PART FIVE

PHARMACEUTICAL MICROBIOLOGY

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39
Fundamentals of microbiology

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CHAPTER CONTENTS

INTRODUCTION

Microorganisms are ubiquitous in nature and are vital components in the cycle of life. The majority are free-living organisms growing on dead or decaying matter whose prime function is the turnover of organic materials in the environment. Pharmaceutical microbiology, however, is concerned with the relatively small group of biological agents that cause human disease, spoil prepared medicines, or which can be used to produce compounds of medical interest.

In order to understand microorganisms more fully, scientists have grouped together living organisms of similar characteristics into taxonomic units. The most fundamental division is between prokaryotic and eukaryotic cells, which differ in a number of respects (Table 39.1) but particularly in the arrangement of their nuclear material. Eukaryotic cells contain chromosomes, which are separate from the cytoplasm and contained within a limiting nuclear membrane, i.e. they possess a true nucleus. Prokaryotic cells do not possess a true nucleus and their nuclear material is free within the cytoplasm, although it may be aggregated into discrete areas called nuclear bodies. Prokaryotic organisms make up the lower forms of life and include Eubacteria and Archaeobacteria. Eukaryotic cell types embrace all the higher forms of life, of which only the fungi will be dealt with in this chapter.

One characteristic shared by all microorganisms is the fact that they are small; however, it is a philosophical argument whether all infectious agents can be regarded as living. Some are little more than simple chemical entities incapable of any free-living existence. Viroids, for example, are small circular, single-stranded RNA molecules not complexed with protein. One particularly well studied viroid has only 359 nucleotides (one-10th the size of the smallest known virus) and yet causes a disease in potatoes. Prions are small, self-replicating proteins devoid of any nucleic acid. The prion associated with Creutzfeld–Jakob disease in humans, scrapie in sheep and bovine spongiform encephalitis in cattle has only 250 amino acids and is highly resistant to inactivation by normal sterilization procedures.

Viruses are more complex than viroids or prions, possessing both protein and nucleic acid. Despite being among the most dangerous infectious agents known, they are still not regarded as living. Table 39.2 shows the major groups of viruses infecting humans.

Table 39.1 Differences between prokaryotic and eukaryotic organisms

Structure	Prokaryotes	Eukaryates
Cell wall structure	Usually contains peptidoglycan	Peptidoglycan absent
Nuclear membrane	Absent	Present. Possess a true nucleus
Nucleolus	Absent	Present
Number of chromosomes	One	More than one
Mitochondria	Absent	Present
Mesosomes	Present	Absent
Ribosomes	70S	80S

VIRUSES

Viruses are obligate intracellular parasites with no intrinsic metabolic activity, being devoid of ribosomes and energy-producing enzyme systems. They are thus incapable of leading an independent existence and cannot be cultivated on cell-free media, no matter how nutritious. The size of human viruses ranges from the largest poxviruses, measuring about 300 nm (1 nm = 10^{-9} m), to the picornaviruses, such as the poliovirus which is approximately 20 nm. When one considers that a bacterial coccus measures 1000 nm in diameter it can be appreciated that only the very largest virus particles may be seen under the light microscope, and electron microscopy is required for visualizing the majority. It will also be apparent that few of these viruses are large enough to be retained on the (0.2 μm) membrane filters used to sterilize thermolabile liquids.

Viruses consist of a core of nucleic acid (either DNA, as in vaccinia virus, or RNA as in poliovirus) surrounded by a protein shell or capsid. Most DNA viruses have linear double-stranded DNA, but in the case of the parvovirus it is single stranded. The majority of RNA-containing viruses contain one molecule of single-stranded RNA, although in reoviruses it is double stranded. The protein capsid comprises 50–90% of the weight of the virus, and as nucleic acid can only synthesize approximately 10% its own weight of protein the capsid must be made up of a number of identical protein molecules. These individual protein units are called capsomeres and are not in themselves symmetrical, but are arranged around the nucleic acid core in characteristic sym-

Table 39.2 The major groups of viruses that infect humans

Family	Capsid	Nucleic acid	Envelope	Example
Adenoviridae	Icosahedral	dsDNA	No	Human adenovirus
Arenaviridae	Helical	ssRNA	Yes	Lassa fever virus
Flaviviridae	Icosahedral	ssRNA	Yes	Yellow fever virus Hepatitis C virus
Hepadnaviridae	Icosahedral	dsDNA	No	Hepatitis B virus
Herpesviridae	Icosahedral	dsDNA	Yes	Herpes simplex virus Cytomegalovirus Varicella zoster virus
Orthomyxoviridae	Helical	ssRNA	Yes	Influenza virus
Papoviridae	Icosahedral	dsDNA	No	Papillomavirus
Paramyxoviridae	Helical	ssRNA	Yes	Respiratory syncytial virus Measles virus Mumps virus
Picornaviridae	Icosahedral	ssRNA	No	Rhinovirus Poliovirus Coxsackie virus
Poxviridae	Complex	dsDNA	Yes	Molluscum contagiosum Vaccinia virus Variola virus
Reoviridae	Icosahedral	dsRNA	No	Rotavirus Colorado tick fever virus
Retroviridae	Icosahedral	ssRNA	Yes	HIV
Rhabdoviridae	Helical	ssRNA	Yes	Rabies virus
Togaviridae	Icosahedral	ssRNA	Yes	Rubella virus

metrical patterns. Additionally, many of the larger viruses possess a lipoprotein envelope surrounding the capsid arising from the membranes within the host cell. In many instances the membranes are virus modified to produce projections outwards from the envelope, such as haemagglutinins or neuraminidase. The enveloped viruses are often called ether sensitive, as ether and other organic solvents may dissolve the membrane.

The arrangement of the capsomeres can be of a number of types:

- Helical – the classic example is tobacco mosaic virus (TMV), which resembles a hollow tube with capsomeres arranged in a helix around the central nucleic acid core. Other examples include mumps and influenza virus.
- Icosahedral – these often resemble spheres on cursory examination, but when studied more closely they are made up of icosahedra that have 20 triangular faces, each containing an identical number of capsomeres. Examples include the poliovirus and adenovirus.
- Complex – the poxviruses and bacterial viruses (bacteriophages) make up a group whose members have a geometry that is individual and complex.

