefore unpredictable.

Hypersensitivity reactions range in severity from fatal anaphylaxis, in which there is widespread tissue oedema, airway obstruction and cardiovascular collapse, to minor and reversible hypersensitivity reactions such as skin eruptions and drug fever. Such reactions are more likely in those with a history of hypersensitivity to the drug, and are more frequent in patients with previous allergic diseases such as childhood eczema or asthma. It is important to question patients closely concerning hypersensitivity reactions before prescribing, as it precludes the use of all compounds within a class, such as the sulphonamides or tetracyclines, while cephalosporins and carbapenems should be used only with caution in patients who are allergic to penicillin, because these agents are structurally related. They should be avoided entirely in those who have had a previous severe hypersensitivity reaction to penicillin.

Drug toxicity is often dose-related and may affect a variety of organs or tissues. For example, the aminoglycosides are both nephrotoxic and ototoxic to varying degrees; therefore, dosaging should be individualized and the serum assayed, especially where renal function is abnormal, to avoid toxic effects and non-therapeutic drug concentrations. An example of dose-related toxicity is chloramphenicol-induced bone marrow suppression. Chloramphenicol interferes with the normal maturation of bone marrow stem cells and high concentrations may result in a steady fall in circulating red and white cells and also platelets. This effect is generally reversible with dose reduction or drug withdrawal. This dose-related toxic reaction of chloramphenicol should be contrasted with idiosyncratic bone marrow toxicity which is unrelated to dose and occurs at a much lower frequency of

approximately 1:40 000 and is frequently irreversible, ending fatally. Toxic effects may also be genetically determined. For example, peripheral neuropathy may occur in those who are slow acetylators of isoniazid, while haemolysis occurs in those deficient in the red cell enzyme glucose-6-phosphate dehydrogenase, when treated with sulphonamides, primaquine, quinolones or nitrofurantoin.

2.7 Superinfection

Anti-infective drugs not only affect the invading organism undergoing treatment but also have an impact on the normal bacterial flora, especially of the skin and mucous membranes. This may result in microbial overgrowth of resistant organisms with subsequent superinfection. One example is the common occurrence of oral or vaginal candidiasis in patients treated with broad-spectrum agents such as ampicillin or tetracycline. A more serious example is the development of pseudomembranous colitis from the overgrowth of toxin-producing strains of *Clostridium difficile* present in the bowel flora following the use of clindamycin or broad-spectrum antibiotics, though any antimicrobial can precipitate this condition. *C. difficile*-associated diarrhoea is managed by drug withdrawal and oral vancomycin, or oral/intravenous metronidazole. Intravenous immunoglogbulin is occasionally used in severe cases, and rarely, colectomy (excision of part or whole of the colon) may be necessary. Once established, *C.difficile* infection is transmissible, particularly in the hospital setting; isolation of symptomatic patients and strict observation of hygiene practices (e.g. hand washing) are therefore key in preventing outbreaks.

2.8 Chemoprophylaxis

An increasingly important use of antimicrobial agents is that of infection prevention, especially in relationship to surgery. Infection remains one of the most important complications of many surgical procedures, and the recognition that perioperative antibiotics are effective and safe in preventing this complication has proved a major advance in surgery. The principles that underlie the chemoprophylactic use of antibacterials relate to the predictability of infection for a particular surgical procedure, in terms of its occurrence, microbial aetiology and susceptibility to antibiotics. Therapeutic drug concentrations present at the operative site at the time of surgery rapidly reduce the number of potentially infectious organisms and prevent wound sepsis. If prophylaxis is delayed to the postoperative period, then efficacy is markedly impaired. It is important that chemoprophylaxis be limited to the perioperative period, the first dose being administered approximately 1 hour before surgery for injectable agents; for many procedures and operative sites, a single dose is now considered sufficient. Prolonging chemoprophylaxis beyond this period is not cost-effective and increases the risk of adverse drug reactions and superinfection. One of the best examples of the efficacy of surgical prophylaxis is in the area of large-bowel surgery. Before the widespread use of chemoprophylaxis, postoperative infection rates for colectomy were often 30% or higher; these have now been reduced to around 5%.

Chemoprophylaxis has been extended to other surgical procedures where the risk of infection may be low but its occurrence has serious consequences. This is especially true for the implantation of prosthetic joints or heart valves. These are major surgical procedures and although infection may be infrequent its consequences are serious and on balance the use of chemoprophylaxis is cost-effective.

Examples of chemoprophylaxis in the non-surgical arena include the prevention of pneumococcal infection with penicillin V in asplenia or patients with sickle-cell disease, and the prevention of secondary cases of meningococcal meningitis with rifampicin or ciprofloxacin among household contacts of an index case.

3 Clinical use

The choice of antimicrobial chemotherapy is initially dependent on the clinical diagnosis. In some circumstances the clinical diagnosis implies a microbiological diagnosis which may dictate specific therapy. For example, typhoid fever is caused by *Salmonella enterica* serovar Typhi, which is generally sensitive to co-trimoxazole, ceftriaxone and ciprofloxacin. However, for many infections, establishing a clinical diagnosis implies a range of possible microbiological causes and requires laboratory confirmation from samples collected, preferably before antibiotic therapy is begun. Laboratory isolation and susceptibility testing of the causative agent establish the diagnosis with certainty and make drug selection more rational. However, in many circumstances, especially in general practice, microbiological documentation of an infection is not possible. Hence knowledge of the usual microbiological cause of a particular infection and its susceptibility to antimicrobial agents is essential for effective drug prescribing. The following section explores a selection of the problems associated with antimicrobial drug prescribing for a range of clinical conditions.

3.1 Respiratory tract infections

Infections of the respiratory tract are among the commonest of infections, and account for much consultation in general practice and a high percentage of acute hospital admissions. They are divided into infections of the upper respiratory tract, involving the ears, throat, nasal sinuses and the trachea, and the lower respiratory tract (LRT), where they affect the airways, lungs and pleura.

3.1.1 Upper respiratory tract infections

Acute pharyngitis presents a diagnostic and therapeutic dilemma. The majority of sore throats are caused by a variety of viruses; fewer than 20% are bacterial and hence potentially responsive to antibiotic therapy. However, antibiotics are widely prescribed and this reflects the difficulty in discriminating streptococcal from non-streptococcal infections clinically in the absence of microbiological documentation. Nonetheless, *Strep. pyogenes* is the most important bacterial pathogen and this responds to oral penicillin. However, up to 10 days' treatment is required for its eradication from the throat. This requirement causes problems with compliance as symptomatic improvement generally occurs within 2–3 days.

Although viral infections are important causes of both otitis media and sinusitis, they are generally self-limiting. Bacterial infections may complicate viral illnesses, and are also primary causes of ear and sinus infections. *Streptococcus pneumoniae* and *Haemophilus influenzae* are the commonest bacterial pathogens. Amoxicillin is widely prescribed for these infections as it is microbiologically active, penetrates the middle ear and sinuses, is well tolerated and has proved effective.

3.1.2 Lower respiratory tract infections

Infections of the LRT include pneumonia, lung abscess, bronchitis, bronchiectasis and infective complications of cystic fibrosis. Each presents a specific diagnostic and therapeutic challenge, which reflects the variety of pathogens involved and the frequent difficulties in establishing an accurate microbial diagnosis. The laboratory diagnosis of LRT infections is largely dependent upon culturing sputum. Unfortunately this may be contaminated with the normal bacterial flora of the upper respiratory tract during expectoration. In hospitalized patients, the empirical use of antibiotics before admission substantially diminishes the value of sputum culture and may result in overgrowth by non-pathogenic microbes, thus causing difficulty with the interpretation of sputum culture results. Alternative diagnostic samples include needle aspiration of sputum directly from the trachea or of fluid within the pleural cavity. Blood may also be cultured and serum examined for antibody responses or microbial antigens. In the community, few patients will have their LRT infection diagnosed microbiologically and the choice of antibiotic is based on clinical diagnosis.

3.1.2.1 Pneumonia

The range of pathogens causing acute pneumonia includes viruses, bacteria and, in the immunocompromised host, parasites and fungi. Table 14.2 summarizes these pathogens and indicates drugs appropriate for their treatment. Clinical assessment includes details of the evolution of the infection, any evidence of a recent viral infection, the age of the patient and risk factors such as corticosteroid therapy or pre-existing lung disease. The extent of the pneumonia, as assessed clinically or by X-ray, is also important.

Streptococcus pneumoniae remains the commonest cause of pneumonia and still responds well to penicillin despite a global increase in isolates showing reduced susceptibility to this agent. So-called 'respiratory quinolones' such as levofloxacin and moxifloxacin, which exhibit increased activity against Gram positive organisms compared to ciprofloxacin, are an alternative. A number of atypical infections may cause pneumonia and include *Mycoplasma pneumoniae*, *Legionella pneumophila*, psittacosis and occasionally Q fever. With psittacosis there may be a history of contact with parrots or budgerigars; while legionnaires' disease has often been acquired during hotel holidays in the Mediterranean area. The atypical pneumonias, unlike pneumococcal pneumonia, do not respond to penicillin. Legionnaires' disease is treated with erythromycin and, in the presence of severe pneumonia, rifampicin is added to the regimen. Mycoplasma infections are best treated with either erythromycin or tetracycline, while the latter drug is indicated for both psittacosis and Q fever.

3.1.2.2 Lung abscess

Destruction of lung tissue may lead to abscess formation and is a feature of aerobic Gram-negative bacillary and *Staph. aureus* infections. In addition, aspiration of oropharyngeal secretion can lead to chronic low-grade sepsis with abscess formation and the expectoration of foul-smelling sputum that characterizes anaerobic sepsis. The latter condition responds to high-dose penicillin, which is active against most of the normal oropharyngeal flora, while metronidazole may be appropriate for strictly anaerobic infections. In the case of aerobic Gram-negative bacillary sepsis, aminoglycosides, with or

Table 14.2 Microorganisms responsible for pneumonia and the therapeutic agent of choice

Pathogen	Drug(s) of choice
Streptococcus pneumoniae	Penicillin
Staphylococcus aureus (MSSA)	Flucloxacillin ± fusidic acid
Staphylococcus aureus (MRSA)	Vancomycin or linezolid
Haemophilus influenzae	Cefotaxime or ciprofloxacin
Klebsiella pneumoniae	Cefotaxime ± gentamicin
Pseudomonas aeruginosa	Ceftazidime ± gentamicin or piperacillin-tazobactam ± gentamicin
Mycoplasma pneumoniae	Erythromycin or tetracycline
Legionella pneumophila	Erythromycin ± rifampicin
Chlamydia psittaci	Tetracycline
Mycobacterium tuberculosis	Rifampicin + isoniazid + ethambutol + pyrazinamide[a]
Herpes simplex, varicella/zoster	Aciclovir
Candida spp.	Fluconazole or echinocandins (caspofungin, anidulafungin)
Aspergillus spp.	Amphotericin B or broad-spectrum triazoles (e.g. voriconazole)
Anaerobic bacteria	Penicillin or metronidazole

[a]Reduce to two drugs after 6–8 weeks.

without a broad-spectrum cephalosporin, are the agents of choice. Acute staphylococcal pneumonia is an extremely serious infection and requires treatment with high-dose flucloxacillin alone or in combination with fusidic acid.

3.1.2.3 Cystic fibrosis

Cystic fibrosis is a multisystem congenital abnormality that often affects the lungs and results in recurrent infections, initially with *Staph. aureus*, subsequently with *H. influenzae* and eventually leads on to recurrent *Pseudomonas aeruginosa* infection. The last organism is associated with copious quantities of purulent sputum that are extremely difficult to expectorate. *Ps. aeruginosa* is a co-factor in the progressive lung damage that is eventually fatal in these patients. Repeated courses of antibiotics are prescribed and although they have improved the quality and longevity of life, infections caused by *Ps. aeruginosa* are difficult to treat and require repeated hospitalization and administration of parenteral antibiotics such as an aminoglycoside, either alone or in combination with an antipseudomonal penicillin or cephalosporin. The dose of aminoglycosides tolerated by these patients is often higher than in normal individuals and is associated with larger volumes of distribution for these and other agents. Some benefit may also be obtained from inhaled aerosolized antibiotics. Unfortunately drug resistance may emerge and makes drug selection more dependent upon laboratory guidance.

3.2 Urinary tract infections

Urinary tract infection is a common problem in both community and hospital practice. Although occurring throughout life, infections are more common in pre-school girls and women during their childbearing years, although in the elderly the sex distribution is similar. Infection is predisposed by factors that impair urine flow. These include congenital abnormalities, reflux of urine from the bladder into the ureters, kidney stones and tumours and, in males, enlargement of the prostate gland. Bladder catheterization is an important cause of urinary tract infection in hospitalized patients.

3.2.1 Pathogenesis

In those with structural or drainage problems the risk exists of ascending infection to involve the kidney and occasionally the bloodstream. Although structural abnormalities may be absent in women of childbearing years, infection can become recurrent, symptomatic and extremely distressing. Of greater concern is the occurrence of infection in the pre-school child, as normal maturation of the kidney may be impaired and may result in progressive damage which presents as renal failure in later life.

From a therapeutic point of view, it is essential to confirm the presence of bacteriuria (a condition in which there are bacteria in the urine), as symptoms alone are not a reliable method of documenting infection. This applies particularly to bladder infection, where the symptoms of burning micturition (dysuria) and frequency can be associated with a variety of non-bacteriuric conditions. Patients with symptomatic bacteriuria should always be treated. However, the necessity to treat asymptomatic bacteriuric patients varies with age and the presence or absence of underlying urinary tract abnormalities. In the preschool child it is essential to treat all urinary tract infections and maintain the urine in a sterile state so that normal kidney maturation can proceed. Likewise in pregnancy there is a risk of infection ascending from the bladder to involve the kidney. This is a serious complication and may result in premature labour. Other indications for treating asymptomatic bacteriuria include the presence of underlying renal abnormalities such as stones, which may be associated with repeated infections caused by *Proteus* spp.

3.2.2 Drug therapy

The antimicrobial treatment of urinary tract infection presents a number of interesting challenges. Drugs must be selected for their ability to achieve high urinary concentrations and, if the kidney is involved, adequate tissue concentrations. Safety in childhood or pregnancy is important as repeated or prolonged medication may be necessary. The choice of agent will be dictated by the microbial aetiology and susceptibility findings, because the latter can vary widely among Gram-negative enteric bacilli, especially in patients who are hospitalized. Table 14.3 shows the distribution of bacteria causing urinary tract infection in the community and in hospitalized patients. The greater tendency towards infections caused by *Klebsiella* spp. and *Ps. aeruginosa* should be noted as antibiotic sensitivity is more variable for these pathogens. Drug resistance has increased substantially in recent years and has reduced the value of formerly widely prescribed agents such as the sulphonamides and ampicillin.

Uncomplicated community-acquired urinary tract infection presents few problems with management. Drugs such as trimethoprim, ciprofloxacin and ampicillin are widely used. Cure rates are close to 100% for ciprofloxacin, about 80% for trimethoprim and about 50% for ampicillin—to which resistance has been steadily increasing. Treatment for 3 days is generally satisfactory and is usually accompanied by prompt control of symptoms. Single-dose therapy with amoxicillin 3 g has also been shown to be effective in selected individuals. Alternative agents include nitrofurantoin, nalidixic acid and norfloxacin, although these are not as well tolerated. Oral cephalosporins and co-amoxiclav are also used.

Table 14.3 Urinary tract infection—distribution of pathogenic bacteria in the community and hospitalized patients

Organism	Community (age <65 yr)	Community (age 65 yr+)	Hospital
	(%)	(%)	(%)
Escherichia coli	77	67	56
Enterococci	4	6	10
Klebsiella spp.	4	6	7
Proteus mirabilis	4	4	6
Pseudomonas aeruginosa	2	3	4
Staphylococcus spp.	2	–	3
Enterobacter spp.	–	3	3

It is important to demonstrate the cure of bacteriuria with a repeat urine sample collected 4–6 weeks after treatment, or sooner should symptoms fail to subside. Recurrent urinary tract infection is an indication for further investigation of the urinary tract to detect underlying pathology that may be surgically correctable. Under these circumstances it also is important to maintain the urine in a sterile state. This can be achieved with repeated courses of antibiotics, guided by laboratory sensitivity data. Alternatively, long-term chemoprophylaxis for periods of 6 months to control infection by either prevention or suppression is widely used. Trimethoprim is the most commonly prescribed chemoprophylactic agent and is given as a single nightly dose. This achieves high urinary concentrations throughout the night and generally ensures a sterile urine. Nitrofurantoin is an alternative agent.

Infection of the kidney demands the use of agents that achieve adequate tissue as well as urinary concentrations. As bacteraemia (a condition in which there are bacteria circulating in the blood) may complicate infection of the kidney, it is generally recommended that antibiotics be administered parenterally. Although ampicillin was formerly widely used, drug resistance is now common and agents such as cefotaxime, co-amoxiclav or ciprofloxacin are often preferred, because the aminoglycosides, although highly effective and preferentially concentrated within the renal cortex, carry the risk of nephrotoxicity.

Infections of the prostate tend to be persistent, recurrent and difficult to treat. This is in part due to the more acid environment of the prostate gland, which inhibits drug penetration by many of the antibiotics used to treat urinary tract infection. Agents that are basic in nature, such as erythromycin, achieve therapeutic concentrations within the gland but unfortunately are not active against the pathogens responsible for bacterial prostatitis. Trimethoprim, and quinolones, however, are useful agents as they are preferentially concentrated within the prostate and active against many of the causative pathogens. It is important that treatment be prolonged for several weeks, as relapse is common.

3.3 Gastrointestinal infections

The gut is vulnerable to infection by viruses, bacteria, parasites and occasionally fungi. Virus infections are the most prevalent but are not susceptible to chemotherapeutic intervention. Bacterial infections are more readily recognized and raise questions concerning the role of antibiotic management. Parasitic infections of the gut are beyond the scope of this chapter.

Bacteria cause disease of the gut as a result of either mucosal invasion or toxin production or a combination of the two mechanisms, as summarized in Table 14.4. Treatment is largely directed at replacing and maintaining an adequate intake of fluid and electrolytes. Antibiotics are generally not recommended for infective gastroenteritis, but deserve consideration where they have been demonstrated to abbreviate the acute disease or to prevent complications including prolonged gastrointestinal excretion of the pathogen where this poses a public health hazard.

It should be emphasized that most gut infections are self-limiting. However, attacks can be severe and may result in hospitalization. Antibiotics are used to treat severe *Campylobacter* and *Shigella* infections; erythromycin and ciprofloxacin, respectively, are the preferred agents. Such treatment abbreviates the disease and eliminates gut excretion in *Shigella* infection. However, in severe *Campylobacter* infection the data are currently equivocal, although the clinical impression favours the use of erythromycin for severe infections. The role of antibiotics for *Campylobacter* and *Shigella* infections should be contrasted with gastrointestinal salmonellosis, for which antibiotics are contraindicated as they do not abbreviate symptoms, are associated with more prolonged gut excretion and introduce the risk of adverse drug reactions. However, in severe salmonellosis, especially at extremes of age, systemic toxaemia and bloodstream infection can occur and under these circumstances treatment with either ciprofloxacin or trimethoprim is appropriate.

Typhoid and paratyphoid fevers (known as enteric fevers), although acquired by ingestion of salmonellae, *Sal. enterica* serovar Typhi and *Sal. enterica* serovar Paratyphi, respectively, are largely systemic infections and antibiotic therapy is mandatory; ciprofloxacin is now the drug of choice although trimethoprim or chloramphenicol are satisfactory alternatives. Prolonged gut excretion of *Sal. enterica* serovar Typhi is a well-known complication of typhoid fever and is a major public health hazard in developing countries. Treatment with ciprofloxacin or high-dose ampicillin can eliminate the gall bladder excretion which is the major site of persistent infection in carriers. However, the presence of gallstones reduces the chance of cure.

Cholera is a serious infection causing epidemics throughout Asia. Although a toxin-mediated disease, largely controlled with replacement of fluid and electrolyte losses,

Table 14.4 Bacterial gut infections—pathogenic mechanisms

Origin	Site of infection	Mechanism
Campylobacter jejuni	Small and large bowel	Invasion
Salmonella spp.	Small and large bowel	Invasion
Shigella spp.	Large bowel	Invasion ± toxin
Escherichia coli		
enteroinvasive	Large bowel	Invasion
enterotoxigenic	Small bowel	Toxin
Clostridium difficile	Large bowel	Toxin
Staphylococcus aureus	Small bowel	Toxin
Vibrio cholerae	Small bowel	Toxin
Clostridium perfringens	Small bowel	Toxin
Yersinia spp.	Small and large bowel	Invasion
Bacillus cereus	Small bowel	Invasion ± toxin
Vibrio parahaemolyticus	Small bowel	Invasion + toxin

tetracycline has proved effective in eliminating the causative vibrio from the bowel, thereby abbreviating the course of the illness and reducing the total fluid and electrolyte losses.

Traveller's diarrhoea may be caused by one of many gastrointestinal pathogens (Table 14.4). However, enterotoxigenic *Escherichia coli* is the most common pathogen. While it is generally short-lived, traveller's diarrhoea can seriously mar a brief period abroad, be it for holiday or business purposes. Although not universally accepted, the use of short-course trimethoprim or quinolone such as ciprofloxacin can abbreviate an attack in patients with severe disease.

3.4 Skin and soft tissue infections

Infections of the skin and soft tissue commonly follow traumatic injury to the epithelium but occasionally may be blood-borne. Interruption of the integrity of the skin allows ingress of microorganisms to produce superficial, localized infections which on occasion may become more deep-seated and spread rapidly through tissues. Skin trauma complicates surgical incisions and accidents, including burns. Similarly, prolonged immobilization can result in pressure damage to skin from impaired blood flow. It is most commonly seen in patients who are unconscious.

Microbes responsible for skin infection often arise from the normal skin flora, which includes *Staph. aureus*. In addition *Strep. pyogenes*, *Ps. aeruginosa* and anaerobic bacteria are other recognized pathogens. Viruses also affect the skin and mucosal surfaces, either as a result of generalized infection or localized disease as in the case of herpes simplex. The latter is amenable to antiviral therapy in selected patients, although for the majority of patients, virus infections of the skin are self-limiting.

Strep. pyogenes is responsible for a range of skin infections: impetigo is a superficial infection of the epidermis which is common in childhood and is highly contagious; cellulitis is a more deep-seated infection which spreads rapidly through the tissues to involve the lymphatics and occasionally the bloodstream; erysipelas is a rapidly spreading cellulitis commonly involving the face, which characteristically has a raised leading edge due to lymphatic involvement. Necrotizing fasciitis is a more serious, rapidly progressive infection of the skin and subcutaneous structures including the fascia and musculature. Despite early diagnosis and high-dose intravenous antibiotics, this condition is often life-threatening and may require extensive surgical debridement of devitalized

tissue and even limb amputation to ensure survival. A fatal outcome is usually the result of profound toxaemia and bloodstream spread. Penicillin is the drug of choice for all these infections, usually in combination with other agents such as an aminoglycoside and metronidazole in the case of necrotizing fasciitis; in severe instances parenteral administration is appropriate. The use of topical agents, such as tetracycline, to treat impetigo may fail as drug resistance is now recognized.

Staph. aureus is responsible for a variety of skin infections which require therapeutic approaches different from those of streptococcal infections. Staphylococcal cellulitis is indistinguishable clinically from streptococcal cellulitis and responds to flucloxacillin, but generally fails to respond to penicillin owing to penicillinase (β-lactamase) production. In hospital-acquired infection, and occasionally in community practice, MRSA must be considered as a possibility, particularly where the patient is known to be colonized. *Staph. aureus* is an important cause of superficial, localized skin sepsis which varies from small pustules to boils and occasionally to a more deeply invasive, suppurative skin abscess known as a carbuncle. Antibiotics are generally not indicated for these conditions. Pustules and boils settle with antiseptic soaps or creams and often discharge spontaneously, whereas carbuncles frequently require surgical drainage. *Staph. aureus* may also cause postoperative wound infections, sometimes associated with retained suture material, and settles once the stitch is removed. Antibiotics are only appropriate in this situation if there is extensive accompanying soft tissue invasion. Rarely, strains of *Staph. aureus* may express a toxin complex known as Panton-Valentine Leukocidin (PVL); these strains can cause severe sepsis and an often fatal necrotizing pneumonia in young, otherwise fit, patients. The treatment for such infections usually aims to minimize toxin production using protein synthesis inhibitors such as clindamycin plus rifampicin, in combination with linezolid.

Anaerobic bacteria are characteristically associated with foul-smelling wounds. They are found in association with surgical incisions following intra-abdominal procedures and pressure sores, which are usually located over the buttocks and hips where they become infected with faecal flora. These infections are frequently mixed and include Gram-negative enteric bacilli, which may mask the presence of underlying anaerobic bacteria. The principles of treating anaerobic soft tissue infection again emphasize the need for removal of all foreign and devitalized material. Antibiotics such as metronidazole or clindamycin should be considered where tissue invasion has occurred.

The treatment of infected burn wounds presents a number of peculiar facets. Burns are initially sterile, especially when they involve all layers of the skin. However, they rapidly become colonized with bacteria whose growth is supported by the protein-rich exudate. Staphylococci, *Strep. pyogenes* and, particularly, *Ps. aeruginosa* frequently colonize burns and may jeopardize survival of skin grafts and occasionally, and more seriously, result in bloodstream invasion. Treatment of invasive *Ps. aeruginosa* infections requires combined therapy with an aminoglycoside, such as gentamicin or tobramycin, and an antipseudomonal agent, such as ceftazidime or piperacillin. This produces high therapeutic concentrations which generally act in a synergistic manner. The use of aminoglycosides in patients with serious burns requires careful monitoring of serum concentrations to ensure that they are therapeutic yet non-toxic, as renal function is often impaired in the days immediately following a serious burn.

3.5 Central nervous system infections

The brain, its surrounding covering of meninges and the spinal cord are subject to infection, which is generally blood-borne but may also complicate neurosurgery, penetrating injuries or direct spread from infection in the middle ear or nasal sinuses. Viral meningitis is the most common infection but is generally self-limiting. Occasionally destructive forms of encephalitis occur; an example is herpes simplex encephalitis. Bacterial infections include meningitis and brain abscesses and carry a high risk of mortality, while in those who recover, residual neurological damage or impairment of intellectual function may follow. This occurs despite the availability of antibiotics active against the responsible bacterial pathogens. Fungal infections of the brain, although rare, are increasing in frequency, particularly among immunocompromised patients who either have underlying malignant conditions or are on potent cytotoxic drugs.

The treatment of bacterial infections of the central nervous system highlights a number of important therapeutic considerations. Bacterial meningitis is caused by a variety of bacteria although their incidence varies with age. In the neonate, *E. coli* and group B streptococci account for the majority of infections, while in the preschool child *H. influenzae* was the commonest pathogen before the introduction of a highly effective vaccine. *Neisseria meningitidis* has a peak incidence between 5 and

15 years of age, while pneumococcal meningitis is predominantly a disease of adults.

Ceftriaxone is the drug of choice for the treatment of group B streptococcal, meningococcal and pneumococcal infections but, as discussed earlier, CSF concentrations of penicillin are significantly influenced by the intensity of the inflammatory response. To achieve therapeutic concentrations within the CSF, high dosages are required, and in the case of pneumococcal meningitis should be continued for 10–14 days. Resistance among *Strep. pneumoniae* to penicillin has increased worldwide; in travellers returning from endemic areas, vancomycin may be indicated. Alternative agents include meropenem.

Resistance of *H. influenzae* to ampicillin has increased in the past two decades and varies geographically. Thus, it can no longer be prescribed with confidence as initial therapy, and cefotaxime or ceftriaxone are now the preferred alternatives. However, once laboratory evidence for β-lactamase activity is excluded, ampicillin can be safely substituted.

E. coli meningitis carries a mortality of greater than 40% and reflects both the virulence of this organism and the pharmacokinetic problems of achieving adequate CSF antibiotic levels. The broad-spectrum cephalosporins such as cefotaxime, ceftriaxone or ceftazidime have been shown to achieve satisfactory therapeutic levels and are the agents of choice to treat Gram-negative bacillary meningitis. Treatment again must be prolonged for periods ranging from 2 to 4 weeks.

Brain abscess presents a different therapeutic challenge. An abscess is locally destructive to the brain and causes further damage by increasing intracranial pressure. The infecting organisms are varied but those arising from middle ear or nasal sinus infection are often polymicrobial and include anaerobic bacteria, microaerophilic species and Gram-negative enteric bacilli. Less commonly, a pure *Staph. aureus* abscess may complicate blood-borne spread. Brain abscess is a neurosurgical emergency and requires drainage. However, antibiotics are an important adjunct to treatment. The polymicrobial nature of many infections demands prompt and careful laboratory examination to determine optimum therapy. Drugs are selected not only on their ability to penetrate the blood–brain barrier and enter the CSF but also on their ability to penetrate the brain substance. Metronidazole has proved a valuable alternative agent in such infections, although it is not active against microaerophilic streptococci, which must be treated with high-dose benzylpenicillin. The two are often used in combination. Chloramphenicol is an alternative agent.

3.6 Fungal infections

Fungal infections are divided into superficial or deep-seated infections. Superficial infections affect the skin, nails or mucosal surfaces of the mouth or genital tract. In contrast, deep-seated fungal diseases may target the lung or disseminate via the bloodstream to organs such as the brain, spleen, liver or skeletal system.

The fungal infections of the skin and nails include tinea pedis (athlete's foot), tinea capitis and tinea corporis (ringworm), *Candida* intertrigo (usually groin and submammary regions) and pityriasis (*Malassezia*). A variety of topical and systemic antifungal agents are available. The imidazole class of drugs includes clotrimazole and miconazole, which are highly effective topically. Systemic antifungals used to treat superficial fungal infections include griseofulvin and terbinafine, which is an allylamine. Both agents are ineffective in the treatment of deep-seated fungal infections that may be caused by yeasts (*Cryptococcus neoformans*), yeast-like fungi (*Candida* spp.) or the filamentous fungi (*Aspergillus* spp). These produce a variety of syndromes for which different antifungal agents are indicated (Table 14.5). The polyenes include amphotericin B, which after many years remains the agent of choice for the treatment of a wide variety of life-threatening fungal diseases which often complicate cancer chemotherapy, organ transplantation

Table 14.5 Treatment recommendations for selected deep-seated fungal infections

Infection	Preferred treatment	Alternative treatment
Candida spp.	Fluconazole	Amphotericin B, echinocandins
Cryptococcus neoformans	Fluconazole	Amphotericin B ± flucytosine
Aspergillus spp.	Amphotericin B	Itraconazole, voriconazole, caspofungin
Mucormycosis	Amphotericin B	–

and immunodeficiency diseases, such as AIDS. Nephrotoxicity is common but can be avoided by careful dosaging or the use of liposomal formulations. The second major class of systemic antifungals is the triazoles, which include fluconazole and newer, broader-spectrum agents such as itraconazole, voriconazole and posaconazole. These are extremely well tolerated but may interact with a number of drugs and drug classes such as the sulphonylureas, antihistamines and lipid-lowering agents among others. The echinocandins (caspofungin, anidulafungin) are the newest class of antifungal agents, and are increasingly used to treat invasive fungal infections; they have a fungicidal action against *Candida* spp., and are fungistatic against many other organisms, including *Aspergillus* spp.

3.7 Medical device associated infections

A wide variety of medical devices are increasingly used in clinical practice. These range from vasculature and urinary catheters, prosthetic joints and heart valves, shunts and stents for improving the flow of CSF, blood or bile according to their site of use, to intracardiac patches and vascular pumps. Unfortunately infection is the most frequent complication of their use and may result in the need to replace or remove the device, sometimes with potentially life-threatening and fatal consequences.

Infections are often caused by organisms arising from the normal skin flora, which gain access at the time of insertion of the device. *Staph. epidermidis* is among the most frequent of isolates. Following attachment to the surface of the device, the organisms undergo multiplication with the formation of extracellular polysaccharide material (glycocalyx) which contains slowly replicating cells to form a biofilm. Microorganisms within a biofilm are less vulnerable to attack by host defences (phagocytes, complement and antibodies) and are relatively insusceptible to antibiotic therapy despite the variable ability of drugs to penetrate the biofilm.

Management approaches have therefore emphasized the need for prevention through the addition of good sterile technique at the time of insertion. Manufacturers have also responded by using materials and creating surface characteristics of implanted materials inclement to microbial attachment. Likewise the use of prophylactic antibiotics at the time of insertion of deep-seated devices such as joint and heart valve prostheses has further reduced the risk of infection. Once a medical device becomes infected, management is difficult. Treatment with agents such as flucloxacillin, vancomycin and most recently linezolid is often unsuccessful and the only course of action is to remove the device.

4 Antibiotic policies

4.1 Rationale

The plethora of available antimicrobial agents presents both an increasing problem of selection to the prescriber and difficulties for the diagnostic laboratory as to which agents should be tested for susceptibility. Differences in antimicrobial activity among related compounds are often of minor importance but can occasionally be of greater significance and may be a source of confusion to the non-specialist. This applies particularly to large classes of drugs, such as the penicillins and cephalosporins, where there has been an explosion in the availability of new agents in recent years. Guidance, in the form of an antibiotic policy, has a major role to play in providing the prescriber with a range of agents appropriate to his/her needs and should be supported by laboratory evidence of susceptibility to these agents.

In recent years, increased awareness of the cost of medical care has led to a major review of various aspects of health costs. The pharmacy budget has often attracted attention as, unlike many other hospital expenses, it is readily identifiable in terms of cost and prescriber. Thus, an antibiotic policy is also seen as a means whereby the economic burden of drug prescribing can be reduced or contained. There can be little argument with the recommendation that the cheaper of two compounds should be selected where agents are similar in terms of efficacy and adverse reactions. Likewise, generic substitution is also desirable provided that there is bio-equivalence. It has become increasingly impractical for pharmacists to stock all the formulations of every antibiotic currently available, and here again an antibiotic policy can produce significant savings by limiting the amount of stock held. A policy based on a restricted number of agents also enables price reduction on purchasing costs through competitive tendering. The above activities have had a major influence on containing or reducing drug costs, although these savings have often been lost as new and often expensive preparations become available, particularly in the field of biological and anticancer therapy.

Another increasingly important argument in favour of an antibiotic policy is the occurrence of drug-resistant bacteria within an institution. The presence of sick patients and the opportunities for the spread of microorganisms can produce outbreaks of hospital infection.

The excessive use of selected agents has been associated with the emergence of drug-resistant bacteria which have often caused serious problems within high-dependency areas, such as intensive care units or burns units where antibiotic use is often high. One oft-quoted example is the occurrence of a multiple antibiotic-resistant *K. aerogenes* within a neurosurgical intensive care unit in which the organism became resistant to all currently available antibiotics and was associated with the widespread use of ampicillin. By prohibiting the use of all antibiotics, and in particular ampicillin, the resistant organism rapidly disappeared and the problem was resolved.

Currently the most important hospital-acquired pathogen is methicillin-resistant *Staph. aureus*, which is responsible for a range of serious infections such as pneumonia, postoperative wound infection and skin infections which may in turn be complicated by bloodstream spread. The use of vancomycin and teicoplanin has escalated as a consequence, and in turn has been linked to the emergence of vancomycin-resistant enterococci. Likewise, the increased prevalence of ESBL-producing Gram-negative pathogens in intensive care settings often leads to increased usage of carbapenems, and a corresponding rise in carbapenem-resistant *Klebsiella* and *Pseudomonas* species on these units.

In formulating an antibiotic policy, it is important that the susceptibility of microorganisms be monitored and reviewed at regular intervals. This applies not only to the hospital as a whole, but to specific high-dependency units in particular. Likewise general practitioner samples should also be monitored. This will provide accurate information on drug susceptibility to guide the prescriber as to the most effective agent.

4.2 Types of antibiotic policies

There are a number of different approaches to the organization of an antibiotic policy. These range from a deliberate absence of any restriction on prescribing to a strict policy whereby all anti-infective agents must have expert approval before they are administered. Restrictive policies vary according to whether they are mainly laboratory-controlled, by employing restrictive reporting, or whether they are mainly pharmacy-controlled, by restrictive dispensing. In many institutions it is common practice to combine the two approaches.

4.2.1 Free prescribing

The advocates of a free prescribing policy argue that strict antibiotic policies are both impractical and limit clinical freedom to prescribe. It is also argued that the greater the number of agents in use the less likely it is that drug resistance will emerge to any one agent or class of agents. However, few would support such an approach, which is generally an argument for mayhem.

4.2.2 Restricted reporting

Another approach that is widely practised in the UK is that of restricted reporting. The laboratory, largely for practical reasons, tests only a limited range of agents against bacterial isolates. The agents may be selected primarily by microbiological staff or following consultation with their clinical colleagues. The antibiotics tested will vary according to the site of infection, as drugs used to treat urinary tract infections often differ from those used to treat systemic disease.

There are specific problems regarding the testing of certain agents such as the cephalosporins, where the many different preparations have varying activity against bacteria. The practice of testing a single agent to represent first-generation, second-generation or third-generation compounds is questionable, and with the new compounds susceptibility should be tested specifically to that agent. By selecting a limited range of compounds for use, sensitivity testing becomes a practical consideration and allows the clinician to use such agents with greater confidence.

4.2.3 Restricted dispensing

As mentioned above, the most draconian of all antibiotic policies is the absolute restriction of drug dispensing pending expert approval. The expert opinion may be provided by either a microbiologist or infectious disease specialist. Such a system can only be effective in large institutions where staff are available 24 hours a day. This approach is often cumbersome, generates hostility and does not necessarily create the best educational forum for learning effective antibiotic prescribing.

A more widely used approach is to divide agents into those approved for unrestricted use and those for restricted use. Agents on the unrestricted list are appropriate for the majority of common clinical situations. The restricted list may include agents where microbiological sensitivity information is essential, such as for vancomycin and certain aminoglycosides. In addition, agents that are used infrequently but for specific indications, such as parenteral amphotericin B, are also restricted in use. Other compounds that may be expensive and used for specific indications, such as broad-spectrum β-lactams in the treatment of *Ps. aeruginosa* infections, may also be justifiably included on the restricted list. Items omitted

from the restricted or unrestricted list are generally not stocked, although they can be obtained at short notice as necessary.

Such a policy should have a mechanism whereby desirable new agents are added as they become available and is most appropriately decided at a therapeutics committee. Policing such a policy is best effected as a joint arrangement between senior pharmacists and microbiologists. This combined approach of both restricted reporting and restricted prescribing is extremely effective and provides a powerful educational tool for medical staff and students faced with learning the complexities of modern antibiotic prescribing.

4.2.4 The antimicrobial stewardship team

In an attempt to ensure antimicrobials are prescribed appropriately in hospitals, antimicrobial stewardship teams have emerged to advise and educate staff while monitoring compliance with prescribing policies as well as ensuring good standards of patient management. Typically these teams comprise, at their core, a consultant in infectious diseases and/or clinical microbiology, and a senior pharmacist specializing in infectious diseases, and may also include infection control practitioners. The team takes a lead in reviewing the therapy of individual patients and setting treatment plans, and, often as part of a wider team, will coordinate the writing and review of antibiotic treatment policies. Other responsibilities may include the education and training of clinical staff, the audit of how well prescribers are adhering to the carefully written policies, and the provision of feedback to prescribers. Evidence suggests that this multidisciplinary approach, aligned with targeted and timely feedback, can improve adherence to prescribing policy, reduce drug expenditure, and improve patient outcomes. Antibiotic stewardship is discussed further in Chapter 15.

5 Further reading

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15 Antibiotic prescribing and antibiotic stewardship

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1 The need for antimicrobial stewardship

In testimony to a US government committee in June 2010, the Infectious Diseases Society of America (IDSA) stated that

> Most commonly used antibiotics cost only a few dollars for a typical course of treatment . . . (and) a single course of antibiotics has the potential to protect and preserve many quality years of life for many people. No other type of medicine can claim such an achievement at such a price.

These statements were made as part of the argument presented by the IDSA to promote both antibiotic research and appropriate use ('stewardship') of antibiotics.

The wider point being made was that antibiotics have, since their discovery in the 1940s, revolutionized medicine, and many of the procedures that are taken for granted now—transplantation, cancer treatment, the care of premature babies and several forms of surgery—would be impossible without them. Yet, unfortunately, largely because they have been taken for granted, the antibiotics we already possess are becoming less effective as a result of bacterial resistance, and the prospects for producing new antibiotics currently look bleak. It was recognized right from the start of the antibiotic era that bacteria had the potential to develop resistance to antimicrobial drugs, but it was quite some time before the perception of antibiotic resistance changed from one in which it was regarded as unusual to one where it was expected; in other words, a recognition that long-term efficacy was the exception, and resistance was the rule.

As the 20th century drew to a close the emergence of the antibiotic-resistant pathogens described in Chapter 13 brought with it both the spectre of untreatable infections where the organisms responsible were resistant to all available agents and a growing sense of urgency to take steps to preserve the usefulness of the antibiotics we currently have. The situation was exacerbated by a reduction in the number of new antibiotics coming into clinical use. Figure 15.1 shows that this number has continued to decline steadily for the last 20 years—a trend that would be difficult to reverse in the short term simply because several of the major international pharmaceutical companies are moving out of antimicrobials as a research and development area. Unfortunately, antibiotics have, in a sense, become victims of their own success: the more effective an antimicrobial is, the shorter the likely duration of treatment, so the lower the pay-back to the company that developed it. Many courses of antibiotic

Hugo and Russell's Pharmaceutical Microbiology, Eighth Edition. Edited by Stephen P. Denyer, Norman Hodges, Sean P. Gorman, Brendan F. Gilmore.

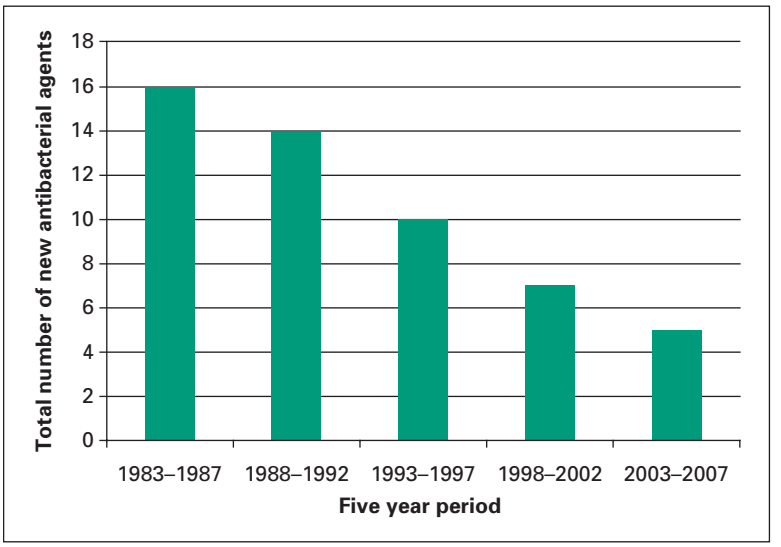

Figure 15.1 Number of new antibacterial agents licensed for use by the FDA, 1983–2007. Spellberg (2007). Reproduced with permission of the University of Chicago Press.

treatment last for a week or less, so the sales accruing from them are far inferior to those from drugs treating chronic conditions like diabetes and hypertension. This fact, together with (1) increasing pressure to use antibiotics sparingly anyway, (2) the expectation that the drug will ultimately become less effective due to resistance and (3) difficulties in establishing clinical trials for antibiotics that satisfy the US Food and Drugs Administration (FDA) criteria, all combined to create a climate in which antibiotics became an unattractive commercial proposition (except in the case of HIV/AIDS therapies that have to be taken throughout the patient's life).

Certainly the problem of growing antibiotic resistance had been recognized for some time before the end of the 20th century and policies designed to improve the quality of antibiotic prescribing and restrict resistance in hospitals became progressively more common in Europe and North America from the 1970s onwards. However, the increasing frequency in the new millennium of infections due to the so-called 'ESKAPE' pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella* species, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and extended spectrum β-lactamase-producing strains of *Escherichia coli* and *Enterobacter* species) together with the drying up of the pipeline of new antibiotics from the pharmaceutical industry has further increased the tempo of measures to preserve what is increasingly being seen as a precious, and perhaps irreplaceable, resource that society has a duty to pass on to future generations rather than squander. These measures include the '10 × 20' initiative to establish an international research effort to develop 10 new antibiotics by the year 2020, and the STAAR (Strategies to Address Antimicrobial Resistance) Act, which is, at the time of writing, being considered by the US Congress.

The cost of antibiotic resistance should, of course, be measured primarily in terms of the suffering that results from the failure of antibiotics to cure infections against which they were formerly effective. The antibiotic-resistant pathogens responsible for these infections can arise both in the home or the hospital environment, but it is in the latter that they are, by far, more common and problematic. Hospital-acquired infections account for a substantial number of deaths each year and the treatment of such infections is time-consuming, difficult and costly. In its testimony to Congress as part of the consultation process accompanying the STAAR Act, the IDSA stated that antibiotic-resistant infections acquired *within* hospitals were responsible for 90 000 deaths each year in the USA and cost the healthcare system between $21 and $34 billion annually. There are less obvious consequences of resistance too: when the first-line drugs cease to be effective it is sometimes necessary to revert to alternatives that are more toxic. *Acinetobacter* infections are a good example of this situation because the organism is naturally multidrug resistant and the incidence of isolates resistant to all first-line antibiotics has risen in the USA from 5% to 40% in 10 years, so now colistin, a drug that

became virtually obsolete in the 1960s because of the significant risk of kidney damage is, for many patients, the most likely antibiotic choice.

There is widespread agreement that greater use of antibiotics predisposes to the development of resistance. The strength of the link between use and resistance varies from one antibiotic to another, but for many antibiotics the connection is irrefutable. However, the situation is far from simple: there is substantial evidence, for example, that heavy use of one antibiotic may be a risk factor for the acquisition of infections by organisms resistant to other, unrelated antibiotics—heavy cephalosporin use has been shown to increase the risk of vancomycin-resistant enterococci, and fluoroquinolone use has been associated with the prevalence of meticillin-resistant *Staph. aureus* (MRSA). The selective pressure created by the use of one antibiotic will often select for resistance in others because plasmids within the bacterial cell may carry resistance genes for multiple antibiotics from different chemical groups. If, for example, an organism possessed a plasmid with genes for both rifampicin and gentamicin resistance, constant exposure to gentamicin would represent a selective pressure that afforded an advantage to that organism so, not only would the incidence of isolates with gentamicin resistance be expected to rise, but so too would the incidence of rifampicin resistant isolates.

Arguments for curtailing antibiotic use in order to restrict resistance development have been supported by audits and surveys of antibiotic prescribing and costs. It has been estimated that up to 50% of antibiotic prescribing is either inappropriate (wrong drug, duration, dose, etc.) or unnecessary (not required at all). Antibiotic consumption varies widely throughout Europe: a 2005 survey showed that France, for example, used three times as much antibiotics per head of population as the Netherlands, but this difference was not justified by higher infection rates or better cure rates, so logic suggests that part of the antibiotic use in France was unnecessary. Inappropriate prescribing and consumption, together with the fact that antibiotics can represent up to 30% of a hospital pharmacy budget, have provided further impetus for measures designed to achieve more prudent prescribing. Such thinking is not new, however; it was the rationale for the introduction of antibiotic policies in the 1970s and 1980s which, in addition to setting out the general standards for safe and appropriate prescribing, advised on the selection of antibiotics for specific infections, for special situations like surgical prophylaxis and for the treatment of specific groups of patients, e.g. the newborn, those with poor kidney function and the immunocompromised. What has changed since then is the recognition of the need for a much broader approach to the problem—in other words, the need for a comprehensive antimicrobial stewardship strategy that incorporates, but also extends, the policies formulated in the last century.

There is not a universally accepted definition of, or agreement upon, what constitutes an antimicrobial stewardship programme. For most people the term applies particularly, or even exclusively, to the manner in which antibiotics are used and distributed in hospitals. However, some see it in a much broader sense as a range of initiatives which, together, impact upon antibiotic resistance but are not necessarily even confined to antibiotic-related practices in the hospital or the home. The great majority of the annual global production of antibiotics is not used in the treatment of human or animal infection anyway. Most of the antibiotic output of the international pharmaceutical industry is used as a food additive to increase weight gain in cattle, pigs and poultry—some estimates put this proportion as high as 80%—and yet more is used in plant production, but this fraction is ill-defined. Although antibiotics that are used to treat human infections have been banned as growth promoters in Europe for many years it is still a common practice in many countries, and even the legitimate veterinary use of antibiotics is considerable: the total volume of antibiotics used in the UK for agricultural purposes in 2007 was 387 tonnes. Curtailment of the use of antibiotics for growth promotion—which many see as inappropriate and a likely contributor to resistance development—together with better-targeted and -promoted use of vaccines (that would reduce the need for antibiotics), better diagnostic agents which would more rapidly and accurately identify the infecting organism and so inform the selection of the best antibiotic, better epidemiological data and computer analysis to provide early warnings of resistance trends, and changes in other medical practices like the early removal of catheters and cannulas which are, themselves, a means by which pathogens can enter the body, might all be seen as part of a stewardship programme. But from the perspective of controlling the incidence and spread of antibiotic-resistant organisms within a hospital it cannot be overemphasized that a comprehensive infection control programme is of paramount importance (see Chapter 16) and the best results are achieved when data from antibiotic stewardship and infection control can be linked and analysed together.

It is generally accepted that the principal goals of a stewardship programme are to:

- Improve patient outcomes
- Lessen the risk of adverse effects
- Reduce resistance levels, or at least slow the rate of resistance development
- Improve cost-effectiveness.

Following the international financial crisis that began in 2007 it is likely, at least in many European countries, that the last of these goals will receive particular attention, so it is worth emphasizing that stewardship programmes can be self-financing. Although there is an initial start-up cost, there is substantial evidence that this is rapidly recovered by cost savings resulting from reductions in antimicrobial use that some reports have estimated to vary from 22 to 36%.

2 Components of antimicrobial stewardship programmes

There are no nationally or internationally accepted guidelines on the structure of a stewardship programme, so they vary from country to country and even from one hospital to another within a geographical region. Indeed, even the titles vary, so terms like 'good antimicrobial practice programmes', 'antibiotic management programmes' and 'antibiotic control programmes' have all been used. There is, however, a measure of agreement on the personnel comprising an antimicrobial stewardship team. They are multidisciplinary and usually consist of:

- An infectious diseases physician
- A clinical pharmacist with infectious diseases training
- A medical microbiologist
- An infection control professional
- A hospital epidemiologist
- An information technology specialist.

In the USA the IDSA and the Society for Heathcare Epidemiology of America (SHEA) set forth stewardship guidelines in 2007 that have been widely adopted both in the USA and elsewhere; but in Europe the situation is less uniform. Here, an expert group reported in 2000 that little was known about which stewardship measures were employed in Europe and which were optimal. As a consequence, the European Commission Concerted Action Antibiotic Resistance Prevention and Control (ARPAC) survey of stewardship practices in 263 hospitals through 32 European countries was undertaken, and it reported in 2009. Table 15.1 lists both the components of the US guidelines and the six key indicators of stewardship that were the subject of the ARPAC survey.

Before considering in more detail the elements of a stewardship programme, it is worth explaining the meaning of some of the terms used in Table 15.1. In this context a prospective audit means a review of the future delivery of healthcare to ensure that best practice is being carried out, so a prospective audit with intervention and feedback is a process in which the use of antibiotics is monitored and suggestions made for improvement, where necessary, *while the course of treatment is still in progress.* The feedback element is the provision of information to the prescriber about the drug in question, e.g. local resistance patterns and dose information based upon pharmacokinetic data.

Most hospitals have a drugs and therapeutics committee (DTC; sometimes also known as a pharmacy and therapeutics committee) whose function is to evaluate drugs for inclusion in a hospital formulary on the basis of their efficacy, toxicity and cost. The intention is to avoid the unnecessary stocking of multiple drugs from the same class which have similar or identical properties; few hospitals stock every available aminoglycoside or third-generation cephalosporin, for example. An antibiotics committee would be a subgroup of the DTC with responsibility for selecting the drugs for inclusion in the antibiotics formulary. The strategy of formulary restriction and preauthorization could limit the availability of certain antibiotics simply by excluding them from the formulary (although they could usually be obtained in exceptional circumstances if required). The preauthorization element would simply be a requirement to seek the recommendation (or even written approval) of senior clinical staff when prescribing antibiotics in a restricted category—usually those drugs whose effectiveness needs to be preserved because of their value in treating infections for which few drugs are effective, e.g. linezolid or daptomycin, or antibiotics which may predispose to other problems, e.g. clindamycin because of its link to *Clostridium difficile* outbreaks.

The ARPAC stewardship indicator that the hospital should have a strategic management goal of improving prescribing has important implications for the provision of resources and granting the authority to implement decisions. Establishing a stewardship programme costs money: staff time must be available for (1) the preparation of the antibiotics policy document and formulary, (2) intervention and feedback, and (3) monitoring of antibiotic use and resistance patterns. It is easier to gain approval for the necessary budget allocation if there is a

Table 15.1 The components of antimicrobial stewardship programmes

Core strategies and elements of a stewardship programme as recommended by the ISDA and SHEA	Key indicators of stewardship as identified by the ARPAC survey
Two core strategies (not necessarily mutually exclusive):	**The hospital had:**
Prospective audit with intervention and feedback	An antibiotic committee
Formulary restriction and preauthorization	A written antibiotic policy
Elements supplementing these strategies:	A written antibiotic formulary
Education	A formulary that included a restricted antibiotic list
Guidelines and clinical pathways	A drugs and therapeutic committee
Antimicrobial order forms	A strategic management goal of improving prescribing
De-escalation of therapy	
Parenteral to oral conversion	
Dose optimization	
Antimicrobial cycling	
Combination therapy	

ISDA, Infectious Diseases Society of America; SHEA, Society for Healthcare Epidemiology of America.

publically stated management goal of improving prescribing. Where preauthorization for selected antibiotics is a part of the programme, the greater the authority bestowed on the person(s) making the recommendations, the greater the effectiveness of the policy has been shown to be.

3 The effectiveness of stewardship strategies

How effective a particular action is will be determined by the parameter used to measure it. In the case of stewardship programmes almost all of the individual components of the two IDSA/SHEA strategies (Table 15.1) can be shown to reduce resistance development, adverse effects or costs, but there are few actions that result in favourable changes in all three criteria.

Both of the core strategies of prospective audit and formulary restriction are active measures and, as such, are more effective than passive ones. Prospective audit/intervention has been shown to reduce inappropriate use of antibiotics, achieve cost savings and, in some cases, restricted the isolation of particular antibiotic-resistant organisms. Formulary restriction/preauthorization has similarly been shown to reduce antibiotic consumption, and this may be immediate and significant, but its long-term impact on restricting resistance development is not proven. Education of patients to dissuade them from pressurizing prescribers for antibiotics—particularly for colds and flu or other viral infections—is now well established, but education of prescribers (conference presentations, teaching sessions, e-mail alerts and bulletins) is a passive approach which, by itself, has been shown to have only a marginal and short-term impact. Clinical pathways (also known as critical care pathways or care maps) are intended to reduce the variability both in the quality of care and in patient outcomes by the adoption of defined, standardized and sequenced procedures for patients with specific conditions—in this context, infections. The use of a clinical pathway that includes a specified antibiotic regimen in the treatment of community-acquired pneumonia, for example, has been shown to be capable of reducing the duration of hospital stay and duration of antibiotic therapy, and there is strong evidence that practice guidelines and clinical pathways incorporating local resistance patterns can generally improve antimicrobial utilization.

Four other elements of the IDSA/SHEA that have been shown to afford clear benefits are the use of antimicrobial order forms, de-escalation of therapy, parenteral to oral conversion and optimized dosing. Order forms are particularly useful when antibiotics are prescribed for prophylactic purposes to reduce the incidence of infection following surgery. In this situation there is a tendency to continue the course of treatment for an

unnecessarily long period after the operation, and studies have shown that order forms with a default stop date have diminished drug consumption with no adverse effect. When hospital treatment for an infection begins, it is often the case that the organism responsible has not been identified, so initial treatment is empirical or 'blind' and, in order to maximize the probability of inhibiting the pathogen, it may involve the use of either a broad-spectrum antibiotic, or of two or more different drugs. Once the organism has been identified it is good practice to replace broad-spectrum antibiotics (or redundant components of a combination) with a drug having more specific activity, because the continued unnecessary use of broad-spectrum therapy contributes to selection of resistant pathogens. Converting from intravenous to oral therapy affords a benefit primarily in terms of cost reduction but may also permit earlier removal of intravenous lines which facilitate the establishment of infections by skin pathogens like *Staph. epidermidis*. Oral antibiotics are usually cheaper than intravenous ones, quite simply because the use of the latter is largely restricted to hospitals anyway, so their manufacturers have to recover the development costs from lower lifetime sales. Optimized dosing is, as the name implies, modifying the antibiotic dose to suit an individual patient's circumstances. Factors that may influence the dose, and hence the effectiveness of the therapy, include the patient's physical characteristics (weight, age, immune status, renal function), the site of infection and the pharmacokinetics of the drug which determine its access to, and concentration at, that infection site.

Two procedures that have been advocated as means of restricting resistance development are cycling of antibiotics and routinely using them in combination, particularly for infections with a long time-course of treatment. However, there is little evidence to support the use of routine cycling—the planned replacement of one specific antibiotic, or category of antibiotics, with another at predetermined intervals—as a means of controlling resistance. True cycling involves the return into use of the original antibiotic after a specified time, and although the first switch may lead to a reduced incidence of resistance to the first drug, some studies have shown that when it is subsequently reintroduced the original resistance level is quickly restored. Using antibiotics in combination as a means of restricting resistance development is well established and of undoubted benefit in the treatment of both tuberculosis, for which the duration of therapy is typically 6 months, and in HIV/AIDS which requires lifelong treatment. During such long time periods the number of pathogen replication cycles is so large, and in these two examples the mutation frequency is so high, that the probability of a resistant mutant arising and being selected as the predominant strain at the site of infection is significant, so using two or more agents with different modes of action is both logical and effective. However, the same logic has been applied to other, relatively short-term, infections without clear evidence of benefit.

4 Monitoring of antibiotic resistance

To get the most out of a newly implemented stewardship programme (or a change to an existing one) it is necessary to have the means by which to measure its effect. It is, therefore, necessary to plan in advance what parameters will be measured and what will be the baseline data against which the changes will be judged. Some of the parameters might include, but are not restricted to:

- Antibiotic consumption and costs, both in total and by specific drug class
- Costs associated with prescribing potentially toxic antibiotics, e.g. gentamicin and vancomycin blood level monitoring
- Rates of resistance to specific antibiotics by problem pathogens
- Pharmacy interventions to advise on inappropriate antibiotic use
- The incidence of hospital-acquired infections.

One of the problems that has dogged reviewers trying to assess the extent of the benefits of stewardship programmes is that in many of the cases reported in the medical literature multiple changes to an established programme have been introduced together, or they have overlapped in time so that evaluating the contribution of each change has been difficult. It is, therefore, worthwhile deciding in advance when a new policy or practice will be implemented and when its effect will be assessed. The input of information technology specialists and hospital epidemiologists to a stewardship management team becomes important since they, together, can decide how the data will be recorded and analysed to best effect.

The more sophisticated antibiotic control and information systems do not simply record data on antibiotic consumption, cost and resistance, but are capable of relating infection control data to antibiotic use and would be expected to draw attention to situations where a change in use of a particular antibiotic was associated

with increasing isolation of a particular pathogen. Such an association does not, of course, mean that one *caused* the other, but it does raise staff awareness of that potential. Computer-assisted surveillance of hospital-acquired infection (HAI) has been shown, in some cases, to be more effective than manual monitoring and reporting; as long ago as 1986 one study reported that 90% of antibiotic-resistant HAIs were detected by computer compared to 76% manually. There is also the potential to improve antibiotic prescribing by minimizing the risk of adverse effects when information systems provide patient-specific warnings on allergies, immune and renal functions and the potential for interaction with the patient's other drugs.

It is not surprising, perhaps, that there are marked differences in antibiotic resistance patterns from one country to another. This is illustrated in Figure 15.2, which shows that penicillin resistance in *Streptococcus pneumoniae* can vary from less than 5% in some European countries to more than 50% in others. Smaller, but nevertheless significant, variations may also arise between different regions of a single country; Figure 15.3 shows the corresponding *Strep. pneumoniae* data for the UK from the national Heath Protection Agency. However, data on local resistance patterns are of paramount importance and well-structured monitoring programmes should be capable of identifying unforeseen consequences of changes in antibiotic use such as that arising when a preapproval policy for cephalosporins was introduced in a New York hospital in an attempt to control cephalosporin resistance in *Klebsiella* species. The policy did achieve a 71% reduction in ceftazidime-resistant *Klebsiella* isolated in intensive care units, but monitoring revealed a concomitant rise in imipenem use and a 69% increase in imipenem-resistant *Ps. aeruginosa* that was attributed largely to the preapproval policy.

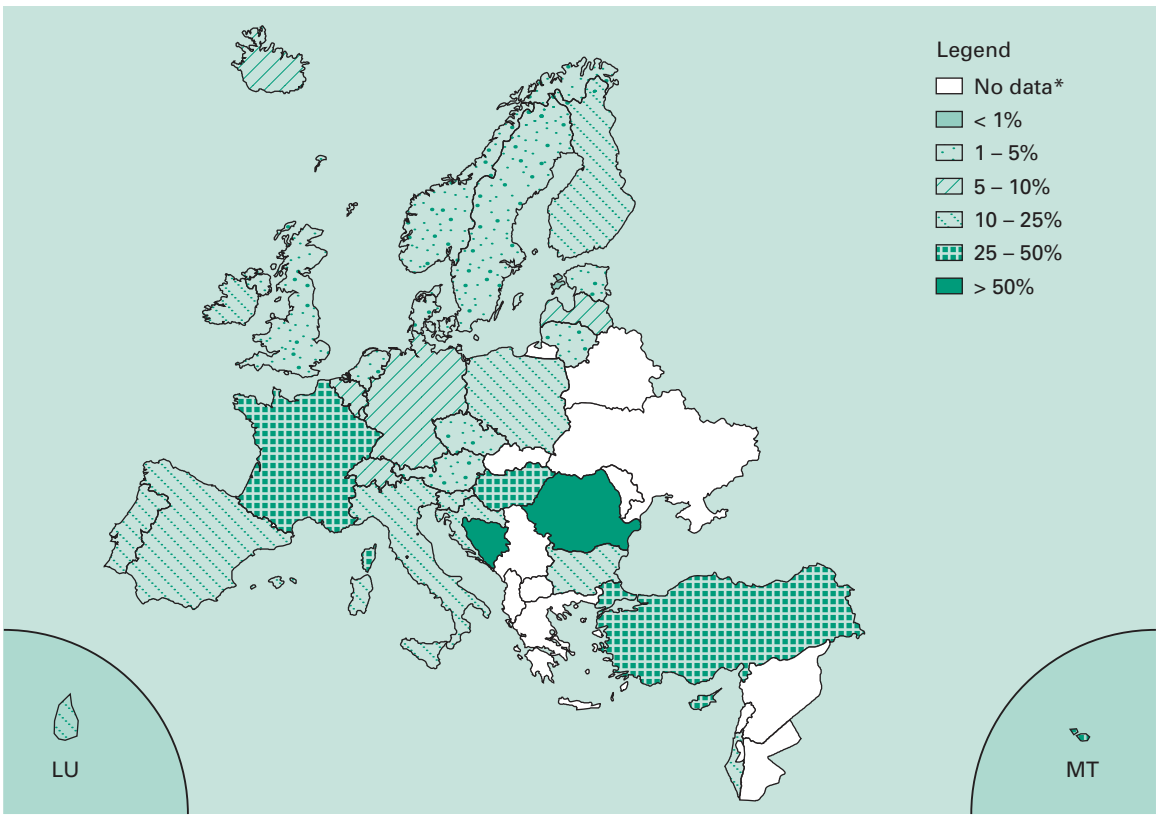

Figure 15.2 Incidence of penicillin-resistant *Strep. pneumoniae* in Europe, 2008. (From the European Antimicrobial Resistance Surveillance System; reproduced with permission.)

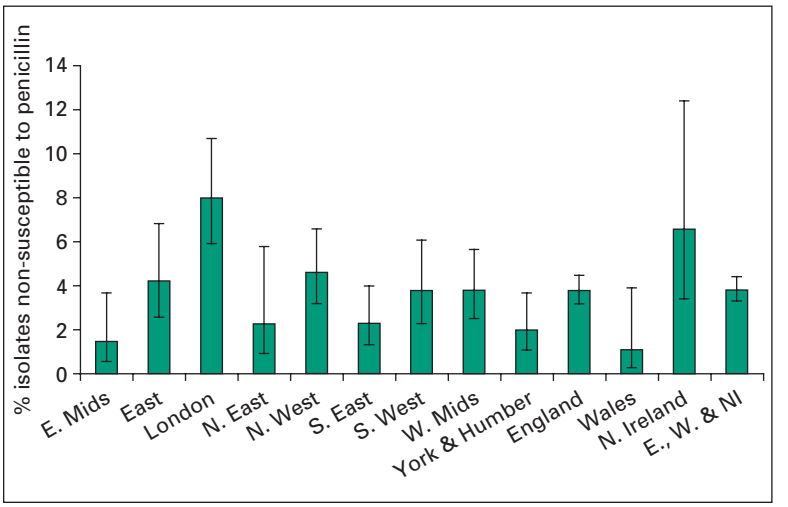

Figure 15.3 Incidence of *Strep. pneumoniae* in different regions of the UK, 2007. (From the UK Health Protection Agency; reproduced with permission).

5 The impact on resistance of antibiotic availability to the public

The ease of availability of antibiotics to the general public varies throughout the world. In Europe the Council of the European Union recommended in 2001 that member states should support the prudent use of antibiotics by restricting systemic antibacterial agents to prescription-only availability. In many other countries, though, antibiotics are supplied without a prescription—either because the practice is not illegal, or because the law is not enforced. Even in the USA this is so: in its testimony to Congress referred to in the first sentence of this chapter the IDSA posed the question 'Antibiotics' true value: "precious resource" or a "giveaway marketing tool"?' They were referring to the practice 'where grocery stores and pharmacies give prescribed antibiotics away for free as a marketing ploy to lure customers into their stores'. Self-medication with antibiotics (obtained via the internet or simply by patients using left-over antibiotics prescribed at an earlier date for unrelated infections) is another practice that contributes to uncontrolled and often inappropriate antibiotic use and so contributes to the resistance problem.

In the UK there has been a slow movement of topical anti-infective agents from prescription-only medicine (POM) to pharmacy-only (P) status; thus, clotrimazole, chloramphenicol and aciclovir creams, and others, have all undergone this reclassification, but in late 2008 azithromycin became the first systemic antibiotic available in pharmacies without prescription, albeit for the defined purpose of treating chlamydial infection. This change in policy by the UK Medicines and Healthcare products Regulatory Agency (MHRA) received a mixed reception. It was welcomed by some as a step towards the desirable objectives of increasing patient choice and encouraging patients to take responsibility for their own healthcare, but executive officers of the British Society of Antimicrobial Chemotherapy strongly criticized the change as a retrograde step that is likely to lead to increased use without professional advice and, as a consequence, increased resistance. The situation was exacerbated by proposals for similar reclassification of other antibiotics, e.g. trimethoprim and nitrofurantoin, both for the treatment of cystitis, but in March 2010 the UK government stepped in to halt MHRA reconsideration of the trimethoprim application.

Apart from the likely increase in use leading to increased resistance, one of the strongest arguments against the availability of antibiotics without prescription is that it would even remove the means by which the consumption of a particular antibiotic could be monitored and correlated with any resistance trends—prescriptions can be counted, but sales are not! The ability to operate such surveillance systems is an integral component of stewardship programmes, so any change in the legal status of systemic antibiotics to make them available without prescription would not only flout the EU recommendation but undermine the government's stated support for prudent antibiotic use. It would seem that the decision to halt MHRA consideration of the proposal to reclassify trimethoprim means that the UK government has heeded the warnings that can be summarized

in the following quotation from the Chief Medical Officer's report for 2008:

> The potency of one of the key weapons in the medical armoury is being eroded. The harm caused by each unnecessary prescription is not visible at the time, and so society fails to take action that is necessary to stop the problem worsening. Correcting this situation will require a paradigm shift in thinking. The effectiveness of antibiotics should be seen as a common and collective public good. Every antibiotic expected by a patient, every unnecessary prescription written by a doctor, every uncompleted course of antibiotics, and every inappropriate or unnecessary use in animals or agriculture is potentially signing a death warrant for a future patient.

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16 Public health microbiology: infection prevention and control

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1 Introduction

The critical and defining element of all infections and infectious diseases is their capacity to spread. The fact that infection can spread from person to person (patient to patient) by direct contact, or by the hands and clothes of healthcare workers, or by contamination of equipment or the environment, means that there is a public health and infection control aspect to all infections. For every patient who acquires an infection there is a need for specific treatment for that patient, but we also need to address the two infection control issues—from where (or from whom) has the infection come, and how can we minimize the risk of it spreading to others. This is the subject of infection prevention and control.

Among the broad range of infections, those that develop as complications of other illnesses or of the treatment of those illnesses, possibly by cross-infection but also by infection from the patient's own bacterial flora,

Hugo and Russell's Pharmaceutical Microbiology, Eighth Edition. Edited by Stephen P. Denyer, Norman Hodges, Sean P. Gorman, Brendan F. Gilmore.

are a major concern to health services throughout the world. These are variously known as hospital acquired infections, hospital cross-infections, or more generally now, *healthcare-associated infections* (HCAIs) to reflect the fact that they occur in patients throughout health and social care settings, not just those in hospital. An increasingly elderly population with a range of chronic medical conditions and the tremendous success of modern medical and surgical practice in increasing life expectancy and treating previously fatal conditions means that health services have to deal with many patients who are highly vulnerable to infection. Invasive clinical procedures are an essential part of most medical care, as are treatment requirements causing significant immunosuppression, both of which are key risks for the patient developing an HCAI.

One of the main responsibilities of all clinicians is that they should, as a priority, not harm their patients through the investigations and treatment they give. Clearly, with modern medical and surgical practice, all risks cannot be eliminated, but the risks must be minimized, in particular the risk of these vulnerable patients acquiring an infection as a direct result of their healthcare. Hence there is a specific focus in all health services on the prevention and control of HCAI.

2 Healthcare-associated infections—definitions and range

HCAIs encompass a considerable variety of infections of different body sites and systems caused by a wide range of bacteria and some viruses and fungi. The types of infection reflect all the body sites and systems that can be the subject of medical intervention.

2.1 Wound and soft tissue infections

Any wound, accidental or surgical, breaches one of the key barriers to infections—the skin. With accidental wounds, contamination with dirt, soil and environmental bacteria may be inevitable, but with 'deliberate' surgical wounds, every effort must be made to minimize the risk of postoperative wound/surgical site infection (SSI; the term reflects the importance of infection at any part of the surgical site, not only the obvious external wound). These SSIs may come from the patient's own normal body flora (e.g. from the intestinal bacteria after abdominal surgery), from nose or skin carriage of wound pathogens such as *Staphylococcus aureus* by the patient, or by cross-infection from other patients or staff as a result of a breakdown of aseptic procedures and proper clinical care. Protocols for clinical care of surgical wounds aim to minimize the risk of the cross-infection, and antibiotic prophylaxis combined with careful surgical practice aims to minimize the risk from the patient's own endogenous bacteria.

Non-surgical soft tissues sites are also prime sources of HCAI, particularly peripheral ulcers (vascular, diabetic, etc.) and pressure sores (decubitus ulcers) where the initial vascular insufficiency and tissue breakdown provides an ideal environment for bacterial infection.

2.2 Bloodstream infections

Potentially the most severe types of HCAI in terms of outcome are bloodstream infections (bacteraemias). The blood and the cardiovascular system should be sterile and the presence of bacteria in the blood is an alarm signal for a patient's healthcare. Many bacteraemias are part of infectious diseases not linked to healthcare risks (bacterial meningitis, community acquired pneumonia, acute pylonephritis) but others are important complications of healthcare. Almost all hospital treatment in modern medical practice requires invasive procedures with the insertion of various synthetic tubes and prosthetic devices. The insertion of indwelling intravascular catheters and cannulae penetrates the protective layer of the skin and provides a portal of entry for bacteria. Central venous catheters are used increasingly for a range of clinical investigations and treatments and carry a significant risk of infection which starts on the artificial surface of the catheter and then seeds the bloodstream more generally, with the risk of clinical sepsis and infection of cardiovascular structures such as the heart valves (endocarditis) or other metastatic infection sites. Short peripheral intravenous cannulae carry less individual risk of infection, but so many are used in modern clinical practice that they are in fact the source of more HCAI bacteraemias than central catheters. Other local sites of HCAI (wounds, ulcers, urinary tract, respiratory tract) can also lead to bacteraemia.

2.3 Urinary tract infections

The commonest form of HCAI (see below) are urinary tract infections, mostly as a result of indwelling urinary catheters which inevitably become contaminated and colonized with bacteria, then leading to infection of the bladder and the lower urinary tract, then the ureters and potentially the kidneys (pylonephritis). This can be an important cause of bacteraemia.

2.4 Respiratory tract infections

Respiratory infections are some of the most common types of infection in the general community and these can also affect hospital patients, but some clinical conditions and treatments predispose patients to healthcare-associated respiratory tract infections. During the postoperative period surgical patients are particularly vulnerable to pneumonia; they will have undergone endotracheal intubation for their anaesthesia and postoperative discomfort and inactivity may lead to inadequate ventilation of their lungs and inability (or disinclination) to cough, resulting in postoperative pneumonia. This risk is greatly magnified in patients needing intensive care and undergoing prolonged intubation and artificial ventilation. Ventilator-associated pneumonia (VAP) is one of the major challenges to successful intensive care. As well as the local effects of VAP on the respiratory tract, it is also a significant cause of bacteraemia.

2.5 Gastrointestinal tract infections

Infectious diarrhoea is a well-recognized community syndrome. Some of the issues that result in diarrhoea and vomiting in the population at large, such as mass catering leading to food poisoning with *Salmonella* and *Campylobacter* spp. also apply in hospitals and other healthcare and social care settings but other gastrointestinal infections cause particular problems in relation to healthcare settings. Norovirus vomiting and diarrhoea spreads so readily in closed communities that it causes numerous and frequent outbreaks in all health and social care settings and is the commonest cause of ward closures in the NHS in England to control the spread of this virus. One bacterial cause of potentially severe, indeed fatal, diarrhoeal disease that is much more specifically an HCAI is *Clostridium difficile* infection (CDI). This infection generally affects only those whose normal gut bacterial flora has been disturbed by treatment with broad-spectrum antibiotics (or some other infection proven or suspected) which enables the *Cl. difficile* spores to germinate and then the vegetative bacteria to produce their damaging toxins.

3 Microorganisms that cause HCAI

Although some infectious agents causing HCAI are also involved in community acquired infectious diseases (norovirus, *Salmonella* spp., *Staph. aureus*), many HCAIs are caused by organisms particularly associated with healthcare. These bacteria are generally characterized by two properties: they tend to be resistant to many antibiotics and have been selected by pressure of antibiotic usage in healthcare, and they are also generally opportunist pathogens with less capacity to produce illness in otherwise fit and healthy people but able to cause severe and life-threatening infection in those who are vulnerable and most susceptible because of their underlying diseases and/or their treatment. Some, in particular, can be associated with artificial implants and prostheses.

3.1 *Staphylococcus aureus*, including MRSA

Staph. aureus is inextricably linked with human health and disease. Its natural habitat is the human skin particularly the anterior nares and the warm, moist skin folds of the perineum (and groin) and axilla and at any one time about one third of people are colonized with *Staph. aureus*, alternatively known as 'carriers'. The nose is the principle carriage site in most (probably all but not always detectable) carriers with the skin sites also colonized in a smaller number. *Staph. aureus* has always been the most common cause of wound infections (accidental and surgical) and a leading cause of HCAIs. When penicillin was first used clinically in the early 1940s, almost all *Staph. aureus* strains were susceptible to it and it was widely used. By the late 1950s, 95% were resistant to penicillin due to natural selection of penicillinase (β-lactamase)-producing strains. Meticillin (formerly known as methicillin), and the later oxacillin, cloxacillin and flucloxacillin, was developed to resist breakdown by β-lactamase and restore treatment options. Within a year of introduction, the first meticillin-resistant *Staph. aureus* (MRSA) strain was described. MRSA were interesting rarities in the 1960s and through the 1970s. In the 1980s, some MRSA strains (now called epidemic strains; EMRSA with sequential numbers) caused isolated and restricted outbreaks of HCAI which were mostly contained by a 'search and destroy' approach (i.e. isolate and treat the patient; screen all contacts among patients and staff for carriage; give decolonization treatment to any found to be positive). However, in the early 1990s in the UK, two EMRSA strains, 15 and 16, emerged with greater capacity to spread in healthcare settings and to cause a higher proportion of severe disease. Their spread through the healthcare system was not controlled and these strains became a major HCAI problem and the subject of media publicity, pressure group campaigns, and political concern in the early years of the 21st century. These strains were difficult to treat and caused significant morbidity and mortality. However, it must be remembered

that MRSA is not a disease; it is a group of *Staph. aureus* strains that cause a wide range of HCAIs—wound and soft tissue infections, VAP, catheter-associated UTI, as well as the bacteraemias that have been a major focus for surveillance and preventive measures as being the most severe end of the spectrum of MRSA disease.

3.2 *Staphylococcus epidermidis* (coagulase-negative staphylococci)

Everyone carries *Staph. epidermidis*, and sometimes other coagulase-negative staphylococci (CNS), on their skin as part of the normal flora. These CNS are often antibiotic resistant (including being meticillin resistant) but have much less pathogenic potential than *Staph. aureus* for healthy people. However, they are one of the most common causes of HCAI associated with indwelling artificial devices and prosthesis. Despite the emphasis on MRSA bacteraemia, *S. epidermidis* is the most common cause of intravenous line-associated bacteraemia, but with more low-grade or 'grumbling' clinical presentations and fewer severe and fatal infections than MRSA. It is also the commonest cause of late-onset deep infections of implanted prostheses such as hip and knee joints and heart valves. In this respect they cause significant morbidity and treatment often requires removal and replacement of the original implant.

3.3 Gram-negative bacteria

Several distinct groups of Gram-negative bacilli have the capacity to cause opportunist infections in vulnerable patients, particularly those with various forms of immunosuppression. All share a general characteristic of being able to survive in moist environmental conditions and being resistant to a range of antibiotics so that their survival and spread is selected by the widespread use of antibiotics in hospitals and other healthcare settings. This group of opportunist pathogens has long included organisms such as *Pseudomonas aeruginosa* (or other *Pseudomonas* species), *Acinetobacter* spp. and the resistant enterobacterial relatives of *Escherichia coli—Enterobacter, Serratia* and *Klebsiella* spp. All have caused serious infections in immunocompromised patients, e.g. patients undergoing chemotherapy for a malignant disease in whom Gram-negative bacteraemias have a high incidence, often leading to clinical sepsis and death. *Ps. aeruginosa* causes similar infections but also has a long association with pulmonary infection in cystic fibrosis patients.

Two further challenges from Gram-negative bacteria are causing concern with the emergence of new antibiotic resistance markers. The β-lactam antibiotics (penicillins and cephalosporins) have been mainstays of treatment of *E. coli* and related Gram-negative bacteria for many years. Strains of *E. coli* have now emerged that have acquired genes for the production of extended spectrum β-lactamases that break down all β-lactam agents. These ESBL-producing *E. coli* have become widely established in patients in hospitals and in other health and social care settings. Gut colonization is linked to urinary tract infection, especially when the patients are catheterized, and this can then result in bacteraemias that are very difficult to treat. Carbapenen antibiotics (e.g. imipenem) are important for treatment of these resistant Gram-negative HCAIs, so it is of special concern that some carbapenemase-producing *Klebsiella* spp. and other Gram-negative species are now being found in some UK and other European hospitals.

3.4 Glycopeptide-resistant enterococci

Enterococci (*Enterococcus faecalis* and *Ent. faecium*) have been recognized causes of HCAI in immunocompromised patients, e.g. post-transplant (including bone marrow recipients) and cancer chemotherapy. These organisms are intrinsically resistant to many antibiotics and the mainstays of treatment where the glycopeptides vancomycin and teicoplanin. There was particular concern in the early 2000s when glycopeptide-resistant strains of *Ent. faecium* caused small but severe outbreaks in transplant and chemotherapy units. A suggested link to glycopeptide use in agriculture has not been proven, and although such strains still cause some serious infections, they have not become a widespread problem in other patient groups.

3.5 *Clostridium difficile*

Cl. difficile was discovered to be the major cause of antibiotic-associated diarrhoea and colitis simultaneously by groups in the UK and the USA in 1978. Although the subject of much interest and research amongst anaerobe microbiologists and a few infectious diseases physicians, it did not reach popular notoriety until the middle of the first decade of the 21st century. *Cl. difficile* is an anaerobic spore-forming organism found in the gut of many species of animal, including humans. With conventional bacteriological methods, about 3% of healthy people are found to carry *Cl. difficile* in their faeces. However, the figure is much greater in hospitalized patients and in residential nursing and care home residents. Patients with *Cl. difficile* diarrhoea excrete large numbers of spores into their surrounding environment

where they can survive for weeks, months and even years. People with a normal, healthy gut flora can swallow *Cl. difficile* spores without ill effect, but in patients whose normal intestinal flora is disturbed, especially and most commonly by use of broad-spectrum antibiotics, the normal inhibition of *Cl. difficile* germination and growth is removed and *Cl. difficile* grows rapidly. The vegetative *C. difficile* cells attach to the gut mucosa and produce two toxins (A and B) that cause the disease. Toxin A is specifically called an enterotoxin whereas toxin B is a cytotoxin, but they show considerable structural and functional overlap and toxin B in particular can produce severe disease on its own when a strain that is A-negative/B-positive is the cause of the infection. The toxins cause diarrhoea, ranging from mild to profuse and debilitating, and colonic ulceration (which appears as erupting volcanoes on microscopy and biopsy specimens) that can develop into pseudomembranous colitis with, at worst, toxic megacolon and colonic perforation.

The directly attributable mortality in several well-documented outbreaks has been around 10% during the initial stages of infection, but in the cohorts of elderly patients who have had CDI, the overall mortality over a period of 2–3 months can be as high as 40%.

The precipitating factor of antibiotic use and the range of severity of CDI have been recognized since the late 1970s and the infection was well documented in the 1980s in surgical patients from over-use of clindamycin and the cephalosporin antibiotics for surgical prophylaxis (and in immunocompromised groups), but it was not regarded as a priority. In an outbreak amongst elderly patients in hospital in the winter of 1991 in Manchester, there were more than 170 cases with 17 deaths. The patients were on open 'Nightingale' wards and most had received antibiotics for chest infections. For the first time, strain typing (by pyrolysis mass spectrometry at that stage) proved that the outbreak was caused by a single strain spread by cross-infection in the hospital setting. A subsequent Department of Health and Public Health Laboratory Service review and report provided guidance and recommendations that remained relevant a decade later but were not generally applied and acted upon with the rigour that was needed. The number of cases reported rose steadily during the 1990s and reached epidemic proportions by the early 2000s when the general increase was compounded by the emergence (in North America, the UK and Europe) of a new strain designated ribotype 027 with increased virulence and specific resistance to fluoroquinolone antibiotics which were being used increasingly in community and hospital practice. The serious impact of this disease meant that health services in the UK and elsewhere had to take specific and vigorous actions to bring down the unacceptably high incidence of CDI which was compromising the delivery of healthcare. Not only were patients suffering from a serious infection that compromised their underlying medical problems, but the management of these patients placed great pressure on hospital services overall and were a serious drain on health service resources.

3.6 Norovirus

Norovirus infections cause very significant disruption to hospital and social care services. This virus infection causes an explosive vomiting and diarrhoea with rapid onset and little preliminary warning that the patient is about to be ill. The vomitus and diarrhoea are heavily laden with the virus and the aerosols created by both are key to its rapid spread among contacts. The virus can spread directly by aerosol to the mucous membranes (mouth, nose, pharynx) of people in the same area. It will also contaminate items throughout the environment that can then themselves be a source of infection when those items of furniture, fittings, equipment, etc. are handled. The infection has a very high attack rate among those exposed and the postinfection immunity is generally very short-lived, so previous infection provides little protection. Both patients or residents and staff are usually affected in these outbreaks. Control of norovirus infection requires prompt and vigorous infection control precautions.

Fortunately, although unpleasant and debilitating when it occurs, norovirus infection is usually short-lived and recovery is quick in most patients. The acute vomiting and diarrhoea symptoms usually last for only 36–48 hours and most people then have only a few days of convalescence before feeling well again. Staff are usually able to resume work about 72 hours after the vomiting and diarrhoea has ceased, by which time they are no longer infectious. However, in health and social care settings where patients and residents are elderly and often frail, with severe underlying conditions, norovirus can be a more severe disease with significant associated morbidity and some mortality.

4 Scale of the HCAI problem—prevalence and incidence

An assessment of the number of cases of HCAI and the risk of infection for different patient groups is based on

a series of different measurements. Point prevalence studies have been undertaken in many countries to give a snapshot of the number, and the percentage, of patients with any type of HCAI in a particular healthcare setting on a specific day. Prevalence studies in various developed countries over the last decade have shown overall prevalence rates between 5% and 10% (Table 16.1). A prevalence study in the four UK countries and the Republic of Ireland in 2006 showed an overall prevalence of 7.6%, ranging from 4.9% in the Republic of Ireland to 8.2% in England. The low rates in the Republic of Ireland probably reflected a rather younger patient population overall and the higher number in England was linked almost entirely to much higher numbers of cases of CDI (2006 was the height of the epidemic of CDI in hospitals in England). The commonest types of infection were urinary tract infection, followed by skin and soft tissue infections and wound infections, respiratory infections, and gastrointestinal infections (Table 16.2). Bloodstream infections accounted for only 7% of HCAIs but represent the most severe end of the spectrum of disease. MRSA infections of all types accounted for 16% of the HCAIs and CDI was 17% of the total in hospitals in England but only 5% in Wales, Northern Ireland and the Republic of Ireland.

Prevalence studies provide valuable comparisons between hospitals and countries and show the general contribution of the various types of HCAI. However, they do not represent the actual number of cases of different HCAIs over time, i.e. the incidence of infection in different hospitals, wards or patient groups. For example, a point prevalence of 8.2% does not mean that 8.2% of the patients admitted to the hospitals in England developed an HCAI because the infected patients tend to be more seriously ill and have longer stays in hospital both from their underlying illness and as a result of their HCAI; therefore, the incidence is always less than the point prevalence. Incidence is measured by ongoing and continued surveillance of specific infections in which all cases of the infection are recorded and related to the number of patients at risk. The HCAIs that are most commonly subject to surveillance programmes are bacteraemias (bloodstream infections) caused by specific healthcare-associated pathogens such as MRSA or ESBL-producing *E. coli* (because they represent the most severe types of HCAI), SSIs (one of the key indicators of the quality of a surgical service or an individual surgeon), and CDI. Surveillance data are necessary for monitoring infection prevention and control activities at national and regional level, which can provide useful comparisons between hospitals for patients who may choose where they wish to have their treatment. However, surveillance with timely feedback and data is particularly important at local level, within individual hospitals to ensure delivery of a high quality of care. Most surveillance programmes have depended on voluntary reporting of the infections to regional or national programmes but in several countries some high-profile surveillance programmes have been made mandatory to ensure that infection prevention

Table 16.1 Prevalence of healthcare-associated infections (HCAIs): international comparisons

Country	Year	Prevalence of all HCAIs (%)
EU	2007	7.6
UK	2006	7.6
Denmark	2003	8.7
France	2006	5.4
Greece	2000	9.3
Italy	2002	7.5
The Netherlands	2007	6.9
Portugal	2003	8.7
Sweden	2004–6	9.5
USA	2006	5.0–10.0
Canada	2002	10.5

Source: Commissioned research, National Audit Office (2009).

Table 16.2 Comparative prevalence of different types of healthcare-associated infections (HCAIs)

Type of infection	Percentage of all HCAIs
Lower respiratory tract	21
Gastrointestinal (diarrhoea/vomiting)	21
Urinary tract	20
Surgical site	14
Skin and soft tissue	10
Bloodstream (bacteraemia)	7
Others	7

Source: Four-country HCAI prevalence survey, 2006 (Smythe *et al.*, 2008)

and control is made a high priority for health service managers and clinicians alike. Mandatory surveillance of MRSA bacteraemia was introduced in England in 2001, followed CDI, glycopeptide-resistant enterococcal bacteraemia and SSIs in orthopaedic surgery in 2004. Similar approaches have been applied in other UK countries, the Republic of Ireland, European countries and the USA (mostly at an individual state level).

A further development of basic surveillance (i.e. reporting numbers of cases) is the application of enhanced surveillance in which information about patient demographics, risk factors, and outcomes is included. This can then be linked to root cause analysis of individual cases to provide valuable information about target areas for preventive measures.

4.1 What has surveillance told us?

Mandatory surveillance of MRSA bacteraemia in England showed a steady rise in cases to a peak of 7700 in 2004. Public and political concern about MRSA resulted in the government setting a target for a 50% reduction in MRSA bacteraemia over the 3 years 2005–2008. The surveillance data enabled active performance management through all levels of the (national) health service, provided pressures for improved infection prevention and control actions, and showed show the target was being achieved. Information from enhanced surveillance within this programme showed the importance of patients' ages and underlying conditions as particular risk factors, and most significantly showed the importance of intravenous catheters and cannulae, renal dialysis catheters, urinary catheters and chronic wounds and ulcers as sites of MRSA infection leading to bacteraemia.

A similar approach was taken with CDI when mandatory surveillance showed that there were 55 000 cases in patients over 65 years of age in England in 2006; as these represent about 75% of cases, this means that there were around 70 000 cases overall. Guidance on prevention and control measures (based on the 1994 guidance) and a target to reduce the number of cases by 30% by 2011 focused activities in NHS hospitals and the 30% reduction was achieved within the first year (2008–2009).

SSI surveillance in orthopaedic surgery has been a national requirement in all UK countries for several years. This is a type of surgery with relatively low rates of infection (<5%) but surveillance has focused attention on infective complications, shown differences between surgical procedures, and also shown a steady improvement in rates in all the countries. However, the very short length of stay now usual for patients after these operations means that some infections only manifest themselves when the patient is at home. This has shown the need for including some system of postdischarge surveillance for surgical site infection to give a realistic picture of these HCAIs. This approach is being pioneered in the UK and the Republic of Ireland in relation to infections following caesarean section births where there is a clear opportunity for professional input to postdischarge surveillance because the mothers are seen at home by a midwife or health visitor.

4.2 The HCAI challenge

The surveillance programmes in various countries have shown the scale of the challenge set by HCAI in modern health and social care services. Why has this situation developed? There is a strong argument that during the last quarter of the 20th century, infections (including HCAI) had not been regarded as an important part of modern medical practice. There had been a view from the late 1960s onwards that infectious diseases had been conquered and that antibiotics and vaccines provided the answer. The importance of infection prevention and control measures was not given its former position in clinical training of doctors and nurses. Modern medicine was making tremendous progress in the treatment of malignant diseases, cardiovascular disease, transplantation, and chemotherapy, and in the management of chronic diseases. Life expectancy increased markedly and the proportion of very elderly people in the population, with their inevitable healthcare needs, rose rapidly. This created a vulnerable patient population at high risk of infection, but these infections were regarded as incidental nuisances rather than the major risk to a patients' health and potential mortality which they are. Infections were regarded as the province of the infection specialists (medical microbiologists, infection control nurses) who had plenty of interesting work to do but a generally low profile. The situation changed with the advent of the 21st century and the recognition of the human and financial costs of HCAIs in a modern healthcare service.

5 Responsibility for HCAI prevention and control

The responsibility for infection prevention and control is shared across all who have a role in the delivery of health and social care, from the most senior managers to the most junior members of staff at ward or unit level—often described as the 'board to ward' approach. Clinicians

(doctors, nurses and other professional colleagues) have a personal and professional responsibility for the care of their patients. Their responsibility for patient safety includes minimizing the risk of infection by implementing best clinical practice protocols, antibiotic stewardship, etc., and delivering the highest standards of clinical care and treatment for those who develop an infection, to treat those infections and minimize the risk of transmission to others.

Health and social care service managers and overseeing boards have an overarching responsibility for providing the corporate environment in which infection prevention and control has a high priority and all staff know what is expected of them. They need surveillance data and performance audits, coupled with inclusion of infection prevention and control in the individual performance review and personal appraisal of all their staff. In this way, infection prevention and control becomes embedded in the culture of the healthcare organization.

Governments, departments of health and health service national managers are the third party in this partnership. They are responsible for making HCAI prevention and control a top priority throughout health and social care services and holding local managers and boards to account for delivery of a high quality and patient care—and low rates of HCAI. They use performance management arrangements to require high standards from healthcare providers and can set targets for HCAI reductions (or ceilings for those with good records). They decide which HCAIs should be subject to mandatory surveillance. Governments also have the ultimate authority in terms of legislation that may be used to support HCAI prevention and control. The UK government was the first to use this type of legislation in relation to healthcare in England. The Health Act 2006 implemented a statutory code of practice for the prevention and control of HCAI.

This code of practice required all NHS bodies to:

- have appropriate management systems for infection prevention and control
- assess the risks of acquiring HCAI and take actions to reduce such risks
- provide and maintain a clean and appropriate environment for healthcare
- provide information on HCAI to patients and the public
- provide information when patients move from one healthcare body to another
- ensure co-operation between organizations
- provide adequate isolation facilities
- ensure adequate laboratory support
- adhere to policies and protocols applicable to infection prevention and control.

These clinical care protocols included:

- standard(universal) infection control precautions
- aseptic technique
- major outbreak protocols
- isolation of patients
- safe handling and disposal of sharps
- prevention of occupational exposure to blood-borne viruses including prevention of sharps injuries
- management of occupational exposure to blood-borne viruses and postexposure prophylaxis
- closure of wards, departments and premises to new admissions
- disinfection policies
- antimicrobial prescribing policies
- reporting of HCAI to the Health Protection Agency as directed by the Department of Health
- policies for the control of infections with specific alert organisms.

The code of practice also required NHS bodies to ensure (as far as reasonably practicable), that healthcare workers are free of and/or protected from exposure to communicable diseases during the course of their work, and the staff are suitably educated in the prevention and control of HCAI. The latter duty within the code of practice reinforced an essential element for all approaches to prevention and control of HCAI—a requirement for education and training of all healthcare workers on the principles of infection prevention and control and the part they need to play.

The combination of all these elements represents the fact that there is personal and corporate responsibility for HCAI prevention and control at all levels in any health and social care system.

6 Keys to infection prevention and control

There is no single 'silver bullet' to solve the challenge of HCAI. Infection prevention and control requires a combination of actions and activities, each of which provides an essential component to the whole.

6.1 Management and organizational commitment

The commitment of senior management to making infection prevention and control a high priority sets a

culture of a health of social care organization. Management should monitor surveillance and audit data at all levels of the organization ('from board to ward') and ensure that all the staff play their part. This helps general a culture of pride in delivery of a quality service.

6.2 Surveillance

Up-to-date surveillance data on the incidence of key infections should be collected, analysed and returned to the clinical units with minimum delay. In this way the data are seen to be 'real' by the staff responsible for the care of the patients. It is now mandatory in several countries to collect surveillance data on MRSA infections (particularly bacteraemias), CDI, and some type of surgical site infections. Although national data collection is focused on whole hospitals or hospital groups, effective action within hospital depends upon the data being assessed and acted upon in individual wards or other clinical units, The essential nature of surveillance data is encapsulated in the dictum 'you have to measure it to manage it'.

6.3 Clinical protocols

There are two main reasons why patients are at risk of developing an HCAI—their underlying medical condition making them vulnerable to infections and the treatment and clinical interventions they are subjected to. These interventions often include invasive procedures that bypass the normal defences of the skin, urinary and respiratory tract. It is, therefore, essential that clinical staff exercise all due care and attention when performing these procedures. To this end, an approach to clinical practice has been developed variously known as 'care bundles' (in the USA in particular) and 'high-impact interventions' (HII; the UK approach). These bundles are care protocols setting out in a simple bullet-point format the five or six essential elements needed to minimize the infection risk associated with the individual aseptic procedures. The aim is for each element to be performed correctly on every occasion and the care bundle/HII incorporates a simple audit tool for self- or peer-assessment on a frequent and regular basis of the performance of the clinical staff doing those procedures. The procedures that had been the main focus of the bundles comprise the following invasive interventions:

- central intravenous catheter insertion and maintenance (Table 16.3)
- peripheral intravenous cannula insertion and maintenance
- renal dialysis catheter insertion and maintenance
- surgical site infection (wound) care
- care of ventilated patients
- urinary catheter insertion and care.

For the HCAI programme in England these were set out in packages called 'Saving Lives' for secondary care and 'Essential Steps' for primary care use. The packages were complemented by a care bundle for CDI and guidance presented in a similar way based upon key elements for antibiotic prescribing, isolation and cohorting of infected patients and the collection of blood cultures which form the basis of the diagnosis of a bacteraemia.

6.4 Isolation and segregation

One of the most basic and ancient approaches to preventing the spread of infection (or 'contagion' in previous centuries) is isolation of the affected patient. Segregation of infected patients from those vulnerable to infection (including various forms of quarantine) is an essential element of all infection prevention and control practice. It is particular important in HCAIs where the infection often occurs in vulnerable or debilitated patients and in a setting where transmission of infection between patients can occur readily. In many hospitals, the number of single rooms available for patient isolation is limited. The best use of the available rooms should be made for those with infections, but where the capacity of single rooms is exceeded by the number of cases of infection, it may then be necessary and appropriate for patients with the same infection to be nursed together in a cohort ward physically separated from other ward areas and with dedicated staff who do not move between the cohort ward and other clinical areas (i.e. cohorting should apply to nursing staff as well as patients).

6.5 Hand hygiene

One of the main routes of transmission of HCAI pathogens such as MRSA is via the hands of healthcare workers as they move between patients. For many years staff were recommended to wash their hands between patient contacts and especially before and after performing clinical procedures. However, very frequent hand washing is time consuming, can damage the skin if done as often as should be required, and can also be quite impracticable when there is inadequate provision of wash-hand basins for staff. The answer to this problem has been the introduction of alcohol hand rubs that can be used repeatedly and quickly at every point of patient contact. Alcohol is highly effective against vegetative bacteria

Table 16.3 High-impact intervention no. 1: central venous catheter care bundle

Insertion actions	
Catheter type	Single lumen unless indicated otherwise,
	Consider antimicrobial impregnated catheter if duration 1–3 weeks and risk of CR-BSI high
Insertion site	Subclavian or internal jugular
Skin preparation	Preferably use 2% chlorhexidine gluconate in 70% isopropyl alcohol and allow to dry
	If patient has a sensitivity use a single patient use povidone-iodine application
Personal protective equipment	Gloves are single-use items and should be removed and discarded immediately after the care activity
	Eye and face protection is indicated if there is a risk of splashing with blood or body fluids
Hand hygiene	Decontaminate hands before and after each patient contact
	Use correct hand hygiene procedure
Aseptic technique	Gown, gloves and drapes as indicated should be used for the insertion of invasive devices
Dressing	Use a sterile, transparent, semipermeable dressing to allow observation of insertion site
Safe disposal of sharps	Sharps container should be available at point of use and should not be overfilled; do not disassemble needle and syringe; do not pass sharps from hand to hand
Documentation	Date of insertion should be recorded in notes
Ongoing care actions	
Hand hygiene	Decontaminate hands before and after each patient contact
	Use correct hand hygiene procedure
Catheter site inspection	Regular observation for signs of infection, at least daily
Dressing	An intact, dry adherent transparent dressing should be present
Catheter access	Use aseptic technique and swab ports or hub with 2% chlorhexidine gluconate in 70% isopropyl alcohol prior to accessing the line for administering fluids or injections
Administration set replacement	Following administration of blood, blood products—immediately
	Following total parenteral nutrition—after 24 h (72 h if no lipid)
	With other fluid sets—after 72 h
No routine catheter replacement	

CR-BSI, catheter-related bloodstream infection.
Source: DH (2007) *Saving Lives: Reducing Infection, Delivering Clean and Safe Care.* Department of Health, London.

causing HCAI such as MRSA and the Gram-negative bacteria. The hand rubs should be made available at every patient bedside and in every clinical area, and at the entrance to cubicles and single rooms. Personal dispensers can also be carried attached to a belt or the clothing of healthcare staff to use as they move between patients. In many hospitals, alcohol hand rub dispensers have also been placed at the hospital entrance and/or at every ward entrance to emphasize to patients and visitors, as well as staff, the absolute importance of hand hygiene. However, the crucial times and places for hand hygiene relate to direct clinical contact with patients. The hand hygiene campaign based on alcohol hand rubs has been promoted internationally by the World Health Organization (WHO) and in the UK by the National Patient Safety Agency through its 'clean**your**hands' campaign. The WHO campaign highlights the five opportunities (and requirements) for hand hygiene:

- before touching a patient
- before clean/aseptic procedures
- after body fluid exposure/risk
- after touching a patient
- after touching patient surroundings.

The need for effective and appropriate hand hygiene has also been included in all the care bundles/HIIs (see above).

However, in one area of infection prevention and control, hand hygiene with alcohol hand rubs does not replace the absolute requirement for hand washing: this is in relation to diarrhoeal infections caused by norovirus or *Cl. difficile*. Alcohol is not effective against norovirus or against the spores of *Cl. difficile*, so for these common diarrhoeal infections, hand washing is essential before and after each patient contact or contact with the environment around infective patients.

The audit of hand hygiene (alcohol hand rub and hand washing) is an important part of monitoring compliance with clinical protocols for infection prevention and control and should include direct observation of all grades of healthcare staff and also measurement of the volume of alcohol hand rub and liquid soaps used as a general proxy measurement of hand hygiene practice.

6.6 Environmental cleanliness and disinfection

Whenever there are problems or outbreaks of HCAI, there is popular outcry over dirty hospitals, giving the impression that environmental cleanliness equates to prevention of infection. Whereas there is no question that hospitals and other healthcare premises should be clean and that a clean environment promotes good healthcare practice, there is less of a direct correlation between general cleanliness and rates of HCAI. However, there is good evidence that bacteria and viruses from infected patients contaminate the general environment around those patients from where they can be picked up, e.g. on the hands, by other vulnerable patients or by healthcare workers and transmitted to other patients. The evidence is particularly clear with *C. difficile* spores, which can be found on all environmental services in rooms where there are patients with CDI. They are particularly prevalent around toilets, or on commodes, or near bed pan washers.

General hospital cleaning is based on a detergent and water cleaning regimen, but when there are patients with known HCAIs, it is advisable to supplement detergent cleaning with use of environmental disinfectants. This is particularly the case in outbreaks of norovirus or CDI. The most effective disinfectants for these viruses and for the spores of *Cl. difficile* are those based on chlorine-releasing agents (see Chapter 19). These should be used routinely in areas where there are cases of norovirus or CDI.

6.7 Antibiotic prescribing

Good antibiotic stewardship and the prudent use of these valuable drugs is an important part of the prevention and control of HCAI. Most bacterial HCAIs are caused by antibiotic-resistant organisms that flourish under the selective pressure of antibiotic use, and because of the fact that many of the bacteria are resistant to several different types of antibiotic, even the use of individual antibiotics can select for bacteria resistant to a wide range of agents. Furthermore, many of the resistance genes are carried on transferable genetic elements that can transfer among bacterial populations, particularly in a selective environment such as a hospital. There are many examples of links between use of particular antibiotics and cases of HCAI caused by resistant organisms. There are also more general links between use of agents such as the fluoroquinolone antibiotics and the rising incidence of MRSA colonization and infection. With CDI, the link is even more direct with the use of broad-spectrum antibiotics being a key precipitating factor for this disease. Outbreaks of CDI have been linked to widespread use of cephalosporins and, more recently, fluoroquinolone antibiotics when the *Cl. difficile* strains such as ribotype 027 have been specifically resistant to these agents.

