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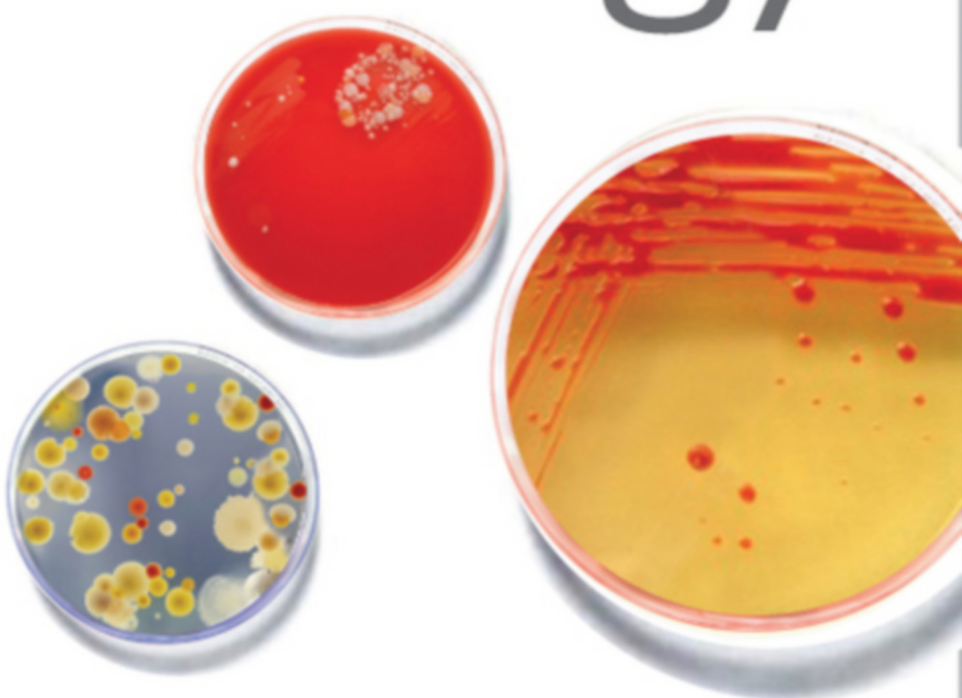
Hugo&Russell's Pharmaceutical Microbiology

8th Edition



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Stephen P Denyer
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Hugo and Russell's

Pharmaceutical Microbiology

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Hugo and Russell's
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