Reproduction of viruses

Because viruses have no intrinsic metabolic capability they require the functioning of the host cell machinery in order to manufacture and assemble new virus particles. The replication of viruses within host cells can be broken down into a number of stages.

Adsorption to host cell

The first step in the infection process involves virus adsorption on to the host cell. This usually occurs via an interaction between protein or glycoprotein moieties on the virus surface with specific receptors on the host cell outer membrane. Different cells possess receptors for different viruses.

Penetration

Enveloped viruses fuse the viral membrane with the host cell membrane and release the nucleocapsid directly into the cytoplasm. Naked virions generally penetrate the cell by phagocytosis.

Uncoating

In this stage the capsid is removed as a result of attack by cellular proteases, and this releases the nucleic acid into the cytoplasm. These first three stages are similar for both DNA and RNA viruses.

Nucleic acid and protein synthesis

The detailed mechanisms by which DNA- and RNA-containing viruses replicate inside the cell is outside the scope of this chapter and the reader is referred to the bibliography for further information. After nucleic acid replication early viral proteins are produced, the function of which is to switch off host cell metabolic activity and direct the activities of the cell towards the synthesis of proteins necessary for the assembly of new virus particles.

Assembly of new virions

Again, there are differences in the detail of how the viruses are assembled within the host cell, but construction of new virions occurs at this stage and up to 100 new virus particles may be produced per cell.

Release of virus progeny

The newly formed virus particles may be liberated from the cell as a burst, in which case the host cell ruptures and dies. Infection with influenza virus results in a lytic response. Alternatively, the virions may be released gradually from the cell by budding of the host cell plasma membrane. These are often called 'persistent' infections, and an example is hepatitis B.

Latent infections

In some instances a virus may enter a cell but not go through the replicative cycle outlined above and the host cell may be unharmed. The genome of the virus is conserved and may become integrated into the host cell genome, where it may be replicated along with the host DNA during cell division. At some later stage the latent virus may become reactivated and progress through a lytic phase, causing cell damage/ death and the release of new virions. Examples of this type of infection are those which occur with the herpes simplex viruses, associated with cold sores, genital herpes and also chicken pox, where the dormant virus may reactivate to give shingles later in life.

Oncogenic viruses

Oncogenic viruses have the capacity to transform the host cell into a cancer cell. In some cases this may lead to relatively harmless, benign growths, such as warts caused by papovavirus, but in other cases more severe, malignant tumours may arise. Cellular transformation may result from viral activation or mutation of normal host genes called proto-oncogenes, or the insertion of viral oncogenes.

Bacteriophages

Bacteriophages (phages) are viruses that attack bacteria but not animal cells. It is generally accepted that the interaction between phage and bacterium is highly specific, and there is probably at least one phage for each species of bacterium. In many cases the infection of a bacterial cell by a phage results in lysis of the bacterium, and such phages are termed virulent. Some phages, however, can infect a bacterium without causing lysis, and in this case the phage DNA becomes incorporated within the bacterial genome. The phage DNA can then be replicated along with the bacterial cell DNA, and this is then termed a prophage. Bacterial cells carrying a prophage are called lysogenic and phages capable of inducing lysogeny are called temperate. Occasionally some of the prophage genes may be expressed, and this will confer on the bacterial cell the ability to produce new proteins. The ability to produce additional proteins as a result of prophage DNA is termed lysogenic conversion.

ARCHAEOBACTERIA

Archaeobacteria are a fascinating group of prokaryotic microorganisms which are frequently found living in hostile environments. They differ in a number of respects from Eubacteria, particularly in the composition of their cell walls. They comprise methane producers, sulphate reducers, halophiles and extreme thermophiles. However, they are of little significance from a pharmaceutical or clinical standpoint and so will not be considered further.

EUBACTERIA

Eubacteria constitute the major group of prokaryotic cells that are of pharmaceutical and clinical significance. They cover a diverse range of microorganisms, from the primitive parasitic rickettsias that share some of the characteristics of viruses, through the more typical free-living bacteria to the branching, filamentous actinomycetes, which at first sight resemble fungi rather than bacteria.

Atypical bacteria

Rickettsiae

The family Rickettsiaceae includes three clinically important genera, *Rickettsia, Coxiella* and *Bartonella*. Although these are prokaryotic cells they differ from most other bacteria both in their structure and in the fact that the majority of species lead an obligate intracellular existence. This means that, with a few exceptions, they cannot be grown on cell free media, although unlike many viruses they do possess some independent enzymes. They have a pleomorphic appearance, ranging from coccoid through to rod-shaped cells, and multiplication is by binary fission. Their cell wall composition bears similarities to that of Gram-negative bacteria and in general they stain this way. The genus *Rickettsia* has a number of species that give rise to human diseases, in particular epidemic typhus (*R. prowazekii*), murine typhus (*R. typhi*) and spotted fevers (various species). These are characterized by transmission via bites from insect vectors, particularly ticks, fleas and lice. *Coxiella burnetii* is the only species in the genus *Coxiella* and this gives rise to a disease called Q fever. Although the source of the disease is infected animals, usually no insect vector is involved and the most common route of transmission is by inhalation of infected dust. *Bartonella quintana* is the causative agent of trench fever which, as the name suggests, occurs typically under conditions of war and deprivation. Each of the infections described here can be treated with doxycycline, although the duration of therapy may vary depending upon the nature of the disease and its severity.

Chlamydiae

These are obligate intracellular parasitic bacteria that possess some independent enzymes but lack the ability to generate ATP. Two cellular forms are identified: a small (0.3 μm) highly infectious elementary body which, after infection, enlarges to give rise to the replicative form called the initial or reticulate body (0.8–1.2 μm). These divide by binary fission within membrane-bound vesicles in the cytoplasm of infected cells. Insect vectors are not required for the transmission of infection. Chlamydiae lack peptidoglycan in their cell walls and have weak Gram-negative characteristics. *Chlamydia trachomatis* is the most important member of the group, being responsible for the disease trachoma, characterized by inflammation of the eyelids, which can lead to scarring of the cornea. This is the most common cause of infectious blindness world wide, and it is estimated that 400 million people are infected, with at least 6 million totally blind. The same species is also recognized as one of the major causes of sexually transmitted disease. *C. psittaci* and *C. pneumoniae* are responsible for respiratory tract infections. Chlamydial infections are responsive to treatment by tetracyclines, either topical or systemic as appropriate.