All healthcare organizations should have antibiotic prescribing protocols to promote and audit good stewardship. This is a requirement of the statutory Code of Practice in England. The guidance in the 'Saving Lives' package recommends that antibiotic stewardship programme should have the following elements:

- a prescribing and management policy for antimicrobials
- a strategy for implementing the policy
- an antimicrobial formulary and guidelines for antimicrobial treatment and prophylaxis
- decision to prescribe should be clinically justified and recorded
- intravenous therapy should only be used for severe infections or where oral antimicrobials are not appropriate
- intravenous antimicrobials should only be used for 2 days before review and switch to an oral agent where possible and appropriate
- all antimicrobial prescriptions should include a stop date—generally a maximum of 5–7 days without represcription
- daily review of antimicrobial treatment
- antimicrobial treatment reviewed on the basis of microbiological results
- minimize the use of broad-spectrum antimicrobials
- a single dose at induction of anaesthesia for most operations where antimicrobial prophylaxis is indicated
- training in implementing antimicrobial prescribing guidance for all prescribers

6.8 Training and education

The implementation of the range of infection prevention and control practices in any health or social care setting can only be successful if there is a comprehensive approach to staff education and training through which all can learn that everyone has a role to play in preventing HCAI. Basic training in infection prevention and control is mandatory for all staff in many hospitals and healthcare settings in the UK, Europe and North America, usually with regular (generally annual) required updates and specialist training for particular professional groups. Completion of this training is generally a requirement for successful appraisal and performance review for all staff.

6.9 Audit

Whereas surveillance of key HCAIs is necessary for monitoring the changing pattern and the incidence of infections, audit of implementation of clinical protocols is equally necessary for maintaining a high level of infection prevention and control practice. There should be regular audits of hand hygiene compliance, adherence to antibiotic prescribing guidelines and the implementation of the clinical protocols in the care bundles/HIIs. The results of these audits should be reviewed in a timely manner at all levels of management in the health and social care organizations so that those implementing the protocols have ownership of the procedures and their effective application. Performance management at all levels depends upon a combination of the data from surveillance of the infections and audit of the clinical practice.

7 Zero tolerance and the principles of infection management

As a result of the implementation of the measures outlined above, the rates of particular HCAIs have fallen significantly in several countries. The application of the central venous catheter care bundle by intensive care units in the USA has resulted in a steep reduction in cases of catheter-related bacteraemia and, in some cases, long periods with no such infections. In England, the package of measures aimed at MRSA bacteraemias resulted in a 65% decrease in cases reported from the 2004 target baseline to 2009. These improvements have enabled the promotion of a zero tolerance approach to HCAI. This does not mean that there will be no infections (this is microbiologically and clinically implausible) but does mean that we can apply a zero tolerance approach to avoidable infections and to poor clinical practice such as inadequate compliance with hand hygiene requirements and imprudent antibiotic prescribing. The aim is to do everything right every time.

The general principles of infection management apply to HCAIs as they do to all types of infection, but it is particularly important to have reliable application of the principles where there is the risk of spread amongst vulnerable patients. Clinical staff need to have a high index of suspicion that a patient may be developing an infection and initiative appropriate confirmative diagnostic tests quickly. There should be prompt isolation of a patient suspected of being infected and specific treatment instituted along with infection prevention and control measures to prevent further spread. As well as these universal principals, there are some specific prevention and control measures aimed at particular infections.

7.1 MRSA

The reductions in MRSA bacteraemia achieved in UK hospitals and elsewhere as part of the targeted approach

has been mostly the result of emphasis on hand hygiene, improved aseptic practices and the care bundles/HII approach for invasive procedures, particularly intravenous central catheter and peripheral cannula insertion and care. Implementation of these measures has been backed by the commitment of managers to reduce infection rates. These general improvements in infection prevention and control procedures would also be expected to help prevent other courses of bacteraemias linked to intravenous catheters and cannulae.

The further measure that is specific to the prevention of MRSA infections overall (wound and soft tissues infections, VAP, etc., as well as bacteraemias) is screening of patients before admission to hospital (when admission is planned or 'elective') or on admission in respect of emergency admissions. The principles behind such screening are that colonization of the nose and/or skin sites generally precedes clinical infection and that a colonized patient (otherwise referred to as a 'carrier') is at risk of developing an MRSA infection themselves and also a potential source of transmission to others. For such screening a swab is taken from the anterior nares, and also from the other skin carriage sites of perineum and axilla if considered appropriate, as well as from any surface lesions such as a chronic ulcer. Laboratory examination of the swab can be based upon convention selective culture for MRSA (which has a minimum turnaround time of 24–48 hours) or more expensive molecular methods based on PCR (polymerase chain reaction) methodology when a rapid result is considered to give significant benefit. Patients found to be colonized with MRSA are generally then given a 'decolonization' or 'suppressive' treatment regimen of nasal mupirocin cream and an antiseptic skin wash and shampoo for 5 days. This is very effective in reducing the bioburden in MRSA colonization in the short term, thus reducing the risk of infection for the individual patient and the risk of transmission to other. However, colonization may recur over a period of several months in 40% or more individuals. Nevertheless, the suppression of colonization will have covered the period of particular vulnerability and the time when they would be more likely to be a source of transmission. Screening and decolonization were part of the 'search and destroy' approach to MRSA infection developed in the UK in the1980s. It was also adopted elsewhere and has continued to be very effective in helping maintain low levels of MRSA infection and low levels of colonization in countries such as the Netherlands, parts of Scandinavia, and Western Australia. This approach was not maintained in most parts of the UK during the1990s but has been reintroduced as part of the MRSA control measures in various ways in the UK countries in more recent years. Screening of all patients admitted to NHS hospitals has been introduced in England, whereas other countries have adopted widespread but more restricted, risk-based approaches to selecting patients for screening. The risk factors are generally age (>65 or 70 years), previous MRSA carriage, previous hospital admission, residence in a nursing home or residential care home, and the presence of a chronic disease. The most appropriate approach will become evident as these different regimens are applied in different healthcare settings.

Screening for *Staph. aureus* more generally, not specifically MRSA, has been used as part of outbreak control measures over many years but has not been adopted on a routine basis. However, modern approaches to MRSA screen could also enable screening for any *Staph. aureus* strain in particular vulnerable groups.

7.2 *Clostridium difficile* infection

The emergence of CDI has been a complication of modern medical care compounded by inadequate attention to antibiotic stewardship and infection prevention and control measures. Elderly patients are the most vulnerable to this infection (75% of cases are in people >65 years old) but severe disease can occur in younger patients also. The major precipitating factor is the use of broad-spectrum antibiotics. In the UK, guidance produced in 1994 was reviewed and an updated document was published in 2009. This recommended the application of a mnemonic protocol (SIGHT) for managing suspected and then proven cases:

- Suspect that a case of diarrhoea maybe infective when there is not a clear alternative cause for the diarrhoea
- Isolate the patient and contact the infection control team
- Gloves and aprons must be worn for all contact with the patient and their environment
- Hand washing with soap and water before and after each contact with the patient and their environment
- Test the stool for *Cl. difficile* toxins by sending a specimen immediately.

CDI itself then needs to be treated as a major diagnosis in its own right (not just a minor complication of the underlying disease). Sufficient isolation capacity is required for single room accommodation of cases, but in outbreak situations it may be necessary to cohort patients in designated CDI isolation wards. Cleanliness and use of sporicidal disinfectants (currently only

chlorine-releasing agents are recommended) are important in CDI control.

There should also be a major focus on antibiotic stewardship. The guidance recommends that hospitals should establish antimicrobial management teams comprising antimicrobial pharmacists, consultant microbiologist, or infectious diseases specialist, and other clinicians as appropriate. The team should develop restricted guidelines to promote the use of narrow-spectrum agents and avoid, where reasonably possible, clindamycin and the second- and third-generation cephalosporins (especially in elderly patients), while minimizing the use of fluoroquinolones, carbapenems and prolonged courses of aninopenicillins.

7.3 Norovirus

It is difficult to prevent norovirus introduction because of the rapid onset of illness, but it should be stressed to all staff, patients and visitors that people should stay away from health and social care settings if they or their families are suffering from this type of vomiting and diarrhoea. When cases occur in health or residential care settings, prompt action is essential. Patients should be isolated at the first signs of the infection (which is often dramatic onset of the vomiting and diarrhoea). Patients or residents and staff who were in the same area should be quarantined to reduce the risk of wider spread as more cases occur in those exposed. Individual cases often trigger outbreaks and as soon as this is recognized to be happening, the ward or residential unit should be closed to further admissions until the outbreak has ended amongst those (patients/residents and staff) already exposed. Because of the extensive environmental contamination, cleaning and disinfection of the affected areas is an important part of control. Areas contaminated with vomitus and faeces, which is common in these infections, should be promptly cleaned and disinfected with chlorine-based disinfectant. When the outbreak is over, patients or residents need to be moved elsewhere and a thorough deep clean of the affected area (ward, unit, etc.) including disinfection with a chlorine-releasing disinfectant should be done before any patients or residents are readmitted.

8 Professional support for infection prevention and control

Throughout this chapter, it has been stressed that infection prevention and control are the responsibility of all staff (clinical, managerial and support staff). However, it is also an area that requires the leadership and expertise of clinicians specifically trained in infection prevention and control. All healthcare and social care organizations should have access to such expertise and should have an infection prevention and control team and committee to deliver the expert service and support for the staff of all the clinical and social care units. The infection control team in hospitals should generally comprise nursing, medical and pharmaceutical professionals with specific training and expertise in infection prevention and control and in antibiotic prescribing. The nurse should be a trained infection prevention and control practitioner and the medical input is generally provided by a consultant medical microbiologist or infectious diseases physician whose training and experience has included the specific area of HCAI prevention and control. The pharmacist member of the team should have specific experience and expertise in antimicrobial prescribing. The team has an important role in outbreak investigation and management but, most importantly, provides the guidance and support for the delivery and effective infection prevention and control throughout the hospital or health and social care organization.

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Part 4 Contamination and infection control

17 Microbial spoilage, infection risk and contamination control

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1 Introduction

Pharmaceutical products used in the prevention, treatment and diagnosis of disease contain a wide variety of ingredients, often in quite complex physicochemical states. Such products must not only meet current good pharmaceutical manufacturing practice (GPMP) requirements for quality, safety and efficacy, but also must be stable and sufficiently attractive to be acceptable to patients. Products made in the pharmaceutical industry today must meet high microbiological specifications; i.e.

Hugo and Russell's Pharmaceutical Microbiology, Eighth Edition. Edited by Stephen P. Denyer, Norman Hodges, Sean P. Gorman, Brendan F. Gilmore.

if not sterile, they are expected to have no more than a minimal microbial population at the time of product release.

Nevertheless, from time to time a few rogue products with an unacceptable level and type of contamination will occasionally escape the quality assurance net. The consequences of such contamination may be serious and far-reaching on several accounts, particularly if contaminants have had the opportunity to multiply to high levels. First, the product may be spoiled, rendering it unfit for use through chemical and physicochemical deterioration of the formulation. Spoilage and subsequent wastage of individual batches usually result in major financial problems for the manufacturer through direct loss of faulty product. Secondly, the threat of litigation and the unwanted, damaging publicity of recalls may have serious economic implications for the manufacturer. Thirdly, inadvertent use of contaminated products may present a potential health hazard to patients, perhaps resulting in outbreaks of medicament-related infections, and ironically therefore contributing to the spread of disease. Most commonly, heavy contamination of product with opportunist pathogens, such as *Pseudomonas* spp., has resulted in the spread of nosocomial (hospital-acquired) infections in compromised patients; less frequently, low levels of contamination with pathogenic organisms, such as *Salmonella*, have attracted considerable attention, as have products contaminated with toxic microbial metabolites, such as mycotoxins in herbal medicines. The consequences of microbial contamination in pharmaceutical products are discussed in more detail below.

2 Spoilage—chemical and physicochemical deterioration of pharmaceuticals

Microorganisms form a major part of the natural recycling processes for biological matter in the environment. As such, they possess a wide variety of degradative capabilities, which they are able to exert under relatively mild physicochemical conditions. Mixed natural communities are often far more effective cooperative biodeteriogens than the individual species alone, and sequences of attack of complex substrates occur where initial attack by one group of microorganisms renders them susceptible to further deterioration by secondary, and subsequent, microorganisms. Under suitable environmental selection pressures, novel degradative pathways may emerge with the capability to attack newly introduced synthetic chemicals (xenobiotics). However, the rates of degradation of materials released into the environment can vary greatly, from half-lives of hours (phenol) to months ('hard' detergents) to years (halogenated pesticides).

The overall rate of deterioration of a chemical depends on its molecular structure; the physicochemical properties of a particular environment; the type and quantity of microbes present; and whether the metabolites produced can serve as sources of usable energy and precursors for the biosynthesis of cellular components, and hence the creation of more microorganisms.

Pharmaceutical formulations may be considered as specialized microenvironments and their susceptibility to microbial attack can be assessed using conventional ecological criteria. Some naturally occurring ingredients are particularly sensitive to attack, and a number of synthetic components, such as modern surfactants, have been deliberately constructed to be readily degraded after disposal into the environment. Crude vegetable and animal drug extracts often contain a wide assortment of microbial nutrients besides the therapeutic agents. This, combined with frequently conducive and unstable physicochemical characteristics, leaves many formulations with a high potential for microbial attack unless steps are taken to minimize it.

2.1 Pharmaceutical ingredients susceptible to microbial attack

• *Therapeutic agents.* Through spoilage, active drug constituents may be metabolized to less potent or chemically inactive forms. Under laboratory conditions, it has been shown that a variety of microorganisms can metabolize a wide assortment of drugs, resulting in loss of activity. Materials as diverse as alkaloids (morphine, strychnine, atropine), analgesics (aspirin, paracetamol), thalidomide (still used in the treatment of some forms of cancer), barbiturates, steroid esters and mandelic acid can be metabolized and serve as substrates for growth. Indeed, the use of microorganisms to carry out subtle transformations on steroid molecules forms the basis of the commercial production of potent therapeutic steroidal agents (see Chapter 26). In practice, reports of drug destruction in medicines are less frequent. There have, however, been some notable exceptions: the metabolism of atropine in eye drops by contaminating fungi; inactivation of penicillin injections by β-lactamase-producing bacteria (see Chapters 11 and 13); steroid metabolism in damp tablets and creams by fungi; microbial hydrolysis of aspirin in suspension by esterase-producing bacteria; and chloramphenicol deactivation

in an oral medicine by a chloramphenicol acetylase-producing contaminant.

• *Surface-active agents. Anionic surfactants*, such as the alkali metal and amine soaps of fatty acids, are generally stable because of the slightly alkaline pH of the formulations, although readily degraded once diluted into sewage. Alkyl and alkylbenzene sulphonates and sulphate esters are metabolized by ω-oxidation of their terminal methyl groups followed by sequential β-oxidation of the alkyl chains and fission of the aromatic rings. The presence of chain branching involves additional α-oxidative processes. Generally, ease of degradation decreases with increasing chain length and complexity of branching of the alkyl chain.

• *Non-ionic surfactants*, such as alkylpolyoxyethylene alcohol emulsifiers, are readily metabolized by a wide variety of microorganisms. Increasing chain lengths and branching again decrease ease of attack. Alkylphenol polyoxyethylene alcohols are similarly attacked, but are significantly more resistant. Lipolytic cleavage of the fatty acids from sorbitan esters, polysorbates and sucrose esters is often followed by degradation of the cyclic nuclei, producing numerous small molecules readily utilizable for microbial growth. Ampholytic surfactants, based on phosphatides, betaines and alkylamino-substituted amino acids, are an increasingly important group of surfactants and are generally reported to be reasonably biodegradable. The cationic surfactants used as antiseptics and preservatives in pharmaceutical applications are usually only slowly degraded at high dilution in sewage. Pseudomonads have been found growing readily in quaternary ammonium antiseptic solutions, largely at the expense of other ingredients such as buffering materials, although some metabolism of the surfactant has also been observed.

• *Organic polymers.* Many of the thickening and suspending agents used in pharmaceutical formulations are subject to microbial depolymerization by specific classes of extracellular enzymes, yielding nutritive fragments and monomers. Examples of such enzymes, with their substrates in parentheses, are: amylases (starches), pectinases (pectins), cellulases (carboxy-methylcelluloses, but not alkylcelluloses), uronidases (polyuronides such as in tragacanth and acacia), dextranases (dextrans) and proteases (proteins). Agar (a complex polysaccharide) is an example of a relatively inert polymer and, as such, is used as a support for solidifying microbiological culture media. The lower molecular weight polyethylene glycols are readily degraded by sequential oxidation of the hydrocarbon chain, but the larger congeners are rather more recalcitrant. Synthetic packaging polymers such as nylon, polystyrene and polyester are extremely resistant to attack, although cellophane (modified cellulose) is susceptible under some humid conditions.

• *Humectants.* Low molecular weight materials such as glycerol and sorbitol are included in some products to reduce water loss and may be readily metabolized unless present in high concentrations (see section 2.3.3).

• *Fats and oils.* These hydrophobic materials are usually attacked extensively when dispersed in aqueous formulations such as oil-in-water emulsions, aided by the high solubility of oxygen in many oils. Fungal attack has been reported in condensed moisture films on the surface of oils in bulk, or where water droplets have contaminated the bulk oil phase. Lipolytic rupture of triglycerides liberates glycerol and fatty acids, the latter often then undergoing β-oxidation of the alkyl chains and the production of odiferous ketones. Although the microbial metabolism of pharmaceutical hydrocarbon oils is rarely reported, this is a problem in engineering and fuel technology when water droplets have accumulated in oil storage tanks and subsequent fungal colonization has catalysed serious corrosion.

• *Sweetening, flavouring and colouring agents.* Many of the sugars and other sweetening agents used in pharmacy are ready substrates for microbial growth. However, some are used in very high concentrations to reduce water activity in aqueous products and inhibit microbial attack (see section 2.3.3). At one time, a variety of colouring agents (such as tartrazine and amaranth) and flavouring agents (such as peppermint water) were kept as stock solutions for extemporaneous dispensing purposes, but they frequently supported the growth of *Pseudomonas* spp., including *Ps. aeruginosa*. Such stock solutions should now be preserved, or freshly made as required by dilution of alcoholic solutions which are much less susceptible to microbial attack.

• *Preservatives and disinfectants.* Many preservatives and disinfectants can be metabolized by a wide variety of Gram-negative bacteria, although most commonly at concentrations below their effective 'use' levels. Growth of pseudomonads in stock solutions of quaternary ammonium antiseptics and chlorhexidine has resulted in infection of patients. *Pseudomonas* spp. have metabolized 4-hydroxybenzoate (parabens) ester preservatives contained in eye-drops and caused serious eye infections, and have also metabolized the preservatives in oral suspensions and solutions. In selecting suitable preservatives for formulation, a detailed knowledge of

the properties of such agents, their susceptibility to contamination and limitations clearly provides invaluable information.

2.2 Observable effects of microbial attack on pharmaceutical products

Microbial contaminants usually need to attack formulation ingredients and create substrates necessary for biosynthesis and energy production before they can replicate to levels where obvious spoilage becomes apparent. Thus, for example, 10^6 microbes will have an overall degradative effect around 10^6 times faster than one cell. However, growth and attack may well be localized in surface moisture films or very unevenly distributed within the bulk of viscous formulations such as creams. Early indications of spoilage are often organoleptic, with the release of unpleasant smelling and tasting metabolites such as 'sour' fatty acids, 'fishy' amines, 'bad eggs', bitter, 'earthy' or sickly tastes and smells. Products may become unappealingly discoloured by microbial pigments of various shades. Thickening and suspending agents such as tragacanth, acacia or carboxymethylcellulose can be depolymerized, resulting in loss of viscosity and sedimentation of suspended ingredients. Alternatively, microbial polymerization of sugars and surfactant molecules can produce slimy, viscous masses in syrups, shampoos and creams, and fungal growth in creams has produced 'gritty' textures. Changes in product pH can occur depending on whether acidic or basic metabolites are released, and become so modified as to permit secondary attack by microbes previously inhibited by the initial product pH. Gaseous metabolites may be seen as trapped bubbles within viscous formulations.

When a complex formulation such as an oil-in-water emulsion is attacked, a gross and progressive spoilage sequence may be observed. Metabolism of surfactants will reduce stability and accelerate 'creaming' of the oil globules. Lipolytic release of fatty acids from oils will lower pH and encourage coalescence of oil globules and 'cracking' of the emulsion. Fatty acids and their ketonic oxidation products will provide a sour taste and unpleasant smell, while bubbles of gaseous metabolites may be visible, trapped in the product, and pigments may discolour it (see Figure 17.1).

2.3 Factors affecting microbial spoilage of pharmaceutical products

By understanding the influence of environmental parameters on microorganisms, it may be possible to manipulate formulations to create conditions which are as unfavourable as possible for growth and spoilage, within the limitations of patient acceptability and therapeutic efficacy. Furthermore, the overall characteristics of a particular formulation will indicate its susceptibility to attack by various classes of microorganisms.

2.3.1 Types and size of contaminant inoculum

Successful formulation of products against microbial attack involves an element of prediction. An understanding of where and how the product is to be used and the challenges it must face during its life will enable the formulator to build in as much protection as possible against microbial attack. When failures inevitably occur from time to time, knowledge of the microbial ecology and careful identification of contaminants can be most useful in tracking down the defective steps in the design or production process.

Low levels of contaminants may not cause appreciable spoilage, particularly if they are unable to replicate in a

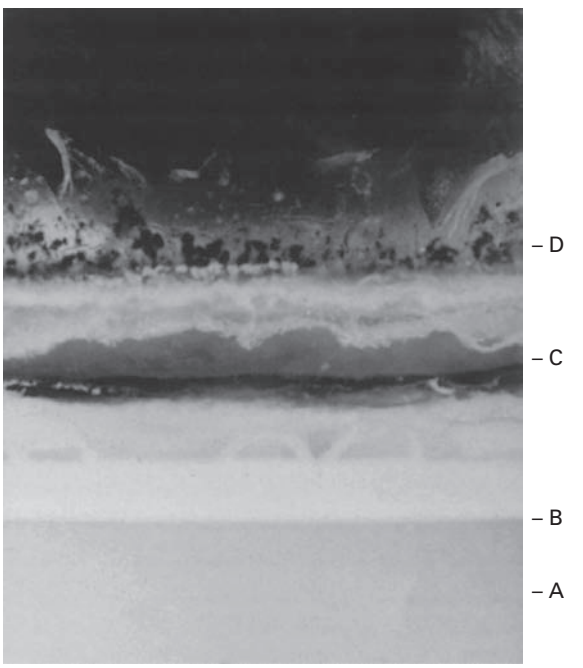

Figure 17.1 Section (×1.5) through an inadequately preserved olive oil, oil-in-water, emulsion in an advanced state of microbial spoilage showing: A, discoloured, oil-depleted, aqueous phase; B, oil globule-rich creamed layer; C, coalesced oil layer from 'cracked' emulsion; D, fungal mycelial growth on surface. Also present are a foul taste and evil smell.

product; however, an unexpected surge in the contaminant bioburden may present an unacceptable challenge to the designed formulation. This could arise if, for example, raw materials were unusually contaminated; there was a lapse in the plant-cleaning protocol; a biofilm detached itself from within supplying pipework; or the product had been grossly misused during administration. Inoculum size alone is not always a reliable indicator of likely spoilage potential. Low levels of aggressive pseudomonads in a weakly preserved solution may pose a greater risk than tablets containing fairly high numbers of fungal and bacterial spores.

When an aggressive microorganism contaminates a medicine, there may be an appreciable lag period before significant spoilage begins, the duration of which decreases disproportionately with increasing contaminant loading. As there is usually a considerable delay between manufacture and administration of factory-made medicines, growth and attack could ensue during this period unless additional steps are taken to prevent it. On the other hand, for extemporaneously dispensed formulations some control can be provided by specifying short shelf-lives, for example 2 weeks.

The isolation of a particular microorganism from a markedly spoiled product does not necessarily mean that it was the initiator of the attack. It could be a secondary opportunist contaminant which had overgrown the primary spoilage organism once the physicochemical properties had been favourably modified by the primary spoiler.

2.3.2 Nutritional factors

The simple nutritional requirements and metabolic adaptability of many common spoilage microorganisms enable them to utilize many formulation components as substrates for biosynthesis and growth. The use of crude vegetable or animal products in a formulation provides an additionally nutritious environment. Even demineralized water prepared by good ion-exchange methods will normally contain sufficient nutrients to allow significant growth of many waterborne Gram-negative bacteria such as *Pseudomonas* spp. When such contaminants fail to survive, it is unlikely to be the result of nutrient limitation in the product but due to other, non-supportive, physicochemical or toxic properties.

Acute pathogens require specific growth factors normally associated with the tissues they infect but which are often absent in pharmaceutical formulations. They are thus unlikely to multiply in them, although they may remain viable and infective for an appreciable time in some dry products where the conditions are suitably protective.

2.3.3 Moisture content: water activity (A_w)

Microorganisms require readily accessible water in appreciable quantities for growth to occur. By measuring a product's water activity, A_w, it is possible to obtain an estimate of the proportion of uncomplexed water that is available in the formulation to support microbial growth, using the formula A_w = vapour pressure of formulation/vapour pressure of water under similar conditions.

The greater the solute concentration, the lower is the water activity. With the exception of halophilic bacteria, most microorganisms grow best in dilute solutions (high A_w) and, as solute concentration rises (lowering A_w), growth rates decline until a minimal growth-inhibitory A_w, is reached. Limiting A_w values are of the order of 0.95 for Gram-negative rods; 0.9 for staphylococci, micrococci and lactobacilli; and 0.88 for most yeasts. Syrup-fermenting osmotolerant yeasts have spoiled products with A_w levels as low as 0.73, while some filamentous fungi such as *Aspergillus glaucus* can grow at 0.61.

The A_w of aqueous formulations can be lowered to increase resistance to microbial attack by the addition of high concentrations of sugars or polyethylene glycols. However, even Syrup BP (67% sucrose; $A_w = 0.86$) has occasionally failed to inhibit osmotolerant yeasts and additional preservation may be necessary. With a continuing trend towards the elimination of sucrose from medicines, alternative solutes which are not thought to encourage dental caries such as sorbitol and fructose have been investigated. A_w can also be reduced by drying, although the dry, often hygroscopic medicines (tablets, capsules, powders, vitreous 'glasses') will require suitable packaging to prevent resorption of water and consequent microbial growth (Figure 17.2).

Tablet film coatings are now available which greatly reduce water vapour uptake during storage while allowing ready dissolution in bulk water. These might contribute to increased microbial stability during storage in particularly humid climates, although suitable foil strip packing may be more effective, albeit more expensive.

Condensed water films can accumulate on the surface of otherwise 'dry' products such as tablets or bulk oils following storage in damp atmospheres with fluctuating temperatures, resulting in sufficiently high localized A_w to initiate fungal growth. Condensation similarly formed on the surface of viscous products such as syrups and creams, or exuded by syneresis from hydrogels, may well permit surface yeast and fungal spoilage.

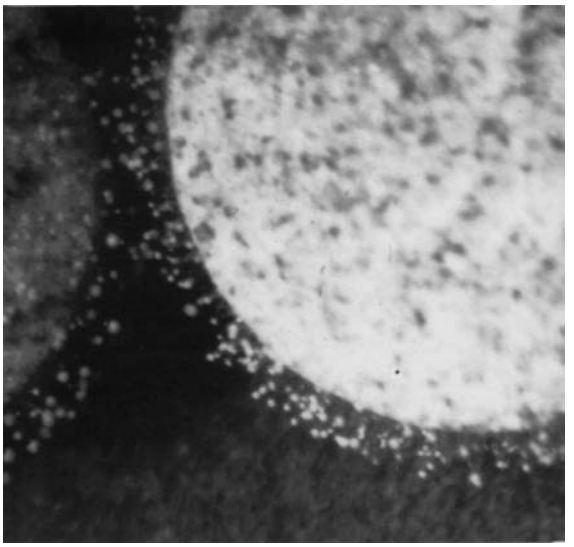

Figure 17.2 Fungal growth on a tablet which has become damp (raised A_w) during storage under humid conditions. Note the sparseness of mycelium, and conidiophores. The contaminant is thought to be a *Penicillium* sp.

2.3.4 Redox potential

The ability of microbes to grow in an environment is influenced by their oxidation–reduction balance (redox potential), as they will require compatible terminal electron acceptors to permit their respiratory pathways to function. The redox potential even in fairly viscous emulsions may be quite high because of the appreciable solubility of oxygen in most fats and oils.

2.3.5 Storage temperature

Spoilage of pharmaceuticals could occur potentially over the range of about −20 °C to 60 °C, although it is much less likely at the extremes. The particular storage temperature may selectively determine the types of microorganisms involved in spoilage. A deep freeze at −20 °C or lower is used for long-term storage of some pharmaceutical raw materials and short-term storage of dispensed total parenteral nutrition (TPN) feeds prepared in hospitals. Reconstituted syrups and multidose eye drop packs are sometimes dispensed with the instruction to 'store in a cool place' such as a domestic fridge (2–8 °C), partly to reduce the risk of growth of contaminants inadvertently introduced during use. Conversely, Water for Injections (EP) should be held at 80 °C or above after distillation and before packing and sterilization to prevent possible regrowth of Gram-negative bacteria and the release of endotoxins.

2.3.6 pH

Extremes of pH prevent microbial attack. Around neutrality bacterial spoilage is more likely, with reports of pseudomonads and related Gram-negative bacteria growing in antacid mixtures, flavoured mouthwashes and distilled or demineralized water. Above pH 8 (e.g. with soap-based emulsions) spoilage is rare. In products with low pH levels (e.g. fruit-juice-flavoured syrups with a pH 3–4), mould or yeast attack is more likely. Yeasts can metabolize organic acids and raise the pH to levels where secondary bacterial growth can occur. Although the use of low pH adjustment to preserve foodstuffs is well established (e.g. pickling, coleslaw, yoghurt), it is not practicable to make deliberate use of this for medicines.

2.3.7 Packaging design

Packaging can have a major influence on microbial stability of some formulations in controlling the entry of contaminants during both storage and use. Considerable thought has gone into the design of containers to prevent the ingress of contaminants into medicines for parenteral administration, because of the high risks of infection by this route. Self-sealing rubber wads must be used to prevent microbial entry into multidose injection containers (Chapter 22) following withdrawals with a hypodermic needle. Wide-mouthed cream jars have now been replaced by narrow nozzles and flexible screw-capped tubes, thereby removing the likelihood of operator-introduced contamination during use of the product. Similarly, hand creams, previously supplied in glass jars, are now packed in closed, disposable dispensers. Where medicines rely on their low A_w to prevent spoilage, packaging such as strip foils must be of water-vapour-proof materials with fully efficient seals. Cardboard outer packaging and labels themselves can become substrates for microbial attack under humid conditions, and preservatives are often included to reduce the risk of damage.

2.3.8 Protection of microorganisms within pharmaceutical products

The survival of microorganisms in particular environments is sometimes influenced by the presence of relatively inert materials. Thus, microbes can be more resistant to heat or desiccation in the presence of polymers such as starch, acacia or gelatin. Adsorption on to naturally occurring particulate material may aid establishment and survival in some environments. There is a

belief, but limited hard evidence, that the presence of suspended particles such as kaolin, magnesium trisilicate or aluminium hydroxide gel may influence contaminant longevity in those products containing them, and that the presence of some surfactants, suspending agents and proteins can increase the resistance of microorganisms to preservatives, over and above their direct inactivating effect on the preservative itself.

3 Hazard to health

Nowadays, it is well recognized that the inadvertent use of a contaminated pharmaceutical product may also present a potential health hazard to the patient. Although isolated outbreaks of medicament-related infections had been reported since the early part of the 20th century, it was only in the 1960s and 1970s that the significance of this contamination to the patient was more fully understood.

Inevitably, the infrequent isolation of true pathogens, such as *Salmonella* spp. and the reporting of associated infections following the use of products contaminated with these organisms (tablets with pancreatin and thyroid extract), attracted considerable attention. More often, the isolation of common saprophytic and non-fastidious opportunist contaminants with limited pathogenicity to healthy individuals has presented a significant challenge to compromised patients.

Gram-negative contaminants, particularly *Pseudomonas* spp., which have simple nutritional requirements and can multiply to significant levels in aqueous products, have been held responsible for numerous outbreaks of infection. For example, while the intact cornea is quite resistant to infection, it offers little resistance to pseudomonads and related bacteria when scratched, or damaged by irritant chemicals; loss of sight has frequently occurred following the use of poorly designed ophthalmic solutions which had become contaminated by *Ps. aeruginosa* and even supported its active growth. Pseudomonads contaminating 'antiseptic' solutions have infected the skin of badly burnt patients, resulting in the failure of skin grafts and subsequent death from Gram-negative septicaemia. Infections of eczematous skin and respiratory infections in neonates have been traced to ointments and creams contaminated with Gram-negative bacteria. Oral mixtures and antacid suspensions can support the growth of Gram-negative bacteria and serious consequences have resulted following their inadvertent administration to patients who were immunocompromised as a result of antineoplastic chemotherapy. Growth of Gram-negative bacteria in bladder washout solutions has been held responsible for painful infections. In more recent times, *Pseudomonas* contamination of TPN fluids during their aseptic compounding in the hospital pharmacy caused the death of several children in the same hospital.

Fatal viral infections resulting from the use of contaminated human tissue or fluids as components of medicines are well recorded. Examples of this include HIV infection of haemophiliacs by contaminated and inadequately treated factor VIII products made from pooled human blood, and Creutzfeldt–Jakob disease (CJD) from injections of human growth hormone derived from human pituitary glands, some of which were infected.

Pharmaceutical products of widely differing forms are known to be susceptible to contamination with a variety of microorganisms, ranging from true pathogens to a motley collection of opportunist pathogens (see Table 17.1). Disinfectants, antiseptics, powders, tablets and other products providing an inhospitable environment to invading contaminants are known to be at risk, as well as products with more nutritious components, such as creams and lotions with carbohydrates, amino acids, vitamins and often appreciable quantities of water.

The outcome of using a contaminated product may vary from patient to patient, depending on the type and degree of contamination and how the product is to be used. Undoubtedly, the most serious effects have been seen with contaminated injected products where generalized bacteraemic shock and in some cases death of patients have been reported. More likely, a wound or sore in broken skin may become locally infected or colonized by the contaminant; this may in turn result in extended hospital bed occupancy, with ensuing economic consequences. It must be stressed, however, that the majority of cases of medicament-related infections are probably not recognized or reported as such. Recognition of these infections presents its own problems. It is a fortunate hospital physician who can, at an early stage, recognize contamination shown as a cluster of infections of rapid onset, such as that following the use of a contaminated intravenous fluid in a hospital ward. The chances of a general practitioner recognizing a medicament-related infection of insidious onset, perhaps spread over several months, in a diverse group of patients in the community, are much more remote. Once recognized, of course, there is a moral obligation to withdraw the offending product; subsequent investigations of the incident therefore become retrospective.

Table 17.1 Contaminants found in pharmaceutical products

Year	Product	Contaminant
1907	Plague vaccine	*Clostridium tetani*
1943	Fluorescein eye drops	*Pseudomonas aeruginosa*
1946	Talcum powder	*Clostridium tetani*
1948	Serum vaccine	*Staphylococcus aureus*
1955	Chloroxylenol disinfectant	*Pseudomonas aeruginosa*
1966	Thyroid tablets	*Salmonella muenchen*
1966	Antibiotic eye ointment	*Pseudomonas aeruginosa*
1966	Saline solution	*Serratia marcescens*
1967	Carmine powder	*Salmonella cubana*
1967	Hand cream	*Klebsiella pneumoniae*
1969	Peppermint water	*Pseudomonas aeruginosa*
1970	Chlorhexidine-cetrimide antiseptic solution	*Pseudomonas cepacia*
1972	Intravenous fluids	*Pseudomonas, Erwinia* and *Enterobacter* spp.
1972	Pancreatin powder	*Salmonella agona*
1977	Contact lens solution	*Serratia* and *Enterobacter* spp.
1981	Surgical dressings	*Clostridium* spp.
1982	Iodophor solution	*Pseudomonas aeruginosa*
1983	Aqueous soap	*Pseudomonas stutzeri*
1984	Thymol mouthwash	*Pseudomonas aeruginosa*
1986	Antiseptic mouthwash	Coliforms
1994	Total parenteral nutrition solution	*Enterobacter cloacae*
1997	Miscellaneous herbal products	*Enterobacter* spp., *Enterococcus faecalis, Clostridium perfringens, Klebsiella pneumonia, Escherichia, Pseudomonas*
2004	Influenza vaccine	Gram-negative bacteria, including *Serratia*

3.1 Microbial toxins

Gram-negative bacteria contain lipopolysaccharides (endotoxins) in their outer cell membranes (Chapter 22); these can remain in an active condition in products even after cell death and some can survive moist heat sterilization. Although inactive by the oral route, endotoxins can induce a number of physiological effects if they enter the bloodstream via contaminated infusion fluids, even in nanogram quantities, or via diffusion across membranes from contaminated haemodialysis solutions. Such effects may include fever, activation of the cytokine system, endothelial cell damage, all leading to septic and often fatal febrile shock.

The acute bacterial toxins associated with food poisoning episodes are not commonly reported in pharmaceutical products, although aflatoxin-producing aspergilli have been detected in some vegetable and herbal ingredients. However, many of the metabolites of microbial

deterioration have quite unpleasant tastes and smell even at low levels, and would deter most patients from using such a medicine.

4 Sources and control of contamination

4.1 In manufacture

Regardless of whether manufacture takes place in industry or on a smaller scale in the hospital pharmacy, the microbiological quality of the finished product will be determined by the formulation components used, the environment in which they are manufactured and the manufacturing process itself. As discussed in Chapter 23, quality must be built into the product at all stages of the process and not simply inspected at the end of manufacture:

- Raw materials, particularly water and those of natural origin, must be of a high microbiological standard.
- All processing equipment should be subject to planned preventive maintenance and should be properly cleaned after use to prevent cross-contamination between batches.
- Cleaning equipment should be appropriate for the task in hand and should be thoroughly cleaned and properly maintained.
- Manufacture should take place in suitable premises, supplied with filtered air, for which the environmental requirements vary according to the type of product being made.
- Staff involved in manufacture should not only have good health but also a sound knowledge of the importance of personal and production hygiene.
- The end-product requires suitable packaging which will protect it from contamination during its shelf-life and is itself free from contamination.

4.1.1 Hospital manufacture

Manufacture in hospital premises raises certain additional problems with regard to contamination control.

4.1.1.1 Water

Mains water in hospitals is frequently stored in large roof tanks, some of which may be relatively inaccessible and poorly maintained. Water for pharmaceutical manufacture requires some further treatment, usually by distillation, reverse osmosis or deionization or a combination of these, depending on the intended use of water. Such processes need careful monitoring, as does the microbiological quality of the water after treatment. Storage of water requires particular care, as some Gram-negative opportunist pathogens can survive on traces of organic matter present in treated water and will readily multiply to high numbers at room temperature. Water should therefore be stored at a temperature in excess of 80 °C and circulated in the distribution system at a flow rate of 1–2 m/s to prevent the build-up of bacterial biofilms in the piping.

4.1.1.2 Environment

The microbial flora of the hospital pharmacy environment is a reflection of the general hospital environment and the activities undertaken there. Free-living opportunist pathogens, such as *Ps. aeruginosa*, can normally be found in wet sites, such as drains, sinks and taps. Cleaning equipment, such as mops, buckets, cloths and scrubbing machines, may be responsible for distributing these organisms around the pharmacy; if stored wet they provide a convenient niche for microbial growth, resulting in heavy contamination of equipment. Contamination levels in the production environment may, however, be minimized by observing good manufacturing practices (GMP), by installing heating traps in sink U-bends, thus destroying one of the main reservoirs of contaminants, and by proper maintenance and storage of equipment, including cleaning equipment. Additionally, cleaning of production units by contractors should be carried out to a pharmaceutical specification.

4.1.1.3 Packaging

Sacking, cardboard, card liners, corks and paper are unsuitable for packaging pharmaceuticals, as they are heavily contaminated, for example with bacterial or fungal spores. These have now been replaced by non-biodegradable plastic materials. In the past, packaging in hospitals was frequently reused for economic reasons. Large numbers of containers may be returned to the pharmacy, bringing with them microbial contaminants introduced during use in the wards. Particular problems have been encountered with disinfectant solutions where residues of old stock have been 'topped up' with fresh supplies, resulting in the issue of contaminated solutions to wards. Reusable containers must therefore be thoroughly washed and dried, and never refilled directly.

Another common practice in hospitals is the repackaging of products purchased in bulk into smaller containers. Increased handling of the product inevitably increases the risk of contamination, as shown by one survey when hospital-repacked items were found to be contaminated twice as often as those in the original pack (Public Health Laboratory Service Report, 1971).

4.2 In use

Pharmaceutical manufacturers may justly argue that their responsibility ends with the supply of a well-preserved product of high microbiological standard in a suitable pack and that the subsequent use, or indeed abuse, of the product is of little concern to them. Although much less is known about how products become contaminated during use, their continued use in a contaminated state is clearly undesirable, particularly in hospitals where it could result in the spread of cross-infection. All multidose products are vulnerable to contamination during use. Regardless of whether products are used in hospital or in the community environment, the sources of contamination are the same, but opportunities for observing it are greater in the former. Although the risk of contamination during product use has been much reduced in recent years, primarily through improvements in packaging and changes in nursing practices, it is nevertheless salutary to reflect upon past reported case histories.

4.2.1 Human sources

During normal usage, patients may contaminate their medicine with their own microbial flora; subsequent use of such products may or may not result in self-infection (Figure 17.3).

Topical products are considered to be most at risk, as the product will probably be applied by hand, thus introducing contaminants from the resident skin flora of staphylococci, *Micrococcus* spp. and diphtheroids but also perhaps transient contaminants, such as *Pseudomonas* or *coliforms*, which would normally be removed with effective hand-washing. Opportunities for contamination may be reduced by using disposable applicators for topical products or by giving oral products by disposable spoon.

In hospitals, multidose products, once contaminated, may serve as a vehicle of cross-contamination or cross-infection between patients. Zinc-based products packed in large stockpots and used in the treatment and prevention of bedsores in long-stay and geriatric patients were reportedly contaminated during use with *Ps. aeruginosa* and *Staphylococcus aureus*. If unpreserved, these products permit multiplication of contaminants, especially if water is present either as part of the formulation, for example in oil/water (o/w) emulsions, or as a film in w/o emulsions which have undergone local cracking, or as a condensed film from atmospheric water. Appreciable numbers of contaminants may then be transferred to other patients when the product is reused. Clearly the economics and convenience of using stockpots need to be balanced against the risk of spreading cross-infection between patients and the inevitable increase in length of the patients' stay in hospital. The use of stockpots in hospitals has noticeably declined over the past two decades or so.

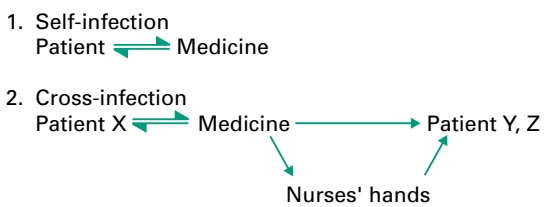

Figure 17.3 Mechanisms of contamination during use of medicinal products.

A further potential source of contamination in hospitals is the nursing staff responsible for medicament administration. During the course of their work, nurses' hands become contaminated with opportunist pathogens which are not part of the normal skin flora but which are easily removed by thorough hand-washing and drying. In busy wards, hand-washing between attending to patients may be overlooked and contaminants may subsequently be transferred to medicaments during administration. Hand lotions and creams used to prevent chapping of nurses' hands may similarly become contaminated, especially when packaged in multidose containers and left at the side of the hand-basin, frequently without lids. Hand lotions and creams should be well preserved and, ideally, packaged in disposable dispensers. Other effective control methods include the supply of products in individual patient's packs and the use of non-touch techniques for medicament administration. The importance of thorough hand-washing in the control of hospital cross-infection cannot be overemphasized. In recent years hospitals have successfully raised the level of awareness on this topic among staff and the general public through widespread publicity and the provision of easily accessible hand disinfection stations on the wards.

4.2.2 Environmental sources

Small numbers of airborne contaminants may settle in products left open to the atmosphere. Some of these will die during storage, with the rest probably remaining at a static level of about 10^2–10^3 colony forming units (CFU) per gram or per millilitre. Larger numbers of waterborne contaminants may be accidentally introduced into topical products by wet hands or by a 'splash-back mechanism' if left at the side of a basin. Such contaminants generally

have simple nutritional requirements and, following multiplication, levels of contamination may often exceed 10^6 CFU/g. In the past this problem has been encountered particularly when the product was stored in warm hospital wards or in hot steamy bathroom cupboards at home. Products used in hospitals as soap substitutes for bathing patients are particularly at risk and soon not only become contaminated with opportunist pathogens such as *Pseudomonas* spp., but also provide conditions conducive to their multiplication. The problem is compounded by stocks kept in multidose pots for use by several patients in the same ward over an extended period of time.

The indigenous microbial population is quite different in the home and in hospitals. Pathogenic organisms are found much more frequently in the latter and consequently are isolated more often from medicines used in hospital. Usually, there are fewer opportunities for contamination in the home, as patients are generally issued with individual supplies in small quantities.

4.2.3 Equipment sources

Patients and nursing staff may use a range of applicators (pads, sponges, brushes and spatulas) during medicament administration, particularly for topical products. If reused, these easily become contaminated and may be responsible for perpetuating contamination between fresh stocks of product, as has indeed been shown in studies of cosmetic products. Disposable applicators or swabs should therefore always be used.

In hospitals today a wide variety of complex equipment is used in the course of patient treatment. Humidifiers, incubators, ventilators, resuscitators and other apparatus require proper maintenance and decontamination after use. Chemical disinfectants used for this purpose have in the past, through misuse, become contaminated with opportunist pathogens, such as *Ps. aeruginosa*, and ironically have contributed to, rather than reduced, the spread of cross-infection in hospital patients. Disinfectants should only be used for their intended purpose and directions for use must be followed at all times.

5 The extent of microbial contamination

Most reports of medicament-borne contamination in the literature tend to be anecdotal in nature, referring to a specific product and isolated incident. Little information is available on the overall risk of products becoming contaminated and causing patient infections when subsequently used. Such information is considered invaluable not only because it may indicate the effectiveness of existing practices and standards, but also because the value of potential improvements in patient quality can be balanced against the inevitable cost of such processes.

5.1 In manufacture

Investigations carried out by the Swedish National Board of Health in 1965 revealed some startling findings on the overall microbiological quality of non-sterile products immediately after manufacture. A wide range of products was routinely found to be contaminated with *Bacillus subtilis*, *Staph. albus*, yeasts and moulds, and in addition large numbers of coliforms were found in a variety of tablets. Furthermore, two nationwide outbreaks of infection in Sweden were subsequently traced to the inadvertent use of contaminated products. Two hundred patients were involved in an outbreak of salmonellosis, caused by thyroid tablets contaminated with *Salmonella bareilly* and *Sal. muenchen* (now known as *Salmonella enterica* subsp. *enterica* serovar Bareilly and *Sal. enterica* serovar Muenchen respectively); and eight patients had severe eye infections following the use of a hydrocortisone eye ointment contaminated with *Ps. aeruginosa*. The results of this investigation had a profound effect on the manufacture of all medicines; not only were they then used as a yardstick to compare the microbiological quality of non-sterile products made in other countries, but also as a baseline upon which international standards could be founded.

Under the UK Medicines Act 1968, pharmaceutical products made in industry were expected to conform to microbiological and chemical quality specifications. The majority of products have since been shown to conform to a high standard, although spot checks have occasionally revealed medicines of unacceptable quality and so necessitated product recall. By contrast, pharmaceutical products made in hospitals were much less rigorously controlled, as shown by several surveys in the 1970s in which significant numbers of preparations were found to be contaminated with *Ps. aeruginosa*. In 1974, however, hospital manufacture also came under the terms of the Medicines Act and, as a consequence, considerable improvements were subsequently seen not only in the conditions and standard of manufacture, but also in the chemical and microbiological quality of finished products. Hospital manufacturing operations were later rationalized. Economic constraints caused a critical evaluation of the true cost of these activities. Competitive purchasing from industry in many cases produced

cheaper alternatives, and small-scale manufacturing was largely discouraged. Where licensed products were available, NHS policy dictated that these were to be purchased from a commercial source and not made locally.

Removal of Crown immunity from the NHS in 1991 meant that manufacturing operations in hospitals were then subject to the full licensing provisions of the Medicines Act 1968, i.e. hospital pharmacies intending to manufacture were required to obtain a manufacturing licence and to comply fully with the EC *Guide to Good Pharmaceutical Manufacturing Practice* (Anon, 1992, revised in 1997, 2002 and 2007). Among other requirements, this included the provision of appropriate environmental manufacturing conditions and associated environmental monitoring. Subsequently, the Medicines Control Agency (MCA) issued guidance in 1992 on certain manufacturing exemptions, by virtue of the product batch size or frequency of manufacture. The need for extemporaneous dispensing of 'one-off' special formulae continued in hospital pharmacies, although this work was largely transferred from the dispensing bench to dedicated preparative facilities with appropriate environmental control. Today hospital manufacturing is concentrated on the supply of bespoke products from a regional centre or small-scale specialist manufacture of those items currently unobtainable from industry. Repacking of commercial products into more convenient pack sizes is, however, still common practice.

5.2 In use

Higher rates of contamination are invariably seen in products after opening and use and, among these, medicines used in hospitals are more likely to be contaminated than those used in the general community. The Public Health Laboratory Service Report of 1971 expressed concern at the overall incidence of contamination in non-sterile products used on hospital wards (327 of 1220 samples) and the proportion of samples found to be heavily contaminated (18% $>10^4$ CFU/g or CFU/ml). Notably, the presence of *Ps. aeruginosa* in 2.7% of samples (mainly oral alkaline mixtures) was considered to be highly undesirable.

By contrast, medicines used in the home are not only less often contaminated but also contain lower levels of contaminants and fewer pathogenic organisms. Generally, there are fewer opportunities for contamination here because individual patients use smaller quantities. Medicines in the home may, however, be hoarded and used for extended periods of time. Additionally, storage conditions may be unsuitable and expiry dates ignored; thus problems other than those of microbial contamination may be seen in the home.

6 Factors determining the outcome of a medicament-borne infection

Although impossible to quantify, the use of contaminated medicines has undoubtedly contributed to the spread of cross-infection in hospitals; undeniably, such nosocomial (hospital-acquired) infections have also extended the length of stay in hospital with concomitant costs. A patient's response to the microbial challenge of a contaminated medicine may be diverse and unpredictable, perhaps with serious consequences. Clinical reactions may not be evident in one patient, yet in another may be indisputable, illustrating one problem in the recognition of medicament-borne infections. Clinical reactions may range from inconvenient local infections of wounds or broken skin, caused possibly from contact with a contaminated cream, to gastrointestinal infections from the ingestion of contaminated oral products, to serious widespread infections such as a bacteraemia or septicaemia, possibly resulting in death, as caused by the administration of contaminated infusion fluids. Undoubtedly, the most serious outbreaks of infection have been seen in the past where contaminated products have been injected directly into the bloodstream of patients whose immunity is already compromised by their underlying disease or therapy.

The outcome of any episode is determined by a combination of several factors, among which the type and degree of microbial contamination, the route of administration and the patient's resistance are of particular importance.

6.1 Type and degree of microbial contamination

Microorganisms that contaminate medicines and cause disease in patients may be classified as true pathogens or opportunist pathogens. Pathogenic organisms like *Clostridium tetani* and *Salmonella* spp. rarely occur in products, but when present cause serious problems. Wound infections and several cases of neonatal death have resulted from use of talcum powder containing *Cl. tetani*. Outbreaks of salmonellosis have followed the inadvertent ingestion of contaminated thyroid and pancreatic powders. On the other hand, opportunist pathogens like *Ps. aeruginosa*, *Klebsiella*, *Serratia* and other free-living organisms are more frequently isolated from

medicinal products and, as their name suggests, may be pathogenic if given the opportunity. The main concern with these organisms is that their simple nutritional requirements enable them to survive in a wide range of pharmaceuticals, and thus they tend to be present in high numbers, perhaps in excess of 10^6–10^7 CFU/g or CFU/ml. The product itself, however, may show no visible sign of contamination. Opportunist pathogens can survive in disinfectants and antiseptic solutions that are normally used in the control of hospital cross-infection, but which, when contaminated, may even perpetuate the spread of infection. Compromised hospital patients, i.e. elderly, burned, traumatized or immunosuppressed patients, are considered to be particularly at risk from infection with these organisms, whereas healthy patients in the general community have given little cause for concern.

The critical dose of microorganisms that will initiate an infection is largely unknown and varies not only between species but also within a species. Animal and human volunteer studies have indicated that the infecting dose may be reduced significantly in the presence of trauma or foreign bodies or if accompanied by a drug having a local vasoconstrictive action.

6.2 Route of administration

As stated previously, contaminated products injected directly into the bloodstream or instilled into the eye cause the most serious problems. Intrathecal and epidural injections are potentially hazardous procedures. In practice, epidural injections are frequently given through a bacterial filter. Injectable and ophthalmic solutions are often simple solutions and provide Gram-negative opportunist pathogens with sufficient nutrients to multiply during storage; if contaminated, a bioburden of 10^6 CFU as well as the production of endotoxins should be expected. TPN fluids, formulated for individual patients' nutritional requirements, can also provide more than adequate nutritional support for invading contaminants. *Ps. aeruginosa*, the notorious contaminant of eye drops, has caused serious ophthalmic infections, including the loss of sight in some cases. The problem is compounded when the eye is damaged through the improper use of contact lenses or scratched by fingernails or cosmetic applicators.

The fate of contaminants ingested orally in medicines may be determined by several factors, as is seen with contaminated food. The acidity of the stomach may provide a successful barrier, depending on whether the medicine is taken on an empty or full stomach and also on the gastric emptying time. Contaminants in topical products may cause little harm when deposited on intact skin. Not only does the skin itself provide an excellent mechanical barrier, but few contaminants normally survive in competition with its resident microbial flora. Skin damaged during surgery or trauma or in patients with burns or pressure sores may, however, be rapidly colonized and subsequently infected by opportunist pathogens. Patients treated with topical steroids are also prone to local infections, particularly if contaminated steroid drugs are inadvertently used.

6.3 Resistance of the patient

A patient's resistance is crucial in determining the outcome of a medicament-borne infection. Hospital patients are more exposed and susceptible to infection than those treated in the general community. Neonates, elderly people, diabetics and patients traumatized by surgery or accident may have impaired defence mechanisms. People suffering from leukaemia and those treated with immunosuppressants are most vulnerable to infection; there is an undeniable case for providing all medicines in a sterile form for these patients.

7 Preservation of medicines using antimicrobial agents: basic principles

7.1 Introduction

An antimicrobial 'preservative' may be included in a formulation to minimize the risk of spoilage and preferably to kill low levels of contaminants introduced during storage or repeated use of a multidose container. However, where there is a low risk of contamination, as with tablets, capsules and dry powders, the inclusion of a preservative may be unnecessary. Preservatives should never be added to mask poor manufacturing processes.

The properties of an ideal preservative are well recognized: a broad spectrum of activity and a rapid rate of kill; selectivity in reacting with the contaminants and not the formulation ingredients; non-irritant and non-toxic to the patient; and stable and effective throughout the life of the product.

Unfortunately, the most active antimicrobial agents are often non-selective in action, interacting significantly with formulation ingredients as well as with patients and microorganisms. Having excluded the more toxic, irritant and reactive agents, those remaining generally have only modest antimicrobial efficacy, and no preservatives are now considered sufficiently non-toxic for use in

highly sensitive areas, e.g. for injection into central nervous system tissues or for use within the eye. A number of microbiologically effective preservatives used in cosmetics have caused a significant number of cases of contact dermatitis, and are thus precluded from use in pharmaceutical creams. Although a rapid rate of kill may be preferable, this may only be possible for relatively simple aqueous solutions such as eye drops or injections. For physicochemically complex systems such as emulsions and creams, inhibition of growth and a slow rate of killing may be all that can be realistically achieved.

In order to maximize preservative efficacy, it is essential to have an appreciation of those parameters that influence antimicrobial activity.

7.2 Effect of preservative concentration, temperature and size of inoculum

Changes in the efficacy of preservatives vary exponentially with changes in concentration. The effect of changes in concentration (concentration exponent, η, Chapter 18) varies with the type of agent. For example, halving the concentration of phenol ($\eta = 6$) gives a 64-fold (2^6) reduction in killing activity, whereas a similar dilution for chlorhexidine ($\eta = 2$) reduces the activity by only fourfold (2^2). Changes in preservative activity are also seen with changes in product temperature, according to the temperature coefficient, Q_{10}. Thus, a reduction in temperature from 30 °C to 20 °C could result in a significantly reduced rate of kill for *Escherichia coli*, fivefold in the case of phenol ($Q_{10} = 5$) and 45-fold in the case of ethanol ($Q_{10} = 45$). If both temperature and concentration vary concurrently, the situation is more complex; however, it has been suggested that if a 0.1% chlorocresol ($\eta = 6$, $Q_{10} = 5$) solution completely killed a suspension of *E. coli* at 30 °C in 10 minutes, it would require around 90 minutes to achieve a similar effect if stored at 20 °C and if slight overheating during production had resulted in a 10% loss in the chlorocresol concentration (other factors remaining constant).

Preservative molecules are used up as they inactivate microorganisms and as they interact non-specifically with significant quantities of contaminant 'dirt' introduced during use. This will result in a progressive and exponential decline in the efficiency of the remaining preservative. Preservative 'capacity' is a term used to describe the cumulative level of contamination that a preserved formulation can tolerate before becoming so depleted as to become ineffective. This will vary with preservative type and complexity of formulation.

7.3 Factors affecting the 'availability' of preservatives

Most preservatives interact in solution to some extent with many of the commonly used formulation ingredients via a number of weak bonding attractions as well as with any contaminants present. Unstable equilibria may form in which only a small proportion of total preservative present is 'available' to inactivate the relatively small microbial mass; the resulting rate of kill may be far lower than might be anticipated from the performance of simple aqueous solutions. However, 'unavailable' preservative may still contribute to the general irritancy of the product. It is commonly believed that where the solute concentrations are very high, and A_w is appreciably reduced, the efficiency of preservatives is often significantly reduced and they may be virtually inactive at very low A_w. The practice of including preservatives in very low A_w products such as tablets and capsules is ill advised, as it only offers minimal protection for the dry tablets; should they become damp, they would be spoiled for other, non-microbial, reasons.

7.3.1 Effect of product pH

In the weakly acidic preservatives, activity resides primarily in the unionized molecules and they only have significant efficacy at pH values where ionization is low. Thus, benzoic and sorbic acids ($pK_a = 4.2$ and 4.75, respectively) have limited preservative usefulness above pH 5, while the 4(*p*)-hydroxybenzoate (parabens) esters with their non-ionizable ester group and poorly ionizable hydroxyl substituent (pK_a *c.*8.5) have a moderate protective effect even at neutral pH levels. The activity of quaternary ammonium preservatives and chlorhexidine probably resides with their cations; they are effective in products of neutral pH. Formulation pH can also directly influence the sensitivity of microorganisms to preservatives (see Chapter 18).

7.3.2 Efficiency in multiphase systems

In a multiphase formulation, such as an oil-in-water emulsion, preservative molecules will distribute themselves in an unstable equilibrium between the bulk aqueous phase and (1) the oil phase by partition, (2) the surfactant micelles by solubilization, (3) polymeric suspending agents and other solutes by competitive displacement of water of solvation, (4) particulate and container surfaces by adsorption and (5) any microorganisms present. Generally, the overall preservative efficiency can be related to the small proportion of pre-

servative molecules remaining unbound in the bulk aqueous phase, although as this becomes depleted some slow re-equilibration between the components can be anticipated. The loss of neutral molecules into oil and micellar phases may be favoured over ionized species, although considerable variation in distribution is found between different systems.

In view of these major potential reductions in preservative efficacy, considerable effort has been directed to devise equations in which one might substitute variously derived system parameters (such as partition coefficients, surfactant and polymer binding constants and oil:water ratios) to obtain estimates of residual preservative levels in aqueous phases. Although some modestly successful predictions have been obtained for very simple laboratory systems, they have proved of limited practical value, as data for many of the required parameters are unavailable for technical grade ingredients or for the more complex commercial systems.

7.3.3 Effect of container or packaging

Preservative availability may be appreciably reduced by interaction with packaging materials. Phenolics, for example, will permeate the rubber wads and teats of multidose injection or eye drop containers and also interact with flexible nylon tubes for creams. Quaternary ammonium preservative levels in formulations have been significantly reduced by adsorption on to the surfaces of plastic and glass containers. Volatile preservatives such as chloroform are so readily lost by the routine opening and closing of containers that their usefulness is somewhat restricted to preservation of medicines in sealed, impervious containers during storage, with short in-use lives once opened.

8 Quality assurance and the control of microbial risk in medicines

8.1 Introduction

Manufacturers of medicinal products must comply with the requirements of their marketing authorization (product licence) and ensure that their products are fit for their intended use in terms of safety, quality and efficacy. A quality management system (QMS) must therefore be in place so that senior management can ensure that the required quality objectives are met through a comprehensively designed and properly implemented system of quality assurance (QA), encompassing both GPMP and quality control (QC).

QA encompasses, in turn, a scheme of management which embraces all the procedures necessary to provide a high probability that a medicine will conform consistently to a specified description of quality. It includes formulation design and development (R&D), GPMP, as well as QC and postmarketing surveillance. As many microorganisms may be hazardous to patients or cause spoilage of formulations under suitable conditions, it is necessary to perform a risk assessment of contamination for each product. At each stage of its anticipated life from raw materials to administration, a risk assessment should be made and strategies should be developed and calculated to reduce the overall risk(s) to acceptably low levels. Such risk assessments are complicated by uncertainties about the exact infective and spoilage hazards likely for many contaminants, and by difficulties in measuring their precise performance in complex systems. As the consequences of product failure and patient harm will inevitably be severe, it is usual for manufacturing companies to make worst-case presumptions and design strategies to cover them fully; lesser problems are also then encompassed. As it must be assumed that all microorganisms may be potentially hazardous for those routes of administration where the likelihood of infection from contaminants is high, then medicines to be given via these routes must be supplied in a sterile form, as is the case with injectable products. It must also be presumed that those administering medicines may not necessarily be highly skilled or motivated in contamination control techniques; additional safeguards to control risks may be included in these situations. This may include detailed information on administration as well as training, in addition to providing a high quality formulation.

8.2 Quality assurance in formulation design and development

The risk of microbial infection and spoilage arising from microbial contamination during manufacture, storage and use could be eliminated by presenting all medicines in sterile, impervious, single-dosage units. However, the high cost of this strategy restricts its use to situations where there is a high risk of consequent infection from any contaminants. Where the risk is assessed as much lower, less efficient but less expensive strategies are adopted. The high risk of infection by contaminants in parenteral medicines, combined with concerns about the systemic toxicity of preservatives, almost always demands sterile single-dosage units. With eye drops for domestic use the risks are perceived to be lower, and sterile multidose products with preservatives to combat

the anticipated in-use contamination are accepted; sterile single-dose units are more common in hospitals where there is an increased risk of infection. Oral and topical routes of administration are generally perceived to present relatively low risks of infection and the emphasis is more on the control of microbial content during manufacture and subsequent protection of the formulation from chemical and physicochemical spoilage.

As part of the design process, it is necessary to include features in the formulation and delivery system that provide as much suitable protection as possible against microbial contamination and spoilage. Owing to potential toxicity and irritancy problems, antimicrobial preservatives should only be considered where there is clear evidence of positive benefit. Manipulation of physicochemical parameters, such as A_w, the elimination of particularly susceptible ingredients (e.g. natural ingredients such as tragacanth powder, used as a thickening agent), the selection of a preservative or the choice of container may individually and collectively contribute significantly to overall medicine stability. For 'dry' dosage forms where their very low A_w provides protection against microbial attack, the moisture vapour properties of packaging materials require careful examination.

Preservatives are intended to offer further protection against environmental microbial contaminants. However, as they are relatively non-specific in their reactivity (see section 7), it is difficult to calculate with any certainty what proportion of preservative added to all but the simplest medicine will be available for inactivating such contamination. Laboratory tests have been devised to challenge the product with an artificial bioburden. Such tests should form part of formulation development and stability trials to ensure that suitable activity is likely to remain throughout the life of the product. They are not normally used in routine manufacturing QC.

Some 'preservative challenge tests' (preservative efficacy tests) add relatively large inocula of various laboratory cultures to aliquots of the product and determine their rate of inactivation by viable counting methods (single challenge tests), while others reinoculate repeatedly at set intervals, monitoring the efficiency of inactivation until the system fails (multiple challenge test). This latter technique may give a better estimate of the preservative capacity of the system than the single challenge approach, but is both time-consuming and expensive. Problems arise when deciding whether the observed performance in such tests gives reliable predictions of real in-use efficacy. Although test organisms should bear some similarity in type and spoilage potential to those met in use, it is known that repeated cultivation on conventional microbiological media (nutrient agar, etc.) frequently results in reduced virulence of strains. Attempts to maintain spoilage activity by inclusion of formulation ingredients in culture media give varied results. Some manufacturers have been able to maintain active spoilage strains by cultivation in unpreserved, or diluted aliquots, of formulations.

The *British Pharmacopoeia* and the *European Pharmacopoeia* describe a single challenge preservative test that routinely uses four test organisms (two bacteria, a yeast and a mould), none of which has any significant history of spoilage potential and which are cultivated on conventional media. However, extension of the basic test is recommended in some situations, such as the inclusion of an osmotolerant yeast if it is thought such in-use spoilage might be a problem. Despite its accepted limitations and the cautious indications given as to what the tests might suggest about a formulation, the test does provide some basic, but useful indicators of likely in-use stability. UK product licence applications for preserved medicines must demonstrate that the formulation at least meets the preservative efficacy criteria of the *British Pharmacopoeia* or a similar test.

The concept of the D-value as used in sterilization technology (Chapter 21) has been applied to the interpretation of challenge testing. Expression of the rate of microbial inactivation in a preserved system in terms of a D-value enables estimation of the nominal time to achieve a prescribed proportionate level of kill. Problems arise, however, when trying to predict the behaviour of very low levels of survivors, and the method has its critics as well as its advocates.

8.3 Good pharmaceutical manufacturing practice (GPMP)

GPMP is concerned with the manufacture of medicines, and includes control of ingredients, plant construction, process validation, production and cleaning (see also Chapter 23). Current GPMP (cGPMP) requirements are found in the Medicines and Healthcare Products Regulatory Agency (MHRA) Rules and Guidance for Pharmaceutical Manufacturers and Distributors, known as the Orange Guide (Anon 2007), and its 20 annexes. QC is that part of GPMP dealing with specification, documentation and assessing conformance to specification.

With traditional QC, a high reliance has been placed on testing samples of finished products to determine the overall quality of a batch. This practice can, however, result in considerable financial loss if non-compliance is

detected only at this late stage, leaving the expensive options of discarding or reworking the batch. Additionally, some microbiological test methods have poor precision and/or accuracy. Validation can be complex or impossible, and interpretation of results can prove difficult. For example, although a sterility assurance level of less than one failure in 10^6 items submitted to a terminal sterilization process is considered acceptable, conventional 'tests for sterility' for finished products (such as that in the *European Pharmacopoeia*) could not possibly be relied upon to find one damaged but viable microbe within the 10^6 items, regardless of allowing for its cultivation with any precision (Chapter 21). Moreover, end-product testing will not prevent and may not even detect the isolated rogue processing failure.

It is now generally accepted that a high assurance of overall product quality can only come from a detailed specification, control and monitoring of *all* the stages that contribute to the manufacturing process. More realistic decisions about conformance to specification can then be made using information from *all* relevant parameters (parametric release method), not just from the results of selective testing of finished products. Thus, a more realistic estimate of the microbial quality of a batch of tablets would be achieved from a knowledge of specific parameters (such as the microbial bioburden of the starting materials, temperature records from granule drying ovens, the moisture level of the dried granules, compaction data, validation records for the foil strip sealing machine and microbial levels in the finished tablets), than from the contaminant content of the finished tablets alone. Similarly, parametric release is now accepted as an operational alternative to routine sterility testing for batch release of some finished sterile products. Through parametric release the manufacturer can provide assurance that the product is of the stipulated quality, based on the evidence of successful validation of the manufacturing process and review of the documentation on process monitoring carried out during manufacturing. Authorization for parametric release is given, refused or withdrawn by pharmaceutical assessors, together with GMP inspectors; the requirements are detailed in Annex 17 of the 2007 Orange Guide.

It may be necessary to exclude certain undesirable contaminants from starting materials, such as pseudomonads from bulk aluminium hydroxide gel, or to include some form of pretreatment to reduce their bioburdens by irradiation, such as for ispaghula husk, herbal materials and spices. For biotechnology-derived drugs produced in human or animal tissue culture, considerable efforts are made to exclude cell lines contaminated with latent host viruses. Official guidelines to limit the risk of prion contamination in medicines require bovine-derived ingredients to be obtained from sources where bovine spongiform encephalopathy (BSE) is not endemic.

By considering the manufacturing plant and its environs from an ecological and physiological viewpoint of microorganisms, it is possible not only to identify areas where contaminants may accumulate and even thrive to create hazards for subsequent production batches, but also to manipulate design and operating conditions in order to discourage such colonization. The facility to clean and dry equipment thoroughly is a very useful deterrent to growth. Design considerations should include the elimination of obscure nooks and crannies (where biofilms may readily become established) and the ability to clean thoroughly in all areas. Some larger items of equipment now have cleaning-in-place (CIP) and sterilization-in-place (SIP) systems installed to improve decontamination capabilities.

It may be necessary to include intermediate steps within processing to reduce the bioburden and improve the efficiency of lethal sterilization cycles, or to prevent swamping of the preservative in a non-sterile medicine after manufacture. Some of the newer and fragile biotechnology-derived products may include chromatographic and/or ultrafiltration processing stages to ensure adequate reductions of viral contamination levels rather than conventional sterilization cycles.

In a validation exercise, it must be demonstrated that each stage of the system is capable of providing the degree of intended efficiency within the limits of variation for which it was designed. Microbial spoilage aspects of process validation might include examination of the cleaning system for its ability to remove deliberately introduced contamination. Chromatographic removal of viral contaminants would be validated by determining the log reduction achievable against a known titre of added viral particles.

8.4 Quality control procedures

While there is general agreement on the need to control total microbial levels in non-sterile medicines and to exclude certain species that have previously proved troublesome, the precision and accuracy of current methods for counting (or even detecting) some microbes in complex products are poor. Pathogens, present in low numbers, and often damaged by processing, can be very difficult to isolate. Products showing active spoilage

can yield surprisingly low viable counts on testing. Although present in high numbers, a particular organism may be neither pathogenic nor the primary spoilage agent, but may be relatively inert, e.g. ungerminated spores or a secondary contaminant which has outgrown the initiating spoiler. Unevenly distributed growth in viscous formulations will present serious sampling problems. The type of culture medium (even different batches of the same medium) and conditions of recovery and incubation may significantly influence any viable counts obtained from products.

An unresolved problem concerns the timing of sampling. Low levels of pseudomonads shortly after manufacture may not constitute a spoilage hazard if their growth is checked. However, if unchecked, high levels may well initiate spoilage.

The *European Pharmacopoeia* has introduced both quantitative and qualitative microbial standards for non-sterile medicines, which may become enforceable in some member states. It prescribes varying maximum total microbial levels and exclusion of particular species according to the routes of administration. The *British Pharmacopoeia* has now included these tests, but suggests that they should be used to assist in validating GPMP processing procedures and not as conformance standards for routine end-product testing. Thus, for a medicine to be administered orally, the total viable count (TVC) should not be more than 10^3 aerobic bacteria or 10^2 fungi per gram or millilitre of product, and there should be an absence of *Escherichia coli*. Higher levels may be permissible if the product contains raw materials of natural origin, as in the case of herbal products where the TVC should not exceed 10^5 aerobic bacteria, 10^4 fungi and 10^3 Enterobacteria and Gram-negatives, with the absence of *E.coli*/gram or millilitre and *Salmonella*/ 10 gram or millilitres.

Most manufacturers perform periodic tests on their products for total microbial counts and the presence of known problem microorganisms; generally these are used for in-house confirmation of the continuing efficiency of their cGPMP systems, rather than as conventional end-product conformance tests. Fluctuation in values, or the appearance of specific and unusual species, can warn of defects in procedures and impending problems.

In order to reduce the costs of testing and shorten quarantine periods, there is considerable interest in automated alternatives to conventional test methods for the detection and determination of microorganisms. Although not in widespread use at present, promising methods include electrical impedance, use of fluorescent dyes and epifluorescence, and the use of 'vital' stains. Considerable advances in the sensitivity of methods for estimating microbial ATP using luciferase now allow the estimation of extremely low bioburdens. The recent development of highly sensitive laser scanning devices for detecting bacteria variously labelled with selective fluorescent probes enables the apparent detection even of single cells.

Endotoxin (pyrogen) levels in parenteral and similar products must be extremely low in order to prevent serious endotoxic shock on administration (Chapter 22). Formerly, this was checked by injecting rabbits and noting any febrile response. Most determinations are now performed using the *Limulus* test in which an amoebocyte lysate from the horseshoe crab (*Limulus polyphemus*) reacts specifically with microbial lipopolysaccharides to give a gel and opacity even at very high dilutions. A variant of the test using a chromogenic substrate gives a coloured end point that can be detected spectroscopically. Tissue culture tests are under development where the ability of endotoxins to induce cytokine release is measured directly.

Sophisticated and very sensitive methods have been developed in the food industry for detecting many other microbial toxins. For example, aflatoxin detection in herbal materials, seedstuffs and their oils is performed by solvent extraction, adsorption onto columns containing antibodies selective for the toxin, and detection by exposure to ultraviolet light.

Although it would be unusual to test for signs of active physicochemical or chemical spoilage of products as part of routine product QC procedures, this may occasionally be necessary in order to examine an incident of anticipated product failure, or during formulation development. Many of the volatile and unpleasant-tasting metabolites generated during active spoilage are readily apparent. Their characterization by high performance liquid chromatography or gas chromatography can be used to distinguish microbial spoilage from other, non-biological deterioration. Spoilage often results in physicochemical changes which can be monitored by conventional methods. Thus, emulsion spoilage may be followed by monitoring changes in creaming rates, pH changes, particle sedimentation and viscosity.

8.5 Postmarket surveillance

Despite extensive development and a rigorous adherence to procedures, it is impossible to guarantee that a medi-

cine will never fail under the harsh abuses of real-life conditions. A proper quality assurance system must include procedures for monitoring in-use performance and for responding to customer complaints. These must be meticulously followed up in great detail in order to decide whether carefully constructed and implemented schemes for product safety require modification to prevent the incident recurring.

9 Overview

Prevention is undoubtedly better than cure in minimizing the risk of medicament-borne infections. In manufacture the principles of GMP must be observed, and control measures must be built in at all stages. Thus, initial stability tests should show that the proposed formulation can withstand an appropriate microbial challenge; raw materials from an authorized supplier should comply with in-house microbial specifications; environmental conditions appropriate to the production process should be subject to regular microbiological monitoring; and finally, end-product analysis should indicate that the product is microbiologically suitable for its intended use and conforms to accepted in-house and international standards.

Based on present knowledge, contaminants, by virtue of their type or number, should not present a potential health hazard to patients when used.

Contamination during use is less easily controlled. Successful measures in the hospital pharmacy have included the packaging of products as individual units, thereby discouraging the use of multidose containers. Unit packaging (one dose per patient) has clear advantages, but economic constraints have prevented this desirable procedure from being realized. Ultimately, the most fruitful approach is through the training and education of patients and hospital staff, so that medicines are used only for their intended purpose. The task of implementing this approach inevitably rests with the clinical and community pharmacists of the future.

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18 Laboratory evaluation of antimicrobial agents

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1 Introduction

Laboratory evaluation of antimicrobial agents remains a cornerstone of clinical microbiology and antimicrobial/biocide discovery and development. The development of robust and reproducible assays for determining microbial susceptibility to antimicrobial agents is of fundamental importance in the appropriate selection of therapeutic agents and biocides for use in infection control, disinfection, preservation and antifouling applications. Such laboratory assays form the basis for high-throughput screening of compounds or biological extracts in the discovery, isolation and development of new antimicrobial drugs and biocides. Such assays facilitate identification of antimicrobial agents from various sources and in lead antimicrobial compound optimization. In the control of human and animal infection, laboratory evaluation of candidate agents yields crucial information which can inform choice of antimicrobial agent(s) where the causative organism is known or suspected. As the number of microorganisms exhibiting resistance to conventional

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antimicrobial agents increases, laboratory evaluation of antimicrobial susceptibility is increasingly important for the selection of appropriate therapeutic antimicrobials. Evaluation of the potential antimicrobial action and nature of the inhibitory or lethal effects of established and novel therapeutic agents and biocides are important considerations in the success of therapeutic interventions and infection/contamination control procedures.

Significant concerns that the extensive use of biocidal agents may be linked to the development of antimicrobial resistance exist. Recent concerns regarding significant global public health issues such as the increasing threat of bioterrorism, the prevalence of healthcare associated infections, severe acute respiratory syndrome (SARS), avian influenza (H5N1) and the 2009 World Health Organization declaration of the swine flu (H1N1) pandemic (the first pandemic of the 21st century) have seen global demand for biocides increase dramatically. In addition, the emergence of new infectious agents (e.g. prions) and the increasing transmission rates of significant blood-borne viruses (e.g. HIV, hepatitis B and C) which may readily contaminate medical instruments or the environment has focused attention on the need for effective and proven disinfecting and sterilizing agents.

Finally, increasing appreciation of the role played by microbial biofilms in human and animal infectious diseases and their ubiquitous distribution in natural ecosystems has led to the development of novel approaches for the laboratory evaluation of antimicrobial susceptibility of microorganisms growing as surface-adhered sessile populations. These studies have demonstrated that microorganisms in the biofilm mode of growth are phenotypically different from their planktonic counterparts and frequently exhibit significant tolerance to antimicrobial challenge (see Chapter 8). This has implications for the environmental control of microorganisms and in the selection of appropriate concentrations of antibiotic or biocide necessary to eradicate them. As such, biofilms may constitute a reservoir of infectious microorganisms which may remain following antimicrobial treatment, even if antimicrobial selection is based on standard laboratory evaluations of antimicrobials which are based on planktonic cultures of microorganisms. Tests for evaluating candidate antimicrobial agents to be used in human and animal medicine as well as environmental biocides remain significant laboratory considerations.

1.1 Definitions

Key terms such as disinfection, preservation, antisepsis and sterilization are defined in Chapters 19 and 21. A number of other important terms used to describe the antimicrobial activity of agents are also commonly used. A *biocide* may be defined as a chemical or physical agent which kills viable organisms, both pathogenic and nonpathogenic. This broad definition clearly *includes* microoganisms, but is not restricted to them. The term *microbicide* is therefore also used to refer specifically to an agent which kills microorganisms (*germicide* may also be used in this context, but generally refers to pathogenic microorganisms). The terms *biocidal*, *bactericidal*, *fungicidal* and *viricidal* therefore describe an agent with killing activity against a specific class or classes of organism indicated by the prefix, whereas the terms *bacteriostatic* and *fungistatic* refer to agents which inhibit the growth of bacteria or fungi (Figure 18.1), but do not necessarily kill them. It should be noted, however, that some microorganisms that appear non-viable and non-cultivable following antimicrobial challenge may be revived by appropriate methods, and that organisms incapable of multiplication may retain some enzymatic activity.

In the laboratory evaluation of antibacterial agents, the terms *minimum inhibitory concentration* (MIC) and *minimum bactericidal concentration* (MBC) are most commonly used. Recently published British Society for Chemotherapy (BSAC) guidelines for the determination of minimum inhibitory concentrations (see Further Reading) define the MIC as the lowest concentration of

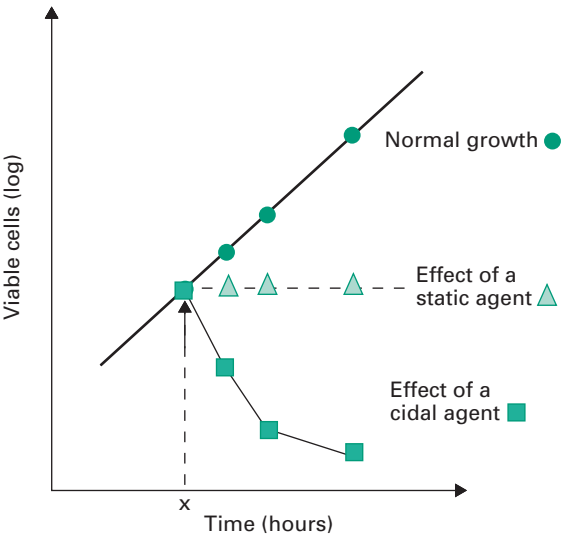

Figure 18.1 Effect on the subsequent growth pattern of inhibitory (static, △) or cidal (□) agents added at time X (the normal growth pattern is indicated by the • line).

antimicrobial which will inhibit the visible growth of a microorganism after overnight cultivation and the MBC as the lowest concentration of antimicrobial that will prevent the growth of a microorganism after subculture onto antibiotic-free media. Generally, MIC and MBC values are recorded in milligrams per litre or per millilitre (mg/L or mg/ml). With most cidal antimicrobials, the MIC and MBC are frequently near or equal in value, although with essentially static agents (e.g. tetracycline), the lowest concentration required to kill the microorganism (i.e. the MBC) is invariably many times the MIC and often clinically unachievable without damage to the human host. As with microbicides, cidal terms can be applied to studies involving not just bacteria but other microbes, e.g. when referring to cidal antifungal agents the term *minimum fungicidal concentration* (MFC) is used. Recently, thanks to developments in the design of high-throughput laboratory screens for biofilm susceptibility, the *minimum biofilm eradication concentration* (MBEC) can be accurately determined for organisms grown as single or mixed species biofilms. The MBEC is the minimum concentration of an antimicrobial agent required to kill a microbial biofilm. For conventional antibiotics and biocides the MBEC value may be 1000-fold higher than the MBC value for the same planktonic microorganisms. Further studies have shown that often no correlation exists between the MIC and the MBEC, indicating the potential limitations of therapeutic antibiotic selection based on determined MIC values.

The term *tolerance* implies the ability of some bacterial strains to survive (without using or expressing resistance mechanisms), but not grow, at levels of antimicrobial agent that should normally be cidal. This applies particularly to systems employing the cell-wall-active β-lactams and glycopeptides, and to Gram-positive bacteria such as streptococci. Normally, MIC and MBC levels in such tests should be similar (i.e. within one or two doubling dilutions); if the MIC/MBC ratio is 32 or greater, the term tolerance is used. Tolerance may in some way be related to the Eagle phenomenon (paradoxical effect), where increasing concentrations of antimicrobial result in reduced killing rather than the increase in cidal activity expected (see Figure 18.2). Tolerance to elevated antimicrobial challenge concentrations is also a characteristic of microbial biofilm populations. Finally, the term *resistance* has several definitions within the literature, however, it generally refers to the ability of a microorganism to withstand the effects of a harmful chemical agent, with the organism neither killed nor inhibited at concentrations to which the majority of strains of that organism

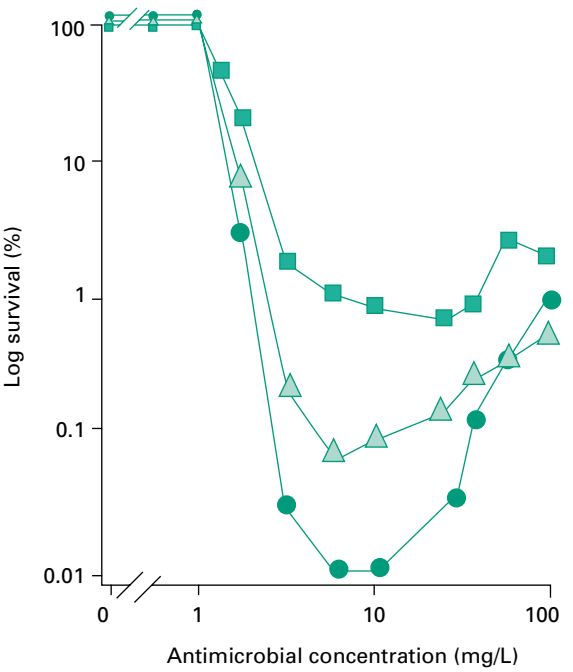

Figure 18.2 Survival of *Enterococcus faecalis* exposed to a fluoroquinolone for 4 hours at 37°C. Three initial bacterial concentrations were studied, 10^7 Cfu/ml (□); 10^6 CFU/ml (△) and 10^5 CFU/ml (○). This clearly demonstrates a paradoxical effect (increasing antimicrobial concentrations past a critical level reveal decreased killing), and the effects of increased inoculum densities on subsequent killing. (Courtesy of Dr Z. Hashmi.)

are susceptible. Resistance mechanisms generally involve modification of the normal target of the antimicrobial agent either by mutation, enzymatic changes, target substitution, antibiotic destruction or alteration, antibiotic efflux mechanisms and restricted permeability to antibiotics.

2 Factors affecting the antimicrobial activity of disinfectants

The activity of antimicrobial agents on a given organism or population of organisms will depend on a number of factors which must be reflected in the tests used to define their efficacy. For example, the activity of a given antimicrobial agent will be affected by nature of the agent, the nature of the challenge organism, the mode of growth of the challenge organism, concentration of agent, size of the challenge population and duration of exposure

of that population to the active agent. Furthermore, environmental/physical conditions (temperature, pH, presence of extraneous organic matter) are also important considerations in modelling the activity of biocidal agents. Laboratory tests for the evaluation of biocidal activity must be carefully designed to take into account these factors which may significantly influence the rate of kill within the microbial challenge population.

The work of Krönig and Paul in the late 1890s, demonstrated that the rate of chemical disinfection was related to both concentration of the chemical agent and the temperature of the system, and that bacteria exposed to a cidal agent do not die simultaneously but in an orderly sequence. This led to various attempts at applying the kinetics of pure chemical reactions (the mechanistic hypothesis of disinfection) to microbe/disinfectant interactions. However, since the inactivation kinetics depend on a large number of defined and undefined variables, such models are often too complicated for routine use. Despite this, the Chick–Watson model (equation 1), based on first-order reaction kinetics, remains the basic rate law for the examination of disinfection kinetics:

$$\frac{dN}{dT} = -k_0 N \qquad (1)$$

where N is the number of surviving microbes after time t and k_0 is the disinfection rate constant. The Chick–Watson model may be further refined to account for biocide concentration (equation 2):

$$\frac{dN}{dT} = -k_1 B^n N \qquad (2)$$

where k_1 is the concentration-independent rate constant, B is the biocide concentration and n is the dilution coefficient. The Chick–Watson model predicts that the number of survivors falls exponentially at a rate governed by the rate constant and the concentration of disinfectant. A general assumption is that the concentration of biocide remains constant throughout the experiment; however, there are a number of situations when this appears not to be the case (sequestering) and may result in observed departures from linear reaction kinetics. The factors influencing the antimicrobial activity of disinfectant agents are discussed below.

2.1 Innate (natural) resistance of microorganisms

The susceptibility of microorganisms to chemical disinfectants and biocides exhibits tremendous variation

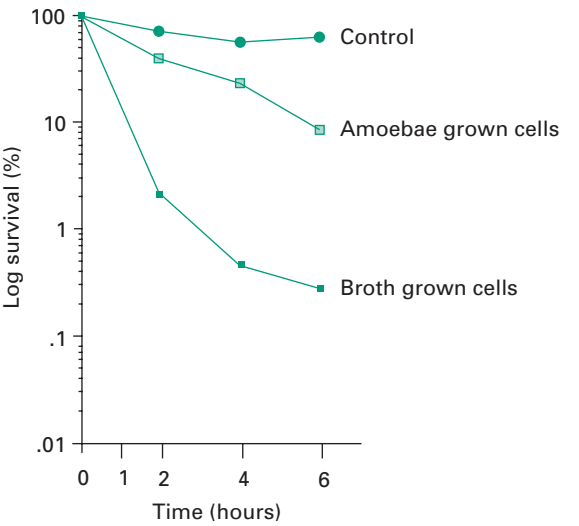

Figure 18.3 Survival of stationary phase broth cultures of *Legionella pneumophila* and amoebae-grown *L. pneumophila* after exposure to 32 mg/L benzisothiazolone (Proxel) at 35 °C in Ringer's solution. (Adapted from Barker *et al.* (1992), *Appl Environ Microbiol*, **58**, 2420–2425, with permission.)

across various classes and species. Bacterial endospores and the mycobacteria (e.g. *Mycobacterium tuberculosis*) possess the most innate resistance, while many vegetative bacteria and some viruses appear highly susceptible (see Chapter 19). In addition, microorganisms adhering to surfaces as biofilms or present within other cells (e.g. legionellae within amoebae), may reveal a marked increase in resistance to disinfectants and biocides (Figure 18.3). Therefore, when evaluating new disinfectants, a suitable range of microorganisms and environmental conditions must be included in tests. The European suspension test (EN 12054) for hospital-related studies and the European/British Standard suspension test (EN 1276) for studies relating to food, industrial, institutional and domestic areas, both include *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Enterococcus hirae* as the challenge organisms to be used in the test. For specific applications, additional strains may be chosen from *Salmonella enterica* serovar Typhimurium, *Lactobacillus brevis* and *Enterobacter cloacae*.

2.2 Microbial density

Many disinfectants require adsorption to the microbial cell surface prior to killing, therefore dense cell populations or sessile populations may sequester all the available

disinfectant before all cells are affected, thus shielding a proportion of the population from the toxic effects of the chemical agent. Therefore, from a practical point of view, the larger the number of microorganisms present, the longer it takes a disinfectant to complete killing of all cells. For instance, using identical test conditions, it has been shown that 10 spores of the anthrax bacillus (*Bacillus anthracis*) were destroyed in 30 minutes, while it took 3 hours to kill 100 000 (10^5) spores. The implications of predisinfection washing and cleaning of objects (which removes most of the microorganisms) becomes obvious. However, when evaluating disinfectants in the laboratory, it must be remembered that unlike sterilization, kill curves with disinfectants may not be linear and the rate of killing may decrease at lower cell numbers (Figure 18.3). Hence a 3-log killing may be more rapidly achieved with 10^8 than 10^4 cells. Johnston *et al.* (2000) demonstrated that even small variations in the initial inoculum size (*Staph. aureus*) had a dramatic effect on log reductions over time, using a constant concentration of sodium dodecyl sulphate (SDS). The authors argue that the presence of microbes quenches the action of the biocide (self-quenching), since cell and membrane components of lysed bacteria (e.g. emulsifiers such as triacylglycerols and phosphatidyl ethanolamine), are similar in action to emulsifiers (such as Tween and lecithin) used in standard biocide quenching/neutralizing agents employed in disinfectant tests. However, this may not hold true across all biocides where similar inoculum size dependency of disinfection is observed (see Russell *et al.*, 1997). Initial bioburden/cell numbers must, therefore, be standardized and accurately quantified in disinfectant efficacy (suspension) tests and agreement reached on the degree of killing required over a stipulated time interval (see Table 18.1).

Table 18.1 Methods of recording viable cells remaining after exposure of an initial population of 1 000 000 (10^6) CFU to a cidal agent

Viable count remaining (CFU)	Log survival (%)	Log killing	% killing
100 000 (10^5)	10	1-log	90
10 000 (10^4)	1	2-log	99
1000 (10^3)	0.1	3-log	99.9
100 (10^2)	0.01	4-log	99.99
10 (10^1)	0.001	5-log	99.999

2.3 Disinfectant concentration and exposure time

The effects of concentration or dilution of the active ingredient on the activity of a disinfectant are of major importance. With the exception of iodophors, the more concentrated a disinfectant, the greater its efficacy, and the shorter the time of exposure required to destroy the population of microorganisms, i.e. there is an exponential relationship between potency and concentration. Therefore, a graph plotting the $\log_{10}$ of the death time (i.e. the time required to kill a standard inoculum) against the $\log_{10}$ of the concentration is typically a straight line, the slope of which is the concentration exponent (η). Expressed as an equation:

$$\eta = \frac{(\text{Log death time at conc}^{n}\ C_2) - (\text{Log death time at conc}^{n}\ C_1)}{\text{Log}\, C_1 - C_2} \quad (3)$$

Thus η can be obtained from experimental data either graphically or by substitution in equation (3) (see Table 18.2).

It is important to note that dilution does not affect the cidal attributes of all disinfectants in a similar manner. For example, mercuric chloride with a concentration exponent of 1 will be reduced by the power of 1 on dilution, and a threefold dilution means the disinfectant activity will be reduced by the value 3^1, i.e. to a third of its original activity. Phenol, however, has a concentration exponent of 6, so a threefold dilution in this case will mean a decrease in activity of 3^6 or 729 times less active than the original concentration. Thus, the likely dilution experienced by the disinfectant agent in use must be given due consideration when selecting an appropriate biocidal agent for a given application.

2.4 Physical and chemical factors

Known and proven influences include temperature, pH and mineral content of water ('hardness').

2.4.1 Temperature

As with most chemical/biochemical reactions, the cidal activity of most disinfectants increases with increase in temperature, since temperature is a measure of the kinetic energy within a reaction system. Increasing the kinetic energy of a reaction system increases the rate of reaction by increasing the number of collisions between reactants per unit time. This process is observed up to an optimum

Table 18.2 Concentration exponents, η, for some disinfectant substances

Antimicrobial agent	η
Hydrogen peroxide	0.5
Silver nitrate	0.9–1.0
Mercurials	0.03–3.0
Iodine	0.9
Crystal violet	0.9
Chlorhexidine	2
Formaldehyde	1
QACs	0.8–2.5
Acridines	0.7–1.9
Formaldehyde donors	0.8–0.9
Bronopol	0.7
Polymeric biguanides	1.5–1.6
Parabens	2.5
Sorbic acid	2.6–3.2
Potassium laurate	2.3
Benzyl alcohol	2.6–4.6
Aliphatic alcohols	6.0–12.7
Glycolmonophenyl ethers	5.8–6.4
Glycolmonoalkyl ethers	6.4–15.9
Phenolic agents	4.0–9.9

QAC, quaternary ammonium compound.

temperature, beyond which reaction rates fall again, due to thermal denaturation of some component(s) of the reaction. As the temperature is increased in arithmetical progression the rate (velocity) of disinfection increases in geometrical progression. Results may be expressed quantitatively by means of a temperature coefficient, either the temperature coefficient per degree rise in temperature (θ), or the coefficient per 10 °C rise (the Q_{10} value) (Hugo & Russell, 1998). As shown by Koch, working with phenol and anthrax (*B. anthracis*) spores over 120 years ago, raising the temperature of phenol from 20 °C to 30 °C increased the killing activity by a factor of 4 (the Q_{10} value).

The value of θ may be calculated from the equation:

$$\theta^{(T_1-T_2)} = t_1 / t_2 \qquad (4)$$

where t_1 is the extinction time at T_1 °C, and t_2 the extinction at T_2 °C (i.e. T_1 + 1 °C).

Q_{10} values may be calculated easily by determining the extinction time at two temperatures differing by exactly 10°C. Then:

$$Q_{10} = \frac{\text{Time to kill at } T^\circ}{\text{Time to kill at } (T+10)^\circ} \qquad (5)$$

It is also possible to plot the rate of kill against the temperature.

While the value for Q_{10} of chemical and enzyme-catalysed reactions lies in a narrow range (between 2 and 3), values for disinfectants vary widely, e.g. 4 for phenol, 45 for ethanol, and almost 300 for ethylene glycol monoethyl ether. Clearly, relating chemical reaction kinetics to disinfection processes is potentially dangerous. Most laboratory tests involving disinfectant-like chemicals are now standardized to 20 °C, i.e. around ambient room temperatures.

2.4.2 pH

Effects of pH on antimicrobial activity can be complex. As well as directly influencing the survival and rate of growth of the microorganism under test, changes in pH may affect the potency of the agent and its ability to interact with cell surface sites. In a many cases (where the biocidal agent is an acid or a base), the ionization state (or degree of ionization) will depend on the pH. As is the case with some antimicrobials (e.g. phenols, acetic acid, benzoic acid), the non-ionized molecule is the active state (capable of crossing the cell membrane/partitioning) and alkaline pHs which favour the formation of ions of such compounds will decrease the activity. For these biocidal agents a knowledge of the molecule's pK_a is important in predicting the pH range over which activity can be observed, since in situations where the pH of the system equals the pK_a of the biocide molecule, ionized and unionized species are in equilibrium. Others, such as glutaraldehyde and quaternary ammonium compounds (QACs), reveal increased cidal activity as the pH rises and are best used under alkaline conditions, possibly due to enhanced interaction with amino groups on microbial biomolecules. The pH also influences the properties of the bacterial cell surface, by increasing the proportion of anionic groups and hence its interaction with cidal molecules. Since the activity of many disinfectants requires adherence to cell surfaces; increasing the external pH renders cell surfaces more negatively charged and enhances the binding of cationic compounds such as chlorhexidine and QACs.

2.4.3 Divalent cations

The presence of divalent cations (e.g. Mg^{2+}, Ca^{2+}), for example in hard water, has been shown to exert an antagonistic effect on certain biocides while having an additive effect on the cidal activity of others. Metal ions such as Mg^{2+} and Ca^{2+} may interact with the disinfectant itself to form insoluble precipitates and also interact with the microbial cell surface and block disinfectant adsorption sites necessary for activity. Biguanides, such as chlorhexidine, are inactivated by hard water. Hard water should always be employed for laboratory disinfectant and antiseptic evaluations to reflect this, with recommended formulae employing various concentrations of $MgCl_2$ and $CaCl_2$ solutions, available from the World Health Organization and the British Standard (BS EN 1276). On the other hand, cationic compounds may disrupt the outer membrane of Gram-negative bacteria and facilitate their own entry.

2.5 Presence of extraneous organic material

The presence of extraneous organic material such as blood, serum, pus, faeces or soil is known to affect the cidal activity of many antimicrobial agents. Therefore, it is necessary to determine the likely interaction between organic matter and the disinfectant by including this parameter in laboratory evaluations of their activity. In order to simulate 'clean' conditions (i.e. conditions of minimal organic contaminant), disinfectants are tested in hard water containing 0.3 g/L bovine albumin, with the albumin being used to mimic 'dirty' conditions. This standardized method replaces earlier approaches, some of which employed dried human faeces or yeast to mimic the effects of blood, pus or faeces on disinfectant activity. Disinfectants whose activities are particularly attenuated in the presence of organic contaminant include the halogen disinfectants (e.g. sodium hypochlorite) where the disinfectant reacts with the organic matter to form inactive complexes, biguanides, phenolic compounds and QACs. The aldehydes (formaldehyde and glutaraldehyde) are largely unaffected by the presence of extraneous organic contaminants. Organic material may also interfere with cidal activity by adherence to the microbial cell surface and blockade of adsorption sites necessary for disinfectant activity. For practical purposes and to mirror potential in-use situations, disinfectants should be evaluated under both clean and dirty conditions. Alternatives to albumin have also been suggested, for example sheep blood or mucin.

3 Evaluation of liquid disinfectants

3.1 General

Phenol coefficient tests were developed in the early 20th century when typhoid fever was a significant public health problem and phenolics were used to disinfect contaminated utensils and other inanimate objects. Details of such tests can be found in earlier editions of this book. However, as non-phenolic disinfectants became more widely available, tests that more closely paralleled the conditions under which disinfectants were being used (e.g. blood spills) and which included a more diverse range of microbial types (e.g. viruses, bacteria, fungi, protozoa) were developed. Evaluation of a disinfectant's efficacy was based on its ability to kill microbes, i.e. its cidal activity, under environmental conditions mimicking as closely as possible real life situations. As an essential component of each test was a final viability assay, removal or neutralization of any residual disinfectant (to prevent 'carryover' toxicity) became a significant consideration.

The development of methods to evaluate disinfectant activity in diverse environmental conditions and to determine suitable in-use concentrations/dilutions to be used led to the development by Kelsey, Sykes and Maurer of the so-called *capacity-use dilution test* which measured the ability of a disinfectant at appropriate concentrations to kill successive additions of a bacterial culture. Results were reported simply as pass or fail and not a numerical coefficient. Tests employed disinfectants diluted in hard water (clean conditions) and in hard water containing organic material (yeast suspension to simulate dirty conditions), with the final recovery broth containing 3% Tween 80 as a neutralizer. Such tests are applicable for use with a wide variety of disinfectants (see Kelsey & Maurer, 1974). Capacity tests mimic the practical situations of housekeeping and instrument disinfection, where surfaces are contaminated, exposed to disinfectant, recontaminated and so forth. The British Standard (BS 6907:1987) method for estimation of disinfectants used in dirty conditions in hospitals by a modification of the original Kelsey–Sykes test is the most widely employed capacity test in the UK and Europe. In the USA, effectiveness test data for submission must be obtained by methods accepted by the Association of Official Analytical Chemists, known collectively as Disinfectant Effectiveness Tests (DETs).

However, the best information concerning the fate of microbes exposed to a disinfectant is obtainable by

counting the number of viable cells remaining after exposure of a standard suspension of cells to the disinfectant at known concentration for a given time interval—*suspension tests*. Viable counting is a facile technique used in many branches of pure and applied microbiology. Assessment of the number of viable microbes remaining (survivors) after exposure allows the killing or cidal activity of the disinfectant to be expressed in a variety of ways, e.g. percentage kill (e.g. 99.999%), as a $\log_{10}$ reduction in numbers (e.g. 5-log killing), or by $\log_{10}$ survival expressed as a percentage. Examples of such outcomes are shown in Table 18.1.

Unfortunately, standardization of the methodology to be employed in these efficacy tests has proven difficult, if not impossible, to obtain, as has consensus on what level of killing represents a satisfactory and/or acceptable result. It must be stressed, however, that unlike tests involving chemotherapeutic agents where the major aim is to establish antimicrobial concentrations that inhibit growth (i.e. MICs), disinfectant tests require determinations of appropriate cidal levels. Levels of killing required over a given time interval tend to vary depending on the regulatory authority concerned. While a 5-log killing of bacteria (starting with 10^6 CFU/ml) has been suggested for suspension tests, some authorities require a 6-log killing in simulated use tests. With viruses, a 4-log killing tends to be an acceptable result, while with prions it has been recommended that a titre loss of 10^4 prions should be regarded as an indication of appropriate disinfection provided that there has been adequate prior cleaning. With simulated use tests, cleaning followed by appropriate disinfection should result in a prion titre loss of at least 10^7.

3.2 Antibacterial disinfectant efficacy tests

Various regulatory authorities in Europe (e.g. European Standard or Norm, EN; British Standards, BS; Germany, DGHM; France, AFNOR) and North America (e.g. Food and Drug Administration, FDA; Environmental Protection Authority, EPA; Association of Official Analytical Chemists, AOAC) have been associated with attempts to produce some form of harmonization of disinfectant tests. Perhaps the most readily accessible and recent guide to the methodology of possible bactericidal, tuberculocidal, fungicidal and viricidal disinfectant efficacy tests, is that of Kampf and colleagues (2002). This publication summarizes and provides references to various EN procedures (e.g. prEN 12054).

3.2.1 Suspension tests

While varying to some degree in their methodology, most of the proposed procedures tend to employ a standard suspension of the microorganism in hard water containing albumin (dirty conditions) and appropriate dilutions of the disinfectant—so-called suspension tests. Tests are carried out at a set temperature (usually around room temperature or 20 °C), and at a selected time interval samples are removed and viable counts are performed following neutralization of any disinfectant remaining in the sample. Neutralization or inactivation of residual disinfectant can be carried out by dilution, or by addition of specific agents (see Table 18.3). Using viable counts, it is possible to calculate the concentration of disinfectant required to kill 99.999% (5-log kill) of the original suspension. Thus 10 survivors from an original population of 10^6 cells represents a 99.999% or 5-log kill. As bacteria may initially decline in numbers in diluents devoid of additional disinfectant, results from tests incorporating disinfectant-treated cells can be compared with results from simultaneous tests involving a non-disinfectant-containing system (untreated cells). The bactericidal effect B_E can then be expressed as:

$$B_E = \log N_C - \log N_D \qquad (6)$$

where N_C and N_D represent the final number of CFU/ml remaining in the control and disinfectant series, respectively.