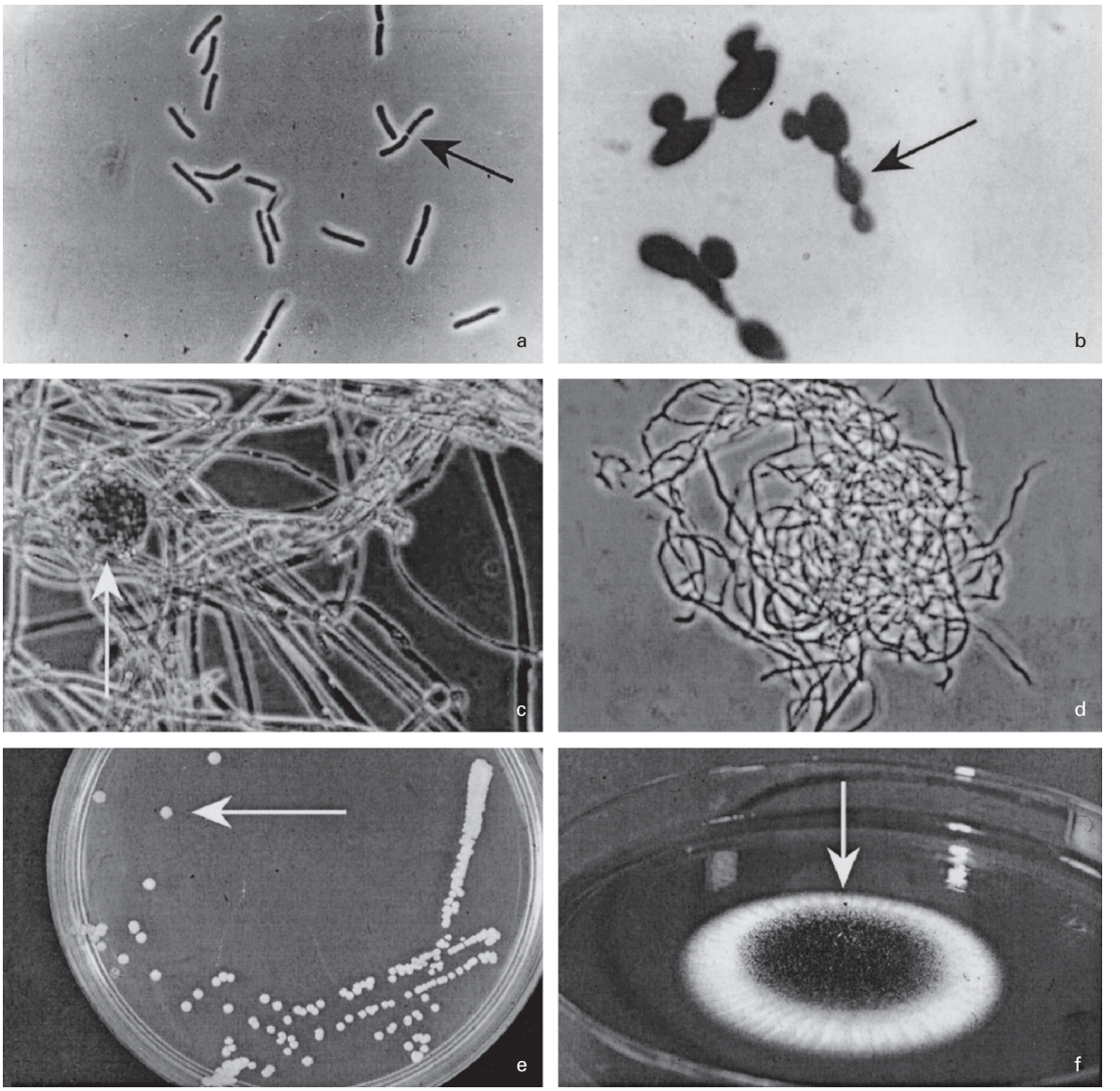

Figure 2.1 (a) A growing culture of *Bacillus megaterium* in which cells about to divide by binary fission display constrictions (arrowed) prior to separation. (b) A growing culture of the yeast *Saccharomyces cerevisiae* displaying budding (arrowed). (c) The mould *Mucor plumbeus* exhibiting the typical 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

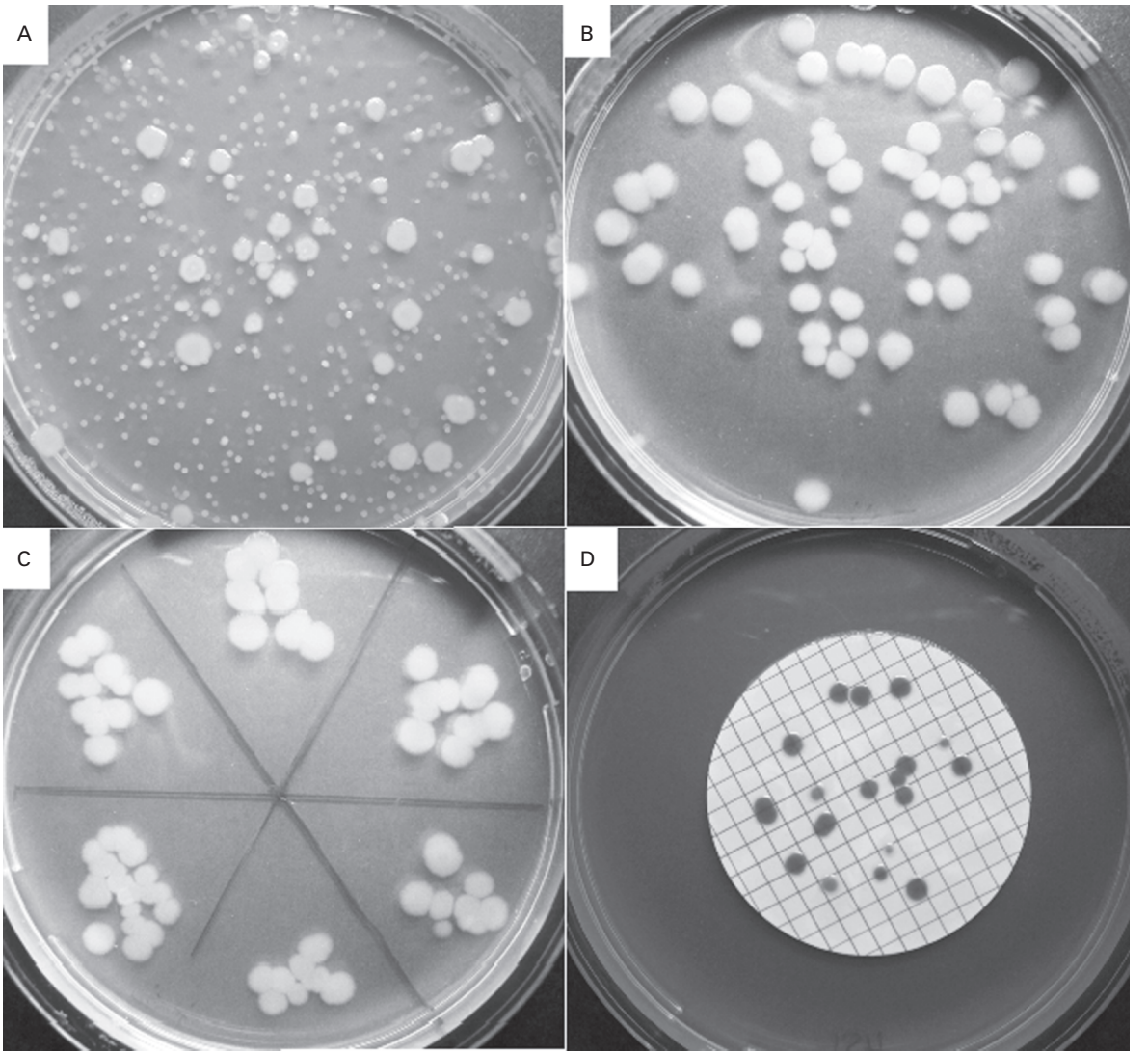

Figure 2.2 Viable counts of bacteria: (A) Pour plate method using *Bacillus subtilis*; the colonies on the surface of the agar are growing larger than those within the agar due to greater oxygen availability. (B) Surface spread, and (C) surface drop (Miles Misra) methods using *Bacillus subtilis*. (D)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