Mycoplasmas

The mycoplasmas are a group of very small (0.3–0.8 μm) prokaryotic microorganisms which are capable of growing on cell-free media but which lack cell walls. The cells are surrounded by a double-layered plasma membrane that contains substantial amounts of phospholipids and sterols. This structure has no rigidity owing to the absence of peptidoglycan, and so the cells are susceptible to osmotic lysis. The lack of peptidoglycan is also the reason for these bacteria being resistant to the effects of cell wall-acting antibiotics such as the penicillins, and also the enzyme lysozyme. Members of this group are pleomorphic, varying in shape from coccoid to filamentous. Most are facultative anaerobes capable of growth at 35°C, and on solid media produce colonies with a characteristic 'fried egg' appearance. They contain a number of genera, of which the most important from a clinical point of view are *Mycoplasma* and *Ureaplasma*. *M. pneumoniae* is a major cause of respiratory tract infections in children and young adults, whereas *U. urealyticum* has been implicated in non-specific genital infections. Despite being resistant to the β-lactam antibiotics, these infections can be effectively treated using either tetracyclines or erythromycin.

Actinomycetes

Many of the macroscopic features of the actinomycetes are those that are more commonly found

among the filamentous fungi, but they are indeed prokaryotic cells. They are a diverse group of Gram-positive bacteria morphologically distinguishable from other bacteria because they have a tendency to produce branching filaments and reproductive spores. *Nocardia* contain a number of species that have been shown to be pathogenic to humans, but they occur principally in tropical climates. Reproduction in this genus is by fragmentation of the hyphal strands into individual cells, each of which can form a new mycelium. The genus *Streptomyces* contains no human pathogens, but most species are saprophytic bacteria found in the soil. They are aerobic microorganisms producing a non-fragmenting, branching mycelium that may bear spores. The reason for their pharmaceutical importance is their ability to produce a wide range of therapeutically useful antibiotics, including streptomycin, chloramphenicol, oxytetracycline, erythromycin and neomycin.

Typical bacteria

Shape, size and aggregation

Bacteria occur in a variety of shapes and sizes, determined not only by the nature of the organisms themselves but also by the way in which they are grown (Fig. 39.1). In general, bacterial dimensions lie in the range 0.75–5 μm (1000 μm = 1 mm). The most common shapes are the sphere (coccus) and the rod (bacillus). Some bacteria grow in the form of rods with a distinct curvature, e.g. vibrios are rod-shaped cells with a single curve resembling a comma, whereas a spirillum possesses a partial rigid spiral; spirochaetes are longer and thinner and exhibit a number of turns, and are also more flexible. Rod-shaped cells occasionally grow in the form of chains but this is dependent upon growth conditions rather than a characteristic of the species. Cocci, however, show considerable variation in aggregation, which is characteristic of the species. The plane of cell division and the strength of adhesion of the cells determine the extent to which they aggregate after division. Cocci growing in pairs are called diplococci, those in four tetrads, and in groups of eight sarcina. If a chain of cells is produced resembling a string of beads this is termed a streptococcus, whereas an irregular cluster similar in appearance to a bunch of grapes is called a staphylococcus. In many cases this is sufficiently characteristic to give rise to the name of the bacterial genus, e.g. *Staphylococcus aureus, Streptococcus pneumoniae.*

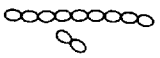
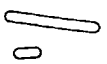
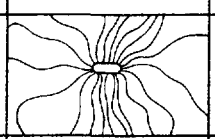
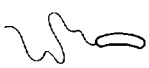
Genus		Approximate dimensions (μm)
Staphylococcus Irregular clusters of spherical cells. Resemble bunch of grapes. Non-motile		0.5–1.5
Streptococcus Spherical or ovoid cells occurring in pairs or in chains. Non-motile		<2.0
Neisseria Small Gram negative cocci. Occur in pairs with adjacent sides flattened. Non-motile		0.6–1.0
Lactobacillus Shape variable between long and slender to short coccobacillus. Non-motile, chain formation common		0.5–0.8 × 2–9
Escherichia Short rods, motile by peritrichous flagella		1.1–1.5 × 2–6
Bacillus Large endospore-forming rods. Motile by lateral flagella (not shown). Gram-positive		0.3–2.2 × 1.2–7.0
Vibrio Short curved or straight rods. Sometimes 'S' shaped. Motile by single polar flagellum		0.5 × 1.5–3.0
Spirochaeta Thin, flexible, helically coiled cells. Motile, possess axial fibrils (not shown)		0.2–0.75 × 5–500
Spirillum Long, slender cells in rigid spirals. Number of turns varies. Motile, bipolar flagellation		0.2–1.7 × 0.5–60
Streptomyces Slender, non-septate branching filaments. Form reproductive spores. Non-motile		0.5–2.0 (diameter)

Fig. 39.1 Morphology of different bacterial genera.

Anatomy

Figure 39.2 is a diagrammatic representation of a typical bacterial cell. The various components are described below.

Capsule Many bacteria produce extracellular polysaccharides, which may take the form of either a