Unfortunately, viable count procedures are based on the assumption that one colony develops from one viable cell or one CFU. Such techniques are, therefore, not ideal for disinfectants (e.g. QACs such as cetrimide) that promote clumping in bacterial suspensions, although the latter problem may be overcome by adding non-ionic surface active agents to the diluting fluid.

3.2.2 In-use and simulated use tests

Apart from suspension tests, in-use testing of used medical devices, and simulated use tests involving instruments or surfaces deliberately contaminated with an organic load and the appropriate test microorganism have been incorporated into disinfectant testing protocols. An example is the in-use test first reported by Maurer in 1972. It is used to determine whether the disinfectant in jars, buckets or other containers in which potentially contaminated material (e.g. lavatory brushes, mops) has been placed contain living microorganisms, and in what numbers. A small volume of fluid is withdrawn from the

Table 18.3 Neutralizing agents for some antimicrobial agents

Antimicrobial agent	Neutralizing and/or inactivating agent[b]
Alcohols	None (dilution)
Alcohol-based hand gels	Tween 80, saponin, histidine and lecithin
Amoxycillin	β-Lactamase from *Bacillus cereus*[c]
Antibiotics (most)	None (dilution, membrane filtration,[d] resin adsorption[e])
Benzoic acid	Dilution or Tween 80[f]
Benzyl penicillin	β-Lactamase from *Bacillus cereus*
Bronopol	Cysteine hydrochloride
Chlorhexidine	Lubrol W and egg lecithin or Tween 80 and lecithin (Letheen)
Formaldehyde	Ammonium ions
Glutaraldehyde	Glycine
Halogens	Sodium thiosulphate
Hexachlorophane	Tween 80
Mercurials	Thioglycollic acid (-SH compounds)
Phenolics	Dilution or Tween 80
QACs	Lubrol W and lecithin or Tween 80 and lecithin (Letheen)
Sulphonamides	*p*-Aminobenzoic acid

[a] Other than dilution.
[b] D/E neutralizing media—adequate for QACs, phenols, iodine and chlorine compounds, mecurials, formaldehyde and glutaraldehyde (see Rutala, 1999).
[c] Other appropriate enzymes can be considered, e.g. inactivating or modifying enzymes for chloramphenicol and aminoglycosides, respectively.
[d] Filter microorganisms on to membrane, wash, transfer membrane to growth medium.
[e] Resins for the absorption of antibiotics from fluids are available.
[f] Tween 80 (polysorbate 80).
Source: adapted from Hugo & Russell (1998).

in-use container, neutralized in a large volume of a suitable diluent, and viable counts are performed on the resulting suspension. Two plates are involved in viable count investigations, one of which is incubated for 3 days at 32 °C (rather than 37 °C, as bacteria damaged by disinfectants recover more rapidly at lowered temperatures), and the other for 7 days at room temperature. Growth of one or two colonies per plate can be ignored (a disinfectant is not usually a sterilant), but 10 or more colonies would suggest poor and unsatisfactory cidal action.

Simulated use tests involve deliberate contamination of instruments, inanimate surfaces, or even skin surfaces, with a microbial suspension. This may either be under clean conditions or may utilize a diluent containing organic material (e.g. albumin) to simulate dirty conditions. After being left to dry, the contaminated surface is exposed to the test disinfectant for an appropriate time interval. The microbes are then removed (e.g. by rubbing with a sterile swab), resuspended in suitable neutralizing medium, and assessed for viability as for suspension tests. New products are often compared with a known comparator compound (e.g. 1 minute application of 60% v/v 2-propanol for hand disinfection products—see EN1500) to show increased efficacy of the novel product.

3.2.3 Problematic bacteria

Mycobacteria are hydrophobic in nature and, as a result, exhibit an increased tendency to clump or aggregate in aqueous media. It may be difficult, therefore, to prepare homogeneous suspensions devoid of undue cell clumping (which may contribute to their resistance to chemical disinfection). As *Mycobacterium tuberculosis* is very slow growing, more rapidly growing species such as *M. terrae, M. bovis* or *M. smegatis* can be substituted in tests (as representative of *M. tuberculosis*). Recent global public health concerns regarding the increasing incidences of tuberculosis (including co-infections with HIV) in developing, middle-tier and industrialized nations brings into sharp focus the necessity for representative evaluations of agents with potential tuberculocidal activity. This is particularly true given the high proportion of cases classified as multidrug resistant tuberculosis (MDR-TB). Apart from vegetative bacterial cells, bacterial or fungal spores can also be used as the inoculum in tests. In such cases, incubation of plates for the final viability determination should be continued for several days to allow for germination and growth.

Compared with suspended (planktonic) cells, bacteria on surfaces as biofilms are invariably phenotypically

more tolerant to antimicrobial agents. With biofilms, suspension tests can be modified to involve biofilms produced on small pieces of an appropriate glass, metal or polymeric substrate, or on the bottom of microtitre tray wells. After being immersed in, or exposed to, the disinfectant solution for the appropriate time interval, the cells from the biofilm are removed, e.g. by sonication, and resuspended in a suitable neutralizing medium. Viable counts are then performed on the resulting planktonic cells. Reduction in biomass following antimicrobial challenge can be monitored using a standard crystal violet staining technique, however, viable counting permits evaluation of rate of kill. The Calgary Biofilm Device, discussed in section 9.1, permits the high-throughput screening of antimicrobial agents against biofilms grown on 96 polycarbonate pegs in a 96-well microtitre plate. Some important environmental bacteria survive in nature as intracellular parasites of other microbes, e.g. *Legionella pneumophila* within the protozoan *Acanthamoeba polyphaga*. Biocide activity is significantly reduced against intracelluar legionellae (see Figure 18.3). Disinfectant tests involving such bacteria should therefore be conducted both on planktonic bacteria and on suspensions involving amoebae-containing bacteria. With the latter, the final bacterial viable counts are performed after suitable lysis of the protozoan host. The legionella/protozoa situation may also be further complicated by the fact that the microbes often occur as biofilms.

3.3 Other microbe disinfectant tests

Suspension-type efficacy tests can also be performed on other microbes, e.g. fungi, viruses, using similar techniques to that described above for bacteria, although significant differences obviously occur in parts of the tests.

3.3.1 Antifungal (fungicidal) tests

In order for disinfectants to claim fungicidal activity, or for the discovery of novel fungicidal activities, a range of standard tests have been devised. Perhaps the main problem with fungi concerns the question of which morphological form of fungi to use as the inoculum. Unicellular yeasts can be treated as for bacteria, but whether to use spores (which may be more resistant than the vegetative mycelium) or pieces of hyphae with the filamentous moulds, has yet to be fully resolved. Spore suspensions (in saline containing the wetting agent Tween 80) obtained from 7-day-old cultures are presently recommended. The species to be used may be a known environmental strain and likely contaminant, such as *Aspergillus niger*, or a pathogen, such as *Trichophyton mentagrophyes*, other strains such as *Penicillium variabile* are also employed. Clearly the final selection of organism will vary depending on the perceived use for the disinfectant under test. In general, spore suspensions of at least 10^6 CFU/ml have been recommended. Viable counts are typically performed on a suitable media (e.g. malt extract agar, sabouraud dextrose agar) with incubation at 20 °C for 48 hours or longer. EN 1275:1997 regulations for fungicidal activity require a minimum reduction in viability by a factor of 10^4 within 60 minutes; test fungi were *Candida albicans* and *A. niger*. Further procedures may be obtained by reference to EN 1650:1998 (quantitative suspension test for evaluation of fungicidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas) and AOAC Fungicidal activity of disinfectants (955.17).

3.3.2 Antiviral (viricidal) tests

The evaluation of disinfectants for viricidal activity is a complicated process requiring specialized training and facilities; viruses are obligate intracellular parasites and are therefore incapable of independent growth and replication in artificial culture media. They require some other system employing living host cells. Suggested test viruses include rotavirus, adenovirus, poliovirus, herpes simplex viruses, HIV, pox viruses and papovavirus, although extension of this list to include additional blood-borne viruses such as hepatitis B and C, and significant animal pathogens (e.g. foot and mouth disease virus) could be argued, given the potential impact on public health or the economy of a nation.

Briefly, the virus is grown in an appropriate cell line that is then mixed with water containing an organic load and the disinfectant under test. After the appropriate time, residual viral infectivity is determined using a tissue culture/plaque assay or other system (e.g. animal host, molecular assay for some specific viral component). Such procedures are costly and time-consuming, and must be appropriately controlled to exclude factors such as disinfectant killing of the cell system or test animal. A reduction of infectivity by a factor of 10^4 has been regarded as evidence of acceptable viricidal activity (prEN 14476). For viruses that cannot be grown in the laboratory (e.g. hepatitis B), naturally infected cells/tissues must be used. Further test procedures are detailed in British Standard BS EN 13610 (quantitative suspension test for the evaluation of viricidal activity against bacteriophages of chemical disinfectants used in food and industrial areas). The use of bacteriophage as model viruses in this procedure

most likely reflects their ease of growth and survivor enumeration via standard plaque assay on host bacterial lawns grown on solid media.

3.3.3 Prion disinfection tests

Prions are a unique class of acellular, proteinaceous infectious agent, devoid of an agent-specific nucleic acid (DNA or RNA). Infection is associated with the abnormal isoform of a host cellular protein called prion protein (PrP^c). Prions exhibit unusually high resistance to conventional chemical and physical decontamination methods, presenting a unique challenge in infection control. Although numerous published studies on prion inactivation by disinfectants are available in the literature, inconsistencies in methodology make direct comparison difficult. For example, strain differences of prion (with respect to sensitivity to thermal and chemical inactivation), prion concentration in tissue homogenate, exposure conditions and determination of log reductions from incubation period assays instead of end-point titrations. Furthermore, since most studies of prion inactivation have been conducted with tissue homogenates, the protective effect of the tissue components may offer some protective role and contribute to resistance to disinfection approaches. Despite this, a consistent picture of effective and ineffective agents has emerged and is summarized in Table 18.4. Although most disinfectants are inadequate for the elimination of prion infectivity, agents such as sodium hydroxide, a phenolic formulation, guanidine thiocyanate and chlorine have all been shown to be effective.

4 Evaluation of solid disinfectants

Solid disinfectants usually consist of a disinfectant substance diluted by an inert powder. Phenolic substances adsorbed onto kieselguhr (diatomite) form the basis of many disinfectant powders; another widely used solid disinfectant is sodium dichloroisocyanurate. Other disinfectant or antiseptic powders used in medicine include acriflavine and compounds with antifungal activity such as zinc undecenoate or salicylic acid mixed with talc. These disinfectants may be evaluated by applying them to suitable test organisms growing on a solid agar medium. Discs may be cut from the agar and subcultured for enumeration of survivors. Inhibitory activity is evaluated by dusting the powders onto the surface of seeded agar plates, using the inert diluents as a control. The extent of growth is then observed following incubation.

5 Evaluation of air disinfectants

The decontamination and disinfection of air is an important consideration for both infection and contamination control. A large number of important infectious diseases are spread via microbial contamination of the air. This cross-infection can occur in a variety of situations (hospitals and care facilities, airplanes, public and institutional buildings), while stringent control of air quality with respect to airborne contaminants and particulates is critical for contamination control in many aseptic procedures. With the increasing public concern regarding the perceived heightened threat of bioterrorism, effective air disinfection procedures have been reviewed as a potential counter-measure. The microorganisms themselves may be contained in aerosols, or may occur as airborne particles liberated from some environmental source, e.g. agitation of spore-laden bed linen, decaying vegetation, etc. Disinfection of air can be carried out by increased ventilation, filtration of air through high-efficiency particulate air (HEPA) filters, chemical aerosol/vapour/fumigation or by ultraviolet germicidal irradiation (UVGI). Although UVGI disinfectant approaches have demonstrated efficacy against a range of airborne pathogens and contaminating organisms, it is often more practical to use some form of chemical vapour or aerosol to kill them. The use of formaldehyde vapour is the most commonly employed agent for fumigation procedures (not strictly air disinfection), although vaporized hydrogen peroxide may be used as an alternative agent. Due to the potential for formation of carcinogenic bis(chloromethyl) ether when used with hydrochloric acid and chlorine containing disinfectants, formaldehyde should not be used with hypochlorites.

The work of Robert Koch in the late 1880s demonstrated that the numbers of viable bacteria present in air can be assessed by simply exposing plates of solid nutrient media to the air. Indeed, this same process is still exploited in environmental monitoring in the form of settle plates. Any bacteria that fall on to the plates after a suitable exposure time can then be detected following an appropriate period of incubation. These gravitational methods are obviously applicable to many microorganisms, but are unsuitable for viruses. However, more meaningful data can be obtained if force rather than gravity is used to collect airborne particles. A stream of air can be directed on to the surface of a nutrient agar plate (impaction; slit sampler) or bubbled through an appropriate buffer or culture medium (liquid impingement). Various commercial impactor samplers are

Table 18.4 Efficacy of Chemical Agents in Prion Inactivation

Ineffective (≤3 log10 reduction within 1 hour)	Effective (>3 log10 reduction within 1 hour of temperatures 20 °C—55 °C)
Acetone	Alkaline detergent (specific formulations)
Alcohol, 50–100%	Enzymatic detergent (specific form^{n})
Ammonia, 1.0 M	Chlorine >1,000 ppm
Alkaline detergent (specific formulations)	Copper, 0.5 mmol/L and H_20_2, 100 mmol/L
Chlorine dioxide, 50 ppm	Guanidine thiocyanate, >3 M
Formaldehyde, 3.7%	Peracetic acid, 0.2%
Glutaraldehyde, 5%	Phenolic disinfectant (specific form^{n}), >0.9%
Hydrochloric acid, 1.0 N	QAC (specific formulation)
Hydrogen peroxide, 0.2%–60%	Hydrogen peroxide, 59%
Iodine, 2%	SDS, 2% and acetic acid, 1%
Ortho-phthaladehyde, 0.55%	Sodium hydroxide, ≥1 N
Peracetic acid, 0.2%–19%	Sodium metaperiodate, 0.01 M
Phenol/phenolics (conc^{n} variable)	
Potassium permanganate, 0.1%–0.8%	
QAC (specific formulation)	
Sodium dodecyl sulfate (SDS) 1%–5%	
Sodium deoxycholate, 5%	
Enzymatic detergent (specific formulations)	
Triton X-100, 1%	
Urea, 4–8 M	

Processes may be listed in both columns (ineffective/effective), due to different testing parameters or testing methods. All experiments conducted without prior cleaning. Adapted from Rutala & Weber, 2010

available. Filtration sampling, where the air is passed through a porous membrane, which is then cultured, can also be used. For experimental evaluation of potential air disinfectants, bacterial or fungal airborne 'suspensions' can be created in a closed chamber, and then exposed to the disinfectant, which may be in the form of radiation, chemical vapour or aerosol. The airborne microbial population is then sampled at regular intervals using an appropriate forced-air apparatus such as the slit sampler. With viruses, the air can be bubbled through a suitable liquid medium, which is then subjected to some appropriate virological assay system. In all cases, problems arise in producing a suitable airborne microbial 'suspension' and in neutralizing residual disinfectant, which may remain in the air.

6 Evaluation of preservatives

Preservatives are widely employed in the cosmetic and pharmaceutical industries as well as in a variety of other manufacturing industries. The addition of preservatives to pharmaceutical formulations to prevent microbial growth and subsequent spoilage, to retard product deterioration and to restrain growth of contaminating microorganisms is commonplace for non-sterile pharmaceutical formulations as well as low-volume aseptically prepared formulations intended for multiple use from one container. Indeed, adequate preservation (and validation of effectiveness) is a legal requirement for certain formulations. Effective preservation prevents microbial and, as a consequence, related chemical, physical and aesthetic spoilage that could otherwise render the formulation unacceptable for patient use, therapeutically ineffective or harmful to the patient (due to presence of toxic metabolites, microbial toxins). The factors which influence the activity of the cidal agent employed as a preservative are largely those which affect disinfectant activity (described in section 2.4), however, when considering the activity of the cidal agent the interactions with formulation components (adsorption to suspended particles, oil–water partitioning, etc.) should be considered as

additional factors which can potentially attenuate the preservative activity.

While the inhibitory or cidal activity of the chemical to be used as the preservative can be evaluated using an appropriate *in vitro* test system (see sections 3.2.1 and 8.1.2), its continued activity when combined with the other ingredients in the final manufactured product must be established. Problems clearly exist with some products, where partitioning into various phases may result in the absence of preservative in one of the phases, e.g. oil-in-water emulsions where the preservative may partition only into the oily phase, allowing any contaminant microorganisms to flourish in the aqueous phase. In addition, one or more of the components may inactivate the preservative. Consequently, suitably designed simulated use challenge tests involving the final product are, therefore, required in addition to direct potency testing of the pure preservative. In the challenge test, the final preserved product is deliberately inoculated with a suitable environmental microorganism which may be fungal (e.g. *C. albicans* or *A. niger*)or bacterial (e.g. *Staph. aureus, E. coli, Ps. aeruginosa*). For oral preparations with a high sucrose content, the osmophilic yeast *Zygosaccharomyces rouxii* is a recommended challenge organism. The subsequent survival (inhibition), death or growth of the inoculum is then assessed using viable count techniques. Different performance criteria are laid down for injectable and ophthalmic preparations, topical preparations and oral liquid preparations in the *British Pharmacopoeia* (Appendix XVI C) and the *European Pharmacopoeia*, which should be consulted for full details of the experimental procedures to be used. In some instances, the range and/or spectrum of preservation can be extended by using more than one preservative at a time. Thus a combination of parabens (*p*-hydroxybenzoic acid) with varying water solubilities may protect both the aqueous and oil phases of an emulsion, while a combination of Germall 115 and parabens results in a preservative system with both antibacterial (Germall 115) and antifungal (parabens) activity.

7 Rapid evaluation procedures

In most of the tests mentioned above, results are not available until visible microbial growth occurs, at least in the controls. This usually takes 24 hours or more. The potential benefits of rapid antimicrobial susceptibility screening procedures are obvious, particularly in aggressive infections or rapidly progressing nosocomial infections of immunocompromised patients where appropriate antimicrobial selection is critical. To date only a few 'rapid' methods for detecting microbial viability or growth are presently employed in assessing the efficacy of antimicrobials. These include epifluorescent and bioluminescence techniques. The former relies on the fact that when exposed to the vital stain acridine orange and viewed under UV light, viable cells fluoresce green or greenish yellow, while dead cells appear orange. Live/dead staining of sessile bacterial populations has the potential to yield important data with respect to antimicrobial susceptibility, but requires skilled personnel and specialized microscopy equipment.

With tests involving liquid systems the early growth of viable cells can be assessed by some light-scattering processes, blood culture techniques have classically used the production of CO_2 as an indicator of bacterial metabolism and growth. In addition, the availability of molecular techniques, such as quantitative PCR, may be useful in demonstrating the presence or growth of microorganisms that are slow or difficult to culture under usual laboratory conditions, e.g. viruses. This may obviate the need to neutralize residual disinfectant with some assays.

Recently, rapid colorimetric assays for antimicrobial susceptibility have been developed including the commercially available Vitek and Vitek2 systems (Biomerieux) and colourimetric tests based on the extracellular reduction of tetrazolium salts 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium (WST-8) and 2,3-bis[2-methyloxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide (XTT). These latter studies have demonstrated the potential for the tetrazolium salts WST-8 and XTT to be used in the rapid, accurate and facile screening of antimicrobial susceptibility and MIC determination in a range of bacteria, including staphylococci, extended β-lactamase producing clinical isolates (*E. coli*, *Ent. faecalis*) and *Ps. aeruginosa* (see Tunney *et al.*, 2004). Using this method, MIC values in agreement with those obtained using standard methods were obtained after 5 hours.

8 Evaluation of potential chemotherapeutic antimicrobials

Unlike tests for the evaluation of disinfectants, where determination of cidal activity is of paramount importance, tests involving potential chemotherapeutic agents (antibiotics) invariably have determination of MIC as their main focus. Tests for the bacteriostatic activity of

antimicrobial agents are valuable tools in predicting antimicrobial sensitivity/tolerance in individual patient samples and for detection and monitoring of resistant bacteria. However, correlation between MIC and therapeutic outcome are frequently difficult to predict, especially in chronic biofilm-mediated infections. The determination of MIC values must be conducted under standardized conditions, since deviation from standard test conditions can result in considerable variation in data.

8.1 Tests for bacteriostatic activity

The historical gradient plates, ditch-plate and cup-plate techniques (see Hugo & Russell, 1998) have been replaced by more quantitative techniques such as disc diffusion (Figure 18.4), broth and agar dilution, and E-tests (Figure 18.5). All employ chemically defined media (e.g. Mueller-Hinton or Iso-Sensitest) at a pH of 7.2–7.4, and in the case of solid media, agar plates of defined thickness. Regularly updated guidelines have been provided by the National Committee for Clinical Laboratory Standards (NCCLS) and are widely used in many countries, although the British Society for Antimicrobial Chemotherapy has produced its own guidelines and testing procedures (Andrews, 2009).

8.1.1 Disc tests

These are really modifications of the earlier cup or ditch-plate procedures where filter-paper discs impregnated with the antimicrobial replace the antimicrobial-filled cups or wells. For disc tests, standard suspensions (e.g. 0.5 McFarland standard) of log-phase growth cells are prepared and inoculated on to the surface of appropriate agar plates to form a lawn. Commercially available filter-paper discs containing known concentrations of antimicrobial agent (it is possible to prepare your own discs for use with novel drugs) are then placed on the dried lawn and the plates are incubated aerobically at 35 °C for 18 hours. The density of bacteria inoculated on to the plate should produce just confluent growth after incubation. Any zone of inhibition occurring around the disc is then measured, and after comparison with known standards, the bacterium under test is identified as susceptible or resistant to that particular antibiotic. For novel agents, these sensitivity parameters are only available after extensive clinical investigations are correlated with laboratory-generated data. Disc tests are basically qualitative; however, the diameter of the zone of inhibition may be correlated to MIC determination through a linear regression analysis (Figure 18.6).

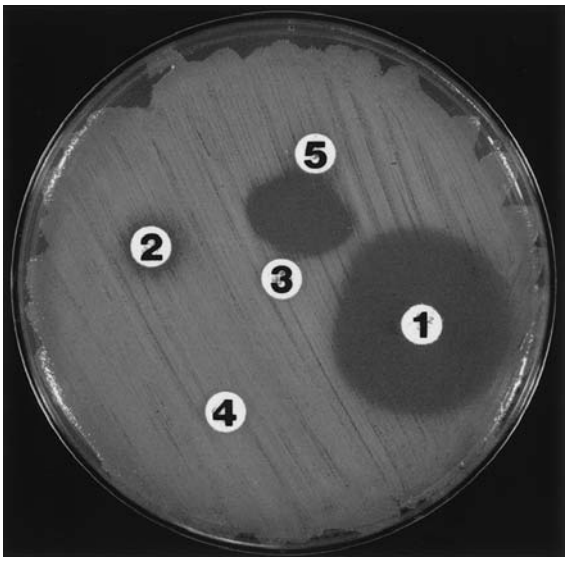

Figure 18.4 Disc test with inhibition zones around two (1, 2) of five discs. The zone around disc 1 is clear and easy to measure, whereas that around disc 2 is indistinct. Although none of the antimicrobials in discs 3, 4 or 5 appear to inhibit the bacterium, synergy (as evidenced by inhibition of growth between the discs) is evident with the antimicrobials in discs 3 and 5. Slight antagonism of the drug in disc 1 by that in disc 3 is evident.

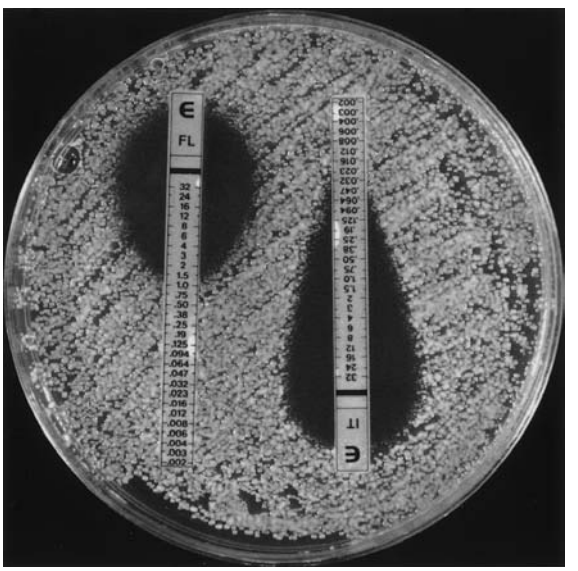

Figure 18.5 E-test on an isolate of *Candida albicans*. Inhibition zone edges are distinct and the MICs for itraconazole (IT) and fluconazole (FL) (0.064 mg/L and 1.5 mg/L, respectively) are easily decipherable.

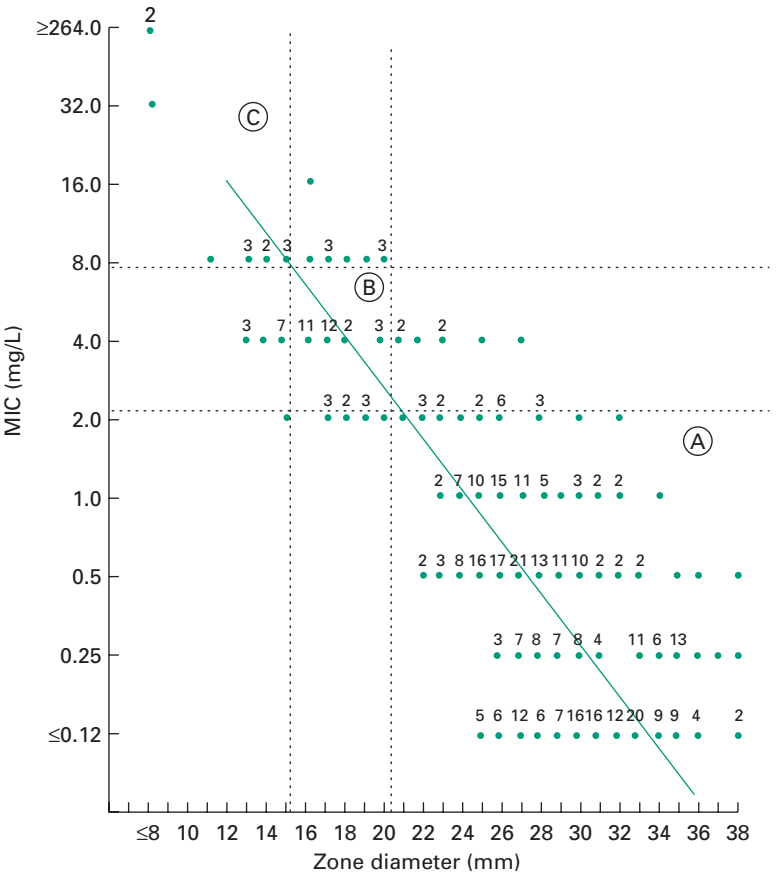

Figure 18.6 A scattergram and regression line analysis correlating zone diameters and MICs. The breakpoints of susceptible (MIC ≤ 2.0 mg/L, zone diameter ≥ 21 mm) and resistant (MIC ≥ 8.0 mg/L, zone ≤ 15 mm) are shown by the dotted lines. For a complete correlation between MICs and zone diameter, all susceptible, intermediate and resistant isolates should fall in boxes A, B and C, respectively. Errors (correlations outside these boxes) occur. (Courtesy of Dr Z. Hashmi.)

Although subtle variations of the disc test are used in some countries, the basic principles behind the tests remain similar and are based on the original work of Bauer and colleagues (Kirby–Bauer method). Some techniques employ a control bacterial isolate on each plate so that comparisons between zone sizes around the test and control bacterium can be ascertained (i.e. a disc potency control). Provided that discs are maintained and handled as recommended by the manufacturer, the value of such controls becomes debatable and probably unnecessary. Control strains of bacteria are available which should have inhibition zones of a given diameter with stipulated antimicrobial discs. Use of such controls endorses the suitability of the methods (e.g. medium, inoculum density, incubation conditions) employed. For slow-growing microorganisms, the incubation period can be extended. Problems arise with disc tests where the inoculum density is inappropriate (e.g. too low, resulting in an indistinct edge to the inhibition zone following incubation), or where the edge is obscured by the sporadic growth of cells within the inhibition zone, i.e. the initial inoculum although pure contains cells expressing varying levels of susceptibility—so-called *heterogeneity*. As the distance from the disc increases, there is a logarithmic reduction in the antimicrobial concentration; the result is that small differences in zone diameter with antimicrobials (e.g. vancomycin) which diffuse poorly through solid media may represent significantly different MICs. Possible synergistic or antagonistic combinations of antimicrobials can often be detected using disc tests (Figure 18.4).

8.1.2 Dilution tests

These usually employ liquid media but can be modified to involve solid media. Doubling dilutions, usually in the range 0.008–256 mg/L of the antimicrobial under test, are prepared in a suitable broth medium, and a volume of log-phase cells is added to each dilution to result in a final

cell density of around 5×10^5 CFU/ml. After incubation at 35°C for 18 hours, the concentration of antimicrobial contained in the first clear tube is read as the MIC. Needless to say, dilution tests require a number of controls, e.g. sterility control, growth control, and the simultaneous testing of a bacterial strain with known MIC to show that the dilution series is correct. Endpoints with dilution tests are usually sharp and easily defined, although 'skipped' wells (inhibition in a well with growth either side) and 'trailing' (a gradual reduction in growth over a series of wells) may be encountered. The latter is especially evident with antifungal tests (see below). Nowadays, the dilution test for established antimicrobials has been simplified by the commercial availability of 96-well microtitre plates which have appropriate antimicrobial dilutions frozen or lyophilized onto wells in the plate. The appropriate antibacterial suspension (in 200–400 µl volumes) is simply added to each well, the plate is incubated as before, and the MIC is read.

Dilution tests can also be carried out using a series of agar plates containing known antimicrobial concentrations. Appropriate bacterial suspensions are inoculated on to each plate and the presence or absence of growth is recorded after suitable incubation. Most clinical laboratories now employ agar dilution breakpoint testing methods. These are essentially truncated agar dilution MIC tests employing only a small range of antimicrobial concentrations around the critical susceptible/resistant cut-off levels. Many automated identification and sensitivity testing machines now use a liquid (broth) variant of the agar breakpoint procedure. Similar breakpoint antimicrobial concentrations are used with the presence or absence of growth being recorded by some automated procedure (e.g. light-scattering, colour change) after a suitable incubation period.

8.1.3 E-tests

Perhaps the most convenient and presently accepted method of determining bacterial MICs, however, is the E (Epsilometer)-test. The concept and execution of the E-test is similar to the disc diffusion test except that a linear gradient of lyophilized antimicrobial in twofold dilutions on nylon carrier strips on one side are used instead of the filter-paper impregnated antimicrobial discs. On the other side of the nylon strip are a series of lines and figures denoting MIC values (Figure 18.5). The nylon strips are placed antimicrobial side down on the freshly prepared bacterial lawn and, after incubation, the MIC is determined by noting where the ellipsoid (pear-shaped) inhibition zone crosses the strip (Figure 18.5). For most microorganisms, there appears to be excellent correlation between dilution and E-test MIC results. As with standard disc diffusion tests, resistant strains may be isolated from within the zone of inhibition.

8.1.4 Problematic bacteria

With some of the emerging antimicrobial-resistant bacterial pathogens, e.g. vancomycin-resistant enterococci (VRE), meticillin-resistant *Staph. aureus* (MRSA), vancomycin-intermediate *Staph. aureus* (VISA), the standard methodology described above may fail to detect the resistant phenotype. This is due to a variety of factors including heterogeneous expression of resistance (e.g. MRSA, VISA), poor agar diffusion of the antimicrobial (e.g. vancomycin) and slow growth of resistant cells (e.g. VISA). Disc tests are unsuitable for VRE, which should have MICs determined by E-test or dilution techniques. With MRSA, a heavier inoculum should be used in tests and 2–4% additional salt (NaCl) included in the medium with incubation for a full 48 hours. Reducing the incubation temperature to 30 °C may also facilitate detection of the true MIC value. Although 100% of MRSA cells may contain resistance genes, the phenotype may only be evident in a small percentage of cells under the usual conditions employed in sensitivity tests. Expression is enhanced at lower temperatures and at higher salt concentrations. With VISA, MIC determinations require incubation for a full 24 hours or more because of the slower growth rate of resistant cells.

8.2 Tests for bactericidal activity

MBC testing is required for the evaluation of novel antimicrobials. The MBC is the lowest concentration (in mg/L) of antimicrobial that results in 99.9% or more killing of the bacterium under test. The 99.9% cut-off is an arbitrary *in vitro* value with 95% confidence limits that has uncertain clinical relevance. MBCs are determined by spreading 0.1 ml (100 µl) volumes of all clear (no growth) tubes from a dilution MIC test onto separate agar plates (residual antimicrobial in the 0.1 ml sample is 'diluted' out over the plate). After incubation at 35 °C overnight (or longer for slow-growing bacteria), the numbers of colonies growing on each plate are recorded. The first concentration of drug that produces <50 colonies after subculture is considered the MBC. This is based on the fact that with MICs, the initial bacterial inoculum should result in about 5×10^5 CFU/ml. Inhibition, but not killing of this inoculum, should therefore result in the growth of 50 000 bacteria from the 0.1 ml sample. A 99.9% (3-log) kill would result in no more than 50 colo-

nies on the subculture plate. With most modern antibacterial drugs, the concentration that inhibits growth is very close to the concentration that produces death, e.g. within one or two dilutions. In general, only MICs are determined for such drugs.

8.3 Tests for fungistatic and fungicidal activity

As fungi have become more prominent human pathogens, techniques for investigating the susceptibility of isolates to the growing number of antifungal agents have been developed. These have been largely based on the established bacterial techniques (disc, dilution, E-test) mentioned above, with the proviso that the medium used is different (e.g. use of RPMI 1640 plus 2% dextrose) and that the inoculum density (yeast cells or spores) used is reduced (*c.*10^4 CFU/ml). With yeast disc and E-tests, a lawn producing just separated/distinct colonies is preferable to confluent growth (see Figure 18.5). Addition of methylene blue (0.5 mg/ml) to media may improve the clarity of inhibition zone edges. Problems of 'tailing' or 'trailing' in dilution tests, and indistinct inhibition zone edges are often seen in tests involving azoles and yeasts and appear in some way related to the type of buffer employed in the growth medium. However, their presence has prompted studies into evaluating the use of other techniques as an indicator of significant fungistasis—e.g. 50% reduction in growth (rather than complete inhibition) as the end point, use of a dye (e.g. Alamar blue) colour change to indicate growth, and sterol (ergosterol) quantitation. Most of these are presently outside the scope of most routine laboratories.

As with MBC estimations, MFC evaluation is an extension of the MIC test. At the completion of the MIC test (e.g. 72 hours for filamentous fungi), 20 ml are subcultured on to a suitable growth medium from each optically clear microtitre tray well and the growth control well. These plates are then incubated at 35 °C until growth is evident on the growth control subculture (24–48 hours). The MFC is the lowest drug concentration showing no growth or fewer than three colonies per plate to obtain approximately 99–99.5% killing activity.

8.4 Evaluation of possible synergistic antimicrobial combinations

The potential interaction between two antimicrobials can be demonstrated using a variety of laboratory procedures, e.g. 'chequerboard' MIC assays where the microorganism is exposed to varying dilutions of each drug alone and in combination, disc diffusion tests (see Figure 18.4) and kinetic kill curve assays. With the former, results can be plotted in the form of a figure called an *isobologram* (see Figure 18.7).

8.4.1 Kinetic kill curves

In the case of kill curves, the microorganism is inoculated into tubes containing a single concentration of each antimicrobial alone, the same concentrations of each antimicrobial in combination, and no antimicrobial—i.e. four tubes. All tubes are then incubated and viable counts are performed at regular intervals on each system. With results plotted on semilogarithmic paper, synergy is defined as a greater than 100-fold increase in killing of the combination compared with either drug alone. Antagonism is defined as at least a 100-fold decrease in killing of the combination when compared with the most active agent alone, while an additive or autonomous combined effect results in a less than 10-fold change from that seen with the most active single drug. Both chemotherapeutic agents and disinfectants are amenable to kill curve assays.

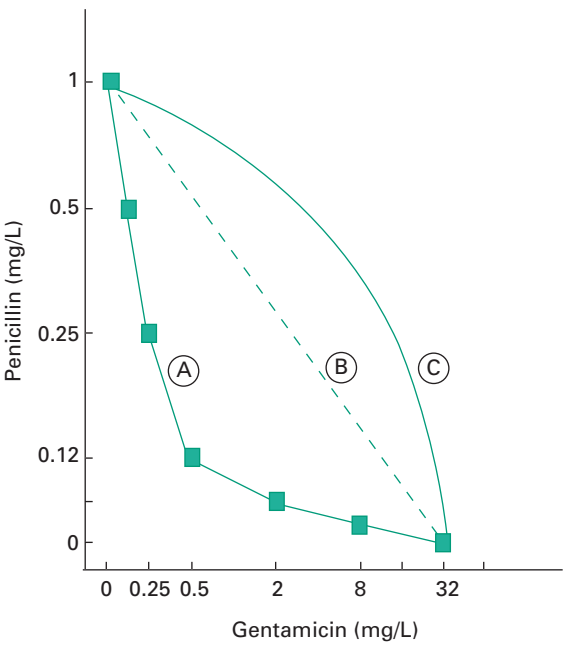

Figure 18.7 Diagrammatic representation of MIC values obtained with two synergistic antimicrobials, penicillin and gentamicin. The resulting graph or isobologram (A) is obtained by linking MIC values for each drug alone and in various dilution combinations. The MIC values for penicillin and gentamicin alone are 1.0 mg/L and 32 mg/L, respectively. The slope of the isobologram for purely additive or antagonistic combinations is shown by B and C, respectively.

9 Tests for biofilm susceptibility

As discussed in Chapter 8 and earlier in this chapter, biofilms present an additional problem to antimicrobial testing as the biofilm may be resistant to more than 1000 times the MIC concentration of antimicrobial. Antimicrobial testing of biofilms under standardized conditions has really only become available since 1999 and the development of the MBEC Assay (Calgary Biofilm Device). Previous techniques for biofilm susceptibility testing suffered from a lack of replicates, as in the case of flow cell technology, or the need for continuous pumping of fluids and bacteria that presented a leakage and contamination risk that was not tolerable in a diagnostic laboratory. Forming biofilms directly in 96-well plates provided replicate numbers, but in this assay system the initial inoculum could not be calculated and the efficacy of treatment was based not on viable cell counts but on a dye absorbance assay that could be measuring a change in extracellular matrix rather than a change in viable bacterial cell number. The MBEC assay placed pegs protruding from the lid of the plate into each well of a 96-well plate. Shear force created by gyration of the plate initiated bacterial adhesion to the peg and biofilm formation, the density of which could be determined by sonication of the biofilm back into a suspension culture and enumeration of viable cell number by standard plate counts. The peg-borne biofilms could then be used as a biofilm inoculation in a standard 96-well MIC assay, only in this case the susceptibility of a biofilm rather than a planktonic population would be determined. Following antimicrobial exposure bacteria would again be sonicated from the pegs and counted to determine the biofilm MIC (BMIC), biofilm bactericidal concentration (BMBC) and biofilm eradication concentration (MBEC) in a highly standardized and reproducible assay based on existing MIC technology. Mycobacteria and fungi can be assayed using a similar format allowing the biofilm susceptibility of these organisms to be tested.

9.1 Synergy biofilm assays

The reduced susceptibility of biofilms to antimicrobials often results in the effective *in vitro* drug concentration to far exceed a safe or achievable dose. Combinations of drugs or drugs and other cofactors are proving to be more effective against biofilms than single drug therapies. Synergies in biofilm testing have been defined on formulas based on the American Society for Microbiology standards. The calculation of synergy as defined in Harrison *et al.* (2008) where the mathematical definition of synergy was based on the sum of the fraction bactericidal concentration (FBC) value or FBC index for each combination of antimicrobial agents. Therefore in a two-component assay of agents A+B synergy would be defined as follows:

$$\text{FBC agent A} = \frac{\text{MBC}_b \text{ of agent A in combination}}{\text{MBC}_b \text{ of agent A alone}}$$

$$\text{FBC agent B} = \frac{\text{MBC}_b \text{ of agent B in combination}}{\text{MBC}_b \text{ of agent B alone}}$$

$$\sum \text{FBC} = \text{FBC agent A} + \text{FBC agent B} \qquad (7)$$

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19 Chemical disinfectants, antiseptics and preservatives

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1 Introduction

Disinfectants, antiseptics and preservatives are chemical agents that have the ability to destroy or inhibit the growth of microorganisms. They play a major role in medical and health care but also have widespread use in the management of livestock, the environment, paints and coatings, plastics, pharmaceutical, food and beverage manufacture, textiles, the catering industry and consumer products. The term *biocide* is increasingly used to describe this group of chemical agents and this term is used widely throughout European Union (EU) directives.

1.1 European Union regulation

Regulation of biocides continues to develop within the EU and many other countries wherein guidance is defined for both manufacturers and users. Specific guidance documents agreed between the EU Commission services and the competent authorities of the Member States include, importantly, the Biocidal Products Directive 98/8/EC and the related directives: Medicinal Products for Human Use Directive 2001/83/EC and the Veterinary Medicinal Products Directive 2001/82/EC. Additionally, 'agricultural pesticides', are regulated by the Plant Protection Products Directive 91/414/EC.

The term 'biocidal product' (98/8/EC) encompasses active substances and preparations containing one or more active substances, put up in the form in which they are supplied to the user, intended to destroy, deter, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means.

The European Commission has proposed a new Regulation of the European Parliament and of the Council concerning the placing on the market and use of biocidal products. The proposed European Regulation will, over a period of time, revise and replace the current regulatory framework for the marketing and use of biocidal products contained in the Biocidal Products Directive (BPD) 98/8/EC (as amended). The BPD is currently transposed into domestic UK law by the Biocidal Products Regulations (BPR) 2001 ([SI 2001/880] as amended). The UK Government Health and Safety Executive (HSE) engages in negotiations with all 27 Member States of the EU, the European Commission and the European Parliament in the development of a new directly-acting EU law. The new draft regulation which is scheduled to come into effect on 1 January 2013 proposes several important changes to the current approach to dealing with biocides, including:

- Extending the scope of the regime to cover treated articles and materials containing biocides
- Adopting a Community authorization scheme for certain types of products
- Requiring mandatory data-sharing of some animal testing data
- Reducing the burden of data collection requirements
- Harmonizing fee structures across member states

1.2 Definitions

1.2.1 Disinfectant and disinfection

Disinfection is the process of removing microorganisms, including pathogens, from the surfaces of inanimate objects. The British Standards Institution (BSI) further defines disinfection as not necessarily killing all microorganisms but reducing them to a level acceptable for a defined purpose, for example, a level that is harmful neither to health nor to the quality of perishable goods. Chemical disinfectants are capable of different levels of action (Table 19.1). The term *high-level disinfection* indicates destruction of all microorganisms but not necessarily bacterial spores; *intermediate-level disinfection* indicates destruction of all vegetative bacteria including *Mycobacterium tuberculosis* but may exclude some resistant viruses and fungi and implies little or no sporicidal activity; *low-level disinfection* can destroy most vegetative bacteria, fungi and viruses, but this will not include spores and some of the more resistant microorganisms. Some high-level disinfectants have good sporicidal activity and are described as *liquid chemical sterilants* or *chemosterilants* to indicate that they can effect a complete kill of all microorganisms, as in sterilization. In defining each of these disinfection l evels the activity and outcome is determined by correct use of the disinfectant product in regard to concentration, time of contact and prevailing environmental conditions as described in subsequent sections of this chapter.

1.2.2 Antiseptic and antisepsis

Antisepsis is defined as destruction or inhibition of microorganisms on living tissues having the effect of limiting or preventing the harmful results of infection. It is not a synonym for disinfection (BSI). The chemicals used are applied to skin and mucous membranes, so as well as having adequate antimicrobial activity they must not be toxic or irritating for these tissues. Antiseptics are mostly

Table 19.1 Levels of disinfection attainable when products are used according to manufacturer's instructions

	Low	Disinfection level Intermediate	High
Microorganisms killed	Most vegetative bacteria Some viruses Some fungi	Vegetative bacteria including *M. tuberculosis* Most viruses including hepatitis B virus (HBV), Most fungi	All microorganisms unless extreme challenge or resistance exhibited
Microorganisms surviving	*M. tuberculosis* Bacterial spores prions	Bacterial spores Prions	Extreme challenge of resistant bacterial spores Prions

used to reduce the microbial population on the skin before surgery or on the hands to help prevent spread of infection by this route. Antiseptics are sometimes formulated as products containing significantly lower concentrations of agents used for disinfection.

1.2.3 Preservative and preservation

Preservatives are included in pharmaceutical and many other types of formulations, both to prevent microbial spoilage of the product and to minimize the risk to the consumer of acquiring an infection when the preparation is administered. Preservatives must be able to limit proliferation of microorganisms that may be introduced unavoidably into non-sterile products such as oral and topical medications during their manufacture and use. In sterile products, where multiuse preparations remain available, preservatives should kill any microbial contaminants introduced inadvertently during use. It is essential that a preservative is not toxic in relation to the intended route of administration of the preserved preparation. Preservatives tend to be employed at very low concentrations and consequently levels of antimicrobial action also tend to be of a lower order than for disinfectants or antiseptics. This is illustrated by the *European Pharmacopoeia* requirements for preservative efficacy where a degree of bactericidal activity is necessary, although this should be obtained within a few hours or over several days of microbial challenge depending on the type of product to be preserved. Other terms are considered in Chapter 18.

1.3 Economic aspects

The international antimicrobial chemical market, particularly in disinfectants, is expected to grow significantly over the coming years, on the basis of concerns about bacterial and other pathogenic threats and the increasing emphasis on hygiene in the home and workplace. The turnover of the US antimicrobial chemical industry is estimated to be in the region of $2 billion per annum. Key disinfectant products in use contain aldehydes, iodophors, nitrogen compounds (quaternary ammonium compounds and amine compounds), organometallics, organosulphurs, chloroisocyanurates and phenolics. There are around 250 chemicals that have been identified as active components of microbicidal products in the EU.

The aim of this chapter is to introduce the range of chemicals in common use and to indicate their activities and applications.

2 Factors affecting choice of antimicrobial agent

Choice of the most appropriate antimicrobial compound for a particular purpose depends on many factors and the key parameters are described.

2.1 Properties of the chemical agent

The process of killing or inhibiting the growth of microorganisms using an antimicrobial agent is basically that of a chemical reaction and the rate and extent of this reaction will be influenced by concentration of agent, temperature, pH and formulation. The influence of these factors on activity is considered in Chapter 18, and is referred to in discussing the individual agents in section 3. Tissue toxicity influences whether a chemical can be

used as an antiseptic or preservative, and this limits the range of agents for these applications or necessitates the use of lower concentrations of the agent. This is discussed further in section 2.5.

2.2 Microbiological challenge

The types of microorganism present and the levels of microbial contamination (the *bioburden*) both have a significant effect on the outcome of treatment. If the bioburden is high, long exposure times and/or higher concentrations of antimicrobial may be required. Microorganisms vary in their sensitivity to the action of chemical agents. Some organisms merit attention either because of their resistance to disinfection (for further discussion see Chapter 20) or because of their significance in cross-infection or nosocomial (hospital-acquired) infections. Of particular concern is the significant increase in resistance to disinfectants resulting from microbial growth in biofilm form rather than free suspension (see Chapter 8). Microbial biofilms form readily on available surfaces, posing a serious problem for hospital infection control committees in advising suitable disinfectants for use in such situations.

The efficacy of an antimicrobial agent must be investigated by appropriate capacity, challenge and in-use tests to ensure that a standard is obtained which is appropriate to the intended use (Chapter 18). In practice, it is not usually possible to know which organisms are present on the articles being treated. Thus, it is necessary to categorize agents according to their antimicrobial activity and for the user to be aware of the level of antimicrobial action required in a particular situation (see Table 19.1).

2.2.1 Vegetative bacteria

At in-use concentrations, chemicals used for disinfection should be capable of killing bacteria and other organisms expected in that environment within a defined contact period. This includes 'problem' organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) and species of *Listeria*, *Campylobacter* and *Legionella*. Antiseptics and preservatives are also expected to have a broad spectrum of antimicrobial activity but at their in-use concentrations, after exerting an initial biocidal (killing) effect, their main function may be biostatic (inhibitory). Gram-negative bacilli, which are a major causes of nosocomial infections, are often more resistant than Gram-positive species. *Pseudomonas aeruginosa,* an opportunistic pathogen (see also Chapter 7), has gained a reputation as the most resistant of the Gram-negative organisms. However, problems mainly arise when a number of additional factors such as heavily soiled articles or diluted or degraded disinfectant solutions are employed.

2.2.2 *Mycobacterium tuberculosis*

M. tuberculosis (the tubercle bacillus) and other mycobacteria are resistant to many bactericides. Resistance is either (1) intrinsic, mainly due to reduced cellular permeability or (2) acquired, due to mutation or the acquisition of plasmids (Chapter 13). Tuberculosis remains an important public health hazard, and indeed the annual number of tuberculosis cases is rising in many countries. The greatest risk of acquiring infection is from the undiagnosed patient. Equipment used for respiratory investigations can become contaminated with mycobacteria if the patient is a carrier of this organism. It is important to be able to disinfect the equipment to a safe level to prevent transmission of infection to other patients (Table 19.2).

2.2.3 Bacterial spores

Bacterial spores can exhibit significant resistance to even the most active chemical disinfectant treatment. The majority of disinfectants have no useful sporicidal action in a pharmaceutical context, which relates to disinfection of materials, instruments and environments that are likely to be contaminated by the spore-forming genera *Bacillus* and *Clostridium*. However, certain aldehydes, halogens and peroxygen compounds display very good activity under controlled conditions and are sometimes used as an alternative to physical methods for sterilization of heat-sensitive equipment. In these circumstances, correct usage of the agent is of paramount importance, as safety margins are lower in comparison with physical methods of sterilization (Chapter 21).

Clostridium difficile is a particularly problematic contaminant in hospital environments, resulting in high levels of morbidity and mortality. In addition to stringent hand-washing, meticulous environmental disinfection procedures must be in place, e.g. using solutions of 5.25–6.15% sodium hypochlorite for routine disinfection. When high-level disinfection of *Cl. difficile* is required, 2% glutaraldehyde, 0.55% *o*-phthalaldehyde and 0.35% peracetic acid are effective.

Table 19.2 Antibacterial activity of commonly used disinfectants and antiseptics

Class of compound	Activity against		General level[a] of antibacterial activity
	Mycobacteria	Bacterial spores	
Alcohols			
Ethanol/isopropyl	+	–	Intermediate
Aldehydes			
Glutaraldehyde	+	+	High
o-Phthalaldehyde	+	+	High
Formaldehyde	+	+	High
Biguanides			
Chlorhexidine	–	–	Intermediate
Halogens			
Hypochlorite/chloramines	+	+	High
Iodine/iodophor	+	+	Intermediate, problems with *Ps. aeruginosa*
Peroxygens			
Peracetic acid	+	+	High
Hydrogen peroxide	+	+	High
Phenolics			
Clear soluble fluids	+	–	Intermediate
Chloroxylenol	–	–	Low
Bisphenols	–	–	Low, poor against *Ps. aeruginosa*
Quaternary ammonium compounds			
Benzalkonium	–	–	Intermediate
Cetrimide	–	–	Intermediate

[a] Activity expected per manufacturer's instructions and will depend on environmental conditions and bioburden.

The antibacterial activity of some disinfectants and antiseptics is summarized in Table 19.2.

2.2.4 Fungi

The vegetative fungal form is often as sensitive as vegetative bacteria to chemical antimicrobial agents. Fungal spores (conidia and chlamydospores; see Chapter 4) may be more resistant, but this resistance is of much lesser magnitude than that exhibited by bacterial spores. The ability to rapidly destroy pathogenic fungi such as the important nosocomial pathogen *Candida albicans*, filamentous fungi such as *Trichophyton mentagrophytes*, and spores of common spoilage moulds such as *Aspergillus niger* is put to advantage in many applications of use. Many disinfectants have good activity against these fungi (Table 19.3). In addition, ethanol (70%) is rapid and reliable against *Candida* species.

2.2.5 Viruses

Susceptibility of viruses to antimicrobial agents can depend on whether the viruses possess a lipid envelope. Non-lipid viruses are frequently more resistant to disinfectants and it is also likely that such viruses cannot be readily categorized with respect to their sensitivities to antimicrobial agents. These viruses are responsible for many nosocomial infections, e.g. rotaviruses, picornaviruses and adenoviruses (see Chapter 5) and it may be necessary to select an antiseptic or disinfectant to suit specific circumstances. Certain viruses, such as Ebola and Marburg, which cause haemorrhagic fevers, are highly

Table 19.3 Antifungal activity of disinfectants and antiseptics

Antimicrobial agent	Time (min) to give >99.99% kill[a] of		
	Aspergillus niger	*Trichophyton mentagrophytes*	*Candida albicans*
Phenolic (0.36%)	<2	<2	<2
Chlorhexidine gluconate (0.02%, alcoholic)	<2	<2	<2
Iodine (1%, alcoholic)	<2	<2	<2
Povidone-iodine (10%, alcoholic and aqueous)	10	<2	<2
Hypochlorite (0.2%)	10	<2	5
Cetrimide (1%)	<2	20	<2
Chlorhexidine gluconate (0.05%) + cetrimide (0.5%)	20	>20	>2
Chlorhexidine gluconate (0.5%, aqueous)	20	>20	>2

[a] Initial viable counts *c.*1×10^6/ml in suspension test.

infectious and their safe destruction by disinfectants is of paramount importance. Hepatitis A is an enterovirus considered to be one of the most resistant viruses to disinfection.

There is much concern for the safety of personnel handling articles contaminated with pathogenic viruses such as hepatitis B virus (HBV) and HIV. Disinfectants must be able to treat rapidly and reliably accidental spills of blood, body fluids or secretions from HIV-infected patients. Such spills may contain levels of HIV as high as 10^4 infectious units/ml. Fortunately, HIV is inactivated by most chemicals at in-use concentrations. However, the recommendation is to use high-level disinfectants (see Table 19.2) for decontamination of HIV- or HBV-infected reusable medical equipment. For patient care areas, cleaning and disinfection with intermediate-level disinfectants is satisfactory. Flooding with a liquid germicide is required only when large spills of cultured or concentrated infectious agents have to be dealt with.

The World Health Organization (WHO) and epidemiologists in many countries track outbreaks of influenza, especially in relation to potential epidemic and pandemic situations arising. The H1N1 outbreak in 2009 generated considerable concern. As an influenza A virus, however, it is susceptible to a large number of disinfectant products when they are used on hard, non-porous surfaces that may be contaminated. Although no research has been conducted on the susceptibility of 2009 H1N1 influenza virus to chlorine and other disinfectants in swimming pools and spas, studies have demonstrated that free chlorine levels of 1–3 mg/L (1–3 ppm) are adequate to disinfect avian H5N1 influenza virus.

2.2.6 Protozoa

Acanthamoeba spp. can cause acanthamoeba keratitis with associated corneal scarring and loss of vision in wearers of soft contact lenses. The cysts of this protozoan present a particular problem in respect of lens disinfection. The chlorine-generating systems in use are generally inadequate. Polyaminopropyl biguanide with or without chlorhexidine (0.003%) and polyhexamethylene biguanide (0.0005%) both show ability as an acanthamoebicide in combating 10^3 levels of cysts. Hydrogen peroxide -based disinfection is considered completely reliable and consistent in producing an acanthamoebicidal effect.

2.2.7 Prions

Prions are generally considered to be the infectious agents most resistant to chemical disinfectants and sterilization processes; strictly speaking, however, they are not microorganisms because they have no cellular structure nor do they contain nucleic acids. As small proteinaceous infectious particles they are a unique class of infectious agent causing spongiform encephalopathies such as bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt–Jakob disease (CJD) in humans. There is considerable concern about the transmission of these agents from infected animals or patients. Risk of infectivity is highest in brain, spinal cord and eye tissues. Prions are considered resistant to most disinfectant procedures. For heat-resistant medical instruments that come into

contact with high infectivity tissues or high-risk contacts, immersion in sodium hydroxide (1 M) or sodium hypochlorite (20 000 ppm available chlorine) for 1 hour is advised in WHO guidelines and this must be followed by further treatment including autoclaving, cleaning and routine sterilization. Recently, a formulation of 0.2% sodium docecyl sulphate, 0.3% NaOH in 20% n-propanol, has achieved potent decontamination of steel carriers contaminated with PrPTSE, the biochemical marker for prion infectivity, from 263K scrapie hamsters (5.5 $\log_{10}$ units reduction) or patients with sporadic or variant Creutzfeldt–Jacob disease. No low-temperature sterilization technology is effective.

2.3 Intended application

The intended application of the antimicrobial agent, whether for preservation, antisepsis or disinfection, will influence its selection and also affect its performance. For example, in medicinal preparations the ingredients in the formulation may antagonize preservative activity. The risk to the patient will depend on whether the antimicrobial is in close contact with a break in the skin or mucous membranes or is introduced into a sterile area of the body.

In disinfection of instruments, the chemicals used must not adversely affect the instruments, e.g. cause corrosion of metals, affect clarity or integrity of lenses, or change the texture of synthetic polymers. Many materials such as fabrics, rubber and plastics are capable of adsorbing certain disinfectants, e.g. quaternary ammonium compounds (QACs) are adsorbed by fabrics, while phenolics are adsorbed by rubber, the consequence of this being a reduction in the concentration of active compound. A disinfectant can only exert its effect if it is in contact with the item being treated. Therefore, access to all parts of an instrument or piece of equipment is essential. For small items, total immersion in the disinfectant must also be ensured.

2.4 Environmental factors

Organic matter can have a drastic effect on antimicrobial capacity either by adsorption or chemical inactivation, thus reducing the concentration of active agent in solution or by acting as a barrier to the penetration of the disinfectant. Blood, body fluids, pus, milk, food residues or colloidal proteins, even when present in small amounts, all reduce the effectiveness of antimicrobial agents to varying degrees, and some are seriously affected. In their normal habitats, microorganisms have a tendency to adhere to surfaces and are thus less accessible to the chemical agent. Some organisms are specific to certain environments and their destruction will be of paramount importance in the selection of a suitable agent, e.g. *Legionella* in cooling towers and non-potable water supply systems, *Listeria* in the dairy and food industry and HBV in blood-contaminated articles.

Dried organic deposits may inhibit penetration of the chemical agent. Where possible, objects to be disinfected should be thoroughly cleaned. The presence of ions in water can also affect activity of antimicrobial agents, thus water for testing biocidal activity can be made artificially 'hard' by addition of ions.

These factors can have very significant effects on activity and are summarized in Table 19.4.

2.5 Toxicity of the agent

In choosing an antimicrobial agent for a particular application some consideration must be given to its toxicity. Increasing concern for health and safety is reflected in the Control of Substances Hazardous to Health (COSHH) Regulations that specify the precautions required in handling toxic or potentially toxic agents. In respect of disinfectants these regulations affect, particularly, the use of phenolics, formaldehyde and glutaraldehyde. Toxic volatile substances, in general, should be kept in covered containers to reduce the level of exposure to irritant vapour and they should be used with an extractor facility. Limits governing the exposure of individuals to such substances are now listed, e.g. 0.7 mg/m^3 (0.2 ppm) glutaraldehyde for both short- and long-term exposure. Many disinfectants including the aldehydes, glutaraldehyde less so than formaldehyde, may affect the eyes, skin (causing contact dermatitis) and induce respiratory distress. Face protection and impermeable nitrile rubber gloves should be worn when using these agents. Table 19.4 lists the toxicity of many of the disinfectants in use and other concerns of toxicity are described below for individual agents.

The COSHH Regulations specify certain disinfectants that contain active substances not supported under the BPD that had to be phased out by 2006. Specified disinfection procedures applied to laboratories in relation to spills and routine use state that certain phenolic agents (including 2,4,6-trichlorophenol and xylenol) can no longer be employed in disinfectant products.

Because of the historically high number of occupational asthma cases caused by glutaraldehyde (an alkylating agent) products in chemical disinfection of endoscopes, an HSE report (2007) sought alternatives to this agent. The report recommended the preferential use of an oxidizing agent such as a chlorine-based or

Table 19.4 Properties of commonly used disinfectants and antiseptics

Class of compound	Effect of organic matter	pH optimum	Toxicity and OES*	Other factors
Alcohols				
Ethanol	Slight		Avoid broken skin, eyes	Poor penetration, good cleansing properties, flammable
			OES: 1000 ppm/1900 mg/m^3, 8 h only	
Isopropanol	Slight		OES: 500 ppm/1225mg/m^3, 10 min; 400 ppm/980 mg m^3, 8 h	
Aldehydes				
Glutaraldehyde	Slight	pH 8	Respiratory complaints and contact dermatitis reported	Non-corrosive, useful for heat- sensitive instruments
			Eyes, sensitivity	Use in well-ventilated area. Gloves, goggles and apron worn for preparation
			OES: 0.2 ppm/0.7mg/m^3, 10 min only	
Formaldehyde	Moderate		Respiratory distress, dermatitis	
			MEL: 2 ppm/2.5 mg/m^3, 10 min and 8 h	
Biguanides	Severe	pH 7–8	Avoid contact with eyes and mucous membranes	Incompatible with soap and anionic detergents
Chlorhexidine			Sensitivity may develop	Inactivated by hard water, some materials and plastic
Chlorine compounds	Severe	Acid/neutral pH	Irritation of skin, eyes and lungs	Corrosive to metals
Hypochlorite			OES: 1 ppm/3 mg/m^3, 10 min; 0.5 ppm/1.5 mg/m^3, 8 h	Dichloroisocyanurate likely to produce Cl gas when used to disinfect acidic urines
Hydrogen peroxide	Slight/moderate	Acid/neutral pH	May irritate skin and mucous membranes	May develop high pressure in container

(*continued*)

Table 19.4 (*continued*)

Class of compound	Effect of organic matter	pH optimum	Toxicity and OES*	Other factors
			OES: 2 ppm/3 mg/m^3, 10 min; 1 ppm, 8 h	
Iodine preparations	Severe	Acid pH	Eye irritation. Tincture or KI (Lugol's iodine)	May corrode metals
			OES: 0.1 ppm/1 mg/m^3, 10 min only	
Phenolics				
Clear soluble fluids	Slight	Acid pH	Protect skin and eyes	Absorbed by rubber/plastic
Black/white fluids	Moderate/severe		Very irritant. Greatly reduced by dilution May irritate skin	
Chloroxylenol	Severe		OES: 10 ppm/38 mg/m^3, 10 min; 5 ppm/19 mg/m^3, 8 h	Absorbed by rubber/plastic
QACs	Severe	Alkaline pH	Avoid contact with eyes	Incompatible with soap and anionic detergents
Cetrimide				Absorbed by fabrics
Benzalkonium chloride				

MEL, maximum exposure limit; OES: occupational exposure standard; QAC, quaternary ammonium compound.
MELs are the time-weighted average upper limits of a substance permitted in the breathing zone of a person. OES levels are the maximum concentrations of a substance in air to which individuals may be exposed during their working life and at which present knowledge indicates there will be no ill effects. Often similar to 8 h MEL levels. (MELs have now been replaced by workplace exposure limits, WELs.)
Source: HSE (2007).

Table 19.5 HSE Recommendations (2007) for endoscopy disinfection

Disinfectant agent	COSHH Essentials Hazard Group	COSHH Essentials Control Approach
Chlorine	A (low hazard)	1 (general ventilation)
Peroxygen	A (low hazard)	1 (general ventilation)
Peracetic acid	C (medium hazard)	3 (containment)
o-Phthalaldehyde	C (medium hazard)	3 (containment)
Glutaraldehyde	E (special case)	4 (special case)

HSE, Health and Safety Executive; COSHH, Control of Substances Hazardous to Health.
Based on COSHH Essentials (HSE 2009).

peroxygen-based product rather than a product containing an alkylating agent. However, it was recognized that consideration must be given to incompatibility of disinfectants with endoscope construction materials in some cases (Table 19.5).

In all situations where the atmosphere of a workplace is likely to be contaminated by disinfectant, sampling and analysis of the atmosphere may need to be carried out on a periodic basis with a frequency determined by conditions.

3 Types of compound

The following section presents, in alphabetical order by chemical grouping, the agents most often employed for disinfection, antisepsis and preservation. This information is summarized in Table 19.6.

3.1 Acids and esters

Antimicrobial activity, within a pharmaceutical context, is generally found only in the organic acids. These are weak acids and will, therefore, dissociate incompletely to give the three entities HA, H^+ and A^- in solution. As the undissociated form, HA, is the active antimicrobial agent, the ionization constant, K_a, is important and the pK_a of the acid must be considered, especially in formulation of the agent.

3.1.1 Benzoic acid

This is an organic acid, C_6H_5COOH, which is included, alone or in combination with other preservatives, in many pharmaceuticals. Although the compound is often used as the sodium salt, the non-ionized acid is the active substance. A limitation on its use is imposed by the pH of the final product as the pK_a of benzoic acid is 4.2 at which pH 50% of the acid is ionized. It is advisable to limit use of the acid to preservation of pharmaceuticals with a maximum final pH of 5.0 and if possible less than 4.0. Concentrations of 0.05–0.1% are suitable for oral preparations. A disadvantage of the compound is the development of resistance by some organisms, in some cases involving metabolism of the acid resulting in complete loss of activity. Benzoic acid also has some use in combination with other agents, salicylic acid for example, in the treatment of superficial fungal infections.

3.1.2 Sorbic acid

This compound is a widely used preservative as the acid or its potassium salt. The pK_a is 4.8 and, as with benzoic acid, activity decreases with increasing pH and ionization. It is most effective at pH 4 or below. Pharmaceutical products such as gums, mucilages and syrups are usefully preserved with this agent.

3.1.3 Sulphur dioxide, sulphites and metabisulphites

Sulphur dioxide has extensive use as a preservative in the food and beverage industries. In a pharmaceutical context, sodium sulphite and metabisulphite or bisulphite have a dual role, acting as preservatives and antioxidants.

3.1.4 Esters of *p*-hydroxybenzoic acid (parabens)

A series of alkyl esters (Figure 19.1) of *p*-hydroxybenzoic acid was originally prepared to overcome the marked pH dependence on activity of the acids. These parabens, the methyl, ethyl, propyl and butyl esters, are less readily ionized, having pK_a values in the range 8–8.5, and exhibit good preservative activity even at pH levels of 7–8, although optimum activity is again displayed in acidic solutions. This broader pH range allows extensive and successful use of the parabens as pharmaceutical preservatives. They are active against a wide range of fungi but are less so against bacteria, especially the pseudomonads which may utilize them as a carbon source. They are frequently used as preservatives of emulsions, creams and lotions where two phases exist. Combinations of

Table 19.6 Examples of the main antimicrobial groups as antiseptics, disinfectants and preservatives

Antimicrobial agent	Antiseptic activity		Disinfectant activity		Preservative activity	
	Concentration	Typical formulation/ application	Concentration	Typical formulation/ application	Concentration	Typical formulation/ application
Acids and esters e.g. benzoic acid, parabens					0.05–0.1% 0.25%	For oral and topical formulations
Alcohols[a] e.g. ethyl or isopropyl	50–90% in water	Skin preparation	50–90% in water	Clean surface preparation		
Aldehydes e.g. glutaraldehyde	10%	Gel for warts	2.0%	Solution for instruments		
Biguanides e.g. chlorhexidine[b] (gluconate, acetate, etc.)	0.02%	Bladder irrigation	0.05%	Storage of instruments, clean instrument disinfection (30 min)	0.0025%	Solution for hard contact lenses
	0.2%	Mouthwash			0.01%	Eye drops
	0.5% (in 70% alcohol)	Skin preparation				
	1.0%	Dusting powder, cream, dental gel	0.5% (in 70% alcohol)	Emergency instrument disinfection (2 min)		
	4.0%	Preoperative scrub in surfactant				
Chlorine e.g. hypochlorite	≤0.5%avCl_2	Solution for skin and wounds	1–10%	Solution for surfaces and instruments		
Hydrogen peroxide	1.5%	Stabilized cream	3.0%	Disinfection of soft contact lenses		
	3–6%	Solution for wounds and ulcers, mouthwash				

Iodine compounds e.g. free iodine, povidone- iodine	1.0%	Aqueous or alcoholic(70%) solution	10.0%	Aqueous or alcoholic solution		
	1.0%	Mouthwash				
	2.5%	Dry powder spray				
	7.5%	Scalp and skin cleanser				
	10%	Preoperative scrub, fabric dressing				
Phenolics e.g. clear soluble phenolics, chloroxylenol	0.5%	Dusting powder	1–2%	Solution		
	1.3%	Solution				
	2.0%	Skin cleanser				
QACs e.g. cetyltrimethyl ammonium bromide (cetrimide)	0.1%	Solution for wounds and burns	0.1%	Storage of instruments	0.01%	Eye drops
			1.0%	Instruments (1h)		
	0.5%	Cream				
	1.0%	Skin solution				

[a] Also used in combination with other agents, e.g. chlorhexidine, iodine.
[b] Several forms available having x% chlorhexidine and 10x% cetrimide.
QAC, quaternary ammonium compound.

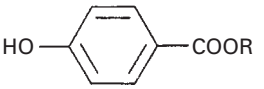

Figure 19.1 *p*-Hydroxybenzoates (R is methyl, ethyl, propyl, butyl, or benzyl).

esters are most successful for this type of product in that the more water-soluble methyl ester (0.25%) protects the aqueous phase, whereas the propyl or butyl esters (0.02%) give protection to the oil phase. Such combinations are also considered to extend the range of activity. As inactivation of parabens occurs with non-ionic surfactants due care should be taken in formulation with both materials.

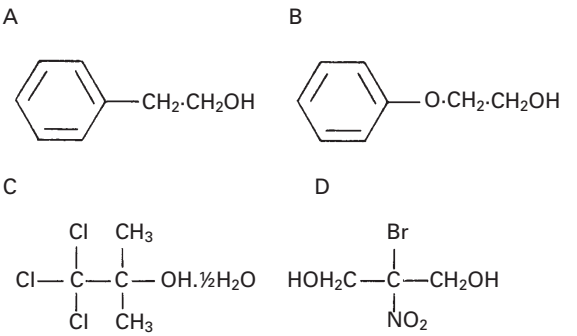

Figure 19.2 Structural formulae of alcohols used in preserving and disinfection: (A) 2-phenylethanol; (B) 2-phenoxyethanol, (C) chlorbutol (trichloro-tert-butanol); (D) bronopol (2-bromo-2-nitropropan-1,3-diol).

3.2 Alcohols

3.2.1 Alcohols used for disinfection and antisepsis

The aliphatic alcohols, notably ethanol and isopropanol, are used for disinfection and antisepsis. They are bactericidal against vegetative forms, including *Mycobacterium* species, but are not sporicidal. Overall cidal activity drops sharply below 50% concentration. Alcohols have poor penetration of organic matter and their use is, therefore, restricted to clean conditions. They possess properties such as a cleansing action and volatility, are able to achieve a rapid and large reduction in skin flora and have been widely used for skin preparation before injection or other surgical procedures. The risk of transmission of infection due to poor hand hygiene has been attributed to lack of compliance with hand-washing procedures. An alcohol hand-rub offers a rapid, easy-to-use alternative that is more acceptable to personnel and is frequently recommended for routine use. However, the contact time of an alcohol-soaked swab with the skin prior to venepuncture is so brief that it is thought to be of doubtful value.

Ethanol (CH_3CH_2OH) is widely used as a disinfectant and antiseptic. The presence of water is essential for activity, hence 100% ethanol is relatively ineffective. Concentrations between 60% and 95% are bactericidal and a 70% solution is usually employed for the disinfection of skin, clean instruments or surfaces. At higher concentrations, e.g. 90%, ethanol is also active against fungi and most lipid-containing viruses, including HIV, though less so against non-lipid-containing viruses. Ethanol is also a popular choice in pharmaceutical preparations and cosmetic products as a solvent and preservative, but it is not recommended for cleaning class II recirculating safety cabinets; ethanol vapours are flammable and the lower explosive limit (LEL) is easily attained. Mixtures with other disinfectants, e.g. with formaldehyde (100 g/L), are more effective than alcohol alone.

Isopropyl alcohol (isopropanol, $CH_3.CHOH.CH_3$) has slightly greater bactericidal activity than ethanol but is also about twice as toxic. It is less active against viruses, particularly non-enveloped viruses, and should be considered a limited-spectrum viricide. Used at concentrations of 60–70%, it is an acceptable alternative to ethanol for preoperative skin treatment and is also employed as a preservative for cosmetics.

3.2.2 Alcohols as preservatives

The aralkyl alcohols and more highly substituted aliphatic alcohols (Figure 19.2) are used mostly as preservatives. These include:

- *Benzyl alcohol* ($C_6H_5CH_2OH$). This has antibacterial and weak local anaesthetic properties and is used as an antimicrobial preservative at a concentration of 2%, although its use in cosmetics is restricted.
- *Chlorbutol* (chlorobutanol; trichlorobutanol; trichloro-t-butanol) is typically used at a concentration of 0.5% and is employed as a preservative in injections and eye drops. It is unstable, decomposition occurring at acid pH during autoclaving, while alkaline solutions are unstable at room temperature.
- *Phenylethanol* (phenylethyl alcohol; 2-phenylethanol), having a typical in-use concentration of 0.25–0.5%, is reported to have greater activity against Gram-negative organisms and is usually employed in conjunction with another agent.
- *Phenoxyethanol* (2-phenoxyethanol). Typical in-use concentration: 1%. It is more active against *Ps. aeruginosa*

than against other bacteria and is usually combined with other preservatives such as the hydroxybenzoates to broaden the spectrum of antimicrobial activity.

• *Bronopol* (2-bromo-2-nitropropan-1,3-diol). Typical in-use concentration: 0.01–0.1%. It has a broad spectrum of antibacterial activity, including *Pseudomonas* species. The main limitation on the use of bronopol is that when exposed to light at alkaline pH, especially if accompanied by an increase in temperature, solutions decompose, turning yellow or brown. A number of decomposition products including formaldehyde are produced. In addition, nitrite ions may be produced and react with any secondary and tertiary amines present forming nitrosamines, which are potentially carcinogenic.

3.3 Aldehydes

A number of aldehydes possess broad-spectrum antimicrobial properties, including sporicidal activity. These highly effective biocides can be employed in appropriate conditions as chemosterilants.

3.3.1 Glutaraldehyde

Glutaraldehyde ($CHO(CH_2)_3CHO$) has a broad spectrum of antimicrobial activity and rapid rate of kill, most vegetative bacteria being killed within a minute of exposure, although bacterial spores may require 3 hours or more. The kill rate depends on the intrinsic resistance of spores, which may vary widely. It has the further advantage of not being affected significantly by organic matter. The glutaraldehyde molecule possesses two aldehyde groupings which are highly reactive and their presence is an important component of biocidal activity. The monomeric molecule is in equilibrium with polymeric forms, and the physical conditions of temperature and pH have a significant effect on this equilibrium. At a pH of 8, biocidal activity is greatest but stability is poor due to polymerization. In contrast, acid solutions are stable but considerably less active, although as temperature is increased, there is a breakdown in the polymeric forms which exist in acid solutions and a concomitant increase in free, active dialdehyde, resulting in better activity. In practice, glutaraldehyde is generally supplied as an acidic 2% or greater aqueous solution, which is stable on prolonged storage. This is then 'activated' before use by addition of a suitable alkalizing agent to bring the pH of the solution to its optimum for activity. The activated solution will have a limited shelf life, of the order of 2 weeks, although more stable formulations are available. Glutaraldehyde is employed mainly for the cold liquid chemical sterilization of medical and surgical materials that cannot be sterilized by other methods. Endoscopes, including for example arthroscopes, laparascopes, cystoscopes and bronchoscopes, may be decontaminated by glutaraldehyde treatment (see section 2.5 concerning toxicity issues). Times employed in practice for high-level disinfection are often considerably less than the many hours recommended by manufacturers to achieve sterilization. The contact time for sterilization can be as long as 10 hours. Times for general disinfection generally range from 20–90 minutes at 20 °C depending on formulation and concentration.

3.3.2 *Ortho*-phthalaldehyde

Ortho-phthalaldehyde (OPA) is a relatively recent addition to the aldehyde group of high-level disinfectants. This agent has demonstrated excellent mycobactericidal activity with complete kill of *M. tuberculosis* within 12 minutes at room temperature. OPA has several other advantages over glutaraldehyde. It requires no activation, is considerably less irritant to the eyes or nasal passages and has excellent stability over the pH range 3–9. It can be used for disinfection of endoscopes (Table 19.5).

3.3.3 Formaldehyde

Formaldehyde (HCHO) can be used in either the liquid or the gaseous state for disinfection purposes. In the vapour phase it has been used for decontamination of isolators, safety cabinets and rooms. The combination of formaldehyde vapour with low-temperature steam (LTSF) has been employed for the sterilization of heat-sensitive items (Chapter 21). Formaldehyde vapour is highly toxic and potentially carcinogenic if inhaled, thus its use must be carefully controlled. It is not very active at temperatures below 20 °C and requires a relative humidity of at least 70%. The agent is not supplied as a gas but either as a solid polymer, paraformaldehyde, or a liquid, formalin, which is a 34–38% aqueous solution. The gas is liberated by heating or mixing the solid or liquid with potassium permanganate and water. Formalin, diluted 1 : 10 to give 4% formaldehyde, may be used for disinfecting surfaces. In general, however, solutions of either aqueous or alcoholic formaldehyde are too irritant for routine application to skin, while poor penetration and a tendency to polymerize on surfaces limit its use as a disinfectant for pharmaceutical purposes.

3.3.4 Formaldehyde-releasing agents

Various formaldehyde condensates have been developed to reduce the irritancy associated with formaldehyde while maintaining activity, and these are described as

formaldehyde-releasing agents or masked-formaldehyde compounds.

Noxythiolin (*N*-hydroxy *N*-methylthiourea) is supplied as a dry powder and on aqueous reconstitution slowly releases formaldehyde and *N*-methylthiourea. The compound has extensive antibacterial and antifungal properties and has been used both topically and in accessible body cavities as an irrigation solution and in the treatment of peritonitis. Polynoxylin (poly-[methylenedi(hydroxymethyl)urea]) is a similar compound available in gel and lozenge formulations.

Taurolidine (bis-(1,1-dioxoperhydro-1,2,4-thiadiazinyl-4) methane) is a condensate of two molecules of the amino acid taurine and three molecules of formaldehyde. It is more stable than noxythiolin in solution and has similar uses.

3.4 Biguanides

3.4.1 Chlorhexidine

Chlorhexidine is an antimicrobial agent first synthesized in 1954. The chlorhexidine molecule, a bisbiguanide, is symmetrical with a hexamethylene chain linking two biguanide groups, each with a *para*-chlorophenyl radical (Figure 19.3).

Chlorhexidine base is not readily soluble in water; therefore its freely soluble salts, acetate, gluconate and hydrochloride, are used in formulation. Chlorhexidine exhibits the greatest antibacterial activity at pH 7–8 where it exists exclusively as a dication. The cationic nature of the compound results in activity being reduced by anionic compounds, including soap, due to the formation of insoluble salts. Anions to be wary of include bicarbonate, borate, carbonate, chloride, citrate and phosphate, with avoidance of hard water if possible. Deionized or distilled water should preferably be used for dilution purposes. Reduction in activity will also occur in the presence of blood, pus and other organic matter. Chlorhexidine has widespread use, in particular as an antiseptic. It has significant antibacterial activity, although Gram-negative bacteria are less sensitive than Gram-positive organisms. A concentration of 0.0005% prevents growth of, for example, *Staph. aureus,* whereas 0.002% prevents growth of *Ps. aeruginosa*. Reports of pseudomonad contamination of aqueous chlorhexidine solutions have prompted the inclusion of small amounts of ethanol or isopropanol. Chlorhexidine is ineffective at ambient temperatures against bacterial spores and *M. tuberculosis*. Limited antifungal activity has been demonstrated, which unfortunately restricts its use as a general preservative. Skin sensitivity has occasionally been reported although, in general, chlorhexidine is well tolerated and non-toxic when applied to skin or mucous membranes and is an important preoperative antiseptic.

3.4.2 Polyhexamethylene biguanides

The antimicrobial activity of the bisbiguanide chlorhexidine exceeds that of monomeric biguanides. This stimulated the development of polymeric biguanides containing repeating biguanide groups linked by hexamethylene chains. One such compound is a commercially available heterodisperse mixture of polyhexamethylene biguanides (PHMB, polyhexanide) having the general formula shown in Figure 19.4.

Within the structure, *n* varies with a mean value of 5.5. The compound has a broad spectrum of activity against Gram-positive and Gram-negative bacteria and has low toxicity. PHMB is employed as an antimicrobial agent in various ophthalmic products.

3.5 Halogens

Chlorine and iodine have been used extensively since their introduction as disinfecting agents in the early 19th century. Preparations containing these halogens, such as Dakin's solution and tincture of iodine, were early inclusions in many pharmacopoeias and national formu-

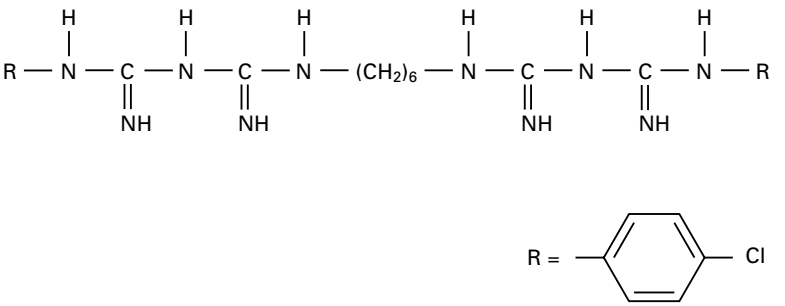

Figure 19.3 Chlorhexidine.

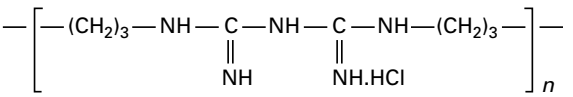

Figure 19.4 Polyhexamethylene biguanide (PHMB).

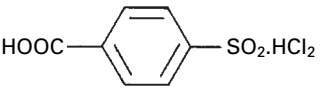

Figure 19.5 Halazone.

laries. More recent formulations of these elements have improved activity, stability and ease of use.

3.5.1 Chlorine

A large number of antimicrobially active chlorine compounds are commercially available, one of the most important being liquid chlorine. This is supplied as an amber liquid made by compressing and cooling gaseous chlorine. The terms liquid and gaseous chlorine refer to elemental chlorine, whereas the word 'chlorine' itself is normally used to signify a mixture of OCl^-, Cl_2, HOCl and other active chlorine compounds in aqueous solution. The potency of chlorine disinfectants is usually expressed in terms of parts per million (ppm) or percentage of available chlorine (avCl).

3.5.2 Hypochlorites

Hypochlorites (bleach) are the oldest and remain the most useful of the chlorine disinfectants, being readily available, inexpensive and compatible with most anionic and cationic surface-active agents. They exhibit a rapid kill against a wide spectrum of microorganisms, including fungi and viruses. High levels of available chlorine will enable eradication of mycobacteria and bacterial spores. Their disadvantages are that they are corrosive, suffer inactivation by organic matter and can become unstable. Hypochlorites are available as powders or liquids, most frequently as the sodium or potassium salts of hypochlorous acid (HOCl). Sodium hypochlorite exists in solution as follows:

$$NaOCl + H_2O \rightleftharpoons HOCl + NaOH \quad (1)$$

Undissociated hypochlorous acid is a strong oxidizing agent and its potent antimicrobial activity is dependent on pH as shown:

$$HOCl \rightleftharpoons H^+ + OCl^- \quad (2)$$

At low pH the existence of HOCl is favoured over OCl^- (hypochlorite ion). The relative microbicidal effectiveness of these forms is of the order of 100 : 1. By lowering the pH of hypochlorite solutions the antimicrobial activity increases to an optimum at about pH 5. However, this is concurrent with a decrease in stability of the solutions. This problem may be alleviated by addition of NaOH (see equation 1) in order to maintain a high pH during storage for stability. The absence of buffer allows the pH to be lowered sufficiently for activity on dilution to use-strength. It is preferable to prepare use-dilutions of hypochlorite on a daily basis.

Undiluted bleach stored at room temperature in a closed container has a shelf life of about 6 months. Storage of stock or working solutions of bleach in open containers causes release of chlorine gas, especially at elevated temperatures, and this considerably weakens the antimicrobial activity of the solution. Working solutions should be prepared on a daily basis.

3.5.3 Organic chlorine compounds

A number of organic chlorine, or chloramine, compounds are now available for disinfection and antisepsis. These are the *N*-chloro (=*N*-Cl) derivatives of, for example, sulphonamides giving compounds such as chloramine-T and dichloramine-T, and halazone (Figure 19.5), which may be used for the disinfection of contaminated drinking-water.

A second group of compounds, formed by *N*-chloro derivatization of heterocyclic compounds containing a nitrogen in the ring, includes the sodium and potassium salts of dichloroisocyanuric acid (e.g. NaDCC). These are available in granule or tablet form and, in contrast to hypochlorite, are very stable on storage if protected from moisture. In water they will give a known chlorine concentration. The antimicrobial activity of the compounds is similar to that of the hypochlorites when acidic conditions of use are maintained. It is, however, important to note that where inadequate ventilation exists, care must be taken not to apply the compound to acidic fluids or large spills of urine in view of the toxic effects of chlorine production. The HSE has set the occupational exposure standard (OES) short-term exposure limit at 1 ppm (see also section 2.5).

3.5.4 Chloroform

Chloroform (trichloromethane, $CHCl_3$) has a narrow spectrum of activity. It has been used extensively as a

preservative of pharmaceuticals since the 19th century, although more recently it has had limitations placed on its use. Marked reductions in concentration may occur through volatilization from products, resulting in the possibility of microbial growth.

3.5.5 Iodine

Iodine has a wide spectrum of antimicrobial activity. Gram-negative and Gram-positive organisms, bacterial spores (on extended exposure), mycobacteria, fungi and viruses are all susceptible. The active agent is the elemental iodine molecule, I_2. As elemental iodine is only slightly soluble in water, iodide ions are required for aqueous solutions such as Aqueous Iodine Solution, BP 1988 (Lugol's Solution) containing 5% iodine in 10% potassium iodide solution. Iodine (2.5%) may also be dissolved in ethanol (90%) and potassium iodide (2.5%) solution to give Weak Iodine Solution, BP 1988 (Iodine Tincture).

The antimicrobial activity of iodine is less dependent than chlorine on temperature and pH, although alkaline pH should be avoided. Iodine is also less susceptible to inactivation by organic matter. Disadvantages in the use of iodine in skin antisepsis are staining of skin and fabrics coupled with possible sensitizing of skin and mucous membranes.

3.5.6 Iodophors

In the 1950s iodophors (*iodo* meaning iodine and *phor* meaning carrier) were developed, to eliminate the disadvantages of iodine while retaining its antimicrobial activity. These allowed slow release of iodine on demand from the complex formed. Essentially, four generic compounds may be used as the carrier molecule or complexing agent. These give polyoxymer iodophors (i.e. with propylene or ethyene oxide polymers), cationic (quaternary ammonium) surfactant iodophors, non-ionic (ethoxylated) surfactant iodophors and polyvinylpyrrolidone iodophors (PVP-I or povidone-iodine). The non-ionic or cationic surface-active agents act as solubilizers and carriers, combining detergency with antimicrobial activity. The former type of surfactant, especially, produces a stable, efficient formulation, the activity of which is further enhanced by the addition of phosphoric or citric acid to give a pH below 5 on use-dilution. The iodine is present in the form of micellar aggregates which disperse on dilution, especially below the critical micelle concentration (cmc) of the surfactant, to liberate free iodine.

When iodine and povidone are combined, a chemical reaction takes place forming a complex between the two. Some of the iodine becomes organically linked to povidone, although the major portion of the complexed iodine is in the form of tri-iodide. Dilution of this iodophor results in a weakening of the iodine linkage to the carrier polymer with concomitant increases in elemental iodine in solution and antimicrobial activity.

The amount of free iodine the solution can generate is termed the 'available iodine'. This acts as a reservoir for active iodine, releasing it when required and therefore largely avoiding the harmful side effects of high iodine concentration. Consequently, when used for antisepsis, iodophors should be allowed to remain on the skin for 2 minutes to obtain full advantage of the sustained-release iodine.

Cadexamer-I_2 is an iodophor similar to povidone-iodine. It is a 2-hydroxymethylene cross-linked (1–4) α-D-glucan carboxymethyl ether containing iodine. The compound is used especially for its absorbent and antiseptic properties in the management of leg ulcers and pressure sores where it is applied in the form of microbeads containing 0.9% iodine.

3.6 Heavy metals

Mercury and silver have antibacterial properties and preparations of these metals were among the earliest used antiseptics; however, they have been largely replaced by less toxic compounds. Silver has enjoyed a renaissance recently as an antimicrobial frequently incorporated in urethral catheters for the prevention of device-related infection. Various forms of silver are employed such as nanoparticulate silver, silver halides, silver oxide and combinations such as silver–palladium. A hard surface disinfectant formulation based on silver dihydrogen citrate is shown to be effective against a wide range of bacteria, fungi and viruses using as little as 30 ppm silver.

3.6.1 Mercurials

The organomercurial derivatives thiomersal and phenylmercuric nitrate or acetate (PMN or PMA) (Figure 19.6) have been primarily employed as preservatives. Use

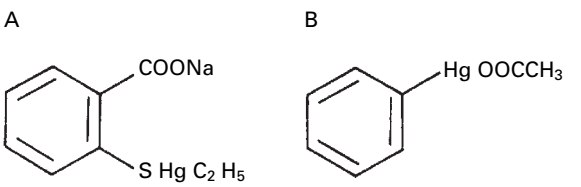

Figure 19.6 Organomercurials: (A) thiomersal (sodium ethylmercurithiosalicylate); (B) phenylmercuric acetate.

of both compounds has declined considerably as a result of concerns about mercury toxicity and risk of hypersensitivity or local irritation. They are absorbed from solution by rubber closures and plastic containers to a significant extent.

3.7 Hydrogen peroxide and peroxygen compounds

Hydrogen peroxide and peracetic acid are high-level disinfectants because of their production of the highly reactive hydroxyl radical. They have the added advantage that their decomposition products are non-toxic and biodegradable. The germicidal properties of hydrogen peroxide (H_2O_2) have been known for more than a century, but use of low concentrations of unstable solutions did little for its reputation. However, stabilized solutions are now available and because of its unusual properties and antimicrobial activity, hydrogen peroxide has a valuable role for specific applications. Its activity against the protozoan *Acanthamoeba*, which can cause keratitis in contact lens wearers, has made it popular for disinfection of soft contact lenses. Concentrations of 3–6% are effective for general disinfection purposes. At high concentrations (up to 35%) and increased temperature, hydrogen peroxide is sporicidal. Use has been made of this in vapour-phase hydrogen peroxide decontamination of equipment and enclosed spaces (Chapter 21).

Peracetic acid (CH_3COOOH) is the peroxide of acetic acid and is a more potent biocide than hydrogen peroxide, with excellent rapid biocidal activity against bacteria, including mycobacteria, fungi, viruses and spores. It can be used in both the liquid and vapour phases and is active in the presence of organic matter. It is finding increasing use at concentrations of 0.2–0.35% as a chemosterilant of medical equipment such as flexible endoscopes. Its disadvantages are that it is corrosive to some metals. It is also highly irritant and must be used in an enclosed system. The combination of hydrogen peroxide and peracetic acid is synergistic and is marketed as a cold sterilant for dialysis machines.

3.8 Phenols

Phenols (Figure 19.7) are widely used as disinfectants and preservatives. They have good antimicrobial activity and are rapidly bactericidal but generally are not sporicidal. Their activity is markedly diminished by dilution and is also reduced by organic matter. They are more active at acid pH. Major disadvantages include their caustic effect on skin and tissues and their systemic toxicity. The more highly substituted phenols are less toxic and can be used as preservatives and antiseptics; however, they are also less active than the simple phenolics, especially against Gram-negative organisms. To improve their poor aqueous solubility, phenolic disinfectants are often formulated with soaps, synthetic detergents, and/or solvents.

3.8.1 Phenol (carbolic acid)

Phenol (Figure 19.7A) no longer plays any significant role as an antibacterial agent. It is largely of historical interest, as it was used by Lister in the 1860s as a surgical antiseptic and has been a standard for comparison with other disinfectants in tests such as the Rideal–Walker test.

3.8.2 Clear soluble fluids, black fluids and white fluids

Phenols obtained by distillation of coal or petroleum can be separated by fractional distillation according to their boiling point range into phenols, cresols, xylenols and high boiling point tar acids. As the boiling point increases bactericidal activity increases and tissue toxicity decreases, but there is increased inactivation by organic matter and decreased water solubility.

Clear soluble fluids are produced from cresols or xylenols. The preparation known as Lysol (Cresol and Soap Solution BP 1968) is a soap-solubilized formulation of cresol (Figure 19.7B) that has been widely used as a general-purpose disinfectant but has largely been superseded by less irritant phenolics. A higher boiling point fraction consisting of xylenols and ethylphenols (Figure 19.7C and D) produces a more active, less corrosive product that retains activity in the presence of organic matter. A variety of proprietary products for general disinfection purposes are available.

Black fluids and white fluids are prepared by solubilizing the high boiling point tar acids. Black fluids are homogeneous solutions that form an emulsion on dilution with water, whereas white fluids are finely dispersed stable emulsions. Both types of fluid have good bactericidal activity. Preparations are very irritant and corrosive to skin; however, they are relatively inexpensive and are useful for household and general disinfection purposes.

3.8.3 Synthetic phenols

Many derivatives of phenol are now made by a synthetic process. A combination of alkyl or aryl substitution and halogenation of phenolic compounds has produced useful derivatives. Two of the best known chlorinated derivatives are *p*-chloro-*m*-cresol (chlorocresol, Figure 19.7E) which was frequently employed as a preservative at a concentration of 0.1%, and *p*-chloro-*m*-xylenol

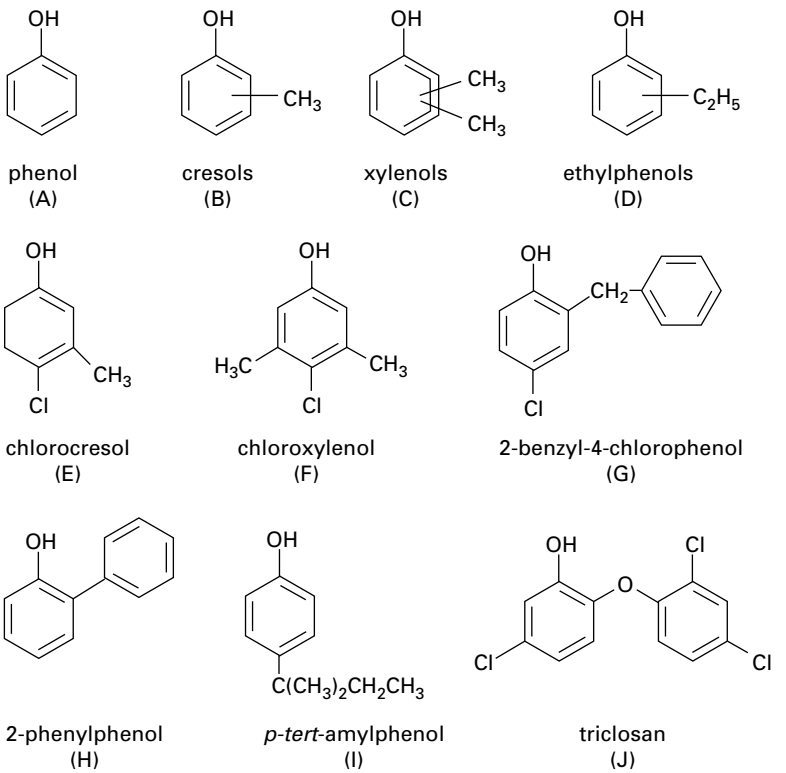

Figure 19.7 Structures of some common phenols possessing antimicrobial activity.

(chloroxylenol, Figure 19.7F) which is sometimes used for skin disinfection. Chloroxylenol is sparingly soluble in water and must be solubilized, for example, in a suitable soap solution in conjunction with terpineol or pine oil. Its antimicrobial capacity is weak and is reduced by the presence of organic matter. Other phenol derivatives of note are: 2-benzyl-4-chlorophenol (Figure 19.7G), 2-phenylphenol (Figure 19.7H) and *p*-tert-amylphenol (Figure 19.7I).

3.8.4 Bisphenols

Bisphenols are composed of two phenolic groups connected by various linkages. Triclosan (Figure 19.7J) is the most widely used. It has been incorporated into medicated soaps, lotions and solutions and is also included in household products such as plastics and fabrics. There is some concern about bacterial resistance developing to triclosan.

3.9 Surface-active agents

Surface-active agents or *surfactants* are classified as anionic, cationic, non-ionic or ampholytic according to the ionization of the hydrophilic group in the molecule. A hydrophobic, water-repellent group is also present. Within the various classes a range of detergent and disinfectant activity is found. The anionic and non-ionic surface-active agents, for example, have strong detergent properties but exhibit little or no antimicrobial activity. They can, however, render certain bacterial species more sensitive to some antimicrobial agents, possibly by altering the permeability of the outer envelope. Ampholytic or amphoteric agents can ionize to give anionic, cationic and zwitterionic (positively and negatively charged ions in the same molecule) activity. Consequently, they display both the detergent properties of the anionic surface-active agents and the antimicrobial activity of the cationic agents. They are used quite extensively in Europe for presurgical hand-scrubbing, medical instrument disinfection and floor disinfection in hospitals.

Of the four classes of surface-active agents the cationic compounds play the most important role in an antimicrobial context.

3.9.1 Cationic surface-active agents

The cationic agents used for their antimicrobial activity all fall within the group known as the quaternary ammo-

nium compounds (QACs, quats or onium ions). These are organically substituted ammonium compounds (Figure 19.8A) where the R substituents are alkyl or heterocyclic radicals to give compounds such as benzalkonium chloride (Figure19.8B), cetyltrimethylammonium bromide (cetrimide) (Figure 19.8C) and cetylpyridinium chloride (Figure 19.8D). Inspection of the structures of these compounds (Figure 19.8B and C) indicates that a chain length in the range C_8–C_{18} in at least one of the R substituents is a requirement for good antimicrobial activity. In the pyridinium compounds (Figure 19.8D), three of the four covalent links may be satisfied by the nitrogen in a pyridine ring. Several 'generational' changes have arisen in the development of QACs. Compounds such as alkyldimethylbenzyl ammonium chloride, alkyldimethylethylbenzyl ammonium chloride and didecyldimethylammonium chloride have roles in disinfection where HIV and HBV are present. Polymeric quaternary ammonium salts such as polyquaternium 1 are finding increasing use as preservatives.

The QACs are most effective against microorganisms at neutral or slightly alkaline pH and become virtually inactive below pH 3.5. Not surprisingly, anionic agents greatly reduce the activity of these compounds. Incompatibilities have also been recorded with non-ionic agents, possibly due to the formation of micelles. The presence of organic matter such as serum, faeces and milk will also seriously affect activity.

QACs exhibit greatest activity against Gram-positive bacteria, with a lethal effect observed using concentrations as low as 0.0005%. Gram-negative bacteria are more resistant, requiring a level of 0.0033%, or higher still if *Ps. aeruginosa* is present. A limited antifungal activity is exhibited and they have no useful sporicidal activity. This relatively narrow spectrum of activity limits the usefulness of the compounds, but as they are generally well tolerated and non-toxic when applied to skin and mucous membranes they have considerable use in treatment of wounds and abrasions. Benzalkonium chloride and cetrimide are employed extensively in surgery, urology and gynaecology as aqueous and alcoholic solutions and as creams. In many instances they are used in conjunction with a biguanide disinfectant such as chlorhexidine. The detergent properties of the QACs also provide a useful activity, especially in hospitals, for general environmental cleaning.

3.10 Other antimicrobials

The full range of chemicals that can be shown to have antimicrobial properties is beyond the scope of this chapter. The agents included in this section have limited use or are of historic interest.

3.10.1 Diamidines

The activity of diamidines is reduced by acid pH and in the presence of blood and serum. Propamidine and dibromopropamidine, as the isethionate salts, have been employed as antimicrobial agents in eye drops (0.1%) for amoebic infection and for topical treatment of minor infections.

3.10.2 Dyes

Crystal violet (Gentian violet), brilliant green and malachite green are triphenylmethane dyes used to stain bacteria for microscopic examination. They have a static activity but are no longer applied topically for the treatment of infections because of carcinogenicity.

The acridine dyes acriflavine and aminacrine have been employed for skin disinfection and treatment of infected wounds or burns but are slow acting and mainly bacteriostatic.

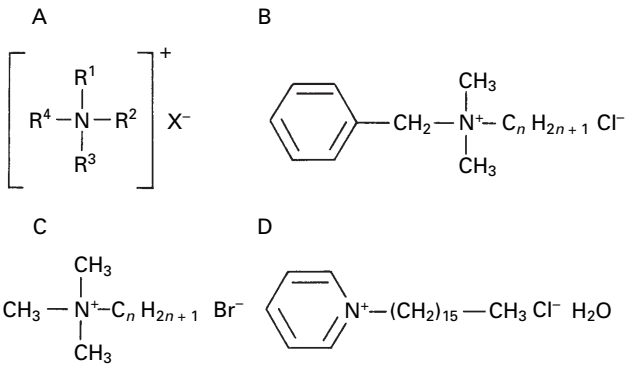

Figure 19.8 Quaternary ammonium compounds (QACs): (A) general structure of QACs; (B) benzalkonium chloride (n = 8–18); (C) cetrimide (n = 12–14 or 16); (D) cetylpyridinium chloride.

3.10.3 Quinoline derivatives

The quinoline derivatives of pharmaceutical interest are little used now. The compound most frequently used is dequalinium chloride, a bisquaternary ammonium derivative of 4-aminoquinaldinium which was formulated as a lozenge for the treatment of oropharyngeal infections.

3.11 Antimicrobial combinations and systems

There is no ideal disinfectant, antiseptic or preservative. All chemical agents have their limitations in terms of either their antimicrobial activity, resistance to organic matter, stability, incompatibility, irritancy, toxicity or corrosivity. To overcome the limitations of an individual agent, formulations consisting of combinations of agents are available. For example, ethanol and isopropanol have been combined with chlorhexidine, QACs, sodium hypochlorite and iodine to produce more active preparations. The combination of chlorhexidine and cetrimide is also considered to improve activity. QACs and phenols have been combined with glutaraldehyde and formaldehyde so that the same effect can be achieved with lower, less irritant concentrations of the aldehydes. Some combinations are considered to be synergistic, e.g. hydrogen peroxide and peroxygen compounds. Care must be taken in deciding on disinfectant combinations, as the concentration exponents associated with each component of a disinfectant combination will have a considerable effect on the degree of activity (Chapter 18).

Research into the resistance of microbial biofilms provides potential for improving elimination of this problematical microbial mode of growth. Bacteria often use a communication system, quorum sensing (QS), to regulate virulence factor production and the formation of biofilms. Increased understanding of how chemicals can block QS could help provide effective prevention and elimination of biofilm-related infection. The incorporation of antimicrobial agents into materials that form working and contact surfaces or those of medical devices and implants has been positive but much further developmental research is required. Such 'bioactive' surfaces can be formed, for example, by incorporation of silver salts and alloys, biguanides and triclosan, and have the ability to reduce infection arising from microbial adherence and biofilm formation.