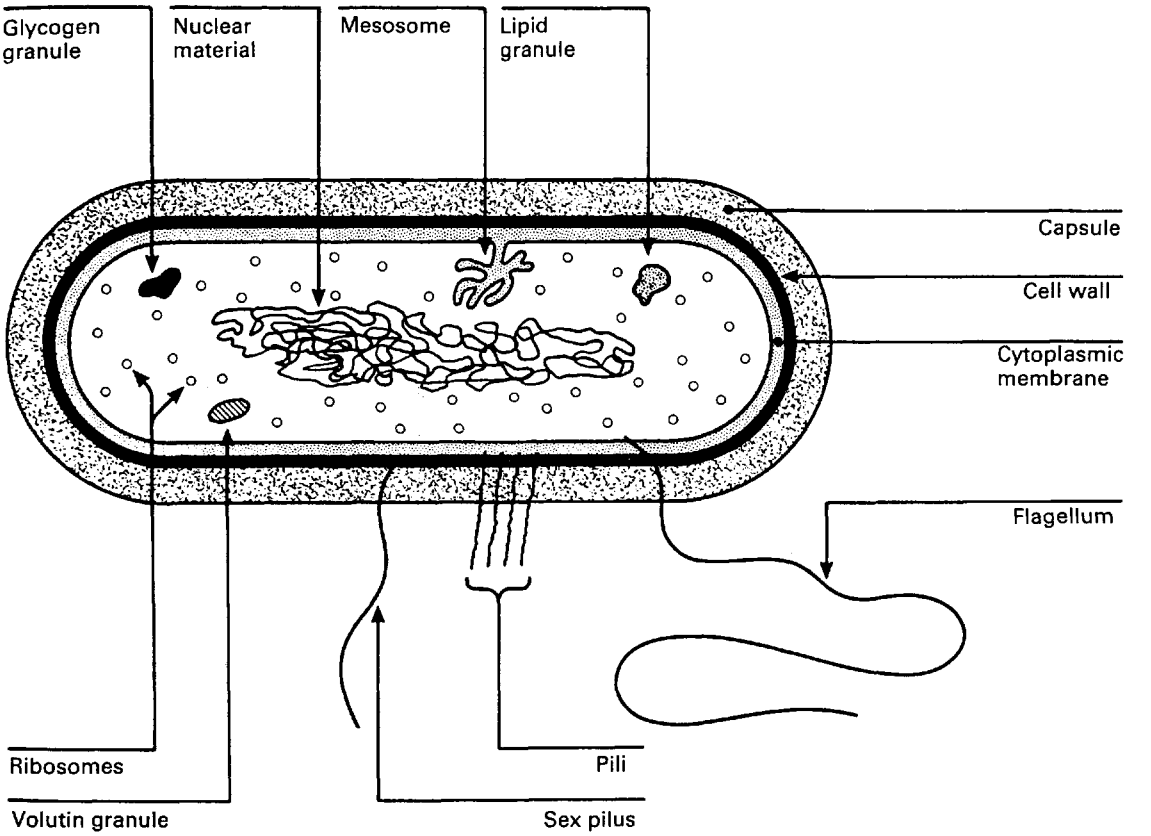


Fig. 39.2 Diagrammatic representation of a typical bacterial cell.

discrete capsule, firmly adhered to the cell, or a more diffuse layer of slime. Not all bacteria produce a capsule, and even those that can will only do so under certain circumstances. Many encapsulated pathogens, when first isolated, give rise to colonies on agar which are smooth (S), but subculturing leads to the formation of rough colonies (R). This S → R transition is due to loss in capsule production. Reinoculation of the R cells into an animal results in the resumption of capsule formation, indicating that the capacity has not been lost irrevocably.

The function of the capsule is generally regarded as protective, as encapsulated cells are more resistant to disinfectants, desiccation and phagocytic attack. In some organisms, however, it serves as an adhesive mechanism, for example *Streptococcus mutans* is an inhabitant of the mouth that metabolizes sucrose to produce a polysaccharide capsule enabling the cell to adhere firmly to the teeth. This is the initial step in the formation of dental plaque, which is a complex array of microorganisms and organic matrix that adheres to the teeth and ultimately leads to decay. The substitution of sucrose by glucose prevents capsule formation and hence eliminates plaque.

A similar picture emerges with *Staph. epidermidis*. This bacterium forms part of the normal microflora of the skin and until recently was thought of as non-pathogenic. With the increased usage of indwelling medical devices coagulase-negative staphylococci, in particular *Staph. epidermidis*, have emerged as the major cause of device-related infections. The normal microbial flora have developed the ability to produce extracellular polysaccharide, which enables the cells to form resistant biofilms attached to the devices. These biofilms are very difficult to eradicate and have profound resistance to antibiotics and disinfectants. It is now apparent that the dominant mode of growth for aquatic bacteria is not planktonic (free swimming) but sessile, i.e. attached to surfaces and covered with protective extracellular polysaccharide or glycocalyx.

Cell wall Bacteria can be divided into two broad groups by the use of the Gram staining procedure (see later for details), which reflects differences in cell wall structure. The classification is based upon

the ability of the cells to retain the dye methyl violet after washing with a decolourizing agent such as absolute alcohol. Gram-positive cells retain the stain whereas Gram-negative cells do not. As a *very rough* guide, the majority of small rod-shaped cells are Gram negative. Most large rods, such as the Bacillaceae, lactobacilli and actinomycetes, are Gram positive. Similarly, most cocci are Gram positive, although there are notable exceptions, such as the Neisseriaceae.

Bacteria are unique in that they possess peptidoglycan in their cell walls. This is a complex molecule with repeating units of *N*-acetylmuramic acid and *N*-acetylglucosamine (Fig. 39.3). This extremely long molecule is wound around the cell and crosslinked by polypeptide bridges to form a structure of great rigidity. The degree and nature of crosslinking vary between bacterial species. It imparts to the cell its characteristic shape and has principally a protective function. Peptidoglycan (also called murein or mucopeptide) is the site of action of a number of antibiotics, such as penicillin, bacitracin, vancomycin and cycloserine. The enzyme lysozyme is also capable of hydrolysing the β, 1–4 linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine.

Figure 39.4 shows simplified diagrams of a Gram-positive and a Gram-negative cell wall. The Gram-positive cell wall is much simpler in layout, containing peptidoglycan interspersed with teichoic acid polymers. These latter are highly antigenic but do not provide structural support. Functions attributed to teichoic acids include the regulation of enzyme activity in cell wall synthesis, sequestration

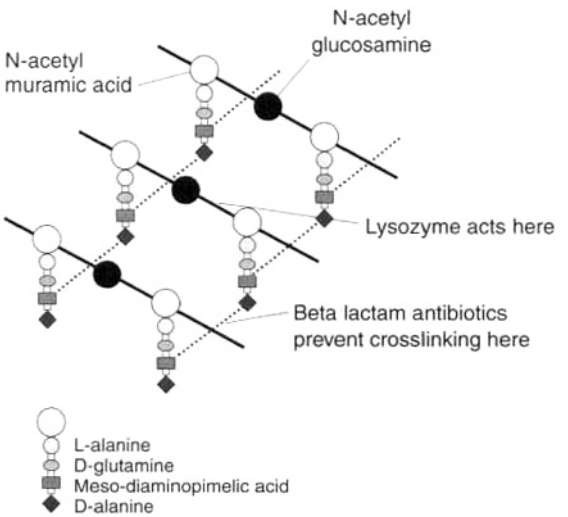


Fig. 39.3 Peptidoglycan

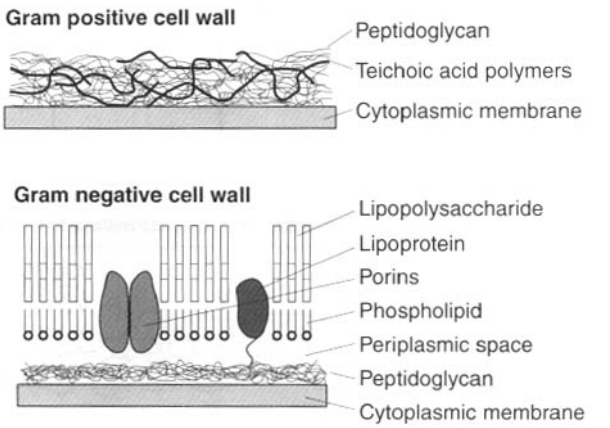


Fig. 39.4 Structural components of bacterial cell walls.

of essential cations, cellular adhesion, and mediation of the inflammatory response in disease. In general, proteins are not found in Gram-positive cell walls. Gram-negative cell walls are more complex, comprising a much thinner layer of peptidoglycan surrounded by an outer bilayered membrane. This outer membrane acts as a diffusional barrier and is the main reason why many Gram-negative cells are much less susceptible to antimicrobial agents than are Gram-positive cells. The lipopolysaccharide component of the outer membrane can be shed from the wall upon cell death. It is a highly heat-resistant molecule known as endotoxin, which has a number of toxic effects on the human body, including fever and shock. For this reason it is important that solutions for injection or infusion are not just sterile but are also free from endotoxins.

Cytoplasmic membrane The cytoplasmic membranes of most bacteria are very similar and are composed of protein, lipids, phospholipids and a small amount of carbohydrate. The components are arranged into a bilayer structure with a hydrophobic interior and a hydrophilic exterior. The cytoplasmic membrane has a variety of functions:

- It serves as an osmotic barrier.
- It is selectively permeable and is the site of carrier-mediated transport.
- It is the site of ATP generation and cytochrome activity.
- It is the site of cell wall synthesis.
- It provides a site for chromosome attachment.

The cytoplasmic membrane has very little tensile strength and the internal hydrostatic pressure of up to 20 bar forces it firmly against the inside of the cell wall. Treatment of bacterial cells with lysozyme may remove the cell wall and, as long as the conditions are

isotonic, the resulting cell will survive. These cells are called protoplasts and, as the cytoplasmic membrane is now the limiting structure, the cell assumes a spherical shape. Protoplasts of Gram-negative bacteria are difficult to obtain because the layer of lipopolysaccharide protects the peptidoglycan from attack. In these cases mixtures of EDTA and lysozyme are used and the resulting cells, which still retain fragments of cell envelope, are termed spheroplasts.

Nuclear material The genetic information necessary for the functioning of the cell is contained within a single circular molecule of double-stranded DNA. When unfolded this would be about 1000 times as long as the cell itself, and so exists within the cytoplasm in a considerably compacted state. It is condensed into discrete areas called chromatin bodies, which are not surrounded by a nuclear membrane. Rapidly dividing cells may contain more than one area of nuclear material, but these are copies of the same chromosome, not different chromosomes, and arise because DNA replication proceeds ahead of cell division.

Mesosomes These are irregular invaginations of the cytoplasmic membrane which are quite prominent in Gram-positive bacteria but less so in Gram-negative bacteria. They are thought to have a variety of functions, including cross-wall synthesis during cell division and furnishing an attachment site for nuclear material, facilitating the separation of segregating chromosomes during cell division. They have also been implicated in enzyme secretions and may act as a site for cell respiration.

Ribosomes The cytoplasm of bacteria is densely populated with ribosomes, which are complexes of RNA and protein in discrete particles 20 nm in diameter. They are the sites of protein synthesis within the cell and the numbers present reflect the degree of metabolic activity of the cell. They are frequently found organized in clusters called polyribosomes or polysomes. Prokaryotic ribosomes have a sedimentation coefficient of 70S, compared to 80S ribosomes of eukaryotic cells. This distinction aids the selective toxicity of a number of antibiotics. The 70S ribosome is made up of RNA and protein and can dissociate into one 30S and one 50S subunit.

Inclusion granules Certain bacteria tend to accumulate reserves of materials after active growth has ceased, and these become incorporated within the cytoplasm in the form of granules. The most common are glycogen granules, volutin granules (containing polymetaphosphate) and lipid granules (containing poly β-hydroxybutyric acid). Other granules, such as sulphur and iron, may also be found in the more primitive bacteria.

Flagella A flagellum is made up of protein called flagellin and it operates by forming a rigid helix that turns rapidly like a propeller. This can propel a motile cell up to 200 times its own length in 1 second. Under the microscope bacteria can be seen to exhibit two kinds of motion, swimming and tumbling. When tumbling the cell stays in one position and spins on its own axis, but when swimming it moves in a straight line. Movement towards or away from a chemical stimulus is referred to as chemotaxis. The flagellum arises from the cytoplasmic membrane and is composed of a basal body, hook and filament. The number and arrangement of flagella depends upon the organisms and varies from a single flagellum (monotrichous) to a complete covering (peritrichous).

Pili (fimbriae) These are smaller than flagella and are not involved in motility. A number of different types of pili have been identified, of which the most important are the common pili and the F-pili. The common pili are found all over the surface of certain bacteria and are believed to be associated with adhesiveness and pathogenicity. They are also antigenic. F-pili are larger and of a different structure to common pili, and are involved in the transfer of genetic information from one cell to another. This is of major importance in the transfer of drug resistance between cell populations.

Endospores Under conditions of specific nutrient deprivation some genera of bacteria, in particular *Bacillus* and *Clostridium*, undergo a differentiation process and change from an actively metabolizing vegetative form to a resting spore form. The process of sporulation is not a reproductive mechanism as found in certain actinomycetes and filamentous fungi, but serves to enable the organism to survive periods of hardship. A single vegetative cell differentiates into a single spore. Subsequent encounter with favourable conditions results in germination of the spore and the resumption of vegetative activities.