Other means are available to potentiate the activity of disinfectants. Ultrasonic energy in combination with suitable disinfectants such as aldehydes and biguanides has been demonstrated to be useful in practice and ultraviolet radiation increases the activity of hydrogen peroxide. Superoxidized water provides an extremely active disinfectant with a mixture of oxidizing species produced from the electrolysis of saline. The main products are hypochlorous acid (144 mg/L) and free chlorine radicals. The antimicrobial activity is rapid against a wide range of microorganisms in the absence of organic matter.

4 Disinfection policies

The aim of a disinfection policy is to control the use of chemicals for disinfection and antisepsis and give guidelines on their use. The preceding descriptions within this chapter of the activities, advantages and disadvantages of the many disinfectants available allow considerable scope for choice and inclusion of agents in a policy to be applied to such areas as industrial plant, walls, ceilings, floors, air, cleaning equipment and laundries and to the extensive range of equipment in contact with hospital patients.

The control of microorganisms is of prime importance in hospital and industrial environments. Where pharmaceutical products (either sterile or non-sterile) are manufactured, contamination of the product may lead to its deterioration and to infection in the user. In hospital there is the additional consideration of patient care, therefore protection from nosocomial (hospital-acquired) infection and prevention of cross-infection must also be covered. Hospitals will have a disinfection policy and the degree of adherence to, and implementation of, the policy content will require stringent monitoring. A specialized infection control committee or similar, comprising a number of specialized personnel such as the pharmacist, the consultant medical microbiologist and senior nurse responsible for infection control, should formulate a suitable policy (Chapter 16). This core team may usefully be expanded to include, for example, a physician, a surgeon, nurse teachers and nurses from several clinical areas, the sterile services manager and the domestic superintendent; purchasing. This expanded committee will meet regularly to help with the implementation of the policy and reassess its efficiency. Tables 19.2–19.4 indicate the susceptibility of various microorganisms to the range of agents available and Table 19.6 presents examples of the range of formulations available. Although scope exists for choice of disinfectant in many of the areas covered by a policy, in certain instances specific recommendations are made as to the type, concentration and usage of disinfectant.

Categories of risk (to patients) may be assigned to equipment coming into contact with a patient, dictating

Table 19.7 Disinfection policies—classification of equipment according to risk

Risk level	Examples	Classification	Objective	Decontamination
High risk: critical items	Surgical instruments, implants, catheters	Objects which enter a sterile tissue or system	Sterility—all microorganisms killed including bacterial spores	Thermal or gaseous sterilization preferable. Chemical sterilization with aldehyde or peroxygen with extensive contact times
Intermediate risk: semicritical items	Endoscopes, cystoscopes, respiratory and anaesthesia equipment	Objects in contact with mucous membranes or broken skin	Free of all viable microorganisms except bacterial spores	High level disinfection with aldehyde or peroxygen; contact times up to 30 min
Low risk: non-critical items	Blood pressure cuffs, food utensils, furniture, floors	Objects in contact with intact skin but not mucous membranes	Some microorganisms remaining	Low-level disinfection using alcohols, chlorine, iodophor, QACs

QAC, quaternary ammonium compound.

the level of decontamination required and degree of concern (Table 19.7). *High-risk* (critical) items have close contact with broken skin or mucous membranes or are those introduced into a sterile area of the body and should, themselves, be sterile; they include instruments, gloves, catheters, syringes and needles. Liquid chemical disinfectants should only be used if heat or other methods of sterilization are unsuitable. *Intermediate-risk* (semicritical) items are in close contact with skin or mucous membranes and disinfection will normally be applied. Endoscopes, respiratory and anaesthetic equipment, wash bowls, bed-pans and similar items are included in this category. *Low-risk* (non-critical) items or areas include those detailed earlier such as walls and floors, which are not in close contact with the patient. Cleaning is obviously important with disinfection being required, for example, in the event of contaminated spillage.

5 References and further reading

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20 Non-antibiotic antimicrobial agents: mode of action and resistance

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1 Introduction

The group of agents which comprises antiseptics, disinfectants, chemical sterilants and preservatives (often collectively called biocides) have frequently been classified as non-specific protoplasmic poisons. Such a broad generalization is, however, far from the true position.

It is often convenient to consider the modes of action of biocides in terms of their targets within the bacterial cell, in particular the region of the cell in which their activity is deemed to predominate. Thus agents have been described variously as cell wall, membrane and cytoplasm-active. This characterization, whilst having the benefits of simplicity, does not necessarily describe their mechanism of action; this is best classified by effects on functional structures and cellular processes. The range and complexity of the reactions involved will become apparent from this account and Table 20.1, and it is worth emphasizing here that many of these substances exhibit concentration-dependant dual or even multiple effects. More detailed treatments of the subject will be found in the references at the end of this chapter.

Hugo and Russell's Pharmaceutical Microbiology, Eighth Edition. Edited by Stephen P. Denyer, Norman Hodges, Sean P. Gorman, Brendan F. Gilmore.

Table 20.1 Cellular targets for non-antibiotic antibacterial agents

Target site		Biocide								
		Alcohols	Anilides (TCS, TCC)	Bronopol	Biguanides (chlorhexidine, PHMB)	Ethylene/ propylene oxide	Formaldehyde	Glutaraldehyde	Hexachlorophane	Hydrogen peroxide, peracetic acid
1	Cell wall						+	+		
2	Cytoplasmic membrane									
2.1	Action on membrane potentials		+						+	
2.2	Action on membrane enzymes					++		++		+
2.2.1	Electron transport chain								+	
2.2.2	Adenosine triphosphatase				+					
2.2.3	Enzymes with thiol groups			+		+		++		+
2.3	Action on general membrane permeability	+	+		+					
3	Cytoplasm									
3.1	General coagulation	+++			+++			++	+++	
3.2	Ribosomes									+
3.3	Nucleic acids					+				+
3.4	Thiol groups			+		+		+		+
3.5	Amino groups					+	++	+		+

(*continued*)

Table 20.1 (*continued*)

Target site		Biocide								
		Hypochlorites, chlorine-releasers	Isothiazolones	Mercury II salts, organic mercurials	Orthophthaldehyde	Parabens	Phenols	β-propiolactone	QACs	Silver salts
1	Cell wall	+		+			+			
2	Cytoplasmic membrane									
2.1	Action on membrane potentials					+	+			
2.2	Action on membrane enzymes	+			++					
2.2.1	Electron transport chain									
2.2.2	Adenosine triphosphatase									
2.2.3	Enzymes with thiol groups	+	+	+	++			+		+
2.3	Action on general membrane permeability					+	++		+	
3	Cytoplasm									
3.1	General coagulation			+++	+++		+++		+++	+++
3.2	Ribosomes			+						
3.3	Nucleic acids	+						+		
3.4	Thiol groups	+		+	++			+		+
3.5	Amino groups	+			+					

Crosses, indicating activity, which appear in several rows for a given compound, demonstrate the multiple actions for the compound concerned. This activity is nearly always concentration-dependent, and the number of crosses indicates the order of concentration at which the effect is elicited, i.e. +, elicited at low concentrations, +++, elicited at high concentrations;

QACs, quaternary ammonium compounds; PHMB, polyhexamethylene biguanides; TCS, tetrachlorosalicylanilide; TCC, trichlorocarbanilide.

2 Mechanisms of interaction

For a chemical to exhibit antimicrobial activity it usually has to undergo a sequence of events that begins with adsorption on to the microbial cell surface. This initial uptake is a physicochemical phenomenon which can be generally characterized into one of several uptake isotherms (Figure 20.1); it bears a relationship to the concentration exponent (Chapter 19) which describes the influence of concentration on activity (Table 20.2). In the many cases where the chemical has an intracellular site of action, adsorption must be followed by passage through porin channels in Gram-negative cells (Chapter 3), diffusion across, or into, the lipid-rich cytoplasmic membrane, and finally, interaction with proteins, enzymes, nucleic acids or other targets within the cytoplasm. These processes are markedly influenced by the physicochemical characteristics of the biocide, e.g. ionization constant and lipid solubility, so the wide diversity of structures exhibited by biocide molecules (Chapter 19) complicates the prediction of antimicrobial potency and explanation of their mechanisms of action. Despite this, it is important to recognize that there is a basis upon which the mode of action might be deduced, because there are certain molecular features of biocides that are associated with activity against particular cellular targets.

3 Antimicrobial effects

Antimicrobial activity is often strongly influenced by the affinity of the biocide for structural or molecular components of the cell, and this, in turn, may depend upon the attraction of dissimilar charges or on hydrophobic interactions. Antimicrobial drugs whose active species is positively charged, e.g. quaternary ammonium compounds (QACs) and chlorohexidine, display an affinity for the negative charges of sugar residues on the microbial cell surface or phosphate groups on the membrane(s); adsorption of these biocides, and thus their antimicrobial activity, is increased as the pH rises and the cell surface becomes more electronegative. Antimicrobial chemicals possessing a long alkyl chain, on the other hand, may integrate into the hydrophobic region of phospholipid molecules within the membrane and so cause membrane disruption and fatal permeability changes. Further examples of structure–activity relationships are afforded by aldehydes, particularly glutaraldehyde, which is an electrophile that is able to react with molecules possessing thiol (SH) or amino groups, e.g. proteins. This reaction, too, increases with pH, so aldehydes are more active in alkaline conditions. Biocides containing heavy metal ions, e.g. silver or mercury, also damage or inactivate enzymes and structural proteins by virtue of

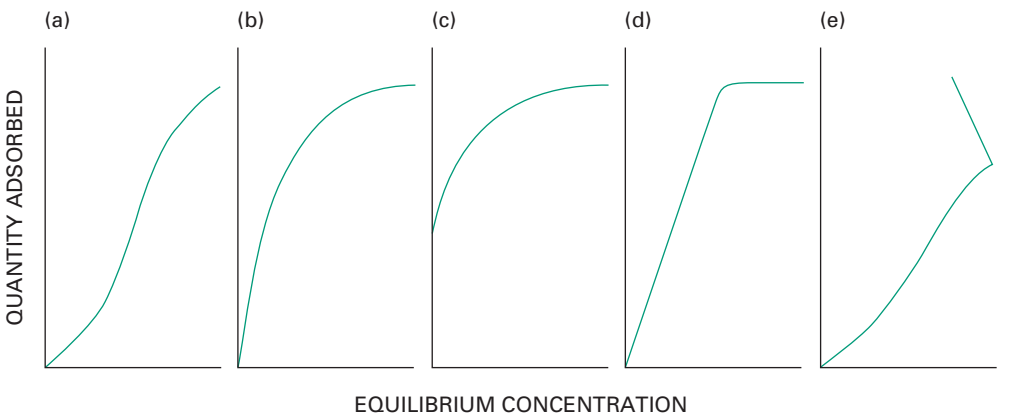

Figure 20.1 Typical uptake isotherms associated with the initial biocide-bacterium interaction. (a) S-shape, cooperative sorption occurs as applied biocide concentration increases; (b) L-shape, Langmuir uptake with biocide molecules orientated at a fixed number of binding sites; (c) H-shape, special form of the L-shape isotherm indicative of high affinity uptake; (d) C-shape, constant partition of biocide from solution until bacterial surface is fully saturated; (e) Z-shape, enhanced uptake following breakdown in cell structure at a critical applied biocide concentration.

Table 20.2 Relationship between biocide concentration exponent, uptake isotherm and mechanism of interaction

Concentration exponent	Predominant uptake isotherm	Type of interaction
<2.0	H or L-shape	Strong chemical or ionic bonding with target site, frequently promoting cytoplasm leakage or membrane disruption; uptake limited by binding sites available. Typical examples include QACs and chlorhexidine
2.0–4.0	S-shape	Initial biocide uptake enhanced by cooperative adsorption, often with specific orientation of adsorbed molecules. A mixed group of biocides typically exercising both chemical and physical effects on cellular components; examples include some phenols and lipophilic acids
>4.0	C, S or Z-shape	Weakly physical interaction, often with partition into lipophilic components of the cell envelope; a plateau in uptake can be reached when the envelope is fully saturated although new sorption sites may be generated with some biocides at a critical applied concentration (Z-shape). Typical examples include phenols and 2-phenoxyethanol which are active as membrane disruptors and proton conductors

interactions with thiol groups. A number of phenols and bisphenols incorporate a hydroxyl group that is capable of generating a labile proton, i.e. they are weak acids. A weakly acidic nature combined with significant lipid solubility are properties associated with uncoupling agents, i.e. those molecules that can disrupt the proton-motive force that is responsible for oxidative phosphorylation in the cell. It is thought that these molecules dissolve in the lipid bilayer of the membrane and act as proton conductors by virtue of their ionizability (section 4.4.2). This property, possessed by biocides such as phenoxythanol and fentichlor, results in the failure of many important energy-requiring processes in the cell, including the concentration and retention of sugars and amino acids.

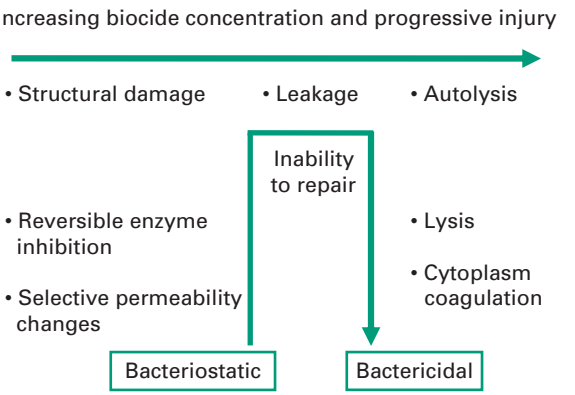

Figure 20.2 Antibacterial events: progression from bacteriostatic to bactericidal lesions.

4 Mechanisms of action

In any consideration of mechanism of action, due regard should be given to the initial health of the organism, duration of contact with the biocide, and the concentration of biocide employed. Antibacterial effects may progress from early, sublethal events to multiple lesions of bactericidal consequence. Figure 20.2 identifies events in order of severity, but should not be interpreted as defining the normal progression of cell injury. As disclosed in the following sections, the biocide interaction may induce particular lesions over others; this will most certainly be in a concentration-dependant manner.

4.1 Oxidation reactions

Biocides with oxidizing (electron-withdrawing) ability are widely used as disinfectants and chemical sterilants, and include the halogens (chlorine, hypochlorites, bromine, iodophors) and peroxygens (hydrogen peroxide, peracetic acid and chlorine dioxide). They can exert specific effects on essential microbial macromolecules

causing, variously: strand breakage and adduct formation on DNA and RNA with disruption of replication, transcription and and translocation processes; degradation of, particularly, unsaturated fatty acids leading to loss of membrane fluidity and subsequent reduced functionality of membrane-bound proteins; and specific modifications to amino acid residues, most notably disulphide bonds, leading to changes in protein primary structure and conformation with consequent disruption of structural enzymic functions. An accumulation of these effects can be particularly devastating to the microbial cell.

4.2 Cross-linking reactions

The aldehydes formaldehyde, glutaraldehyde and orthophthalaldehyde, and the sterilant alkylating agents ethylene oxide and propylene oxide, are both highly reactive chemical classes. The alkylating agents exhibit particularly strong reactions with guanine residues causing cross-linking between DNA strands, inhibiting DNA unwinding and RNA translation. The amino, carboxyl, sulphydryl and hydroxyl groups of structural or enzymic proteins are also susceptible to alkylation, causing cross-links between adjacent amino acid chains and also with other amino acid-containing structures such as peptidoglycan. The aldehydes are generally more specific with greatest effect against the amino groups of surface-exposed lysine or hydroxylysine residues of proteins, again causing extensive cross-linking.

In all instances, progressive cross-linking leads to macromolecule malfunction causing inhibition or arrest of essential cell functions. It is safe to say that there is no single fatal reaction but that death results from the accumulated effect of many reactions in a manner similar to oxidizing agents (section 4.1).

4.3 Coagulation

The cross-linking reactions identified in section 4.2 give rise to macromolecule denaturation which can be recognized under electron microscopy as intracellular coagulation. Coagulative effects are not unique to aldehydes and alkylating agents, however, and high concentrations of disinfectants such as chlorhexidine, phenol, ethanol and mercuric salts will also coagulate the cytoplasm. This most likely arises from the precipitation of protein caused by a variety of interactions including ionic and hydrophobic bonding and the disruption of hydrogen bonds.

4.4 Disruption of functional structures

The integrity and functions of the bacterial cell are dependant upon critical macromolecular structural arrangements including within the cell wall and cytoplasmic membrane (Chapter 3). A number of biocides can have a profound effect on these organelles.

4.4.1 Cell wall

This structure is the traditional target for a group of antibiotics which includes the penicillins (Chapter 11), but a little-noticed report which appeared in 1948 showed that low concentrations of disinfectant substances caused cell wall lysis such that a normally turbid suspension of bacteria became clear. It is thought that these low concentrations of disinfectant cause enzymes whose normal role is to synthesize the cell wall to reverse their role in some way and effect its disruption or lysis. In the original report, these low concentrations of disinfectants (formalin, 0.12%; phenol, 0.32%; mercuric chloride, 0.0008%; sodium hypochlorite, 0.005% and merthiolate, 0.0004%) caused lysis of *Escherichia coli*, streptococci, and staphylococci.

Divalent cations, in addition to their role as enzyme cofactors, also stabilize cell wall, membrane and ribosomal structures. In particular, magnesium serves to link the lipopolysaccharide (LPS) of Gram-negative bacteria to the outer membrane. Chelators, particularly ethylenediamine tetraacetic acid (EDTA), have been used to disrupt this link and cause the release of LPS into the medium. The loss of outer membrane integrity and subsequent permeabilization has been exploited in the potentiation of biocides, including combinations of EDTA with chloroxylenol, cetrimide, phenylethanol and the parahydroxy benzoic acid esters (Chapter 19).

4.4.2 Cytoplasmic membrane

The bacterial cytoplasmic membrane consists of an impermeable, negatively-charged, fluid phospholipid bilayer incorporating an organized array of membrane-associated proteins. Through the membrane-bound electron transport chain aerobically, or the membrane-bound adenosine triphosphatase (ATPase) anaerobically, the bacterium succeeds in maintaining a transmembrane gradient of electrical potential and pH such that the interior of the cell is negative and alkaline. This *proton motive force*, as it is called, drives a number of energy-requiring functions which include the synthesis of ATP, the coupling of oxidative processes to phosphorylation, a metabolic sequence called *oxidative phosphorylation,* and the transport and concentration in the cell of metabolites such as sugars and amino acids. This, put briefly, is the basis of the chemiosmotic theory linking metabolism to energy-requiring processes.

Certain chemical substances have been known for many years to uncouple oxidation from phosphorylation and to inhibit active transport, and for this reason they are named *uncoupling agents*. They are believed to act by partitioning into the membrane and rendering it permeable to protons, hence short-circuiting the potential gradient or protonmotive force. Some examples of antibacterial agents which owe at least a part of their activity to this ability are tetrachlorosalicylanilide (TCS), tricarbanilide, trichlorocarbanilide (TCC), pentachlorophenol, di-(5-chloro-2-hydroxyphenyl) sulphide (fentichlor), 2-phenoxyethanol, and lipophilic acids and esters.

The membrane, as well as providing a dynamic link between metabolim and transport, serves to maintain the pool of metabolites within the cytoplasm. A general increase in membrane permeability brought about by the association and likely insertion of biocide molecules into the lipid bilayer was recognized early as being one effect of many disinfectant substances.

Treatment of bacterial cells with appropriate concentrations of such substances as cetrimide and other QACs, chlorhexidine, polyhexamethylene biguanides, phenol and hexylresorcinol causes a leakage of a group of characteristic chemical species. The potassium ion, being a small entity, is the first substance to appear when the cytoplasmic membrane is damaged. Amino acids, purines, pyrimidines and pentoses are examples of other substances which will leak from treated cells. If the action of the drug is not prolonged or exerted only in low concentration, the damage may be reversible and leakage may only induce bacteriostasis. There is however, evidence that a depletion of intracellular potassium caused by membrane damage can lead to the activation of latent ribonucleases and the consequent breakdown of RNA. Several biocides, including cetrimide and some phenols, are known to cause the release of nucleotides and nucleosides following an autolytic process. This is irreversible and has been proposed as an autocidal (suicide) process, committing the injured cell to death (Denyer & Stewart, 1998).

Surface-associated proteins within the membrane fulfil a number of important roles including wall biosynthesis, nutrient transport and respiration. Usually enzymes, these macromolecules are often topologically organized and uniquely exposed to disruption by biocidal agents. Thus, hexachlorophane inhibits the electron transport chain in bacteria, chlorhexidine has been shown to inhibit ATPase, and thiol-containing membrane dehydrogenases are highly susceptible to mercury-containing antibacterials, silver, 2-bromo-2-nitropropan-1,3-diol (bronopol) and isothiazolinones.

5 Enhancing activity

Mention has already been made of the use of permeabilizing chelators to enhance the penetration of biocides to their target (section 4.4.1). Much effort has also been expended in the search for synergistic combinations of biocides which, when added together, will greatly amplify the bactericidal effect. While theoretically possible, and potentially predictable from mechanism of action studies, in practice this effect is elusive; combinations of phenylmercuric acetate with benzalkonium chloride, lipophilic weak acids with fatty alcohols, and chlorocresol with phenylethanol have been reported. The most likely route to enhancing activity lies with optimizing biocide formulations to ensure maximum availability of the active moiety—particularly important in situations of pH-dependency or poor water solubility. In the area of pharmaceutical and cosmetic preservation, constructive use of formulation ingredients, each with some intrinsic antimicrobial activity, has successfully built on the activity of the original preservative agent to create a cumulative bactericidal effect, an approach called 'hurdle technology'.

6 Mechanisms of resistance to biocides

Bacterial resistance to biocides has been reported since the 1950s, notably with QACs, biguanides and phenolics. Overall there has been more documented evidence of bacterial resistance to antiseptics than to disinfectants (Table 20.3). It is worth mentioning that some bacteria surviving in biocidal formulations have been associated with outbreaks and pseudo-outbreaks of infection (Weber *et al.*, 2007). Bacterial resistance to all known preservatives has also been reported (Chapman *et al.*, 1998). Recently, much interest has focused on bacteria surviving high-level disinfection, which is usually employed for the disinfection of medical devices. Thus bacteria surviving exposure to the in-use (high) concentration of highly reactive biocides (e.g. glutaraldehyde, chlorine dioxide, hydrogen peroxide) have been isolated and studied. In 2009, a large outbreak of atypical mycobacteria in at least 38 hospitals in Brazil was reported. These mycobacteria were traced to endoscope contamination and were resistant to 2% w/v glutaraldehyde but also to the clinical concentration of front-line antibiotics against mycobacteria (Duarte *et al.*, 2009). This was the first time that biocide resistance was linked to antibiotic resistance, nosocomial infection and a large infection outbreak.

Table 20.3 Examples of bacterial resistance in biocidal products

Biocides	Resistant bacteria
Used for disinfection	
Ethanol	*Bacillus cereus**
Glutaraldehyde	*Mycobacterium chelonae**, *Methylbacterium mesophilicum**, atypical mycobacteria*
Formaldehyde	*Pseudomonas aeruginosa**, *Stenotrophomonas maltophilia*, *Klebsiella oxytoca**
QAC	*Burkholderia cepacia**, *Serratia marcescens**, *Achromobacter xylosoxydans**, *Pseudomonas aeruginosa**
Phenolics	*Pseudomonas* spp., *Pseudomonas aeruginosa**, *Alcaligenes faecalis*
Used for antisepsis	
Alcohols	*Bacillus cereus**, *Burkholderia cepacia*
Chlorhexidine	*Pseudomonas* spp., *Burkholderia cepacia*, *Flavobacterium meningosepticum*, *Serratia marcescens*, *Rastonia pickettii**, *Achromobacter xylosoxidans*
Chlorhexidine with cetrimide	*Pseudomonas multivorans, Stenotrophomonas maltophilia*
Benzalkonium chloride	*Pseudomonas* spp.*, *Achromobacter* spp., *Enterobacter aerogenes*, *Pseudomonas kingii*, *Burkholderia cepacia**, *Serratia marcescens, Mycobacterium chelonae, Mycobacterium abscessus*
Chloroxylenol	*Serratia marcescens*
Povidone iodine	*Burkholderia cepacia**, *Pseudomonas putida*
Polyoxamer-iodine	*Pseudomonas aeruginosa*
Triclosan	*Serratia marcescens*

* Bacteria associated with pseudo-outbreak or outbreak.
Adapted from Weber *et al.* (2007).

Over the last 10 years, much progress has been made in understanding the mechanisms conferring resistance to biocides in bacteria. Interestingly, some mechanisms that were thought to occur only with antibiotics have now been described with biocides.

6.1 General mechanisms

A number of mechanisms conferring some level of resistance to biocide exposure have been documented. Traditionally mechanisms of resistance have been divided into intrinsic and acquired resistance. *Intrinsic* (or *innate*) *resistance* is a natural property of the bacteria and provides some explanation as to why some bacteria are less susceptible than others. Intrinsic mechanisms often involve a structural difference, for example, a difference in the permeability of the bacterial membrane to biocides, but also the expression of chromosomal genes such as those encoding for an efflux pump or a degradative enzyme. *Acquired resistance* refers to the acquisition of a new property by the bacteria through mutation and genetic transfer; such a property can be the mutation of a target site or the transfer of a gene encoding for an efflux pump or a degradative enzyme. It should be noted that when gene transfer occurs, often several genes present on the same conjugative plasmid or transposon can be transferred to a recipient cell at the same time. In this case the term *co-resistance* is often used to denote the simultaneous acquisition of a number of genes conferring resistance to a number of antimicrobials, both biocides and antibiotics.

Bacterial resistance to a biocide arises often from the presence of several mechanisms that work together to

decrease the detrimental concentration of the biocide to a level that is no longer harmful for the bacterium (Table 20.4). The expression of only one mechanism confers low-level resistance often measured as an increase in minimum inhibitory concentration (MIC), but rarely high-level resistance, which can be measured as an increase in minimum bactericidal concentration. Finally, a distinction can be made between mechanisms expressed by a single bacterium and the mechanisms of resistance that arise from a community of bacteria such as in bacterial biofilms.

6.1.1 Changes in cell permeability

The decrease in biocide penetration arising from changes in cell permeability is well established and has been described with different bacterial genera, notably with Gram-negative bacteria and mycobacteria. It is also the case with bacterial endospores, which are discussed later in this chapter. In Gram-negative bacteria, the outer membrane, and notably the composition of LPS, offers some protection to the cell, by reducing biocide penetration. The role of LPS has been exemplified by researchers with the use of permeabilizing agents and notably ion chelators such as EDTA. EDTA contributes to the removal (loss) of LPS from the outer membrane by scavenging dications involved in the stabilization of LPS in the membrane (section 4.4.1). By losing LPS, the outer membrane becomes more permeable and biocides can penetrate better resulting in enhanced activity. A change in the structure of the outer membrane following a change in

Table 20.4 Possible mechanisms of bacterial resistance to biocides

Effect	Mechanisms	Biocides (examples)
Barrier to penetration	Cell envelope: spore coat; mycobacterial envelope (intrinsic)	QACs, biguanides, phenolics, aldehydes (except orthophthalaldehyde)
	Outer membrane: lipopolysaccharide; cation content (intrinsic/acquired/ environmental)	
	Porins: reduction in number, size (intrinsic/ acquired/ environmental)	QACs, biguanides
	Peptidoglycan/mycoylarabinogalactan (intrinsic)	Glutaraldehyde, QACs
	Biofilm: cell population—reduced diffusion	As above, plus oxidizing agents
Decreased accumulation	Efflux (intrinsic/acquired)	QACs, phenolics, chlorhexidine, silver
	Degradation/modification of the biocide (intrinsic/acquired)	Silver, organomercurials, glutaraldehyde, formaldehyde, phenolics, oxidizing agents
Adaptation	Modification of targets (acquired)	Triclosan
	Overproduction of targets/amplification (acquired)	Triclosan
By-pass metabolic activity	Increase in pyruvate synthesis and fatty acid production via an altered metabolic pathway (intrinsic/acquired)	Triclosan
Communication	Gene transfer	Possibly all
	Quorum sensing	Unknown
	Extracellular induction components	Triclosan, QAC
Selective pressure	Selection of insusceptible bacteria (changes in a complex population)	Triclosan, QAC, biguanides, aldehydes, oxidizing agents

QACs, quaternary ammonium compounds.
Adapted from Maillard (2007).

protein, fatty acid or phospholipid composition has been associated with a decrease in the efficacy of cationic biocides. In particular, a decrease in the number of porin proteins as a result of biocide exposure has been associated with high-level resistance to QACs in pseudomonads. Recently, a change in surface charge in *Pseudomonas aeruginosa* has been associated with a decrease in susceptibility to QACs.

In mycobacteria, the lipid-rich outer cell wall (responsible for the waxy appearance of the colonies), and particularly the presence of a mycoylacylarabinogalactan layer and the composition of the arabinogalactan/arabinomannan within the cell wall, account for a reduction in biocide penetration; increasing the permeability of the mycobacterial outer cell wall, for example with ethambutol, enhances the activity of biocides and antibiotics.

6.1.2 Efflux

Efflux pumps are cross-membrane proteins which pump out various substrates including biocides and antibiotics. A large number of efflux pumps in bacteria have been identified and have been divided into five main classes depending on their structure and activity: the small multidrug resistance (SMR) family; the major facilitator superfamily (MFS); the ATP-binding cassette (ABC) family; the resistance-nodulation-division (RND) family; and the multidrug and toxic compound extrusion (MATE) family (Figure 20.3).

The role of efflux pumps is to remove harmful substances from the bacterial cytoplasm, including biocides (e.g. QACs and phenolics), to levels that are not damaging for the cell. The quantity of antimicrobial pumped out depends upon the number of pumps present, their expression and efficacy. Some studies have shown that high-level resistance can be achieved by efflux, for instance against the bisphenol triclosan, but usually, the effect is an increase in MIC.

6.1.3 Enzymatic inactivation

Enzymatic degradation plays a role in reducing the harmful concentration of a biocide and has been observed with aldehydes (e.g. aldehyde dehydrogenase), oxidizing agents (e.g. catalases, superoxide dismutase, hydroxyperoxidases), phenolics and parabens. In the case of metallic salts such as silver, the ionic form is reduced to the inactive metal. The role of enzymatic inactivation has not been widely studied, but it is unlikely that a bacterial enzyme will contribute to high-level resistance to a biocide.

6.1.4 Modification of target site

To date, resistance conferred by the modification of a biocide target site has only been observed with triclosan. At a low concentration this bisphenol interacts specifically with a bacterial enoyl-acyl reductase carrier protein, which is involved in the synthesis of fatty acid. Triclosan has been shown to interact with a number of structurally related enzymes in many bacterial genera. A modification of the enzyme confers a low-level resistance to triclosan, although some studies claim that a high-level resistance has been observed. This is unlikely, since at a high concentration triclosan interacts with multiple target sites to bring about a bactericidal effect.

6.1.5 Change in metabolic pathway

A change in metabolic pathway that confers resistance to a biocide is a relatively new concept that was thought to occur only with sulphonamides. However, bacterial adaptation to triclosan, as measured by an extended lag phase of growth followed by a normal exponential phase, has been observed in several bacterial genera. The recent use of a microarray in *Salmonella enterica* serovar Typhimurium enabled the identification of a 'triclosan resistance network' including an alternative pathway to the production of pyruvate and fatty acid. In *Staphylococcus aureus* showing reduced sensitivity to triclosan, a change in lipid composition of the cell membrane was associated with altered expression of various genes involved in fatty acid metabolism. Low-level QAC resistance in *Serratia marcescens* may arise from a change in synthetic or metabolic pathways.

6.2 Induction of resistance

The induction of antimicrobial resistance in bacteria following biocide exposure is a relatively recent concern which is particularly pertinent to the increasing number of commercially available products containing a low concentration of a biocide. This low, often subinhibitory, concentration can induce the expression of resistance mechanisms that can confer bacterial survival to biocide and/or antibiotic exposure. Low-level resistance as measured by an increase in MIC has often been observed. Prior to the use of genomics, proteomics and metabolomics, induction of resistance in bacteria was observed with an increase in lag phase of growth and a decrease in growth rate, an overexpression of efflux pumps and the production of guanosine 5′-diphosphate 3′-diphosphate (ppGpp). An increase in DNA repair was also associated with an increase in bacterial survival following biocide exposure.

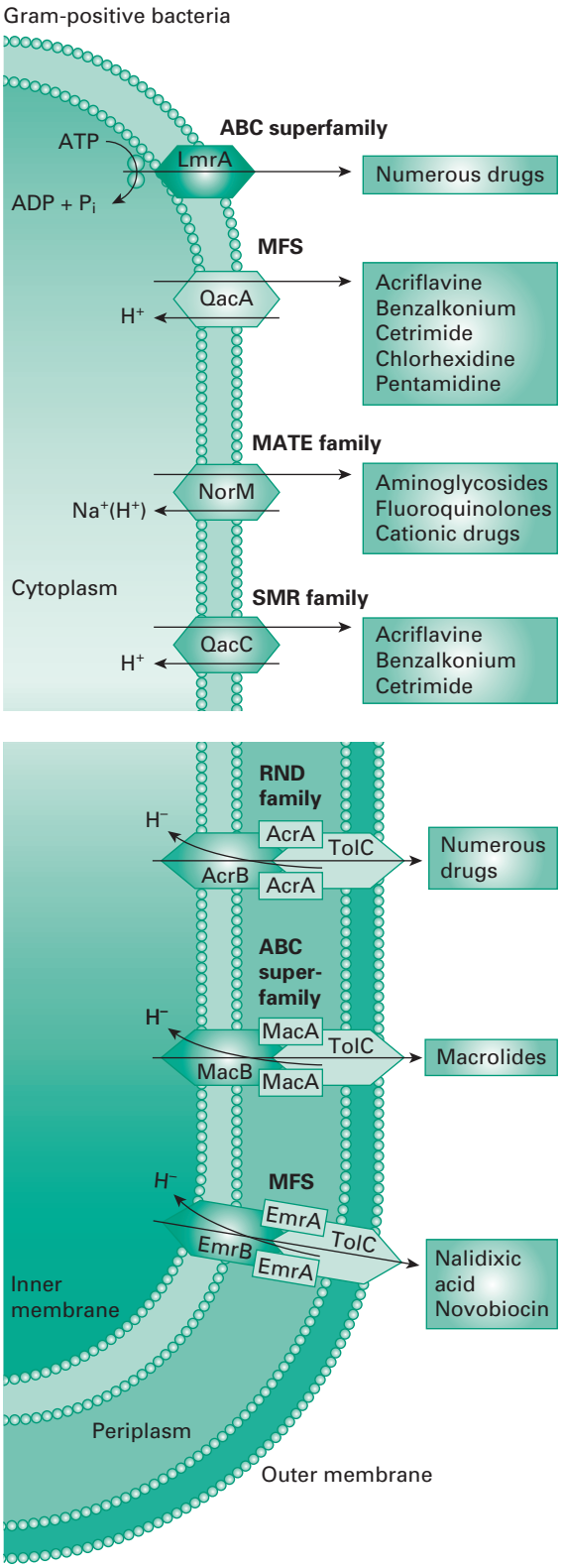

Figure 20.3 Examples of efflux pumps in bacteria. Note the wide range of substrates used by these pumps. (From Piddock, L.J.V. (2006) Multidrug resistance efflux pumps—not just for resistance. *Nat Rev Microbiol*, **4**, 629–636. Reprinted by permission from Macmillan Publishers Ltd, *Nature Reviews Microbiology*.)

More recently, a change in expression of regulons commanding a number of responses, such as a stress type of response (and repair mechanism), increased efflux, change in membrane composition, and a change in metabolic and synthetic pathways, has been recorded following exposure to a low concentration of biocides. Such a global response is of concern as it also confers a decreased susceptibility to antibiotics, and recent evidence suggests it might also lead to overexpression of virulence determinants.

It can be noted that the concentration of a biocide that promotes mutation in bacteria might be low. Indeed, a number of investigations have observed that the mutation rate increases in the presence of an active efflux system. The effect of bacterial mutation in the development of resistance has not been widely investigated, with the exception of triclosan. It is also possible that biocides that interact with the bacterial genome (e.g. dyes, oxidizing agents) might produce a higher mutation rate.

6.3 Dissemination of resistance

Surprisingly, the dissemination of biocide resistance mechanisms between bacteria has been little studied. The acquisition of new genetic determinants, notably by the process of conjugation, is of concern as it is often dependent on the presence of large transferable plasmids, and transposons which encode for many genes including bacterial resistance to antibiotics and virulence factors. When several resistance genes are transferred at the same time, the term coresistance is used. For example, the resistance to the QAC benzalkonium chloride in *Staphylococcus aureus* has been associated with the presence of plasmids containing *qac*, *bla* and *tet* resistant genes (encoding for efflux pumps and a β-lactamase). A number of mechanisms of resistance such as efflux and degradative enzymes have been documented to be transferred between bacteria. The extent of such dissemination is difficult to measure, although it is thought to readily occur in bacterial communities such as a biofilm.

Equally important to the horizontal transfer of resistance is the maintenance of resistant determinants (plasmids) following the continuous presence of biocides. Although this issue has not been widely studied, it is of interest with the use of biocidal products which are documented to leave a residual concentration of biocide on surfaces, antimicrobial surfaces, and the continuous presence of biocides in certain applications, such as drinking-water chlorination.

6.4 Bacterial spores

The formation of a spore is a mechanism of bacterial survival when growth conditions are detrimental for the vegetative form. Such adverse conditions include lack of food but also the presence of biocides and other detrimental physical and chemical conditions. The spore structure is unique and confers upon the spore high-level resistance to biocides. Hence only a few biocides, mainly highly reactive ones such as aldehydes, oxidizing agents and chlorine-releasing agents, are sporicidal, while others such as biguanides, QACs and phenolics are sporostatic despite their bactericidal effect on the vegetative bacteria. It should also be noted that sporicides take time to kill spores, usually a minimum of 5 minutes contact, and at a high concentration; aldehydes such as glutaraldehyde and formaldehyde need a much longer contact time and, in the case of orthophthalaldehyde, a raised temperature.

6.4.1 Sporulation and germination

Sporulation, a process in which a bacterial spore develops from a vegetative cell (Chapter 3), involves seven stages (I–VII); of these, stages IV–VII (cortex and coat development) are the most important in relation to the development of biocide resistance. Resistance to biocidal agents develops during sporulation and may be an early, intermediate or late/very late event. For example, resistance to chlorhexidine occurs at an intermediate stage, at about the same time as heat resistance, whereas decreasing susceptibility to glutaraldehyde is a very late event.

Bacillus spore coatless mutants and chemically-induced coatless spores have shown the role of the spore coats in limiting access of biocides to the spore core. The cortex also acts as a barrier to some extent.

During germination and/or outgrowth, metabolism and biosynthetic processes increase and cells regain their sensitivity to antibacterial agents. Some inhibitors act at the germination stage (e.g. phenolics, parabens), whereas others such as chlorhexidine and the QACs do not affect germination but inhibit outgrowth. Glutaraldehyde, at low concentrations, is an effective inhibitor of both stages.

6.4.2 Spore structure

The spore structure (Chapter 3) and the interaction between a biocide and the spore have been particularly well documented in the genus *Bacillus*. The spore core (protoplast, sometimes refered to as the germ cell) is enclosed within a cell wall which is surrounded by the

cortex and several spore coats. Sometimes an exosporium may surround the spore.

The spore core is the target site of sporicides since it is the location of RNA, DNA, dipicolinic acid (DPA) and most of the calcium, potassium, manganese and phosphorus. Also present are substantial amounts of low molecular weight basic proteins, the small acid-soluble spore proteins (SASPs) which are rapidly degraded during germination. SASPs, comprising about 10–20% of the protein in the dormant spore, exist in two forms (α/β and γ) and are essential for expression of spore resistance to ultraviolet radiation and also appear to be involved in resistance to some biocides e.g. hydrogen peroxide. Spores (α^-/β^-) deficient in α/β-type SASPs are much more peroxide-sensitive than are wild-type (normal) spores. It has been proposed that in wild-type spores DNA is saturated with α/β-type SASPs and is thus protected from free radical damage.

6.5 Bacterial biofilms

Bacteria are generally associated with surfaces in a complex community called biofilms (see also Chapter 8). Following attachment to a surface, a bacterium will go through a series of metabolic and phenotypic changes, leading to the formation of microcolonies embedded within a matrix of secreted exopolysaccharides. Bacteria in biofilms have been shown to be less susceptible to antibiotics and biocides than planktonic bacteria. There are several biocide resistance mechanisms, all contributing to a 'biofilm-associated phenotype': reduction in biocide penetration, reduced bacterial metabolism, quiescence, enzymatic inactivation and efflux (Table 20.5).

Biocides have been observed to change the composition of a complex biofilm, composed of different bacterial genera or/and species. For example, polyhexamethylene biguanides (PHMB), chlorhexidine and Bardac (a QAC) have been shown to select for pseudmonads to the detriment of Gram-positive bacteria. The bisphenol triclosan was shown to reduce the genera diversity of a complex waste drain biofilm and to decrease the overall susceptibility of the remaining population.

6.6 Misuse and abuse of biocides

The indiscriminate use of biocides in an increasing number of applications and, notably, the use of

Table 20.5 Mechanisms of resistance of bacterial biofilms

Resistance mechanisms	Observation
Establishing a reduced local biocide concentration	Diffusion gradient Non-specific neutralizing interaction with cell constituents Lysed bacterial community offering mechanistic inactivation as a result of increased organic load
Enhanced bacterial insusceptibility	Degradation of antimicrobial agent Efflux (more effective against lower concentrations) Early stress-response
Slow growth/metabolism	A local chemical gradient (reduced nutrients/O_2) can retard growth rate, mitigating against biocide injury
Selection for increased resistance	Formation of pockets of surviving bacteria Dormant cells (which regrow rapidly in the presence of exudates released from lysed community)
Acquisition of new resistance determinants	Increased genetic exchange
Intrinsic resistance	Nature of microorganisms (i.e. some being more resistant than others)

From Maillard & Denyer (2009).

sub-optimal low concentrations has fuelled the debate on emerging bacterial cross-resistance to antibiotics used for human and animal medicine. This is based on *in vitro* evidence that some mechanisms conferring a decrease in biocide susceptibility can also lead to resistance to therapeutic concentrations of antibiotics. Some of the most common mechanisms involved include expression and over-expression of efflux pumps and changes in cell permeability and metabolism. However, there is no useful rule of thumb to predict cross-resistance between biocide and antibiotic resistance in bacteria. In addition, emerging cross-resistance following biocide exposure *in situ* has not been widely reported. The most significant study to date was reported in 2009 and concerned an outbreak in 38 hospitals of an isolate of *Mycobacterium massiliense* resistant to 2% glutaraldehyde and to antimycobacterial therapeutic antibiotics.

7 Viricidal activity of biocides

It is generally accepted that viruses can be divided into two groups according to their susceptibility to biocides. Lipophilic viruses that possess a viral envelope derived from their host (e.g. HIV, herpes simplex virus, influenza virus) are the most susceptible to biocides. The hydrophilic viruses comprise all the non-enveloped viruses and differ tremendously in size and structure. Among these, the small non-enveloped viruses such as the picornaviruses (e.g. poliovirus, hepatitis A virus, foot-and-mouth disease virus) are often considered to be the least susceptible to biocide exposure, although some larger viruses such as adenoviruses and rotaviruses can also be quite resilient. Overall, the viricidal activity of biocides has been little studied and often conflicting information can be found in the peer-reviewed literature. Discrepancies in reported viricidal activity often can be traced to the difference in efficacy test methodology used, and notably the lack of an appropriate neutralizer to quench the activity of the biocides (see Chapter 18). In general terms if the membrane-active biocides such as biguanides, phenolics, QACs and alcohols have a good efficacy against enveloped viruses, their activity against non-enveloped viruses is limited. A recent study, however, indicated that the limitation in activity of the biguanide PHMB was caused by the formation of viral aggregates.

One of the biggest challenges for biocide activity against viruses is that viruses on surfaces are often associated with soiling and fomites. Such an association allows viral survival (notably for enveloped viruses) on surfaces for long periods of time, as fomites/soiling appear to protect viruses from desiccation. In addition, these organic materials can protect viruses from detrimental chemical agents such as biocides. Viricidal tests do not always consider viruses embedded in an organic load.

In terms of mechanisms of action, the goal of a viricide should be the destruction of the viral nucleic acid. In reality, very few biocides have been shown to affect the viral genome; cationic biocides and alcohols damage the viral envelope releasing an intact viral capsid and genome, although they have been shown to affect the capsid in some instances, but not the viral genome. Biocides that have been shown to interact and break open the capsid (e.g. chlorine-releasing agents) might not have a damaging effect on the viral nucleic acid. To date, only a few biocides (i.e. mainly oxidizing agents) have been observed to damage the viral nucleic acid within the capsid.

The main mechanism of viral resistance to biocides is the formation of viral aggregates before or during biocide exposure. These clumps protect some viruses from the damaging effect of biocides that fail to penetrate deep within these clumps. Chlorine-releasing agents (e.g. hypochlorite) and PHMB have been shown to produce viral aggregates, limiting their viricidal efficacy. To some extent a change in capsid configuration has been shown to alter the susceptibility of viruses to a lower concentration of biocides (e.g. glutaraldehyde). Finally, multiplicity reactivation, a process that has been observed only *in vitro*, concerns the reassembly of intact viruses that have been structurally damaged by a biocide intervention, but where the genome remains intact. This process, together with the suggestion that the viral genome of certain viruses (e.g. hepatitis B virus) can remain infectious, emphasizes the importance for viricides to destroy the viral nucleic acid.

8 Biocides and protozoa

The activity of biocides has been described in a number of amoebae, notably in *Giardia* spp. and *Cryptosporidium* spp., which are major waterborne pathogens, and *Acanthamoeba* spp., a pathogen mainly associated with contact lenses and contact lens solutions. Trophozoites (the actively-growing form) of *Acanthamoeba* spp. have been shown to be susceptible to low concentrations of chlorhexidine, PHMB, QACs, oxidizing agents (hydrogen peroxide, chlorine dioxide, peracetic acid, ozone), chlorine-releasing agents and isothiazolinones.

Glutaraldehyde might possess only a poor trophocidal activity. In general higher concentrations and much longer contact times are needed to achieve a cysticidal activity (e.g. hydrogen peroxide 3% for 4 hours) and the concentration of biocide (e.g. PHMB, QAC) used for the disinfection of contact lenses might be ineffective against *Acanthamoeba* cysts. Other biocides such as iodine and bromine and the isothiazolinones have been shown to have no activity against *Acanthamoeba* cysts. It should be noted that differences in inactivation using the same biocides and parameters have been observed against cysts of different species.

Since most biocides have a poor cysticidal efficacy at a low concentration, the combination of biocides or the formulation excipients can become important. For example, a combination of chlorhexidine and thiomersal and/or EDTA has been shown to be cycticidal within 24 hours. QAC cysticidal activity can be improved when combined with tributyltin neodecanoate. A combination of hydrogen peroxide (3%) with catalase and potassium iodide (50 μM) was shown to enhance significantly cysticidal activity against *A. polyphaga*.

The mechanisms of action of biocides against trophozoites are similar to those observed on bacterial structures. For example, cationic biocides have been shown to damage the cytoplasmic membrane and to induce pentose leakage in *Acanthamoeba* spp.

Amoebal trophozoites undergo encystation when exposed to detrimental conditions, which include biocide (e.g. diamidines, chlorhexidine) exposure. Cysts are a dormant form which enable survival for many years in the environment. They are a dehydrated structure with a double wall composed of cellulose and relatively small numbers of proteins. The outer ectocyst wall is composed mainly of protein and lipid containing materials and the inner endocyst wall contains cellulose.

Encystation is a relatively rapid process that can be divided into three principal stages: induction, during which cellular components are degraded; immature cysts, during which the first cell wall is synthesized; and mature cysts, during which the second cell wall is formed.

The composition and morphological aspects of the cyst wall vary between species and depend upon the composition of the media used during encystation.

The double cyst wall represents a permeability barrier for biocides (e.g. chlorhexidine, PHMB, diamidines). In addition, the metabolically-dormant nature of the cyst might affect the cysticidal activity of biocides. It is thus not surprising that cysts represent a challenge for disinfection (Table 20.6). This is now of particular concern following a report that protozoal cysts can protect intracellular bacterial pathogens from disinfection.

9 Biocides and fungi

Fungi and their spores are a major potential source of contamination in pharmaceutical product preparation and aseptic processing as they are ubiquitous in the environment. The activity of biocides against fungi has not been widely documented (Table 20.6). It is often assumed that the interactions of biocides with fungal and yeast cells can be extrapolated from what is known of the interactions of these agents against bacteria. However, the fungal cell wall is fundamentally different from that of the bacteria and little is known about its capacity to impede the penetration of biocides. The interactions of QACs and biguanides with the fungal cell have been studied to some extent. Available information tentatively links cell wall glucan, wall thickness and consequent relative porosity to the sensitivity of *Saccharomyces cerevisiae* to chlorhexidine. Moulds tend to be less susceptible to biocides than yeasts, although more evidence is needed since only a limited number of fungal genera have been investigated.

Fungi have been shown to possess additional mechanisms of resistance to biocide intervention. The expression of degradative enzymes notably against metallic salts (e.g. copper, mercury) has been documented. In *S. cerevisiae* the production of hydrogen sulphide combining with heavy metal results in insoluble sulphides which are better tolerated by the microorganism. The expression of formaldehyde dehydrogenase to decrease the effect of formaldehyde has also been reported in *Penicillium* species. The presence of efflux pumps has now been widely reported in fungi, although their role in biocide resistance has been little investigated.

10 Inactivation of prions

Prions are the cause of transmissible spongiform encephalopathies, a group of fatal neurological diseases such as scrapie, Creutzfeld–Jakob disease (CJD), new variant Creutzfeld–Jakob disease (vCJD), bovine spongiform encephalopathy (BSE), kuru and Gerstman–Straussler–Sckeinker syndrome (GSS). It is now widely accepted that prions are an abnormal, protease-resistant form of a normal harmless host protein (PrP). The prion protein undergoes a conformational change from four α-helices

Table 20.6 Susceptibility of different types of microorganisms to biocides

Microorganisms[a]	Examples	Comments
Prions	Scrapie, Creutzfeld–Jakob disease (CJD), new variant CJD	Highly resistant to conventional microbicides due to their proteinaceous nature
Bacterial endospores	*Bacillus* spp., *Geobacillus* spp., *Clostridium difficile*	Bacillus used as biological indicators for sterilization processes due to their high intrinsic resistance
Protozoal oocysts	Cryptosporidium	Particularly challenging for water disinfection, associated with infection outbreaks
Mycobacteria	*M. chelonae, M. avium intracellulare, M. tuberculosis, M. terrae*	Environmental mycobacteria, *M. chelonae* and *M. massiliense* might show capacity to develop resistance to repeated microbicide exposure, and might become a challenge for high-level disinfection
Small non-enveloped viruses	Picornaviruses, papillomaviruses	
Protozoal cysts	*Giardia* spp., *Acnathamoeba* spp.	Might harbour pathogenic bacteria and thus aid their survival when exposed to microbicides
Fungal spores	*Aspergillus* spp.	There is very little information on microbicide activity against fungal spores
Gram-negative bacteria	*Pseudomonas* spp., *Burkholderia* spp., *Esherichia coli*, *Acinetobacter* spp.	*Pseudomonas* spp. and *Burkholderia* spp. are particularly challenging for preservative systems; pathogenic *E. coli* (e.g. O157) associated with surface contamination often embedded within organic matter
Moulds	*Aspergillus* spp.	Very little information on microbicide activity against moulds
Yeasts	*Candida albicans*, *Saccharomyces cerevisiae*	Yeasts usually considered more susceptible than moulds. Overall very little information available on yeast susceptibility to microbicides
Protozoa	*Acanthamoeba* spp., *Giardia* spp.	Important to control for water disinfection and contact lens disinfection
Large, non-enveloped viruses	Adenoviruses, rotaviruses	Certain rotaviruses more resilient; viruses often associated with soiling
Gram-positive bacteria	Staphylococci, streptococci, enterococci	Less complex cell walls than Gram-negative bacteria, generally allowing easier biocide access to target sites
Enveloped viruses	HIV, HSV, influenza, RSV	Viruses on surfaces often associated with fomites

[a] Listed in order of resistance to biocides from high to low.

to an infectious β-sheet form. These conformational changes cause degeneration of nervous tissue which, under the microscope, exhibits a sponge-like appearance. Prions are found in association with a wide range of tissues and are of particular significance in a pharmaceutical context because of the need to decontaminate surgical or other hospital equipment that has been in contact with such diseased tissue. Prions are very stable in the environment, may form aggregates, and are highly resistant to conventional disinfection and sterilization methodologies.

Prions are considered highly resistant to various types of biocidal products (formulations) (Table 20.6); this includes strong acids (e.g. 8 M hydrochloric acid for 1 hour), alkylating agents (e.g. glutaraldehyde, β-propiolactone), iodine and iodophors, phenolics, alcohols, oxidizing agents in their liquid form (e.g. hydrogen peroxide, peracetic acid) and proteolytic enzymes. Mild detergents were also reported inactive although sodium dodecyl sulphate (SDS) has shown some activity.

Alkali (e.g. 1 M NaOH for 1 hour) is usually effective against prions and as such has been widely used in the laboratory, industrial and clinical environments. However, alkali efficacy might depend on the prion's nature (host), and residual prion infectivity following treatment has been documented. More aggressive treatments combining alkali and gravity-displacement autoclaving at 121 °C for 30 minutes have been used. The use of sodium hypochlorite containing 20 000 ppm of available chlorine for 1 hour has been recommended for use in practice. Sodium dichloroisocyanurate (NaDCC) might not be equally effective against some prion proteins, as some infectivity following treatment has been reported. Hydrogen peroxide in a gaseous form has been shown to be active against prions, although reported efficacy to date depends much on the type of vapourized hydrogen peroxide generator used.

Formulations play an important role in prion decontamination. Complex formulations containing liquid hydrogen peroxide and copper have been shown to be active against prions. The combination of alkali, chelating agents, surfactants and various buffers have been particularly effective as they combined prion removal from surfaces and prion inactivation. A combination of proteinase K, pronase and SDS has been shown to degrade PrP^{res} material from highly concentrated vCJD-infected brain preparations. Slight changes in formulations might bring a loss of efficacy against prions, however, and new formulations need to be carefully assessed for antiprion activity.

11 Conclusion

Biocides are valuable compounds used in a wide range of applications. They exercise a variety of mechanisms of action, often in multiplicity, which can lead to their successful and widespread application in preservation and disinfection. It should also be noted that biocides are used in complex formulations where excipients might affect microbicidal efficacy. There is a renewed interest in biocide use with the rising impact of antibiotic resistance in bacteria and the perceived decline in hygiene standards in healthcare facilities. The number of products commercially available containing a biocide is increasing, which is a concern when the concentration of a biocide is sub-optimal. Bacterial resistance to biocides has now been widely described, with novel mechanisms, such as alterations in a metabolic pathway, still emerging. The use of genomic, proteomic, transcriptomic and metabolomic tools has helped secure a better understanding of the mechanisms involved, but also, and importantly, a better understanding of the induction of resistance as a result of biocide exposure. Resistant bacteria can be readily isolated from clinical settings. The efficacy of a biocidal product can be hampered by a number of external factors such as level of soiling, type of surface, temperature and contact time, not forgetting the nature of the target microorganism. The lack of understanding of these factors combined with inappropriate in-use concentrations of a biocide in certain applications (e.g. antimicrobial surfaces, impregnated textiles) might be conducive not only to the selection of emerging microbial resistance to biocides but also to antibiotics. The concentration of a microbicide is paramount for its lethal activity.

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21 Sterilization procedures and sterility assurance

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1 Introduction

Sterilization is an essential stage in the processing of any product destined for parenteral administration, or for contact with broken skin, mucosal surfaces, or internal organs, where the threat of infection exists. In addition, the sterilization of microbiological materials, soiled dressings and other contaminated items is necessary to minimize the health hazard associated with these articles.

Sterilization processes involve the application of a biocidal agent or physical microbial removal process to a product or preparation with the object of killing or removing all microorganisms. These processes may involve elevated temperature, reactive gas, irradiation or filtration through a microorganism-proof filter. The success of the process depends on a suitable choice of treatment conditions, e.g. temperature and duration of exposure. It must be remembered, however, that with all articles to be sterilized there is a potential risk of product damage, which for a pharmaceutical preparation may result in reduced therapeutic efficacy, stability or patient acceptability. Thus, there is a need to achieve a balance between the maximum acceptable risk of failing to achieve sterility and the maximum level of product damage that is acceptable. This is best determined from a knowledge of the properties of the sterilizing agent, the properties of the product to be sterilized and the nature of the likely contaminants. A suitable sterilization process may then be selected to ensure maximum microbial kill/removal with minimum product deterioration.

2 Sensitivity of microorganisms

The general pattern of resistance of microorganisms to biocidal sterilization processes is independent of the type of agent employed (heat, radiation or gas), with vegetative forms of bacteria and fungi, along with the larger viruses, showing a greater sensitivity to sterilization processes than small viruses and bacterial or fungal spores. The choice of suitable reference organisms for testing the efficiency of sterilization processes (see section 12.3) is therefore made from the most durable bacterial spores; these are usually represented by *Bacillus stearothermophilus* for moist heat, certain strains of *B. subtilis* for dry heat and gaseous sterilization, and *B. pumilus* for ionizing radiation.

Ideally, when considering the level of treatment necessary to achieve sterility a knowledge of the type and total number of microorganisms present in a product, together with their likely response to the proposed treatment, is necessary. Without this information, however, it is usually assumed that organisms within the load are no more resistant than the reference spores or than specific resistant product isolates. In the latter case, it must be remembered that resistance may be altered or lost entirely by repeated laboratory subculture and the resistance characteristics of the maintained strain must be regularly checked.

A sterilization process may thus be developed without a full microbiological background to the product, instead being based on the ability to deal with a 'worst case' condition. This is indeed the situation for official sterilization methods, which must be capable of general application, and modern pharmacopoeial recommendations are derived from a careful analysis of experimental data on bacterial spore survival following treatments with heat, ionizing radiation or gas.

However, the infectious agents responsible for spongiform encephalopathies (prions, see Chapters 5 and 20) such as bovine spongiform encephalopathy (BSE) and Creutzfeldt–Jakob disease (CJD) exhibit exceptional degrees of resistance to many lethal agents. Recent work has even cast doubt on the adequacy of the process of 18 minute exposure to steam at 134–138 °C which has been recommended for the destruction of prions (and which far exceeds the lethal treatment required to achieve adequate destruction of bacterial spores).

2.1 Survivor curves

When exposed to a killing process, populations of microorganisms generally lose their viability in an exponential fashion, independent of the initial number of organisms. This can be represented graphically with a 'survivor curve' drawn from a plot of the logarithm of the fraction of survivors against the exposure time or dose (Figure 21.1). Of the typical curves obtained, all have a linear portion which may be continuous (plot A), or may be modified by an initial shoulder (B) or by a reduced rate of kill at low survivor levels (C). Furthermore, a short activation phase, representing an initial increase in viable count, may be seen during the heat treatment of certain bacterial spores. Survivor curves have been employed principally in the examination of heat sterilization methods, but can equally well be applied to any biocidal process.

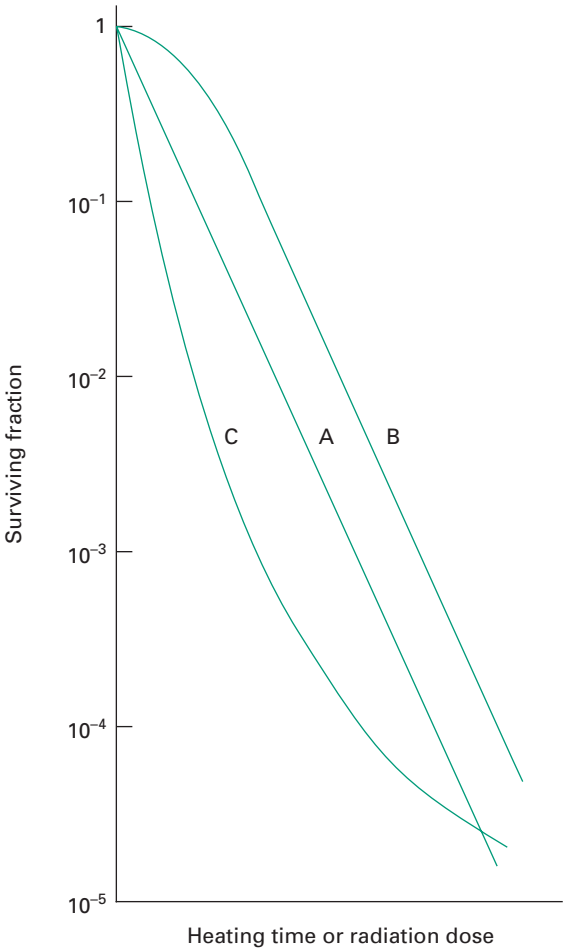

Figure 21.1 Typical survivor curves for bacterial spores exposed to moist heat or gamma radiation.

2.2 Expressions of resistance

2.2.1 *D*-value

The resistance of an organism to a sterilizing agent can be described by means of the *D*-value. For heat and radiation treatments, respectively, this is defined as the time taken at a fixed temperature or the radiation dose required to achieve a 90% reduction in viable cells (i.e. a 1 log cycle reduction in survivors; Figure 21.2A). The calculation of the *D*-value assumes a linear type A survivor curve (Figure 21.1), and must be corrected to allow for any deviation from linearity with type B or C curves. Some typical *D*-values for resistant bacterial spores are given in Table 21.1.

2.2.2 *Z*-value

For heat treatment, a *D*-value only refers to the resistance of a microorganism at a particular temperature. In order to assess the influence of temperature changes on thermal resistance, a relationship between temperature and log *D*-value can be developed, leading to the expression of a *Z*-value, which represents the increase in temperature needed to reduce the *D*-value of an organism by 90% (i.e. 1 log cycle reduction; Figure 21.2B). For bacterial spores used as biological indicators for moist heat (*B. stearothermophilus*) and dry heat (*B. subtilis*) sterilization processes, mean *Z*-values are given as 10 °C and 22 °C, respectively. The *Z*-value is not truly independent of temperature but may be considered essentially constant over the temperature ranges used in heat sterilization processes.

2.3 Sterility assurance

The term 'sterile', in a microbiological context, means no surviving organisms whatsoever. Thus, there are no degrees of sterility; an item is either sterile or it is not, and so there are no levels of contamination which may be considered negligible or insignificant and therefore acceptable. From the survivor curves presented, it can be seen that the elimination of viable microorganisms from a product is a time-dependent process, and will be influenced by the rate and duration of biocidal action and the initial microbial contamination level. It is also evident from Figure 21.2A that true sterility, represented by zero survivors, can only be achieved after an infinite exposure period or radiation dose. Clearly, then, it is illogical to claim, or expect, that a sterilization procedure will guarantee sterility. Thus, the likelihood of a product being produced free of microorganisms is best expressed in terms of the probability of an organism surviving the treatment process, a possibility not entertained in the absolute term 'sterile'. From this approach has arisen the concept of sterility assurance or a microbial safety index which gives a numerical value to the probability of a single surviving organism remaining to contaminate a processed product. For pharmaceutical products, the most frequently applied standard is that the probability, poststerilization, of a non-sterile unit is no more than 1 in 1 million units processed (i.e. $\leq 10^{-6}$). The sterilization protocol necessary to achieve this with any given organism of known *D*-value can be established from the inactivation factor (IF) which may be defined as:

$$IF = 10^{t/D}$$

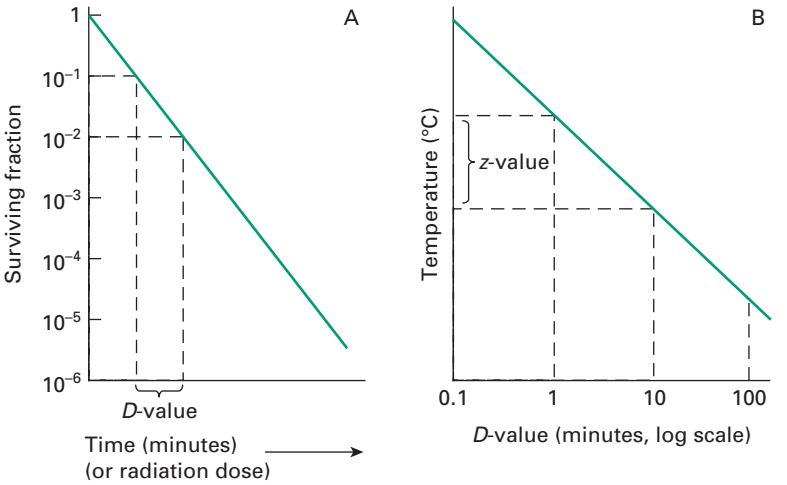

Figure 21.2 Determination of: (A) *D*-value; (B) *Z*-value.

Table 21.1 Inactivation factors (IF) for selected sterilization protocols and their corresponding biological indicator (BI) organisms

Sterilization protocol	BI organism	*D*-value	Log IF
Moist heat (121 °C for 15 min)	*B. stearothermophilus*	1.5 min	10
Dry heat (160 °C for 2 h)	*B. subtilis var. niger*	Max. 3 min	Min. 40
Irradiation (25 kGy)	*B. pumilus*	1.9 kGy	13.2

where *t* is the contact time (for a heat or gaseous sterilization process) or dose (for ionizing radiation) and *D* is the *D*-value appropriate to the process employed.

Thus, for an initial burden of 10^2 spores an inactivation factor of 10^8 will be needed to give the required sterility assurance of 10^{-6} (Figure 21.3). The sterilization process will therefore need to produce sufficient lethality to achieve an 8 log cycle reduction in viable organisms; this will require exposure of the product to eight times the *D*-value of the reference organism (8*D*). In practice, it is generally assumed that the contaminant will have the same resistance as the relevant biological indicator spores unless full microbiological data are available to indicate otherwise. The inactivation factors associated with certain sterilization protocols and their biological indicator organisms are given in Table 21.1.

3 Sterilization methods

A sterilization process should always be considered a compromise between achieving good antimicrobial activity and maintaining product stability. It must, therefore, be validated against a suitable test organism and its efficacy continually monitored during use. Even so, a limit will exist as to the type and size of microbial challenge that can be handled by the process without significant loss of sterility assurance. Thus, sterilization must not be seen as a 'catch-all' or as an alternative to Good Manufacturing Practice but must be considered as only the final stage in a programme of microbiological control. The *European Pharmacopoeia* recognizes five methods for the sterilization of pharmaceutical products: (1) steam sterilization (heating in an autoclave); (2) dry heat; (3) ionizing radiation; (4) gaseous sterilization; and (5) filtration. In addition, other approaches involving steam and formaldehyde and UV light have evolved for use in certain situations. For each method, the possible permutations of exposure conditions are numerous, but experience and product stability requirements have generally served to limit this choice. Nevertheless, it should be remembered that even the recommended methods and regimens do not necessarily demonstrate equivalent biocidal potential (see Table 21.1), but simply offer

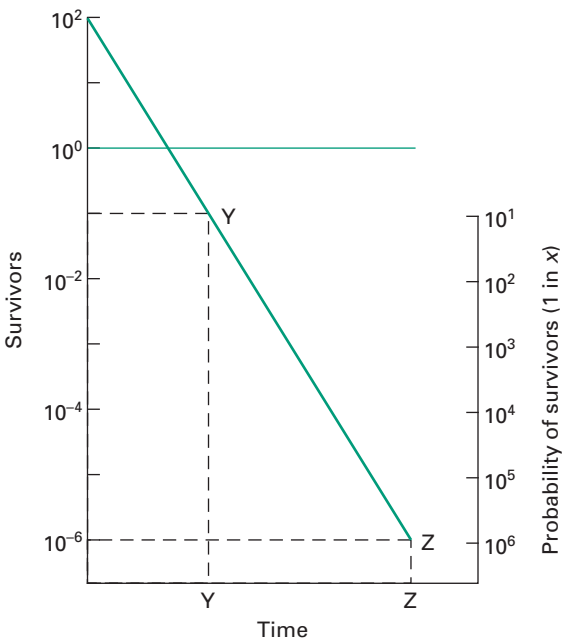

Figure 21.3 Sterility assurance. At Y, there is (literally) 10^{-1} bacterium in one bottle, i.e. in 10 loads of single containers, there would be one chance in 10 that one load would be positive. Likewise, at Z, there is (literally) 10^{-6} bacterium in one bottle, i.e. in 1 million (10^6) loads of single containers, there is one chance in 1 million that one load would be positive.

alternative strategies for application to a wide variety of product types. Thus, each should be validated in their application to demonstrate that the minimum required level of sterility assurance can be achieved (sections 2.3 and 9).

In the following sections, factors governing the successful use of these sterilizing methods will be covered and their application to pharmaceutical and medical products considered. Methods for monitoring the efficacy of these processes are discussed in section 12.

4 Heat sterilization

Heat is the most reliable and widely used means of sterilization, affording its antimicrobial activity through destruction of enzymes and other essential cell constituents. These lethal events proceed most rapidly in a fully hydrated state, thus requiring a lower heat input (temperature and time) under conditions of high humidity where denaturation and hydrolysis reactions predominate, rather than in the dry state where oxidative changes take place. This method of sterilization is limited to thermostable products, but can be applied to both moisture-sensitive and moisture-resistant items for which dry (160–180 °C) and moist (121–134 °C) heat sterilization procedures are respectively used. Where thermal degradation of a product might possibly occur, it can usually be minimized by selecting the higher temperature range, as the shorter exposure times employed generally result in a lower fractional degradation.

4.1 Sterilization processes

In any heat sterilization process, the articles to be treated must first be raised to sterilization temperature and this involves a heating-up stage. In the traditional approach, timing for the process (the holding time) then begins. It has been recognized, however, that during both the heating-up and cooling-down stages of a sterilization cycle (Figure 21.4), the product is held at an elevated temperature and these stages may thus contribute to the overall biocidal potential of the process.

A method has been devised to convert all the temperature–time combinations occurring during the heating, sterilizing and cooling stages of a moist heat (steam) sterilization cycle to the equivalent time at 121 °C. This involves following the temperature profile of a load, integrating the heat input (as a measure of lethality), and converting it to the equivalent time at the standard temperature of 121 °C. Using this approach, the overall lethality of any process can be deduced and is defined as the *F*-value; this expresses heat treatment at any temperature as equal to that of a certain number of minutes at 121 °C. In other words, if a moist heat sterilization process has an *F*-value of *x*, then it has the same lethal effect on a given organism as heating at 121 °C for *x* minutes, irrespective of the actual temperature employed or of any fluctuations in the heating process due to heating and cooling stages. The *F*-value of a process will vary according to the moist heat resistance of the reference organism; when the reference spore is that of *B. stearothermophilus* with a *Z*-value of 10 °C, then the *F*-value is known as the F_0-value.

A relationship between *F*- and *D*-values, leading to an assessment of the probable number of survivors in a load following heat treatment, can be established from the following equation:

$$F = D(\log N_0 - \log N)$$

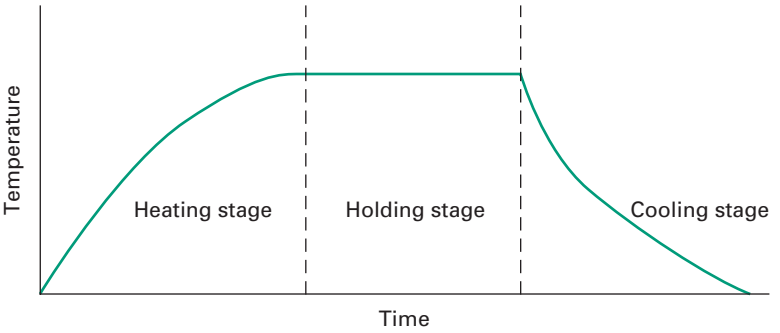

Figure 21.4 Typical temperature profile of a heat sterilization process.

where D is the D-value at 121 °C, and N_0 and N represent, respectively, the initial and final number of viable cells per unit volume.

The F-concept has evolved from the food industry and principally relates to the sterilization of articles by moist heat. Because it permits calculation of the extent to which the heating and cooling phases contribute to the overall killing effect of the autoclaving cycle, the F-concept enables a sterilization process to be individually developed for a particular product. This means that adequate sterility assurance can be achieved in autoclaving cycles in which the traditional pharmacopoeial recommendation of 15 minutes at 121 °C is not achieved. The holding time may be reduced below 15 minutes if there is a substantial killing effect during the heating and cooling phases, and an adequate cycle can be achieved even if the 'target' temperature of 121 °C is not reached. Thus, F-values offer both a means by which alternative sterilizing cycles can be compared in terms of their microbial killing efficiency, and a mechanism by which overprocessing of marginally thermolabile products can be reduced without compromising sterility assurance. The *European Pharmacopoeia* emphasizes that when a steam sterilization cycle is designed on the basis of F_0 data, it may be necessary to perform continuous and rigorous microbiological monitoring of the bioburden (section 10) during routine manufacturing in order consistently to achieve an acceptable sterility assurance level.

F_0 values may be calculated either from the area under the curve of a plot of autoclave temperature against time constructed using special chart paper on which the temperature scale is modified to take into account the progressively greater lethality of higher temperatures, or by use of the equation:

$$F_0 = \Delta t \Sigma 10^{(T-121)/Z}$$

where Δt is the time interval between temperature measurements, T is the product temperature at time t, and Z is (assumed to be) 10 °C.

Thus, if temperatures were being recorded from a thermocouple at 1.00 minute intervals then $\Delta t = 1.00$, and a temperature of, for example, 115 °C maintained for 1 minute would give an F_0 value of 1 minute $\times 10^{(115-121)/10}$, which is equal to 0.25 minutes. In practice, such calculations could easily be performed on the data from several thermocouples within an autoclave using suitable software, and, in a manufacturing situation, these would be part of the batch records. Application of the F-value concept has been largely restricted to steam sterilization processes, although there is a less frequently employed, but direct parallel in dry heat sterilization (see section 4.3).

4.2 Moist heat sterilization

Moist heat has been recognized as an efficient biocidal agent from the early days of bacteriology, when it was principally developed for the sterilization of culture media. It now finds widespread application in the processing of many thermostable products and devices. In the pharmaceutical and medical sphere it is used in the sterilization of dressings, sheets, surgical and diagnostic equipment, containers and closures, and aqueous injections, ophthalmic preparations and irrigation fluids, in addition to the processing of soiled and contaminated items (Chapter 22).

Sterilization by moist heat usually involves the use of steam at temperatures in the range 121–134 °C, and while alternative strategies are available for the processing of products unstable at these high temperatures, they rarely offer the same degree of sterility assurance and should be avoided if at all possible. The elevated temperatures generally associated with moist heat sterilization methods

Table 21.2 Pressure–temperature relationships and antimicrobial efficacies of alternative steam sterilization cycles

Temperature	Holding time	Steam pressure		Inactivation factor[a]
(°C)	(min)	(kPa)	(psi)	(decimal reductions)
115	30	69	10	5
121	15	103	15	10
126	10	138	20	21
134	3	207	30	40

[a]Calculated for a spore suspension having a D_{121} of 1.5 min and a Z-value of 10 °C.

can only be achieved by the generation of steam under pressure.

By far the most commonly employed standard temperature/time cycles for bottled fluids and porous loads (e.g. surgical dressings) are 121 °C for 15 minutes and 134 °C for 3 minutes, respectively. Not only do high-temperature–short time cycles often result in lower fractional degradation, they also afford the advantage of achieving higher levels of sterility assurance due to greater inactivation factors (Table 21.2). Before the publication of the 1988 *British Pharmacopoeia* the 115 °C for 30 minute cycle was considered an acceptable alternative to 121 °C for 15 minutes, but it is no longer considered sufficient to give the desired sterility assurance levels for products which may contain significant concentrations of thermophilic spores.

4.2.1 Steam as a sterilizing agent

To act as an efficient sterilizing agent, steam should be able to provide moisture and heat efficiently to the article to be sterilized. This is most effectively done using saturated steam, which is steam in thermal equilibrium with the water from which it is derived, i.e. steam on the phase boundary (Figure 21.5). Under these circumstances, contact with a cooler surface causes condensation and contraction, drawing in fresh steam and leading to the immediate release of the latent heat, which represents approximately 80% of the total heat energy. In this way, heat and moisture are imparted rapidly to articles being sterilized and dry porous loads are quickly penetrated by the steam.

Steam for sterilization can either be generated within the sterilizer, as with portable bench or 'instrument and utensil' sterilizers, in which case it is constantly in contact with water and is known as 'wet' steam, or can be supplied under pressure (350–400 kPa) from a separate boiler as 'dry' saturated steam with no entrained water droplets. The killing potential of 'wet' steam is the same as that of 'dry' saturated steam at the same temperature, but it is more likely to soak a porous load, creating physical difficulties for further steam penetration. Thus, major industrial and hospital sterilizers are usually supplied with 'dry' saturated steam and attention is paid to the removal of entrained water droplets within the supply line to prevent introduction of a water 'fog' into the sterilizer.

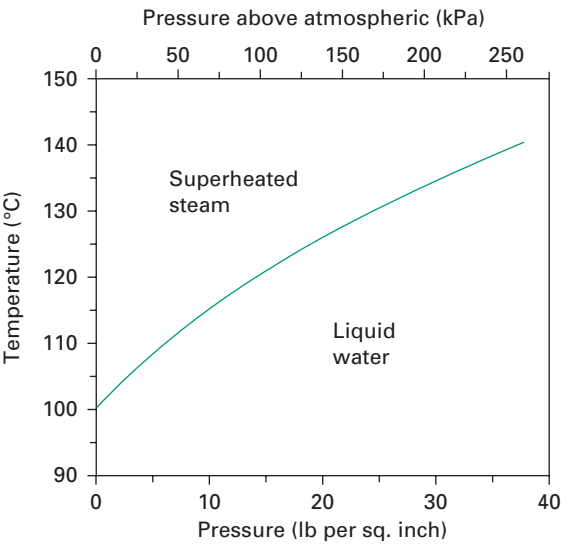

Figure 21.5 Pressure–temperature diagram for water at the phase boundary.

If the temperature of 'dry' saturated steam is increased, then, in the absence of entrained moisture, the relative humidity or degree of saturation is reduced and the steam becomes superheated (Figure 21.5). During sterilization this can arise in a number of ways, for example by overheating the steam-jacket (see section 4.2.2), by using too dry a steam supply, by excessive pressure reduction during passage of steam from the boiler to the sterilizer chamber, and by evolution of heat of hydration when steaming overdried cotton fabrics. Superheated steam behaves in the same manner as hot air as condensation and release of latent heat will not occur unless the steam is cooled to the phase boundary temperature. Thus, it proves to be an inefficient sterilizing agent and, although a small degree of transient superheating can be tolerated, a maximum acceptable level of 5 °C superheat is set, i.e. the temperature of the steam is never greater than 5 °C above the phase boundary temperature at that pressure.

The relationship between temperature and pressure holds true only in the presence of pure steam; adulteration with air contributes to a partial pressure but not to the temperature of the steam. Thus, in the presence of air the temperature achieved will reflect the contribution made by the steam and will be lower than that normally attributed to the total pressure recorded. Addition of further steam will raise the temperature but residual air surrounding articles may delay heat penetration or, if a large amount of air is present, it may collect at the bottom of the sterilizer, completely altering the temperature profile of the sterilizer chamber. It is for these reasons that efficient air removal is a major aim in the design and operation of a boiler-fed steam sterilizer.

4.2.2 Sterilizer design and operation

Steam sterilizers, or *autoclaves* as they are also known, are stainless steel vessels designed to withstand the steam pressures employed in sterilization. They can be: 'portable' sterilizers, which generally have internal electric heaters to produce steam and are used for small pilot or laboratory-scale sterilization and for the treatment of instruments and utensils; or large-scale sterilizers for routine hospital or industrial use, operating on 'dry' saturated steam from a separate boiler (Figure 21.6). Because of their widespread use within pharmacy this latter type will be considered in greatest detail.

There are two main types of large sterilizers, those designed for use with porous loads (i.e. dressings) and generally operated at a minimum temperature of 134 °C, and those designed as bottled fluid sterilizers employing a minimum temperature of 121 °C. The stages of operation are common to both and can be summarized as air removal and steam admission, heating-up and exposure, and drying or cooling. Many modifications of design exist and in this section only general features will be considered. Fuller treatments of sterilizer design and operation can be found in the relevant Department of Health technical memorandums (DH 1995, DH 1997).

4.2.2.1 General design features

Steam sterilizers are constructed with either cylindrical or rectangular chambers, with preferred capacities ranging from 400 to 800 L. They can be sealed by either a single door or by doors at both ends (to allow through-passage of processed materials; see Chapter 23). During sterilization the doors are held closed by a locking mechanism which prevents opening when the chamber is under pressure and until the chamber has cooled to a preset temperature, typically 80 °C.

In the larger sterilizers the chamber may be surrounded by a steam jacket which can be used to heat the autoclave chamber and promote a more uniform temperature throughout the load. The same jacket can also be filled with water at the end of the cycle to facilitate cooling and thus reduce the overall cycle time. The chamber floor slopes towards a discharge channel through which air and condensate can be removed. Temperature is monitored within the opening of the discharge channel and by thermocouples in dummy packages; jacket and chamber pressures are followed using pressure gauges. In hospitals and industry, it is common practice to operate sterilizers on an automatic cycle, each stage of operation being controlled by a timer responding to temperature- or pressure-sensing devices.

The stages of operation are as follows.

1 *Air removal and steam admission.* Air can be removed from steam sterilizers either by downward displacement with steam, evacuation or a combination of the two. In the downward displacement sterilizer, the heavier cool air is forced out of the discharge channel by incoming hot steam. This has the benefit of warming the load during air removal, which aids the heating-up process. It finds widest application in the sterilization of bottled fluids where bottle breakage may occur under the combined stresses of evacuation and high temperature. For more air-retentive loads (i.e. dressings), however, this technique of air removal is unsatisfactory and mechanical evacuation of the air is essential before admission of the steam. This can either be to an extremely high level (e.g. 2.5 kPa) or can involve a period of pulsed evacuation and

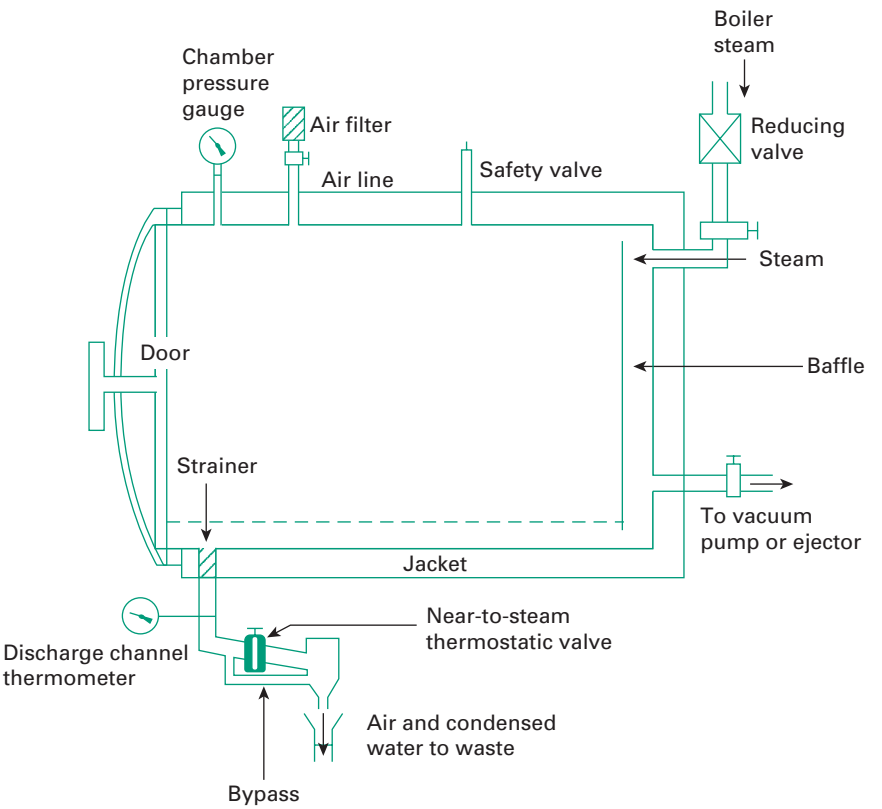

Figure 21.6 Main constructional features of a large-scale steam sterilizer (autoclave).

steam admission, the latter approach improving air extraction from dressings packs. After evacuation, steam penetration into the load is very rapid and heating-up is almost instantaneous. It is axiomatic that packaging and loading of articles within a sterilizer be so organized as to facilitate air removal.

During the sterilization process, small pockets of entrained air may still be released, especially from packages, and this air must be removed. This is achieved in a porous load autoclave with a near-to-steam thermostatic valve incorporated in the discharge channel. The valve operates on the principle of an expandable bellows containing a volatile liquid which vaporizes at the temperature of saturated steam thereby closing the valve, and condenses on the passage of a cooler air-steam mixture, thus reopening the valve and discharging the air. Condensate generated during the sterilization process can also be removed by this device. Small quantities of air will not, however, lower the temperature sufficiently to operate the valve and so a continual slight flow of steam is maintained through a bypass around the device in order to flush away residual air.

It is common practice to package sterile fluids, especially intravenous fluids, in flexible plastic containers. During sterilization these can develop a considerable internal pressure in the airspace above the fluid and it is therefore necessary to maintain a proportion of air within the sterilizing chamber to produce sufficient overpressure to prevent these containers from bursting (air ballasting). In sterilizers modified or designed to process this type of product, air removal is therefore unnecessary but special attention must be paid to the prevention of air 'layering' within the chamber. This is overcome by the inclusion of a fan or through a continuous spray of hot water within the chamber to mix the air and steam. Air ballasting can also be employed to prevent bottle breakage.

2 *Heating-up and exposure.* When the sterilizer reaches its operating temperature and pressure the sterilization stage begins. The duration of exposure may include a heating-up time in addition to the holding time and this

will normally be established using thermocouples in dummy articles.

3 *Drying or cooling.* Dressings packs and other porous loads may become dampened during the sterilization process and must be dried before removal from the chamber. This is achieved by steam exhaust and application of a vacuum, often assisted by heat from the steam-filled jacket if fitted. After drying, atmospheric pressure within the chamber is restored by admission of sterile filtered air.

For bottled fluids the final stage of the sterilization process is cooling, and this needs to be achieved as rapidly as possible to minimize thermal degradation of the product and to reduce processing time. In modem sterilizers, this is achieved by circulating water in the jacket that surrounds the chamber or by spray-cooling with retained condensate delivered to the surface of the load by nozzles fitted into the roof of the sterilizer chamber. This is often accompanied by the introduction of filtered, compressed air to minimize container breakage due to high internal pressures (air ballasting). Containers must not be removed from the sterilizer until the internal pressure has dropped to a safe level, usually indicated by a temperature of less than 80 °C. Occasionally, spray-cooling water may be a source of bacterial contamination and its microbiological quality must be carefully monitored.

4.3 Dry heat sterilization

The lethal effects of dry heat on microorganisms are due largely to oxidative processes, which are less effective than the hydrolytic damage which results from exposure to steam. Thus, dry heat sterilization usually employs higher temperatures in the range 160–180 °C and requires exposure times of up to 2 hours depending on the temperature employed.

Again, bacterial spores are much more resistant than vegetative cells and their recorded resistance varies markedly depending on their degree of dryness. In many early studies on dry heat resistance of spores their water content was not adequately controlled, so conflicting data arose regarding the exposure conditions necessary to achieve effective sterilization. This was partly responsible for variations in recommended exposure temperatures and times in different pharmacopoeias.

Dry heat application is generally restricted to glassware and metal surgical instruments (where its good penetrability and non-corrosive nature are of benefit), non-aqueous thermostable liquids and thermostable powders (see Chapter 22). In practice, the range of materials that are actually subjected to dry heat sterilization is quite limited, and consists largely of items used in hospitals. The major industrial application is in the sterilization of glass bottles which are to be filled aseptically, and here the attraction of the process is that it not only achieves an adequate sterility assurance level, but that it may also destroy bacterial endotoxins (products of Gram-negative bacteria, also known as pyrogens, that cause fever when injected into the body). These are difficult to eliminate by other means. For the purposes of depyrogenation of glass, temperatures of approximately 250 °C are used.

The *F*-value concept that was developed for steam sterilization processes has an equivalent in dry heat sterilization although its application has been limited. The F_H designation describes the lethality of a dry heat process in terms of the equivalent number of minutes exposure at 170 °C, and in this case a *Z*-value of 20 °C has been found empirically to be appropriate for calculation purposes; this contrasts with the value of 10 °C which is typically employed to describe moist heat resistance.

4.3.1 Sterilizer design

Dry heat sterilization is usually carried out in a hot-air oven which comprises an insulated polished stainless steel chamber, with a usual capacity of up to 250 L, surrounded by an outer case containing electric heaters located in positions to prevent cool spots developing inside the chamber. A fan is fitted to the rear of the oven to provide circulating air, thus ensuring more rapid equilibration of temperature. Shelves within the chamber are perforated to allow good airflow. Thermocouples can be used to monitor the temperature of both the oven air and articles contained within. A fixed temperature sensor connected to a chart or digital recorder provides a permanent record of the sterilization cycle. Appropriate door-locking controls should be incorporated to prevent interruption of a sterilization cycle once begun.

Recent sterilizer developments have led to the use of dry heat sterilizing tunnels where heat transfer is achieved by infrared irradiation or by forced convection in filtered laminar airflow tunnels. Items to be sterilized are placed on a conveyer belt and pass through a high-temperature zone (250–300+ °C) over a period of several minutes.

4.3.2 Sterilizer operation

Articles to be sterilized must be wrapped or enclosed in containers of sufficient strength and integrity to provide good poststerilization protection against contamination. Suitable materials are paper, cardboard tubes or aluminium containers. Container shape and design

must be such that heat penetration is encouraged in order to shorten the heating-up stage; this can be achieved by using narrow containers with dull, non-reflecting surfaces. In a hot-air oven, heat is delivered to articles principally by radiation and convection; thus, they must be carefully arranged within the chamber to avoid obscuring centrally placed articles from wall radiation or impending air flow. The temperature variation within the chamber should not exceed ±5 °C of the recorded temperature. Heating-up times, which may be as long as 4 hours for articles with poor heat-conducting properties, can be reduced by preheating the oven before loading. Following sterilization, the chamber temperature is usually allowed to fall to around 40 °C before removal of sterilized articles; this can be accelerated by the use of forced cooling with filtered air.

5 Gaseous sterilization

The chemically reactive gases ethylene oxide [$(CH_2)_2O$] and formaldehyde [(methanal, H.CHO)] possess broad-spectrum biocidal activity, and have found application in the sterilization of reusable surgical instruments, certain medical, diagnostic and electrical equipment, and the surface sterilization of powders. Sterilization processes using ethylene oxide sterilization are far more commonly used on an international basis than those employing formaldehyde.

Ethylene oxide treatment can also be considered as an alternative to radiation sterilization in the commercial production of disposable medical devices (Chapter 22). These techniques do not, however, offer the same degree of sterility assurance as heat methods and are generally reserved for temperature-sensitive items.

The mechanism of antimicrobial action of the two gases is assumed to be through alkylation of sulphydryl, amino, hydroxyl and carboxyl groups on proteins and imino groups of nucleic acids. At the concentrations employed in sterilization protocols, type A survivor curves (section 2.1, Figure 21.1) are produced, the lethality of these gases increasing in a non-uniform manner with increasing concentration, exposure temperature and humidity. For this reason, sterilization protocols have generally been established by an empirical approach using a standard product load containing suitable biological indicator test strips (section 12.3). Concentration ranges (given as weight of gas per unit chamber volume) are usually of the order of 800–1200 mg/L for ethylene oxide and 15–100 mg/L for formaldehyde, with operating temperatures in the region of 45–63 °C and 70–75 °C, respectively. Even at the higher concentrations and temperatures, the sterilization processes are lengthy and therefore unsuitable for the resterilization of high-turnover articles. Further delays occur because of the need to remove toxic residues of the gases before release of the items for use. In addition, because recovery of survivors in sterility tests is more protracted with gaseous sterilization methods than with other processes, an extended quarantine period may also be required.

As alkylating agents, both gases are potentially mutagenic and carcinogenic (as is the ethylene chlorohydrin that results from ethylene oxide reaction with chlorine); they also produce symptoms of acute toxicity including irritation of the skin, conjunctiva and nasal mucosa. Consequently, strict control of their atmospheric concentrations is necessary and safe working protocols are required to protect personnel. Table 21.3 summarizes the comparative advantages afforded by ethylene oxide and low-temperature steam and formaldehyde (LTSF) processes.

Table 21.3 Relative merits of ethylene oxide and low-temperature steam and formaldehyde (LTSF) processes

Advantages of ethylene oxide over LTSF	Advantages of LTSF over ethylene oxide
Wider international regulatory acceptance	Less hazardous because formaldehyde is not flammable and is more readily detected by smell
Better gas penetration into plastics and rubber	The gas is obtained readily from aqueous solution (formalin) which is a more convenient source than gas in cylinders
Cycle times may be shorter	
Relatively slow to form solid polymers (with the potential to block pipes, etc.)	
With long exposure times it is possible to sterilize at ambient temperatures	
Very low incidence of product deterioration	

5.1 Ethylene oxide

Ethylene oxide gas is highly explosive in mixtures of more than 3.6% v/v in air; in order to reduce this explosion hazard it is usually supplied for sterilization purposes as a 10% mix with carbon dioxide, or as an 8.6% mixture with HFC 124 (2-chloro-1,1,1,2 tetrafluoroethane), which has replaced fluorinated hydrocarbons (freons). Alternatively, pure ethylene oxide gas can be used below atmospheric pressure in sterilizer chambers from which all air has been removed.

The efficacy of ethylene oxide treatment depends on achieving a suitable concentration in each article and this is assisted greatly by the good penetrating powers of the gas, which diffuses readily into many packaging materials including rubber, plastics, fabric and paper. This is not without its drawbacks, however, as the level of ethylene oxide in a sterilizer will decrease due to absorption during the process and the treated articles must undergo a desorption stage to remove toxic residues. Desorption can be allowed to occur naturally on open shelves, in which case complete desorption may take many days, e.g. for materials like PVC, or it may be encouraged by special forced-aeration cabinets where flowing, heated air assists gas removal, reducing desorption times to between 2 and 24 hours.

Organisms are more resistant to ethylene oxide treatment in a dried state, as are those protected from the gas by inclusion in crystalline or dried organic deposits. Thus, a further condition to be satisfied in ethylene oxide sterilization is attainment of a minimum level of moisture in the immediate product environment. This requires a sterilizer humidity of 30–70% and frequently a preconditioning of the load at relative humidities of more than 50%.

5.1.1 Sterilizer design and operation

An ethylene oxide sterilizer consists of a leak-proof and explosion-proof steel chamber, normally of 100–300 L capacity, which can be surrounded by a hot-water jacket to provide a uniform chamber temperature. Successful operation of the sterilizer requires removal of air from the chamber by evacuation, humidification and conditioning of the load by passage of subatmospheric-pressure steam followed by a further evacuation period and the admission of preheated vaporized ethylene oxide from external pressurized canisters or single-charge cartridges. Forced gas circulation is often employed to minimize variations in conditions throughout the sterilizer chamber. Packaging materials must be air-, steam- and gas-permeable to permit suitable conditions for sterilization to be achieved within individual articles in the load. Absorption of ethylene oxide by the load is compensated for by the introduction of excess gas at the beginning or by the addition of more gas as the pressure drops during the sterilization process. The same may also be true for moisture absorption, which is compensated for by supplementary addition of water to maintain appropriate relative humidity.

After treatment, the gases are evacuated either directly to the outside atmosphere or through a special exhaust system. Filtered, sterile air is then admitted either for a repeat of the vacuum/air cycle or for air purging until the chamber is opened. In this way, safe removal of the ethylene oxide is achieved, reducing the toxic hazard to the operator. Sterilized articles are removed directly from the chamber and arranged for desorption. The operation of an ethylene oxide sterilizer should be monitored and controlled automatically. A typical operating cycle for pure ethylene oxide gas is shown in Figure 21.7.

5.2 Formaldehyde

Formaldehyde gas for use in sterilization is produced by heating formalin (37% w/v aqueous solution of formaldehyde) to a temperature of 70–75 °C with steam, leading to the process known as LTSF. Formaldehyde has a similar toxicity to ethylene oxide and although absorption to materials appears to be lower, similar desorption routines are recommended. A major disadvantage of formaldehyde is low penetrating power, and this limits the packaging materials that can be employed to principally paper and cotton fabric.

5.2.1 Sterilizer design and operation

An LTSF sterilizer is designed to operate with subatmospheric-pressure steam. Air is removed by evacuation and steam is admitted to the chamber to allow heating of the load and to assist in air removal. The sterilization period starts with the release of formaldehyde by vaporization from formalin (in a vaporizer with a steam-jacket) and continues through either a simple holding stage or through a series of pulsed evacuations and steam and formaldehyde admission cycles. The chamber temperature is maintained by a thermostatically controlled water jacket, and steam and condensate are removed via a drain channel and an evacuated condenser. At the end of the treatment period formaldehyde vapour is expelled by steam flushing and the load is dried by alternating stages of evacuation and admission of sterile, filtered air. A typical pulsed cycle of operation is shown in Figure 21.8.

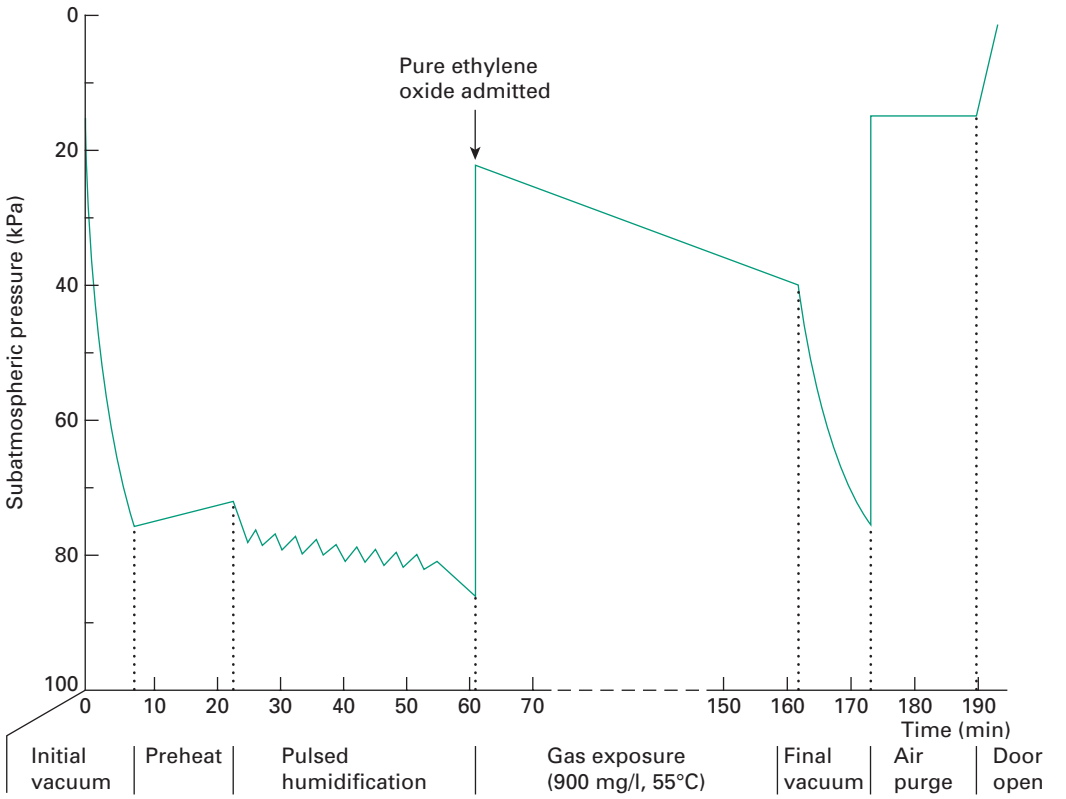

Figure 21.7 Typical operating cycle for pure ethylene oxide gas.

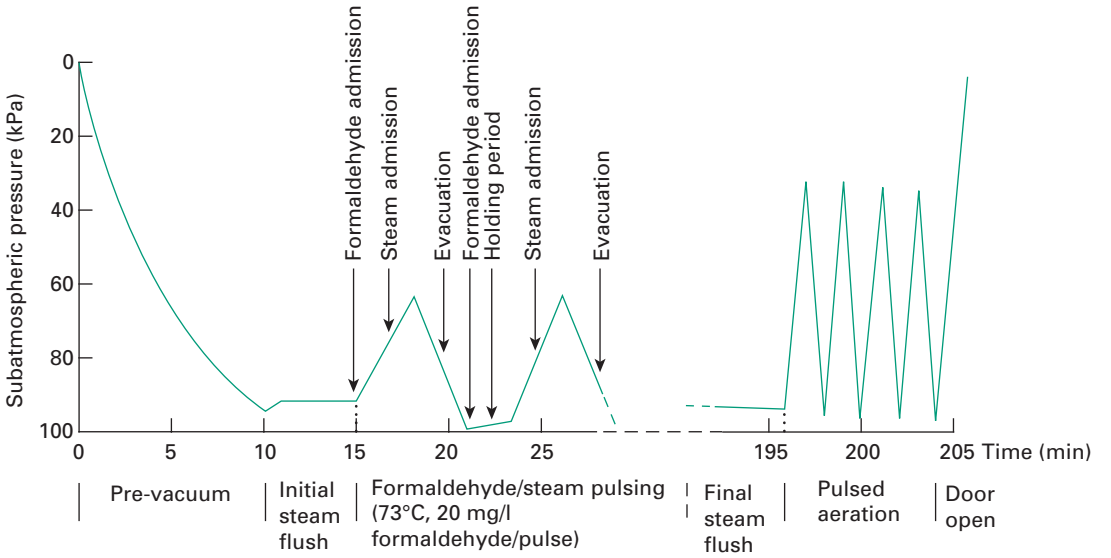

Figure 21.8 Typical operating cycle for low-temperature steam and formaldehyde treatment.

5.3 Peroxygen compounds

Hydrogen peroxide (H_2O_2), 30–35%w/v, and peracetic acid (CH_3CO_3H), 3.5%w/v, are used as highly effective oxidizing agents to kill microorganisms (Chapters 19 and 20). The liquids are heated to vaporize them and are held in a sealed chamber where all surfaces which come into contact with the vapour will be sterilized. These methods are widely used for the sterilization of equipment used for aseptic preparation and manufacture such as isolators, with the vaporized hydrogen peroxide (VHP) often used for the routine decontamination of cleanrooms.

6 Radiation sterilization

Several types of radiation find a sterilizing application in the manufacture of pharmaceutical and medical products, principal among which are accelerated electrons (particulate radiation), gamma rays and UV light (both electromagnetic radiations). The major target for these radiations is believed to be microbial DNA, with damage occurring as a consequence of ionization and free-radical production (gamma rays and electrons) or excitation (UV light). This latter process is less damaging and less lethal than ionization, and so UV irradiation is not as efficient a sterilization method as electron or gamma irradiation. As mentioned earlier (section 2), vegetative bacteria generally prove to be the most sensitive to irradiation (with notable exceptions, e.g. *Deinococcus* (*Micrococcus) radiodurans*), followed by moulds and yeasts, with bacterial spores and viruses as the most resistant (except in the case of UV light, where mould spores prove to be most resistant). The extent of DNA damage required to produce cell death can vary and this, together with the ability to carry out effective repair, probably determines the resistance of the organism to radiation. With ionizing radiations (gamma ray and accelerated electrons), microbial resistance decreases with the presence of moisture or dissolved oxygen (as a result of increased free-radical production) and also with elevated temperatures.

Radiation sterilization with high-energy gamma rays or accelerated electrons has proved to be a useful method for the industrial sterilization of heat-sensitive products. However, undesirable changes can occur in irradiated preparations, especially those in aqueous solution where radiolysis of water contributes to the damaging processes. In addition, certain glass or plastic (e.g. polypropylene, PTFE) materials used for packaging or for medical devices can also suffer damage. Thus, radiation sterilization is generally applied to articles in the dried state; these include surgical instruments, sutures, prostheses, unit-dose ointments, plastic syringes and dry pharmaceutical products (Chapter 22). With these radiations, destruction of a microbial population follows the classic survivor curves (see Figure 21.1) and a *D*-value, given as a radiation dose, can be established for standard bacterial spores (e.g. *B. pumilus*) permitting a suitable sterilizing dose to be calculated. In the UK it is usual to apply a dose of 25 kGy (2.5 Mrad) for pharmaceutical and medical products, although lower doses are employed in the USA and Canada.

UV light, with its much lower energy, causes less damage to microbial DNA. This, coupled with its poor penetrability of normal packaging materials, renders UV light unsuitable for sterilization of pharmaceutical dosage forms. It does find applications, however, in the sterilization of air, for the surface sterilization of aseptic work areas, and for the treatment of manufacturing-grade water.

6.1 Sterilizer design and operation

6.1.1 Gamma ray sterilizers

Gamma rays for sterilization are usually derived from a cobalt-60 (^{60}Co) source (caesium-137 may also be used), with a half-life of 5.25 years, which on disintegration emits radiation at two energy levels of 1.33 and 1.17 MeV. The isotope is held as pellets packed in metal rods, each rod carefully arranged within the source and containing up to 20 kCi (740×10^{12} Bq) of activity; these rods are replaced or rearranged as the activity of the source either drops or becomes unevenly distributed. A typical ^{60}Co installation may contain up to 1 MCi (3.7×10^{16} Bq) of activity. For safety reasons, this source is housed within a reinforced concrete building with walls some 2 m thick, and it is raised from a sunken water-filled tank only when required for use. Control devices operate to ensure that the source is raised only when the chamber is locked and that it is immediately lowered if a malfunction occurs. Articles being sterilized are passed through the irradiation chamber on a conveyor belt or monorail system and move around the raised source, the rate of passage regulating the dose absorbed (Figure 21.9).

Radiation monitors are continually employed to detect any radiation leakage during operation or source storage, and to confirm a return to satisfactory background levels within the sterilization chamber following operation. The dose delivered is dependent upon source strength and exposure period, with dwell times typically up to 20 hours. The difference in radiation susceptibilities of microbial cells and humans may be gauged from the

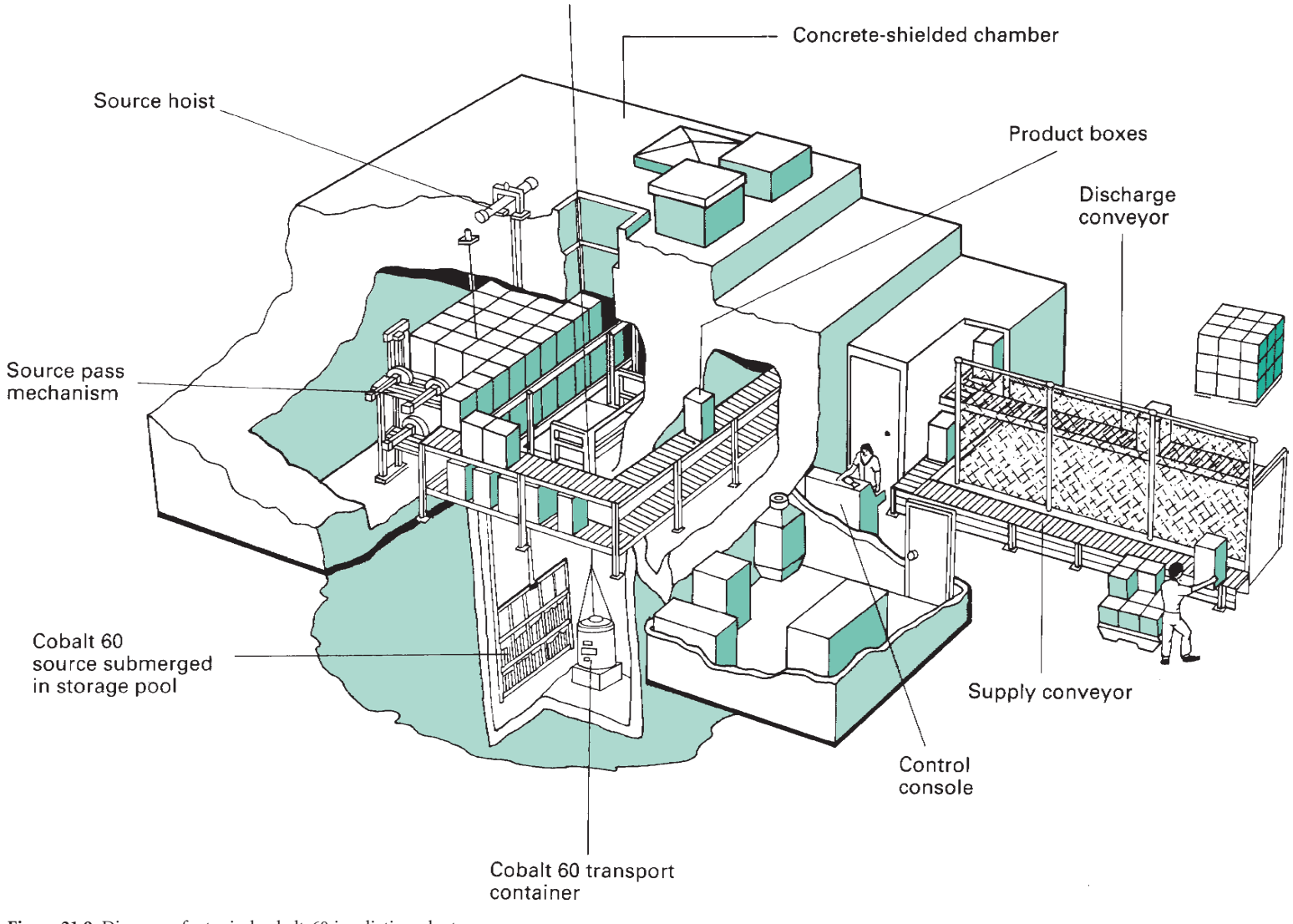

Figure 21.9 Diagram of a typical cobalt-60 irradiation plant.

fact that a lethal human dose would be delivered by an exposure of seconds or minutes.

6.1.2 Electron accelerators

Two types of electron accelerator machine exist, the electrostatic accelerator and the microwave linear accelerator, producing electrons with maximum energies of 5 MeV and 10 MeV, respectively. Although higher energies would achieve better penetration into the product, there is a risk of induced radiation and so they are not used. In the first, a high-energy electron beam is generated by accelerating electrons from a hot filament down an evacuated tube under high potential difference, while in the second, additional energy is imparted to this beam in a pulsed manner by a synchronized travelling microwave. Articles for treatment are generally limited to small packs and are arranged on a horizontal conveyor belt, usually for irradiation from one side but sometimes from both. The sterilizing dose is delivered more rapidly in an electron accelerator than in a ^{60}Co plant, with exposure times for sterilization usually amounting to only a few seconds or minutes. Varying extents of shielding, depending upon the size of the accelerator, are necessary to protect operators from X-rays generated by the bremsstrahlung effect.

6.1.3 Ultraviolet irradiation

The optimum wavelength for UV sterilization is around 260 nm. A suitable source for UV light in this region is a mercury lamp giving peak emission levels at 254 nm. These sources are generally wall- or ceiling-mounted for air disinfection, or fixed to vessels for water treatment. Operators present in an irradiated room should wear appropriate protective clothing and eye shields.

7 Filtration sterilization

The process of filtration is unique among sterilization techniques in that it removes, rather than destroys, microorganisms. Further, it is capable of preventing the passage of both viable and nonviable particles and can thus be used for both the clarification and sterilization of liquids and gases. The principal application of sterilizing-grade filters is the treatment of heat-sensitive injections and ophthalmic solutions, biological products and air and other gases for supply to aseptic areas (see Chapters 22 and 23).

Filters may also be required in industrial applications where they become part of venting systems on fermenters, centrifuges, autoclaves and freeze-driers. Certain types of filter (membrane filters) also have an important role in sterility testing, where they can be employed to trap and concentrate contaminating organisms from solutions under test. These filters are then placed on a solid nutrient medium or in a liquid medium and incubated to encourage colony growth or turbidity (section 13.1).

The major mechanisms of filtration are sieving, adsorption and trapping within the matrix of the filter material. Of these, only sieving can be regarded as absolute as it ensures the exclusion of all particles above a defined size. It is generally accepted that synthetic membrane filters, derived from cellulose esters or other polymeric materials, approximate most closely to sieve filters; while fibrous pads, sintered glass and sintered ceramic products can be regarded as depth filters relying principally on mechanisms of adsorption and entrapment. Some of the characteristics of filter media are summarized in Table 21.4. The potential hazard of microbial multiplication within a depth filter and subsequent contamination of the filtrate (microbial grow-through) should be recognized.

7.1 Filtration sterilization of liquids

In order to compare favourably with other methods of sterilization, the microorganism removal efficiency of filters employed in the processing of liquids must be high. For this reason, membrane filters of 0.2–0.22 μm nominal pore diameter are chiefly used, while sintered filters are used only in restricted circumstances, i.e. for the processing of corrosive liquids, viscous fluids or organic solvents. It may be tempting to assume that the pore size is the major determinant of filtration efficiency and two filters of 0.2 μm pore diameter from different manufacturers will behave similarly. This is not so, because, in addition to the sieving effect, trapping within the filter matrix, adsorption and charge effects all contribute significantly towards the removal of particles. Consequently, the depth of the membrane, its charge and the tortuosity of the channels are all factors which can make the performance of one filter far superior to that of another. The major criterion by which filters should be compared, therefore, is their titre reduction values, i.e. the ratio of the number of organisms challenging a filter under defined conditions to the number penetrating it. In all cases, the filter medium employed must be sterilizable, ideally by steam treatment; in the case of membrane filters this may be for once-only use, or, in the case of larger industrial filters, a small, fixed number of resterilizations; sintered filters may be

Table 21.4 Some characteristics of membrane and depth filters

Characteristic	Membrane	Depth
Absolute retention of microorganisms greater than rated pore size	+	–
Rapid rate of filtration	+	–
High dirt-handling capacity	–	+
Grow-through of microorganisms	Unlikely	+
Shedding of filter components	–	+
Fluid retention	–	+
Solute adsorption	–	+
Good chemical stability	Variable (depends on membrane)	+
Good sterilization characteristics	+	+

+, applicable; –, not applicable.

resterilized many times. Filtration sterilization is an aseptic process and careful monitoring of filter integrity is necessary as well as final product sterility testing (section 13).

Membrane filters, in the form of discs, can be assembled into pressure-operated filter holders for syringe mounting and in-line use or vacuum filtration tower devices. Filtration under pressure is generally considered most suitable, as filling at high flow rates directly into the final containers is possible without problems of foaming, solvent evaporation or air leaks. To increase the filtration area, and hence process volumes, several filter discs can be used in parallel in multiple-plate filtration systems or, alternatively, membrane filters can be fabricated into plain or pleated cylinders and installed in cartridges. Membrane filters are often used in combination with a coarse-grade fibreglass depth prefilter to improve their dirt-handling capacity.

7.2 Filtration sterilization of gases

The principal application for filtration sterilization of gases is in the provision of sterile air to aseptic manufacturing suites, hospital isolation units and some operating theatres. Filters employed generally consist of pleated sheets of glass microfibres separated and supported by an aluminium framework; these are employed in ducts, wall or ceiling panels, overhead canopies, or laminar airflow cabinets (Chapter 23). These high-efficiency particulate air (HEPA) filters can remove up to 99.997% of particles more than 0.3 μm in diameter and thus are acting as depth filters. In practice, their microorganism removal efficiency is rather better as the majority of bacteria are found associated with dust particles and only the larger fungal spores are found in the free state. Air is forced through HEPA filters by blower fans, and prefilters are used to remove larger particles to extend the lifetime of the HEPA filter. The operational efficiency and integrity of a HEPA filter can be monitored by pressure differential and airflow rate measurements, and dioctylphthalate smoke particle penetration tests.

Other applications of filters include sterilization of venting or displacement air in tissue and microbiological culture (carbon filters and hydrophobic membrane filters); decontamination of air in mechanical ventilators (glass fibre filters); treatment of exhausted air from microbiological safety cabinets (HEPA filters); and the clarification and sterilization of medical gases (glass wool depth filters and hydrophobic membrane filters).

8 New sterilization technologies

Heat is the means of terminal sterilization that is preferred by the regulatory authorities because of its relative simplicity and the high sterility assurance that it affords. However, a significant number of traditional pharmaceutical products and many recently developed biotechnology products are damaged by heat, as are many polymer-based medical devices and surgical implants; for such products alternative sterilization processes must be adopted. Whilst radiation is a viable option for many dry materials, radiation-induced damage is common in aqueous drug solutions, and gaseous methods are also inappropriate for liquids. Aseptic manufacture from individually sterilized ingredients is a suitable solution to the problem of making sterile thermolabile products, but it affords a lower degree of sterility assurance than steam sterilization and is both time-consuming and expensive. For these reasons alternative sterilization strategies have

been developed in recent years. Two processes that have progressed to the stage of commercial exploitation are those employing high-intensity light and low-temperature plasma. It must be stressed, however, that although the need to develop alternative strategies for the terminal sterilization of protein- or nucleic acid-containing biotechnology products is one of the stimuli for the investigation of new methods in general, these particular processes are unsuitable for such products.

8.1 High-intensity light

UV light has long been known to have the potential to kill all types of microorganisms, but its penetrating power is so poor that it has found practical application only in the decontamination of air (e.g. in laminar-flow workstations and operating theatres), shallow layers of water and surfaces. UV light does not penetrate metal at all, nor glass to any useful degree, but it will penetrate those polymers that do not contain unsaturated bonds or aromatic groups (e.g. polyethylene and polypropylene, but not polystyrene, polycarbonate or polyvinyl chloride). High-intensity light sterilization is based on the generation of short flashes of broad-wavelength light from a xenon lamp that has an intensity almost 100 000 times that of the sun; approximately 25% of the flash is UV light. The procedure has been applied to the sterilization of water and studied as a means of terminal sterilization for injectables in UV-transmitting plastic ampoules in a blow–fill–seal operation. Although pulsed light is unlikely to be useful for coloured solutions or those that contain solutes with a high UV absorbance, it is likely that the procedure will be readily applicable not only to water but to some simple solutions of organic molecules, e.g. dextrose-saline injection.

8.2 Low-temperature plasma

Plasma is a gas or vapour that has been subjected to an electrical or magnetic field which causes a substantial proportion of the molecules to become ionized. It is thus composed of a cloud of neutral species, ions and electrons in which the numbers of positive and negatively charged particles are equal. Plasmas may be generated from many substances but those from chlorine, glutaraldehyde and hydrogen peroxide have been shown to possess the greatest antimicrobial activity.

Low-temperature plasma is a method of sterilization that is applicable to most of the items and materials for which ethylene oxide is used, i.e. principally medical devices rather than drugs; it cannot be used to sterilize liquids, powders and certain fabrics. Commercial plasma sterilizers, which have been available since the early 1990s, typically consist of a sterilization chamber of about 75 L; this is evacuated, then filled with hydrogen peroxide vapour which is subsequently converted to a plasma by application of an electric field. An alternative commercial plasma sterilizer utilizes alternating cycles of peracetic acid vapour and a plasma containing oxygen, hydrogen and an inert carrier gas. The cycle times are typically from 60 to 90 minutes and the operating temperatures are less than 50 °C. Major benefits of plasma sterilization include elimination of the requirement to remove toxic gases at the end of the cycle (in contrast to ethylene oxide and LTSF processes); there is also no requirement for the treated device to be aired to remove residual gas, and there is no significant corrosion or reduction in sharpness of exposed surgical instruments.

9 Sterilization control and sterility assurance

To be labelled 'sterile', a product must be free of viable microorganisms. To achieve this, the product, or its ingredients, must undergo a sterilization process of sufficient microbiocidal capacity to ensure a minimum level of sterility assurance. It is essential that the required conditions for sterilization be achieved and maintained through every operation of the sterilizer. Some examples of typical conditions employed in sterilization are shown in Table 21.5.

Historically, the quality control of sterile products consisted largely, or in some cases, even exclusively, of a sterility test, to which the product was subjected at the end of the manufacturing process. However, a growing awareness of the limitations of sterility tests in terms of their ability to detect low concentrations of microorganisms has resulted in a shift in emphasis from a crucial dependence on end-testing to a situation in which the conferment of the status 'sterile' results from the attainment of satisfactory quality standards throughout the whole manufacturing process. In other words, the quality is 'assured' by a combination of process monitoring and performance criteria; these may be considered under four headings:

- Bioburden determinations (section 10)
- Environmental monitoring (section 11)
- Validation and in-process monitoring of sterilization procedures (section 12)
- Sterility testing (section 13).

Table 21.5 Examples of typical conditions employed in the sterilization of pharmaceutical and medical products

Sterilization method	Conditions
Moist heat (autoclaving)	121 °C for 15 min
	134 °C for 3 min
Dry heat	160 °C for 120 min
	170 °C for 60 min
	180 °C for 30 min
Ethylene oxide	Gas concentration:
	600–1200 mg/L
	45–63 °C
	30–70% relative humidity
	1–4 h sterilizing time
Low-temperature steam and formaldehyde	Gas concentration:
	15–100 mg/L
	Steam admission to 73 °C
	40–180 min sterilizing time depending on type of process
Peracetic acid	3.5% w/v
	450 °C
	Duration dependent on chamber size and local validation
Hydrogen peroxide	30–35% w/v (8.6 mg/L)
	600 °C
	1 h per 30 m^3
Irradiation	25 kGy (2.5 Mrad) dose
Gamma rays or accelerated electrons	
Filtration	≤0.22 µm pore size, sterile membrane filter

In well-understood and well-characterized sterilization processes (e.g. heat and irradiation), where physical measurements may be accurately made, sterility can be assured by ensuring that the manufacturing process as a whole conforms to the established protocols for the first three of the above headings. In this case the process has satisfied the required parameters thereby permitting parametric release (i.e. release based on process data) of the product without recourse to a sterility test (see Chapter 22).

10 Bioburden determinations

The term *bioburden* is used to describe the concentration of microorganisms in a material; this may be either a total number of organisms per millilitre or per gram, regardless of type, or a breakdown into such categories as aerobic bacteria or yeasts and moulds. Bioburden determinations are normally undertaken by the supplier of the raw material, whose responsibility it is to ensure that the material supplied conforms to the agreed specification, but they may also be checked by the recipient. The maximum permitted concentrations of contaminants may be those specified in various pharmacopoeias or the levels established by the manufacturer during product development.

The level of sterility assurance that is achieved in a terminally sterilized product is dependent on the design of the sterilization process itself and on the bioburden immediately prior to sterilization (Chapters 22 and 23). However, the adoption of high standards for the quality of the raw materials is not, in itself, a strategy that will ensure that the product has an acceptably low bioburden immediately prior to sterilization. It is necessary also to ensure that the opportunities for microbial contamination during manufacture are restricted (see below), and that those organisms that are present initially do not normally find themselves in conditions conducive to growth. It is for these reasons that manufacturing processes are designed to utilize adverse temperatures, extreme pH values and organic solvent exposures in order to prevent an increase in the microbial load. For example, water is the most common, and potentially the most significant, source of contamination in the manufactured product, and maintenance of water at elevated temperatures is commonly employed as a means of limiting the growth of organisms such as *Pseudomonas* spp., which can proliferate during storage, even in distilled or deionized water. Precautions such as these ensure that chemically synthesized raw materials have bioburdens that are generally much lower than those found in 'natural' products of animal, vegetable or mineral origin.

11 Environmental monitoring

The levels of microbial contamination in the manufacturing areas (Chapter 23) are monitored on a regular basis to confirm that the numbers do not exceed specified limits. The concentrations of bacteria and of yeasts/moulds in the atmosphere may be determined either by use of 'settle plates' (Petri dishes of suitable media exposed for fixed periods, on which the colonies are counted after incubation) or by use of air samplers which cause a known volume of air to be passed over an agar surface. Similarly, the contamination on surfaces, including manufacturing equipment, may be measured using swabs or contact plates (also known as Rodac—replicate organism detection and counting—plates) which are specially designed Petri dishes slightly overfilled with agar, which, when set, projects very slightly above the plastic wall of the dish. This permits the plate to be inverted on to or against any solid surface, thereby allowing transfer of organisms from the surface on to the agar.

Less commonly, environmental monitoring can extend also to the operators in the manufacturing area whose clothing, e.g. gloves or face masks, may be sampled in order to estimate the levels and types of organisms that may arise as product contaminants from those sources.

12 Validation and in-process monitoring of sterilization procedures

There are several definitions of 'validation' but, in simple terms, the word means demonstrating that a process will consistently produce the results that it is intended to. Thus, with respect to sterile products, validation would be necessary for each of the individual aspects of the manufacturing process, e.g. environmental monitoring, raw materials quality assessment, the sterilization process itself and the sterility testing procedure. Of these, it is the sterilization process that is likely to be subject to the most detailed and complex validation procedures, and these will be used to exemplify the factors to be considered. A typical validation procedure for a steam sterilization process is likely to incorporate most, or all, of the following features:

- Calibration and testing of all the physical instruments used to monitor the process, e.g. thermocouples, pressure gauges and timers
- Production of evidence that the steam is of the desired quality (e.g. that the chamber temperature is that expected for pure steam at the measured pressure)
- Conduct of leak tests and steam penetration tests using both an empty chamber and a chamber filled with the product to be sterilized in the intended load conformation
- Use of biological indicators either alone or in combination with bioburden organisms to demonstrate that the sterilization cycle is capable of producing an acceptable level of sterility assurance under 'worst case' conditions
- Production of data to demonstrate repeatability of the above (typically for three runs)
- Testing of software associated with parametric and operational monitoring
- Comprehensive documentation of all of these aspects.

There are different approaches to the demonstration of adequate sterility assurance in steam sterilization depending upon the thermostability and knowledge of the presterilization bioburden. Where the product is known to be stable, an overkill approach may be adopted in which biological indicators (section 12.3) containing 10^6 test organisms are inactivated in half the proposed exposure time (thus achieving a 12-log reduction and a sterility assurance level of 10^{-6} in the full exposure period). For a marginally thermostable product the cycle could be validated on the basis of measurements of the worst case bioburden level and the heat resistance of the known bioburden organisms; such an approach would necessitate rigorous control of the bioburden during routine manufacturing. In the UK, biological indicators are used primarily in validation rather than routine monitoring of heat sterilization processes, although their use in routine manufacturing may be required in other countries. Chemical indicators of sterilization (section 12.2) are more convenient to use than biological indicators, but as they provide no direct measure of the efficacy of the process in terms of microbial killing they are considered to be less useful. In certain instances these are no longer routinely used. Physical measurements of temperature, pressure, time, relative humidity, etc. are of such fundamental importance to the assurance of sterility that records of these parameters are retained for each batch of sterilized product.

12.1 Physical indicators

In heat sterilization processes, a temperature record is made of each sterilization cycle with both dry and moist heat (i.e. autoclave) sterilizers; this chart/digital record

forms part of the batch documentation and is compared against a master temperature record (MTR). It is recommended that the temperature be taken at the coolest part of the loaded sterilizer. Further information on heat distribution and penetration within a sterilizer can be gained by the use of thermocouples placed at selected sites in the chamber or inserted directly into test packs or bottles. For gaseous sterilization procedures, elevated temperatures are monitored for each sterilization cycle by temperature probes, and routine leak tests are performed to ensure gas-tight seals. Pressure and humidity measurements are recorded. Gas concentration is measured independently of pressure rise, often by reference to weight of gas used. In radiation sterilization, a plastic (often Perspex) dosimeter which gradually darkens in proportion to the radiation absorbed gives an accurate measure of the radiation dose and is considered to be the best technique currently available for following the radiosterilization process.

Sterilizing filters are subject to a bubble point pressure test, which is a technique employed for determining the pore size of filters, and may also be used to check the integrity of certain types of filter device (membrane and sintered glass; section 7) immediately after use. The principle of the test is that the wetted filter, in its assembled unit, is subjected to an increasing air or nitrogen gas pressure differential. The pressure difference recorded when the first bubble of gas breaks away from the filter is related to the maximum pore size. When the gas pressure is further increased slowly, there is a general eruption of bubbles over the entire surface. The pressure difference here is related to the mean pore size. A pressure differential below the expected value would signify a damaged or faulty filter. A modification to this test for membrane filters involves measuring the diffusion of gas through a wetted filter at pressures below the bubble point pressure (diffusion rate test); a faster diffusion rate than expected would again indicate a loss of filter integrity. In addition, a filter is considered ineffective when an unusually rapid rate of filtration occurs.

Efficiency testing of HEPA filters used for the supply of sterile air to aseptic workplaces (Chapter 23) is normally achieved by the generation upstream of dioctylphthalate (DOP) or sodium chloride particles of known dimension followed by detection in downstream filtered air. Retention efficiency is recorded as the percentage of particles removed under defined test conditions. Microbiological tests are not normally done.

12.2 Chemical indicators

Chemical monitoring of a sterilization process is based on the ability of heat, steam, sterilant gases and ionizing radiation to alter the chemical and/or physical characteristics of a variety of chemical substances. Ideally, this change should take place only when satisfactory conditions for sterilization prevail, thus confirming that the sterilization cycle has been successfully completed. In practice, however, the ideal indicator response is not always achieved and so a necessary distinction is made between (1) those chemical indicators which integrate several sterilization parameters (i.e. temperature, time and saturated steam) and closely approach the ideal; and (2) those which measure only one parameter and consequently can only be used to distinguish processed from unprocessed articles. Thus, indicators which rely on the melting of a chemical substance show that the temperature has been attained but not necessarily maintained.

Chemical indicators generally undergo melting or colour changes, the relationship of this change to the sterilization process being influenced by the design of the test device (Table 21.6). It must be remembered, however, that the changes recorded do not necessarily correspond to microbiological sterility and consequently the devices should never be employed as sole indicators in a sterilization process. Nevertheless, when included in strategically placed containers or packages, chemical indicators are valuable monitors of the conditions prevailing at the coolest or most inaccessible parts of a sterilizer.

12.3 Biological indicators

Biological indicators (BIs) for use in thermal, chemical or radiation sterilization processes consist of standardized bacterial spore preparations which are usually in the form either of suspensions in water or culture medium or of spores dried on paper, aluminium or plastic carriers. As with chemical indicators, they are usually placed in dummy packs located at strategic sites in the sterilizer. Alternatively, for gaseous sterilization these may also be placed within a tubular helix (Line–Pickerill) device. After the sterilization process, the aqueous suspensions or spores on carriers are aseptically transferred to an appropriate nutrient medium, which is then incubated and periodically examined for signs of growth. Spores of *B. stearothermophilus* in sealed ampoules of culture medium are used for steam sterilization monitoring, and these may be incubated directly at 55 °C; this eliminates the need for an aseptic transfer. Aseptic transfers are also avoided by the use of self-contained units where the spore

Table 21.6 Examples of chemical indicators that have been used for monitoring sterilization processes

Sterilization method	Principle	Device	Parameter(s) monitored
Heat			
Autoclaving or dry heat	Temperature-sensitive coloured solution	Sealed tubes partly filled with a solution which changes colour at elevated temperatures; rate of colour change is proportional to temperature, e.g. Browne's tubes	Temperature, time
Dry heat only	Temperature-sensitive chemical	Usually a temperature-sensitive white wax concealing a black marked or printed (paper) surface; at a predetermined temperature the wax rapidly melts, exposing the background mark(s)	Temperature
Heating in an autoclave only	Steam-sensitive chemical	Usually an organic chemical in a printing ink base impregnated into a carrier material. A combination of moisture and heat produces a darkening of the ink, e.g. autoclave tape. Devices of this sort can be used within dressings packs to confirm adequate removal of air and penetration of saturated steam (Bowie–Dick test)	Saturated steam
	Capillary principle (Thermalog S)	Consists of a blue dye in a waxy pellet, the melting-point of which is depressed in the presence of saturated steam. At autoclaving temperatures, and in the continued presence of steam, the pellet melts and travels along a paper wick forming a blue band the length of which is dependent upon both exposure time and temperature	Temperature, saturated steam, time
Gaseous sterilization			
Ethylene oxide (EO)	Reactive chemical	Indicator paper impregnated with a reactive chemical which undergoes a distinct colour change on reaction with EO in the presence of heat and moisture. With some devices rate of colour development varies with temperature and EO concentration	Gas concentration, temperature, time (selected devices); NB a minimum relative humidity (rh) is required for device to function
	Capillary principle (Thermalog G)	Based on the same 'migration along wick' principle as Thermalog S. Optimum response in a cycle of 600 mg/L EO, temperature 54 °C, rh 40–80%. Lower EO levels and/or temperature will slow response time	Gas concentration, temperature, time (selected cycles)
Low-temperature steam and formaldehyde	Reactive chemical	Indicator paper impregnated with a formaldehyde-, steam- and temperature-sensitive reactive chemical which changes colour during the sterilization process	Temperature, time (selected cycles)
Radiation sterilization			
	Radiochromic chemical	Plastic devices impregnated with radiosensitive chemicals which undergo colour changes at relatively low radiation doses	Only indicate exposure to radiation
	Dosimeter device	Acidified ferric ammonium sulphate or ceric sulphate solutions respond to irradiation by dose-related changes in their optical density	Accurately measure radiation doses

Table 21.7 Biological indicators (BIs) recommended by the *European Pharmacopoeia* (2009) for monitoring sterilization processes

Sterilization process	Species	Inoculum size	D-value
Steam sterilization (121 °C)	*Bacillus stearothermophilus*	$>5 \times 10^5$	>1.5 min
Dry heat (160 °C)	*Bacillus subtilis* var *niger*	$>1 \times 10^5$	1–3 min
Hydrogen peroxide and peracetic acid	*Bacillus stearothermophilus*	$>5 \times 10^5$	–
Ethylene oxide (EO)	*Bacillus subtilis* var *niger*	$>5 \times 10^5$	>2.5 min at 54 °C, 60% relative humidity and 600 mg/L EO
Formaldehyde	*Bacillus subtilis* var *niger*	$>5 \times 10^5$	–
Ionizing radiation	*Bacillus pumilus*	$>1 \times 10^7$	1.9 kGy

strip and nutrient medium are present in the same device ready for mixing after use.

The bacterial species to be used in a BI must be selected carefully, as it must be non-pathogenic and should possess above-average resistance to the particular sterilization process. Resistance is adjudged from the spore destruction curve obtained upon exposure to the sterilization process; recommended BI spores and their decimal reduction times (*D*-values; section 2.2.1) are shown in Table 21.7. Great care must be taken in the preparation and storage of BIs to ensure a standardized response to sterilization processes. Indeed, while certainly offering the most direct method of monitoring sterilization processes, it should be realized that BIs may be less reliable monitors than physical methods and they are not recommended for routine use, except in the case of gaseous sterilization.

One of the long-standing criticisms of BIs is that the incubation period required in order to confirm a satisfactory sterilization process imposes an undesirable delay on the release of the product. This problem has been overcome, with respect to steam sterilization at least, by the use of a detection system in which a spore enzyme, α-glucosidase (reflective of spore viability), converts a non-fluorescent substrate into a fluorescent product in as little as 1 hour.

Filtration sterilization requires a different approach from biological monitoring, the test effectively measuring the ability of a filter to produce a sterile filtrate from a culture of a suitable organism. For this purpose, *Serratia marcescens*, a small Gram-negative rod-shaped bacterium (minimum dimension 0.5 µm), has been used for filters of 0.45 µm pore size, and a more rigorous test involving *Brevundimonas diminuta* (formerly *Pseudomonas diminuta*) having a minimum dimension of 0.3 µm is applied to filters of 0.22 µm pore size. The latter filters are defined as those capable of completely removing *Brev. diminuta* from suspension. In this test, using this organism, a realistic inoculum level must be adopted, as the probability of bacteria appearing in the filtrate rises as the number of *Brev. diminuta* cells in the test challenge increases; a standardized inoculum size of 10^7 cells cm^{-2} is normally employed. The extent of the passage of this organism through membrane filters is enhanced by increasing the filtration pressure. Thus, successful sterile filtration depends markedly on the challenge conditions. Such tests are used as part of the filter manufacturer's characterization and quality assurance process, and a user's initial validation procedure. They are not employed as a test of filter performance in use.

13 Sterility testing

A sterility test is essentially a test which assesses whether a sterilized pharmaceutical or medical product is free from contaminating microorganisms by incubation of either the whole or a part of that product with a nutrient medium. It thus becomes a destructive test and is of questionable suitability for testing large, expensive or delicate products or equipment. Furthermore, by its very nature such a test is a statistical process in which part of a batch is sampled and the chance of the batch being passed for use then depends on the sample passing the

sterility test. Random sampling should be applied to products that have been processed and filled aseptically. With products sterilized in their final containers, samples should be taken from the potentially coolest or least sterilant-accessible part of the load.

A further limitation is that which is inherent in a procedure intended to demonstrate a negative. A sterility test is intended to demonstrate that no viable organisms are present, but failure to detect them could simply be a consequence of the use of unsuitable media or inappropriate cultural conditions. To be certain that no organisms are present it would be necessary to use a universal culture medium suitable for the growth of any possible contaminant and to incubate the sample under an infinite variety of conditions. Clearly, no such medium or combination of media are available and, in practice, only media capable of supporting non-fastidious bacteria, yeasts and moulds are employed. Furthermore, in pharmacopoeial tests, no attempt is made to detect viruses, which on a size basis, are the organisms most likely to pass through a sterilizing filter. Nevertheless, the sterility test does have an important application in monitoring the microbiological quality of filter-sterilized, aseptically filled products and does offer a final check on terminally sterilized articles. In the UK, test procedures laid down by the *European Pharmacopoeia* must be followed; this provides details of the sample sizes to be adopted in particular cases. The principles of these tests are discussed below.

13.1 Methods

Three alternative methods are available when conducting sterility tests:

- The *direct inoculation* method involves introducing test samples directly into nutrient media. The *European Pharmacopoeia* recommends two media: (1) fluid mercaptoacetate medium (also known as fluid thioglycollate medium), which contains glucose and sodium mercaptoacetate (sodium thioglycollate) and is particularly suitable for the cultivation of anaerobic organisms (incubation temperature 30–35 °C); and (2) soyabean casein digest medium (also known as tryptone soya broth), which will support the growth of both aerobic bacteria (incubation temperature 30–35 °C) and fungi (incubation temperature 20–25 °C). Other media may be used provided that they can be shown to be suitable alternatives. Limits are placed upon the ratio of the weight or volume of added sample relative to the volume of culture medium so as to avoid reducing the nutrient properties of the medium or creating unfavourably high osmotic pressures within it.
- *Membrane filtration* is the technique recommended by most pharmacopoeias and, consequently, the method by which the great majority of products are examined. It involves filtration of fluids through a sterile membrane filter (pore size ≤0.45 μm), any microorganism present being retained on the surface of the filter. After washing *in situ*, the filter is divided aseptically and portions are transferred to suitable culture media which are then incubated at the appropriate temperature for the required period of time. Water-soluble solids can be dissolved in a suitable diluent and processed in this way and oil-soluble products may be dissolved in a suitable solvent, e.g. isopropyl myristate.
- A sensitive method for detecting low levels of contamination in intravenous infusion fluids involves the addition of a *concentrated culture medium* to the fluid in its original container, such that the resultant mixture is equivalent to single strength culture medium. In this way, sampling of the entire volume is achieved.

With the techniques discussed above, the media employed should previously have been assessed for nutritive (growth-supporting) properties and a lack of toxicity using specified organisms. It must be remembered that any survivors of a sterilization process may be damaged and thus must be given the best possible conditions for growth.

As a precaution against accidental contamination, product testing must be carried out under conditions of strict asepsis using, for example, a laminar airflow cabinet to provide a suitable environment.

The *European Pharmacopoeia* indicates that it is necessary to conduct control tests that confirm the adequacy of the facilities by sampling of air and surfaces and carrying out tests using samples 'known' to be sterile (negative controls). In reality, this means samples that have been subjected to a very reliable sterilization process, e.g. radiation, or samples that have been subjected to a sterilization procedure more than once. In order to minimize the risk of introducing contaminants from the surroundings or from the operator during the test itself, isolators are often employed which physically separate the operator from the materials under test.

13.2 Antimicrobial agents

Where an antimicrobial agent comprises the product or forms part of the product, for example as a preservative, its activity must be nullified in some way during sterility testing so that an inhibitory action in preventing the growth of any contaminating microorganisms is overcome. This is achieved by the following methods.

13.2.1 Specific inactivation

An appropriate inactivating (neutralizing) agent (Table 21.8) is incorporated into the culture media. The inactivating agent must be non-toxic to microorganisms, as must any product resulting from an interaction of the inactivator and the antimicrobial agent.

Although Table 21.7 lists only benzylpenicillin and ampicillin as being inactivated by β-lactamase (from *B. cereus*), other β-lactams may also be hydrolysed by β-lactamases. Other antibiotic-inactivating enzymes are also known (Chapter 13) and have been considered as possible inactivating agents, e.g. chloramphenicol acetyltransferase (inactivates chloramphenicol) and enzymes that modify aminoglycoside antibiotics.

13.2.2 Dilution

The antimicrobial agent is diluted in the culture medium to a level at which it ceases to have any activity, for example phenols, cresols and alcohols (see Chapter 18). This method applies to substances with a high dilution coefficient, η.

13.2.3 Membrane filtration

This method has traditionally been used to overcome the activity of antibiotics for which there are no inactivating agents, although it could be extended to cover other products if necessary, e.g. those containing preservatives for which no specific or effective inactivators are available. Basically, a solution of the product is filtered through a hydrophobic-edged membrane filter that will retain any contaminating microorganisms. The membrane is washed *in situ* to remove any traces of antibiotic adhering to the membrane and is then transferred to appropriate culture media.

Table 21.8 Inactivating agents (neutralizing agents)

Inhibitory agents	Inactivating agents
Phenols, cresols	None (dilution)
Alcohols	None (dilution)
Parabens	Dilution and Tween
Mercury compounds	-SH compounds
Quaternary ammonium compounds	Lecithin + Lubrol W; Lecithin + Tween (Letheen)
Benzylpenicillin[a], Ampicillin	β-Lactamase from *Bacillus cereus*
Other antibiotics[a]	None (membrane filtration)
Sulphonamides	*p*-Aminobenzoic acid

[a]See text.

13.3 Positive controls

It is essential to show that microorganisms will actually grow under the conditions of the test. For this reason positive controls have to be carried out; in these, the ability of small numbers of suitable microorganisms to grow in media in the presence of the sample is assessed. The microorganism used for positive control tests with a product containing or comprising an antimicrobial agent must, if at all possible, be sensitive to that agent, so that growth of the organism indicates a satisfactory inactivation, dilution or removal of the agent. The *European Pharmacopoeia* suggests the use of designated strains of *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa* as appropriate aerobic organisms, *Clostridium sporogenes* as an anaerobe and *Candida albicans* or *Aspergillus niger* as fungi.

In practice, a positive control (medium with added test sample) and a negative control (medium without it) are inoculated simultaneously, and the rate and extent of growth arising in each should be similar. However, the negative control without the test sample, is, in effect, exactly the same as the growth promotion control that is also described in the test procedure, so, for the organisms concerned, it is not necessary to do both.

All the controls may be conducted either before, or in parallel with, the test itself, providing that the same batches of media are used for both. If the controls are carried out in parallel with the tests and one of the controls gives an unexpected result, the test for sterility may be declared invalid, and, when the problem is resolved, the test may be repeated.

13.4 Specific cases

Specific details of the sterility testing of parenteral products, ophthalmic and other non-injectable preparations, and surgical sutures will be found in the *European Pharmacopoeia*. These procedures cannot conveniently be applied to items like surgical dressings and medical devices because they are too big. In such cases the most convenient approach is to immerse the whole object in culture medium in a sterile flexible bag, but care must be

taken to ensure that the liquid penetrates to all parts and surfaces of the material.

13.5 Sampling

A sterility test attempts to infer the state (sterile or non-sterile) of a batch from the results of an examination of part of a batch, and is thus a statistical operation. Suppose that p represents the proportion of infected containers in a batch and q the proportion of non-infected containers, then, $p + q = 1$ or $q = 1 - p$.

Suppose also that a sample of two items is taken from a large batch containing 10% contaminated containers. The probability of a single item taken at random being contaminated is $p = 0.1$ (10% = 0.1), whereas the probability of such an item being non-contaminated is given by $q = 1 - p = 0.9$. The probability of both items being contaminated is $p^2 = 0.01$, and of both items being non-contaminated, $q^2 = (1 - p)^2 = 0.81$. The probability of obtaining one contaminated item and one non-contaminated item is $1 - (0.01 + 0.81) = 0.18 = 2pq$.

In a sterility test involving a sample size of n containers, the probability p of obtaining n consecutive 'steriles' is given by $q^n = (1 - p)^n$. Values for various levels of p (i.e. proportion of infected containers in a batch) with a constant sample size are given in Table 21.9, which shows that the test cannot detect low levels of contamination. Similarly, if different sample sizes are employed (also based on $(1 - p)^n$) it can be shown that as the sample size increases, the probability of the batch being passed as sterile decreases.

It can be seen from the above that a sterility test can only show that a proportion of the products in a batch is sterile. Thus, the correct conclusion to be drawn from a satisfactory test result is that the batch has passed the sterility test not that the batch is sterile.

13.6 Retests

Under certain circumstances a sterility test may be repeated, but the only justification for repeating the test is unequivocal evidence that the first test was invalid; a retest cannot be viewed as a second opportunity for the batch to pass when it has failed the first time. Circumstances that may justify a retest would include, for example, failure of the air filtration system in the testing facility which might have permitted airborne contaminants to enter the product or media during testing, non-sterility of the media used for testing, or evidence that contamination arose during testing from the operating personnel or a source other than the sample under test.

14 The role of sterility testing

The techniques discussed in this chapter comprise an attempt to achieve, as far as possible, the continuous monitoring of a particular sterilization process. The sterility test on its own provides no guarantee as to the sterility of a batch; however, it is an additional check and, as it will detect gross failure, continued compliance with the test does give confidence as to the efficacy of a sterilization or aseptic process. Failure to carry out a sterility test, despite the major criticism of its inability to detect other than gross contamination, may have important legal and moral consequences.

15 References and further reading

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Table 21.9 Sampling in sterility testing

	Infected items in batch (%)					
	0.1	1	5	10	20	50
p	0.001	0.01	0.05	0.1	0.2	0.5
q	0.999	0.99	0.95	0.9	0.8	0.5
Probability (P), of drawing 20† consecutive sterile items[a]	0.98	0.82	0.36	0.12	0.012	<0.00001

[a]Calculated from $P = (1 - p)^{20} = q^{20}$; 20 is the sample size required by the *European Pharmacopoeia* for batches of >500 items.

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Part 5 Pharmaceutical production

22 Sterile pharmaceutical products

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Hugo and Russell's Pharmaceutical Microbiology, Eighth Edition. Edited by Stephen P. Denyer, Norman Hodges, Sean P. Gorman, Brendan F. Gilmore.

1 Introduction

Parenteral drug delivery systems and many medicinal products, such as dressings and sutures, must be sterile in order to avoid the possibilities of microbial degradation or infection occurring as a result of their use. Sterility is also important for any material or instrument likely to contact broken skin or internal organs. Although pathogenic bacteria, fungi or viruses pose the most obvious danger to a patient, it should be also realized that microorganisms usually regarded as non-pathogenic and which inadvertently gain access to body cavities in sufficient numbers may cause a severe, possibly fatal infection. Consequently, injections, ophthalmic preparations, irrigation fluids, dialysis solutions, sutures and ligatures, implants, and certain surgical dressings, as well as instruments necessary for their use or administration, must be presented in a sterile condition.

Although there is always a chance of an idiosyncratic reaction between a medicine and a patient caused by sensitivity, allergic reaction or unwanted side effects, for sterile products there is the added requirement that they must be free of viable microorganisms. This consequently means the product should be manufactured in a manner that reduces to the lowest likelihood the risk of microbial contamination. Thus a sterile product should not contain viable bacteria, yeasts or fungi, nor other microorganisms such as rickettsiae, mycoplasmas, protozoa or viruses. The absence of prion particles is also desirable but difficult to demonstrate (see Chapter 2). Sterilization processes concentrate on the destruction or removal of microorganisms. Each process is designed to remove the most problematic microorganism (i.e. the smallest bacteria in filtration or the most heat-resistant bacterial spores in heat sterilization processes) on the basis that, once a sterilization process has been chosen, elimination of the most problematic species will have led to the elimination of all less resistant microorganisms.

The principles behind the sterilization processes are described in Chapter 21. The choice of method is determined largely by the ability of the formulation and container to withstand the physical stresses applied during the sterilization process. All products intended for sterilization should be manufactured under clean conditions and therefore will be of low microbial content (bioburden) prior to sterilization. Under these conditions, the sterilization process will not be overtaxed and will generally be within the safety limits needed to provide the required level of sterility assurance (Chapter 21). The next section emphasizes parenteral products, but the practices described apply to many other types of sterile product.

2 Types of sterile product

The most obviously recognized sterile pharmaceutical preparations are injections. These vary from very small-volume antigenic products to large-volume, total parenteral nutrition products. Other sterile products include ophthalmic preparations, creams and dusting powders. This section describes their formulation and packaging and the constraints imposed by sterilization on stability, formulation and packaging of some of the more common sterile products.

2.1 Injections

Injections may be aqueous solutions, oily solutions (because of poor aqueous solubility or the necessity for a prolongation of drug activity), aqueous suspensions or oily suspensions. They may be aseptically produced or terminally sterilized in their final containers (Chapter 23). Those drugs that are unstable in solution may be presented as a freeze-dried (lyophilized) powder. The choice of final packaging should not determine the method of sterilization.

2.1.1 Formulation philosophy

An injection must be manufactured under conditions that result in a product containing the minimum possible levels of particles and pyrogenic substances (section 4.4). Its formulation and packaging must maintain physical and chemical stability throughout the production processes, the intended shelf life and during administration. To achieve this, excipients such as buffers and antioxidants may be required to ensure chemical stability, and solubilizers, such as propylene glycol or polysorbates, may be necessary for drugs with poor aqueous solubility to maintain the drug in solution. Table 22.1 lists some chemical constituents of common injections and ophthalmic preparations.

Many injections are formulated as aqueous solutions, with Water for Injections (see Chapter 23) as the vehicle. Their formulation depends on several factors including the aqueous solubility of the active ingredient, the dose, its thermal stability, the route of administration, and whether the product is to be offered as a multiple-dose product (i.e. with doses removed on different occasions) or as a single-dose form (as the term suggests, only one dose per container). Most injections are prepared in

Table 22.1 Some examples of excipients used in formulations and reasons for their inclusion

Product	Excipients	Reason for inclusion
Diazepam injection	Ethanol	Cosolvent
	Propylene glycol	Cosolvent
Insulin isophane injection (Humalin, Eli-Lilly)	m-Cresol	Preservative
	Glycerol	Tonicity modifier
	Phenol	Preservative
	Protamine sulphate	Forms insulin complex
	Dibasic sodium phosphate	Buffer
	Zinc oxide	Adjusts zinc content
	Hydrochloric acid	pH adjustment
	Sodium hydroxide	pH adjustment
Promethazine hydrochloride injection USP	Disodium edetate	Chelating agent
	Sodium metabisulphite	Antioxidant
	Phenol	Preservative
	Sodium acetate	Buffer
	Acetic acid	Buffer
Minims chloramphenicol eye drops	Borax	Buffer
	Boric acid	Buffer
Minims prednisolone sodium phosphate eye drops	Disodium edetate	Chelating agent
	Sodium chloride	Tonicity adjustment
	Sodium dihydrogen phosphate	Buffer
	Sodium hydroxide	pH adjustment

single-dose form but this is mandatory for certain routes, e.g. spinal injections where the intrathecal route is used, and large-volume intravenous infusions. Multiple-dose injections may require the inclusion of a suitable preservative to prevent contamination following the removal of each dose. Injections used for several routes, including the intrathecal and intracardiac routes, must not contain a preservative because of potential long-term damage to the patient. A review of the preservatives used in parenteral products has been given by Meyer & Shi (2009).

Some types of injections must be isotonic with blood serum. This applies particularly to large-volume intravenous infusions if at all possible; hypotonic solutions may cause lysis of red blood corpuscles and thus must not be used for this purpose. Conversely, hypertonic solutions can be employed; these induce shrinkage, but not lysis, of red cells, which recover their shape later. Intraspinal injections must also be isotonic to reduce pain at the site of injection; so should intramuscular and subcutaneous injections. Adjustment to isotonicity can be determined from either the depression of freezing point or from sodium chloride equivalents. The depression of the freezing point depends on the number of dissolved particles (molecules or ions) present in a solution. The equation:

$$W = (0.52 - a)/b \quad (1)$$

where W is the percentage (w/v) of adjusting substance, a is the freezing point of unadjusted solution and b is the depression of the freezing point of water induced by 1% w/v of adjusting substance, allows the determination of how much adjusting substance is required to produce isotonicity with blood plasma.

Alternatively the sodium chloride equivalent, which is produced by dividing the value for the depression of freezing point produced by a solution of the substance by the corresponding value of a solution of sodium chloride of the same strength, may be used. Fuller details of each method may be found in the *Pharmaceutical Codex* (1994).

2.1.2 Intravenous infusions

Intravenous infusions consist of large-volume injections or drips (500 ml or more) that are infused at various rates (50–500 ml/h) into the venous system. They are generally sterilized in an autoclave. Examples include isotonic solutions of sodium chloride or glucose that are used to maintain fluid and electrolyte balance, for replacement of extracellular body fluids (e.g. after surgery or prolonged periods of fluid loss), as a supplementary energy source (1 L of 5% w/v glucose yields 714 kJ of energy) or as a vehicle for drugs. Other important examples are blood products, which are collected and processed in sterile containers, and plasma substitutes, e.g. dextrans and degraded gelatin. Dextrans are glucose polymers in which the glucose monomers are joined by 1–6-α links; they are produced by certain bacteria of the genus *Leuconostoc*, e.g. *Leuconostoc mesenteroides*.

2.1.2.1 Intravenous additives

A common hospital practice is to add drugs to infusions immediately before administration. Regularly used additives include potassium chloride, lidocaine (lignocaine), heparin, certain vitamins and antibiotics. Potentially this can be a hazardous practice. For instance, the drug may precipitate in the infusion fluid because of the pH (e.g. amphotericin) or the presence of calcium salts (e.g. thiopentone); the drug may degrade rapidly (e.g. ampicillin in 5% w/v glucose); multiple additions may lead to precipitation of one or both of the drugs or to accelerated degradation; and finally, drug loss may occur because of sorption by the container. For instance, insulin is adsorbed by glass or by polyvinyl chloride (PVC); glyceryl trinitrite and diazepam are absorbed by PVC. Apart from these problems, if the addition is not carried out under strict aseptic conditions the fluid can become contaminated with microorganisms during the procedure. Thus any addition should be made in a laminar-flow workstation or isolator, and the fluid should, ideally, be administered within 24 hours of preparation.

Another approach to the problem of providing an intravenous drug additive service is to add the drug to a small volume (50–100 ml) infusion in a collapsible plastic container and store the preparation at −20 °C in a freezer. The infusion can be removed when required and thawed rapidly in a microwave oven. Many antibiotics are stable for several months when stored in minibags at −20 °C and are unaffected by the thawing process. Other antibiotics, e.g. ampicillin, degrade even when frozen.

2.1.2.2 Total parenteral nutrition

Total parenteral nutrition (TPN) is the use of mixtures of amino acids, vitamins, electrolytes, trace elements and an energy source (glucose and fat) in the long-term feeding of patients who are unconscious or unable to take food. All or most of the ingredients to feed a patient for 1 day are combined aseptically in one large (3 L capacity) collapsible plastic bag, the contents of which are infused over a 12–24 hour period. Transfer of amino acid, glucose and electrolyte infusions, and the addition of vitamins and trace elements must be carried out with great care under aseptic conditions to avoid microbial contamination. These solutions often provide good growth conditions for bacteria and moulds. Fats are administered as oil-in-water emulsions comprising small droplets of a suitable vegetable oil (e.g. soyabean) emulsified with egg lecithin and sterilized by autoclaving. In many cases, the fat emulsion is added to the 3 L bag. Thus TPN fluids are complex mixtures and a multitude of potential interactions, both chemical and physicochemical, may occur between their individual components resulting in decomposition, creaming, precipitation or even the formation of toxic by-products. Trace elements, calcium, vitamins and lipids are particularly prone to affecting the stability.

Although many vitamins may be administered as a single dose at various time intervals, many of the patient's requirements will be found in what is basically an emulsion formulation, prepared aseptically and thus with no terminal sterilization. The product usually contains both essential and non-essential amino acids rather than fully formed protein, and energy is provided at a ratio of 0.6–1.1 MJ per gram of protein nitrogen. A mixture of carbohydrate (glucose) and fat (as an emulsion) provides the energy and electrolytes, trace elements and vitamins are included as required. Thus the TPN fluid is prepared to suit the individual patient's needs. The fact that the product contains so many ingredients makes TPN fluids extremely difficult to prepare, and once vitamins are added, their chemical instability reduces the shelf life. During preparation, TPN fluids are compounded from individual solutions or emulsions. Generally, the bulk of the final volume is derived from glucose solutions, amino acid solutions and fat emulsions; small-volume solutions are added to these before filling. During compounding, electrolytes are added to the amino acid solutions and phosphate salts to the dextrose (glucose) solutions, which are then mixed and the lipid emulsions added. This order of mixing is adopted because the pH

of glucose solutions decreases due to degradation during their sterilization and addition of emulsions to this low pH solution might cause emulsion instability. The mixing of the amino acids with the glucose solutions provides a vehicle with some degree of buffering capacity. Calcium might precipitate as the phosphate if its salts were to be added directly to the phospholipid emulsion. Vitamins are added to the lipid emulsion or to the bag immediately before use.

A number of other difficulties may be encountered. Polyunsaturated acids are subject to hydrolysis. Any residual air might cause oxidation of labile vitamins, e.g. vitamin C. Lipids (the fat emulsions and fat-soluble vitamins formulated as an emulsion) may extract plasticizer from a plastic container, especially if the bag is based on PVC. Any electrolytes may compromise emulsion stability by altering the electrochemistry around the dispersed oil droplets, thus allowing the droplets to move closer to each other (due to a disruption of the Stern layer) and coalesce; a less noticeable problem would be changes in the globule size. Additionally, the plastic bag might absorb the oil-soluble vitamins and care has to be taken in the selection of the container to avoid moisture loss. As a final example of the complex nature of TPN fluids, amino acids may undergo the Maillard reaction with glucose, resulting in discoloration. An account of the clinical aspects of TPN can be found in Harper & Lamerton (2009).

2.1.3 Small-volume injections

This category includes single-dose injections, usually of 1–2 ml but as high as 50 ml, dispensed in borosilicate glass ampoules, plastic (polyethylene or polypropylene) ampoules or, rarely, multiple-dose glass vials of 5–25 ml capacity stoppered with a rubber closure through which a hypodermic needle can be inserted, e.g. insulins, vaccines. The closure is designed to reseal after withdrawal of the needle. It is unwise to include too many doses in a multiple-dose container because of the risk of microbial contamination during repeated use. Preservatives must be added to injections in multiple-dose containers to prevent contamination during withdrawal of successive doses. However, preservatives may not be used in injections in which the total volume to be injected at one time exceeds 15 ml. This may occur if the solubility of a drug is such that a therapeutic dose can only be achieved in this volume of solvent. There is also an absolute prohibition on the inclusion of preservatives in intra-arterial, intracardiac, intrathecal or subarachnoid, intracisternal and peridural injections, and various ophthalmic injections.

2.1.3.1 Small-volume oily injections

Certain small-volume injections are available where the drug is dissolved in a viscous oil because it is insoluble in water and therefore a non-aqueous solvent is used. In addition, drugs in non-aqueous solvents provide a depot effect, e.g. for hormones. The intramuscular route of injection must be used. The vehicle may be a metabolizable fixed oil such as arachis oil or sesame oil (but not a mineral oil) or an ester, such as ethyl oleate, which is also capable of being metabolized. The latter is less viscous and therefore easier to administer, but the depot effect is of shorter duration. The drug is normally dissolved in the oil, filtered under pressure and distributed into ampoules. After sealing, the ampoules are sterilized by dry heat. A preservative is probably ineffective in such a medium and therefore offers very little protection against contamination in a multiple-dose oily injection.

2.1.4 Freeze-dried products

In brief, freeze-drying (lyophilization) consists of preparing the drug solution (with buffers and cryoprotectants), filtering through a bacteria-proof filter, dispensing into containers, removing water in a freeze-drier, then capping and closing the containers. Many biotechnology products are freeze-dried.

Freeze-drying is an aseptic process whereby water is removed from a frozen product mainly by sublimation, i.e. by the conversion of ice directly into the vapour state without the intermediary of liquid water. It is a batch process, of relatively long duration, and is used frequently for drugs of poor stability. Drugs are reconstituted into solution immediately prior to injection. The process consists of three stages:

- *freezing*, which slows down degradation and solidifies the product
- *primary drying*, whereby energy is provided to the system and a vacuum applied to expedite the removal of moisture at subambient temperatures
- *secondary drying*, whereby the product is heated to remove the last traces (2%) of water.

A number of characteristics of the formulation control the behaviour of the product during the lyophilization cycle. These include the glass transition temperature and the collapse temperature. The maintenance of sterility and retention of the appropriate *sterility assurance level*

(SAL) is implicit in the freeze-drier design (Pikal, 2007). Although membrane-filtered sterile solutions may be used to fill containers to be placed into the freeze-drier, other measures to maintain sterility are also employed. These include using steam sterilization of the drier; gaseous sterilization has not been widely adopted. The temperature of shelves is regulated using a circulating fluid such as dimethylsiloxane oil. Electronics and computerization have led to the accumulation of better data for validation. Stoppering systems allow the successful sealing of the containers and gas entering the drier may be filtered to effect sterilization.

2.1.5 Packaging, closures and blow-fill technology

The packaging and closures must prevent loss of vehicle, excipient or drug during sterilization and storage. Additionally, ingress of microorganisms must be prevented. The packaging must not contribute any significant amounts of extractable chemicals to the contents, e.g. vulcanizing agents from rubbers or plasticizers from PVC infusion containers.

2.1.5.1 Glass containers

Single-dose injections are usually packed in glass ampoules containing 1, 2 or 5 ml of product. To ensure removal of the correct dose volume by syringe and needle, it is necessary to add an appropriate overage to the ampoule. Thus a 1 ml ampoule will actually contain 1.1 ml of product and a 2 ml ampoule should contain 2.15 ml of product.

Many injectables are sealed with a rubber closure held on by an aluminium screw-cap or crimp-on ring. The rubber should be non-fragmenting, not release soluble extractives, and be sufficiently soft and pliable to seal around the needle inserted immediately prior to use. Although filled bottles are sterilized by autoclaving, it is still possible for the infusions in glass bottles to become contaminated with microorganisms through the seal before use. For instance, during the final part of the autoclave cycle, bottles may be spray-cooled with water to hasten the cooling process. However, if there is a poor fit between bottle lip and rubber plug (a skirted inset type is used) it is possible for the spray-cooling water to spread by capillary movement between bottle thread and screw-cap and even to enter the bottle contents. Failure may also result from any imperfection of the bottle or plug. Microorganisms may gain access to the product within the containers during storage if hairline cracks (due to bad handling or rough treatment) are present which permit fluid seepage. Finally, contamination may occur during use (1) if poor aseptic techniques are applied when setting up the infusion, (2) via an ineffective air inlet (which allows replacement of the infused fluid with air in glass bottles), or (3) when changing the giving set or bottle.

Three types of glass are suitable for use in the manufacture of containers for injectable preparations. These are a neutral borosilicate glass, a sulphated soda glass and a soft, moderately hydrolytic resistant glass. The glasses are classified by their hydrolytic resistance. The choice for a container depends on the properties of the solution they are used to package. The advantages of glasses as container materials include their chemical resistance, the fact that they do not absorb or leach organic materials, their impermeability to water vapour and other gases, their transparency, their ability to form rigid strong stable containers which resist puncture, their ability to hold a vacuum and their overall stability to moist heat or dry heat sterilization. However, glass containers may break and crack during the sterilization process, they are attacked by alkaline solutions (and so may be a problem with, for example, sodium citrate bladder irrigation), they are heavy and require venting during administration of their contents.

2.1.5.2 Closures

Closures are made of a polymer and their formulation include curing agents, activators, antioxidants, plasticizers, fillers and pigments. They have to be selected with the drug product in mind to avoid chemical incompatibility and possible reaction with the ingredients in the product formulation. Sorption of the preservative from multiple-dose formulations has frequently been a problem and closures may therefore require saturation with the ingredients in the product prior to packaging.

Closures should be flexible, to conform to the shape of the vial; resilient, so as to reseal after each needle puncture; tough, so that low fragmentation levels occur when punctured; non-thermoplastic, so that the heat sterilization process is tolerated; and chemically compatible with the drug formulation. Early closures were sulphur-based, and easily cured with accelerators to speed up the curing rate. Unfortunately, a high degree of water-extractable by-products could be taken up by the product that they were intended to protect. Consequently they have been replaced by modern polymer formulations with low extract curatives. Bromobutyl and chlorobutyl rubbers show superior performance although special polymers,

e.g. nitrile rubbers, are used for mineral oil products. Problems of incompatibility may be overcome by film bonding a fluorocarbon barrier film to the surfaces of the closures.

2.1.5.3 Plastic containers

Most infusions are now packed in plastic containers. The plastic material should be pliable, thermoresistant, transparent and non-toxic. The plastics may contain antioxidants, stabilizers, lubricants, plasticizers, fillers and colorants. Suitable materials are PVC (which may present a problem with moisture loss) and polyethylene. The former is transparent and very pliable, allowing the pack to collapse as the contents are withdrawn (consequently no air inlet is required). These packs are also amenable to the inclusion of ports into the bag, allowing greater safety during use. Such ports may be protected by sterile overseals.

Two problems arise: (1) the possibility of toxic extractives, e.g. diethyl phthalate, from the plastic entering the fluid if poor quality PVC is used, and (2) moisture permeability leading to loss of water if the packs are not protected by a water-impermeable outer wrap. Bags of high-quality polyethylene are readily moulded (although separate ports cannot be included), translucent and free from potential toxic extractives. Again, these packs normally collapse readily during infusion. An important advantage of all plastic packs is that the containers are hermetically sealed prior to autoclaving and therefore spray-cooling water cannot enter the pack unless there is seal failure, an easily detected occurrence. However, autoclaving of plastic bags is more complex than that of bottled fluids because a steam/air mixture is necessary to prevent bursting of the bags when heated (air ballasting); adequate mixing of the steam and air is therefore required to prevent layering of gases inside the chamber.

2.1.5.4 Blow-fill technology

Blow-fill technology is an aseptic process whereby the container is formed from thermoplastic granules, filled with sterile solution and sealed, all within one automatic operation. The bulk solution should have a low bioburden and is delivered to the machine through a filling system that has been previously sanitized and steam sterilized *in situ*. Concern has been expressed that the machine itself may generate particles. The plastic granules are composed usually of polyethylene, polypropylene or one of their copolymers and are heat extruded at about 200 °C into a tube. The two halves of a mould close around this tube and seal the base. The required quantity of sterile fluid is filled into the container, which is then sealed. Products packed in this way include intravenous solutions, and small-volume parenteral, ophthalmic and nebulizer solutions. The technique offers lower costs than conventional packaging.

2.1.5.5 Cartridges and ready-to-use syringes

Small-volume injections may also be packaged in cartridges or directly into disposable syringes. The latter are immediately available for use but have a high cost of production and their fixed content may lead to waste of material that remains uninjected after single use. Cartridges are lower cost and may be fitted into injection pens; many insulin products are produced in this manner because of their low waste, ease of use and not requiring the patient to draw a dose volume into a separate syringe. Cartridges have a plunger stopper at one end of a cylindrical glass body containing the product for injection, and the other end is sealed with a rubber-lined crimp cap. Processing steps include preparing the bulk sterile solution for injection, washing and siliconizing the plunger stoppers, caps and glass cartridges, inserting the plunger stopper, filling and closing. The product is then sterilized, but care has to be taken that the internal pressures that develop during the autoclave cycle do not force the cartridge plunger out of the cartridge. The industry is developing a range of devices designed to breach the skin's defences to allow transdermal delivery (Arora *et al.*, 2008). These include microneedle arrays and needle-free injections.

2.1.6 Quality control of ampoules and infusion containers

2.1.6.1 Particulate contamination

Because of the possible clinical consequences (such as granuloma of the lung) of injecting solid particles into the bloodstream, the number of particles present in injections and other solutions used in body cavities must be restricted. The *British Pharmacopoeia* (2010) states that injectable preparations which are solutions 'when examined under suitable conditions of visibility are clear and practically free from particles'. It also sets limits for subvisible particles in injections based on the principle of light blockage. Not more than 100 particles/ml greater than 5 μm and not more than 50 particles/ml greater than 10 μm should be generally obtained. The *British Pharmacopoeia* (2010) describes a microscopic method for determination of the particulate contamination of injections and intravenous infusions. The counting

methods should estimate extraneous particles, but not bubbles, that are unintentionally present in the solutions. If the method provides a means for identifying and detecting the particles, insight may be gained into their possible origin. Filtration and observation using light microscopy have clear advantages, including simplicity and allowing the operator to visualize the particles.

All parenterally injected solutions should be checked for particulate contamination, but the above procedure is clearly impractical as a bulk screening exercise. Those products contaminated with particulate matter should be rejected. In practice, all products may be tested individually by a human observer against split white/black screens and/or under polarized light for obvious particulate contamination, and again there is a method described in pharmacopoeias based on the split-screen technique. Nowadays optical control equipment can take over this arduous and boring task.

2.1.6.2 Integrity of seals

The integrity of sealing of ampoules should be assessed on an individual basis. Two techniques are available that depend on dye ingress under vacuum or electronic means. With dye intrusion, the ampoules are submerged in a dye solution and under an applied vacuum. Any container that has cracks in its structure or is not sealed will admit the dye when the vacuum is reduced. On washing, badly sealed ampoules will be coloured. This technique underestimates the problem of bad sealing. In the alternative technique, high-frequency spark testing, the presence of a leak causes a change in a high-frequency electrical signal placed across the ampoule. The method is limited to aqueous products with a high conductivity. It is a very sensitive technique and detects weak seals not detected by the dye test. In reality, both tests should be used in parallel.

2.2 Non-injectable sterile fluids

There are many other types of solution in a sterile form, for use particularly in hospitals.

2.2.1 Non-injectable water

This is sterile water, not necessarily of injectable water standards, which is used widely during surgical procedures for wound irrigation, moistening of tissues, washing of surgeons' gloves and instruments during use and, when warmed, as a haemostat. Isotonic saline may also be used. Topical water (as it is often called) is prepared in 500 ml and 1 l polyethylene or polypropylene containers with a wide neck and tear-off cap to allow for ease of pouring.

2.2.2 Urological (bladder) irrigation solutions

These are used for rinsing of the urinary tract to aid tissue integrity and cleanliness during or after surgery. Either water or glycine solution is used, the latter eliminating the risk of intravascular haemolysis when electrosurgical instruments are used. These are sterile solutions produced in collapsible or semirigid plastic containers of up to 3 L capacity.

2.2.3 Peritoneal dialysis and haemodialysis solutions

Peritoneal dialysis solutions are admitted into the peritoneal cavity as a means of removing accumulated waste or toxic products following renal failure or poisoning. They contain electrolytes and glucose (1.4–7% w/v) to provide a solution equivalent to potassium-free extracellular fluid; lactate or acetate is added as a source of bicarbonate ions. Slightly hypertonic solutions are usually employed to avoid increasing the water content of the intravascular compartment. A more hypertonic solution containing a higher glucose concentration is used to achieve a more rapid removal of water. In fact, the peritoneal cavity behaves as if it were separated from the body organs by a semipermeable membrane. Warm peritoneal solution (up to 5 L) is perfused into the cavity for 30–90 minutes and then drained out completely. This procedure can then be repeated as often as required. As the procedure requires larger volumes, these fluids are commonly packed in 2.5 L containers. It is not uncommon to add drugs (for instance potassium chloride or heparin) to the fluid prior to use.

Haemodialysis is the process of circulating a patient's blood through a machine via tubing composed of a semipermeable material such that waste products permeate into the dialysing fluid and the blood then returns to the patient. Haemodialysis solutions need not be sterile but must be free from heavy bacterial contamination.

2.2.4 Inhaler solutions

In cases of severe asthmatic attacks, bronchodilators and steroids for direct delivery to the lungs may be needed in large doses. This is achieved by direct inhalation via a nebulizer device; this converts a liquid into a mist or fine spray. The drug is diluted in small volumes of Water for Injections before loading into the reservoir of the machine. This vehicle must be sterile and preservative-free and is therefore prepared as a terminally sterilized unit dose in polyethylene nebules.

2.3 Ophthalmic preparations

2.3.1 Design philosophy

Medication intended for instillation on to the surface of the eye is formulated in aqueous solution as eye drops or lotion or in an oily base as an ointment. Because of the possibility of eye infection occurring, particularly after abrasion or damage to the corneal surface, all ophthalmic preparations must be sterile. As there is a very poor blood supply to the anterior chamber, defence against microbial invasion is minimal; furthermore, it appears to provide a particularly good environment for growth of bacteria. As well as being sterile, eye products should also be relatively free from particles that might cause damage to the cornea. However, unlike aqueous injections the recommended vehicle is purified water because the presence of pyrogens is not clinically significant.

Another type of sterile ophthalmic product is the contact lens solution. However, unlike the other types this is not used for medication purposes but merely as wetting, cleaning and soaking conditions for contact lenses.

2.3.2 Eye drops

Some typical excipients for eye drops are given in Table 22.1. Eye drops are presented for use in (1) sterile single-dose plastic sachets (often termed Minims) containing 0.3–0.5 ml of liquid, (2) multiple-dose amber fluted eye dropper bottles including the rubber teat as part of the closed container or supplied separately, or (3) plastic bottles with integral dropper. A breakable seal indicates that the dropper or cap has not been removed prior to initial use. Although a standard design of bottle is used in hospitals, many proprietary products are manufactured in plastic bottles designed to improve safety and care of use. The maximum volume in each container is limited to 10 ml. Because of the likelihood of microbial contamination of eye dropper bottles during use (arising from repeated opening or contact of the dropper with infected eye tissue or the hands of the patient), it is essential to protect the product with a preservative (Matthews & Skinner, 2006). Eye drops for surgical theatre use should be supplied in single-dose containers.

Examples of preservatives are phenylmercuric nitrate or acetate (0.002% w/v), chlorhexidine acetate (0.01% w/v), thiomersal (0.01% w/v) and benzalkonium chloride (0.01% w/v). Chlorocresol is too toxic to the corneal epithelium, but 8-hydroxyquinoline and thiomersal may be used in specific instances. The principal consideration in relation to antimicrobial properties is the activity of the bactericide against *Pseudomonas aeruginosa*, a major source of serious nosocomial eye infections. There is some concern over the toxicity of mercurials, and their use is becoming less common. Although benzalkonium chloride is probably the most active of the recommended preservatives, it cannot always be used because of its incompatibility with many compounds commonly used to treat eye diseases, nor should it be used to preserve eye drops containing anaesthetics. As benzalkonium chloride reacts with natural rubbers, silicone or butyl rubber teats should be substituted and products should not be stored for more than 3 months after manufacture because silicone rubber is permeable to water vapour. As with all rubber components, the rubber teat should be pre-equilibrated with the preservative before use. Thermostable eye drops and lotions are sterilized at 121 °C for 15 minutes. For thermolabile drugs, filtration sterilization followed by aseptic filling into sterile containers is necessary. Eye drops in plastic bottles are prepared aseptically.

In order to lessen the risk of eye drops becoming heavily contaminated, either by repeated inoculation or by the growth of resistant organisms in the solution, use is restricted to 1 month after the container is first opened. This is usually reduced to 7 days for hospital ward use on one eye of a single patient. The period is shorter in the hospital environment because of the greater danger of contamination by potential pathogens, particularly pseudomonads.

2.3.3 Eye lotions

Eye lotions are isotonic solutions used for washing or bathing the eyes. They are sterilized by autoclaving in relatively large-volume containers (100 ml or greater) of coloured fluted glass with a rubber closure and screw-cap, or packed in plastic containers with a screw-cap or tear-off seal. They may contain a preservative if intended for intermittent domiciliary use for up to 7 days. If intended for first aid or similar purposes, however, no bactericide is included and any remaining solution is discarded after 24 hours.

2.3.4 Eye ointments

Eye ointments are prepared in a semisolid base—e.g. Simple Eye Ointment BP, which consists of yellow soft paraffin (8 parts), liquid paraffin (1 part) and wool fat (1 part). The base is filtered when molten to remove particles and sterilized at 160 °C for 2 hours. The drug is incorporated prior to sterilization if heat-stable, or added aseptically to the sterile base. Finally the product is

aseptically packed in clear sterile aluminium or plastic tubes. As the product contains virtually no water, the danger of bacteria proliferating in the ointment is negligible.

2.3.5 Contact lens solutions

Most contact lenses are worn for optical reasons as an alternative to spectacles. Contact lenses are of two types: hard lenses, which are hydrophobic, and soft lenses, which may be either hydrophilic or hydrophobic. The surfaces of lenses must be wetted before use and wetting solutions are used for this purpose. Hard, and more especially, soft lenses become heavily contaminated with protein material during use and therefore must be cleaned before disinfection. Contact lenses are potential sources of eye infection and, consequently, microorganisms should be removed before the lens is again inserted into the eye. Lenses must also be clean and easily wettable by lachrymal secretions. Contact lens solutions are thus sterile solutions of the various types described below. Apart from achieving their stated functions, either singly or in combination, all solutions must be non-irritating or must protect against microbial contamination during use and storage.

2.3.5.1 Wetting solutions

These are used to hydrate the surfaces of hard lenses after disinfection. As they must also cope with chance contamination, they must contain a preservative as well as a wetting agent. They may be isotonic with lachrymal secretions and be formulated to a pH of about 7.2 for compatibility with normal tears.

2.3.5.2 Cleaning solutions

These are responsible for the removal of ocular debris and protein deposits, and contain a cleaning agent that consists of a surfactant and/or an enzyme product. As they must also cope with chance contamination, they contain a preservative, are isotonic and have a pH of about 7.2.

2.3.5.3 Soaking solutions

These are solutions for disinfection of lenses but also maintain the lenses in a hydrated state. The antimicrobial agents used for disinfecting hard lenses are those used in eye drops (benzalkonium, chlorhexidine, phenylmercuric acetate or nitrate, thiomersal and chlorbutol). Ethylenediamine tetraacetic acid (EDTA) is usually present as a synergist. Benzalkonium chloride and chlorbutol are strongly bound to hydrophilic soft contact lenses and therefore cannot be used in storage solutions for these; chlorhexidine and thiomersal are usually employed. It must be added that the concentrations of all preservatives used in contact lens solutions are lower than those employed in eye drops, to minimize irritancy. Hydrogen peroxide is becoming commonly used but must be inactivated before the lenses are inserted onto the eyes. Finally, heat may be utilized as an alternative method to disinfect soft contact lenses, especially the hydrophilic types. Lenses are boiled in isotonic saline.

2.4 Dressings

Dressings and surgical materials are used widely in medicine, both as a means of protecting and providing comfort for wounds and for many associated activities such as cleaning and swabbing. They may or may not be used on areas of broken skin. If there is a potential danger of infection arising from the use of a dressing then it must be sterile. For instance, sterile dressings must be used on all open wounds, both surgical and traumatic, on burns, and during and after catheterization at a site of injection. It is also important to appreciate that sterile dressings must be packaged in such a way that they can be applied to the wound aseptically.

Dressings are described in the *British Pharmacopoeia* (2010). Methods for their sterilization include autoclaving, dry heat, ethylene oxide and ionizing radiation. Any other effective method may be used. The choice is governed principally by the stability of the dressing constituents to the stress applied and the nature of their components. Most celluloses and synthetic fibres withstand autoclaving, but there are exceptions. For instance, boric acid tenderizes cellulose fibres during autoclaving, and dressings containing waxes cannot be sterilized by moist heat. Certain constituents are also adversely affected on exposure to large doses of gamma radiation. Examples of dressings that are required to be sterile are listed in Table 22.2, together with other dressings and materials that may be sterilized when required.

A very important aspect of the production of dressings is packaging. The packaging material must allow correct sterilization conditions (e.g. permeation of moisture or ethylene oxide), retain the dressing in a sterile condition and allow for its removal without contamination prior to use. All dressings intended for aseptic handling and application must be double wrapped. For steam sterilization they may be individually wrapped in fabric, paper or nylon and sterilized in metal drums, cardboard boxes or bleached Kraft paper. The choice of method also deter-

Table 22.2 Uses of surgical dressings and methods of sterilization

Dressing	Uses	Method of sterilization
Required to be sterile		
Chlorhexidine gauze dressing	Medicated open wound dressing, burns, grafts	Any combination of dry heat, gamma radiation and ethylene oxide
Framycetin gauze dressing	Medicated open wound dressing, burns, grafts	
Knitted viscose primary dressing	Ulcerative and granulating wounds	
Paraffin gauze dressing	Burns, scalds and grafts	
Perforated film absorbent dressing	Postoperative wounds	
Polyurethane foam dressing	Burns, ulcers, grafts, granulating wounds	
Semipermeable adhesive dressing	Adhesive dressing for open wounds, IV sites, stoma care, etc.	
Sodium fusidate gauze dressing	Medicated open wound dressing, burns, grafts	
May be sterile for use in certain circumstances		
Absorbent cotton wool	Swabbing, cleaning, medication application	Any method
Elastic adhesive dressing	Protective wound dressings	Ethylene oxide or gamma radiation
Plastic wound dressings	Protective dressing (permeable or occlusive)	Ethylene oxide or gamma radiation
Absorbent cotton gauze	Absorbent wound dressing	Any method
Gauze pads	Swabbing, dressing, wound packing	Any method
Absorbent viscose wadding	Wound cleaning, swabbing, skin antiseptic	Any method

mines the design of the autoclave cycle. Providing that adequate steam penetration is assured, dressings may be sterilized in downward displacement autoclaves which rely on displacement of air by steam. However, high prevacuum autoclaves in which virtually all the air is removed before the admission of steam are much more commonly employed. This method ensures rapid heating up of dressings, reduces the time needed to achieve sterilization (e.g. 134 °C for 4 minutes) and shortens the overall sterilization cycle.

A recent development is the use of spray-on dressings. A convenient type is an acrylic polymer dissolved in ethyl acetate and packed as an aerosol. This should be self-sterilizing. The film after application is able to maintain the sterility of a clean wound for up to 2 weeks. However, they can only be used on clean, relatively dry wounds.

2.5 Implants

Implants are small, sterile cylinders of drug, inserted beneath the skin or into muscle tissue to provide slow absorption and prolonged action therapy. This is principally based on the fact that such drugs, invariably hormones, are almost insoluble in water and yet the implant provides a rate of dissolution sufficient for a therapeutic effect. Implants are manufactured from the pure drug made into tablet form by compression or fusion. No other ingredient can be included because this may be insoluble or toxic, or, most importantly, may influence the rate of drug release. Copolymers such as polylactic acid/polyglycolic acid may be used as the implant matrix to provide a controlled rate of drug delivery.

Compression of sterile drugs must be conducted under aseptic conditions using sterile machine parts and

materials. After manufacture, the outer surface of the implant is sterilized by immersion in 0.002% w/v phenylmercuric nitrate at 75 °C for 12 hours. After the surface has been dried, each implant is placed aseptically into a sterile glass vial with a cotton wool plug at both ends. This prevents damage and reduces the risk of glass specules, formed when the vial is opened, adhering to the implant. This compression process is not ideal and fusion processes may be used provided that the drug is heat-stable. The pure drug is melted at 5–10 °C above its melting temperature and poured into moulds. The interior of the implant will be automatically sterilized by this process if the melting temperature is high enough. It is also possible to dry heat sterilize the implant after packaging provided that the melting temperature is above 160 °C. Clearly, it is easier to manufacture sterile implants by fusion as the process does not require presterilized ingredients or aseptic processing. The implant hardness is also very convenient.

2.6 Absorbable haemostats

The reduction of blood loss during or after surgical procedures where suturing or ligature is either impractical or impossible can often be accomplished by the use of sterile, absorbable haemostats. These consist of a soft pad of solid material packed around and over the wound that can be left *in situ* and absorbed by body tissues over a period of time, usually up to 6 weeks. The principal mechanism of action of these is their ability to encourage platelet fracture because of their fibrous or rough surfaces, and to act as a matrix for complete blood clotting. Four products commonly used are oxidized cellulose, absorbable gelatin sponge, human fibrin foam and calcium alginate.

2.6.1 Oxidized cellulose

This consists of cellulose material that has been partially oxidized. White gauze is the most common form, although lint is also used. It can be absorbed by the body in 2–7 weeks, depending on the size. Its action is based principally on a mechanical effect and it is used in the dry state. As it inactivates thrombin, its activity cannot be enhanced by thrombin incorporation.

2.6.2 Absorbable gelatin foam

This insoluble foam is produced by whisking warm gelatin solution to form a uniform foam, which is then dried. It can be cut into suitable shapes, packed in metal or paper containers and sterilized by dry heat (150 °C for 1 hour). Moist heat destroys the physical properties of the material. Immediately before use, it can be moistened with normal saline containing thrombin. It behaves as a mechanical haemostat, providing the framework on which blood clotting can occur.

2.6.3 Human fibrin foam

This is a dry sponge of human fibrin prepared by clotting a foam of human fibrinogen solution with human thrombin. It is then freeze-dried, cut into shapes and sterilized by dry heat at 130 °C for 3 hours. Before use it is saturated with thrombin solution. Blood coagulation occurs in contact with the thrombin in the interstices of the foam.

2.6.4 Calcium alginate

This is composed of the sodium and calcium salts of alginic acid formed into a powder of fibrous material and sterilized by autoclaving. It aids clotting by forming a sodium–calcium alginate complex in contact with tissue fluids, acting principally as a mechanical haemostat. It is relatively slowly absorbed and some residues may occasionally remain in the tissues.

2.7 Surgical ligatures and sutures

The use of strands of material to tie off blood or other vessels (ligature) and to stitch wounds (suture) is an essential part of surgery. Both absorbable and non-absorbable materials are available for this purpose.

2.7.1 Sterilized surgical catgut

This consists of absorbable strands of collagen derived from mammalian tissue, particularly (despite its name) the intestines of sheep. Because of its source, it is particularly prone to bacterial contamination, and even anaerobic spores may be found in such material. Sterilization is therefore a particularly difficult process. As collagen is converted to gelatin when exposed to moist heat, autoclaving cannot be used. The official method is to pack the 'plain' catgut strands (up to 350 cm in length) on a metal spindle in a glass or other suitable container with a tubing fluid, the purpose of which is to maintain both flexibility and tensile strength after sterilization. Probably the most suitable method is to expose the material to gamma radiation. There is minimal loss of tensile strength and the container can be overwrapped before sterilization to provide a sterile container surface for opening aseptically. The alternative method involves placing the coiled suture immersed in a tubing fluid (commonly 95% ethyl alcohol with or without 0.002% w/v phenylmercuric nitrate) and storing for sufficient time to ensure sterilization. The

outer surface of the vial must be sterilized before opening to avoid contamination of the suture when removed. Therefore the vial is immersed in 1% w/v formaldehyde in ethanol before use. It cannot be heated. A non-official method of sterilization is to immerse the catgut in a non-aqueous solvent (naphthalene or toluene) and heat at 160 °C for 2 hours. The catgut becomes hard and brittle during the process, and is aseptically transferred to an aqueous tubing fluid to restore its flexibility and tensile strength.

Catgut is packed in single threads up to 350 cm in length of various thicknesses related to tensile strength in single-use glass or plastic containers that cannot be resealed after use. Any remaining material should be discarded. Hardened catgut is prepared by treating strands with certain agents to prolong resistance to digestion. If hardened with chromium compounds, the material is known as chromicized catgut.

2.7.2 Non-absorbable types

Sutures and ligatures are also made from many materials not absorbed by the body tissues. These consist of uniform strands of metal or organic material that will not cause any tissue reactions and are capable of being sterilized. Depending on the physical stability of each material, they are preferably sterilized by autoclaving or gamma radiation. They are packed in single-dose sachets, either dry or surrounded by a preserving fluid with or without a bactericide. The different materials are described in the *British Pharmacopoeia* (2010); they include linen (adversely affected by gamma rays), nylon (either monofilament or plaited), silk and polypropylene.

2.8 Instruments and equipment

The method chosen for sterilization of instruments (see Table 22.3) depends on the nature of the components and the design of the item. The wide range of instruments that may be required in a sterile condition includes syringes (glass or plastic disposable), needles, giving sets, metal surgical instruments (e.g. scalpels, scissors, forceps), rubber gloves, catheters, etc. Relatively complicated equipment such as pressure transducers, pacemakers, kidney dialysis equipment, incubators and aerosol machine parts may also be sterilized. Artificial joints could also be included in the vast range of items required in a sterile condition in modern medical practice. The choice of method depends largely on the physical stability of the items and the appropriate technique in particular situations. For instance, incubators necessitate a chemical method of sterilization. On the other hand, even delicate instruments like pressure transducers are now available that can withstand autoclaving.

3 Sterilization considerations

Sterilization processes are discussed in detail in Chapter 21. However, it is axiomatic that whatever method is chosen, the process should not cause damage to the product. By reference mostly to moist heat sterilization processes (the reader should remember that there are parallel approaches to other methods of sterilization) this section illustrates the factors that must be considered in the design of a sterilization process.

The simplest method of sterilization, for an aqueous product, is to expose it to the standard moist heat sterilization conditions, i.e. holding the product at 121 °C for 15 minutes, a process termed overkill. These conditions are quite severe and therefore milder conditions might be considered, i.e. a lower holding temperature than 121 °C, or a shorter holding period than 15 minutes for a product prone to degradation. The minimum holding period for moist heat sterilization might be considered to be 8 minutes at 121 °C. However, in reality a slightly shorter holding period may be satisfactory if the lethality of the whole autoclave cycle (including heat-up and cooling phases) is calculated using F_0 values (Chapter 21) and shown to afford the requisite SAL. F_0 values of 8 minutes or more are normally considered satisfactory. Use of lower temperatures and times gives an autoclave process partly based on the initial bioburden and partly on the known stability of the product.

3.1 Decision trees

Where it is not possible to sterilize a product in its final container by terminal heat sterilization at 121 °C for 15 minutes, decisions have to be made to use an alternative method. The options include filtration in combination with aseptic processing, but readers should note that aseptic processing by itself is not a method of sterilization, rather of preventing contamination of the product whilst it is manufactured from individually sterilized components.

The European Agency for the Evaluation of Medicinal Products in 2000 produced an Annex for Guidance on Development Pharmaceuticals (CPMP/QWP/155/96) showing decision trees for the selection of sterilization methods. The tree for the sterilization choices for aqueous products is shown in Figure 22.1. The initial premise is

Table 22.3 Methods* commonly used to sterilize or disinfect equipment[a]

Equipment	Method of treatment	Sterilization or disinfection	Preferred method	Comments
Syringes (glass)	Dry heat	Sterilization	Dry heat using assembled syringes	Autoclave not recommended; difficulty with steam penetration unless plungers and barrels sterilized separately
Syringes (glass), dismantled	Moist heat	Sterilization		
Syringes (disposable)	Gamma radiation Ethylene oxide[b]	Sterilization	Gamma radiation	Possibility of 'crazing' of syringes after ethylene oxide
Needles (all metal)	Dry heat	Sterilization	Dry heat	
Needles (disposable)	Gamma radiation	Sterilization	Gamma radiation	
	Ethylene oxide[b]			
Metal instruments (including scalpels)	Autoclave Dry heat	Sterilization	Dry heat	Cutting edges should be protected from mechanical damage during the process
Disposable instruments	Gamma radiation	Sterilization	Gamma radiation	
	Ethylene oxide[b]			
Rubber gloves	Autoclave Gamma radiation Ethylene oxide[b]	Sterilization	Gamma radiation	If autoclave used, care should be taken with drying at end of process. Little oxidative degradation when high-vacuum autoclave used
Administration (giving) sets	Gamma radiation Ethylene oxide[b]	Sterilization	Gamma radiation	
Respirator parts	Moist heat (autoclave)	Sterilization	Sterilization by dry heat where possible	Chemicals not recommended; may be microbiologically ineffective, may present hazard to patient safety by compromising the safety devices on the machine
	Moist heat (low-temperature steam, or hot water at 80 °C)[c]	Disinfection		
Dialysis machines	Chemical[d]	Disinfection	Formalin	Ethylene oxide not recommended in NHS for practical reasons
Fragile heat-sensitive equipment	Ethylene oxide[b]	Sterilization	Ethylene oxide under expert supervision	
	Chemical	Disinfection		

[a] Disposable equipment should not be resterilized or reused.
[b] Ethylene oxide sterilization is a difficult process to control and the UK Department of Health discourages its use in hospitals.
[c] Low-temperature steam with formaldehyde is of value in the sterilization/disinfection of some heat-sensitive materials.
[d] Chemical agents, e.g. glutaraldehyde, hypochlorite.

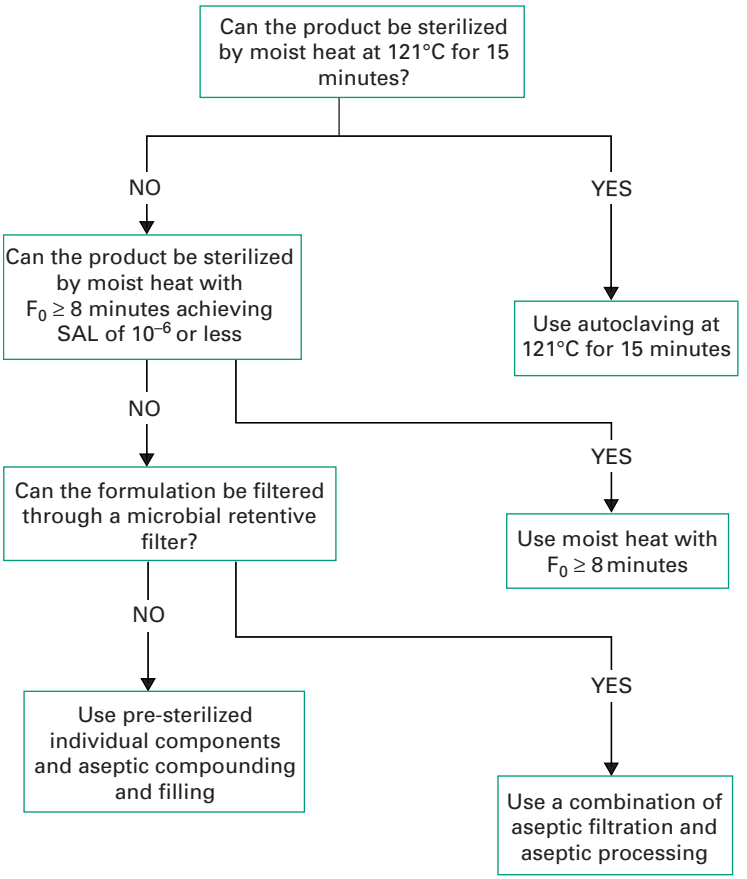

Figure 22.1 Decision tree for sterilization choices for aqueous products. (From EMA (2000), CPMP/QWP/054/98.)

that if the products may be sterilized at 121 °C for 15 minutes, that process should be used. The next alternative is that if the product is stable when an F_0 of 8 minutes or more can be used, then the reduced moist heat process should be undertaken. If heat processes are unsuitable (an $F_0 < 8$ minutes will not achieve the necessary SAL), then filtration through a microbial filter should be chosen as the process to render the product sterile. If that process cannot be utilized, then presterilizing of stable components and aseptic compounding and filling must be considered. The described methods generally show decreasing levels of sterility assurance on moving down the tree. It is therefore imperative to remember that the highest level of sterility assurance is achieved in conjunction with the lowest presterilization bioburden. The use of inappropriate heat-labile packaging material cannot by itself be the reason for the use of aseptic processing, and any manufacturer should use the best sterilization method achievable for a given formulation before selecting the packaging material. The manufacture of biotechnology products, which are typically heat-labile peptides, proteins or nucleic acids, will provide a challenge as their overall stability dictates their positioning near the bottom of the decision tree. They may require sterilization by submicron (<0.1 µm) filtration and filling and finishing using aseptic processes. The overall SAL for terminally sterilized products should be less than 10^{-6} and for aseptically produced products less than 10^{-3}.

Figure 22.2 gives the decision tree for sterilization choices for non-aqueous liquid, semisolid or dry powder products. Intermediary decisions, based on radiation and not found with aqueous products, may be seen in the tree.

3.2 Problems of drug stability

Certain issues of product instability may be resolved by formulation or careful selection of vehicle. Aminophylline injection, for example, is a solution of the drug in Water for Injections free from carbon dioxide, as the presence

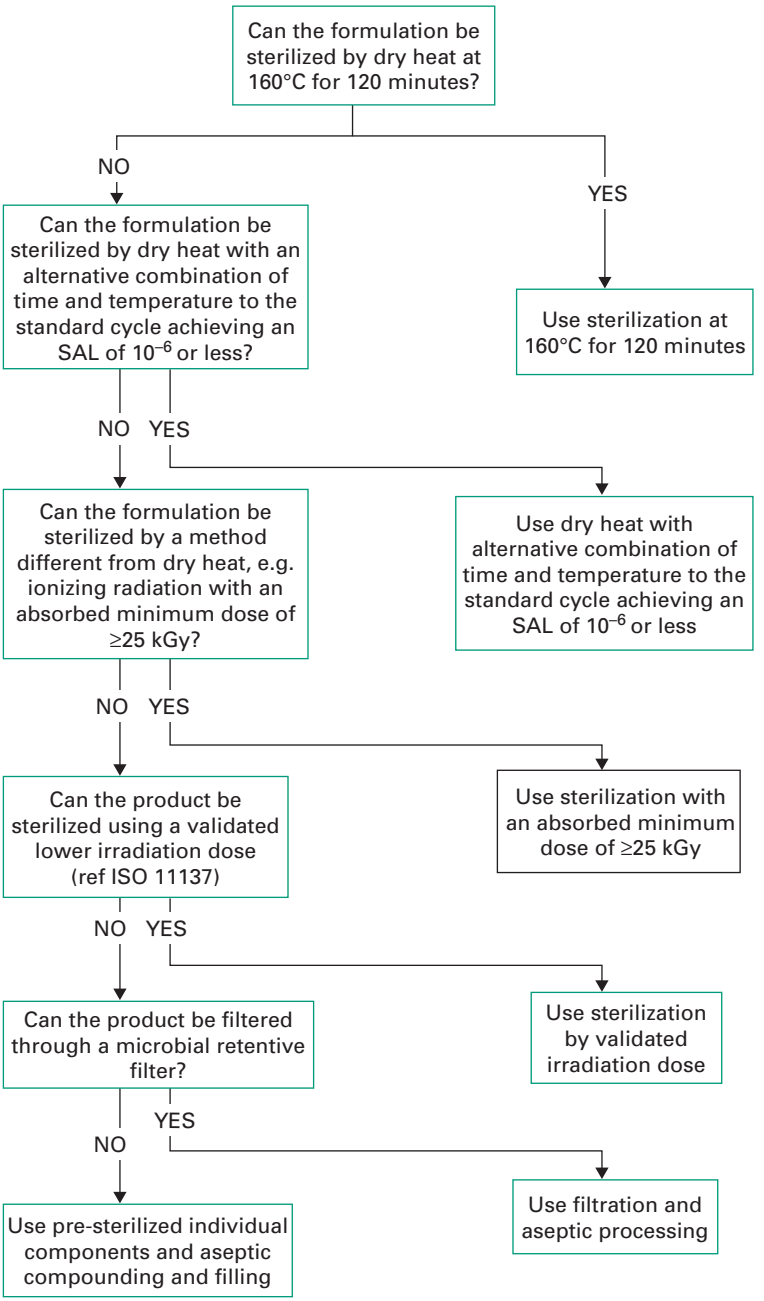

Figure 22.2 Decision tree for sterilization choices for non-aqueous liquid, semisolid or dry powder products. (From EMA (2000), CPMP/QWP/054/98.)

of this gas causes precipitation of the active ingredient. Similarly, promethazine injection is a solution of the active ingredient in Water for Injections free from dissolved air, as the presence of oxygen would cause promethazine oxidation. Removal of these gases can be accomplished by prior boiling; additionally, the product may be packed under an atmosphere of nitrogen to eliminate oxygen from the headspace in the ampoule.

Formulations may be further stabilized by the inclusion of inactive ingredients with specific functions.

Although the *British Pharmacopoeia* (2010) describes chloramphenicol eye drops as a sterile solution of chloramphenicol in purified water, normally the system is buffered for stability with a boric acid/sodium borate buffer (see Table 22.1). Sodium metabisulphite may be found in many products as an antioxidant to prevent degradation of the active, examples being promethazine injection and adrenaline injection. The presence of antimicrobial preservatives may be found in multiple-dose products, to prevent microbial growth following contamination during use. Many of these formulation considerations relate to stability of the product during storage, but an understanding of thermostability is required for the selection of the appropriate sterilization process.

The choice of sterilization method depends on the thermostability of the active ingredient. Moist heat sterilization can only be applied to drugs that are heat-stable in aqueous solution and are not subject to hydrolysis. Where aqueous solutions are so unstable that chemical stabilization is impossible, consideration should be given to sterilization of the drug itself by dry heat processes (160 °C for 2 hours or its equivalent at higher temperatures) in its final container and dissolution immediately before use by the addition of sterile Water for Injections BP. For drugs which are both thermolabile and unstable in aqueous solution, a sterile solution of the drug may be freeze-dried in its final container and is again reconstituted as above just before use. Examples include many antibiotics and Hyaluronidase Injection BP.

4 Quality control and quality assurance of sterile products

It is not the aim of this section to review the entirety of quality control of sterile products or of the chemical assays and requirements of drugs and excipients prior to formulation. Only those techniques with importance either to microbiology or the confirmation of sterility of the final product are introduced here.

4.1 Bioburden

It should be obvious from previous sections that a successful sterilization process is dependent on a product having a low presterilization bioburden. This will also be true of the individual ingredients, which must have low levels of microbial contamination, or else there is a danger that the contaminants will find their way into the final product or be a source of pyrogens (see section 4.4). Sterilization should normally be considered as the removal of the bioburden, but the high heat resistance of bacterial endotoxins means that successful steam sterilization does not necessarily guarantee that the product will pass a pharmacopoeial endotoxin test; dead bacteria are likely to remain pyrogenic.

Underestimating the level of microbial contamination prior to the terminal sterilization process will lead to a miscalculation of the sterilization dose requirements to achieve the desired SAL. The bioburden must be maintained within certain limits to justify the chosen sterilization process. When a higher number of organisms or more resistant microorganisms are encountered during manufacture of batches than was determined during the initial validation, those batches must be assumed not to be sterile. The bioburden is an estimate of the total viable count of microorganisms present before sterilization, and a knowledge of the resistance characteristics of these organisms is often an integral part of the sterility assurance calculation. To build some degree of safety into the sterilization process the sterilization conditions should be set to destroy all the bioburden by assuming that *all* the contaminating microorganisms are the most resistant of the species identified in that bioburden. Sterility assurance, as implied in the schemes shown in Figures 22.1 and 22.2, can only be achieved with a low bioburden and with fully validated, correctly functioning sterilizers.

4.2 The test for sterility

The broad basis of the test for sterility is that it examines samples of the final product for the presence of microorganisms. Theoretically, the test for sterility should be applied to all products that are designated as sterile. However, the test does not examine all samples in a batch, and its results can only be considered valid if all items in a batch are treated similarly (*British Pharmacopoeia*, 2010). Clearly, for products which are terminally sterilized this might seem a reasonable assumption but only if there is uniform heat distribution in an autoclave or hot air oven or uniform delivery of a radiation dose. With aseptically produced products there are dangers because not all items in a batch may have been treated similarly. A successful test only shows that no microbial contamination was found in the samples examined under the test conditions. Extension of the result to a whole batch requires the assurance that every unit in the batch was manufactured in such a manner that it would also have passed the test with a high degree of probability. This highlights the weakness of the test for sterility and why the controls of sterilization processes are very important

and probably of greater assurance in confirming the sterility of a batch. The test, however, remains one of few analytical methods that examine a product for sterility; the practical aspects of sterility tests are considered in Chapter 21 and the limitations of sterility testing have been discussed by Moldenhaur & Sutton (2004).

4.3 Parametric release

As there are significant limitations with the test for sterility, many authorities place considerable reliance on the validation and reliable performance of sterilizers and their sterilization cycles. Parametric release takes this reliance a step further by allowing batches of terminally sterilized products to be released without being subjected to the test for sterility. The sterilization cycle will be validated to have a SAL of 10^{-6} or less as the minimum safety factor. Validation studies would include heat distribution, heat penetration, bioburden, container closure and cycle lethality studies. For a product to be subject to parametric release, presterilization bioburden testing on each batch would be completed, and the comparative resistance of isolated spore-formers checked. Each cycle would include the use of chemical or biological indicators. It is hoped that these actions will provide a significantly higher level of assurance of sterility than provided by the test for sterility. This requires confirmation that each part of the manufacturing process has been satisfactorily completed, the initial presterilization bioburden is within agreed limits, that the controls for the sterilizing cycle were satisfactory and that the correct time cycles were achieved. In practice parametric release should only be used when experience has been gained on a reliably controlled and adequately validated process and where a relationship has been proved between end-product testing and in-process monitoring.

Clearly, reproducibility, regular monitoring and documentation are required. However, parametric release would imply abandoning the sterility test, an option that many manufacturers have not yet adopted, possibly because of the fear of litigation based on the premise that any sterile product would, if tested, have passed the test for sterility.

4.4 Pyrogens

The discovery that aqueous solutions may lead to an increase in body temperature when injected into a patient dates back to the 19th century. The agents responsible for this fever were termed 'pyrogens'. In theory a pyrogen is any substance that, when injected into a mammal, elicits a rise in body temperature, and substances produced by some Gram-positive bacteria, mycobacteria, fungi and also viruses conform to this definition. The most common pyrogens, however, and those of major significance to the pharmaceutical industry, are produced by Gram-negative bacteria and are known as *endotoxins*; they are lipopolysaccharides (LPS) found in the cell envelope (Chapter 3). The presence of pyrogens in aqueous solutions was first demonstrated by injection into rabbits whose body temperature was recorded. More sensitive methods have since been developed, mostly based on the discovery that a fraction of the horseshoe crab blood reacts with LPS as a clotting agent.

Two pharmacopoeial limit tests exist. That for pyrogens uses rabbits to assess pharmacological activity and therefore the presence of pyrogens of all kinds. The test for bacterial endotoxins uses lysed amoebocytes (blood cells) of the horseshoe crab and is therefore termed the *Limulus* amoebocyte lysate (LAL) test. This may be extended to many drug and device products and clearly will be developed in the future to assess the presence of endotoxins in biotechnology products.

4.4.1 Physiological effects of pyrogens

The most characteristic effect following injection of pyrogens into humans is a rise of body temperature, but it is only one of a number of dose-dependent diverse effects. Pyrogens elevate the circulating levels of inflammatory cytokines, which may be followed by fever, blood coagulation, hypotension, lymphopenia, neutrophilia, elevated levels of plasma cortisol and acute-phase proteins. Low doses of pyrogens induce asymptomatic inflammatory reactions. Moderate doses induce fever and changes in plasma composition. Injection of high pyrogenic doses results in shock, characterized by cardiovascular dysfunction, vasodilation, vasoconstriction, endothelium dysfunction and multiple organ dysfunction or failure and death.