Endospores are very much more resistant to heat, disinfectants, desiccation and radiation than are vegetative cells, making them difficult to eradicate from foods and pharmaceutical products. Heating at 80°C for 10 minutes would kill most vegetative bacteria, whereas some spores will resist boiling for several hours. The sterilization procedures now routinely used for pharmaceutical products are thus designed specifically with reference to the destruction of the bacterial spore. The mechanism of this extreme heat resistance was a perplexing issue for many years, and at one time it was thought to be due to the presence of a unique spore component, dipicolinic acid (DPA). This compound is found

only in bacterial spores, where it is associated in a complex with calcium ions. The isolation of heat-resistant DPA-less mutants, however, led to the demise of this theory. Spores do not have water content appreciably different from that of vegetative cells, but the distribution within the different compartments is unequal and this is thought to generate the heat resistance. The central core of the spore houses the genetic information necessary for growth after germination, and this becomes dehydrated by expansion of the cortex against the rigid outer protein coats. Water is thus squeezed out of the central core. Osmotic pressure differences also help to maintain this water imbalance.

The sequence of events involved in sporulation is illustrated in Figure 39.5 and is a continuous process, although for convenience it is divided into six stages. The complete process takes about 8 hours, although this may vary depending on the species and the conditions used. Occurring simultaneously with the morphological changes are a number of biochemical events that have been shown to be associated with specific stages and occur in an exact sequence. One important biochemical event is the production of antibiotics. Peptides possessing antimicrobial activity have been isolated from the majority of *Bacillus* species and many of these have found pharmaceutical applications. Examples of

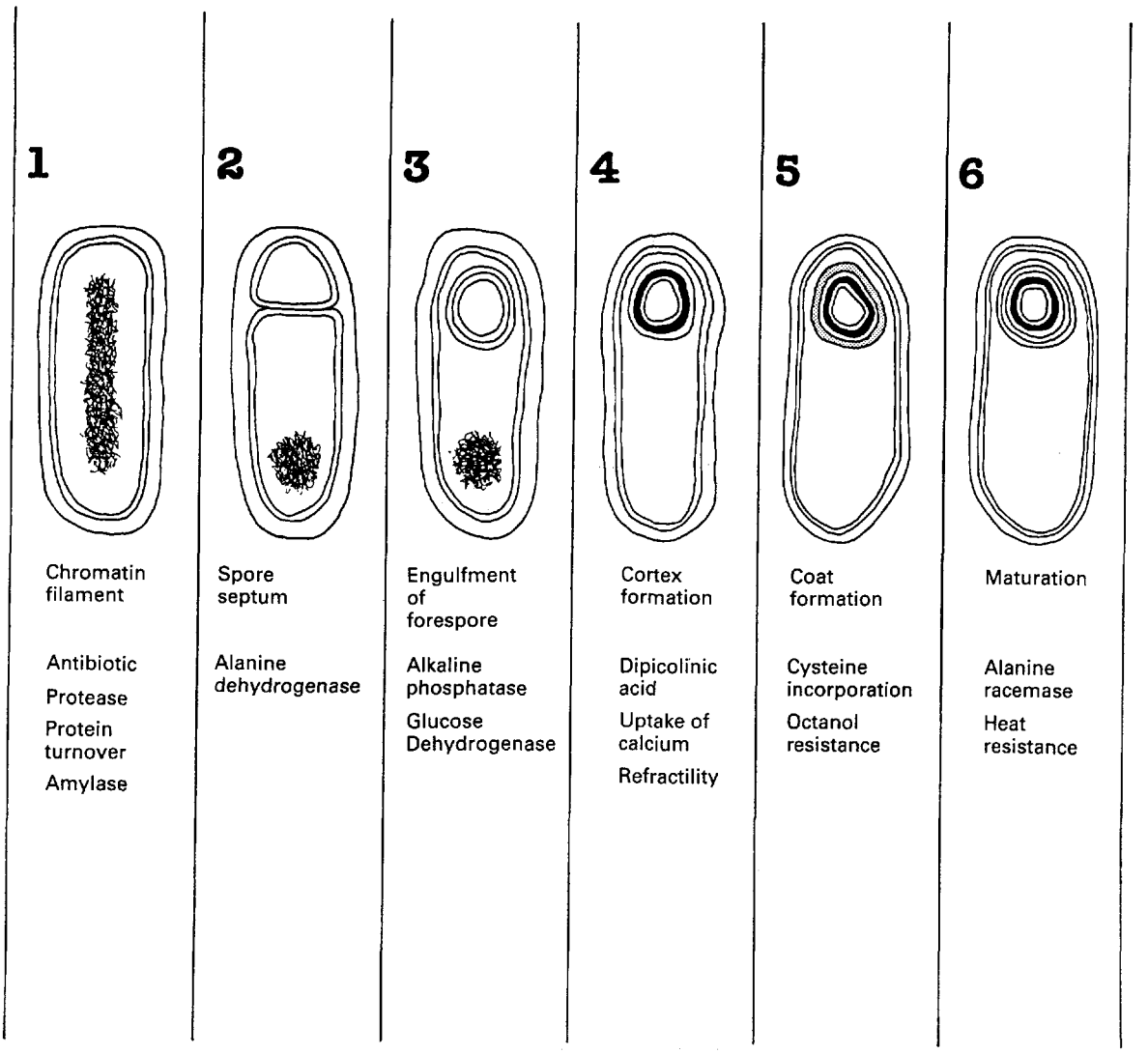


Fig. 39.5 Morphological and biochemical changes during spore formation.

antibiotics include bacitracin, polymyxin and gramicidin. Similarly, the proteases produced by *Bacillus* species during sporulation are used extensively in a wide variety of industries.

Microscopy and staining of bacteria

Bacterial cells contain about 80% water by weight and this results in a very low refractility, i.e. they are transparent when viewed under ordinary transmitted light. Consequently, in order to visualize bacteria under the microscope the cells must be killed and stained with some compound that scatters the light or, if live preparations are required, special adaptations must be made to the microscope. Such adaptations are found in phase-contrast, dark-ground and differential-interference contrast microscopy.

The microscopic examination of fixed and stained preparations is a routine procedure in most laboratories but it must be appreciated that not only are the cells dead, but they may also have been altered morphologically by the often quite drastic staining process. The majority of stains used routinely are basic dyes, i.e. the chromophore has a positive charge and this readily combines with the abundant negative charges present both in the cytoplasm in the form of nucleic acids and on the cell surface. These dyes remain firmly adhered even after washing with water. This type of staining is called simple staining and all bacteria and other biological material are stained the same colour. Differential staining is a much more useful process as different organisms or even different parts of the same cell can be stained distinctive colours.