4.4.2 Characteristics of bacterial endotoxin

The release of LPS from bacteria takes place after death and lysis. Many Gram-negative bacteria, e.g. *Escherichia coli* and *Proteus, Pseudomonas, Enterobacte*r and *Klebsiella* species produce pyrogenic LPS which is composed of two main parts: a hydrophilic polysaccharide chain with antigenic regions, and a hydrophobic lipid group termed lipid A which is responsible for many of the biological activities. The molecular size of the polysaccharide chain is very variable, and consequently the molecular weight of the LPS may vary from a few thousand to several million daltons. LPS is unusually thermostable and insen-

sitive to pH changes. Molecules are able to withstand 120 °C for over 3 hours. Extremes of pH are required for rapid destruction of the LPS.

4.4.3 Sources

The sources of pyrogens in parenteral products include water used at the end stages of the purification and crystallization of the drug or excipients; water used during processing; packaging components; and the chemicals, raw materials or equipment used in the preparation of the product. The presence of endotoxins on devices may be attributed to water in the manufacturing process, the washing of components such as filter media to be used for the manufacture of filters, or the washing/rinsing of tubing or other plastic devices prior to their sterilization. Additionally, if the drug is biologically produced, incomplete removal of the microorganisms during purification can result in high endotoxin levels.

4.4.4 Measurement of pyrogens

Pyrogens have traditionally been assessed using rabbits which are stored in carefully controlled conditions and whose temperature is monitored before the administration of the test product. The *British Pharmacopoeia* (2010) describes a test initially based on three rabbits; the number is progressively increased if the results fall between the two values (Table 22.4). Samples of the product under test are injected into the marginal ear vein at a dose no greater than 10 ml/kg. The animals are monitored for the 3 hour period immediately after injection, at 30 minute intervals. The test assumes that the maximum rise in temperature will be detected in this 3 hour period immediately after injection. Table 22.4 describes the criteria for pass or fail as the number of rabbits used increases to the maximum of 12.

A number of limitations of the rabbit pyrogen test are recognized. Repeated use of animals leads to endotoxin tolerance. There is low reactivity to the endotoxin produced by certain species, e.g. *Legionella*. There is also variability in control results when identical standardized endotoxin preparations are used, which is probably related to interlaboratory factors and variations due to seasons, rabbit species and other biological sources. Care must be taken in testing radiopharmaceuticals, and certain drugs may themselves elicit a rise in temperature on administration. The test is therefore inadequate for radiopharmaceuticals, cancer chemotherapeutic agents, hypnotics and narcotics, vitamins, steroids and some antibiotics. The presence of pyrogens may be hidden by the pharmacological activity of the product's components. Finally the rabbit test is insufficiently sensitive to detect endotoxin in intrathecal products where only low levels of pyrogens are acceptable.

Table 22.4 Increases of temperature used to determine outcome of pyrogen tests

Number of rabbits	Material passes if summed response does not exceed	Material fails if summed response exceeds
3	1.15 °C	2.65 °C
6	2.80 °C	4.30 °C
9	4.45 °C	5.95 °C
12	6.60 °C	6.60 °C

EMA (2009) is to encourage the replacement of the rabbit test with the monocyte activation test (Hoffmann, 2005) for plasma-derived medicinal products. Human monocytes from cultured cell lines mimic the human fever reaction *in vitro* by producing cytokines. Cytokine release can be determined, usually using enzyme-linked immunoassay (ELISA).

4.4.5 Measurement of bacterial endotoxins

The LAL test is considerably more sensitive than the pyrogen test. As mentioned above, although the *Legionella* endotoxin is not very pyrogenic to rabbits it is easily detected by the LAL test. It has been estimated that there is a 1000-fold difference in sensitivity between the two tests, but the LAL test only detects endotoxins of Gram-negative bacteria and not all pyrogens. However, the LAL test may be used for radiopharmaceuticals.

LAL test reagent comes from the American horseshoe crab *Limulus polyphemus.* The endotoxin-induced coagulation of its blood is based on an enzyme-mediated interaction of LAL with endotoxins. The reagents are obtained from the blood of freshly captured horseshoe crabs whose amoebocytes are concentrated, washed and lysed with endotoxin-free water. The LAL is separated from the remaining cellular debris and its activity optimized using metallic cations, pH adjustment and additives and then freeze-dried. Certain preparations interfere with the interaction between LAL and endotoxin. Chemical inhibitors may cause chelation of the divalent cations necessary for the reaction, protein denaturation or inappropriate pH changes. Physical inhibition may result from adsorption of endotoxin or be caused by viscosity of the product. Even the type

of glassware may affect the test. Siliconized glassware or plastic can inhibit gel-clot formation, or prevent accurate spectrophotometric readings of the reaction end-point.

The samples of products are incubated with LAL at 37 °C. If endotoxins are present a solid gel forms, indicating the presence of endotoxins. The *British Pharmacopoeia* (2010) describes six separate methodologies for the test for endotoxin. These are (A) gel-clot limit test; (B) gel-clot: semi quantitative; (C) turbidimetric kinetic method; (D) chromogenic kinetic method; (E) chromogenic end-point method; and (F) turbidimetric end-point method. There are checks for interfering factors. Any validated method may be used, but the gel-clot method is the referee test in the case of dispute. Coloured products cannot be tested by turbidimetric and chromogenic methods, as precipitate formation may be mistaken for a positive response.

Kinetic LAL methods are claimed to increase the efficiency of large-scale testing, probably important when validation of depyrogenation cycles or preparation of components for aseptic processing are required. For all procedures, test validation must be conducted to rule out interference, which may be either inhibition or enhancement. Depyrogenated glassware must be used throughout.

The gel-clot method is most commonly used. The test is conducted by adding the LAL reagent to an equal volume of test solution, agitating and storing at 37 °C for 1 hour when the end-point is determined by inversion of the tubes. If a solid clot remains intact, the product is considered to contain endotoxins. Chromogenic methods utilize colorimetry but do not depend on the clottable protein. A synthetic substrate is used that contains an amino acid sequence similar to that of coagulogen, the clottable protein. The activated proclotting enzyme cleaves a *p*-nitroanilide chromophore from the synthetic substrate and the colour produced is proportional to the amount of endotoxin. The turbidimetric LAL method is based on the fact that an increase in endotoxin concentration will cause a proportional increase in turbidity caused by the precipitation of the clottable protein, coagulogen. The optical density is read spectrophotometrically either at a fixed time or constantly for kinetic assays as turbidity develops. The kinetic methods depend on the relationship between the logarithm of the response and the logarithm of the endotoxin concentration. The end-point methods relate endotoxin levels to the quantity of chromophore released or the amount of precipitation.

4.4.6 Endotoxins in parenteral pharmaceuticals

The limits for endotoxin are based on the dose of the product. Put simply, the endotoxin limit, EL, which represents the maximum amount of endotoxin that is allowed in a specific dose, is inversely related to the dose of the drug; it may be assessed from the following equation (*United States Pharmacopeia*, 2010):

$$EL = K/M \qquad (2)$$

where K is the threshold human pyrogenic dose of endotoxin per kg body weight and M is the maximum human dose of the product in kg body weight that would be administered in a single 1 hour period. M recognizes that the pharmacological effects of endotoxin are dose-dependent. The endotoxin limit is the level at which a product is adjudged pyrogenic or non-pyrogenic. Gel-clot reagent sensitivities are generally in the range 0.015–0.5 EU/ml. As examples of endotoxin limits, the *United States Pharmacopeia* (2010) states limits of no more than 0.5 EU/ml for Dextrose Infusion, no more than 5 EU/mg promethazine in Promethazine Injection USP, no more than 10 EU/mg of mitomycin in Mitomycin for Injection USP and no more than 24 EU/mg warfarin sodium in Warfarin Sodium for Injection. The *British Pharmacopoeia* (2010) has a limit of 0.25 IU/ml in Glucose Intravenous Infusion; this value is similar for many BP intravenous infusions. As another example, insulin should contain no more than 10 IU/mg of endotoxin. The endotoxin limit for drugs gaining access to the cerebrospinal fluid is reduced to 0.2 EU/kg because the intrathecal route is the most toxic route for endotoxins.

4.4.7 Depyrogenation and the production of apyrogenic products

Pyrogens and endotoxins are difficult to remove from products once present and it is easier to keep components relatively endotoxin-free rather than to remove them from the final product. Rinsing or dilution is one way of eliminating pyrogenic activity provided that the rinsing fluid is apyrogenic. Closures and vials should be washed with pyrogen-free water before sterilization. Pyrogens in vials or glass components may be destroyed by dry heat sterilization at high temperatures. A recommended condition for depyrogenation of glassware and equipment is heating at 250 °C for 45 minutes. Pyrogens are also destroyed at 650 °C in 1 minute or at 180 °C in 4 hours. The *British Pharmacopoeia* (2010) states that dry heat at temperatures above 220 °C may be used for the depyrogenation of glassware. Sterilizing tunnels are designed

not only to sterilize at 250–300 °C but also to remove pyrogens (Chapter 21). These processes equate to incineration, although removal by washing, also termed dilution, may be used. Filtration, irradiation or ethylene oxide treatment have limited value in reducing pyrogen or endotoxin loads.

The removal of pyrogens from Water for Injections (Chapter 24) may be effected by distillation or reverse osmosis. Distillation is the most reliable method for removing endotoxin. Care has to be taken to avoid splashing in the still as pyrogens have been carried over in droplets. Another source of endotoxins is the Water for Injection system. Generally, circulating hot water at temperatures above 75 °C provides an environment that is not conducive to microbial growth and thus the formation of endotoxin. Circulating water at approximately 60 °C causes some concern as some Gram-negative organisms, e.g. *Legionella pneumophila*, will survive and grow at 57 °C. The water-producing systems may be sanitized by circulating water at 75–80 °C.

Pyrogen-free water can be produced using an ultrafiltration membrane with a nominal molecular weight limit that is low enough to ensure the removal of endotoxins under all conditions. Hollow fibre polysulphone membranes can be sanitized with sodium hydroxide, which efficiently destroys pyrogens. A nominal molecular weight limit of 5000 Da should efficiently remove endotoxins. However, many endotoxin-producing microorganisms multiply in ambient temperature Water for Injection systems, especially reverse osmosis (RO) systems, in which the filters are not absolute and may be used in series in order to manufacture pyrogen-free water.

5 Acknowledgement

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23 Principles of good manufacturing practice

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1 Introduction

Regulatory authorities require that pharmaceutical products be manufactured according to the principles of good manufacturing practice (GMP) (also referred to as current good manufacturing practice, cGMP). Such authorities include the European Union (EU), the UK Medicines and Health Care Products Regulatory Agency (MHRA) and the US Food and Drug Administration (FDA). Products manufactured in the UK for the US market must satisfy the FDA. GMP guidelines were first given statutory authority in the USA and published in the UK in 1971 (see Immel, 2000 and Sharp, 2009 for a more detailed history).

Compliance must not, however, be seen as a regulatory burden. Failure in GMP can have massive consequences for the well-being of the patient and the finances of the manufacturer. For the manufacturer it can lead to litigation, losses associated with recall of product or loss of

Hugo and Russell's Pharmaceutical Microbiology, Eighth Edition. Edited by Stephen P. Denyer, Norman Hodges, Sean P. Gorman, Brendan F. Gilmore.

licence with attendant bad publicity. Furthermore, an incident in 2004 of failure in GMP for vaccine manufacture at a UK company led to a major shortage of flu vaccine in the USA.

Some products, such as injections, must be sterile (see Chapter 22), while others, such as oral drugs, need not be sterile but must be free from pathogens that can be contracted via the oral route (British Pharmacopoeia Commission, 2010, Appendix XVI D). More space in the literature is dedicated to quality of sterile products than that of non-sterile products, but this reflects the *additional* quality assurance requirements compared to those for non-sterile products (Sharp, 2000; Butson & Hawitt, 2008).

The manufacture of sterile products is carried out in both industry and hospitals. In the latter, batches tend to be much smaller, sometimes only one item, and the products are stored for a much shorter time, usually less than 24 hours (Beaney, 2005).

This chapter summarizes measures for the control during manufacture of one important feature of product quality: the level of microbial contamination. The chapter is designed to complement and be read in conjunction with Chapters 7, 17, 19, 21 and 22. It is not intended as a manual, but as an explanation of the principles involved, and more detailed information can be found in Section 8 (References and further reading).

2 Definitions

Several terms used in industrial and hospital production must be defined to enable the reader to follow this chapter. These definitions are given in sections 2.1–2.6. More detailed definitions are to be found in *The Rules and Guidance for Pharmaceutical Manufacturers and Distributors* (2007). The inter-relationship between quality assurance (QA), GMP, quality control (QC) and in-process control is shown in Figure 23.1.

The UK Orange Guide (*The Rules and Guidance for Pharmaceutical Manufacturers and Distributors*, 2007) emphasizes the fundamental point:

> Quality assurance is particularly important, and this type of manufacture must strictly follow carefully established and validated methods of preparation and procedure. Sole reliance for sterility or other quality aspects must not be placed on any terminal sterilization process or finished product test.

The difficulty in demonstrating quality is that the tests carried out are designed to show the absence of quality.

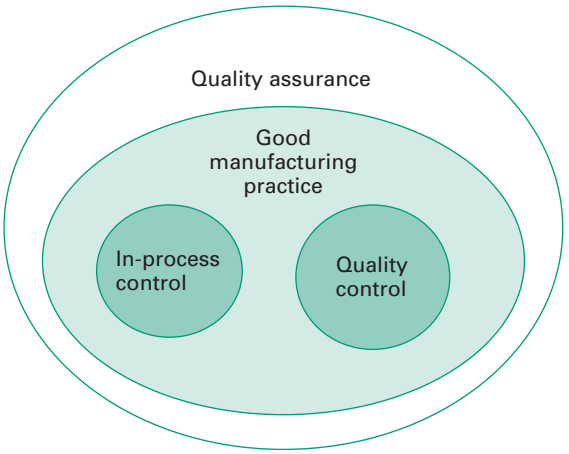

Figure 23.1 The inter-relationship between quality assurance, good manufacturing practice, quality control and in-process control.

For example, the test for sterility (Chapter 21) involves taking samples and testing for microorganisms. If 20 samples are tested, 3.4% of the batch needs to be contaminated to have a 50% chance of detecting that contamination. That level of contamination represents gross failure of GMP and problems would normally be detected by environmental monitoring. Indeed, it is not unknown for a batch to pass a sterility test but be rejected due to problems detected during environmental monitoring (section 5.2). Therefore it is important that a product be manufactured in a suitable environment by a procedure that minimizes the possibility of contamination occurring. At the end of this process the tests can be performed as an *additional* measure of quality.

2.1 Quality

There are many definitions of *quality* (see Sharp, 2000). For the purpose of pharmaceutical products the term *quality* is usually taken to mean *fitness for purpose*. Not only must the product have the desired therapeutic properties, it must also be safe for administration by the route intended. Sharp (2000, 2001) discussed several meanings of quality but summarizes it as follows:

> in a nutshell, it is fit to be given to a patient in the confidence that it will have the desired effects and not damage him or her, in any way, through faults in manufacture.

2.2 Manufacture

Manufacture is the complete cycle of production of a medical product. This cycle includes the acquisition of all

raw materials, their processing into a final product, and subsequent packaging and distribution.

2.3 Quality assurance (QA)

Quality assurance is a wide ranging concept covering all matters which individually or collectively influence the quality of a product. It is the total sum of the procedures needed to ensure the fitness of a pharmaceutical product for its intended use. QA incorporates GMP plus other factors.

2.4 Good manufacturing practice (GMP)

GMP is that part of QA which is aimed at ensuring the product is consistently manufactured to a quality appropriate for its intended use and to meet the requirements of the regulatory authorities. GMP requires that: (1) the manufacturing process is fully defined before it begins; and (2) the necessary facilities are provided. In practice, this means that:

- Personnel must be adequately trained..
- Suitable premises and equipment must be employed.
- Correct materials must be used.
- Approved procedures must be adopted.
- Suitable storage and transport facilities must be available.
- Appropriate records must be made.

The reasons for GMP are (Sharp, 2001):

- the poor chance of the patient detecting that anything is wrong
- the weakness of product testing because:
  - we can only test samples
  - we cannot test for everything
- the dangers to patients of even only a small number of defective or wrongly labelled items in a batch (and it is very difficult to detect a small number of defectives).

It is about getting things right *all along the line*.

2.5 Quality control (QC)

QC is that part of GMP concerned with sampling, specifications and testing, as well as the organization, documentation and release procedures which ensure that the necessary and relevant tests are carried out, and that materials are not released for use, nor products released for sale or supply, until their quality has been judged satisfactory. For sterile products QC includes testing for sterility and pyrogens (see Chapters 21 and 22). *The Rules and Guidance for Pharmaceutical Manufacturers and Distributors* (2007) states that QC is not confined to laboratory operations, but must be involved in all decisions which may affect the quality of the product. The independence of QC from production is considered fundamental to the satisfactory operation of QC.

2.6 In-process control

This comprises any test on a product, the environment or the equipment that is used during the manufacturing process. An example of this is testing that an autoclave is functioning correctly (Gardner & Peel, 1998).

2.7 Validation

A documented programme that provides a high degree of assurance that a specific process, method or system will consistently produce a result meeting predetermined acceptance criteria.

3 Control of microbial contamination during manufacture: general aspects

A pharmaceutical product may become contaminated by a number of means and at several points during manufacture. There are several ways in which this risk can be minimized. Any such measures require an understanding of the risks involved (Chapter 17).

3.1 Risk assessment

GMP is informed by past mistakes and case studies have been valuable (Friedman, 2004). However a proactive approach is required. Nowadays a manufacturer is expected to demonstrate to the regulatory authorities that an extensive risk assessment has been carried out. Risk analysis must comply with ICH 9Q (EMEA, 2006) and is underpinned by a sound understanding of the process and of the microbial ecology of the environment and ingredients.

Several methods are employed (see EMEA, 2006; Kirupakar, 2007), including hazard analysis critical control points (HACCP), failure mode and effects analysis (FMEA), fault tree analysis (FTA), risk ranking and filtering (RRF) and hazard operability analysis (HAZOP). Only HACCP and FMEA are discussed here.

3.1.1 Hazard analysis critical control points (HACCP)

HACCP has been widely used in the food industry and is becoming more commonly used in the pharmaceutical industry (McCullogh, 2007; Sharp, 2000; WHO, 2003; Whyte, 2010). The original HACCP had seven steps:

1 Conduct a hazard analysis and identify preventive measures for each step of the process.
2 Determine the critical control points.
3 Establish critical limits.
4 Establish a system to monitor the critical control points.
5 Establish the corrective action to be taken when monitoring indicates that the critical control points are not in a state of control.
6 Establish a system to verify that the HACCP procedure is working effectively.
7 Establish a record-keeping system.

However, HACCP has been modified so that it can be applied quantitatively not only to microbiology but also to pyrogens and particles (Tidswell, 2004; Whyte, 2010).

3.1.2 Failure mode and effects analysis (FMEA)

FMEA was first used in the engineering industry (Stamatis, 2003). It involves breaking the process down into many discrete steps. For each step scales are set for severity, occurrence and detection. The scores are multiplied and compared to an informed score at which risk becomes unacceptable.

3.2 Environmental cleanliness and hygiene

Microorganisms may be transferred to a product from working surfaces, fixtures and equipment. Pooled stagnant water is a frequent source of contamination. Thus it is essential that all working areas are kept clean, dry and tidy. Any cracks where microorganisms may accumulate must be eliminated. All walls, floors and ceilings should be easy to clean. This entails impervious and washable surfaces, free from open joints or ledges. Coving should be used at junctions between walls and floors or ceilings. All services such as pipes, light fittings and ventilation points should be sited so that inaccessible recesses are avoided. A rigorous disinfection policy must be in place (Chapter 19; Pharmig 2006). All equipment must be easy to dismantle and clean and should be inspected for cleanliness before use.

Fall-out of dust- and droplet-borne microorganisms from the atmosphere is an obvious route for contamination. 'Clean' air (section 4.1.4) is therefore a prerequisite during manufacturing processes and the spread of dust during manufacture or packaging must be avoided. Microorganisms may thrive in certain liquid preparations and creams and ointments (Chapter 22). The manufacture of such products should, as far as possible, be in a closed system; this serves a dual purpose as it also prevents evaporative loss.

Personnel are another source of potential contamination. High standards of personal hygiene are essential. Operatives should be free from communicable disease and open lesions on exposed body surfaces. To ensure high standards of personal cleanliness, adequate handwashing and hand-disinfecting facilities and protective garments, including headgear and gloves, must be provided. Staff should be trained in the principles of GMP and in the practice (and theory) of the tasks assigned. Staff employed in the manufacture of sterile products should also receive training in basic microbiology.

3.3 Quality of starting materials

Raw materials account for a high proportion of the microorganisms introduced during the manufacture of pharmaceuticals, and the selection of materials of good microbiological quality aids in the control of contamination levels in both products and the environment. It is, however, common to have to accept raw materials which have some non-pathogenic microorganisms present and this must be considered during risk assessment. Whatever the means of prevention of growth or survival by chemical or in-process treatment, it should be regarded as critical and controlled accordingly (Sharp 2000).

Untreated raw materials that are derived from a natural source usually support an extensive and varied microflora. Products from animal sources such as gelatin, desiccated thyroid, pancreas and cochineal may be contaminated with animal-borne pathogens. For this reason some statutory bodies such as the *British Pharmacopoeia* require freedom of such materials from *Escherichia coli* and *Salmonella* spp. at a stated level before they can be used in the preparation of pharmaceutical products. The microflora of materials of plant origin such as gum acacia and tragacanth, agar, powdered rhubarb and starches may arise from those indigenous to plants and may include bacteria such as *Erwinia* spp., *Pseudomonas* spp., *Lactobacillus* spp., *Bacillus* spp., streptococci, moulds such as *Cladosporium* spp., *Alternaria* spp. and *Fusarium* spp. and non-mycelated yeasts, or those introduced during cultivation. For example, the use of untreated sewage as a fertilizer may result in animal-borne pathogens such as *Salmonella* spp. being present. Some refining processes modify the microflora of raw materials; for example, drying may concentrate the level of spore-forming bacteria and some solubilizing processes may introduce waterborne bacteria such as *Escherichia coli*. Synthetic raw materials are usually free from all but incidental microbial contamination.

The storage condition of raw materials, particularly hygroscopic substances, is important, and as a minimum

water activity (A_w) of 0.70 is required for osmophilic yeasts, 0.80 for most spoilage moulds and 0.91 for most spoilage bacteria, precautions should be taken to ensure that dry materials are held below these levels (Chapter 17). Some packaging used for raw materials, such as unlined paper sacks, may absorb moisture and may itself be subject to microbial deterioration and so contaminate the contents; for this reason polythene-lined sacks are preferable. Some liquid or semisolid raw materials contain preservatives, but others such as syrups depend upon osmotic pressure to prevent the growth of osmophiles, which are often present. With this type of material it is important that they are held at a constant temperature, as any variation may result in evaporation of some of the water content followed by condensation and dilution of the surface layers to give an A_w value which may permit the growth of osmophiles and spoil the syrup.

The use of natural products with a high non-pathogenic microbial count is possible if a sterilization stage is included either before or during the manufacturing process. Such sterilization procedures (see also Chapter 21) may include heat treatment, filtration, irradiation, recrystallization from a bactericidal solvent such as an alcohol, or for dry products, where compatible, ethylene oxide gas. If the raw material is only a minor constituent and the final product is adequately preserved either by low A_w, chemically or by virtue of its pH, sugar or alcohol content, an in-process sterilization stage may not be necessary. If, however, the product is intended for parenteral or ophthalmic use a sterilization stage is essential.

The handling of contaminated raw materials as described previously may increase the airborne contamination level, and if there is a central dispensing area precautions may be necessary to prevent airborne cross-contamination, as well as that from contaminated measuring and weighing equipment. This presents a risk for all materials but in particular those stored in the liquid state where contamination may result in the bulk being spoiled.

3.4 Water

Many grades of water are used in pharmaceutical manufacturing (Table 23.1). Water for manufacturing may be potable mains water, water purified by ion exchange, reverse osmosis or distillation, or water for injection purposes (EMEA, 2002).

Most types of water are derived from municipal supplies. Such water is treated, sometimes by filtration, and always by chemicals, usually chlorine, to render it free from coliforms. This water is, however, not sterile. Its microbial and chemical content varies from region to region and the microbial count can increase on storage.

Water used for parenteral products, known as Water for Injections or Water For Injection (WFI), must be virtually apyrogenic (Chapter 22). The *British Pharmacopoeia* (British Pharmacopoeia Commission, 2010) and the *US Pharmacopeia* (2009a) specify an endotoxin level of no more than 0.25 IU/ml for WFI. In Europe such water is usually produced in a still specially designed to prevent pyrogens from being mechanically carried over into the distillate. In other countries reverse osmosis may also be used (US Pharmacopoeia, 2009a), but in

Table 23.1 Types of water for sterile manufacture

Type	Properties	Use
Mains (potable)	Not sterile. Contains ions, chlorine	Initial washing if rinsed with purified water
Purified water	Potable water purified by distillation, ion exchange, reverse osmosis	Not sterile Washing containers
Water for injections BP	Distilled water, free from pyrogens (some countries allow reverse osmosis)	
Water for injections in bulk		Final rinse Solutions to be sterilized
Sterile water for injections	Autoclaved in suitable container	Sterile solutions

Europe reverse osmosis is not approved (EMEA, 2002). WFI can be used immediately for the preparation of injections, provided it is sterilized within 4 hours of water collection. Alternatively, the water can be kept for longer periods at a temperature above 65 °C (typically 80 °C) to prevent bacterial growth with consequent pyrogen production. Ultraviolet radiation may be useful for treating WFI in order to reduce the bacterial count, but this must not be regarded as a sterilization process (Chapter 21). A more detailed account of water for pharmaceutical use may be found in EMEA (2002) and *US Pharmacopeia* (2009b).

3.5 Process design

The manufacturing process must be fully defined and capable of providing, with the facilities available, a product that is microbiologically acceptable and conforms to specifications. The process must be fully validated before starting to ensure that it is suitable for routine production operations. Processes and procedures must also be subject to frequent reappraisal and should be re-evaluated when any significant changes are made in the equipment or materials used.

3.6 Quality control and documentation

The lower the microbiological count of the starting materials, the more readily the quality of the product can be controlled. Microbiological standards should be set for all raw materials as well as microbial limits for in-process samples and the final product. Microbiological quality assurance also covers the validation of cleaning and disinfectant solutions and the monitoring of the production environment by microbial counts. This monitoring should be carried out while normal production operations are in progress. In addition, sterile manufacture requires extra safeguards. Operators must be adequately trained and their aseptic technique monitored both by observation and microbiological testing. Air filter and sterilizer efficiency must also be evaluated (Chapter 21), whilst sterility testing (Chapter 21) and, where necessary testing for pyrogens (Chapter 22), are the final tests on the finished product.

Documentation is a vital part of quality assurance. Details of starting materials, packaging materials, and intermediate, bulk and finished products should be recorded so that the history of each batch may be traced. Distribution records must be kept. This information is of paramount importance in the event that a defective batch has to be recalled.

3.7 Packaging, storage and transport

Packaging serves a number of functions; it keeps the contents in, it should keep contaminants out and is labelled to permit identification of its contents. The product is contained within primary packaging. In industry these packages are then placed inside secondary packaging for storage and transport. This secondary packaging may take the form of cartons, boxes, trays or shrink wrapping.

Consideration must be given to both the fabric of the packaging and its cleaning, and to the actual process of packaging. Where terminal sterilization is carried out, the packaging must be suitable for the process. Packaging of aseptically processed products into a sterile container (section 3.7) must be carried out in a grade A environment (Table 23.2).

Packaging material has a dual role and acts both to contain the product and to prevent the entry of microorganisms or moisture which may result in spoilage, and it is therefore important that the source of contamination is not the packaging itself. The microflora of a packaging material is dependent upon both its composition and storage conditions. This, and a consideration of the type of pharmaceutical product to be packed, determines whether a sterilization treatment is required.

Glass containers are sterile on leaving the furnace, but are often stored in dusty conditions and packed for transport in cardboard boxes. As a result they may contain mould spores of *Cladosporium* spp., *Penicillium* spp., *Aspergillus* spp. and bacteria such as *Bacillus* spp. and *Micrococcus* spp. which originate from the cardboard, although it can be treated to remove these contaminants. It is commonplace either to air-blow or wash glass containers to remove any glass spicules or dust which may be present, and it is often advantageous to include

Table 23.2 Air quality for cleanrooms

Grade	At rest		In operation	
	Maximum permitted number of particles/m^3 equal to or above			
	0.5 µm	5 µm	0.5 µm	5 µm
A	3520	20	3520	20
B	3520	29	352 000	2900
C	352 000	2900	3 520 000	29 000
D	3 520 000	29 000	Not defined	

a disinfection stage if the product is a liquid or semisolid preparation. Plastic bottles that are either blow- or injection-moulded have a very low microbial count and may not require disinfection. They may, however, become contaminated with mould spores if they are transported in a non-sanitary packaging material such as unlined cardboard. Packaging materials that have a smooth, impervious surface, free from crevices or interstices, e.g. cellulose acetate, polyethylene, polypropylene, polyvinyl chloride (PVC), and metal foils and laminates, all have a low surface microbial count.

Closure liners of pulpboard or cork, unless specially treated with a preservative, foil or wax coating, are often a source of mould contamination for liquid or semisolid products. A closure with a plastic flowed-in liner is less prone to introduce or support microbial growth than one stuck in with an adhesive, particularly if the latter is based on a natural product such as casein. Closures can be sterilized by either formaldehyde or ethylene oxide gas if required.

In the case of injectables and ophthalmic preparations which are manufactured aseptically but do not receive a sterilization treatment in their final container the packaging has to be sterilized (Figure 23.2b). Dry heat at 170 °C is often used for vials and ampoules. Containers and closures may also be sterilized by moist heat, chemicals and irradiation, but consideration of the destruction or removal of bacterial pyrogens may be necessary (see Chapter 22). Regardless of the type of sterilization, the process must be validated and critical control points or other risk assessment parameters (section 3.1) must be established.

4 Manufacture of sterile products

Methods of sterilization are discussed in Chapter 21 and the various types of sterile product are described in Chapter 22. For production purposes an important distinction exists between sterile products which have been terminally sterilized (Figure 23.2a) and those which have not. Terminal sterilization involves the product being sealed in its container and then sterilized, usually by heat, but ionizing radiation or, less commonly, ethylene oxide may be employed. Such a product must be manufactured in a clean area (sections 4.1.1–4.1.8). A product which cannot be terminally sterilized is prepared aseptically (Figure 23.2b) from previously sterilized materials or by sterile filtration; in either case, aseptic filling is a post-sterilization step. Strict aseptic conditions are required throughout (section 4.1).

Vaccines, consisting of dead microorganisms, microbial extracts or inactivated viruses (see Chapter 24) may be filled in the same premises as other sterile medicinal products, so the completeness of killing or removal of live organisms must be validated before processing. Separate premises are needed for the filling of live or attenuated vaccines and for the preparation of other products derived from live organisms. Non-sterile products and sterile products must not be processed in the same area.

4.1 Clean and aseptic areas: general requirements

4.1.1 Design of premises

Sterile production should be carried out in a purpose-built unit separated from other manufacturing areas and thoroughfares. The unit should be designed to encourage separation of each stage of production but should ensure a safe and organized workflow. A plan of such a facility is shown in Figure 23.3. Sterilized products held in quarantine pending sterility test results (Chapter 21: Sharp, 1997) must be kept separate from those awaiting sterilization.

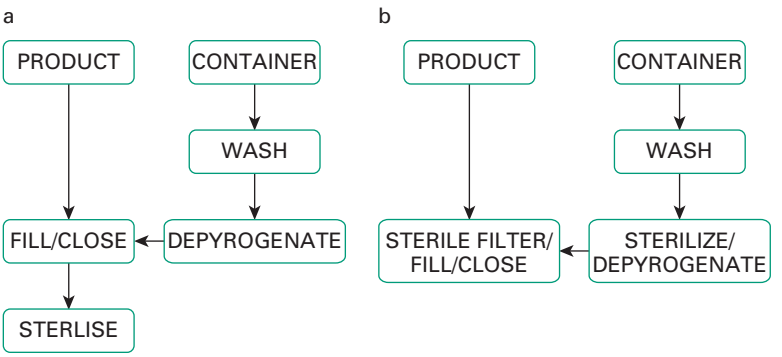

Figure 23.2 A comparison of (a) terminal sterilization and (b) aseptic processing in sterile manufacturing.

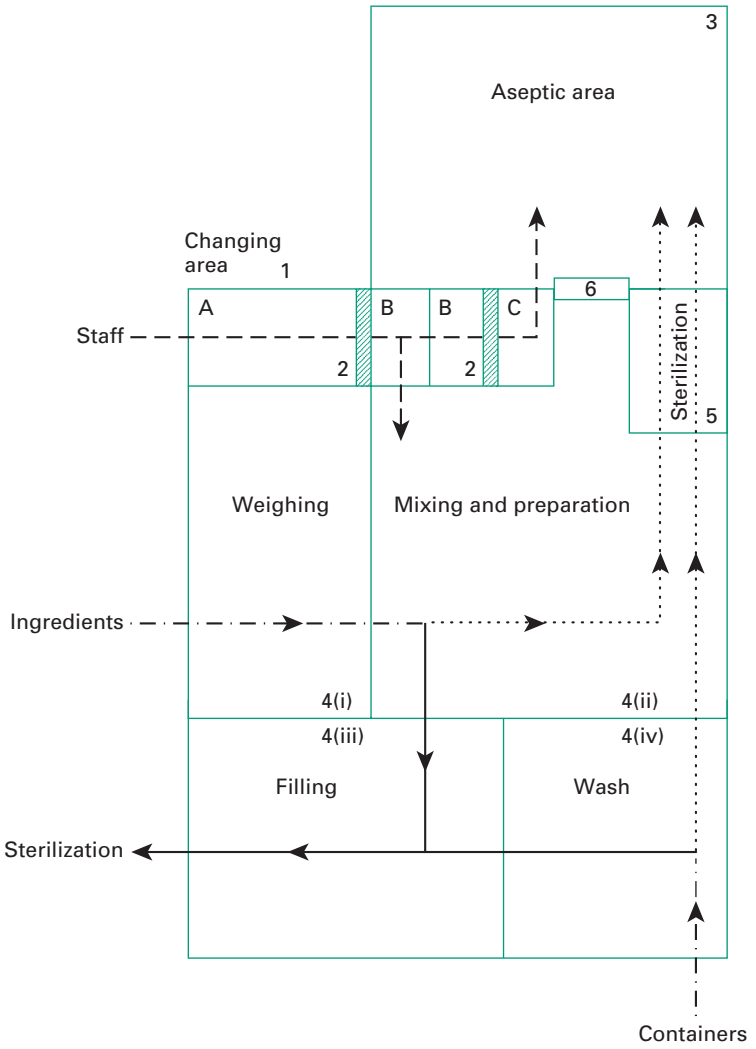

Figure 23.3 Example of a diagrammatic representation of the layout and workflow of a sterile products manufacturing unit. **1**, the changing area in this example is built on the black (A)–grey (B)–white (C) principle; passage into the clean area is through A and B (see section 3.1.6) whereas entry to the aseptic area is first through A and B followed by C (see section 3.2.2). **2**, Dividing step-over sill. **3**, For details of aseptic area requirements, see text; a laminar airflow work station would be included in this area. **4i–4iv**, These areas are clean areas. In filling rooms for terminally sterilized products, care should be exercised to protect containers from airborne contamination. The final rinse point (i.e. where the containers are finally washed) should be sited as near as possible to the filling point. **5**, Articles which are to be transferred directly to the aseptic area from elsewhere must be sterilized by passage through a double-ended sterilizer. Solutions manufactured in the clean area may be brought into the aseptic area through a sterilizing-grade membrane filter. **6**, Double-doored hatchway through which presterilized articles may be passed into the aseptic area (see section 3.2.3). Note: inspection, holding and final packaging areas have been omitted. Direction of workflow: ——→——, for terminally sterilized products; - - -→- - -, for aseptically prepared products; —·→·—, shared stages of preparation.

4.1.2 Internal surfaces, fittings and floors

Particulate, as well as microbial, contamination must be prevented. To this end all surfaces must be smooth and impervious in order to: (1) prevent accumulation of dust or other particulate matter; and (2) permit easily repeated cleaning and disinfection. Smooth rounded coving should be used where the wall meets the floor and the ceiling.

Suitable flooring may be provided by welded sheets of PVC; cracks and open joints which might harbour dirt and microorganisms must be avoided. The preferred surfaces for walls are plastic, epoxy-coated plaster, plastic fibreglass or glass-reinforced polyester. Often the final finish for the floor, wall and ceiling is achieved using continuous welded PVC sheeting. False ceilings should be adequately sealed to prevent contamination from the space above. Use should be made of well-sealed glass panels, especially in dividing walls, to ensure good visibility and allow satisfactory supervision. Doors and windows should be flush with the walls. Windows should not be openable.

Internal fittings such as cupboards, drawers and shelves should be kept to a minimum. They must be sited where they do not interfere with the laminar flow of the filtered air supply. Stainless steel or laminated plastic are the preferred materials for such fittings. Stainless steel

trolleys may be used to transport equipment and materials within the clean and aseptic areas but must remain confined to their respective units. Equipment must be designed so that it may be easily cleaned and sterilized or disinfected.

4.1.3 Services

Clean and aseptic areas must be adequately illuminated; lights are best housed in translucent panels set in a false ceiling. Electrical switches and sockets must be flush with the wall or fitted outside. When required, gases should be pumped in from outside the unit. Pipes and ducts, if they must be brought into the clean area, must be sealed through the walls. Additionally, in order to prevent dust accumulation, pipes and ducts must be boxed in or readily cleanable. Alternatively, they may be sited above false ceilings.

Sinks should be of stainless steel with no overflow, and water must be of at least potable quality. Wherever possible, drains should be avoided. If installed they must be fitted with effective, readily cleanable traps and with air breaks to prevent backflow. Any floor channels should be open, shallow and cleanable and connected to drains outside the area; they should be monitored microbiologically. Sinks and drains should be excluded from aseptic areas except where radiopharmaceuticals are being processed when sinks are a requirement.

4.1.4 Air supply

Areas for sterile manufacture are classified according to the required characteristics of the environment. Each operation requires an appropriate level of microbial and particulate cleanliness; four grades (Table 23.2) are specified in *The Rules and Guidance for Pharmaceutical Manufacturers and Distributors* (2007). Environmental quality is substantially influenced by the air supplied to the manufacturing environment. The grades of air required for specific manufacturing activities are listed in Table 23.3.

Filtered air (Chapter 22) is used to achieve the necessary standards; this should be maintained at positive pressure throughout a clean or aseptic area, with the highest pressure in the most critical rooms (aseptic or clean filling rooms) and a progressive reduction through the preparation and changing rooms (Figure 23.4); a minimum pressure differential of 10 kPa is normally required between each class of room. A minimum of 20 changes of air per hour is usual in clean and aseptic rooms. The air inlet points should be situated in or near the ceiling, with the final filters placed as close as possible to the point of input to the room. Equipment or furnishings must be sited so as not to interfere with laminar flow.

The greatest risk of contamination of a product comes from its immediate environment. Additional protection is needed both in the filling area of the cleanroom and in the aseptic suite. This can be provided by a workstation

Table 23.3 Operations carried out in the various grades of air

Grade	Examples of operations
For terminally sterilized products	
A	Filling of products, when unusually at risk
B	Background environment to grade A preparation areas
C	Preparation of solutions, when unusually at risk Filling of products
D	Preparation of solutions and components for subsequent filling
For aseptic processes	
A	Aseptic preparation and filling
B	Background environment to grade A preparation areas
C	Preparation of solutions to be filtered
D	Handling of components after washing Background for an isolator

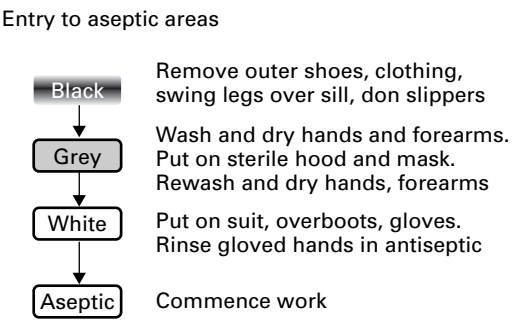

Figure 23.4 Entry into aseptic area.

supplied with a unidirectional flow of filtered sterile air. This is known as a laminar flow cabinet. Displacement of air may be vertical or horizontal with a typical homogeneous air flow of 0.45 m/s at the working position. Consequently airborne contamination is not added to the work space, and any generated by manipulation is swept away by the laminar air currents. A fuller description of high efficiency particulate air (HEPA) filters in laminar flow cabinets is given by Gardner and Peel (1998).

The efficacy of the filters through which the air is passed should be monitored at predetermined intervals. Air quality may be monitored for bacteria and fungi by slit sampler or settle plate. Particles are measured using a discrete airborne particle counter. The latest edition of *The Rules and Guidance for Pharmaceutical Manufacturers and Distributors* (2007) states that particles must be monitored continuously in a grade A area and recommends it for grade B areas. It should be noted that grade A air is not the purest that can be obtained; four even cleaner grades are used in the electronics industry (ISO14644-1).

4.1.5 Clothing

Clothing worn in a clean area must be of non-shedding fibres; polyester is a suitable fabric. Airborne contamination, both microbial and particulate, is reduced when trouser suits, close-fitting at the neck, wrists and ankles, are worn. Clean suits should be provided once a day, but fresh headwear, overshoes and powder-free gloves are necessary for each working session. Special laundering facilities are desirable. Additional requirements for aseptic rooms are discussed in section 5.1.

4.1.6 Changing facilities

Entry to a clean or aseptic area should be through a changing room fitted with interlocking doors; this design acts as an airlock to prevent influx of air from the outside. This route is for personnel only, not for the transfer of materials and equipment. Staff entering the changing room should already be clad in the standard factory or hospital protective clothing. For entry into a clean area, passage through the changing room should be from a 'black' to a 'grey' area, via a dividing step-over sill (Figure 23.4). Movement through these areas and finally into the cleanroom is permitted only when observing a strict protocol, whereby outer garments are removed in the 'black' area and cleanroom trouser suits donned in the 'grey' area. Only after hand-washing in a sink fitted with elbow- or foot-operated taps may the operator enter the cleanroom.

The changing procedure for entry to an aseptic area is described in section 5.1.2.

4.1.7 Cleaning and disinfection

A strict, validated disinfection policy is necessary if microbial contamination is to be kept to a minimum. Cleaning agents used include alkaline detergents and ionic and non-ionic surfactants. A wide range of chemical disinfectants is available (Chapter 19). Clear, soluble phenolics are commonly used for interior services and fittings. Disinfectants for working surfaces are alcohols (70% ethanol or isopropanol) or, less commonly, chlorine-based agents such as hypochlorites. Skin may be disinfected with cationic detergents such as cetrimide or chlorhexidine, usually formulated with 70% alcohol to avoid the need for rinsing. Gloved hands may be disinfected with these detergents or 70% alcohol. The former have the advantage of offering residual activity. Rotation of different disinfectants reduces the risk of the emergence of resistant strains, but such rotation should be validated. In-use dilutions must not be used unless sterilized. Disinfectants and detergents for use in grade A/B areas must be sterile prior to use and formulated with water for injections. Modern sprays are fitted with devices to prevent air being sucked back, extending the life of the disinfectant. As mentioned in section 4.1.2, smooth polished surfaces are more readily cleaned. Floors and horizontal surfaces should be cleaned and disinfected daily, walls and ceilings as often as required, but the interval should not exceed 1 month. Regular microbiological monitoring should be carried out to determine the efficacy of disinfection procedures. Records must be kept and immediate remedial action taken should normal levels for that area be exceeded.

4.1.8 Operation

The number of persons involved in sterile manufacture should be kept to a minimum to avoid the inevitable turbulence and shedding of particles and organisms associated with the operatives. All operations should be undertaken in a controlled and methodical manner as excessive activity may increase turbulence and particle shedding.

Containers made from fibrous materials such as paper, cardboard and sacking are generally heavily contaminated (especially with moulds and bacterial spores) and should not be taken into clean areas. Ingredients which must be brought into clean areas must first be transferred to suitable metal or plastic containers. Containers and closures for terminally sterilized products

must be thoroughly cleaned before use and should undergo a final washing and rinsing process in apyrogenic distilled water (which has been passed through a bacteria-proof membrane filter) immediately prior to filling. Containers and closures for use in aseptic manufacture must, in addition, be sterilized after washing and rinsing in preparation for aseptic filling (Figure 23.2).

5 Aseptic areas

5.1 Additional requirements

Additional requirements for aseptic areas, over and above those discussed in sections 4.1.1–4.1.8, are discussed below.

5.1.1 Clothing

Requirements in addition to those in section 4.1.5 are needed for aseptic areas. The operative is a potential source of microorganisms and it is imperative that steps are taken to prevent this contamination. The operative must wear sterile protective headwear totally enclosing hair and beard, spectacles, powder-free rubber or plastic gloves (often two pairs are worn), a non-fibre-shedding facemask (to prevent the release of droplets) and footwear. A suitable garment is a one- or two-piece trouser suit. Fresh sterile clothing should be provided each time a person enters an aseptic area.

5.1.2 Entry to aseptic areas

Entry to an aseptic suite is usually through a 'black–grey–white' changing procedure (Figure 23.4), where white represents the highest level of cleanliness. Movement from 'black' to 'white' is via two changing rooms, the 'grey' area also serving as an entry to the cleanroom (Figure 23.4 and section 4.1.6). There are several types of entry system in use. More details may be found in Whyte (2010).

5.1.3 Equipment and operation

Any articles entering the aseptic area should ideally be sterilized, but may be disinfected. In order to achieve this, articles should be transferred via a double-ended sterilizer or hatch (*i.e.* with a door at each end). If they are not to be discharged directly to the aseptic area, they should be (1) double-wrapped before sterilization; (2) transferred immediately after sterilizing into a clean environment until required; (3) transferred from this clean environment via a double-doored hatch (where the outer wrapping is removed) to the aseptic area (where the inner wrapper is removed at the workbench). Hatches and sterilizers must be designed so that only one door may be opened at any one time. Solutions manufactured in the cleanroom may be brought into the aseptic area through a sterile 0.22 μm membrane filter.

Workbenches, including laminar flow units, and equipment, should be disinfected immediately before and after each work session. Equipment must be of the simplest design possible for the operation being performed.

Aseptic manipulations must be carried out in the grade A air of a laminar flow cabinet or isolator. Speed, accuracy and economy of movement are essential features of good aseptic technique. It is therefore essential that workers are well trained and motivated and familiar with the task in hand. Observation and microbiological monitoring of the operator and of the environment are very important. Under no circumstances must living microorganisms, including those used for vaccine preparation (Chapter 24) and for biological monitoring be introduced into the aseptic area.

5.2 Environmental monitoring

Monitoring of the environment is essential during manufacturing. It ensures that environmental requirements are being met and also helps spot trends.

Air is monitored for particles (section 4.1.4) and microorganisms. Microorganisms are usually sought using settle plates or active samplers, such as the slit-to-agar sampler. Settle plates rely on organisms falling from the atmosphere and settling onto an exposed agar plate. After a specified time (usually 4 hours) the plate will be covered and incubated. A slit-to-agar sampler draws in a specified volume of air, forcing organisms onto the surface of an agar plate. This latter method is able to give a viable count per volume, but organisms may be damaged and hence rendered non-viable by the capture process. Limits of viable counts for different grades of air are shown in Table 23.4. One of the limitations of traditional microbial detection is the time taken to culture bacteria and fungi. There is a great deal of interest in developing rapid (Denyer, 2007) or instantaneous (Jiang, 2009) methods of microbial detection.

The nature of contamination can be informative. For example, the presence of *Staphylococcus* spp. suggests human-borne contamination. The adequacy of changing facilities and gowning would then be checked. In contrast, *Bacillus* spores would suggest environmental contamination and the entry of equipment into the cleanroom would be scrutinized.

Table 23.4 Recommended limits for microbial contamination

Grade	Air sample (cfu m–3)	Settle plates (90 mm) (cfu/4 h)	Contact plates (55 mm) (cfu/plate)	Glove print (5 fingers) (cfu/glove)
A	<1	<1	<1	<1
B	10	5	5	5
C	100	50	25	–
D	200	100	50	–

Glove prints are taken by applying four fingers and a thumb to an agar plate. This ensures that disinfection of gloves is adequate. Surfaces may be monitored by swapping or by using contact plates. The latter have the advantage of providing a quantitative measure of surface contamination, but there is a risk of leaving agar deposits on the surface (Butson & Hawitt, 2008).

5.3 Eliminating human intervention

The greatest source of contamination in the cleanroom comes from the operating staff (Whyte & Hejab, 2007). Movement of staff can increase particle shedding and disrupting laminar airflow. It is not surprising, therefore, that modern practices seek to minimize or even eliminate humans from the aseptic production area. This can be achieved by the use of automation, of isolators and of restricted access barriers (RABs).

All aseptic packaging should be carried out in a grade A environment with a grade B background (Table 23.2). Advances in technology now permit the production of self-contained workstations, or isolators, which incorporate many of the design principles of cleanrooms and laminar flow cabinets.

5.3.1 Isolators

The isolator both protects the product from contamination by the operator and the operator from any hazardous materials. Direct interaction between the operator and the product is minimized by providing a grade A laminar flow of air with a positive pressure, the internal space being accessed by means of a glove/sleeve system (Figure 23.5). A grade D background (Table 23.2) is considered adequate for such operations. A fuller account of isolators is given by Midcalf *et al.* (2004).

Figure 23.5 An isolator.

5.3.2 Restricted access barrier systems

Restricted access barrier systems (RABS) provide a level of control intermediate between an isolator and a cleanroom (Agalloco & Akers, 2006). They allow for easier intervention than an isolator but require a grade B background.

5.3.3 Blow–fill–seal technology

Blow–fill–seal units are purpose-built pieces of equipment which carry out these three steps in a continuous process within a controlled environment. Containers are formed from thermoplastic granules and blown to form containers which are then filled and heat-sealed. These units are fitted with a grade A air shower and operated in a grade C environment for aseptic manufacture and a grade D background for products which are to be terminally sterilized.

6 Guide to Good Pharmaceutical Manufacturing Practice

Between 1971 and 1983 the essential features of GMP were covered in the UK by three editions of the *Guide to Good Pharmaceutical Manufacturing Practice*, frequently referred to as the 'Orange Guide.' This guide was prepared by the UK Medicines Inspectorate in consultation with industrial, hospital, professional and other interested parties. The principles of this national guide were subsequently assimilated into the EC *Guide to Good Manufacturing Practice for Medicinal Products* in 1989 and are now published as *Rules and Guidance for Pharmaceutical Manufacturers and Distributors* (2007) by the MHRA. The FDA has published *FDA Requirements for cGMP Compliance* (2007). Two important publications from the Pharmaceutical Press are *Quality in the Manufacture of Medicines and Other Health Care Products* (Sharp, 2000) and *Quality Assurance of Aseptic Preparation Services* (Beaney, 2005), which discusses manufacturing in hospitals.

Compliance with GMP is one of the major factors considered by the licensing authority when examining an application for a licence to manufacture under the Medicines Act (1968). Similar codes exist in the USA and other countries.

7 Conclusions

GMP is not just a process; it is a way of thinking. All staff should be well trained and motivated and be working to a common goal: the production of a pharmaceutical product of a quality that is safe for the patient. The procedures should not be seen as a chore or burden to make work more difficult, but essential steps in the production of a safe, satisfactory product. Self-inspection and external audit of procedures are important processes in maintaining standards of cleanliness. Even after manufacture and distribution it is vital that the products are used properly, especially multiuse containers that are subject to potential in-use contamination.

The manufacture of non-sterile products requires that certain standards of cleanliness, personal hygiene, production methods and storage must be met. Many such products are for oral and topical use and one might wonder why such stringent parameters need be in place. However, there have been controlled hospital studies and case reports associating these products with nosocomial (hospital-acquired) infection (Chapter 16). Furthermore, methods of controlling pathogens also control spoilage organisms (Chapter 17), which could cause the industry considerable expense. Spoilage organisms can alter the aesthetic qualities (such as smell, taste and appearance), physical properties (pH, viscosity) and efficacy of the product, in addition to producing toxins.

Greater stringency is required for terminally sterilized products. Such environmental and process controls might seem overzealous, but it is better to minimize risk at all stages rather than to rely on final product testing (section 2.5). The lower the bioburden, the easier it is to achieve the required sterility assurance level with the terminal sterilization process (Chapter 21). It is also important to exclude pyrogens and particulate matter which would not be removed by the sterilization process.

Where products are processed aseptically, even higher standards of cleanliness are necessary. The importance of the knowledge and commitment of the operatives cannot be overemphasized, both in hospital and industry. Most reported incidents of defective products have been traced to human rather than technological error.

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There are two relevant computer-aided learning (CAL) packages produced by COACS (www.coacs.com, accessed 1 November 2009): *Good Manufacturing Practice* and *Pharmaceutical Microbiology Assessment*.

A range of GMP training videos is available from *MVI/Micron Training*, Portsmouth (http://www.mvitraining.com/, accessed 1 November 2009).

24 The manufacture and quality control of immunological products

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1 Introduction

Immunological products comprise a group of pharmaceutical preparations of varied composition but with a common pharmacological purpose: the modification of the immune status of the recipient, either to provide immunity to infectious disease, or in the case of *in vivo* diagnostics, to provoke an indication of immune status usually signifying previous exposure to the sensitizing agent. The immunological products that are currently available are of the following types: vaccines; *in vivo* diagnostics; immune sera; human immunoglobulins; monoclonal antibodies; and antibody-targeted therapeutics and diagnostics. For the purpose of this chapter, cell biology-derived immunomodifiers with a non-specific action, e.g. cytokines or chemokines, are not included.

Vaccines are by far the most important immunological products. They have enabled the control or eradication of numerous infectious diseases affecting humans and

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their domesticated animals. For example, the systematic application of smallpox vaccine, deployed under the aegis of the World Health Organization (WHO), achieved the eradication of one of the most devastating infections. Similarly, the universal application of poliomyelitis vaccine has brought poliomyelitis to the verge of eradication. Diphtheria, tetanus, pertussis (whooping cough), measles and rubella vaccines have been applied worldwide through national or UNICEF-sponsored healthcare programmes and have virtually eliminated these diseases in those countries in which there have been the resources and the will to deploy them effectively. Vaccines that provide protection against many other infections are available for use in appropriate circumstances. Some, such as hepatitis B and conjugate vaccines against *Haemophilus influenzae* b (Hib), meningococci and pneumococci, have had a huge impact on morbidity and mortality wherever they have been applied.

The range of disorders that may be prevented or treated by vaccines has enlarged considerably beyond infectious diseases. Vaccines are currently undergoing evaluation for several other purposes including therapy of cancer; prevention of allergies; desensitization of allergic patients; fertility control; and treatment of addictions.

In vivo diagnostics such as tuberculins, mallein, histoplasmin, coccidioidin and brucellin, are used to demonstrate an immune response, and hence previous exposure, to specific pathogens as an aid to diagnosis. Allergen skin test diagnostics are used to indicate sensitization to materials of biological origin that may be present in the environment or in specific products. Others, such as the Schick test (diphtheria) toxin are used to detect the presence of protective immunity. Because of their clinical and pharmaceutical limitations, the trend has been to phase out these preparations, and tuberculins (as purified protein derivative, PPD) are now by far the most important of this group.

Immune sera, which were once very widely used in the prophylaxis and treatment of many infections, have more limited use today. Vaccines and antibiotics have superseded some and lack of proven therapeutic benefit has caused others to be relegated to immunological history. However, some still play an important role in the management of specific conditions. Thus, diphtheria and botulinum antitoxins prepared in horses remain the only specific treatments for diphtheria and botulism respectively. Equine tetanus antitoxin is still used as an effective prophylactic in some parts of the world, although largely replaced by human tetanus immunoglobulin in developed countries. Similarly, antivenins prepared in horses, sheep, goats or other animals against the venoms of snakes, spiders, scorpions and marine invertebrates still provide the only effective treatment for venomous bites and stings and are important therapeutic agents in some parts of the world.

Human immunoglobulins have important but limited uses, for example in the prophylaxis of hepatitis A, hepatitis B, tetanus and varicella zoster. Additional specific immunoglobulins against diphtheria and botulism toxins are under development and vaccinia immunoglobulin may be reintroduced. Monoclonal antibodies to bacterial endotoxin, to cytokines involved in the pathogenesis of septic shock and to specific infectious agents have been developed and evaluated clinically but have yet to enter into general use. Monoclonal antibodies against specific cell receptors have undergone a rapid development and are employed successfully in cancer therapy and are under development for treatment of autoimmune disease. Immune sera and human immunoglobulins depend for their protective effects on their content of antibodies derived, in the case of immune sera, from immunized animals and, in the case of immunoglobulins, from humans who have been immunized or who have high antibody titres as a consequence of prior infection. The form of immunity conferred is known as passive immunity and is achieved immediately but is limited in its duration to the time that protective levels of antibodies remain in the circulation (see also Chapter 10).

Vaccines achieve their protective effects by stimulating the immune system of the recipient to produce T-cells and/or antibodies that impede the attachment of infectious agents, promote their destruction or neutralize their toxins. This form of protection, known as *active immunity*, develops in the course of days following infection and in the case of many vaccines develops adequately only after two or three doses of vaccine have been given at intervals of days or weeks. Once established, this immunity can last for years but it may need to be reinforced by booster doses of vaccine given at relatively long intervals. The immunogenicity of some vaccines can be improved by formulating them with *adjuvants*. These are a heterogeneous group of substances which enhance the immune response. Aluminium hydroxide gel (hydrated aluminium oxide) and aluminium phosphate are the only ones currently in general use in human vaccines. A much wider range of substances including oily emulsions, saponin, immune- stimulating complexes (ISCOMS), monophosphoryl lipid A, CpG motif contained in oligodeoxynucleotide (CpG-ODN) and others are used in

veterinary vaccines, and some are under investigation for use in human vaccines.

Different types of infectious agent require preferential mobilization of different arms of the immune response. For example, toxigenic bacterial infections require the production of toxin neutralizing antibodies whereas intracellular bacterial infections such as tuberculosis require cell-mediated responses involving mixed T-lymphocytes and activated macrophages, whereas many viral infections will require neutralizing antibody and cytotoxic T-cell responses for effective protection. Achieving the appropriate response can be difficult and in the past has had to be approached empirically. This is why most successful viral vaccines have been based on live attenuated strains, which simulate natural infection. Non-living vaccines have been effective against many bacterial infections but markedly less so against those requiring sustained cell-mediated responses. The development of more selective vaccine adjuvants and delivery systems promises to put the future process of vaccine design on a more rational basis.

A property common to vaccines, immune sera and human immunoglobulins is their high specificity of action. Usually each provides immunity to only one infection, although in some cases cross-protection can occur, e.g. BCG protects against both tuberculosis and leprosy. Where it is necessary to protect against more than one type of agent, monospecific preparations can be combined. For example, botulism antitoxin usually covers types A, B and E; meningococcal polysaccharide vaccine may cover groups A, C, W125 and Y; and pneumococcal polysaccharide vaccine usually covers 23 serotypes. Heterologous preparations may also be combined as in measles/mumps/rubella and diphtheria/tetanus/pertussis vaccines. With the increasing number of vaccines for infants and young children, the trend is to produce more complex combinations such as diphtheria /tetanus/pertussis/hepatitis B/inactivated polio/Hib vaccine, to minimize the number of injections. The possible additive or interactive effects of the various components on the immune system have raised concerns about the safety of such combinations. While some evidence of reduced responses to certain components has been obtained, there is little to support suggestions of serious adverse effects from current combinations.

In addition to the three main types of immunological products that are widely available, more specialized preparations include: synthetic peptide immune response modifiers such as those used to block T-cell responses in multiple sclerosis; monoclonal antibodies for cancer therapy or diagnosis; and hybrid toxins containing a bacterial or plant toxin subunit attached to an antibody or human cell receptor-binding protein, and also intended mainly for cancer therapy. These have rather limited applications and for the most part, are designed to suppress or exploit the specificity of immune responses rather than to stimulate them.

Principles of immunity are discussed in Chapter 9, and Chapter 10 describes a vaccination and immunization programme.

2 Vaccines

The vaccines currently used for the prevention of infectious diseases of humans are all derived, directly or indirectly, from pathogenic microorganisms. The basis of vaccine manufacture thus consists of procedures which produce from infectious agents, their components or their products, immunogenic preparations that are devoid of pathogenic properties but which, nonetheless, can still induce a protective response in their recipients. The methods that are used in vaccine manufacture are constrained by technical limitations, cost, problems of delivery to the recipient/patient, by regulatory issues and, most of all, by the biological properties of the pathogens from which vaccines are derived. Those vaccines currently in use in conventional immunization programmes are of several readily distinguishable types.

2.1 Types of vaccines

2.1.1 Live vaccines

These are preparations of live bacteria, viruses or other agents which, when administered by an appropriate route, cause subclinical or mild infections. In the course of such an infection the components of the microorganisms in the vaccine evoke an immune response which provides protection against the more serious natural disease. Live vaccines have a long history, dating from the development of smallpox vaccine. Initially, material from mild cases of smallpox was used for inoculation. This process of 'variolation' was hazardous and could produce fatalities and secondary smallpox cases. A much safer alternative was introduced in 1796 by the Gloucestershire physician Edward Jenner, following observations made by Benjamin Jesty, a local farmer, that an attack of the mild condition known as cowpox (probably a rodent

pox) protected milkmaids from smallpox during epidemics of this dreaded disease. For many years the cowpox vaccine was propagated by serial transfer from person to person and at some point evolved into a distinctive virus, vaccinia, with some features of both cowpox and smallpox viruses but quite probably derived from a now extinct poxvirus. Vaccinia was eventually used to eradicate smallpox. Its significance was that it could stimulate a high degree of immunity to smallpox while producing only a localized infection in the recipient.

The natural occurrence of cross-protective organisms of low pathogenicity seems to be a rare event and attenuated strains have usually had to be selected by laboratory manipulation. Thus the bacille Calmette–Guérin (BCG) strain of *Mycobacterium bovis* used to protect against human tuberculosis caused by the related species *M. tuberculosis*, was produced by many sequential subcultures on ox bile medium. This process resulted in deletion of many genes present in virulent *M. bovis*, including some essential for pathogenicity. Similarly, treatment of a virulent strain of *Salmonella enterica* serovar Typhi with nitrosoguanidine, which produced multiple mutations, gave rise to the live attenuated typhoid vaccine strain Ty21A. More recently developed attenuated strains of *S. enterica* serovar Typhi and *Vibrio cholerae* have been selected by directed mutagenesis processes which can produce defined mutations in specific genes.

Perhaps surprisingly, nearly all of the most successful attenuated viral vaccine strains in current use were produced by empirical methods long before the genetic basis of pathogenesis by the specific pathogen was understood. Thus, attenuated strains of polio virus for use as a live, oral vaccine (Sabin) were selected by growth of viruses isolated from human cases under cultural conditions that did not permit replication of neuropathogenic virus. Comparable procedures were used to select the attenuated virus strains that are currently used in live measles, mumps, rubella and yellow fever vaccines. A more recent approach has been to use genetic reassortants to produce live rotavirus vaccines.

Now, attenuated strains of pathogens can be selected by deliberate selective modification of genes responsible for encoding factors determining pathogenesis, such as toxins or immunomodulators, or metabolites essential for *in vivo* growth. Live vaccine strains can also be genetically modified by incorporating genes that encode protective antigens of other infectious agents. Several of these are under evaluation at present e.g. for vaccination against malaria and tuberculosis.

2.1.2 Killed vaccines

Killed vaccines are suspensions of bacteria, viruses or other pathogenic agents that have been killed by heat or by disinfectants such as phenol, ethanol or formaldehyde. Killed microorganisms obviously cannot replicate and cause an infection and so it is necessary for each dose of a killed vaccine to contain sufficient antigenic material to stimulate a protective immune response. Killed vaccines therefore usually have to be relatively concentrated suspensions. Even so, such preparations are often rather poorly protective, possibly because of partial destruction of protective antigens during the killing process or inadequate expression of these during *in vitro* culture. At the same time, because they contain all components of the microorganism they can be somewhat toxic. It is thus often necessary to divide the total amount of vaccine that is needed to induce protection into several doses that are given at intervals of a few days or weeks. Such a course of vaccination takes advantage of the enhanced 'secondary' response that occurs when a vaccine is administered to an individual person whose immune system has been sensitized ('primed') by a previous dose of the same vaccine. The best-known killed vaccines are whooping cough (pertussis), typhoid, cholera, plague, inactivated polio vaccine (Salk type) and rabies vaccine. The trend now is for these rather crude preparations to be phased out and replaced by better-defined subunit vaccines containing only relevant protective antigens, e.g. acellular pertussis and typhoid Vi polysaccharide vaccines.

2.1.3 Toxoid vaccines

Toxoid vaccines are preparations derived from the toxins that are secreted by certain species of bacteria. In the manufacture of such vaccines, the toxin is separated from the bacteria and treated chemically to eliminate toxicity without eliminating immunogenicity, a process termed 'toxoiding'.

A variety of reagents have been used for toxoiding, but by far the most widely employed and generally successful has been formaldehyde. Under carefully controlled conditions this reacts preferentially with the amino groups of proteins although many other functional groups potentially may be affected. Ideally the toxoided protein will be rendered non-toxic but retain its immunogenicity.

The treated toxins are sometimes referred to as formol toxoids. Toxoid vaccines are very effective in the prevention of those diseases such as diphtheria, tetanus, botulism and clostridial infections of farm animals, in which

the infecting bacteria produce disease through the toxic effects of secreted proteins which enzymically modify essential cellular components. Many of the clostridial toxins are lytic enzymes with very specific substrates such as neural proteins. Detoxification is also required for the pertussis toxin component of acellular pertussis vaccines.

Anthrax adsorbed vaccine is not toxoided but relies on the use of cultural conditions that favour production of the protective antigen (binding and internalization factor) rather than the lethal factor (protease) and oedema factor (adenyl cyclase) components of the toxin. Selective adsorption to aluminium hydroxide or phosphate also slows release of residual toxin.

2.1.4 Bacterial cell component vaccines

Rather than use whole cells, which may contain undesirable and potentially reactogenic components such as lipopolysaccharide endotoxins, a more precise strategy is to prepare vaccines from purified protective components. These are of two main types, proteins and capsular polysaccharides. Often more than one component may be needed to ensure protection against the full range of prevalent serotypes. The potential advantage of such vaccines is that they evoke an immune response only to the component, or components, in the vaccine and thus induce a response that is more specific and effective. At the same time, the amount of unnecessary material in the vaccine is reduced and with it the likelihood of adverse reaction. Vaccines that have been based on one or more capsular polysaccharides include Hib vaccine; the *Neisseria meningitidis* ACWY vaccines; the 23-valent pneumococcal polysaccharide vaccine; and the typhoid Vi vaccine. These have the disadvantage that they are T-cell-independent antigens and thus do not evoke immunological memory or effective protective responses in the very young. This problem can be overcome by chemically coupling the polysaccharides to T-cell-dependent protein carriers.

The pertussis vaccine is another example where, traditionally, whole bacterial cells have been used, but recent developments have led to an acellular pertussis vaccine that may contain detoxified toxin, either alone or combined with several other bacterial antigens.

2.1.5 Conjugate vaccines

The performance of certain types of antigen that give weak or inappropriate immune responses can often be improved by chemically conjugating them to more immunogenic carriers. Among others, polysaccharide–protein, peptide–protein, protein–protein, lipid–protein and alkaloid–protein conjugate vaccines may be prepared in this way. These have a wide range of applications, including prevention of infection, tumour therapy, fertility control and treatment of addictions. This approach has been very successful against infections caused by bacteria that produce polysaccharide capsules. The latter are T-independent antigens and induce weak responses without immunological memory. They are particularly ineffective in the very young.

2.1.6 Viral subunit vaccines

Three viral subunit vaccines are widely available, two influenza vaccines and a hepatitis B vaccine. The influenza vaccines are prepared by treating intact influenza virus particles from embryonated hens' eggs infected with influenza virus with a surface-active agent such as a non-ionic detergent. This disrupts the virus particles, releasing the virus subunits. The two types that are required in the vaccine, haemagglutinin and neuraminidase, can be recovered and concentrated by centrifugation methods. The hepatitis B vaccine was, at one time, prepared from hepatitis B surface antigen (HbsAg) obtained from the blood of carriers of hepatitis B virus. This very constrained source of antigen has been replaced by production in yeast or mammalian cells that have been genetically engineered to express HbsAg during fermentation. The human papillomavirus (HPV) vaccines for prevention of genital warts and cervical cancer also contain recombinant viral proteins.

For further information see Ada (1994), Ellis (1999), Mizrahi (1990), Pastoret *et al.* (1997), Perlmann & Wigzell (1999), Plotkin, Orenstein & Offit (2008) and Powell & Newman (1995).

2.2 The seed lot system

The starting point for the production of all microbial vaccines is the isolation of the appropriate infectious agent. Such isolates have usually been derived from human infections and in some cases have yielded strains suitable for vaccine production very readily; in other instances a great deal of manipulation and selection in the laboratory have been needed before a suitable strain has been obtained. For example, bacterial strains may need to be selected for high toxin yield or production of abundant capsular polysaccharide; viral strains may need to be selected for stable attenuation or good growth in cell cultures.

Once a suitable strain is available, the practice is to grow, often from a single viable unit, a substantial volume

of culture which is distributed in small amounts in a large number of ampoules and then stored at −70 °C or below, or freeze-dried. This is the original seed lot. From this seed lot, one or more ampoules are used to generate the working seed from which a limited number of batches of vaccine are generated. These are first examined exhaustively in laboratory and animal tests and then, if found to be satisfactory, tested for safety and efficacy in clinical trials. Satisfactory results in the clinical trials validate the seed lot as the material from which batches of vaccine for routine use can subsequently be produced.

It is important that the full history of the seed is known, including the nature of the culture media used to propagate the strain since isolation. If at all possible, media prepared from animal products should be avoided. If this is not practicable, media components must be from sources certified free of transmissible spongiform encephalopathy (TSE) agents.

2.3 Production of the bacteria and the cellular components of bacterial vaccines

The bacteria and cellular components needed for the manufacture of most bacterial vaccines are prepared in laboratory media by well-established fermentation methods. The end-product of the fermentation, the harvest, is processed to provide a concentrated and purified bulk lot of vaccine component that may be conveniently stored for long periods or even sold to other manufacturers prior to further processing. It is important that the materials, equipment, facilities and working practices are of a standard acceptable for the manufacture of pharmaceutical products. The requirements for this are defined as Good Manufacturing Practice (GMP). Guidelines on the basic requirements have been published by WHO (1999a, 1999b).

2.4 Fermentation

The production of a bacterial vaccine batch begins with the recovery of the bacterial seed contained in an ampoule of the seed lot stored at −70 °C or below, or freeze-dried. The resuscitated bacteria are first cultivated through one or more passages in preproduction media. Then, when the bacteria have multiplied sufficiently, they are used to inoculate a batch of production medium. Again, all media used must be from sources certified free of TSEs. Wherever possible, medium components of animal origin, especially human and ruminant, should be avoided.

The production medium is usually contained in a large fermenter, the contents of which are continuously stirred. Usually the pH and the oxidation/reduction potential of the medium are monitored and adjusted throughout the growth period to provide conditions that will ensure the greatest bacterial yield. In the case of rapidly growing bacteria the maximum yield is obtained after about a day but in the case of bacteria that grow slowly, e.g. *M. bovis* BCG, the maximum yield may not be reached before 2 weeks. At the end of the growth period the contents of the fermenter, which are known as the *harvest*, are ready for the next stage in the production of the vaccine

2.4.1 Processing of bacterial harvests

The harvest is a very complex mixture of bacterial cells, metabolic products and exhausted medium. In the case of a live attenuated vaccine it should be innocuous, and all that is necessary is for the bacteria to be separated and resuspended under aseptic conditions in an appropriate diluent, possibly for freeze-drying. In a vaccine made from a virulent strain of pathogen the harvest may be intensely dangerous and great care is necessary in the subsequent processing. Adequate containment will be required and for class 3 pathogens such as *S. enterica* serovar Typhi or *Yersinia pestis* or bulk production of bacterial toxins, dedicated facilities that will provide complete protection for the operators and the environment are essential.

- *Killing*. This is the process by which heat and disinfectants are used to render the live bacteria in the culture non-viable and harmless. Heat and/or formalin or thiomersal are used to kill the cells of *Bordetella pertussis* used to make whole-cell pertussis vaccines, whereas phenol was used to kill the *V. cholerae* and the *S. enterica* serovar Typhi cells used in the now obsolete whole-cell cholera and typhoid vaccines.
- *Separation*. The process by which the bacterial cells are separated from the culture fluid and soluble products. Centrifugation using either a batch or continuous flow process, or ultrafiltration, is commonly used. Precipitation of the cells by reducing the pH has been used as an alternative. In the case of vaccines prepared from cells, the supernatant fluid is discarded and the cells are resuspended in a saline diluent; where vaccines are made from a constituent of the supernatant fluid, the cells are discarded.
- *Fractionation*. This is the process by which components are extracted from bacterial cells or from the medium in which the bacteria are grown and obtained in a purified form. The polysaccharide antigens of *N.meningitidis* are usually separated from the bacterial cells by treatment with hexadecyltrimethylammonium bromide followed by

extraction with calcium chloride and selective precipitation with ethanol. Those of *Streptococcus pneumoniae* are usually extracted with sodium deoxycholate, deproteinized and then fractionally precipitated with ethanol. The purity of an extracted material may be improved by resolubilization in a suitable solvent and reprecipitation. These procedures are often supplemented with filtration through membranes or ultrafilters with specific molecular size cut-off points. After purification, a component may be freeze-dried, stored indefinitely at low temperature and, as required, incorporated into a vaccine in precisely weighed amount at the blending stage.

• *Detoxification.* The process by which bacterial toxins are converted to harmless toxoids. Formaldehyde is used to detoxify the toxins of *Corynebacterium diphtheriae*, *Clostridium botulinum* and *Cl. tetani.* The detoxification may be performed either on the whole culture in the fermenter or on the purified toxin after fractionation. Traditionally the former approach has been adopted, as it is much safer for the operator. However, the latter gives a purer product. The pertussis toxin used in acellular vaccines may be detoxified with formaldehyde, glutaraldehyde, or both, hydrogen peroxide or tetranitromethane. In the case of genetically detoxified pertussis toxin, a treatment with a low concentration of formaldehyde is still performed to stabilize the protein.

• *Further processing.* This may include physical or chemical treatments to modify the product. For example polysaccharides may be further fractionated to produce material of a narrow molecular size specification. They may then be activated and conjugated to carrier proteins to produce glycoconjugate vaccines. Further purification may be required to eliminate unwanted reactants and by-products. These processes must be done under conditions that minimize extraneous microbial contamination. If sterility is not achievable then strict bioburden limits are imposed.

• *Adsorption.* This describes the adsorption of the components of a vaccine on to a mineral adjuvant or carrier (aluminium hydroxide or aluminium phosphate; rarely calcium phosphate). Their effect is to increase the immunogenicity and decrease the toxicity, local and systemic, of a vaccine. Diphtheria vaccine, tetanus vaccine, diphtheria/tetanus vaccine and diphtheria/tetanus/pertussis (whole-cell or acellular) vaccine, are generally prepared as adsorbed vaccines.

• *Conjugation.* The linking of a vaccine component that induces an inadequate immune response with a vaccine component that induces a good immune response. For example, the immunogenicity for infants of the capsular polysaccharide of *H. influenzae* type b is greatly enhanced by the conjugation of the polysaccharide with diphtheria or tetanus toxoid, or with the outer-membrane protein of *N. meningitidis*. More recently, in attempts to improve efficacy, protein carriers that themselves induce a protective immune response against the pathogen have been conjugated to the capsular polysaccharide e.g. Panton–Valentine leucocidin conjugated to *Staph. aureus* capsular polysaccharide.

2.5 Production of the viruses and the components of viral vaccines

Viruses replicate only in living cells, so the first viral vaccines were necessarily made in animals: smallpox vaccine in the dermis of calves and sheep, and rabies vaccines in the spinal cords of rabbits and brains of mice. Such methods are no longer used in advanced vaccine production; the only intact animal hosts that are still used are embryonated hens' eggs. Almost all the virus that is needed for viral vaccine production is obtained from cell cultures infected with virus of the appropriate strain.

2.5.1 Growth of viruses

Embryonated hens' eggs are still the most convenient hosts for growth of the viruses that are needed for influenza and yellow fever vaccines. Influenza viruses accumulate in high titre in the allantoic fluid of the eggs, and yellow fever virus accumulates in the nervous system of the embryos. A few bacterial vaccines, e.g. against rickettsial and chlamydial agents, are also prepared in embryonated eggs. It is important to use eggs from disease-free flocks and emphasis is placed on screening the latter for various avian viruses. The allantoic fluid or embryos must be harvested under conditions that minimize extraneous microbial contamination.

Where cell cultures are used for virus production, they must be of known origin, obtained from validated sources and shown to be free of extraneous agents. The media used in their production should not contain components of human or animal origin, unless the latter are from TSE-free sources.

2.5.2 Processing of viral harvests

The processing of the virus-containing material from infected embryonated eggs may take one of several forms. In the case of influenza vaccines the allantoic fluid is centrifuged to provide a concentrated and partially purified suspension of virus. This concentrate is treated with organic solvent or detergent to split the virus into its

components when split virion or surface antigen vaccines are prepared. The chick embryos used in the production of yellow fever vaccine are homogenized in sterile water to provide a virus-containing pulp. Centrifugation then precipitates most of the embryonic debris and leaves much of the yellow fever virus in an aqueous suspension. Further purification can then be performed as required.

Cell cultures provide infected fluids that contain little debris and can generally be satisfactorily clarified by filtration. Because most viral vaccines made from cell cultures consist of live attenuated virus, there is no inactivation stage in their manufacture. There are, however, two important exceptions: inactivated poliomyelitis virus vaccine is inactivated with dilute formaldehyde or β-propiolactone and rabies vaccine is inactivated with β-propiolactone. The preparation of these inactivated vaccines also involves a concentration stage—by adsorption and elution of the virus in the case of poliomyelitis vaccine and by ultrafiltration in the case of rabies vaccine. When processing is complete the bulk materials may be stored until needed for blending into final vaccine. Because of the lability of many viruses, however, it is necessary to store most purified materials at temperatures of −70 °C.

2.6 Blending

Blending is the process in which the various components of a vaccine are mixed to form a final bulk. It is undertaken in a large, closed vessel fitted with a stirrer and ports for the addition of constituents and withdrawal of the final blend. When bacterial vaccines are blended, the active constituents usually need to be greatly diluted and the vessel is first charged with the diluents, usually containing a preservative. Thiomersal has been widely used in the past but is now being phased out and replaced by phenoxyethanol or alternatives. A single-component final bulk is made by adding bacterial suspension, bacterial component or concentrated toxoid in such quantity that it is at the required concentration in the final product. A multiple-component final bulk of a combined vaccine is made by adding each required component in sequence. When viral vaccines are blended, the need to maintain adequate antigenicity or infectivity may preclude dilution, and tissue culture fluids, or concentrates made from them, are often used undiluted or, in the case of multicomponent vaccines, merely diluted one with another. After thorough mixing, a final bulk may be divided into a number of moderate-sized volumes to facilitate handling.

2.7 Filling and drying

As vaccine is required to meet orders, bulk vaccine is distributed into single-dose ampoules or into multidose vials as necessary. Vaccines that are filled as liquids are sealed and capped in their containers, whereas vaccines that are provided as dried preparations are freeze-dried before sealing.

The single-component bacterial vaccines are listed in Table 24.1. For each vaccine, notes are provided of the basic material from which the vaccine is made, the salient production processes and tests for potency and for safety. The multicomponent vaccines that are made by blending together two or more of the single-component vaccines are required to meet the potency and safety requirements for each of the single components that they contain. The best-known of the combined bacterial vaccines is the adsorbed diphtheria, tetanus and pertussis vaccine (DTPer/Vac/Ads) that is used to immunize infants, and the adsorbed diphtheria and tetanus vaccine (DT/Vac/Ads) that is used to reinforce the immunity of school entrants. The trend is to produce increasingly complex combinations, and hepta- and octavalent preparations are now available.

The single-component viral vaccines are listed in Table 24.2, with notes similar to those provided with the bacterial vaccines. The only combined viral vaccine that is widely used is the measles, mumps and rubella vaccine (MMR Vac). In a sense however, both the inactivated (Salk) poliovaccine (Pol/Vac (inactivated)) and the live (Sabin) poliovaccine (Pol/Vac (oral) are combined vaccines in that they are both mixtures of virus of each of the three serotypes of poliovirus. Influenza vaccines, too, are combined vaccines in that they usually contain components from several virus strains, usually from two strains of influenza A and one strain of influenza B.

2.8 Quality control

The quality control of vaccines is intended to provide assurances of both the probable efficacy and the safety of every batch of every product. It is achieved in three ways: (1) in-process control; (2) final product control; and (3) requirements that for each product the starting materials, intermediates, final product and processing methods are consistent.

The results of all quality control tests must be recorded in detail and authorized by a qualified person as, in those countries in which the manufacture of vaccines is regulated by law, they are part of the evidence on which control authorities judge the acceptability or otherwise of each batch of each preparation.

Table 24.1 Bacterial vaccines used for the prevention of infectious disease in humans

Vaccine	Source material	Processing	Potency assay	Safety tests
Anthrax[a]	Medium from cultures of *B. anthracis*	**1** Separation of cells from medium **2** Filtration of supernatant **3** Adsorption of protective antigen complex to Al adjuvant	3 + 3 quantal assay in guinea-pigs using challenge with *B. anthracis*	Exclusion of live *B. anthracis* and of anthrax toxins
BCG[b]	Cultures of live BCG cells in liquid or on solid media	**1** Bacteria centrifuged from medium **2** Resuspension in stabilizer solution **3** Freeze-drying	Viable count; induction of sensitivity to tuberculin in guinea-pigs	Exclusion of virulent mycobacteria; excessive dermal reactivity Exclusion of extraneous microorganisms
Diphtheria (adsorbed)[b]	Cultures of *C. diphtheriae* in liquid medium	**1** Separation and concentration of toxin **2** Conversion of toxin to toxoid **3** Adsorption of toxoid to adjuvant	3 + 3 quantal assay in guinea-pigs using intradermal challenge with diphtheria toxin or serological assay for antitoxin	Inoculation of guinea-pigs to exclude untoxoided toxin Toxin assay in Vero cell cultures
Haemophilus influenzae type b conjugate[b]	Cultures of *H. influenzae* type b Protein carrier (tetanus/diphtheria toxoid, CRM 197)	**1** Separation of capsular polysaccharide **2** Size selection **3** Activation and conjugation with a protein carrier **4** Purification **5** Filtration	Estimation of capsular polysaccharide content and molecular size, free polysaccharide	Absence of unreacted intermediates Endotoxin assay Sterility
Neisseria meningitidis types A, C,W135, Y conjugate[a]	Cultures of *N. meningitidis* of serotypes A, C,W_{135}, Y Carrier protein ; tetanus/diphtheria toxoid, CRM 197	**1** Precipitation with hexadecyl-trimethyammonium bromide **2** Solubilization and Purification **3** Size selection **4** Activation and conjugation **5** Purification **6** Blending **7** Freeze-drying	Estimation of capsular polysaccharide content and molecular size, free polysaccharide	Absence of unreacted intermediates Endotoxin assay Sterility
Pneumococcal polysaccharide conjugate[b]	Cultures of selected serotypes of *Strep. pneumonia* Carrier protein; tetanus toxoid, CRM 197, *H.influenzae* OMP	**1** Precipitation of extracted polysaccharides with ethanol **2** Size restriction **3** Activation and conjugation to carrier protein **4** Purification **5** Blending **6** Filtration	Physicochemical /immunoassay of polysaccharides, molecular size distribution, free saccharide	Absence of unreacted intermediates Absence of pyrogens Sterility

Tetanus (adsorbed)[b]	Cultures of *Cl. tetani* in liquid medium	**1** Conversion of toxin to toxoid **2** Separation and purification of toxoid **3** Adsorption to adjuvant	3 + 3 quantal assay in mice using subcutaneous challenge with tetanus toxin or measurement of serological response	Inoculation of guinea-pigs to exclude presence of untoxoided toxin Sterility
Typhoid Vi capsular polysaccharide antigen[a]	Cultures of *Sal. typhi* grown in liquid medium	Extraction of capsular antigen	Estimation of capsular antigen and molecular size	Endotoxin assay Sterility
Typhoid live vaccine[a]	Cultures of *Sal. typhi* Strain Ty21A	Encapsulation	Estimation of content of live bacteria	Absence of live enteric pathogens
Whooping cough (pertussis) whole cell[b]	Cultures of *Bord. pertussis* grown in liquid or on solid media	**1** Harvest **2** Killing with heat, thiomersal or formalin **3** Resuspension **4** Blending **5** Adsorption	3 + 3 quantal assay in mice using intracerebral challenge with live *Bord. pertussis*	Estimation of bacteria to limit content to 20×10^9 per human dose Weight gain test in mice to exclude excess toxicity Pertussis toxin assay Endotoxin assay Sterility
Whooping cough (pertussis) (acellular)[b]	Cultures of *Bord. pertussis*	**1** Harvest **2** Extraction, detoxification and blending of cell components (pertussis toxin, filamentous haemagglutinin, pertactin, fimbriae) **3** Adsorption to adjuvant	Immunogenicity assay (product specific) or modified quantal assay using intracerebral challenge with live *Bord. pertussis*	Specific toxin and endotoxin assays Sterility

[a]Vaccines used to provide additional protection when circumstances indicate a need.
[b]Vaccines used in conventional immunization schedules.
Diphtheria and pertussis vaccines are seldom used as single-component vaccines but as components of diphtheria/tetanus vaccines and diphtheria/tetanus/pertussis vaccines. Combined diphtheria/tetanus/pertussis/Hib and diphtheria/tetanus/pertussis/Hep B vaccines with or without inactivated polio vaccine are available.
Bacterial vaccines of restricted availability include anthrax, botulism, cholera, plague, Q fever, typhus and tularaemia vaccines.

Table 24.2 Viral vaccines used for the prevention of infectious diseases in humans

Vaccine	Source material	Processing	Potency assay	Safety tests
Hepatitis A[a]	Human diploid cells infected with hepatitis A virus	**1** Separation of virus from cells **2** Inactivation with formaldehyde **3** Adsorption to $Al(OH)_3$ gel	Assay of antigen content by ELISA	Inoculation of cell cultures to exclude presence of live virus
Hepatitis B[a]	Yeast cells genetically modified to express surface antigen	**1** Separation of HbsAg from yeast cells **2** Adsorption to $Al(OH)_3$ gel	Immunogenicity assay or HbsAg assay by ELISA	Test for presence of yeast DNA
Influenza (split virion)[a]	Allantoic fluid from embryonated hens' eggs infected with influenza viruses A and B	**1** Harvest of viruses **2** Disruption with surface-active agent or solvent **3** Blending of components of different serotypes	Assay of haemagglutinin content by single radial diffusion	Inoculation of embryonated hens' eggs to exclude live virus
Influenza (surface antigen)[a]	Allantoic fluid from embryonated hens' eggs infected with influenza viruses A and B	**1** Inactivation and disruption **2** Separation of haemagglutinin and neuraminidase **3** Blending of haemagglutinins and neuraminidase of different serotypes	Assay of haemagglutinin content by single radial diffusion	Inoculation of embryonated hens' eggs to exclude live virus
Measles[b]	Chick embryo cell cultures infected with attenuated measles virus	**1** Clarification **2** Freeze-drying	Infectivity titration in cell cultures	Tests to exclude presence of extraneous viruses
Mumps[b]	Chick embryo cell cultures infected with attenuated mumps virus	**1** Clarification **2** Freeze-drying	Infectivity titration in cell cultures	Tests to exclude presence of extraneous viruses

Poliomyelitis (inactivated)	Human diploid cell cultures infected with each of the three serotypes of poliovirus	**1** Clarification **2** Inactivation with formaldehyde **3** Concentration **4** Blending of virus of each serotype	Estimation of D antigen content	Inoculation of cell cultures and monkey or transgenic mouse spinal cords to exclude live virus
Poliomyelitis (live or oral)[b] (Sabin type)	Cell cultures infected with attenuated poliovirus of each of the three serotypes	**1** Clarification **2** Blending with β-propiolactone	Infectivity titration in cell cultures	Neurovirulence test in monkeys or transgenic mice
Rubella[b] (German measles)	Human diploid cell cultures infected with rabies virus	**1** Clarification **2** Blending with stabilizer **3** Freeze-drying	Infectivity titration in cell cultures	Tests to exclude presence of extraneous viruses
Varicella[a]	Human diploid cell cultures infected with attenuated varicella virus	**1** Clarification **2** Freeze-drying	Infectivity titration in cell cultures	Tests to exclude presence of extraneous viruses
Yellow fever[a]	Aqueous homogenate of chick embryos infected with attenuated yellow fever virus 17D	**1** Centrifugation to remove cell debris **2** Freeze-drying	Infectivity titration in cell cultures by plaque assay	Tests to exclude extraneous viruses

[a]Vaccines used to provide additional protection when circumstances indicate a need.

[b]Vaccines used in conventional immunization programmes. Measles, mumps and rubella vaccines are generally administered in the form of a combined measles/mumps/rubella vaccine (MMR vaccine).

Viral vaccines of restricted application include Congo Crimean haemorrhagic fever vaccine, dengue fever vaccine, Japanese encephalitis B vaccine, rabies vaccine, smallpox vaccine, tick-borne encephalitis vaccine and Venezuelan equine encephalitis vaccine.

ELISA, enzyme-linked immunosorbent assay.

2.8.1 In-process control

In-process quality control is the control exercised over starting materials and intermediates. Its importance stems from the opportunities that it provides for the examination of a product at the stages in its manufacture at which testing is most likely to provide the most meaningful information. The WHO recommendations and national authorities stipulate many in-process controls but manufacturers often perform tests in excess of those stipulated, especially sterility tests (Chapter 21) as, by so doing, they obtain assurance that production is proceeding normally and that the final product is likely to be satisfactory. Numerous examples of in-process control exist for various types of vaccine but three demonstrate the principle.

The quality control of both diphtheria and tetanus vaccines requires that the products are tested for the presence of free toxin, i.e. for specific toxicity due to inadequate detoxification with formaldehyde, at the final product stage. By this stage, however, the toxoid concentrates used in the preparation of the vaccines have been much diluted and, as the volume of vaccine that can be inoculated into the test animals (guinea-pigs) is limited, the tests are relatively insensitive. In-process control, however, provides for tests on the undiluted concentrates and thus increases the sensitivity of the method at least 100-fold.

An example from virus vaccine manufacture is the titration, prior to inactivation, of the infectivity of the pools of live poliovirus used to make inactivated poliomyelitis vaccine. Adequate infectivity of the virus from the tissue cultures is an indicator of the adequate virus content of the starting material and, as infectivity is destroyed in the inactivation process, there is no possibility of performing such an assay after formaldehyde treatment.

A more general example from virus vaccine production is the rigorous examination of tissue cultures to exclude contamination with infectious agents from the source animal or, in the cases of human diploid cells or cells from continuous cell lines, to detect cells with abnormal characteristics. Monkey kidney cell cultures are tested for simian herpes B virus, simian virus 40, mycoplasma and tubercle bacilli. Cultures of human diploid cells and continuous line cells are subjected to detailed karyological examination (examination of chromosomes by microscopy) to ensure that the cells have not undergone any changes likely to impair the quality of a vaccine or lead to adverse effects.

2.8.2 Final product control

2.8.2.1 Assays

Vaccines containing killed microorganisms or their products are generally tested for potency in assays in which the amount of the vaccine that is required to protect animals from a defined challenge dose of the appropriate pathogen, or its product, is compared with the amount of a standard vaccine that is required to provide the same protection. The usual format of the test is the 3 + 3 dose quantal assay that is used to estimate the potency of whole-cell pertussis vaccine (*British Pharmacopoeia*, 2010). Three logarithmic serial doses of the test vaccine and 3 of the standard vaccine are made and each is used to inoculate a group of 16 mice. In the case of both the test vaccine and the standard, the middle dose is chosen on the basis of experience, so that it is sufficient to induce a protective response in about 50% of the animals to which it is given. Each lower dose may then be expected to protect less than 50% of the mice to which it is given and each higher dose to protect more than 50% of the animals. Fourteen days later all of the mice are inoculated ('challenged') with a suitable virulent *Bordetella pertussis* strain and, after a further 14 days, the number of mice surviving in each of the 6 groups is counted. The number of survivors in each group is used to calculate the potency of the test vaccine relative to the potency of the standard vaccine by the statistical method of probit analysis (Finney, 1971). The potency of the test vaccine may be expressed as a percentage of the potency of the standard vaccine. However, as the standard vaccine will have an assigned potency in international units (IU), it is more usual to express the potency of the test vaccine in similar units. Tests similar to that used to estimate the potency of pertussis vaccine are prescribed for the potency determinations of diphtheria vaccine and tetanus vaccines. In these cases the respective bacterial toxins are used as the challenge material (*British Pharmacopoeia*, 2010). Tests that do not involve challenge but involve titration of the antitoxin response *in vitro*, e.g. by ELISA (enzyme-linked immunosorbent assay), are now being adopted.

Vaccines containing live microorganisms are generally tested for potency by determining their content of viable particles. In the case of the most widely used live bacterial vaccine, BCG vaccine, dilutions of vaccine are prepared in a medium which inhibits clumping of cells, and fixed volumes are dropped on to solid media capable of supporting mycobacterial growth. After a fortnight the colonies generated by the drops are counted and the live

count of the undiluted vaccine is calculated. The potency of live viral vaccines is estimated in much the same way except that a substrate of living cells is used. Dilutions of vaccine are inoculated on to tissue culture monolayers in Petri dishes or in plastic trays, and the infective particle count of the vaccine is calculated from the infectivity of the dilutions as indicated by plaque formation, cytopathic effect, haemadsorption or other effect and the dilution factor involved.

2.8.2.2 Safety tests

Because many vaccines are derived from basic materials of intense pathogenicity—the lethal dose of tetanus toxin for a mouse is estimated to be 3×10^{-2} ng—safety testing is of paramount importance. Effective testing provides a guarantee of the safety of each batch of every product and most vaccines in the final container must pass one or more safety tests as prescribed in a pharmacopoeial monograph. This generality does not absolve a manufacturer from the need to perform in-process tests as required, but it is relaxed for those preparations that have a final formulation that makes safety tests on the final product either impractical or meaningless.

Bacterial vaccines are regulated by relatively simple safety tests. Those vaccines composed of killed bacteria or bacterial products must be shown to be completely free from the living microorganisms used in the production process. Inoculation of appropriate bacteriological media with the final product provides an assurance that all organisms have been killed. Those vaccines prepared from toxins, for example, diphtheria and tetanus toxoids, require in addition, a test system capable of revealing inadequately detoxified toxins; this can be done by inoculation of guinea-pigs, which are exquisitely sensitive to both diphtheria and tetanus toxins. A test for sensitization of mice to the lethal effects of histamine is used to detect active pertussis toxin in pertussis vaccines. An improved non-lethal method is also available. The trend is to replace *in vivo* assays by cell culture methods where possible but these do not always emulate *in vivo* effects. Inoculation of guinea-pigs is also used to exclude the presence of abnormally virulent organisms in BCG vaccine. Molecular genetic methods, such as nucleic acid amplification to probe for genes specific to virulent strains, are now available but not yet in routine use for vaccine testing.

Viral vaccines can present problems of safety testing far more complex than those experienced with most bacterial vaccines. With killed viral vaccines the potential hazards are those due to incomplete virus inactivation and the consequent presence of residual live virus in the preparation. The tests used to detect such live virus consist of the inoculation of susceptible tissue cultures and of susceptible animals. The cultures are examined for cytopathic effects, and the animals for symptoms of disease and histological evidence of infection at autopsy. This test is of particular importance in inactivated poliomyelitis vaccines, the vaccine being injected intraspinally into monkeys or mice transgenic for the poliovirus receptor. At autopsy, sections of brain and spinal cord are examined microscopically for the histological lesions indicative of proliferating poliovirus.

With attenuated viral vaccines the potential hazards are those associated with reversion of the virus during production to a degree of virulence capable of causing disease in recipients. To a large extent this possibility is controlled by very careful selection of a stable seed but, especially with live attenuated poliomyelitis vaccine, it is usual to compare the neurovirulence of the vaccine with that of a vaccine known to be safe in field use. The technique involves the intraspinal inoculation of monkeys with both the reference vaccine and the test vaccine followed by comparison of the neurological lesions and symptoms, if any, that are caused. If the vaccine causes abnormalities in excess of those caused by the reference it fails the test. A modification of this test which uses transgenic mice instead of monkeys is now available. An *in vitro* method (MAPREC test) which relies on detecting RNA sequences specific to virulent virus has also been developed. A widespread problem with safety testing of live viral vaccines is that the host specificity of many viruses limits the availability of suitable animal models.

2.8.2.3 Tests of general application

In addition to the tests designed to estimate the potency and to exclude the hazards peculiar to each vaccine there are a number of tests of more general application. These relatively simple tests are as follows.

- *Sterility.* In general, vaccines are required to be sterile. The exceptions to this requirement are smallpox vaccine made from the dermis of animals and bacterial vaccines such as BCG, Ty21A and tularaemia vaccine, which consist of living but attenuated strains. These have a bioburden limit which defines the number of permissible microorganisms but excludes pathogens. WHO recommendations and pharmacopoeial monographs stipulate, for vaccine batches of different size, the numbers of

containers that must be tested and found to be sterile. The preferred method of sterility testing is membrane filtration, as this technique permits the testing of large volumes without dilution of the test media. The test system must be capable of detecting aerobic and anaerobic bacteria and fungi (see Chapter 21).

• *Freedom from abnormal or general toxicity.* The purpose of this simple test is to exclude the presence in a final container of a highly toxic contaminant. Five mice of 17–22 g and two guinea-pigs of 250–350 g are inoculated with one human dose or 1.0 ml, whichever is less, of the test preparation. All must survive for 7 days without signs of illness. Current pharmacopoeial monographs usually do not require this test if another *in vivo* test has been performed on the product.

• *Pyrogenicity or endotoxin content.* The pyrogenicity of a specified dose of product when administered to rabbits can be assayed by a standard pharmacopoeial method but the trend is to replace this with an *in vitro* assay for endotoxin (Chapter 22). The capacity of the product to induce gelation of *Limulus polyphemus* amoebocyte lysate is determined against a reference endotoxin preparation and the result is expressed as IU of endotoxin. For pyrogens other than endotoxin, a monocyte stimulation test is available.

• *Presence of aluminium and calcium.* The quantity of aluminium in vaccines containing aluminium hydroxide or aluminium phosphate as an adjuvant is limited to 1.25 mg per dose and it is usually estimated complexiметrically. The quantity of calcium is limited to 1.3 mg per dose and is usually estimated by atomic absorption spectrometry.

• *Free formaldehyde.* Inactivation of bacterial toxins with formaldehyde may lead to the presence of small amounts of free formaldehyde in the final product. The concentration, as estimated by colour development with acetylacetone, must not exceed 0.02%.

• *Phenol concentration*. When phenol is used to preserve a vaccine its concentration must not exceed 0.25% w/v or, in the case of some vaccines, 0.5% w/v. Phenol is usually estimated by the colour reaction with aminophenazone and hexacyanoferrate.

• *pH.* The potentiometric determination of pH is made by measuring the potential difference between two appropriate electrodes immersed in the solution to be examined: one of these electrodes is sensitive to hydrogen ions and the other is the reference electrode. The pH apparatus is calibrated with the buffer solution of potassium hydrogen phthalate and one other buffer solution of different pH. The pH in the test sample should comply with the limits approved for the particular products.

• *Osmolality*. Osmolality is a practical means of giving an overall measure of the contribution of the various solutes present in a solution to the osmotic pressure of the solution. Osmolality is determined by measurement of the depression of freezing point of the test sample using appropriate apparatus. The osmolality of the test sample should comply with the limits approved for the particular products, for example minimum 240 mOsmol/kg.

3 *In vivo* diagnostics

3.1 Preparation

The most widely used of these are the tuberculins employed to detect sensitization by mycobacterial proteins and hence the possible presence of infection. These are prepared by growing approved strains of *M. tuberculosis* (or *M. bovis* or *M. avium* in preparations intended for veterinary use) in a protein-free medium for several weeks. The culture is then steamed for a prolonged period to kill surviving bacteria and to facilitate release of tuberculoproteins from the cells. The culture supernatant is recovered by centrifugation and further concentrated by evaporation and sterile filtered to make a product known as Old Tuberculin. The crude material may then be standardized against a reference preparation by titration in the skin of guinea-pigs sensitized to *M. tuberculosis*. In practice, further purification is usually performed by precipitation with trichloracetic acid or other protein precipitant to produce purified protein derivative, which is standardized by *in vivo* assay. Concentrated preparations containing 100 000 IU/ml are used to formulate working strengths such as 1000, 100 or 10 IU/ml. These have to be diluted in a medium containing a Tween surfactant to reduce adsorption to glass. The concentrated material can be used for intradermal testing by a multiprong device such as the Heaf or Tine method.

3.2 Quality control

Apart from standardization of potency, which also serves as an identity test, the material must be checked for sterility and for the absence of viable mycobacteria. Because of their slow growth the latter may not be detected by conventional sterility tests and it is usual to perform check tests by guinea pig inoculation, or by prolonged culture on Lowenstein–Jensen medium. The product is also checked for absence of reactogenicity in unsensitized

guinea-pigs and if required by the regulatory authority, for abnormal toxicity.

Analogous intradermal test reagents such as mallein, histoplasmin and coccidioidin, are produced by similar methods. Their use has declined, however, as they, like the tuberculin test, detect previous exposure and sensitization to the antigens of the agent but not necessarily active infection.

4 Immune sera

4.1 Preparation

Immune sera are preparations derived from the blood of animals, usually horses, but mules, donkeys, sheep or goats are also used. The animals must be in good health, free of infections and obtained from sources free of transmissible spongiform encephalopathies, and kept under veterinary supervision. To prepare an immune serum, horses or other animals are injected with a sequence of spaced doses of an antigen until a trial blood sample shows that the injections have induced a high titre of antibody to the injected antigen. An adjuvant may be used if required. A large volume of blood is then removed by venepuncture and collected into a vessel containing sufficient citrate solution to prevent clotting. The blood cells are allowed to settle and the supernatant plasma is drawn off. Alternatively, the blood can be mechanically defibrinated. The crude plasma can be sterilized by filtration and dispensed for use, but it is preferable to fractionate it to separate the immune globulin. This is done by fractional precipitation of the plasma by the addition of ammonium sulphate. The globulin fraction is recovered and treated with pepsin to yield a refined immune product containing the Fab fragment. This refined globulin contains no more than a trace of the albumin and other proteins that were present in the plasma. It is less antigenic, has a longer half-life in the circulation and is less likely to provoke anaphylaxis or serum sickness than whole serum or crude globulin (Harms, 1948). The antibody content of the refined product is determined by specific assay, the product is diluted to the required concentration and transferred into ampoules. Two or more monovalent immune sera may be blended together to provide a multivalent immune serum.

4.2 Quality control

The quality of immune sera is controlled by potency tests and by conventional tests for safety and sterility. The potency tests have a common design in that, in the case of all immune sera, the potency is estimated by comparing the amount of the product that is required to neutralize an effect of a homologous toxin with the amount of a standard preparation that is required to achieve the same effect. Serial dilutions of the immune serum and of a standard preparation are made and to each is added a constant amount of the homologous antigen. Each mixture is then inoculated into a group of animals, usually guinea-pigs or mice, and the dilutions of the immune serum and of the standard, which neutralize the effects of toxin, are noted. As the potencies of the standard preparations are expressed in IU, the potencies of the immune sera are determined in corresponding units per millilitre (*British Pharmacopoeia*, 2010).The quality of globulin fractions is usually monitored by gel electrophoresis to detect contaminating proteins and uncleaved immunoglobulin, and by size-exclusion high-performance liquid chromatography to detect aggregates and small fragments. The immune sera are also tested for contaminating viruses by inoculation on to cell cultures capable of detecting a wide range of viruses relevant to the particular product.