To prepare a film ready for staining the glass microscope slide must be carefully cleaned to remove all traces of grease and dust. If the culture of bacteria is in liquid form then a loopful of suspension is transferred directly to the slide. Bacteria from solid surfaces require suspension with a small drop of water on the slide to give a faintly turbid film. A common fault with inexperienced workers is to make the film too thick. The films must then be allowed to dry in air. When thoroughly dry the film is fixed by passing the back of the slide through a small Bunsen flame until the area is just too hot to touch on the palm of the hand. The bacteria are killed by this procedure and also stuck on to the slide. Fixing also makes the bacteria more permeable to the stain and inhibits lysis. Chemical fixation is commonly carried out using formalin or methyl alcohol: this causes less damage to the specimen but tends to be used principally for blood films and tissue sections.

Differential stains A large number of differential stains have been developed and the reader is referred to the bibliography for more details. Only a few of those available will be discussed here.

Gram's stain By far the most important in terms of use and application is the Gram stain, developed by Christian Gram in 1884 and subsequently modified. The fixed film of bacteria is flooded initially with a solution of methyl violet. This is followed by a solution of Gram's iodine, which is an iodine–potassium iodine complex acting as a mordant, fixing the dye firmly in certain bacteria and allowing easy removal in others. Decolourization is effected with either alcohol or acetone or mixtures of these. After treatment some bacteria retain the stain and appear a dark purple colour and these are called Gram positive. Others do not retain the stain and appear colourless (Gram negative). The colourless cells may be stained with a counterstain of contrasting colour, such as 0.5% safranin, which is red.

This method, although extremely useful, must be used with caution as the Gram reaction may vary with the age of the cells and the technique of the operator. For this reason, known Gram-positive and Gram-negative controls should be stained alongside the specimen of interest.

Ziehl–Neelsen's acid-fast stain Mycobacterium tuberculosis contains within its cell wall a high proportion of lipids, fatty acids and alcohols, which render the bacterium resistant to normal staining procedures. The inclusion of phenol in the dye solution, together with the application of heat, enables the dye (basic fuchsin) to penetrate the cell and, once attached, to resist vigorous decolourization by strong acids, e.g. 20% sulphuric acid. These organisms are therefore called acid fast. Any unstained material can be counterstained with a contrasting colour, e.g. methylene blue.

Fluorescence microscopy Certain materials, when irradiated by short-wave illuminations, e.g. UV light, become excited and emit visible light of a longer wavelength. This phenomenon is termed fluorescence and will persist only for as long as the material is irradiated. A number of dyes have been shown to fluoresce and are useful in that they tend to be specific to various tissues, which can then be demonstrated by UV irradiation and subsequent fluorescence of the attached fluorochrome. Coupling antibodies to the fluorochromes can enhance specificity, and this technique has found wide application in microbiology. As with the staining procedures described above, this technique can only be applied to dead cells. The three following techniques have been developed for the examination of living organisms.

Dark-ground microscopy The usual function of the microscope condenser is to concentrate as much light as possible through the specimen and into the objective. The dark-ground condenser performs the opposite task, producing a cone of light that comes to a focus on the specimen. The rays of light in the cone are at an oblique angle, such that after passing across the specimen they continue without meeting the front lens of the objective, resulting in a dark background. Any objects present at the point of focus scatter the light, which then enters the objective to show up as a bright image against the dark background.

Specimen preparation is more critical, as very dilute bacterial suspensions are required, preferably with all the objects in the same plane of focus. Air bubbles must be absent both from the film and the immersion oil, if used. Dust and grease also scatter light and destroy the uniformly black background required for this technique.

Phase-contrast microscopy This technique allows us to see transparent objects well contrasted from the background in clear detail and is the most widely used image enhancement method used in microbiology. The theory is too complex to explain in detail here, but in essence an annulus of light is produced by the condenser and focused on the back focal plane of the objective. Here a phase plate, comprising a glass disc containing an annular depression, is situated. The direct rays of the light source annulus pass through the annular groove and any diffracted rays pass through the remainder of the disc. Passage of the diffracted light through this thicker glass layer results in retardation of the light, thereby altering its phase relationship to the direct rays and increasing contrast.

Differential-interference contrast microscopy This method uses polarized light and has other applications outside the scope of this chapter, such as detecting surface irregularities in opaque specimens. It offers some advantages over phase-contrast microscopy, notably the elimination of haloes around the object edges, and enables extremely detailed observation of specimens. It does, however, tend to be more difficult to set up.

Electron microscopy The highest magnification available using a light microscope is about 1500 times. This limitation is imposed not by the design of the microscope itself, as much higher magnifications are possible, but by the wavelength of light. An object can only be seen if it causes a ray of light to deflect. If a particle is very small indeed then no deflection is produced and the object is not seen. Visible light has a wavelength between 0.3 and 0.8 μm and objects less than 0.3 μm will not be clearly resolved, i.e. even if the magnification were increased no more detail would be seen. In order to increase the resolution it is necessary to use light of a shorter wavelength, such as UV light. This has been done and resulted in some useful applications but generally, for the purposes of increased definition, electrons are now used and they can be thought of as behaving like very short wavelength light. Transmission electron microscopy requires the preparation of ultrathin (50–60 nm) sections of material mounted on copper grids for support. Because of the severe conditions applied to the specimen during preparation, and the likelihood of artefacts, care must be taken in the interpretation of information from electron micrographs.

Growth and reproduction of bacteria

The growth and multiplication of bacteria can be examined in terms of individual cells or populations of cells. During the cell division cycle a bacterium assimilates nutrients from the surrounding medium and increases in size. When a predetermined size has been reached a cross-wall will be produced, dividing the large cell into two daughter cells. This process is known as binary fission. In a closed environment, such as a culture in a test tube, the rate at which cell division occurs varies according to the conditions, and this manifests itself in characteristic changes in the population concentration. When fresh medium is inoculated with a small number of bacterial cells the number remains static for a short time while the cells undergo a period of metabolic adjustment. This period is called the lag phase (Fig. 39.6) and its length depends on the degree of readjustment necessary. Once the cells are adapted to the environment they begin to divide in the manner described above, and this division occurs at regular intervals. The numbers of bacteria during this period increase in an exponential fashion, i.e. 2, 4, 8, 16, 32, 64, 128 etc., and this is therefore termed the exponential or logarithmic phase. When cell numbers are plotted on a log scale against time a straight line results for this phase.