Table 24.3 lists the immune sera for which there is currently a demand, or a potential need, and indicates their required potencies and the salient features of the potency assay methods.

5 Human immunoglobulins

5.1 Source material

Human immunoglobulins are preparations of the immunoglobulins, principally immunoglobulin G (IgG) subclasses, that are present in human blood. They are derived from the plasma of donated blood and from plasma obtained by plasmapheresis. Normal immunoglobulin, that is immunoglobulin that has relatively low titres of antibodies representative of those present in the population at large, is prepared from pools of plasma obtained from not fewer than 1000 individuals. Specific immunoglobulins, that is immunoglobulins with a high titre of a particular antibody, are usually prepared from smaller pools of plasma obtained from individuals who have suffered recent infections or who have undergone recent immunization and who thus have a high titre of a particular antibody. Each contribution of plasma to a pool is tested for the presence of hepatitis B surface antigen (HBsAg), for antibodies to HIV 1 and 2 and for antibodies to hepatitis C virus in order to identify, and to exclude from a pool, any plasma capable of transmitting infection

Table 24.3 Immune sera used in the prevention or treatment of infections in humans

Immunoserum	Potency assay method	Potency requirement
Botulinum antitoxin	Neutralization of the lethal effects of botulinum toxins A, B and E in mice. Similar assays can also be used to titrate the activity of monoclonal antibodies which can include activity against types A–G	500 IU/ml type A 500 IU/ml type B 50 IU/ml type E
Diphtheria antitoxin	Neutralization of the erythrogenic effect of diphtheria toxin in the skin of guinea-pigs The activity of the sera can also be titrated by ELISA or by neutralization of toxin activity in Vero cell cultures	1000 IU/ml if prepared in other species
Tetanus antitoxin	Neutralization of the paralytic effect of tetanus toxin in mice The antitoxin activity can also be titrated by ELISA	1000 IU/ml for prophylaxis 3000 IU/ml for treatment

In each of the assays of potency the amount of the immune serum and the amount of a corresponding standard antitoxin that are required to neutralize the effects of a defined dose of the corresponding toxin are determined. The two determined amounts and the assigned unitage of the standard antitoxin are then used to calculate the potency of the immune serum in international units (IU).

from donor to recipient. Donors who have been resident for 6 months or more in areas endemic for transmissible spongiform bovine encephalopathy (BSE), which currently includes the UK, are also excluded. The solvent-detergent process, one of the methods used to inactivate enveloped viruses, uses treatment with a combination of tributyl phosphate and octoxinol 10; these reagents are subsequently removed by oil extraction or by solid phase extraction (*British Pharmacopoeia*, 2010).

5.2 Fractionation

The immunoglobulins are obtained from the plasma pools by fractionation methods that are based on ethanol precipitation in the cold with rigorous control of protein concentration, pH and ionic strength (Cohn *et al.*, 1946). The variation of Kistler & Nitschmann (1962) is widely used. Some of the fractionation steps may contribute to the safety of immunoglobulins by inactivating or removing contaminating viruses that have not been detected by tests on the blood donations. The immunoglobulins may be presented either as a freeze-dried or a liquid preparation at a concentration that is at least 10 times that in the initial pooled plasma. Glycine may be added as a stabilizer. Multidose preparations contain an antimicrobial preservative but single-dose preparations do not (*British Pharmacopoeia*, 2010).

5.3 Quality control

The quality control of immunoglobulins includes potency tests and conventional tests for safety and sterility. The potency tests consist of toxin or virus neutralization tests that parallel those used for the potency assay of immune sera, except that for in-process control of some immunoglobulins wider use is made of *in vitro* assays. In addition to the safety and sterility tests, total protein is determined by nitrogen estimations, the protein composition by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis and molecular size by high performance liquid chromatography. The presence of immunoglobulins derived from species other than humans is excluded by precipitin tests. Table 24.4 lists six human immunoglobulins and their requisite potencies and indicates the methods by which the potencies are determined.

6 Monoclonal antibodies

6.1 Preparation

Monoclonal antibodies are immunoglobulins or a fragment of an immunoglobulin, with defined specificity, produced by a single clone of cells. They can be obtained from immortalized B-lymphocytes that are cloned and expanded as continuous cell lines (murine and human monoclonal antibodies) or from rDNA-engineered mammalian or bacterial cell lines (engineered monoclonal antibodies). Production of monoclonal antibodies is based on a seed lot system using a master cell bank and a working cell bank derived from the cloned cells. Two approaches are currently in use: single harvest (produc-

Table 24.4 Immunoglobulins used in the prevention and treatment of infections in humans

Immunoglobulins	Potency assay method	Potency requirement
Normal	Neutralization tests in cell cultures or in animals, or *in vitro* ELISA estimations	Measurable amounts of one bacterial antibody and of one viral antibody for which there are international standards
Hepatitis B	Radioimmunoassay or enzyme immunoassay	Not less than 100 IU/ml
Measles	Neutralization of the infectivity of measles virus for cell cultures	Not less than 50 IU/ml
Rabies	Neutralization of the infectivity of rabies virus for mice	Not less than 150 IU/ml
Tetanus	Neutralization of the paralytic effect of tetanus toxin in mice ELISA estimation of antitoxin	Not less than 50 IU/ml
Varicella/zoster	ELISA in parallel with a standard varicella zoster immunoglobulin	Not less than 100 IU/ml

In each of the assays of potency the amount of the immunoglobulin and the amount of a corresponding standard preparation that are required to neutralize the infectivity or other biological activity of a defined amount of virus or to neutralize a defined amount of a bacterial toxin are determined. The two determined amounts and the assigned unitage of the standard preparation are then used to calculate the potency of the immunoglobulins in international units (IU). ELISA, enzyme-linked immunosorbent assay.

tion at finite passage level) and multiple harvest (continuous-culture production). In the first method, the cells are cultivated up to a defined maximum number of passages or population doublings (in accordance with the stability of the cell line). In the second method, cells are continuously cultivated for a defined period (in accordance with the stability of the system and production consistency). In this case, monitoring is necessary throughout the life of the culture; the required frequency and type of monitoring will depend on the nature of the production system. Bulk harvests can be made by pooling individual harvests before purification. The purification process to remove unwanted host cell derived proteins, nucleic acids and carbohydrates is also designed to remove and/or inactivate non-enveloped and enveloped viruses and other impurities.

6.2 Quality control

The quality of therapeutic monoclonal antibodies is controlled by rigorous characterization of the products by chemical and biological methods. The final product should be tested for bioburden and bacterial endotoxins, purity, integrity and potency by suitable analytical methods, comparing with a reference preparation. The identity test can be done by suitable methods comparing the product with the reference preparation. Molecular size distribution can be determined by size-exclusion chromatography. Depending on the nature of the monoclonal antibody, its microheterogeneity and isoforms, a number of different tests can be used to demonstrate molecular identity and structural integrity. These tests may include peptide mapping, isoelectric focusing, ion-exchange chromatography, hydrophobic interaction chromatography, oligosaccharide mapping, monosaccharide content and mass spectrometry. The purity can be examined by SDS–polyacrylamide gel electrophoresis under reducing and non-reducing conditions or capillary electrophoresis. For monoclonal antibodies with specific antimicrobial activity, an appropriate assay is performed to determine the level of this. Monoclonal antibodies have been developed for postexposure prophylaxis of anthrax (anti-Protective Antigen), botulism (anti-toxins A–G) and plague (anti-V antigen). Monoclonal antibodies directed against the core region of Gram-negative bacterial endotoxins and intended for treatment of sepsis, have met with little success.

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25 Recombinant DNA technology

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1 Introduction: biotechnology in pharmaceutical sciences

The rapid developments in biotechnology and the applications of genetic engineering to practical human problems have allowed the advancement of pharmaceutical biotechnology at a staggering pace. Furthermore, the release of the human genome sequence has also been key for the identification of human genetic diseases and the design of revolutionary approaches for their treatment.

Genetic engineering involves altering DNA molecules outside an organism, making the resultant DNA molecules function in living cells. Many of these cells have been genetically engineered to produce substances that are medically useful to humans. Pharmaceutical biotechnology involves the use of living organisms such as microorganisms to create new pharmaceutical products, or safer and more effective versions of conventionally produced pharmaceuticals, more cost-effectively.

Since the manufacture of the first recombinant pharmaceutical, insulin, there has been a burst in the generation of new recombinant drugs, some of which will be covered later on in this chapter. Furthermore, the use of recombinant DNA technology has spread further allowing the development not only of subunit vaccines,

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such as the one used in the prevention of hepatitis B, but also of attenuated vaccines, vector vaccines and DNA vaccines. One of pharmaceutical biotechnology's great potentials lies in gene therapy, which consists in the modification of the genetic material of living cells to prevent, control or cure disease. It encompasses repairing or replacing defective genes and, for example, making tumours more susceptible to other kinds of treatment.

This chapter aims to describe some essential genetic manipulation techniques and to illustrate, with some key examples, their use for the generation of recombinant pharmaceutical drugs. Applications of recombinant DNA techniques in the diagnosis of diseases will also be covered.

2 Enabling techniques

To understand how recombinant pharmaceuticals are manufactured we first need to review some of the essential DNA manipulation techniques used to generate these products. We will start by looking at ways to cut and join fragments of DNA and then examine step by step how these techniques can be exploited to clone and express genes from eukaryotic and prokaryotic cells.

2.1 Cutting and joining DNA molecules

DNA isolated from any type of cell can be fragmented using restriction endonucleases. These are enzymes produced by microorganisms which cut foreign DNA and can restrict the proliferation of infecting viruses. Some of these enzymes cut at specific points known as *restriction sites* which are palindromic sequences (complementary sequences with identical nucleotide sequences when read in the 5′ to 3′ direction) of various lengths. For example, *Eco*RI (*Escherichia coli* restriction enzyme I) has specificity for the sequence GAATTC and hydrolyses the G-A phosphodiester bond. Enzymes which recognize and cut at restriction sites of 4–8 base pairs (bp) are particularly useful, as the probability of a site appearing in a random DNA fragment is inversely proportional to its length

There are two different types of DNA ends that can be generated using restriction enzymes: cohesive or sticky and blunt ends (Table 25.1). DNA fragments obtained by restriction enzyme digestion can be covalently joined together using the enzyme DNA ligase. There is a limitation, however, with regards to the type of ends this

Table 25.1 Restriction enzymes and the compatibility of digested ends for ligation

Restriction enzyme	Restriction site	Digested ends		Compatibility for ligase	Ligation products
		Sticky			
*Sau*3A	5′-**GATC**-3′ 3′-**CTAG**-5′	5′- 3′-**CTAG**	**GATC**-3′ -5′		5′-GATCC-3′ 3′-**CTAG**G-5′
		Sticky		Compatible	
*Bam*HI	5′-GGATCC-3′ 3′-CCTAGG-5′	5′-G 3′-CCTAG	GATCC-3′ G-5′		5′-G**GATC**-3′ 3′-CCTAG-5′
		Sticky		Incompatible	
*Eco*RI	5′-**GAATTC**-3′ 3′-**CTTAAG**-5′	5′-**G** 3′-**CTTAA**	**AATTC**-3′ **G**-5′		5′-**G**AATTC-3′ 3′-**CTTAA**G-5′
		Sticky		Compatible	
*Eco*RI	5′-GAATTC-3′ 3′-CTTAAG-5′	5′-G 3′-CTTAA	AATTC-3′ G-5′		5′-G**AATTC**-3′ 3′-CTTAA**G**-5′
		Blunt		Incompatible	
*Dra*I	5′-**TTTAAA**-3′ 3′-**AAATTT**-5′	5′-**TTT** 3′-**AAA**	**AAA**-3′ **TTT**-5′		5′-**TTT**ATC-3′ 3′-**AAA**TAG-5′
		Blunt		Compatible	
*Eco*RV	5′-GATATC-3′ 3′-CTATAG-5′	5′-GAT 3′-CTA	ATC-3′ TAG-5′		5′-GAT**AAA**-3′ 3′-CTA**TTT**-5′

enzyme is able to bond together. Only blunt ends generated by some restriction enzymes (e.g. *Dra*I and *Eco*RV) or compatible sticky ends generated by either the same restriction enzymes (e.g. *Eco*RI) or by enzymes that generate complementary overhanging ends (e.g. *Sau*3AI and *Bam*HI) will be bonded by the DNA ligase (Table 25.1).

2.2 Cloning vectors

Genes present in DNA fragments that have been excised with restriction endonucleases can be maintained (replicated) and expressed by inserting them (cloning) into vectors after ligation. These cloning vectors are of different types and are relatively small DNA molecules that have the ability to self-replicate in a host cell. The main type of vectors used for gene cloning are plasmids, cosmids and bacteriophages, which are normally used according to the size of the DNA fragments that need to be cloned.

2.2.1 Cloning of small fragments of DNA

To allow the cloning of small DNA fragments, generally up to 10 kb, plasmids are the main vectors of choice.

2.2.1.1 Plasmids

Plasmids are circular extrachromosomal DNA molecules that replicate independently within cells using their own origin of replication. There are a number of features generally found in plasmids used as cloning vectors:

- *Antibiotic resistance markers*. These are genes coding for proteins that confer resistance to specific antibiotics. These markers therefore allow the selection of hosts carrying the plasmids.
- *Multiple cloning sites* (MCS). These are stretches of DNA designed to contain a number of unique different restriction sites, providing a choice of possible restriction enzymes to be used for the cloning of DNA fragments.
- *Origin of replication.* Required for plasmid replication in a specific host. These DNA sequences also determine the number of copies at which the plasmid will replicate, from just one to several hundreds per cell. Plasmids having more than one host-specific origin of replication are known as shuttle vectors.
- *Insertional inactivation markers*. These facilitate the selection of recombinant from non-recombinant plasmids. The most commonly used is the *lacZ* gene coding for β-galactosidase. This enzyme cleaves 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), an artificial substrate that mimics galactose, which results in the generation of an insoluble blue product. Insertion of a recombinant DNA fragment in *lacZ* will result in the disruption of β-galactosidase production and the inability to cleave X-Gal. Hence colonies from bacteria carrying an intact *lacZ* gene, i.e. from non-recombinant plasmids, appear blue on agar plates containing X-Gal. In contrast, those with a successful insertion in *lacZ*, i.e. harbouring recombinant plasmids, appear white.

Figure 25.1 shows all these features in a simplified diagram of the cloning vector pUC18.

An ideal plasmid for cloning should be small (2–10 kb) and conjugation-defective, i.e. non-self-mobilizable from cell to cell, and produce a selectable phenotype in host cells. It should also contain a large MCS and replicate at a high copy number (>10 copies per cell).

2.2.2 Cloning of large fragments of DNA

Sometimes there is a need to clone large fragments of DNA, for example for the isolation of complete gene

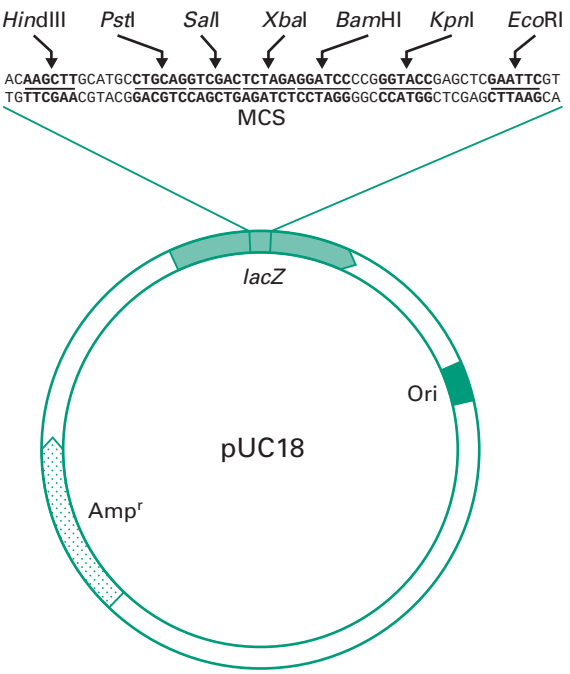


Figure 25.1 Simplified diagram of the plasmid pUC18. *lacZ* represents the insertional inactivation marker coding for β-galactosidase activity. A multiple cloning site (MCS) is present within the *lacZ* gene, allowing the cloning of DNA fragments. 'Ori' represents the origin of replication which, in this case, is functional in *Escherichia coli*, and Amp^r represents an ampicillin resistance marker.

clusters. An example would be the cloning of large eukaryotic genes or genes required for the synthesis of a certain molecule. Sometimes the synthesis of a compound requires more than 10 different genes and these are frequently organized in operons cotranscribed in a single mRNA molecule. To enable the cloning of large genes or full-length operons, vectors such as bacteriophages, cosmids or BACs need to be used.

2.2.2.1 Bacteriophages

The most popular has been the *E. coli* λ (lambda) bacteriophage, which is composed of a tubular protein tail and a protein head packed with approximately 50 kb of double-stranded (ds) DNA (Figure 25.2). After injection of the viral DNA into *E. coli*, bacteriophage λ can multiply and enter a lytic cycle leading to the lysis of the host cell and the subsequent release of a large number of phage particles. Alternatively, injection of the DNA can lead to a lysogenic cycle in which the phage DNA is integrated into the *E. coli* chromosome where it is maintained until the environmental conditions change and is then excised, entering a lytic cycle. Out of the 50 kb that make the λ bacteriophage less than half are essential for its propagation and therefore around 20 kb can be replaced by recombinant DNA, hence their name *λ replacement vectors*. For this reason these are very useful vectors for the generation of genomic libraries. DNA in λ bacteriophages contain at each end small single-stranded (ss) complementary DNA fragments called λ cohesive ends (λ*cos* ends). Recombinant phages can be assembled in a test tube into phage-like particles by enzymes which recognize and process the λ*cos* ends, provided that they are 35–45 kb apart. These enzymes as well as the head and tails required for the assembly process are commercially

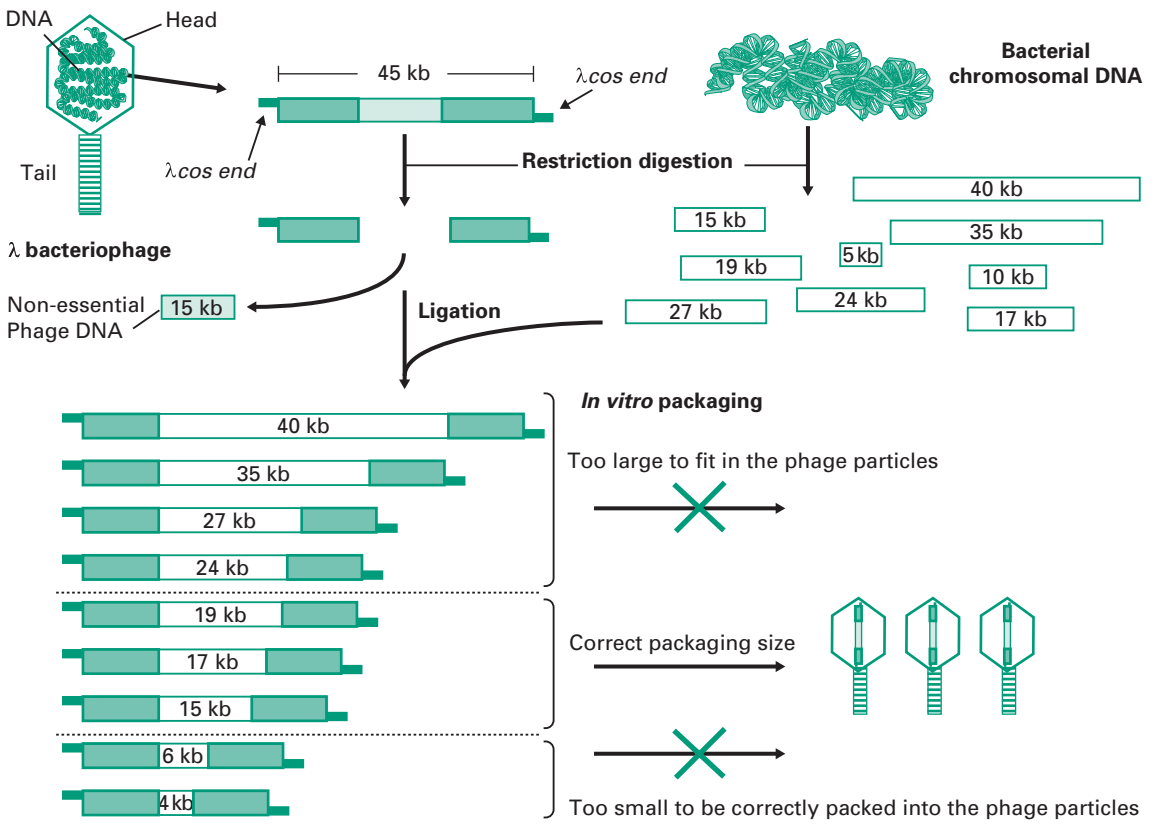


Figure 25.2 Cloning of DNA into λ replacement vectors. The DNA from the λ bacteriophage can be purified from the phage particles and digested with restriction enzymes to remove an internal fragment of around 15 kb which is not required for the life cycle of the bacteriophage. This fragment can be replaced with other fragments of DNA such as those coming from digested bacterial chromosomal DNA. As the λ bacteriophage can only pack DNA fragments flanked by λ*cos* ends which are 35–45 kb apart, any recombinant fragments larger or smaller than that will not be successfully packed.

available as part of *in vitro* packaging kits. The *in vitro* packaging results in the formation of recombinant phages that can be transduced to *E. coli* cells. Transduced *E. coli* will be identified by the formation of lysis plaques in agar plates seeded with a mixture of *E. coli* and recombinant λ bacteriophages.

2.2.2.2 Cosmids and fosmids

Cosmids are plasmids maintained in *E. coli* that have been engineered to carry λ*cos* sequences. This allows their packaging *in vitro* into λ phage particles to be transduced into *E. coli* cells for replication. Once inside the bacterial host the cosmid DNA is circularized by the joining of the λ*cos* ends and thereafter it replicates as a normal plasmid. This implies that the cloned DNA will be available as *E. coli* colonies and not as plaques like with the λ bacteriophages. As less DNA is required for plasmid than for bacteriophage replication, cosmids can carry up to 40 kb of cloned DNA still enabling the packaging into λ phage particles.

Eukaryotic and more particularly mammalian genomic DNA rich in multiple repeated elements can be subject to deletions and rearrangements when cloned in standard multicopy cosmids and replicated in bacteria. This inconvenient can be reduced by using special cosmids in which the multicopy origin of replication has been replaced by the single-copy origin of replication from the *E. coli* F′ factor. These cloning vectors are known as *fosmids*.

2.2.2.3 Bacterial artificial chromosomes

Although cosmids and fosmids enable the cloning of relatively large DNA fragments, the amount of DNA that can be packed into a λ bacteriophage head is limited to around 50 kb. To clone even larger DNA fragments cloning vectors derived from the single-copy *E. coli* F′ factor, similar to the fosmids, have been designed. In this case the λ*cos* ends are absent or not used for packaging and instead the recombinant plasmids are introduced directly into the *E. coli* cells by transformation. This allows the cloning of DNA fragments of several hundreds of kilobases resulting in what are then called *bacterial artificial chromosomes* (BACs).

2.3 Introduction of vector into hosts

For the expression and maintenance of recombinant genes the recombinant vectors harbouring them need to be introduced into suitable hosts. The four main methods used to achieve this are transformation, electroporation, conjugation and transduction.

- *Transformation* is the direct incorporation of DNA into host cells. Bacteria such as *E. coli* can uptake recombinant plasmid DNA when treated with ice-cold $CaCl_2$ until they reach a 'competent' state in which they are ready to take up DNA. These cells are then mixed with the recombinant plasmid and exposed briefly to a heat shock of 42 °C which causes them to take up the DNA.
- *Electroporation* is, however, the most efficient way of introducing DNA not only in bacteria but also in eukaryotic cells. This technique is based on the induction of free DNA uptake by the cells after subjecting them to a strong electric field.
- In some cases *conjugation* can be used as a natural transmission of plasmid DNA from a donor cell to a recipient cell by direct contact through cell–cell junctions. Only plasmid cloning vectors containing conjugative elements can be transferred by conjugation. This procedure requires direct contact between the donor and the recipient cell. Conjugation is not as frequently used as electroporation as most plasmid vectors used for the cloning of recombinant DNA lack conjugative functions, preventing these plasmids from being passed to other cells inadvertently.
- Finally, in *transduction* (prokaryotes) and *transfection* (eukaryotes) the transfer of recombinant non-viral DNA to a cell is achieved by a virus. This is the method of choice for the introduction of recombinant λ bacteriophages, cosmids and fosmids into *E. coli* cells.

2.4 Construction of genomic libraries

Before we study how genomic libraries are made we first need to understand the differences between the genetic organization in eukaryotic and prokaryotic cells. Bacterial genes are uninterrupted sequences of nucleotides encoding the genetic information required for the synthesis of a protein. These genes can sometimes be cotranscribed with adjacent genes of related function into the same mRNA molecule. This set of cotranscribed genes is called an *operon* (Figure 25.3). The mRNA in bacteria does not generally need to be processed before translation, and transcription and translation occur simultaneously.

In contrast, genes from eukaryotic cells contain non-coding sequences called *introns* and coding sequences called *exons*. The former are removed after transcription by a process called 'splicing' that occurs in the nucleus of the cell. In addition the mRNA is subjected to further processing involving the addition of a methylated guanine (M_7Gppp) called CAP on its 5′ end, required for translation, and a polyadenine (poly(A))tail on its 3′ end (Figure 25.3). Mature mRNA is then exported from the nucleus

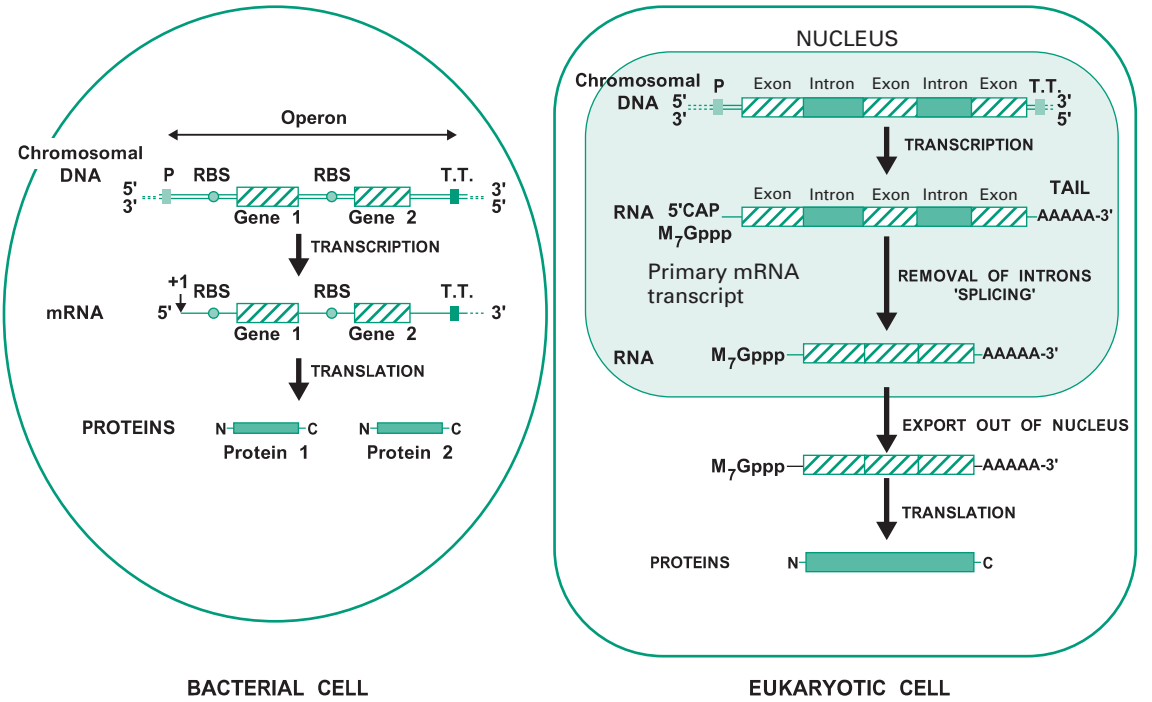


Figure 25.3 Genetic organization in bacteria (prokaryotes) and eukaryotes. In prokaryotes genes can sometimes be grouped in operons and hence transcribed together in a single molecule of mRNA. In these organisms the whole process of transcription and translation takes place simultaneously in the cytoplasm. In contrast, in eukaryotes, genes are organized in single transcriptional units incorporating introns. Upon transcription in the nucleus, eukaryotic mRNA is firstly modified by the addition of a CAP and a poly(A) tail and then by the splicing out of the introns. The mature mRNA is then exported into the cytoplasm where it is translated into proteins. P, promoter; T.T., transcriptional terminator; RBS, ribosome binding site.

into the cytoplasm, where it is translated into proteins. Eukaryotic genes appear to be transcribed individually, as operons have not been described in eukaryotes.

To enable the cloning and isolation of a specific gene(s) from a cell several steps are required. The first consists of choosing the source of genetic material which, in prokaryotic cells, is normally the chromosomal DNA. In contrast, in eukaryotic cells this is more often the mature mRNA, as it is not interrupted by introns and consequently codes for complete, active proteins. The second step consists of the preparation of the purified DNA or RNA for cloning. This step is more straightforward when using prokaryotic DNA (section 2.4.1) than eukaryotic RNA (section 2.4.2). The result will be the construction of a collection of cloned DNA fragments propagated in bacteria that is called the genomic library. This library should ideally contain representatives of every sequence in the chromosome of a prokaryotic cell and every expressed gene in the case of a eukaryotic cell. The final step consists of the screening of the recombinant clones to identify the required gene(s).

2.4.1 Prokaryotic gene libraries: shotgun cloning

The construction of a prokaryotic gene library can be achieved by a technique called 'shotgun cloning' (Figure 25.4). This involves the purification and partial digestion of the genomic (chromosomal) DNA from a prokaryotic organism with restriction endonucleases to produce a random mixture of fragments of different sizes. Chromosomal DNA can also be mechanically sheared and in this case the extremities must be repaired and made blunt with DNA polymerase in the presence of deoxynucleotides (dNTPs). These fragments are then fractionated into different sizes and ligated into a cloning vector appropriately digested. The recombinant vectors are then transformed, in the case of plasmids, or

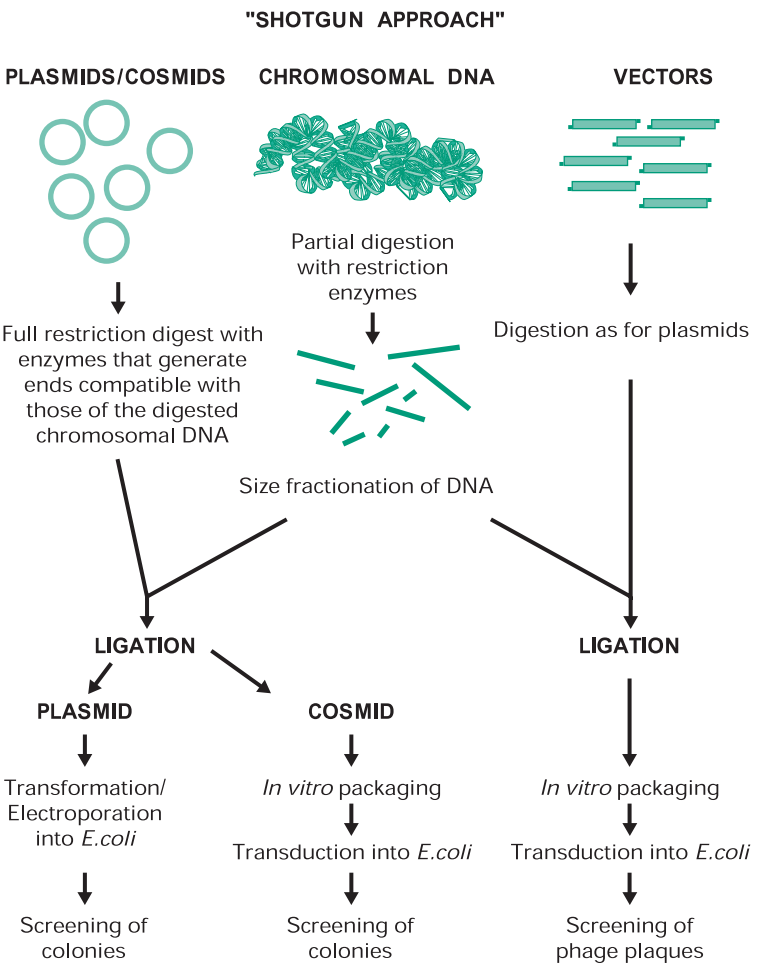


Figure 25.4 Construction of prokaryotic genomic libraries by shotgun cloning. The shotgun approach for the construction of genomic libraries involves the purification and digestion of chromosomal DNA from the prokaryotic organisms, followed by the cloning into a digested suitable vector using DNA ligase. The recombinant vector is then introduced into the host cell using the appropriate method and then the colonies or plaques are screened for the presence of the recombinant gene of interest.

transfected, in the case of bacteriophages and cosmids, into the host cell of choice. The resulting genomic library can then be screened for the presence of the recombinant gene of interest by a number of methods (see section 2.5).

2.4.2 Eukaryotic cDNA gene libraries

The shotgun approach cannot be applied for the construction of eukaryotic gene libraries because of the presence of introns in the DNA, which prevents the direct cloning of functional genes from digested chromosomal DNA. Instead, mature mRNA from the cytoplasm of cells expressing the desired gene is used as the source of genetic material. For example, to make a genomic library containing the insulin gene, RNA from pancreatic cells expressing this gene have to be isolated. Remember that cells show distinct differentiation in different tissues, and express only a small percentage of the whole genome according to their role in the tissue of which they form part. Consequently, it is not possible to purify RNA coding for insulin from, for example, cells of the pituitary gland. Therefore, the cells expressing the gene of interest have to be isolated first, and then their mRNA purified. As mentioned earlier, virtually every eukaryotic mRNA has a poly(A) tail on its 3′ end. This provides a convenient way to isolate mature mRNA from total cellular RNA, most of which (98%) is ribosomal RNA (rRNA) and transfer RNA (tRNA). The total RNA purified from a cell can be passed through an affinity column packed with cellulose linked to deoxythymidine oligonucleotides (oligo(dT)). As the total RNA passes through the column, only the mRNA molecules which have poly(A) tails will bind to the oligo(dT) while the rest will flow through the column to be discarded. The purified mRNA then has to

be converted into ds cDNA (complementary DNA) to enable its cloning into a suitable vector.

2.4.2.1 Synthesis and cloning of cDNA

There are generally two main strategies used for the synthesis of cDNA from mRNA: *replacement synthesis* and *primer adaptor synthesis*. For both strategies the first strand cDNA synthesis is based on the priming of the mRNA with an oligo-dT which anneals to the poly(A) tail of the mRNA molecule and, consequently, with the action of the enzyme reverse transcriptase and in the presence of dNTPs, the synthesis of the first cDNA strand takes place. This results in the formation of a mRNA/cDNA heteroduplex hybrid. The second stage is different for the two strategies mentioned. The most commonly used is the replacement synthesis, which is based on the use of ribonuclease H (RNaseH), an enzyme that cleaves the RNA moiety of RNA/DNA hybrids and has 5′ to 3′ and 3′ to 5′ direction exonuclease activities. This results in partial digestion of the RNA in both directions. The resulting RNA fragments can serve as primers for DNA synthesis using DNA polymerase I. This enzyme, with its 5′ to 3′ direction exonuclease and polymerase activities will fill the nicks and effectively remove the RNA primers. The cDNA fragments synthesized will be joined using DNA ligase. This method causes the loss of some nucleotides at the 5′ end of the mRNA, including the CAP region.

The primer adaptor method for the synthesis of the second strand of cDNA starts with the removal of the RNA strand from the mRNA/cDNA hybrid, by treatment with alkali. This is followed by the addition of a poly(C) tail to the 3′ end of the DNA strand using an enzyme called terminal transferase. This enables the hybridization of a complementary poly(G) primer that will be the starting point for the synthesis of the second cDNA strand by the DNA polymerase (Figure 25.5). This method, in contrast to the replacement synthesis, generates cDNA molecules with a complete 5′ CAP region. However, it requires more steps and the terminal transferase step is difficult to control.

Finally, the cloning of the resulting cDNA is aided by the addition of a poly(C) tail at the 3′ ends of the cDNA fragments using terminal transferase and the ligation of these to a linearized vector containing a complementary poly(G) tail also generated by the terminal transferase.

2.4.3 Comparison between libraries

There are a number of points to take into consideration when choosing a strategy to generate a genomic library. The larger the insert size, the lower the number of clones required to have full representation of an entire genome. To reduce the number of recombinant clones to be screened before the gene of interest is identified, cosmids, λ bacteriophages or BACs should be used as they can take larger DNA inserts. Furthermore, if we want to isolate a large DNA fragment containing, for example, all the genes required for the biosynthesis of an antibiotic, plasmid libraries might not be the right choice. In contrast, if we are trying to isolate a single gene and the extent of the screening is not a problem, plasmid libraries are ideal as they reduce the subcloning steps required to single out the gene of interest. Table 25.2 shows a comparison between the insert sizes taken by different vectors, their hosts and the genomic libraries for which they can be suitable.

2.5 Screening of genomic libraries

Once the genomic library has been generated it is necessary to screen for the gene of interest within thousands of recombinant clones. The choice of screening method will very much depend on the availability of reagents and the information on the target gene to be isolated.

2.5.1 Hybridization screening

This technique is used when some of the DNA sequence for the gene screened for is known, or when a fragment of this gene is available from a previous cloning. Alternatively, a DNA fragment from a closely related gene can be used as a probe for the isolation of the gene of interest. The hybridization technique requires plating the library on a set of agar plates to generate a replica, on nitrocellulose or nylon membranes, of the plaques or colonies, each containing a different recombinant DNA fragment. This process transfers a portion of each plaque or colony to the membranes and is done in such a way that the pattern of plaques/colonies on the original plate is maintained on the filters. The membranes are then hybridized with a radiolabelled DNA probe containing part of the sequence to be isolated from the library. The probe will only bind/hybridize to the recombinant clones containing that sequence. After this process, the membranes are exposed to X-ray film (autoradiography). The presence of dark spots on the films represents the location of colonies containing the target gene. By orienting the film with the original agar plate, the colony/plaque carrying the complementary sequence can be identified and the desired clone isolated. An alternative to the use of radio-isotopes for the probes resides in the use of nucleotides labelled with a molecule such as digoxigenin

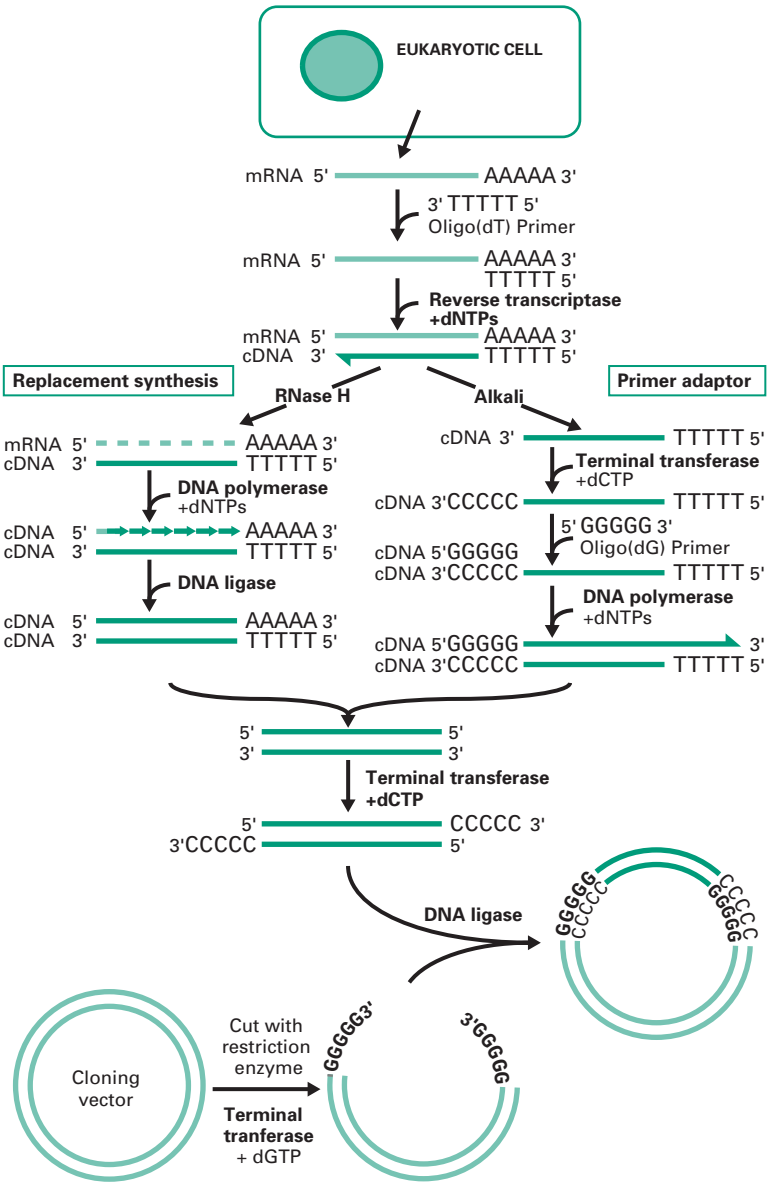


Figure 25.5 Synthesis and cloning of cDNA. Cloning of eukaryotic genes involves the isolation of mRNA from the cytoplasm of the cells expressing the gene of interest. To allow the cloning of the mRNA the synthesis of ds cDNA is first required. This involves the synthesis of the first cDNA strand by reverse transcriptase using an oligo(dT) primer. To generate the second strand of cDNA there are two main methods. The replacement synthesis method involves the generation of nicks in the mRNA strand by the RNaseH followed by the synthesis of the complementary strand, using the RNA fragments generated by the RNaseH as primers, by the DNA polymerase. The DNA fragments generated are then joined together by the DNA ligase. In contrast, the primer adaptor method requires the degradation of the mRNA strand by alkali followed by the addition of a poly(C) tail at the 3′ end of the cDNA strand by the terminal transferase. For the synthesis of the second strand of cDNA, addition of an oligo(dG) primer and DNA polymerase are required. The ds cDNA generated by either method can be cloned into any vector upon addition of poly(C) sticky ends by the terminal transferase, provided that the vector has complementary poly(G) ends created by this enzyme.

(DIG). In that case detection of the hybridized probes is performed with anti-DIG antibodies conjugated to an enzyme such as alkaline phosphatase, which reacts with a substrate to produce chemiluminescence.

2.5.2 Immunological screening

This technique is used when we need to isolate a gene coding for a protein for which there are antibodies available. The success of this technique relies on the expression of the gene of interest, as it requires the synthesis of the target protein from the target recombinant gene. The screening steps are similar to those used for the hybridization screening, with the difference that the membranes containing portions of plaques or colonies have to be incubated with the antibodies that will recognize the target protein. This antibody, called the primary antibody, will bind tightly to those colonies/plaques containing the recombinant gene of interest, provided that the

Table 25.2 Comparison of vectors used for the construction of genomic libraries

Vector	Insert size (kb)	Host	Cloning method suitability
Plasmid	<10	Specified by origin of replication present on the plasmid	Shotgun cloning cDNA cloning
λ Bacteriophages (replacement only)	7–22	*E. coli*	Shotgun cloning
Cosmids and fosmids	25–45	*E. coli*	Shotgun cloning
BACs	up to 750	*E. coli*	Shotgun cloning

protein encoded by this gene has been synthesized. The position of the bound antibody is revealed by incubating the membranes with a labelled antibody (secondary antibody) that recognizes the primary antibody. There are different types of labels for antibodies, all of which can easily be detected.

2.5.3 Protein activity screening

This type of screening is limited to proteins that have a specific activity that can easily be identified within a large population of recombinant clones. Needless to say, to detect a protein activity the gene coding for this protein must be expressed and an active protein must be produced. Understanding of this technique can be helped by illustrating this screening with an example. Suppose we want to isolate a gene coding for a bacterial haemolytic toxin from a genomic library. As we know that this toxin lyses red blood cells, we could plate the library on plates containing agar mixed with blood. Those colonies/plaques expressing the haemolytic toxin could easily be identified by the presence of a haemolytic halo around them resulting from the action of the toxin on the red blood cells.

2.6 Optimizing expression of recombinant genes

The primary objective of pharmaceutical companies involved in the production of recombinant drugs is the maximal expression of recombinant genes to generate large quantities of these drugs. Unfortunately, the cloning of a gene into a vector does not ensure that it will be properly expressed. To improve expression of a cloned gene the different stages that lead to the synthesis of the protein therefore have to be optimized. This is achieved by the use of so-called *expression vectors* (Figure 25.6). Some expression vectors have been designed to produce large quantities of protein in specific cell hosts. For example, the *bacmids* are shuttle expression vectors derived from a baculovirus ds DNA circular genome and are used to transfect insect cells in order to produce large quantities of a recombinant protein in fermenters.

2.6.1 Optimizing transcription

To optimize transcription it must be ensured that the recombinant gene is placed after a promoter (Figure 25.6) that will be recognized by the RNA polymerase of the host cell where the gene is going to be expressed. Two types of promoters can be used: (1) *constitutive promoters*, which are expressed all the time and (2) *inducible promoters*, where expression is turned off during culture growth and turned on, for example, upon the addition of an inducing molecule to the culture, usually shortly before harvesting, when high numbers of bacteria are present in the culture. Inducible promoters are very useful when expressing foreign genes coding for proteins toxic to the bacterial hosts as their premature expression could lead to growth impairments and consequently low yields of recombinant protein.

Furthermore, to ensure that transcription finishes after the 3′ end of the recombinant gene, a *transcriptional terminator* (Figure 25.6) must be placed just downstream of this gene.

2.6.2 Optimizing translation

A key feature that determines whether a gene is going to be efficiently translated by a certain host is the nucleotide sequence of the *ribosome binding site* (RBS), located upstream of the gene (Figure 25.6), which needs to be efficiently recognized by the ribosomes of this host. In addition, the distance between the RBS and the transla-

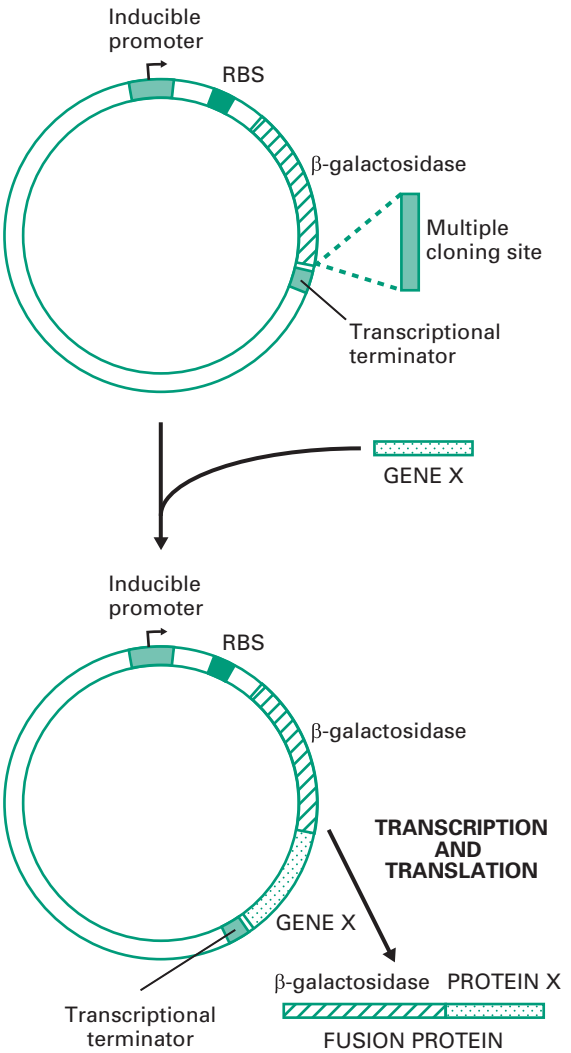


Figure 25.6 Expression vectors and the generation of fusion proteins. Expression vectors have optimized all the signals required for transcription (inducible promoter and transcriptional terminator) and for translation (ribosome binding site). Some of them carry the gene for β-galactosidase with a multiple cloning site that allows the insertion of small genes for the generation of fusion proteins. RBS, ribosome binding site.

tion *start codon* needs to be optimal to enable the right interactions between the mRNA and the ribosomes and start the protein synthesis. There are commercially available vectors carrying sequences for RBSs and translation start codons which are optimally recognized by the ribosomes of the host cells, ensuring that any recombinant genes cloned after the start codon will be maximally translated.

Small proteins are frequently susceptible to proteolytic degradation when expressed in a foreign host. This degradation can be avoided by expressing them fused to a larger protein. This is normally achieved by cloning the small gene downstream of a gene coding for a protein such as β-galactosidase. To obtain the fusion protein (Figure 25.6) it is essential to ensure that the reading frame is conserved and that no translation stop codons are present between the β-galactosidase and the target gene, enabling the ribosomes to read through. Interestingly, affinity columns that will bind the fused polypeptides are available, which facilitates the purification of the recombinant protein by affinity chromatography.

2.6.3 Post-translational modifications

Although high levels of protein production may be achieved by optimizing transcription and translation of a gene, the obtained protein may still need to undergo post-translational modifications before it is active. Some of these modifications include correct disulphide bond formation, proteolytic cleavage of a precursor, glycosylation and additions to amino acids such as phosphorylation, acetylation, sulphation, acylation, etc. Unfortunately, the practical *E. coli* host, in which most recombinant proteins are produced, does not possess the same type of cellular machinery required for these modifications. Recently, *Campylobacter jejuni* has been found to possess an eukaryotic-like system for protein glycosylation and efforts are being made to genetically engineer *E. coli* strains to perform the adequate glycosylation of recombinant proteins as mammalian cells. Hence, it is essential to select a suitable host for the expression of the target gene that can carry out the required post-translational modifications that will enable the synthesis of large amounts of a biologically authentic product. Table 25.3 shows a comparison of a selection of hosts currently used for the expression of recombinant proteins.

2.7 Amplifying DNA: the polymerase chain reaction

The polymerase chain reaction (PCR) is an extremely simple and powerful technique that was perfected by Kary Mullis in the mid-1980s and has revolutionized many studies in molecular biology, currently having applications ranging from forensic studies to the development of new recombinant drugs. This technique allows the generation of large amounts of copies of a specified

Table 25.3 Comparison of different hosts used for the expression of recombinant genes

	Advantages	Disadvantages
Prokaryotic hosts		
Escherichia coli	Easy to grow in large-scale volumes Transcriptional and translational control well known Successfully used in the manufacture of insulin, interferon and human somatotropin	Difficult to achieve export of some proteins into growth medium Degradation of small proteins by proteases Unable to undertake most post-translational modifications, e.g. glycosylation Many proteins retained in the cytoplasm as insoluble aggregates
Bacilli *Bacillus subtilis* *Bacillus brevis*	Many proteins can be exported into growth medium Easy to grow in large-scale volumes	Regulation of gene expression not very well known Lack of high-level expression vectors Unable to carry out most post-translational modifications, e.g. glycosylation
Eukaryotic hosts		
Yeasts *Saccharomyces cerevisiae* *Pichia pastoris* *Hansenula polymorpha*	Easy to grow in large-scale volumes Efficient protein glycosylation Good export of heterologous proteins into growth medium Wide range of high-level expression systems available Recombinant proteins do not form insoluble aggregates in the cytoplasm	Gene expression can be difficult to control Sometimes fails to achieve accurate post-translational modification of recombinant proteins
Insect cells *Spodoptera frugiperda*	High expression levels Free of virus or prion-type agents pathogenic to mammals Can produce accurate glycosylation	Difficult to scale up High-mannose chains can be immunogenic
Mammalian cells	Precise post-translational modification of human proteins Good expression systems available High stability of recombinant proteins	Gene regulation not well known Low protein secretion levels Can harbour infectious agents such as viruses Difficult to scale up
Transgenic animals	Precise post-transcriptional modifications Easy to generate large amount of recombinant protein, e.g. one goat can generate 1 kg of recombinant protein in milk per year Relatively inexpensive	Risk of contamination with infectious agents Products can sometimes be unstable

DNA sequence from a single DNA molecule without the need for cloning.

The PCR exploits certain characteristics of DNA replication, as it uses single-stranded DNA (ss DNA) as a template for the synthesis of complementary new strands in a 5′ to 3′ direction. The ss DNA templates can be generated by heating ds DNA above 90 °C. DNA polymerase synthesizes ds DNA by extending the complementary strand of a template. Hence the DNA polymerase can be directed to synthesize a specific region of DNA by using a synthetic, complementary oligonucleotide primer that will anneal to the template when the temperature is

lowered. The PCR reaction uses a special DNA polymerase (*Taq* DNA polymerase from the thermophilic bacterium *Thermus aquaticus*) that can withstand temperatures above 100 °C and has an optimal activity at 72 °C, which has the advantage of reducing non-specific primer annealing that may occur at lower temperatures.

In PCR both strands of a target DNA serve simultaneously as templates upon the addition of a pair of primers, one for each strand of DNA. A typical PCR amplification is shown in Figure 25.7. Every PCR cycle is normally repeated up to 30 times. The net result of a PCR is that, at the end of n cycles, it will generate 2^n ds DNA copies of a single DNA fragment located between the two primers.

2.7.1 Advantages and limitations of PCR

There are some obvious advantages of using PCR. The main one is specificity, as it allows, using the appropriate primers, the amplification of specific DNA fragments from a population of different cells. It is also a very rapid technique, as it only takes a few hours to amplify a fragment of DNA compared with days using conventional cloning methods. An important feature of PCR is its versatility, as it allows the incorporation of mismatches on the 5′ end of the primers provided that the 3′ end has perfect complementary with the targeted strand. This can be exploited to add restriction sites to enable subsequent cloning of the amplified DNA, or introducing specific mutations into genes. Furthermore, the equipment used for PCR is relatively inexpensive and allows the analysis of a large number of samples at one time. Finally, PCR does not require purified template DNA and can amplify genes from whole cells or tissue samples.

However, there are also a number of limitations to the use of PCR. The designing of primers for this technique requires some knowledge of the DNA sequence to be amplified. Although there are new genetically engineered DNA polymerases that can synthesize large fragments of DNA, there are still some restrictions with regards to the maximum length of DNA that can be amplified. Ideally, fragments of 0.1–3 kb can be easily amplified although this technique, under the appropriate conditions, would amplify larger fragments (up to 20 kb). In addition, the slightest sample contamination can lead to false positive results, which can have detrimental effects when this technique is used in diagnostics. Finally, sometimes there is a risk of non-specific amplification when the primers anneal to sequences similar to the targets, leading to the amplification of the wrong DNA.

2.7.2 Clinical applications of PCR

The development of PCR has revolutionized not only basic research but also different areas of medicine. Table 25.4 lists some of the most important clinical applications of PCR. These types of analysis were practically impossible without PCR owing to the large amount of samples that needed handling, the amount of time required to obtain results or the lack of sensitivity of the available tests.

3 Biotechnology in the pharmaceutical industry

One of the first and most important commercial applications of genetic engineering was the introduction of genes coding for clinically important proteins into bacteria. Because bacterial cells are cheap to grow on a large scale in fermenters, they can synthesize vast amounts of protein from the recombinant genes they carry. This results in a significant reduction in cost and an increase in the availability of these proteins. There are currently a large number of important recombinant drugs available on the market, examples of which are provided in Table 25.5. This chapter covers only the genetic manipulation strategies used to produce some well-known drugs.

3.1 Recombinant human insulin

Recombinant human insulin, used for the treatment of diabetes, was the first drug produced using genetic engineering, in 1982. Before this, animals, notably pigs and cattle, were the only non-human sources of insulin. Animal insulin differs slightly from the human form and, consequently, it can potentially elicit an immune response in humans, making it ineffective. Additionally, the use of recombinant insulin prevents the risks resulting from potential contamination of animal insulin with other hormones or viruses. To understand how insulin is produced using recombinant DNA techniques we first need to review its structure. Figure 25.8 illustrates how insulin is initially synthesized as preproinsulin, a single polypeptide which is processed during export into proinsulin and finally into active insulin**,** once proteolytic cleavage of the connecting sequence for the two A and B insulin chains has occurred. These two chains remain joined through disulphide bonds.

Currently, different approaches are employed to produce recombinant insulin, one of which is shown in Figure 25.8. First of all, two DNA fragments coding for the A or the B insulin chains are synthesized chemically.

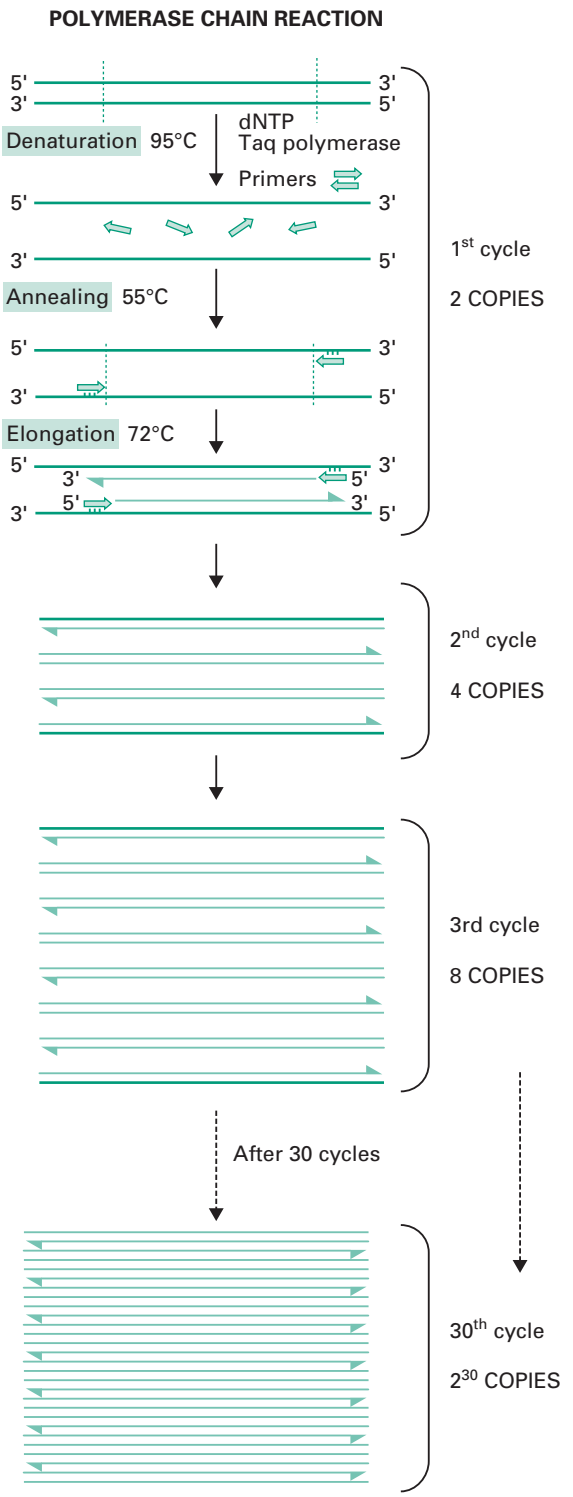


Figure 25.7 The polymerase chain reaction (PCR). A single PCR reaction involves the following steps: (1) Denaturation of the target ds DNA by heating at 95 °C and addition of an excess of two oligonucleotide primers, each complementary to a different strand of the target sequence, Taq DNA polymerase and dNTPs. (2) Annealing of the primers to the DNA strands by decreasing the temperature to 55 °C. (3) Elongation (or synthesis) of the new strand of DNA by the Taq DNA polymerase after increasing the temperature to 72 °C. This cycle can be repeated up to 30 times. In each cycle the number of DNA copies is doubled, hence, at the end of a PCR reaction, we could have 2^{30} copies of an original target DNA molecule.

Each of these synthetic fragments is then individually inserted in a plasmid after the *E. coli* gene coding for β-galactosidase. This enables this bacterium to produce large fusion proteins with the insulin chains tacked on to the end of the β-galactosidase enzyme. These fusion proteins can then be purified from bacterial extracts and the insulin chains released upon treatment with cyanogen bromide, which cleaves peptide bonds following methionine residues. As methionine was inserted at the boundaries between the β-galactosidase and the insulin chains, and there are no methionines present internally within the insulin molecule, treatment with cyanogen bromide results in the cleavage of intact insulin chains from the fusion proteins. The purified A and B insulin chains can be mixed and reconstituted into an active insulin molecule. Currently there are also other methods used to produce recombinant insulin which are based on the generation of single β-galactosidase fusions to the full-length insulin gene containing the coding sequences for both A and B chains. These alternative methods can simplify the manufacturing of this drug.

3.2 Recombinant somatostatin

Somatostatin, also known as the 'antigrowth hormone', modulates the action of growth hormone and is frequently used to treat acromegaly (uncontrolled bone growth). Being a very small peptide, the gene coding for it can easily be chemically synthesized and cloned into a suitable expression vector. As *E. coli* tends to degrade small peptides, the generation of a β-galactosidase–somatostatin fusion protein prevents this degradation. Furthermore, as with insulin, the absence of methionine residues in the somatostatin amino acid sequence allows the insertion of a methionine codon in the junction between β-galactosidase and the somatostatin gene. This enables the subsequent cleavage with cyanogen bromide of the recombinant hormone purified from *E. coli*. The

Table 25.4 Clinical applications of PCR

Application	Examples
Diagnostics of inherited diseases	Duchenne muscular dystrophy Fragile X syndrome Lesch–Nyhan syndrome Tay–Sachs disease Kennedy's disease Cystic fibrosis Haemophilia B
Infectious disease screening	HIV Measles virus Herpes virus Adenovirus *Chlamydia trachomatis* Lyme disease
Forensic examination	Identification of criminals from samples of blood, tissue, hair, etc.
Prenatal screening	Haemophilia Sickle cell anaemia β-Thalassaemia Duchenne muscular dystrophy Batten's disease Sex determination
HLA subtyping	Prevention of insulin-dependent diabetes mellitus
Susceptibility to cardiovascular disease	Mutations in gene coding for angiotensin-converting enzyme (ACE) Mutation in the angiotensinogen gene Mutation in the apolipoprotein CII gene Mutation in the LDL receptor
Susceptibility to cancer	Neoplastic disease Lymph node metastasis in melanoma Acute promyelocytic leukaemia Thyroid cancer Non-Hodgkin's lymphoma

strategy used to generate recombinant somatostatin is shown in Figure 25.9.

3.3 Recombinant somatotropin

Somatotropin, also known as human growth hormone (hGH), consists of 191 amino acids; it is produced in the pituitary gland and regulates growth and development. Regular injections of hGH are given to children with dwarfism caused by the lack of this hormone so that they can reach near normal heights. In this case, unlike with insulin, animal-derived hormones are ineffective and only the human protein works. Because of continuous shortages of pituitaries from human cadavers, the production of recombinant hGH has been imperative. Furthermore, the contamination of children with fatal viruses from the cadavers has been an additional reason for moving away from this source of hormone. As the gene for the hGH is 573 nucleotides long, it was in practice too large to be synthetically produced to generate the recombinant hormone as achieved with insulin and somatostatin. Hence there have been two practical ways of generating recombinant hGH, one of which resulted

Table 25.5 Examples of some commercial clinically important recombinant proteins

Protein	Size/structure	Commercial names/ Company	Expression host	Application
Human insulin	Two peptide chains: A = 21 amino acids B = 30 amino acids	Humulin (Eli Lilly) Humalog (Eli Lilly) Novolin (Novo Nordisk)	*E. coli*	Treatment of diabetes mellitus
Human somatotropin	191 amino acids	Protropin (Genentech) Genotropin (Pharmacia & Upjohn) Humatrope (Eli Lilly) Nutropin (Genetech) Biotropin (Bio-Technology General)	*E. coli*	Treatment of human growth hormone deficiency in children
Interferon α_{2a} and α_{2b}	166 amino acids	Roferon A (Hoffmann-La Roche) Actimmune (Genentech)	*E. coli*	Treatment of various cancers and viral diseases
Interferon γ_{1b}	143 amino acids—glycosylated	Actimmune (Genentech)	*E. coli*	Treatment of chronic granulomatous disease
Tissue plasminogen activator	530 amino acids—glycosylated	Activase (Genentech)	*E. coli* Yeast Animal cells	Treatment of acute mycocardial infarct and pulmonary embolism
Interleukin-2	133 amino acids	Proleukin (Chiron Corporation)	*E. coli* Animal cells	Treatment of kidney carcinoma and metastatic melanoma
Human serum albumin	582 amino acids with 17 disulphide bridges	Albutein (Alpha Therapeutic Corporation)	Yeast	Treatment of hypovolemic shock Adjunct in haemodialysis
Factor VIII	2332 amino acids— glycosylated	Recombinate (Hyland Immuno) Kogenate (Bayer) ReFacto (Wyeth)	Mammalian cells	Treatment of haemophilia
Factor IX	415 amino acids— glycosylated	BeneFIX (Hyland Immuno)	Mammalian cells	Treatment of haemophilia B
Erythropoietin	166 amino acids— glycosylated	Eprex (Jansen-Cilag) NeoRecormon (Roche)	Mammalian cells	Treatment of anaemia associated with dialysis and AZT/AIDS
Hepatitis B surface antigen	Monomer consisting of 226 amino acids	Engerix B (SmithKline Beecham) HB-Vax II (Aventis Pasteur)	Yeast Mammalian cells	Vaccination
Influenza virus hemagglutinin	547 amino acids glycosylated monomer	FluBlock (Protein Sciences Corporation)	Insect cells	Vaccination

in the generation of this hormone with an added methionine at the N-terminus. Figure 25.10 shows these two strategies.

Initially the coding region for hGH was isolated from a cDNA library. The DNA fragment coding for the mammalian signal peptide can then be excised by a restriction enzyme that also removes the first 24 codons of the mature protein. A chemically synthesized DNA fragment containing a methionine codon, to enable translation in *E. coli*, followed by these first 24 codons was therefore ligated to the DNA fragment coding for the remaining amino acids 25–191 of the hGH (Figure 25.10A). The resulting DNA was cloned into an expression vector and transformed into *E. coli* where the recombinant hGH accumulated in the cytoplasm. The recombinant hormone can be isolated from bacterial cell extracts and, in contrast to the non-recombinant protein, carries a methionine residue at the N-terminus.

Figure 25.10B shows an alternative method consisting of the replacement of the mammalian signal peptide for a secretion signal peptide functional in bacteria. This enables the purification of the recombinant hGH from the periplasm of the bacterial cell, reducing the difficulties associated with the purification of recombinant proteins from the cytoplasm. To achieve this, once the mammalian signal peptide has been removed as above, a synthetic DNA molecule containing the missing 24 codons of the hGH, without an added methionine codon, is ligated to the DNA fragment coding for the 25–191 remaining residues. The resulting DNA molecule is then inserted in an expression vector that codes for a bacterial signal peptide in fusion with the hGH gene. Once transformed into *E. coli*, the recombinant hGH is produced and the signal sequence will target the protein for secretion into the periplasmic space where it will accumulate. The periplasmic proteases release the signal peptide, leaving hGH without additional amino acids. The protein can then be easily purified from the periplasmic space after release by hypotonic disruption of the outer membrane.

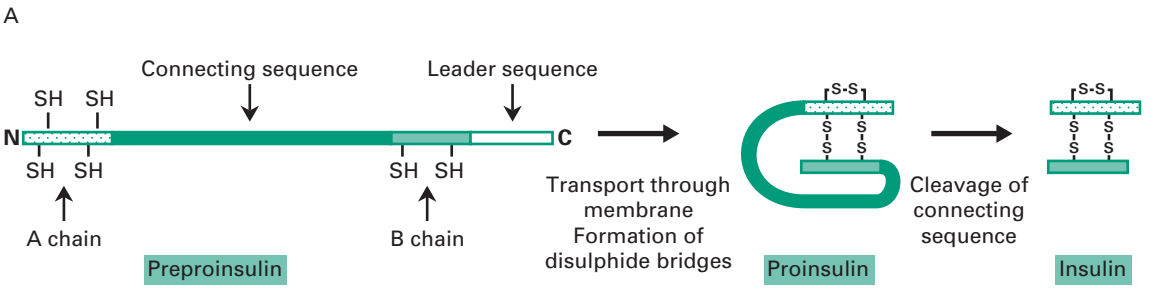
3.4 Recombinant hepatitis B vaccine

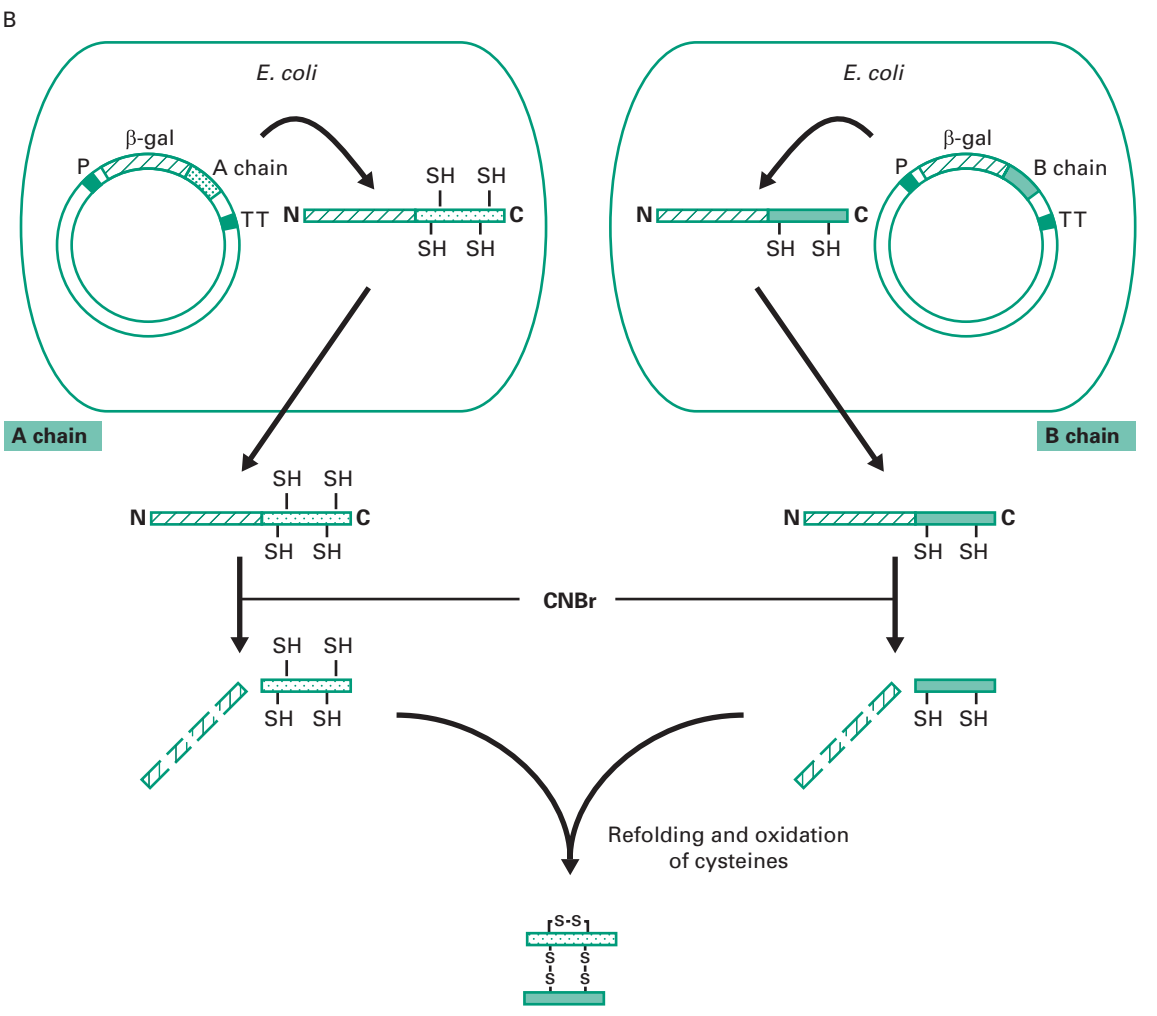
Before the development of recombinant DNA technology, two main strategies were employed for vaccine production: the generation of *inactivated vaccines* consisting of chemically killed derivatives of the infectious agent, and of *attenuated vaccines*, which are altered viruses and bacteria that are avirulent and can no longer cause disease. However, these vaccines were potentially dangerous as they could be contaminated with infectious organisms or revert to a virulent form. To avoid these problems, recombinant DNA technology has enabled the production of *subunit vaccines* consisting solely of immunogenic surface proteins which can elicit immune responses without the risk of infection.

The hepatitis B virus (HBV) vaccine was the first successful subunit vaccine developed. This virus infects the liver and can cause serious damage. This virus has a surface antigen, HBsAg, which is found in blood of infected patients and has been found to elicit a significant immune response. The gene coding for this antigen has been isolated from the virus and cloned into a vector that allows high expression of the HBsAg protein in yeast cells. Figure 25.11 shows the strategy currently used for the generation of recombinant HBV vaccine. In this case, as the 3.2-kb genomic sequence of the HBV virus was known, the gene coding for the HBsAg was directly cloned into a shuttle expression vector that replicates in both, *E. coli* for the genetic manipulation steps, and in yeasts such as *Saccharomyces cerevisiae* for the production of the recombinant antigen. Transcription of the gene encoding HBsAg is driven from a strong yeast promoter and is stopped by a transcriptional terminator present in the vector. The vector also has a leucine biosynthesis marker for selection in yeasts and a tetracycline resistance marker for selection in bacteria. The yeast harbouring this plasmid can grow in fermenters in the absence of leucine, generating large amounts of the antigen that can subsequently be extracted from the cells.

3.5 Recombinant influenza virus vaccines

Influenza viruses type A, B and C cause flu in mammals and birds. The influenza virus type A has been the cause of several pandemics in the past, causing the deaths of millions of people. Its 13.6-kb genome consists of 8 ss linear RNA molecules that code for 11 proteins. Because of the segmented nature of its genome, alleles can easily be swapped between different influenza viruses strains during the co-infection of a host. This makes the virus extremely adaptable and able to escape the immune system by rapidly acquiring novel combinations of its immunogenic proteins. Two of these proteins are located on the surface of the viral particles and define their antigenicity: a hemagglutinin (H or HA) and a neuraminidase (N or NA). To date there are 16 different H antigens (H1–H16) and 9 different N antigens (N1–N9) known, thus the influenza A viruses are classified accordingly as H1N1 (*swine flu* or *Spanish flu*), H5N1 (*bird flu*), H7N7 (*horse flu*), etc. *Seasonal flu* recurs annually and since 2003 it has been caused mostly by variants of the H3N2,





ACTIVE INSULIN

Figure 25.8 Production of recombinant insulin. (A) Insulin consists of two polypeptide chains (chains A and B). It is initially synthesized as part of a larger peptide called *preproinsulin*. The transport across the cell membrane of preproinsulin results in the cleavage of the signal peptide and the formation of disulphide bridges to generate *proinsulin*. Finally, the connecting peptide is cleaved generating the mature insulin. (B) One of the strategies used to make recombinant insulin consists of the cloning of the DNA fragment coding for the A chain and the B chain into two separate expression vectors as β-galactosidase fusions in *E. coli*. The fusion proteins are then purified and the insulin is cleaved with cyanogen bromide (CNBr) after a methionine incorporated at the β-galactosidase and insulin junction. The presence of several methionines in the β-galactosidase results in multiple cleavage of this molecule by CNBr. Finally, the resulting insulin A and B chains are refolded and the cysteines are oxidized for the generation of the active insulin.

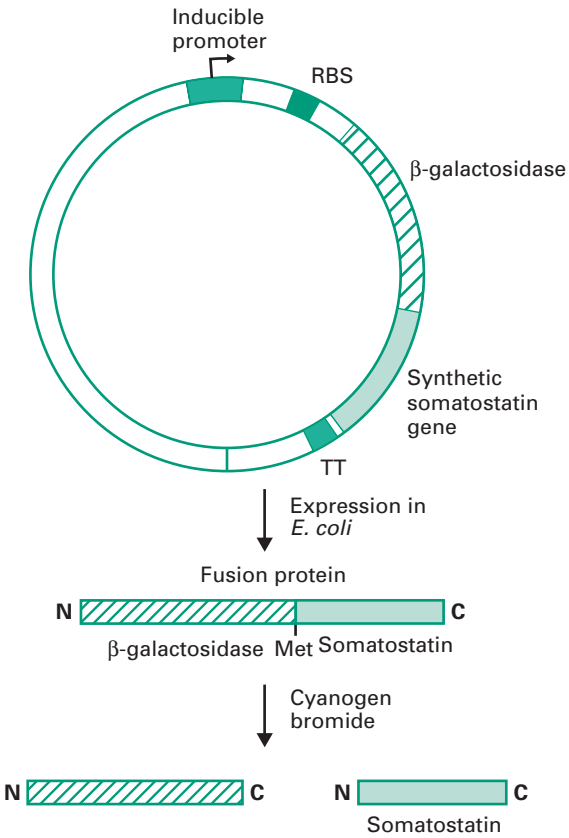


Figure 25.9 Production of recombinant somatostatin. The small size of somatostatin allows the chemical synthesis of the gene coding for it. This gene can be cloned into an expression fused to β-galactosidase. The fusion protein generated in *E. coli* is then purified and the somatostatin polypeptide is released by treatment with cyanogen bromide. RBS, ribosome binding site; TT, transcriptional terminator.

H1N1 and influenza B viruses, the exact types changing over time with developing acquired resistances through the human population by exposure and vaccinations. Therefore, the evolving prevalence of the different serotypes is constantly being monitored with the aim of anticipating the serotype composition of potential pandemic influenza viruses. Because of the short time frame between the identification of a novel strain and the need for a vaccine against it, recombinant biotechnology is essential. As the hemagglutinin is the most rapidly evolving gene product and is crucial for viral attachment and evasion from the immune system recombinant influenza virus vaccines are developed using the gene coding this protein. To achieve this, the RNA is first converted into ds cDNA, cloned and sequenced. This allows the complete identification and genotyping of the virus. Secondly, the gene coding for the hemagglutinin is subcloned into a bacmid expression vector and the recombinant glycoprotein (rHA) is produced in large quantities by transfecting *Spodoptera frugiperda* insect cells growing in fermenters as for the hepatitis B vaccine.

3.6 Production of recombinant antibiotics

A large number of the antibiotics currently used have been isolated from the Gram-positive soil bacterium *Streptomyces*, although many other microorganisms have also been used as sources of antibiotics. The biosynthesis of an antibiotic can sometimes include 10–30 separate enzyme-catalysed reactions, which makes the cloning of all the genes coding for these enzymes very difficult. A strategy used to isolate the complete set of antibiotic biosynthetic genes consists of the transformation of a recombinant gene library from an organism producing the antibiotic into a mutant strain of the same organism unable to produce it. The transformants can be screened for the production of the antibiotic by plating them on to agar plates that have been seeded with a sensitive bacterium. The appearance of halos of growth inhibition around the recombinant colonies indicates the successful cloning of the antibiotic biosynthetic gene cluster. This strategy was successfully used for the cloning and production of the antibiotic undecylprodigiosin from *Streptomyces coelicolor*, as illustrated in Figure 25.12.

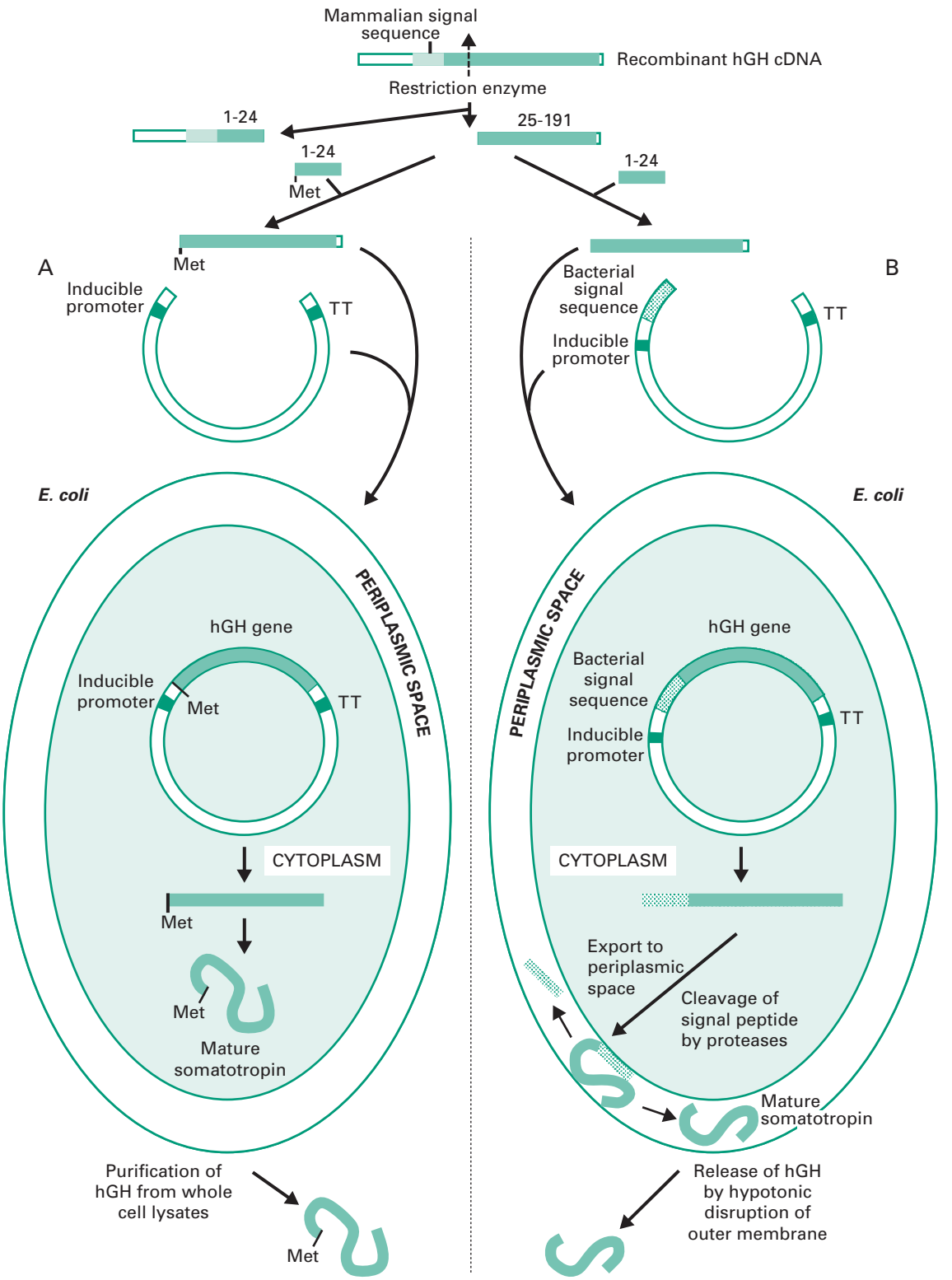

Mammalian signal sequence
Recombinant hGH cDNA
Restriction enzyme
1-24
25-191
1-24
Met
1-24
A
Met
Inducible promoter
TT
B
Bacterial signal sequence
Inducible promoter
TT
E. coli
E. coli
PERIPLASMIC SPACE
hGH gene
Inducible promoter
Met
TT
CYTOPLASM
Met
Met
Mature somatotropin
PERIPLASMIC SPACE
hGH gene
Bacterial signal sequence
Inducible promoter
TT
CYTOPLASM
Export to periplasmic space
Cleavage of signal peptide by proteases
Mature somatotropin
Purification of hGH from whole cell lysates
Met
Release of hGH by hypotonic disruption of outer membrane

Figure 25.10 Two strategies to produce recombinant hGH. These two strategies use as the starting material the recombinant cDNA for hGH which contains the mammalian signal sequence required for the secretion of this protein from mammalian cells. This signal peptide is first removed using a restriction enzyme that cleaves after the nucleotides coding for the first 24 amino acids of the hGH. From this stage two strategies can be followed. (A) A chemically synthesized fragment containing the genetic information for the first 24 amino acids of the hGH, plus a methionine codon in the 5′ end, is ligated to the remaining cDNA fragment coding for amino acids 25–191 and introduced into an expression vector. The hGH expressed from this vector in *E. coli* accumulates in the cytoplasm and is extracted from whole cell lysates. (B) A chemically synthesized fragment also containing the genetic information for the first 24 amino acids of the hGH, but without an added methionine codon, is ligated to the remaining cDNA fragment and cloned into an expression vector immediately after the sequence for a bacterial signal peptide. Consequently, when expressed in *E. coli*, the hGH is tagged on its N-terminus with this signal peptide that drives the export of this protein to the periplasm. Once in the periplasm, the signal peptide is cleaved by proteases and the mature hGH can be released and purified upon hypotonic disruption of the outer membrane. TT, transcriptional terminator.

In some instances, recombinant DNA technology has been successfully used to generate novel antibiotics by introducing in the same organism the genes responsible for the synthesis of two closely related antibiotics. By cross-feeding antibiotic precursors between two close metabolic pathways, novel antibiotics can be generated. This strategy has been very successful in the cross-feeding of antibiotic pathways between different *Streptomyces* spp.

4 New diagnostics using recombinant DNA technology

For many years clinical diagnostic laboratories had limitations in the detection of pathogenic bacteria and parasites due to time constraints in the identification of these agents, as they required to be cultured, a time-consuming procedure detrimental to the patients' health. In addition, many inherited genetic disorders could not be identified owing to the lack of appropriate techniques and the unavailability of the human genome sequence. The rapid developments in molecular biology have enabled modern medicine to overcome some of these problems. Currently, a good diagnostic test must be *specific* for a target molecule, *sensitive* enough to detect minute levels of that molecule, *rapid* and technically *simple*.

This section introduces some molecular diagnostic techniques, based on the detection of specific nucleic acid sequences, currently used in the clinic.

4.1 Diagnosis of infectious diseases

Each pathogenic microorganism contains genetic material that distinguishes it from its host and from other microorganisms. This specific material constitutes a signature that allows the identification of a particular microorganism from a complex mixed population. In the diagnosis of infectious diseases, identification of specific sequences from microbial pathogens will allow appropriate treatment at an early stage as well as prevention of the spread of disease.

The two main techniques used for the diagnosis of infectious disease targeting nucleic acids are hybridization and PCR-based amplification. There are currently specific primers and probes for the detection of more than 100 infectious disease. Table 25.6 shows just a few examples.

4.1.1 DNA hybridization techniques

Nucleic acid hybridization is based on the precise nucleotide base pairing and hydrogen bonding between one strand of nucleotides and a complementary nucleotide sequence. Any diagnostic nucleic acid hybridization consists of three essential elements: a DNA probe, the target DNA and the signal detection system. Recent developments in detection systems and improvements in safety have enabled the use of highly sensitive non-radioactive methods. Figure 25.13 illustrates the general steps required for DNA hybridization using chemiluminescent-based detection. This non-radioactive system achieves signal amplification by enzymatic conversion of a chemiluminescent substrate. First of all, DNA from the biological sample including the target DNA from the pathogen to be identified needs to be extracted. A diagnostic biotin-labelled probe is then mixed with the target DNA bound to a membrane support. The hybridized probe is then incubated with streptavidin, which has multiple sites that avidly bind biotin. Subsequent incubation with biotin conjugated to alkaline phosphatase results in the enzymatic labelling of the bound streptavidin. Finally, addition of a special substrate for the alkaline

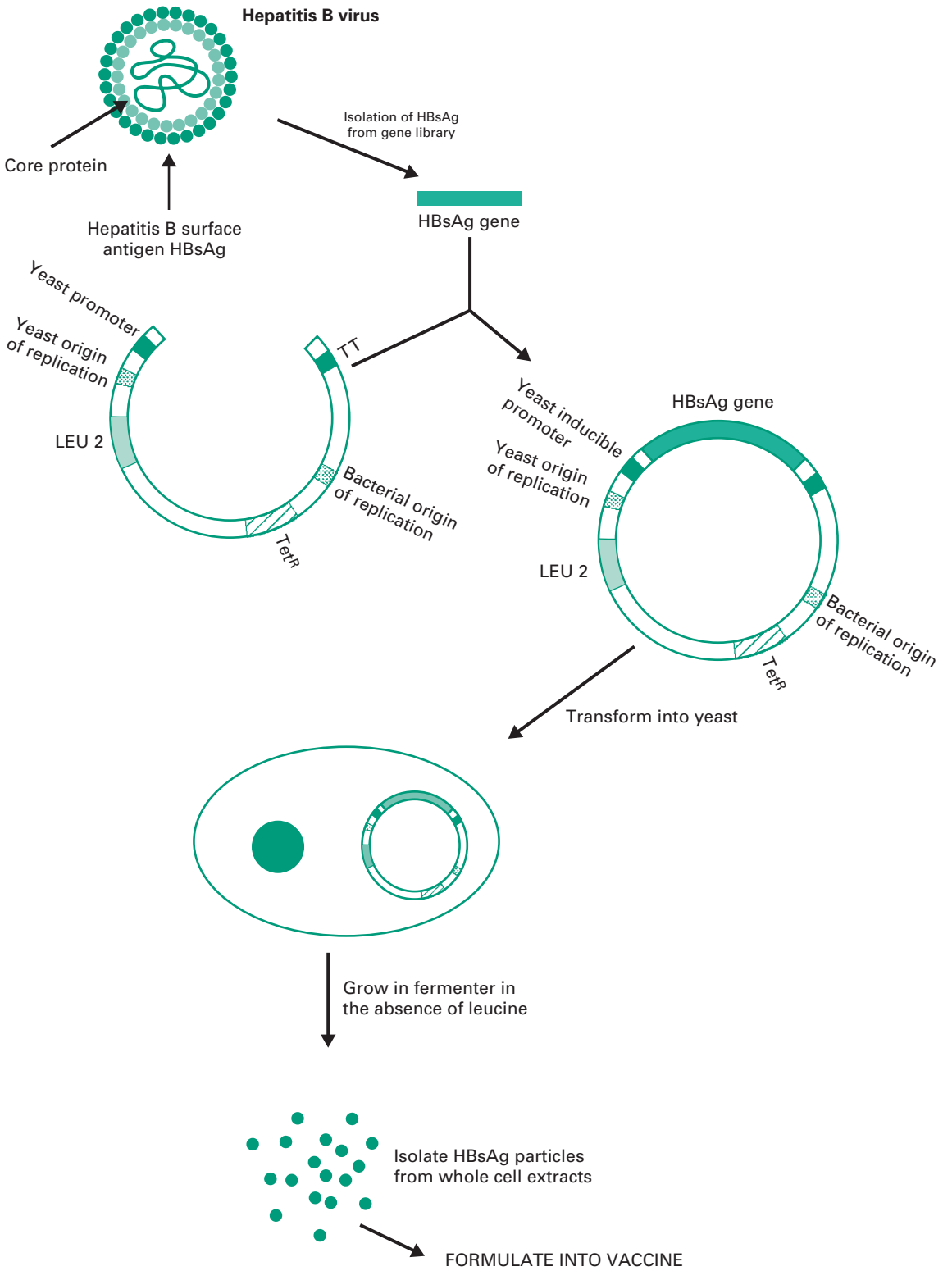

Hepatitis B virus
Core protein
Hepatitis B surface antigen HBsAg
Isolation of HBsAg from gene library
HBsAg gene
Yeast promoter
Yeast origin of replication
LEU 2
TT
Bacterial origin of replication
TetR
Yeast inducible promoter
Yeast origin of replication
HBsAg gene
LEU 2
Bacterial origin of replication
TetR
Transform into yeast
Grow in fermenter in the absence of leucine
Isolate HBsAg particles from whole cell extracts
FORMULATE INTO VACCINE

Figure 25.11 Production of hepatitis B subunit vaccine. The gene for the hepatitis B surface antigen (HBsAg) was isolated from a genomic library and cloned into a shuttle vector that promotes high expression levels of this gene in yeast cells. The presence of a LEU2 marker allows the selection of yeasts containing this plasmid by growing them in the absence of leucine. Recombinant yeast cells expressing the HBsAg are grown in large fermenters and the antigen is purified from whole cell extracts for further formulation into the hepatitis B subunit vaccine.

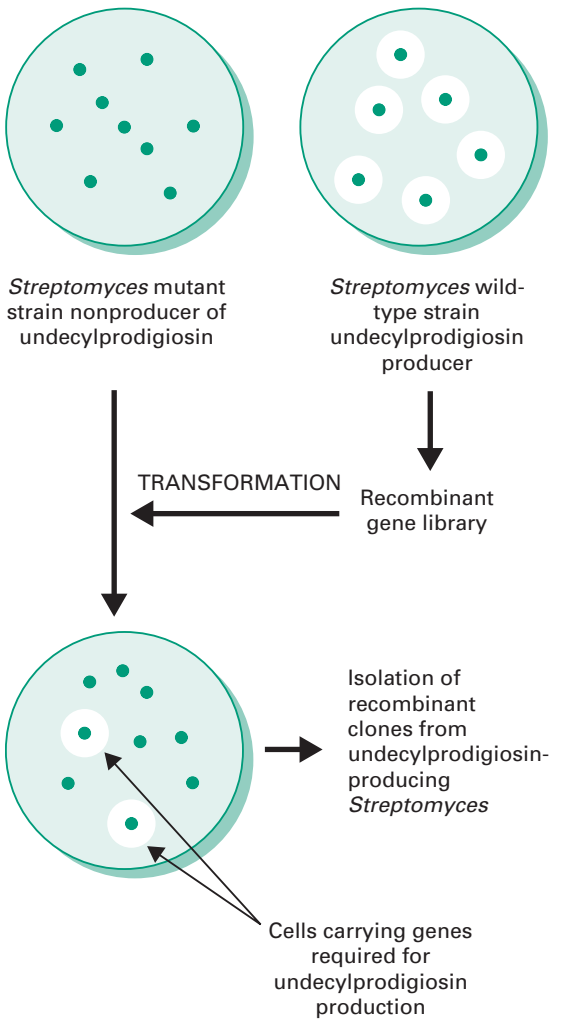


Figure 25.12 Isolation of genes responsible for antibiotic production. For the isolation of the genes required for undecylprodigiosin biosynthesis a *Streptomyces* strain, unable to make this antibiotic, was transformed with a genomic library from a *Streptomyces* undecylprodigiosin-producing strain. The resulting cells were plated onto agar seeded with a bacterium sensitive to undecylprodigiosin. The recombinant genes required for undecylprodigiosin biosynthesis could be isolated from colonies showing a halo of cell lysis around them resulting from the production of this antibiotic.

Table 25.6 Example of infectious diseases currently identified by PCR

Disease	Causative pathogen
Malaria	*Plasmodium falciparum*
Chagas disease	*Trypanosoma cruzi*
Respiratory failure	*Legionella pneumophila*
Food poisoning	*Salmonella enterica* serovar Typhi
Gastritis	*Campylobacter intestinalis*
AIDS	HIV
Gastroenteritis	Enterotoxigenic *Escherichia coli*

phosphatase results in the conversion of this substrate into a product which emits light and which can be detected after exposure to X-ray film or by using sensitive imaging cameras.

The procedure described above can also be scaled down, automated and redesigned to use thousands of sequence-specific probes at once in what are called *microarrays*. In this case non-labelled DNA probes are synthesized and minute droplets are spotted at high density on to glass slides where they will remain bound. The DNA from the biological sample is then labelled at the extremities with a fluorescent dye and after denaturation into single strands it is allowed to anneal with the probes bound on the slide. After washing, labelled DNA fragments which annealed to specific probes are visualized by illuminating the slide with light of the appropriate wavelength to cause the dyes to fluoresce. As microarrays can carry thousands of probes per square centimetre, this is performed by automated scanning devices and dedicated software which identifies the probes producing positive signals. This technique allows the screening at once of a very large number of pathogens or defined genetic markers.

4.1.2 PCR amplification using fluorescent primers

As in many other fields, PCR has brought a revolutionary change in nucleic acid-based diagnosis. In a clinical setting, PCR has many desirable features such as the sen-

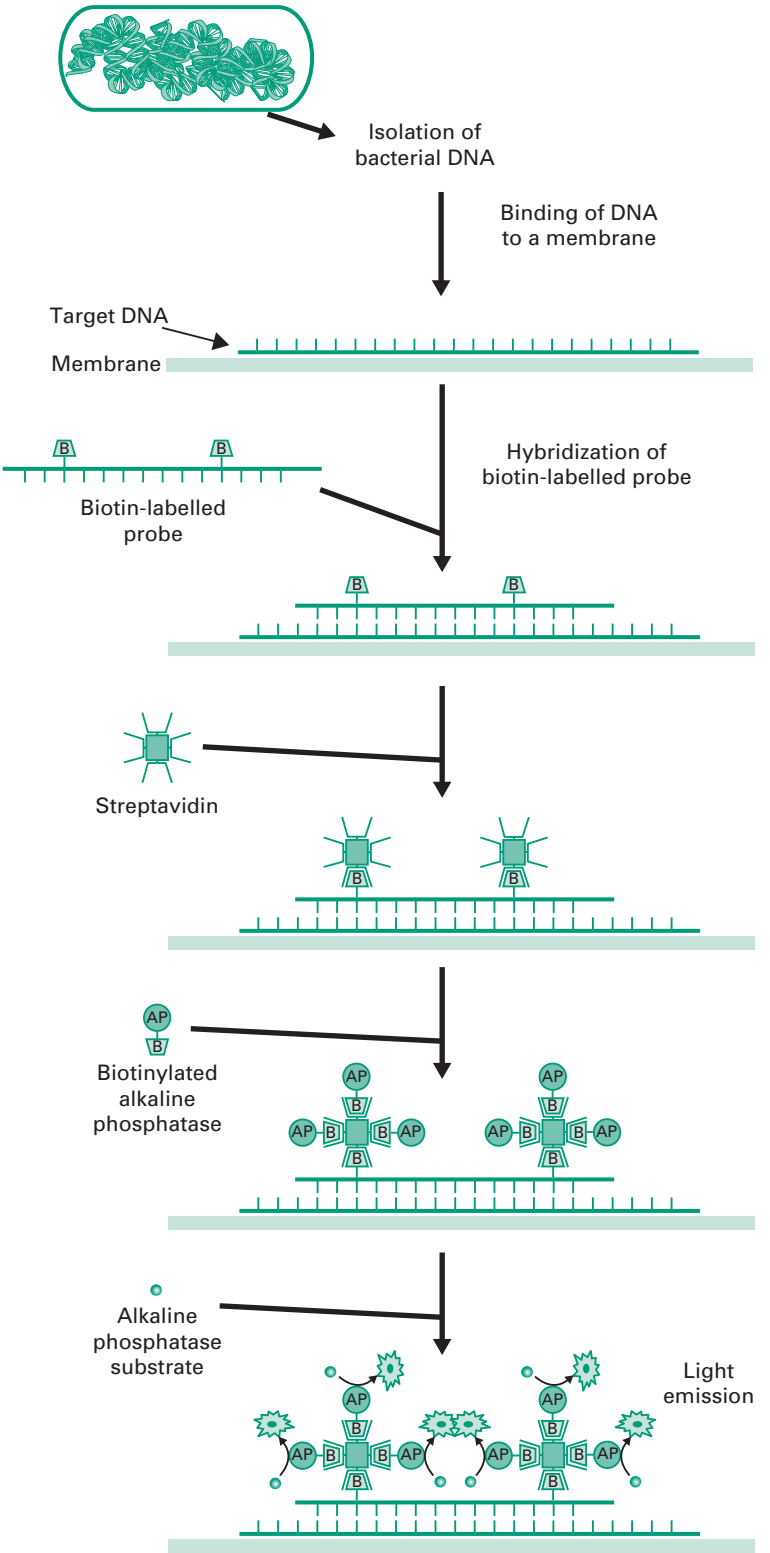


Figure 25.13 Diagnosis using DNA hybridization with biotin-labelled probes. The DNA from the pathogen to be identified is first purified and bound to a membrane. The membrane is then incubated with a diagnostic biotinylated probe. The biotin from the probe will be recognized by streptavidin which has several biotin recognition sites. Hence, subsequent incubation with biotin-labelled alkaline phosphatase results in the recognition of the bound streptavidin. As several molecules of biotin-labelled alkaline phosphatase will bind to a single streptavidin molecule, incubation of the membranes with chemiluminescence substrate for this enzyme will lead to an amplified reaction and the generation of light-emitting products.

sitivity to work with tiny amounts of DNA samples from blood or tissue to achieve a specific and significant amplification of target sequences. Furthermore, the rapidity of this process, as explained previously in this chapter, provides a significant advantage in the early diagnosis and treatment of infectious diseases.

A PCR fluorescence-based technique has been used successfully in the diagnosis of infectious diseases. In this case the PCR primers are labelled with a fluorescent dye that is bound to the 5′ end of each primer. Two main types of fluorescent dyes are normally used: one is fluorescein, which appears green under certain light wavelengths, and the other is rhodamine, which appears red. After PCR amplification of the target sequence with the fluorescent-labelled primers, the primers are removed by chromatographic separation, and the presence of the labelled PCR product is detected. The absence of labelled PCR product is interpreted as the absence of the target DNA sequence.

The procedure can also be applied to detect specific RNAs, for example to detect RNA viruses. In this case the extracted RNA has to be converted first to ds cDNA with reverse transcriptase before the PCR amplification. The procedure is then called reverse transcriptase PCR or RT-PCR.

4.2 Diagnosis of genetic disorders

The use of new diagnostic techniques has allowed individuals to discover whether they or their offspring are at risk of suffering from specific inherited diseases. DNA analysis using PCR has been used for the identification of carriers of hereditary disorders, for prenatal diagnosis of deleterious genetic conditions and for the early diagnosis of these disorders before the manifestations of any symptoms. Table 25.7 shows some examples of genetic human disorders that are currently identified by PCR.

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Table 25.7 Some of the inherited human diseases currently diagnosed by PCR

Haemophilia A and B	Gaucher's disease	Lesch–Nyhan syndrome
Cystic fibrosis	α_1-Antitrypsin deficiency	Maple syrup urine disease
Adenosine deaminase deficiency	β- and δ-Thalassaemia	Retinoblastoma
Fabry's disease	Von Willebrand's disease	Tay–Sachs disease
Familial hypercholesterolaemia	Sickle cell anaemia	Phenylketonuria

Part 6 Current trends and new directions

26 The wider contribution of microbiology to the pharmaceutical sciences

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1 Introduction

There has long been a tendency, particularly in the medical and pharmaceutical fields, to regard microbes as harmful entities to be destroyed. However, as will be described in this chapter, the exploitation of microorganisms and their products has assumed an increasingly prominent role in the diagnosis, treatment and prevention of human diseases. Non-medical uses are also of significance, e.g. the use of bacterial spores (*Bacillus thuringiensis*) and viruses (baculoviruses) to control insect

Hugo and Russell's Pharmaceutical Microbiology, Eighth Edition. Edited by Stephen P. Denyer, Norman Hodges, Sean P. Gorman, Brendan F. Gilmore.

pests, the fungus *Sclerotinia sclerotiorum* to kill some common weeds, and improved varieties of *Trichoderma harzianum* to protect crops against fungal infections, and these will also be explored.

1.1 Early treatment of human disease

The earliest uses of microorganisms to treat human disease can be traced to the belief that the formation of pus in some way drained off noxious humours responsible for systemic diseases. Although the spontaneous appearance of pus in their patients' wounds satisfied most physicians, deliberate contamination of wounds was also practiced. Bizarre concoctions of bacteria such as 'ointment of pigs' dung' and 'herb sclerata' were particularly favoured during the Middle Ages. Early central European and South American civilizations cultivated various fungi for application to wounds. In the 19th century, sophisticated concepts of microbial antagonism were developed following Pasteur's experiments demonstrating inhibition of anthrax bacteria by 'common bacteria' simultaneously introduced into the same culture medium. Patients suffering with diseases such as diphtheria, tuberculosis and syphilis were treated by deliberate infection with what were then thought to be harmless bacteria such as staphylococci, *Escherichia coli* and lactobacilli. Following their discovery in the early part of the last century, bacterial viruses (bacteriophages) were considered as potential antibacterial agents—an idea that soon fell into disuse but has recently been revived (Chapter 27).

1.2 Present-day exploitation

Some of the most important and widespread uses of microorganisms in the pharmaceutical sciences are in the production of antibiotics and vaccines and the use of microorganisms in the recombinant DNA industry. These are described in Chapters 11, 24 and 25. However, there are a variety of other medicinal agents derived from microorganisms including vitamins, amino acids, dextrans, iron-chelating agents and enzymes. Microorganisms as whole or subcellular fractions, in suspension or immobilized in an inert matrix are employed in a variety of assays. Microorganisms have also been used in the pharmaceutical industry to achieve specific modifications of complex drug molecules such as steroids, in situations where synthetic routes are difficult and expensive to carry out, and more recently microorganisms have been employed in their own right as platforms for the discovery of novel therapeutic peptides and proteins.

2 Pharmaceuticals produced by microorganisms

2.1 Dextrans

Dextrans are polysaccharides produced by lactic acid bacteria, in particular members of the genus *Leuconostoc* (e.g. *L. dextranicus* and *L. mesenteroides*), following growth on sucrose. These sugar polymers first came to the attention of industrial microbiologists in sugar refineries where large gummy masses of dextran clogged pipelines. Dextran is essentially a glucose polymer consisting of (1–6)-α-links of high but variable molecular weight (15 000–20 000 000; Figure 26.1). Growth of the dextran producing strain is carried out in large fermenters using media with a low nitrogen but high carbohydrate content. The average molecular weight of the dextrans produced will vary with the strain used. This is important, since the laboratory or clinical utility of the dextran is dependent on a defined molecular weight. Two main methods are employed for obtaining dextrans of a suitable molecular weight. The first involves acid hydrolysis of very high molecular weight polymers, while the second uses small preformed dextrans as templates for the polymerization process. These templates are added to the culture fluid to produce dextrans of shorter chain length. Once formed, dextrans of the required molecular weight are obtained by precipitation with organic solvents prior to formulation.

Dextrans are produced commercially for use as plasma substitutes (plasma expanders), which can be administered by intravenous injection to maintain or restore the blood volume, and for application to ulcerated wounds or to burns where they form a hydrophilic layer that absorbs fluid exudates.

A summary of the properties of the different types of dextrans available is presented in Table 26.1. Dextrans for clinical use as plasma expanders require a molecular weight between 40 000 and 300 000. Polymers below 40 000 molecular weight are excreted too rapidly from the kidneys, while those above 300 000 molecular weight are potentially dangerous because of retention in the body. In practice, infusions containing dextrans of average molecular weights of 40 000, 70 000 and 110 000 are commonly encountered.

Iron dextran injection, containing a complex of iron hydroxide with dextrans of average molecular weight between 5000 and 7000, is used for the treatment of iron-deficiency anaemia in situations where oral therapy is either ineffective or impractical. The sodium salt of sulphuric acid esters of dextran, i.e. dextran sodium sulphate, has

anticoagulant properties comparable with those of heparin and is formulated as an injection for intravenous use.

2.2 Vitamins, amino acids and organic acids

Several chemicals used in medicinal products are produced by fermentation.

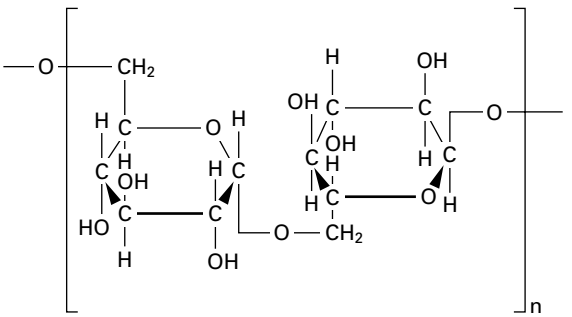


Figure 26.1 Structure of dextran showing (1–6)-α-linkage.

2.2.1 Vitamins

Vitamin B_2 (riboflavin), one of the B group of vitamins, is present in milk, liver, kidneys, cereals and vegetables and is also synthesised by intestinal flora in carbohydrate-rich diets. Vitamin B_2 deficiency, although rare, is characterized by symptoms that include an inflamed tongue, dermatitis and injury to the bone marrow. In genuine cases of malnutrition these symptoms will accompany those induced by other vitamin deficiencies. Riboflavin is produced commercially in significant yields by the moulds *Eremothecium ashbyii* and *Ashbya gossypii* and some bacteria including *B. subtilis* (Table 26.2).

Pernicious anaemia is a fatal disease first reported in 1880, but it was not until 1926 that it was discovered that eating raw liver effected a remission. The 'anti-pernicious' ingredient was subsequently isolated and called vitamin B_{12} or cyanocobalamin. Vitamin B_{12} was initially obtained from liver but during the 1960s it was determined that it could also be obtained as a by-product of microbial metabolism (Table 26.2). Hydroxycobalamin is the form

Table 26.1 Properties and uses of dextrans

Type of dextran[a]	Molecular weight (average)	Product	Sterilization method	Clinical uses
Dextran 40	40 000	10% w/v in 5% w/v glucose injection or 0.9% w/v sodium chloride injection	Autoclave	IV infusion: improves blood flow and tissue function in burns and conditions associated with local ischaemia
Dextran 70	70 000	6% w/v in 5% w/v glucose injection or 0.9% w/v sodium chloride injection	Autoclave	IV: used to produce an expansion of plasma volume in conditions associated with loss of plasma proteins
Dextran 110	110 000	6% w/v in 5% w/v glucose injection or 0.9% w/v sodium chloride injection	Autoclave	IV: as for dextran 70
Iron dextran	5000–7500 (complex with ferric chloride)	Colloidal solution in 0.9% w/v sodium chloride injection	Autoclave	Deep IM or IV (slow infusion): iron-deficiency anaemia (oral therapy ineffective or impractical)
Dextran sodium sulphate		Powder for preparing solution	Autoclave	Anticoagulant (intravenous use of solution)
Chemically cross- linked dextrans		—	—	Water-insoluble: chromatographic techniques (fractionation and purification)

IV, intravenous; IM, intramuscular.

The current *British Pharmacopoeia* and *British National Formulary* should be consulted for further information, including toxic manifestations.

[a]In the USA, dextran injections with average molecular weights of about 75 000 are also available.

Table 26.2 Examples of vitamins, amino acids, antibiotics and organic acids produced by microorganisms

Pharmaceutical	Producer organism	Use
Riboflavin (vitamin B_2)	*Eremothecium ashbyii* *Ashbya gossypii*	Treatment of vitamin B_2 deficiency disease
Cyanocobalamin (vitamin B_{12})	*Propionibacterium freudenreichii* *Propionibacterium shermanii* *Pseudomonas denitrificans*	Treatment of pernicious anaemia
Amino acids, e.g.,		Supplementation of feeds/food; intravenous infusion fluid constituents
Glutamate	*Corynebacterium glutamicum*	
Lysine	*Brevibacterium flavum*	
Antibiotics,[a] e.g.		Antibacterial drugs
Benzylpenicillin	*Penicillium notatum, P. chrysogenum*	
Gentamicin	*Micromonospora purpurea*	
Nystatin	*Streptomyces noursei*	
Organic acids, e.g.		
Citric acid	*Aspergillus niger*	Effervescent products; sodium citrate used as an anticoagulant; potassium citrate used to treat cystitis
Lactic acid	*Lactobacillus delbrueckii* *Rhizopus oryzae*	Calcium lactate is a convenient source of Ca^{2+} for oral administration; constituent of intraperitoneal dialysis solutions
Gluconic acid	*Gluconobacter suboxydans* *Aspergillus niger*	Calcium gluconate is a source of Ca2+ for oral administration; gluconates are used to render bases more soluble, e.g. chlorhexidine gluconate

[a]For further information, see Chapter 11.

of choice for therapeutic use and can be derived either by chemical transformation of cyanocobalamin or directly as a fermentation product.

Biotin, formerly known as vitamin H, is now regarded as another member of the vitamin B family and is found in similar food types. Biotin acts as an essential cofactor in chemical reactions that maintain normal metabolic function. It is also an essential growth factor for some bacteria. Its chemical structure was established in the early 1940s and a practical, highly stereospecific, chemical synthesis enabled D-biotin, identical to that found in yeasts and other cells, to be produced.

2.2.2 Amino acids

Amino acids find applications as ingredients of infusion solutions for parenteral nutrition and individually for the treatment of specific conditions. They are obtained either by fermentation processes similar to those used for antibiotics or in cell-free extracts employing enzymes isolated from bacteria (Table 26.2). Details of the many and varied processes reported in the literature will be found in the appropriate references in the 'Further reading' section at the end of the chapter.

2.2.3 Organic acids

Examples of organic acids (citric, lactic, gluconic) produced by microorganisms, together with pharmaceutical and medical uses, are given in Table 26.2. Citric and lactic acids also have widespread uses in the food and drink and plastics industries. Gluconic acid is also used as a metal-chelating agent in, for example, detergent products.

2.3 Iron-chelating agents

The growth of many microorganisms in iron-deficient growth media results in the secretion of low molecular weight iron-chelating agents called siderophores, which are usually phenolate or hydroxamate compounds. The therapeutic potential of these compounds has generated considerable interest. Uncomplicated iron deficiency can be treated with oral preparations of iron(II) (ferrous) sulphate but such treatment is not without hazard and iron salts remain a common cause of poisoning in children. The accidental consumption of around 3 g of ferrous sulphate by a small child leads to acidosis, coma and heart failure which, if untreated, are fatal. Desferrioxamine B (Figure 26.2), the deferrated form of a siderophore produced by *Streptomyces pilosus*, is a highly effective antidote for the treatment of acute iron poisoning. Desferrioxamine owes its effectiveness both to its high affinity for ferric iron (its binding constant is in excess of 10^{30}) and because the iron–desferrioxamine complex is highly water-soluble and is readily excreted through the kidneys. In haemolytic anaemias such as thalassaemia, desferrioxamine is used together with blood transfusions to maintain normal blood levels of free iron and haemoglobin. Desferrioxamine is prepared as a sterile powder for use as an injection, but it is also administered orally in acute iron poisoning to remove unabsorbed iron from the gut.

Patients with iron overload disorders treated with desferrioxamine may, however, have increased susceptibility to infections. The important role played by iron availability during infections in vertebrate hosts has only been recognized relatively recently. The ability of the host to withhold growth-essential iron from microbial and, indeed, neoplastic invaders while retaining its own access to this metal has led to suggestions that microbial iron chelators or their semisynthetic derivatives may be of use in antimicrobial and anticancer chemotherapy. Preliminary work has shown some encouraging results. The bacterial siderophores parabactin and compound II secreted by *Paracoccus denitrificans* have been shown to inhibit the growth of leukaemia cells in culture and in experimental animals. They also appear capable of inhibiting the replication of RNA viruses. Siderophores such as desferrioxamine may therefore find increasing applications not only in the treatment of iron poisoning and iron-overloaded disease states but also as chemotherapeutic agents.

2.4 Enzymes

Several enzymes have important therapeutic, medical or pharmaceutical uses (Table 26.3). In this section,

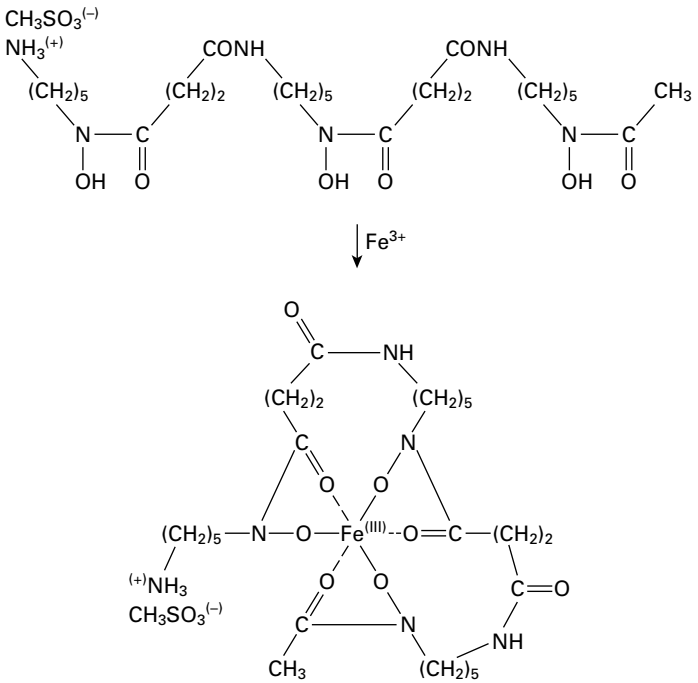


Figure 26.2 Structure of desferrioxamine B and its corresponding iron chelate.

Table 26.3 Clinical uses and other applications of enzymes

Enzyme	Source	Clinical and/or other use	Section(s)
Streptokinase	Certain streptococcal strains	Liquefying blood clots	2.4.1
Streptodornase	Certain streptococcal strains	Liquefying pus	2.4.1
L-Asparaginase	*E. coli or Erwinia* spp.	Cancer chemotherapy	2.4.2
Neuraminidase	*Vibrio cholerae*	Possible: increase immunogenicity of tumour cells	2.4.3
β-Lactamases	*Bacillus cereus* (or other bacteria, as appropriate)	Sterility testing, treatment of penicillin-induced allergic reaction	2.4.4, 5.5
Other antibiotic- modifying or-inactivating enzymes	Some AGAC-resistant bacteria	Sterility testing, assay	5.5
	Some CMP-resistant bacteria	Sterility testing	5.1.2, 5.5
Glucose oxidase	*Aspergillus niger*	Blood glucose analysis	5.6

AGAC, aminoglycoside-aminocyclitol antibiotics (see Chapter 11); CMP, chloramphenicol.

those enzymes used therapeutically will be described. Applications of microbially derived enzymes for antibiotic inactivation in sterility testing and diagnostic assays are discussed in section 5.

2.4.1 Streptokinase and streptodornase

Mammalian blood will clot spontaneously within minutes if allowed to stand, but if left to stand longer the clot begins to dissolve as a result of the action of a proteolytic enzyme called plasmin. Plasmin is normally present as its inactive precursor, plasminogen. Certain strains of streptococci were found to produce a substance that is capable of activating plasminogen (Figure 26.3), a phenomenon that suggested a potential use in liquefying clots, i.e. fibrinolysis. This substance, called streptokinase, was isolated and determined to be an enzyme.

Streptokinase is administered by intravenous or intra-arterial infusion in the treatment of thromboembolic disorders, e.g. pulmonary embolism, deep vein thrombosis and arterial occlusions. It is also used in emergency medicine for acute myocardial infarction.

A second enzyme, streptodornase, present in streptococcal culture filtrates, was observed to liquefy pus. Streptodornase is a deoxyribonuclease that breaks down deoxyribonucleoprotein and DNA, both constituents of pus, resulting in a reduction in pus viscosity. Streptokinase and streptodornase together have been used to facilitate drainage by liquefying blood clots and/or pus in the chest cavity. The combination can also be applied topically to wounds that have excessive suppuration.

Streptokinase and streptodornase are isolated following growth of non-pathogenic streptococcal producer strains in media containing excess glucose. They are obtained as a crude mixture from the culture filtrate and can be prepared relatively free of each other. They are commercially available as either streptokinase injection or as a combination of streptokinase and streptodornase.

2.4.2 L-Asparaginase

L-Asparaginase, an enzyme derived from *E. coli* or *Erwinia chrysanthemi*, converts L-asparagine to aspartic acid and ammonia. In contrast to normal tissue, some tumours have an essential requirement for L-asparagine and L-asparaginase has therefore been investigated as a selective cancer chemotherapeutic. Although L-asparaginase showed early promise in a variety of experimentally induced tumours, it has primarily found utility in humans for the treatment of acute lymphoblastic leukaemia and occasionally for myeloid leukaemia.

2.4.3 Neuraminidase

Many tumours escape immune surveillance through mechanisms including the masking of cell surface antigens by, for example, *N*-acetylneuraminic (sialic) acid

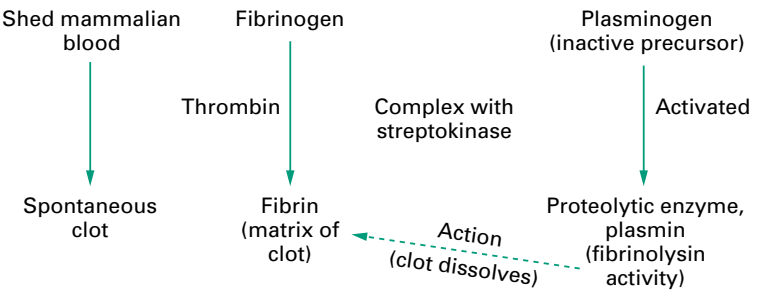


Figure 26.3 Action of streptokinase.

residues. Neuraminidase, derived from *Vibrio cholerae,* has been used experimentally to increase the immunogenicity of tumour cells by stripping sialic acid residues from the outer surface of certain tumour cells resulting in presentation of tumour-specific antigens to the host immune system. In laboratory animals, administration of neuraminidase-treated tumour cells was found to be effective against a variety of mouse leukaemias. Preliminary investigations in acute myelocytic leukaemia patients have suggested that treatment of tumour cells with neuraminidase in combination with conventional chemotherapy may increase remission rates.

2.4.4 β-Lactamases

β-Lactamase enzymes, whilst presenting a considerable therapeutic challenge due to their ability to confer bacterial resistance by inactivating penicillins and cephalosporins (Chapter 13), are nevertheless useful in the sterility testing of certain antibiotics (see section 5.5) and, prior to culture, in inactivating various β-lactams in blood or urine samples in patients undergoing therapy with these drugs. One other important therapeutic application is in the rescue of patients presenting symptoms of a severe allergic reaction following administration of a β-lactamase-sensitive penicillin. In such cases, a highly purified penicillinase obtained from *B. cereus* has been administered either intramuscularly or intravenously and in combination with other supportive measures such as adrenaline and antihistamines.

3 Applications of microorganisms in the partial synthesis of pharmaceuticals

Microorganisms and microbially derived enzymes continue to play a significant role in the production of novel antibiotics. The potential of microorganisms as chemical catalysts, however, was a later development and first realized in the synthesis of industrially important steroids. These reactions assumed increasing importance following the discovery that certain steroids could be formulated as potent therapeutics, e.g. hydrocortisone has anti-inflammatory activity, and derivatives of the steroidal sex hormones are useful as oral contraceptive agents. More recently, chiral inversion of non-steroidal anti-inflammatory drugs (NSAIDs) has also been demonstrated.

3.1 Production of antibiotics

In the antibiotics industry, the hydrolysis of benzylpenicillin to give 6-aminopenicillanic acid by the enzyme penicillin acylase is an important stage in the synthesis of many clinically useful penicillins (Chapters 11). The combination of genetic engineering techniques to produce hybrid microorganisms with significantly higher acylase levels, together with their entrapment in gel matrices, which appears to improve the stability of the hybrids, has resulted in considerable increases in 6-aminopenicillanic acid yields.

A second example is provided by the production by fermentation of cephalosporin C, which is used solely for the subsequent preparation of semisynthetic cephalosporins (Chapter 11).

Furthermore, antibiotics produced by fermentation of various moulds and particularly *Streptomyces* spp., can be utilized by medicinal chemists as starting blocks in the production of what might be more effective antimicrobial compounds.

3.2 Steroid biotransformations

Previously, steroid hormones could only be obtained in small quantities directly from mammals and therefore attempts were made to synthesize them from plant sterols, which can be obtained economically in large quantities. However, adrenocortical steroids are characterized by the presence of an oxygen molecule at position 11 in the

steroid nucleus and although it is relatively easy to hydroxylate a steroidal compound it is extremely difficult to achieve site-specific hydroxylation, such that many of the routes used for synthesizing the desired steroid are lengthy, complex and consequently expensive. This problem was overcome when it was realized that many microorganisms are capable of performing limited oxidations with both stereo- and regio-specificity. By simply adding a steroid to growing cultures of the appropriate microorganism, specific site-directed chemical changes can be introduced into the molecule. In 1952, the first commercially employed process involving the conversion of progesterone to 11α-hydroxyprogesterone by the fungus *Rhizopus nigricans* was introduced (Figure 26.4). This reaction is an important stage in the manufacture of cortisone and hydrocortisone from more readily available steroids. Table 26.4 gives several other examples of microbially directed oxidations that have been or are employed in the manufacture of steroidal drugs.

More recently, microorganisms utilized for biotransformation reactions have been immobilized by entrapment in a polymer gel matrix to avoid the often costly and time-consuming enzyme extraction steps that can result in enzyme inactivation. Immobilization also serves to increase the stability of membrane-associated enzymes that are unstable in the solubilized state, as well as permitting the conversion of water-insoluble compounds like steroids in two-phase water–organic solvent systems.

3.3 Chiral inversion

Several clinically relevant drugs including salbutamol (a β-adrenoceptor agonist), propanolol (a β-adrenoceptor antagonist) and the 2-arylpropionic acids (NSAIDs) are administered in their racemic form but undergo *in vivo* chiral inversion through metabolic transformations by microorganisms that mimic phase I metabolic processes, i.e. functionalization reactions. For example, the activity of NSAIDs (e.g. ibuprofen), resides almost exclusively in the *S*(+) isomers. However, unidirectional chiral inversion from *R*(–) to *S*(+) (Figure 26.5) occurs *in vivo* over a 3-hour period. The *S*(+) form is a more effective inhibitor of prostaglandin synthesis, and enzymes from some fungal species (e.g. *Verticillium lecanii*) convert a racemic mixture into the *S*(+) isomer *in vitro*. Another example

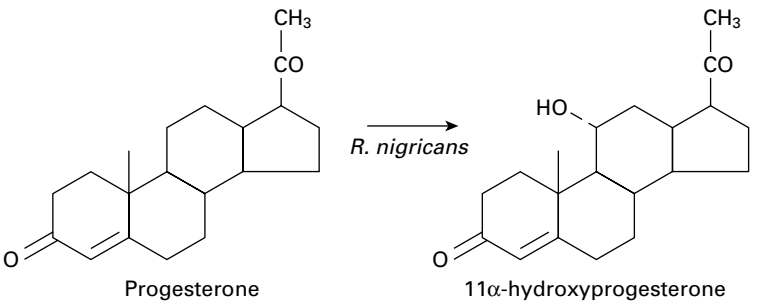


Figure 26.4 Conversion of progesterone to 11α-hydroxyprogesterone by *Rhizopus nigricans*.

Table 26.4 Examples of biological transformations of steroids

Starting material	Product	Type of reaction
Progesterone	11a-Hydroxyprogesterone	Hydroxylation
Compound S[a]	Hydrocortisone	Hydroxylation
11α-Hydroxyprogesterone	D-11α-Hydroxyprogesterone	Dehydrogenation
Hydrocortisone	Prednisolone	Dehydrogenation
Cortisone	Prednisone	Dehydrogenation

[a] Derived from diosgenin by chemical transformation.

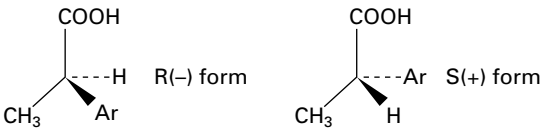


Figure 26.5 Alternative isomeric forms of profens.

is the biotransformation of ±propranolol to *S*(+) propranolol by *R. arrhizus* and *Geotrichum candidum* with around 83% efficiency.

4 Applications of microorganisms in the discovery of pharmaceuticals

Microbial display platforms expressing recombinant polypeptides (peptides, antibodies, enzymes) on their surface are emerging as invaluable tools for the investigation of protein–protein interactions and can serve as biological combinatorial libraries for the discovery of new therapeutics. To date three microbial display platforms have been described: phage, bacterial, and yeast display. All three technologies share the common principle of a direct link between genotype and phenotype affording the identification of the displayed polypeptide by gene sequencing. Of the three technologies described, it is probably phage display that has witnessed the most widespread application.

4.1 Phage display

The filamentous bacteriophage readily accepts relatively large insertions of additional genetic material into its genome, which allows for the display of polypeptides as fusions with bacteriophage coat proteins. Phage libraries containing a repertoire of many billions of viral particles that each display a unique polypeptide sequence can be subjected to an affinity selection process against a target of interest. Those phage clones that display a polypeptide that strongly interacts with the target can be recovered and amplified for further rounds of selection before the direct link between genotype and phenotype is exploited to identify the polypeptide displayed.

Although to date no phage-derived product has reached market, a number of clinical trials are currently under way using polypeptides derived from phage display: DX-88 (Ecallantide, Dyax Corp) is a highly specific and potent inhibitor of kallikrein (K_i ~20–40 pM). Kallikrein is a key molecule in the regulation of inflammatory and blood clotting processes and plays a role in a number of autoimmune and inflammatory conditions. DX-88 has successfully completed phase III trials in the treatment of hereditary angiodema and phase I/II trials for the prevention of blood loss in on-pump coronary artery bypass graft surgery. Compstatin (Potentia Pharmaceutics), a cyclic 13-mer peptide originally identified from phage display, is a selective and potent inhibitor (K_d ~0.13 μM) of C3 protein in the complement cascade and will shortly enter clinical trials for the treatment of age-related macular degeneration.

5 Use of microorganisms and their products in assays

Microorganisms have found widespread uses in bioassays for:

- determining the concentration of compounds (e.g. amino acids, vitamins and some antibiotics) in complex chemical mixtures or in body fluids
- diagnosing diseases
- testing chemicals for potential mutagenicity and carcinogenicity
- monitoring processes involving the use of immobilized enzymes
- sterility testing of antibiotics.

5.1 Antibiotic bioassays

Although antibiotics may be assayed by a variety of methods, the following section will only take into consideration microbiological and radioenzymatic assays.

5.1.1 Microbiological assays

In microbiological assays the response of a growing population of microorganisms to the antimicrobial agent under investigation is measured. The usual methods involve agar diffusion assays in which the drug diffuses into agar seeded with a susceptible microbial population producing a zone of growth inhibition.

In the commonest form of microbiological bioassay used today, samples to be assayed are applied in some form of reservoir (porcelain cup, paper disc or well) to a thin layer of agar seeded with indicator organism. The drug diffuses into the medium and after incubation a zone of growth inhibition forms, in this case as a circle around the reservoir. All other factors being constant, the diameter of the zone of inhibition is essentially related to the concentration of antibiotic in the reservoir.

During incubation the antibiotic diffuses from the reservoir and that part of the microbial population distant

to the influence of the antibiotic increases by cell division. The edge of the area of microbial growth is formed when the minimum concentration of antibiotic that will inhibit the growth of the organism on the plate (critical concentration) reaches, for the first time, a population density too great for it to inhibit. The position of the zone edge is thus determined by the initial population density, the growth rate of the organism and the rate of diffusion of the antibiotic.

In situations where the likely concentration range of the tests will lie within a relatively narrow range (e.g. in determining potency of pharmaceutical preparations) and maximal precision is sought, then a Latin square design with tests and calibrators at two or three levels of concentration may be used. For example, an 8×8 Latin square can be used to assay three samples and one calibrator, or two samples and two calibrators at two concentrations each (over a twofold or fourfold range), with a coefficient of variation of around 3%. Using this technique, parallel dose–response lines should be obtained for the calibrators and the tests at the two dilutions (Figure 26.6). Using such a method, potency can be computed or determined from carefully prepared nomograms.

Conventional plate assays require several hours' incubation and consequently the possibility of using rapid microbiological assay methods has been studied. Two such methods are:

- *Urease assay.* When *Proteus mirabilis* grows in a urea-containing medium it hydrolyses the urea to ammonia and consequently raises the pH of the medium. This production of urease is inhibited by aminoglycoside antibiotics (inhibitors of protein synthesis; Chapter 11). In practice, it is difficult to obtain reliable results by this method.
- *Luciferase assay.* In this technique, firefly luciferase (or similar enzyme) is used to measure small amounts of ATP in a bacterial culture, ATP levels being reduced by the inhibitory action of aminoglycoside antibiotics.

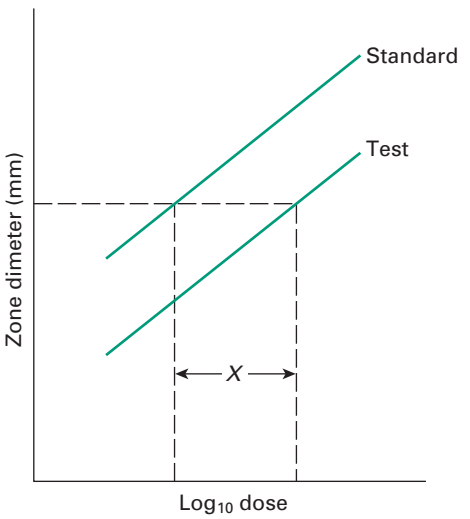


Figure 26.6 Graphical representation of a 2×2 assay response. *X* is the horizontal distance between the two lines. The antilog of *X* gives the relative potency of the standard and test.

5.1.2 Radioenzymatic (transferase) assays

Radioenzymatic assays depend on the fact that bacterial resistance to aminoglycosides, such as gentamicin, tobramycin, amikacin, netilmicin, streptomycin, spectinomycin, and chloramphenicol is frequently associated with the presence of specific enzymes (often coded for by transmissible plasmids), which either acetylate, adenylylate or phosphorylate the antibiotics, thereby rendering them inactive (Chapter 13). Aminoglycosides may be susceptible to attack by aminoglycoside acetyltransferases (AAC), aminoglycoside adenylyltransferases (AAD) or aminoglycoside phosphotransferases (APH). Chloramphenicol is attacked by chloramphenicol acetyltransferases (CAT). Acetyltransferases attack susceptible amino groups and require acetyl coenzyme A, while AAD or APH enzymes attack susceptible hydroxyl groups and require ATP (or another nucleotide triphosphate).

Several AAC and AAD enzymes have been used for assays. The enzyme and the appropriate radio-labelled cofactor ([1-^{14}C] acetyl coenzyme A, or [2-^{3}H] ATP) are used to radiolabel the drug being assayed. The radiolabelled drug is separated from the reaction mixture after the reaction has been allowed to go to completion; the amount of radioactivity extracted is directly proportional to the amount of drug present. Aminoglycosides are usually separated by binding them to phosphocellulose paper, whereas chloramphenicol is usually extracted using an organic solvent.

These types of assay are rapid, taking approximately 2 hours, show good precision and are much more specific than microbiological assays.

5.2 Vitamin and amino acid bioassays

The principle of microbiological bioassays for growth factors such as vitamins and amino acids is quite simple.

Unlike antibiotic assays (see section 5.1) that are based on studies of growth inhibition, these assays are based on growth exhibition. All that is required is a culture medium that is nutritionally adequate for the test microorganism in all essential growth factors except the one being assayed. If a range of limiting concentrations of the test substance is added, the growth of the test microorganism will be proportional to the amount added. A calibration curve of concentration of substance being assayed against some parameter of microbial growth, e.g. cell dry weight, optical density or acid production, can be plotted. One example of this is the assay for pyridoxine (vitamin B_6), which can be assayed using a pyridoxine-requiring mutant of the mould *Neurospora*. Using elegant study designs, it is possible to assay a variety of different growth factors with a single test organism simply by preparing a basal medium with different growth-limiting nutrients. Table 26.5 summarizes some of the vitamin and amino acid bioassays currently available. In practice however, high performance liquid chromatography (HPLC) has replaced bioassays as the method of choice for most amino acids and several B group vitamins.

5.3 Phenylketonuria testing

Phenylketonuria (PKU) is an inborn error of metabolism in which the body is unable to convert surplus phenylalanine (PA) to tyrosine for use in the biosynthesis of, for example, thyroxine, adrenaline and noradrenaline. This results from a deficiency in the liver enzyme phenylalanine 4-mono-oxygenase (phenylalanine hydroxylase).

Table 26.5 Some examples of microorganisms used as bioassays for vitamins

Assay microorganism	Vitamin
Lactobacillus casei	Biotin
L. arabinosus	Calcium pantothenate
L. leichmannii	Cyanocobalamin
L. casei	Folic acid
Saccharomyces uvarum	Inositol
L. arabinosus	Nicotinic acid
Acetobacter suboxydans	Pantothenol
L. casei	Pyridoxal
Neurospora crassa or S. carlsbergiensis	Pyridoxine
L. casei	Riboflavine
L. viridans	Thiamine

A secondary metabolic pathway comes into play in which there is a transamination reaction between PA and α-ketoglutaric acid to produce phenylpyruvic acid (PPVA), a ketone and glutamic acid. Overall, PKU may be defined as a genetic defect in PA metabolism such that there are elevated levels of both PA and PPVA in blood and excessive excretion of PPVA (Figure 26.7).

Control of PKU can be achieved simply by resorting to a low PA-containing diet. However, failure to diagnose PKU will result in mental deficiency, and thus early diagnosis is essential. In 1968, the UK Medical Research Council Working Party on PKU recommended the adoption of the Guthrie test as a convenient method for screening newborn infants. This assay employs *Bacillus subtilis* as the test organism. In minimal culture medium, growth of this bacterium is inhibited by β-2-thienylalanine (Figure 26.8a) and is competitively reversed in the presence of PA (Figure 26.8b) or PPVA. The use of filter-paper discs impregnated with blood or urine permits the detection of elevated levels of PA and PPVA. The test can be quantified by the measurement of the diameter of the growth zone around the filter-paper disc and comparing it with a calibration curve constructed from known concentrations of PA or PPVA (Figure 26.8c). If positive, the Guthrie test provides presumptive evidence for the presence of PKU. This is then confirmed by other chemical means. The test is still in widespread use but is gradually being phased out in favour of modern genetic testing techniques.

5.4 Carcinogen and mutagen testing

A carcinogen is a substance that causes living tissues to become carcinomatous, i.e. to produce a malignant tumour. A mutagen is a chemical (or physical) agent that induces mutation in a human (or other) cell.

Mutagenicity tests are used to screen a wide variety of chemicals for their ability to cause a mutation in the DNA of a cell. Such mutations can occur at either the gene level (a *point mutation*) at individual chromosomes, or at the level of a chromosome set, i.e. a change in the number of chromosomes (*aneuploidy*). Some compounds are only mutagenic or carcinogenic after metabolism (often in the liver). This aspect must, therefore, be considered in designing a suitable test method for such agents (see section 5.4.2).

5.4.1 Mutations at the gene level

Forward mutation refers to mutation of the natural ('wild-type') organism to a more stringent organism. By contrast, reverse (backward) mutation is the return of a

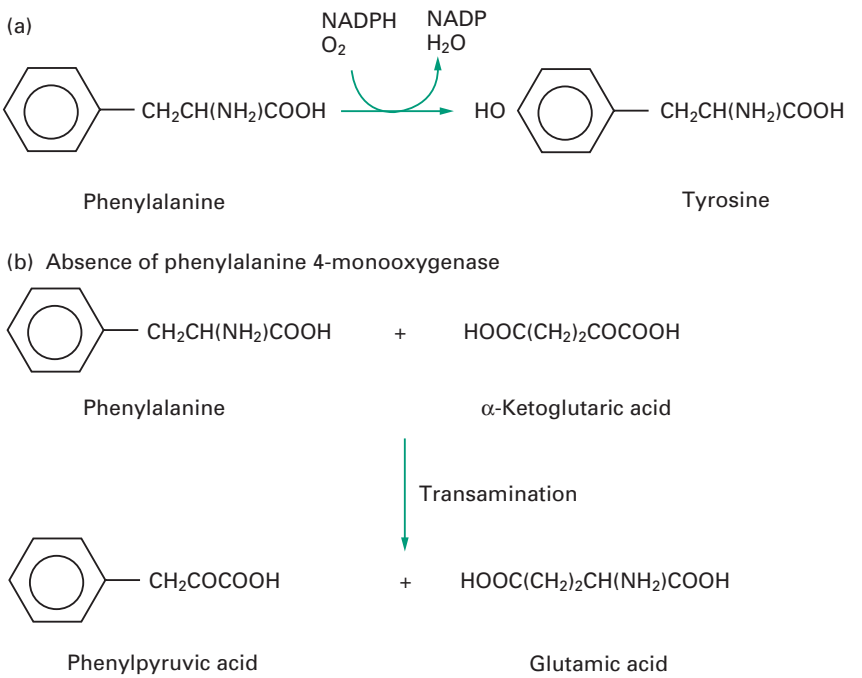


Figure 26.7 (a) Normal metabolism, in which phenylalanine is converted by phenylalanine 4-monooxygenase to tyrosine. (b) Phenylketonuria, in which there is a transamination reaction between phenylalanine and α-ketoglutaric acid. Phenylalanine 4-monooxygenase is absent in about 1 in every 10 000 human beings because of a recessive mutant gene.

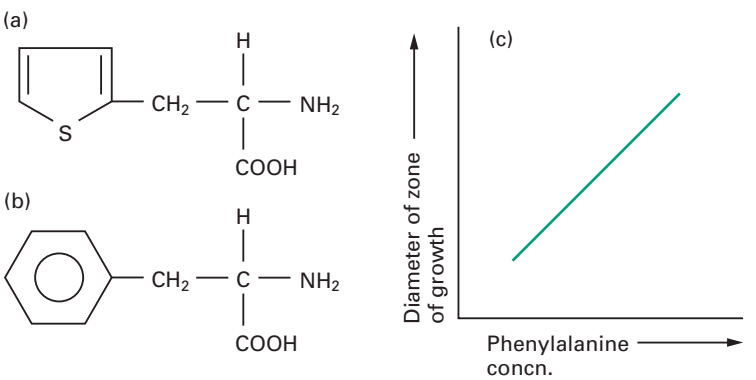


Figure 26.8 (a) β-Thienylalanine, (b) phenylalanine, (c) standard curve in Guthrie test.

mutant strain to the wild-type form, i.e. it is a heritable change in a previously mutated gene that restores the original function of that gene.

There are two types of reverse mutation:

- *Frame-shift.* In these mutants, the gene is altered by the addition or deletion of one or more bases so that the triplex reading frame for RNA is modified;
- *Base-pair.* In these mutants, a single base is altered so that the triplex reading frame is again modified.

These principles of reverse mutation are utilized in one important method, the Ames test (section 5.4.2), which is used to detect compounds that act as mutagens or carcinogens (most carcinogens are mutagens).

5.4.2 The Ames test

The Ames test is used to screen a wide variety of chemicals for potential carcinogenicity or conversely for their potential as cancer chemotherapeutic agents. The

test enables a large number of compounds to be screened rapidly by examining their ability to induce mutagenesis in several specially constructed bacterial mutants derived from *Salmonella enterica* serovar Typhi. The test strains contain mutations in the histidine operon such that they cannot synthesize the amino acid histidine. Two additional mutations increase further the sensitivity of the system. The first is a defect in their lipopolysaccharide structure (Chapter 3) such that they are in fact deep rough mutants possessing only 2-keto-3-deoxyoctonate (KDO) linked to lipid A. This mutation increases the permeability of the mutants to large hydrophobic molecules. The second mutation concerns a DNA excision repair system, which prevents the organism repairing its damaged DNA following exposure to a mutagen.

The assay method involves treatment of a large population of these mutant tester strains with the test compound. Histidine-requiring mutants are used to detect mutagens capable of causing base-pair substitutions (in some strains) or frame-shift mutations (other strains). This can be carried out by incorporating both the test strain and test compound in molten agar (at 45 °C), which is then poured on to a minimal glucose agar plate. Alternatively, the suspected mutagens can be applied to the surface of the top agar as a liquid or as a few crystals. The medium used for the top agar contains a limited concentration of histidine, which permits the bacteria on the plate to undergo several divisions, since for many mutagens some growth is a necessary prerequisite for mutagenesis to occur. After incubation for 2 days at 37 °C the number of 'revertant' colonies can be counted and compared with control plates from which the test compound has been omitted. Each revertant colony is assumed to be derived from a cell that has mutated back to the wild-type and thus can now synthesize its own histidine: see Figure 26.9 for a summary.

A further refinement to the Ames test permits screening of agents that require metabolic activation before their mutagenicity or carcinogenicity is apparent. This is achieved by incorporating into the top agar layer, along with the bacteria, homogenates of liver (commonly rat or human) whose activating enzyme systems have been induced by exposure to polychlorinated biphenyl mixtures. This test is sometimes referred to as the *Salmonella*/microsome assay because the fraction of liver homogenate used, called the S9 fraction, contains predominantly liver microsomes.

It is important to realize that this test is flexible and is still undergoing modification and development. Almost all the known human carcinogens have been tested and shown to be positive. These include agents such as β-naphthylamine, cigarette smoke condensates, aflatoxin B and vinyl chloride, as well as drugs used in cancer treatment such as adriamycin, daunomycin and mitomycin C. Although the test is not perfect for the prediction of mammalian carcinogenicity or mutagenicity and for making definitive conclusions about potential toxicity or lack of toxicity in humans, it nevertheless provides useful screening information rapidly and cheaply. The Ames test remains an important part of a battery of tests, the others of which are non-microbial in nature, for detecting mutagenicity or carcinogenicity.

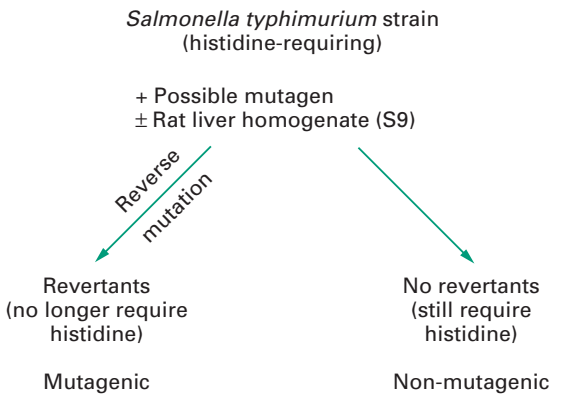


Figure 26.9 Summary of the Ames test.

5.5 Use of microbial enzymes in sterility testing

Sterile pharmaceutical preparations must be tested for the presence of fungal and bacterial contamination before use (see Chapter 21). Of course if the preparation contains an antibiotic it must be removed or inactivated and this is generally achieved by membrane filtration. However, the technique has certain disadvantages including accidental contamination and retention of the antibiotic on the filter followed by subsequent liberation into the nutrient medium.

Enzymatic inactivation of the antibiotic (see also Chapter 13) before testing would provide an elegant solution to this problem. Currently, the only pharmacopoeial method permitted is that of using an appropriate β-lactamase to inactivate penicillins and cephalosporins. Other antibiotics that are susceptible to inactivating enzymes are chloramphenicol (by chloramphenicol acetyltransferase) and the aminoglycosides, e.g. gentamicin, which can be inactivated by phosphorylation, acetylation or adenylylation. A method for acetylating

and consequently inactivating aminoglycosides prior to testing using 3-*N*-acetyltransferase (an enzyme with wide substrate specificity) in combination with acetyl coenzyme A has been described, but this method has yet to be adopted.

5.6 Immobilized enzyme technology

The therapeutic uses of microbially derived enzymes have already been examined (section 2.4). However, enzymes also form the basis of many diagnostic tests used in clinical medicine. For example, glucose oxidase, an enzyme used in blood glucose analysis, is obtained commercially from *Aspergillus niger*. Although the design of glucose blood monitoring test strips has become more refined, the basic design remains the same. Glucose oxidase is sandwiched between polycarbonate and cellulose acetate membranes. When a pinprick volume of blood is applied, the polycarbonate membrane allows the diffusion of glucose into the sandwich but limits entry of larger molecules thus reducing background contaminants. The enzyme oxidizes the glucose, resulting in the formation of hydrogen peroxide. The hydrogen peroxide diffuses through the cellulose membrane where it is measured amperometrically following interaction with a platinum electrode. More recently, several types of implantable glucose oxidase electrodes have been developed experimentally for continuous monitoring of blood glucose levels in diabetics. While there are challenges relating to enzyme inactivation *in vivo*, glucose calibration, and immune response there are, nevertheless, a number of major research efforts in this area and indeed the US Food and Drug Administration (FDA) has recently approved a wireless subcutaneous sensor that continuously monitors patient glucose levels for 7 days. It is likely that biosensors employing immobilized enzymes which are potentially useful for monitoring many substances of clinical importance will become readily available in the not-too-distant future.

6 Use of microorganisms as models of mammalian drug metabolism

The safety and efficacy of a drug must be exhaustively evaluated before its approval for use in the treatment of human diseases. Investigations of the manner in which a drug is metabolized are extremely valuable as they potentially provide information on its mode of action, why it exhibits toxicity and how it is distributed, excreted and stored in the body. Traditionally, drug metabolism studies have relied on the use of animal models and, to a lesser extent, liver microsomal preparations, tissue culture and perfused organ systems. Each of these models has certain advantages and disadvantages. Animals in particular are expensive to purchase and maintain, and there is considerable pressure from animal welfare groups to curb the use of animals in scientific research.

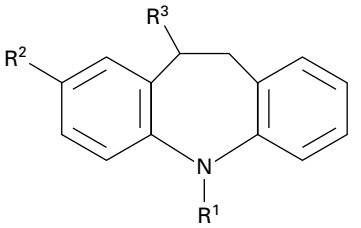
The many similarities between certain microbial enzyme systems and mammalian liver enzyme systems has led to the utilization of various microbial models for the exploration of mammalian drug metabolism. The major advantages of using microorganisms are their ability to produce significant quantities of metabolites that would otherwise be difficult to obtain from animal systems or by chemical synthesis, and the considerable reduction in operating costs compared with animal studies.

Microbial drug metabolism studies are usually carried out by firstly screening a large number of microorganisms for their ability to metabolize a drug substrate. The organism is usually grown in a medium such as peptone glucose in flasks that are shaken to ensure good aeration. Drugs as substrates are generally added after 24 hours of growth and are then sampled for the presence of metabolites at intervals up to 14 days after substrate addition. Once it has been determined that a microorganism can metabolize a drug, the whole process can be scaled up for the production of large quantities of metabolites for the determination of their structure and biological properties.

As an example of this the metabolism of the antidepressant drug imipramine can be considered. In mammalian systems, this is metabolized to five major metabolites: 2-hydroxyimipramine, 10-hydroxyimipramine, iminodibenzyl, imipramine-*N*-oxide and desipramine (Figure 26.10).

For microbial metabolism studies, a large number of fungi are screened, from which several are chosen for the preparative scale production of imipramine metabolites. *Cunninghamella blakesleeana* produces the hydroxylated metabolites 2-hydroxyimipramine and 10-hydroxyimipramine; *Aspergillus flavipes* and *Fusarium oxysporum* f. sp. *cepae* yield the *N*-oxide derivative and iminodibenzyl, respectively; while the pharmacologically active metabolite desipramine is produced by *Mucor griseocyanus* together with the 10-hydroxy and *N*-oxide metabolites. By scaling up this procedure, significant quantities of the metabolites that are formed during mammalian metabolism can be obtained.

Microorganisms thus have considerable potential as tools in the study of drug metabolism. Although they



Imipramine	$R^1 = (CH_2)_3N(CH_3)_2$; $R^2 = R^3 = H$
Desipramine	$R^1 = (CH_2)_3NHCH_3$; $R^2 = R^3 = H$
2-hydroxyimipramine	$R^1 = (CH_2)_3N(CH_3)_2$; $R^2 = OH$; $R^3 = H$
10-hydroxyimipramine	$R^1 = (CH_2)_3N(CH_3)_2$; $R^2 = OH$; $R^3 = H$
Iminodibenzyl	$R^1 = R^2 = R^3 = H$
Imipramine-*N*-oxide	$R^1 = (CH_2)_3N(CH_3)_2$; $R^2 = R^3 = H$ ↓ O

Figure 26.10 Structure of imipramine and its metabolites.

cannot completely replace animals, they are extremely useful as predictive models for initial studies.

7 Microorganisms as therapy

7.1 Bacteriophages

In the early 1900s, before the discovery of penicillin, Felix D'Herelles observed that patients with high titres of bacteriophages in their faeces recovered from dysentery and typhoid fever more rapidly. This paved the way for the commercialization of bacteriophage preparations for a variety of bacterial infections by, for example, the Société Française de Teintures Inoffensives pour Cheveux (The French Society for Safe Hair Colouring) or L'Oréal as it is known today. Following the advent of modern antibiotic therapy in the 1930s the science of phage therapy was all but bankrupted, but the emergence of antibiotic resistance has led to a resurgent interest in research and development of phage therapeutics (Chapter 27) and at least eight commercial enterprises are involved in the development of clinically relevant phage medicines. Most phages have a specific affinity for only a small group of bacteria, predicated by the interaction of phage components with bacterial surface receptors. Upon interaction, the viral DNA is translocated into the bacterial cell for transcription where lytic or lysogenic replication may occur (Chapter 5). Lytic phages replicate and assemble and then 'burst' from the host cell, resulting in cell death. In the lysogenic lifecycle, bacteriophage DNA becomes integrated in the host bacterium's genome. This newly generated material termed a prophage is replicated during cell division. The lysogenic lifecycle is shifted to a lytic one when exposed to some external trigger, such as UV radiation.

Lytic phage are in many ways an ideal antibacterial agent. They are target specific and the existence of more than 1×10^8 species of phage suggests there may be a phage therapeutic for every bacterial species; they kill bacteria rapidly and amplify at the site of infection, and are relatively inexpensive to produce. In addition, the FDA recognizes that humans ingest vast quantities of phages on a daily basis and tacitly accept that they are safe for oral administration. It is perhaps topical administration that has seen the most interest, however, with the application of cocktails of phage to chronic wounds either as simple suspensions or incorporated into some form of dressing system such as a biodegradable polymer infused with phage and antibiotics. The systemic administration of phage therapeutics is complicated by an inadequate knowledge of the pharmacokinetic and pharmacodynamic properties of most phage species, with many studies indicating that the timing of administration is critical for infection control. Recent reports suggest, however, that phages, which are naturally immunostimulatory, may be useful as vaccine delivery vehicles either by vaccinating with phages displaying the antigen or by utilizing phages to deliver a DNA expression cassette integrated into the phage genome.

7.2 Probiotics

The bacterial microflora that colonizes the gastrointestinal (GI) tract is an essential feature of normal human physiology and represents a symbiotic relationship where the bacteria both protect the host against pathogenic microbes and aid in the digestion of food, contributing to the production of essential host nutrients. Under certain conditions (e.g. illness, infection, antibiotic therapy), the bacterial population in the GI tract may be diminished, contributing to disease states. Probiotics are live cultures of 'good' bacteria that are purported to survive transit through the stomach, subsequently colonizing the intestinal mucosa and replacing the diminished natural microflora or displacing pathogenic microorganisms. *Bifidobacteria* and *Lactobacillus* spp. are the most commonly encountered probiotic bacteria, primarily because they are reported to survive the harsh environment of the upper GI tract more readily than other species. Of note, *Bifidobacteria* spp. are amongst the first colonizers of the neonate intestine, as a consequence of both *Bifidobacteria* and prebiotic content in breast

milk, and contribute to defence against pathogenic invaders and maturation of the immune system. Probiotics are generally formulated as capsules or as food supplements particularly as dairy products such as yoghurts. Often probiotic formulations are combined with prebiotics—indigestible oligosaccharides that are fermented by anaerobic bacteria in the gut, yielding metabolic substrates that promote probiotic growth.

Probiotics have been investigated for efficacy in a range of conditions that may be associated with diminished bacterial microflora. Several probiotic species including *Lactobacillus* spp. have shown utility in both the prevention and treatment of nosocomial, antibiotic and traveller's diarrhoea. Long-term treatment with *E. coli* Nissle 1917 (>12 months) is reported to be at least equivalent to mesalazine therapy in preventing relapse in ulcerative colitis. In irritable bowel syndrome (IBS) the results are equivocal but hopeful, with some studies reporting a reduction in symptoms such as abdominal discomfort.

A cautionary note, however: probiotics are increasingly marketed as a 'lifestyle' nutrient to healthy individuals to promote general GI and immune health, despite limited evidence of any significant effect. Nevertheless, research into the benefits of probiotics in both healthy and diseased individuals is ongoing, using recognized and novel probiotic species, and may in the future reap significant reward.

7.3 Toxins

7.3.1 Botulinum toxin

In the late 19th century, a Belgian professor of microbiology, van Ermengem, conducted a serious of experiments to identify the cause of a fatal outbreak of food poisoning, the clinical symptoms of which had been described over a century before by the German physician Justinus Kerner. van Ermengem's endeavours resulted in the identification of botulinum toxin, a potent exotoxin produced by the Gram-positive anaerobic bacterium *Clostridium botulinum*. It is this toxin that is responsible for what is now widely recognized as botulism food poisoning. The symptoms of botulism, which remains a common cause of fatal food poisoning, include GI disturbances, dysphagia, facial paralysis and, depending on the ingested dose, more widespread muscle weakness resulting in possible respiratory paralysis and subsequent death.

In the mid-20th century the work of Burgen and colleagues established that the basic mechanism of action for botulinum toxin is neuromuscular blockade. Now, some 60 years later, we understand that the toxic component of the protein complex is a 150 kDa single-chain polypeptide, consisting of a 100 kDa heavy chain linked to a 50 kDa light chain by a disulphide bridge, which temporarily inhibits acetylcholine release from the presynaptic membrane of cholinergic nerve terminals. Seven different serotypes of the botulinum neurotoxin (A–G), have been identified. The 150 kDa toxic component of these macromolecular protein complexes is relatively homologous, conferring only subtle differences between the mechanisms of action of the serotypes. However the non-toxic proteins within the bacterium-derived botulinum toxin protein complex also differ, depending on the strain of *Cl. botulinum*. Botulinum toxin serotypes therefore possess molecular weights between 300 and 900 kDa.

In the late 1970s and 1980s nanogram quantities of the botulinum toxin were being locally injected, by clinical researchers, into the muscles of human volunteers to induce local paralysis in an attempt to treat various movement disorders. In 1989, after more than a decade of clinical development, the FDA approved the first botulinum toxin therapy. This commercial product contained the botulinum toxin A serotype and was used for the treatment of strabismus, blepharospasm and hemifacial spasm. In 1991 Allergan obtained both the license and the manufacturing facilities to become the sole supplier of botulinum toxin A for clinical therapy and they branded their product Botox.

There are currently five licensed pharmaceutical forms of the botulinum toxin A: Botox, Vistabel, Dysport, Xeomin and Azzalure. These pharmaceutical preparations contain different forms of the toxin, are formulated differently and/or are licensed for different therapeutic indications. For example, Xeomin contains only the 150 kDa light chain region of the toxin and contains human albumin and sucrose as excipients, whereas Botox contains the 900 kDa macromolecular protein complex and contains human albumin and sodium chloride as excipients. Doses of commercial botulinum toxin A preparations are therefore not interchangeable and specific brands should be prescribed for specific clinical indications. Doses are significantly less than the lethal dose for a human, but systemic side effects of the toxin have been observed, albeit rarely. Clinical administration of the toxin relies on multiple localized injections, often in the secondary care setting, directly into the target tissue. However, the therapeutic effect of botulinum toxin is transient, typically 6–12 months, and patients therefore return for treatment at regular intervals.

In the past two decades the use of botulinum toxin A in clinical practice has increased almost exponentially and it is now used to treat a diversity of medical conditions. Specific licensed indications include blepharospasm, cervical dystonia, hemifacial spasm, glabellar lines and hyperhidrosis. However, it has been used more widely, often unlicensed, for a range of clinical indications related to movement disorders, spasticity, ophthalmic disorders, GI disorders, genitourinary disorders, surgical interventions, tendon release in the Ponseti treatment of talipes, and more recently pain. The toxin is even better known for its widespread use in the cosmetic industry. A significant population of patients and cosmetic clients have therefore now been treated with the neurotoxin, and in general it appears to be a safe and effective addition to the therapeutic armoury. Botulinum toxin type B is also available commercially, as Neurobloc, but at present this serotype is used less widely in therapy. The clinical use of botulinum toxin is primarily restricted to conditions that are associated with superficial/accessible tissues, to minimize the risk of systemic uptake, but there is no doubt that the toxin is emerging as a useful therapeutic entity and has already revolutionized the treatment of some conditions including hyperhidrosis.

7.3.2 Cholera toxin

Vibrio cholerae is a bacterial pathogen that colonizes the small intestine leading to cholera, an infection characterized by life-threatening acute diarrhoea. Cholera is endemic in developing countries and in areas where hygiene and sanitary conditions are poor, and even with supportive therapy that includes rehydration and restoration of electrolytes, morbidity and mortality rates remain high. An oligomeric protein (87 kDa) secreted by *V. cholerae* was confirmed as the causative agent of cholera in 1963 by Finkelstein and colleagues and has been termed cholera toxin (CT). CT is a member of the superfamily of AB toxins comprised of a catalytic heterodimeric A subunit (A1 and A2 chains) and a glycolipid receptor binding homopentameric B-subunit connected by a disulphide bond. When *V. cholerae* colonizes the small intestine it secretes CT which subsequently interacts, via the B-subunit, with an enterocyte membrane receptor GM1 (monosialotetrahexosylganglioside) localized in lipid rafts. The CT is then internalized into early endosomes and trafficked to the trans-Golgi network, eventually ending up in the endoplasmic reticulum where protein disulphide isomerase dissociates and unfolds the A1 chain from CT. The A1 chain is then translocated by the Sec61 channel into the cytosol where it interacts with proteins that regulate adenylate cyclase (AC) leading to the constitutive activation of AC. This is accompanied by an increase in intracellular cAMP concentration resulting in phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR). The net consequence is extracellular secretion of chloride ions into the small intestine, producing an osmotic gradient that draws water into the lumen, resulting in diarrhoea.

CT, despite its pathogenicity, has significant immunological properties and has been proposed as a mucosal adjuvant for subunit vaccines where the toxin is co-administered or complexed with the antigen. Mucosally administered vaccines, i.e. oral, nasal, rectal or vaginal, have a number of advantages over the more traditional intravenous vaccines, not least the ability to stimulate mucosal and systemic protection and may perhaps enhance vaccine uptake rates given no needles are involved. The mechanism of adjuvanticity of cholera toxin is controversial and remains to be fully elucidated but evidence suggests the A-subunit, when administered orally, enhances antigen presentation following complex interactions with mucosal cells and cells of the immune system(see Chapter 9). The role of the B-subunit in adjuvanticity is interesting; CT-B does not appear to survive transit through the GI tract but there is growing evidence of autoimmune stimulation following nasal or intravenous administration and therefore the B-subunit may offer utility in the treatment of autoimmune diseases. A number of preclinical *in vivo* studies have been undertaken to ascertain the effectiveness of CT as a mucosal adjuvant for antigens derived from (for example) *Helicobacter pylori*, influenza, tetanus, HIV and *Streptococcus pneumoniae*, and for the treatment of diabetes mellitus. To date, however, it appears that oral doses of CT that elicit adjuvanticity are similar to those that induce diarrhoea amongst other adverse reactions, although efforts to 'detoxify' CT through genetic engineering are under way.

8 Insecticides

Like animals, insects are susceptible to infections, which may be caused by viruses, fungi, bacteria or protozoa. The use of microorganisms to spread diseases to particular insect pests offers an attractive method of biological control, particularly in view of the ever-increasing incidence of resistance to chemical insecticides. However, any microorganism used in this way must be highly virulent, specific for the target pest but non-pathogenic to animals,

humans or plants. It must be economical to produce, stable on storage and preferably rapidly acting. Bacterial and viral pathogens have so far shown the most promise.

Perhaps the best studied, commercially available insecticidal agent is *B. thuringiensis*. This insect pathogen contains two toxins of major importance. The δ-endotoxin is a protein present inside the bacterial cell as a crystalline inclusion within the spore case. This toxin is primarily active against the larvae of lepidopteran insects (moths and butterflies). Its mechanism of action is summarized in Figure 26.11. Commercially available preparations of *B. thuringiensis* are spore–crystal mixtures prepared as dusting powders. They are used primarily to protect commercial crops from destruction by caterpillars and are surprisingly non-toxic to humans and animals. Although the currently available preparation has a rather narrow spectrum of activity, a variant *B. thuringiensis* strain has been isolated and found to produce a different δ-endotoxin with activity against coleopteran insects (beetles) rather than lepidopteran or dipteran (flies and mosquitoes) insects.

The second *B. thuringiensis* toxin, the β-exotoxin, has a much broader spectrum encompassing the Lepidoptera, Coleoptera and Diptera. It is an adenine nucleotide, probably an ATP analogue that acts by competitively inhibiting enzymes that catalyse the hydrolysis of ATP and pyrophosphate. However, this compound is toxic when administered to mammals, so commercial preparations of the *B. thuringiensis* δ-endotoxin are obtained from strains that do not produce the β-exotoxin.

Strains of *B. sphaericus* pathogenic to mosquitoes were isolated several years ago. Strains of this organism with increased toxicity to mosquitoes have been isolated and might have considerable potential as control agents. Commercially available formulations of *B. sphaericus* are now available as larvicides.

Other insect pathogens are being evaluated for activity against insects that are vectors for diseases such as sleeping sickness, as well as those that cause damage to crops. Viruses may well have the greatest potential for insect control as they are host-specific and highly virulent, and one infected insect can release vast numbers of virus particles into the environment. They have already been used with considerable success against the spruce sawfly and pine moth.

9 Bioterrorism

Bioterrorism, or biological terrorism, involves the deliberate release of biological agents such as viruses, bacteria and toxins, to intentionally cause terror, illness or death in a target population. In a climate of increasing political instability and radical fundamentalism there are many concerns over the likelihood of exposure to, and the ability to protect civilians from, such attacks. There are a

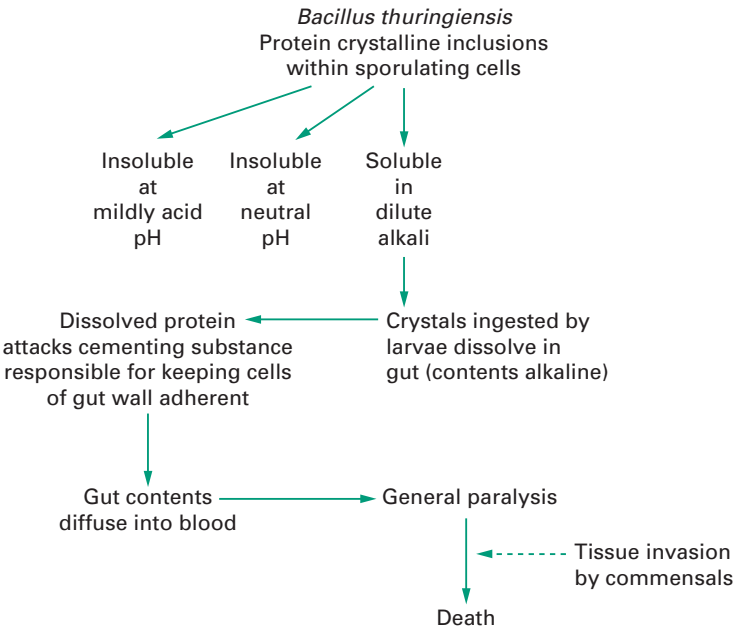


Figure 26.11 Mechanism of action of δ-endotoxin from *B. thuringiensis*.

variety of biological agents that could potentially be used as biological weapons. The US Centers for Disease Control and Prevention (CDC) and the UK Health Protection Agency (HPA) have defined bioterrorism agents into three categories (A, B and C) based largely on their lethality, ease of transmission and ability to cause panic. Category A agents, i.e. those that pose the greatest threat, are *B. anthracis* (anthrax), *Cl. botulinum* toxin (botulism), *Yersinia pestis* (plague), variola major (smallpox), *Francisella tularensis* (tularemia) and the filovirus (Ebola, Marburg) and arenavirus (Lassa, Machupo) strains that cause viral haemorrhagic fevers.

In the event of a biological attack governmental organizations would invoke a preparedness plan that would include environmental decontamination, e.g. hypochlorite solution, and pharmaceutical prophylaxis and/or treatment. Although it is practically, ethically, politically, socially and economically difficult to vaccinate against known bioterror pathogens, it may be possible to stockpile and thereafter disseminate pharmaceutical prophylactics and therapeutics. For example, in the UK the HPA would follow guidance from the Advisory Group for Medical Countermeasures and respond with short-term antibiotic cover with, for example, ciprofloxacin or doxycycline (anthrax, plague) or doxycycline/rifampicin or co-trimoxazole (brucella). Botulinum antitoxin would also be provided for treatment of botulism. Clearly, the effectiveness of this plan in preventing illness and death would depend on the ability to detect the threat and respond rapidly.

In the future, the problem may help to provide the solution as advances in genetic medicine, a science that exploits bacteria and viruses, produce vaccine platforms that can be efficiently stockpiled to protect susceptible populations against bioweapons.

10 Concluding remarks

Microorganisms are not always the killers they are portrayed to be. In fact, humankind has been remarkably adept at harnessing microbes for a variety of purposes. In many instances—e.g. antibiotics by whole or partial synthetic production (Chapter 11) and various forms of vaccines—products have been obtained to turn the tables on infecting organisms. Other products have been used for a variety of purposes (including many non-pharmaceutical or non-medical ones, outside the scope of this chapter). Microorganisms have also been employed for specific assay purposes and different types of chemical transformations, as well as in genetic engineering (Chapter 25). Immobilized microorganisms have now been used with considerable success in the partial synthesis of steroids and antibiotics and in the production of the antiviral compound adenine arabinoside (Chapter 5). In a changing scientific landscape where modern recombinant and molecular technologies are being rapidly adopted, there remains significant scope for the practice of pharmaceutical microbiology and the beneficial harnessing of microbes is likely to continue for many years.

11 Acknowledgement

The authors wish to both acknowledge and pay tribute to the original author of this chapter and indeed this text, A.D. Russell. Denver Russell was a pharmaceutical microbiologist par exemplar. Over a long and distinguished career Denver witnessed the inception of the field of pharmaceutical microbiology and contributed extensively to the fledgling research community producing a body of work that is testament to his international reputation. It has been an honour for us to update Denver's chapter for this edition of the text.

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27 Alternative strategies for antimicrobial therapy

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1 Introduction

Most of us have no notion of what life would be like without antibiotics. Penicillin was discovered in 1928 and after a lengthy developmental period became available for clinical use in the 1940s. Then followed what is often referred to as the golden era of antibiotic discovery which brought us the cephalosporins, the tetracyclines, the macrolides, the aminoglycosides, the 4-quinolones, etc. (Table 27.1). It is interesting to note that the impact of antibiotic discovery was sufficient to halt the development of virtually all other antimicrobial therapies being pursued at that time. The fact that Alexander Fleming in the early years had flagged up the probability of resistance development in bacterial pathogens was largely ignored because of the constant stream of new agents on to the market. There is anecdotal evidence that in 1967 the US Surgeon General stated '. . . it is time to close the book on infectious disease, declare the war against pestilence won . . .'. Whether or not he actually said this is debatable, but it represented a widely held view at the time that the stream of antibiotic discovery would never dry up. However, we now know that is precisely what has happened.

At present we are faced with two major issues—we have seen an inexorable rise in the incidence of antibiotic resistance among the most important bacterial pathogens (dealt with in Chapter 13), while at the same time the development of novel antibacterial antibiotics has all but stopped. Some clinical isolates from species such as *Pseudomonas aeruginosa, Acinetobacter baumannii, Enterococcus faecium* and *Mycobacterium tuberculosis* are resistant to virtually every antibiotic we have at our disposal. It is not appropriate here to discuss the issue of antibiotic resistance, other than to say it is an almost inevitable event occurring as a consequence of the bacterial cell's remarkable adaptability to environmental change. Without this ability to adapt to their environment bacteria would not still be thriving 3.5 billion years after first emerging on to what was at that time an extremely hostile earth. Suffice it to say there is very little we can do to stop this process.

Hugo and Russell's Pharmaceutical Microbiology, Eighth Edition. Edited by Stephen P. Denyer, Norman Hodges, Sean P. Gorman, Brendan F. Gilmore.

Table 27.1 Time line for the discovery and use of naturally occurring and synthetic antibiotics

Time	Antibiotic
1900–1920	Salvarsan
1920–1940	Benzylpenicillin
1940–1960	Sulphanilamide Cephalosporins Streptomycin Tetracycline Chloramphenicol Erythromycin Isoniazid Vancomycin Polymyxin Methicillin
1960–1980	Ampicillin Trimethoprim Sulphamethoxazole Fusidic acid Carbapenems Gentamicin Clindamycin Amoxycillin 4-Quinolones
1980–2000	Mupirocin Teicoplanin Clarithromycin Aztreonam Latamoxef Azithromycin Quinupristin/dalfopristin Ceftazidime Linezolid
2000 to date	Daptomycin Doripenem Tigecycline

The second issue of antibiotic discovery and development is, of course, under our control. We have the scientific ingenuity to devise novel approaches to circumvent the resistance mechanisms put in place by these bacteria. Unfortunately, the driving force behind drug discovery is not based on altruism but has a financial imperative. The cost of bringing a new chemical entity to the market has been estimated as $800 million or more, while the process of development takes about 8 years. Antibiotics simply do not generate sufficient revenue to justify this initial outlay of time and cost, and not surprisingly the focus for the large pharmaceutical companies has switched to those medicines which patients will take long-term such as statins, antidepressants and antihypertensives. The market for antiviral agents, particularly those to treat HIV/AIDS, remains buoyant simply because the patients will be taking the medicines for the remainder of their lives.

All this poses the question, 'How in the near future will clinicians be able to treat patients with infections caused by multiresistant bacterial pathogens?' In reality this is a question which is relevant today but will only become more pressing in the future. Some respected authorities in the field have already expressed the view that we are on the verge of entering a postantibiotic era.

This chapter examines a range of alternative therapeutic strategies available for the management of infectious disease (Table 27.2). It is not the intention to cover each of these in any depth but merely to give an overview of the general situation; the reader is referred to the further reading listed at the end of the chapter for more details. As we will see, a number of the techniques to be described have a very long history, some of them going back many centuries. Some of these have very little chance of ever moving from the fringes of medicine, some may have the potential to be useful but in reality suffer from the same developmental issues as conventional antibiotics, some are niche approaches which may find relevance in specific clinical areas, while a few may offer a realistic alternative, or at least an adjunct, to antibiotics.

2 Essential oils

Essential oils, sometimes termed ethereal or volatile oils, are contained within many plants and impart their characteristic odour. They can be obtained from the plant material by distillation, expression or solvent extraction and have been used throughout the centuries for medicinal and other purposes. In recent times attention has focused on a few of these oils, particularly tea tree oil, because of their reported antimicrobial activity and this will be used as an exemplar of the group as a whole. As the name suggests, they are hydrophobic, which makes them difficult to work with, and being natural products they frequently contain a large number of chemical components.

Table 27.2 Potential alternatives to antibiotics for the treatment of bacterial infections

Alternative therapeutic strategy	Selected references
Essential oils (tea tree oil)	Carson *et al.* (2006)
Honey therapy	Moore *et al.* (2001)
Garlic	Amagase *et al.* (2001)
Probiotics	Mombelli & Gismondo (2000); Hedin *et al.* (2007)
Maggot therapy	Chan *et al.* (2007)
Photodynamic antimicrobial therapy	Wainwright (1998)
Vaccines and immunotherapy	BNF (2009); Döring & Pier (2008)
Silver	Edwards-Jones (2009)
Bacteriophages	Hanlon (2007)
Bacteriophage lysins	Fischetti (2008)

Table 27.3 Composition of tea tree oil as determined by BS ISO 4730:2004

Components	Minimum (%)	Maximum (%)
α-Pinene	1	6
Saninene	Trace	3.5
α -Terpinene	5	13
Limonene	0.5	1.5
p-Cymene	0.5	8
1,8-Cineole	Trace	15
γ-Terpinene	10	28
Terpinolene	1.5	5
Terpinen-4-ol	30	48
α-Terpineol	1.5	8
Aromadendrene	Trace	3
Ledene (syn. viridiflorene)	Trace	3
δ-Cadinene	Trace	3
Globulol	Trace	1
Viridiflorol	Trace	1

Tea tree oil (TTO) is the essential oil obtained from the Australian plant *Melaleuca alternifolia* (although other species may be used). Of its numerous chemical constituents (mainly monoterpenes, sesquiterpenes and their associated alcohols) terpinen-4-ol has been shown to have the major antimicrobial activity. It has general antimicrobial activity against viruses, bacteria and fungi but the main interest in this compound is centred on its activity against meticillin-resistant *Staphylococcus aureus* (MRSA). TTO has been suggested as a potential agent for the nasal and skin decolonization of MRSA in carriers. It has been formulated in a variety of topical preparations and is available over the counter for the treatment of a range of conditions.

One of the many issues surrounding the use of any natural material, especially multicomponent substances such as plant extracts, is that of batch variability and quality control. In the case of TTO there is a British Standard which regulates the composition of the 15 components of the oil and this is identical to ISO 4730:2004 (Table 27.3). The standard demands a lower limit of 30% for the main antimicrobial compound terpinen-4-ol, and other minor components are included in order to make the formulation of an artificial oil more difficult. Unless all preparations conform to the required standards of composition it is very difficult to judge the value of any *in vitro* or *in vivo* tests. The situation is further confused because the nature of the oil makes it problematic to evaluate. Oils are only sparingly soluble in the media used to test antimicrobial activity and when mixed with aqueous solutions result in turbid suspensions; consequently, end-points based on turbidity are difficult to determine, and the hydrophobic nature of the compounds limits their diffusion in agar. Thus in the literature a range of methodologies have been described to evaluate antimicrobial activity, and comparison between them is difficult.

Those *in vitro* studies which have been conducted indicate activity against a broad range of bacteria with similar minimum inhibitory concentrations (MICs) reported regardless of whether the isolate was antibiotic-sensitive or resistant. Generally bacteria are susceptible to concentrations below 1% (v/v) with the main exception being *Ps. aeruginosa* which requires concentrations

approaching 8% (v/v). The mechanism of action of TTO is thought to involve disruption of cellular membranes which will result in loss of intracellular constituents and inhibition of enzyme function. The more complex outer membranes of *Ps. aeruginosa* render that organism more resistant to the effects of TTO.

Clinical studies have evaluated TTO for the treatment of a range of bacterial and fungal infections. There is no doubt that it is effective, although in many cases the treatment was not found to be superior to conventional therapy. Despite being used for many years TTO has exhibited safety issues and is toxic if ingested. It has also been shown to have irritant effects on the skin, although these may be reduced if the oil is properly formulated. Essential oils such as TTO cannot be directly compared to conventional antibiotics since they are used primarily as topical antiseptics. However, in this regard they certainly have some value although more extensive, properly controlled trials are necessary to fully confirm their potential use.

3 Honey therapy

Bees collect nectar (a weak natural sugar solution) and pollen from flowers in their locality, and in their hives it is ultimately transformed into honey. As a consequence of the processing in the hive, sucrose in the honey is converted into fructose and glucose and the enzyme glucose oxidase converts glucose into gluconic acid and hydrogen peroxide. As water evaporates from the honey the sugar concentration increases until it is of the order of 79% w/v. These features act to preserve the honey from microbial degradation. Other components of the honey are dependent on the geographical source of the nectar, and much interest has focused recently on honey collected in the vicinity of the Australian tea tree (*Melaleuca alternifolia*) mentioned above, and the New Zealand manuka (*Leptospermum scoparium*). The honey collected from these areas has been studied widely because of its reported antibacterial and wound-healing properties. These have been attributed to a number of factors, including the following.

- *pH.* This ranges from 3.2 to 4.5, which is below the minimum pH values required for the growth of many common bacteria.
- *Osmotic pressure and water activity.* Honey is a supersaturated sugar solution with a water content of between 15 and 21% w/w. The water availability (A_w) ranges from 0.56 to 0.62, which again is below that required for the growth of most common bacteria.
- *Hydrogen peroxide.* This is an effective antibacterial agent and, although it is rapidly neutralized, the presence of glucose oxidase ensures a constant replenishment.
- *Non-peroxide antibacterial activity.* Many workers have shown that the antimicrobial activity of honey persists even when the hydrogen peroxide has been removed.

The nature of the substance responsible for the non-peroxide activity has been elusive, but recent studies suggest that it may be methylglyoxal, found in some highly active forms of manuka honey. However, many other candidate materials are under investigation.

Results from animal studies, some clinical trials and much anecdotal evidence over centuries suggests that honey may have a role to play in the management of infected wounds due to its antibacterial and wound-healing properties. However, a recent Cochrane review examined 19 published clinical trials in which honey was evaluated in the treatment of acute or chronic wounds. The conclusion reached was that although honey may improve healing times in mild to moderate wounds there was insufficient evidence to support its use in other areas.

4 Garlic

Garlic (*Allium sativum*) has been used as a culinary ingredient for thousands of years, but it has also been shown to possess a range of pharmacological activities leading to uses in cardiovascular disease and cancer. Its use in these conditions is outside the remit of this chapter; however, of interest here is the fact that it has also been reported to have antimicrobial activity against a broad spectrum of bacterial pathogens.

The chemistry of garlic is highly complex and like other plant extracts the material is made up of a large number of compounds. The main sulphur-containing components of intact garlic are γ-glutamyl-*S*-allyl cysteines and *S*-allyl-L-cysteine sulphoxides (of which the main one is known as alliin). When garlic is crushed or damaged, the enzyme alliinase, which is normally contained within vacuoles, comes into contact with the cytosolic cysteine sulphoxides and converts them to thiosulphinates such as allicin. This process is thought to represent a protective effect for the plant because assault by predators (worms, bacteria, etc.) would result in a localized high concentration of these toxic products. Allicin was the component that was first shown to be

responsible for the marked antimicrobial activity of fresh garlic, and on the basis of this a number of garlic-containing products were marketed for their antibacterial effects. However, allicin was subsequently found to be highly unstable and to degrade rapidly within a matter of days to a variety of sulphides in both aqueous and alcoholic solutions. In the context of the plant's protective strategy this makes sense, because having generated a toxic metabolite to counter an insult it is in the plant's best interest to neutralize that toxin as rapidly as possible after the invader has been repelled.

Orally administered allicin is degraded in the stomach acid and is not absorbed from the gut, thus demonstrating that it cannot be responsible for any of the reported *in vivo* antibacterial effects. Attention has thus switched to other components, particularly the allicin breakdown products including diallyl sulphide and diallyl disulphide. Studies in mice have shown that oral dosing of these agents can reduce MRSA viability in blood, liver, kidney and spleen. They also provide immunological protective properties. However, the number of studies conducted is small and the methodologies variable. More comprehensive clinical trials are required together with a rigorous study of the role the individual components play in the overall biological activity.

Garlic suffers from many of the issues raised above for TTO in that the composition of the product varies markedly depending on the source of the raw material, how it is processed to extract the active ingredients, how it is formulated and how it is stored. Inconsistencies reported for the activity of garlic preparations are primarily due to a lack of standardization of the product. As things currently stand, garlic is unlikely to contribute significantly to the resolution of the issue of diminishing antibiotic availability.

5 Probiotics

Probiotics are defined as living microorganisms that confer some health benefits when utilized in sufficient numbers. The use of harmless bacteria to replace pathogenic ones (bacteriotherapy) is not a new concept but has gained renewed interest in recent years. Particularly, they have been strongly marketed as a prophylactic approach to the promotion of well-being. In addition, bacteriotherapy has been used in the treatment of a variety of gastrointestinal conditions (including diarrhoea), respiratory infections, recurrent bacterial vaginitis, recurrent otitis media, streptococcal tonsillitis and infected burns. The microorganisms most frequently used are normal components of the gut microflora and include the lactic acid bacteria such as lactobacilli, lactococci, bifidobacteria, enterococci and streptococci (see Table 27.4). Other bacteria such as *Bacillus* species and yeasts such as *Saccharomyces* may also be utilized. Since each of these organisms possesses different characteristics and requirements for growth it is likely that they do not have a common mechanism of action.

5.1 Gastrointestinal conditions

Probiotics for oral administration are marketed as dairy products such as yoghurt drinks, but also as powders, tablets or capsules. Irrespective of the nature of the product, there is a need to consume in the region of 10^9–10^{11} microorganisms in order to bring about any effect. A number of studies have highlighted issues of inconsistency in the content of viable microorganisms within various products. The precise manner in which probiotics bring about their health benefits is unclear, but a prerequisite property is the ability to colonize the gut. The orally administered organisms must first, therefore,

Table 27.4 Microorganisms commonly used as probiotics

Bacillus subtilis
Bifidobacterium bifidum
B. breve
B. infantis
B. lactis
B. longum
Enterococcus faecium
Escherichia coli Nissle 1917
Lactobacillus acidophilus
L. casei immunitas
L. johnsonii
L. paracasei subsp *paracasei 19*
L. plantarum
L. raffinolactis
L. reuteri
L. rhamnosus
L. salivarius
Leuconostoc spp.
Pediococcus pentoseceus
Streptococcus thermophilus
Saccharomyces boulardii
S. cerevisiae

be able to survive passage through the stomach acid and then compete with the indigenous flora in the intestine. A competitive edge will be provided by the ability to colonize the gut wall and the capacity to produce bacteriocins. Probiotics have been administered rectally to overcome the necessity for acid tolerance, but there is a risk of spreading faecal pathogens.

Probiotics have been found to be of benefit in the treatment of infectious diarrhoea in children. When used as a supplement to breast milk in premature infants they were found to reduce the incidence and severity of necrotizing enterocolitis. A recent review summarizing the available clinical trial data has suggested value in the treatment of ulcerative colitis, but the results for Crohn's disease were disappointing and the authors stated that the use of probiotics could not be recommended on the available evidence.

Clostridium difficile is a Gram positive, spore-forming anaerobic rod which, in certain conditions, can overgrow in the intestinal tract and produce toxins causing diarrhoea and damage to the epithelial lining of the gut. This condition is a particular problem in elderly hospitalized patients who have received courses of broad-spectrum antibiotics. Since the colitis is caused by overgrowth of a pathogenic bacterium in the intestinal tract it would seem to be potentially amenable to probiotic treatment. However, a recent Cochrane review of the current clinical trial data found that only treatment with *Saccharomyces boulardii* had any significant effect. Many hospitals use a range of commercially available probiotic yoghurt preparations but the effectiveness of these is currently unproven.

5.2 Infected burn wounds

Burn wounds are devoid of microorganisms immediately after the injury has been sustained, but fairly quickly thereafter they will become colonized with microflora from the adjacent intact skin and also by environmental microbes. These bacteria are mainly Gram-positive coagulase-negative staphylococci, but they may be gradually replaced by more harmful Gram-negative bacteria such as *Ps. aeruginosa* and *Acinetobacter* spp. over the following few days. These bacteria are notorious for being highly antibiotic resistant and they flourish, often forming biofilms, because the usual defensive properties of intact skin are no longer present. Colonization with these organisms delays healing, can inhibit grafting and may lead to systemic infection. Studies have shown that applications of *Lactobacillus plantarum* were as effective as silver sulphadiazine in the management of infected burn wounds. This may be of relevance in those countries where the costs of conventional antibiotic treatment are prohibitive or where there is a high level of antibiotic resistance.

5.3 Recurrent vaginitis

Many women suffer from vaginitis, caused by either bacteria or *Candida albicans*, which may lead on to urinary tract infections. In these cases the normal vaginal microflora which comprises mainly lactobacilli is overrun by various other, sometimes pathogenic, bacteria or fungi. The usual approach to treatment is the administration of antibiotics, but recurrence is commonplace. The vaginal administration of probiotic cultures has proved to be beneficial in these cases; however, strains of bacteria isolated from dairy products are not always suitable. The lactobacilli should be those that have the capacity to bind specifically to vaginal epithelial cells.

5.4 Acute otitis media

This is a very common condition in young children in which bacteria normally present in the nasopharynx transfer via the eustachian tube into the middle ear. The predominant bacteria are *Streptococcus pneumoniae* and *Haemophilus influenzae*. Antibiotic therapy is frequently unsuccessful and recurrences are common. As we have seen in the examples outlined above, the picture is once again one of undesirable bacteria replacing normal commensal flora and causing disease. Studies have been reported in which children were treated with suspensions of α-haemolytic streptococci sprayed into the ear to inhibit the growth of the invading pathogens and recolonize the infected site. The results showed that the treated patients had significantly better outcomes than the placebo group.

Some workers have highlighted the fact that probiotics should be used with caution in those patients whose immune system is impaired because there have been reported incidences of invasive infections occurring in immunocompromised adults. In addition, *Lactobacillus* bacteraemia has been reported following probiotic therapy in two children who had received multiple courses of antibiotics and who were suffering from other serious underlying conditions.

The currently available data suggest that probiotics may have some value in certain selected conditions, but a great deal more background scientific information is required before this can be moved forward substantially. The approach of using a single culture for a whole spectrum of conditions is untenable. It is clear that the pro-

biotic bacteria will act in a variety ways, producing a range of interfering substances and having different abilities to adhere to epithelial cells. Individual cultures may therefore need to be developed for use in specific conditions and once this has been achieved large-scale trials are needed to verify their value.

6 Maggot therapy

Chronic infected wounds arising from conditions such as diabetes mellitus and peripheral vascular disease are a major problem, and their incidence is increasing. Whereas normal wounds go through well-defined phases of healing, chronic wounds do not heal because of the presence of necrotic tissue, slough, debris and infection. In order to allow the wound to begin to heal it is necessary to physically remove the necrotic burden, in a process known as debridement, and also deal with the infection. Clearing the wound of necrotic and devitalized tissue is normally carried out surgically before application of antimicrobial agents to remove the infecting pathogens. The mechanical techniques used in debridement are varied and can lead to pain and damage to underlying healthy tissue.

The use of maggots to facilitate wound debridement and subsequent healing has been known for several centuries, but its association with modern medicine goes back approximately 70 years. However, the clinical use of maggots dwindled with the advent of antibiotics and has only now experienced a resurgence of interest. The main focus of attention has been in the area of wounds infected with MRSA. The larvae of the blow fly *Lucilia sericata* are grown under sterile conditions and used when they are less than 8 hours old, at which point they are only 1–2 mm in length. Up to 1000 maggots are placed in the wound, covered to prevent escape and left for 1–3 days. The maggots require a moist environment for optimal activity and their secretions contain multiple proteolytic enzymes which degrade necrotic tissue. This digested material is subsequently consumed by the maggots as a source of nutrients, but it has been suggested that there is also a mechanical component to the clearing of necrotic tissue. One of the characteristics of the larvae is that they try to avoid light and as a result of their small size they tend to burrow into deep crevices which may have proved difficult to debride mechanically. The larval secretions contain ammonia which increases the local pH producing an antimicrobial effect, and in addition the maggots ingest the bacteria, thus clearing the site of infection. The presence of the maggots also leads to stimulation of granulation tissue formation and hence promotes healing.

There is now abundant literature which appears to affirm the view that maggot therapy can play an important role in the treatment of chronic infected wounds. However, a large (267 patients) multicentre trial was carried out in the UK recently comparing maggot therapy with a standard debridement technique (hydrogel) for the treatment of chronic infected leg ulcers. The authors found that there was no difference in healing times between the two groups and the larval group experienced more pain. Nonetheless, the larvae did reduce the time to debridement. Some workers have shown that the antimicrobial efficacy of the larvae is dependent on the species of bacterium under investigation. Gram-positive bacteria such as *Staph. aureus* are very susceptible, whereas Gram-negative bacteria such as *Ps. aeruginosa* are more resistant. The precise reasons for this are unclear. Additional hurdles to the widespread use of maggot therapy include patient acceptability and resistance of medical staff to apparently 'old-fashioned' techniques. Hence the case for maggot therapy is equivocal, and more basic research is required to understand why it appears to be effective in some circumstances and not in others.

7 Photodynamic therapy (photoactivated disinfection)

The therapeutic effects of light have been known for thousands of years and the combination of light with various chemicals again dates back to ancient times. It was only at the beginning of the 20th century, however, that the scientific basis of this phenomenon began to be explored. The use of photosensitizing agents to kill microorganisms was first shown in 1900 and it was shortly after this that the term *photodynamic therapy* (PDT) was coined. This chapter only discusses the use of this technique for the killing of microorganisms, although it should be pointed out that PDT has also been used to treat tumours using a combination of photosensitizing porphyrin compounds and laser light.

The most common photosensitizers used for their antimicrobial effects are the thiazine dyes methylene blue and toluidine blue O (also known as tolonium chloride; Figure 27.1). Both of these molecules carry a positive charge and this enables them to interact with the negatively charged outer surfaces of bacteria. In particular, they accumulate at the cytoplasmic membrane and when activated cause lethal damage to that target site. It has

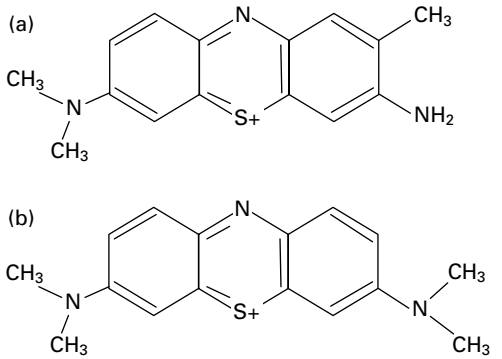

Figure 27.1 Structure of photosensitizers of the thiazine family of dyes: (a) toluidine blue O, (b) methylene blue.

been found that Gram-positive bacteria are more sensitive to PDT than Gram-negative bacteria, because of the different nature of their cell walls (Chapter 3).

The light sources used in the recent past have been lasers which produce light of a defined waveband designed to match the peak excitation wavelength of the photosensitizer used. Lasers also have the advantage of being able to be passed through thin, flexible fibres for use in difficult-to-access areas. More recently, advances in the development of light-emitting diodes (LEDs), particularly in respect of their increasing power, has enabled these to be used under some circumstances in place of lasers with obvious benefits of safety and cost. Toluidine blue O has an absorbance peak of ±11 nm around the maximum and so is suitable for use in conjunction with these systems.

A detailed discussion of the mechanism of antimicrobial action of light-activated photosensitizers is outside the scope of this chapter and the reader is referred to Wainwright (1998) for further information. However, in brief, the photosensitizer which is adsorbed on to the bacterial cell will, when illuminated, give rise to the production of toxic singlet oxygen which is very short-lived but highly destructive. This singlet oxygen is present only when the photosensitizer is excited by light and does not persist once the light is extinguished. The toxic effect is confined to bacteria and the photosensitizer does not appear to have any adverse effects on normal tissue.

PDT is essentially a highly efficient disinfection process which is harmless to surrounding tissues. The limitations of the system are associated with the ability of both the dye and the light to penetrate the matrix in which the bacteria are located. The main use for the technology is therefore to treat localized, surface infections which are not particularly amenable to systemic therapy. It has been used extensively for infections in dentistry, particularly root canal infections, periodontitis and caries, and devices are commercially available for use in dental surgeries. Applications have also been found in dermatology, ophthalmology and ENT infections. It seems likely that the range of conditions appropriate for this technology will grow in the future, although the nature of the process is such that it will be a niche market treating localized, difficult-to-manage infections.

8 Vaccines and immunotherapies

Vaccines to prevent both bacterial and viral diseases have been available for many years and in some cases their use predates that of antibiotics. In fact, for some diseases such as whooping cough and diphtheria they are still the most important form of infection control and highlight the saying that prevention is better than a cure. Table 27.5 illustrates the range of bacterial vaccines currently available in the UK and some of these such as diphtheria, tetanus, pertussis, meningococcal, haemophilus and BCG vaccines (alongside viral vaccines) are an integral part of the healthcare programme for the early years of life (Chapters 10 and 24).

The recent explosion of knowledge in the area of molecular biology has opened up new avenues for the development of effective vaccines. DNA and viral vector vaccines are examples of novel approaches currently being studied. A number of vaccine technologies have been explored recently aimed at *Ps. aeruginosa* which causes severe opportunistic infections of wounds, the respiratory tract, the cornea and the urinary tract. Some patients can be identified as being at particular risk of contracting infections caused by this pathogen and they include those with cystic fibrosis, burns patients and those who are in intensive care, especially if they are mechanically ventilated. These, and others, may therefore benefit from an effective vaccine with a view to preventing infection from occurring. A recent review has argued that from a scientific standpoint it is not a problem to design vaccines containing specific bacterial antigens and demonstrate their effectiveness in animals and preclinical trials. There have however, been issues with conducting meaningful clinical trials, due, among other things, to a lack of patients available at a given time to provide appropriate comparisons. Similar arguments have been put forward about the development of better vaccines to replace BCG for protection against tuberculosis. In this

Table 27.5 Examples of bacterial infections for which vaccines or immunotherapy are currently available (BNF 58, 2009)

Microorganism	Indication	Form
Bordetella pertussis	Whooping cough	Acellular vaccine containing highly purified components of *B. pertussis*. Whole cell vaccine used previously
Bacillus anthracis	Anthrax	Alum precipitate of antigens from *B. anthracis*
Clostridium tetani	Tetanus	Cell-free purified toxin adsorbed onto aluminium hydroxide or aluminium phosphate. Immunoglobulin also available
Corynebacterium diphtheriae	Diphtheria	Adsorbed toxin. Diphtheria antitoxin also available for passive immunization
Clostridium botulinum	Botulism	Trivalent botulism antitoxin
Haemophilus influenzae	Those at risk from invasive haemophilus infection	Capsular polysaccharide of *Haemophilus* type b. Conjugate vaccine (adsorbed).
Neisseria meningitidis	Meningitis	Capsular polysaccharide meningococcal group C conjugate vaccine
Mycobacterium bovis (BCG)	Tuberculosis	Live attenuated cells
Salmonella enterica serovar Typhi	Typhoid	Vi capsular polysaccharide antigen, also live attenuated cells
Streptococcus pneumoniae	Peumococcal infection	Polyvalent capsular antigens. Pneumococcal polysaccharide conjugate vaccine (adsorbed)
Vibrio cholerae	Cholera	Heat and formaldehyde inactivated whole cells and recombinant cholera toxin-B subunit

case the outcome of the investigation would not be known until the clinical trial had been in progress for many years.

9 Silver

It is perhaps not strictly appropriate to discuss the use of silver as an alternative to antibiotics, because silver functions really as an antiseptic biocide more like chlorhexidine or povidone-iodine. However, some reviews have discussed it in this context and so it is included here for completeness. Silver has of course been used over centuries to treat and prevent infections, and like some of the other forms of treatment described in this chapter, its use decreased with the advent of antibiotics. In the form of silver sulphadiazine, however, it has been a mainstay in the treatment of wound, particularly burn, infections for the last 40 years and this is likely to continue. In addition, the number of wound dressings containing silver as an antimicrobial component is increasing. Despite this, there are a limited number of conclusive clinical trials attesting to their efficacy and this situation certainly needs to be addressed.

Silver can generate a number of different ions, i.e. Ag^{o}, Ag^{+}, Ag^{2+} and Ag^{3+}. Elemental silver has no antibacterial activity and the principal active component is the Ag^{+} cation which forms when in contact with aqueous solutions. This ion has wide-ranging detrimental effects on the bacterial cell by disrupting membrane function and enzyme activity. There is activity against a broad range of bacterial pathogens including the important *Staph. aureus* and *Ps. aeruginosa*. Since the silver ion can be quenched by the presence of chloride ions, continued activity relies on the continuous release of further silver ions. Improved activity has been reported for the use of nanocrystalline silver which also appears to have an anti-inflammatory action. Despite its widespread use there are limited reports on the toxicity of silver and the incidence of resistance is currently low.

The worsening situation with regard to antibiotic resistance has brought all potential therapies into sharper

focus and silver is a candidate for increasing use, for example as a coating on medical devices such as urinary catheters and endotracheal tubes to prevent biofilm formation.

10 Bacteriophage therapy

Bacteriophages (phages) are viruses which specifically infect and kill bacteria, but they are unable to attack mammalian cells. In fact, their specificity is even more profound, in that a particular bacteriophage will only infect a given species of bacterium and often is even strain-specific. There are an estimated 10^{31} bacteriophages on the planet, and viral predation accounts for a significant part of carbon recycling in the oceans. These viruses have existed on earth in conjunction with bacteria for nearly 4 billion years and so are just as adept as their prey at changing with their environment.

The structure of a typical bacteriophage is shown in Figure 27.2. The genome is normally double-stranded DNA and this is enclosed within a protein capsid. At the tips of the tail fibres are receptors which recognize attachment sites on the bacterial cell surface. When the phage encounters a host bacterium it attaches via the tail fibres and injects its DNA into the cell. This DNA then takes over the cell's metabolic machinery and the cell devotes itself to the production of new virus particles (*virions*). When the process is complete the infected cell may contain some hundreds of progeny virions and these are liberated from the cell by the action of endolysins, which are virally encoded enzymes acting firstly on the cytoplasmic membrane and then on the peptidoglycan layer. The newly released viruses are then available to attack other host cells within the vicinity. Not all phages go through this lytic cycle. Following infection of the cell some viruses incorporate their DNA within the host cell DNA where it remains as a *prophage*; usually it causes no harm and each time the bacterial cell subsequently divides the viral DNA divides also. These are known as *temperate bacteriophages* and they induce in their host a state of *lysogeny*. This phenomenon can be responsible for conferring on the host cell additional virulence characteristics such as toxin production or resistance. At some later point in time the prophage may break free and embark on a lytic infection cycle similar to that described above.

Bacteriophages were first discovered at the beginning of the 20th century and it was quickly realized that they might have a use in the therapy of bacterial infections in humans. In the early days very little was known about phage biology, particularly their specificity and the presence of temperate phages. Despite this there was considerable success in treating gastrointestinal infections such as dysentery and cholera, wound infections and urinary

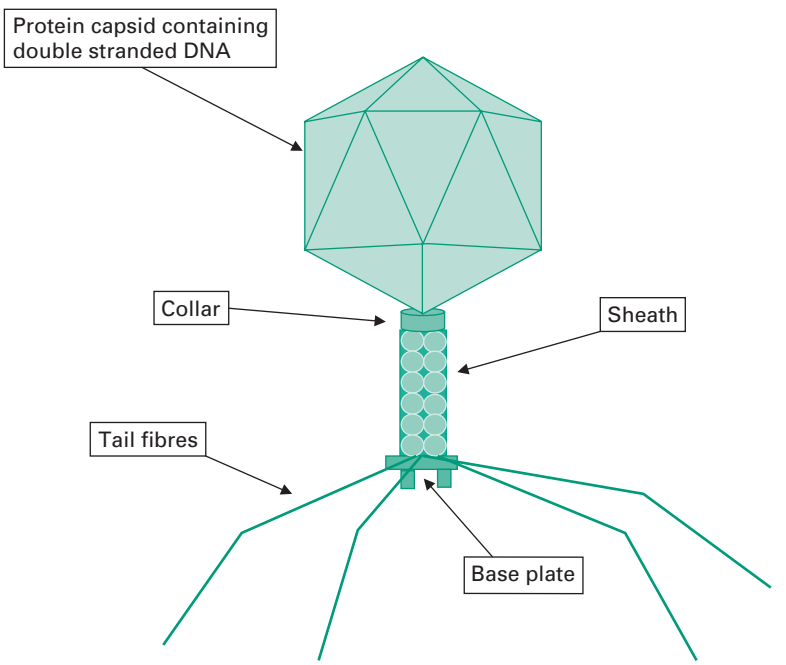

Figure 27.2 Diagram of a typical bacteriophage.

tract infections. This situation was short-lived, however, and as the number of workers in the field increased so the success rate decreased and toxicity issues primarily due to endotoxin release increased. With the emergence of antibiotics in the 1940s the use of phage as a therapy declined to virtually zero except in the former Soviet Union and parts of eastern Europe.

In the intervening period bacteriophages have been studied extensively, not as a means of treating infections but for their role in the molecular biology revolution. As a consequence, we now know a great deal about these viruses and are well placed to revisit their use in therapy. In addition, the former Soviet Union has continued to use phages to treat infections over the past 70 years and so has a vast amount of clinical knowledge available. Given the improved relationship between East and West since the breakdown of the Berlin Wall there is now a greater spirit of cooperation which could be harnessed to our benefit.

Bacteriophages offer a number of therapeutic advantages over the use of antibiotics:

- They are specific in their action, usually attacking only single species of bacteria. This has the advantage that only the infecting pathogen will be eliminated and the host microflora will remain intact. In order to broaden the spectrum of activity it is possible to use mixtures or cocktails of phages of different specificity.
- The mode of action of phages is such that antibiotic-resistant strains are just as susceptible as sensitive strains.
- The pharmacokinetic properties are unique in that after administration the number of phage particles will increase as the virus propagates and hence multiple dosing may be unnecessary.
- The phage can be formulated for administration into a wide range of pharmaceutical products and can be administered orally, topically, and even by injection.
- Very few incidences of side effects or allergic reactions have been reported.
- If resistance occurs then a range of other phages may be available for use, or, if not, it is a relatively simple exercise to obtain more virulent phages from environmental sources.
- Because phage therapy is a platform technology, the cost of bringing new phages to the clinic should not be expensive and the timescales should be short. The technology also lends itself for use in developing countries that do not have access to high-cost health care.

Care has to be taken in the selection of phages for therapy to ensure that they do not contain any toxin or virulence genes nor induce lysogeny.

There are hundreds of papers in the scientific literature describing the beneficial clinical effects of bacteriophages for the treatment of a wide range of bacterial infections. However, most of these emanate from the Soviet Union and are generally regarded to be of very poor quality. This situation needs to be addressed. Interest in phage therapy has increased dramatically in the West over the last 20 years and a number of companies are being formed in order to exploit the technology in the future. The regulatory authorities (including the Food and Drug Administration in the USA) have given approval for the use of bacteriophages in ready-to-eat food and a mixture of phages for the protection of cheeses from contamination by *Listeria monocytogenes* has been given GRAS (Generally Regarded As Safe) approval. Some companies have begun human clinical trials for the phage treatment of *Ps. aeruginosa* ear infections and for the eradication of MRSA from the nasal cavity. There are good indications that phages may play a greater role in the treatment of bacterial infections in the future.

11 Bacteriophage lysins

Bacteriophage lysins were described above as the means by which phages are released from the host cells they have infected. They are generally a two-component enzyme system consisting of *holins* which attack the cytoplasmic membrane and *endolysins* which then degrade the cell wall peptidoglycan. The latter are heat-stable enzymes capable of being isolated and purified, sometimes using recombinant techniques. When small quantities of these purified lysins are applied to cultures of bacteria they achieve extremely rapid lysis and have been reported to give greater than 6-log reduction in a matter of seconds. Characteristics such as this have led to the generation of great interest in these agents as possible alternatives to antibiotics.

The bacteriophage lysins are similar in some ways to enzymes such as lysozyme in that they are able to degrade intact peptidoglycan. This weakens the cell wall and the internal hydrostatic pressure causes the wall to rupture. Access to the peptidoglycan layer is critical, however, and although this is readily achieved in Gram-positive bacteria, the presence of an outer membrane in Gram-negative bacteria makes them resistant to the effects of these enzymes. An interesting feature of the endolysins is that they are specific for the bacteria from which they were generated, thus giving them the same specificity as the bacteriophages. This has some advantages in that the

normal microflora of the body will not be affected; they do not, however, have the self-replication characteristics of phages and so behave like an ordinary drug.

Most antibiotics are small molecules which are not usually immunogenic, but the endolysins are proteins with molecular weights in the region of 25–40 kDa which makes them potentially capable of stimulating an immune response. Animal experimentation has shown that when administered systemically the endolysins have a very short half-life because they are neutralized by the immune system. However, they are still able to produce a satisfactory antimicrobial effect because their action is so rapid.

An interesting idea which has been put forward is to use the endolysins for prophylaxis as a means of reducing the bioburden of mucous membranes. in particular eliminating potential pathogens such as *Staph. aureus* and *Strep. pneumoniae*. Both of these organisms are responsible for secondary bacterial respiratory tract infections following an initial viral infection. It is proposed that removal of these bacteria from the mucous membranes would significantly reduce the chances of secondary infection. The hypothesis has been supported by animal models but requires clinical trials for verification.

12 Conclusion

As the control of bacterial infections with antibiotics becomes ever more difficult we need now to explore the possible alternatives available to us. It is unrealistic to expect these to replace antibiotics and maybe the best we can hope for is strategies to augment their use. Some of the alternatives discussed above can be discounted fairly quickly but others may, if properly managed, become useful adjuncts to antibiotic therapy in the future.

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