During exponential growth (Fig. 39.6) the medium undergoes continuous change, as nutrients are consumed and metabolic waste products excreted. The fact that the cells continue to divide exponentially during this period is a tribute to their physiological adaptability. Eventually, the medium becomes so changed due to either substrate exhaustion or excessive concentrations of toxic products, that it is unable to support further growth. At this

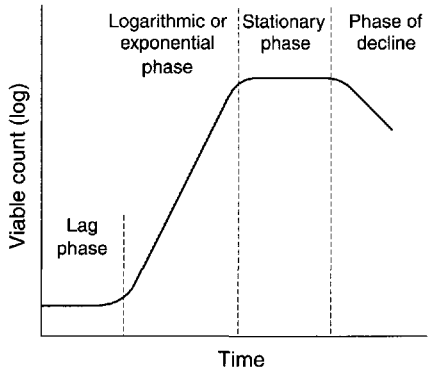


Fig. 39.6 Phases of bacterial growth.

stage cell division slows and eventually stops, leading to the stationary phase. During this period some cells lyse and die whereas others sporadically divide, but the cell numbers remain more or less constant. Gradually all the cells lyse and the culture enters the phase of decline.

It should be appreciated that this sequence of events is not a characteristic of the cell but a consequence of the interaction of the organisms with the nutrients in a closed environment, and does not necessarily reflect the way in which the organism would behave in vivo.

Genetic exchange In addition to mutations, bacteria can alter their genetic make-up by transferring information from one cell to another, either as fragments of DNA or in the form of small extrachromosomal elements (plasmids). Transfer can be achieved in three ways: by transformation, transduction or conjugation.

Transformation When bacteria die they lyse and release cell fragments, including DNA, into the environment. Several bacterial genera (*Bacillus*, *Haemophilus*, *Streptococcus* etc.) are able to take up these DNA fragments and incorporate them into their own chromosome, thereby inheriting the characteristics carried on that fragment. Cells able to participate in transformation are called competent, and the development of competence has been shown in some cases to occur synchronously in a culture under the action of specific inducing proteins.

Transduction Some bacteriophages infect a bacterial cell and incorporate their nucleic acid into the host cell chromosome, with the result that the viral genes are replicated along with the bacterial DNA. In many instances this is a dormant lysogenic state for the phage, but sometimes it is triggered into action and lysis of the cell occurs, with liberation of phage particles. These new phage particles may have bacterial DNA incorporated into the viral genome and this will infect any new host cell. On entering a new lysogenic state the new host cell will replicate the viral nucleic acid in addition to that portion received from the previous host. Bacteria in which this has been shown to occur include *Mycobacterium*, *Salmonella*, *Shigella* and *Staphylococcus*.

Conjugation Gram-negative bacteria such as *Salmonella*, *Shigella* and *Escherichia coli* have been shown to transfer genetic material conferring antibiotic resistance by cellular contact. This process is called conjugation and is controlled by an R-factor plasmid, which is a small circular strand of duplex DNA replicating independently from the bacterial chromosome. R factor comprises a region containing resistance transfer genes that control the formation of sex pili, together with a variety of genes that code for the resistance to drugs. Conjugation is initiated when the resistance transfer genes stimulate the production of a sex pilus and random motion brings about contact with a recipient cell. One strand of the replicating R factor is nicked and passes through the sex pilus into the recipient cell. Upon receipt of this single strand of plasmid DNA the complementary strand is produced and the free ends are joined. For a short time afterwards this cell has the ability to form a sex pilus itself and so transfer the R factor further.

This is by no means an exhaustive discussion of genetic exchange in bacteria and the reader is referred to the bibliography for further information.

Bacterial nutrition Bacteria require certain elements in fairly large quantities for growth and metabolism, including carbon, hydrogen, oxygen and nitrogen. Sulphur and phosphorus are also required but not in such large amounts, and only low concentrations of iron, calcium, potassium, sodium, magnesium and manganese are needed. Some elements, such as cobalt, zinc and copper, are required only in trace amounts and an actual requirement may be difficult to demonstrate.

The metabolic capabilities of bacteria differ considerably and this is reflected in the form in which nutrients may be assimilated. Bacteria can therefore be classified according to their requirements for carbon and energy.

Lithotrophs (synonym: autotrophs) These utilize carbon dioxide as their main source of carbon. Energy is derived from different sources within this group thus:

- Chemolithotrophs (chemosynthetic autotrophs) obtain their energy from the oxidation of inorganic compounds.
- Photolithotrophs (photosynthetic autotrophs) obtain their energy from sunlight.

Organotrophs (synonym: heterotrophs) Organotrophs utilize organic carbon sources and can similarly be divided into:

- Chemoorganotrophs, which obtain their energy from oxidation or fermentation of organic compounds;
- Photo-organotrophs, which utilize light energy.

Oxygen requirements As mentioned above, all bacteria require elemental oxygen in order to build up the complex materials necessary for growth and metabolism, but many organisms also require free oxygen as the final electron acceptor in the breakdown of carbon and energy sources. These organisms are called aerobes. If the organism will only grow in the presence of air it is called a strict aerobe, but most organisms can either grow in its presence or its absence and are called facultative anaerobes. A strict anaerobe cannot grow and may even be killed in the presence of oxygen, because some other compound replaces oxygen as the final electron acceptor in these organisms. A fourth group of microaerophilic organisms has also been recognized which grow best in only trace amounts of free oxygen and usually prefer an increased carbon dioxide concentration.

Influence of environmental factors on the growth of bacteria The rate of growth and metabolic activity of bacteria is the sum of a multitude of enzyme reactions, and so it follows that those environmental factors that influence enzyme activity will also affect growth rate. Such factors include temperature, pH and osmolarity.

Temperature Bacteria can survive wide limits of temperature but each organism will exhibit minimum, optimum and maximum growth temperatures, and on this basis fall into three broad groups:

- Psychrophils: grow best below 20°C but have a minimum about 0°C and a maximum of 30°C. These organisms are responsible for low-temperature spoilage.
- Mesophils: exhibit a minimum growth temperature of 5–10°C and a maximum of 45–50°C. Within this group two populations can be identified: saprophytic mesophils, with an optimum temperature of 20–30°C, and parasitic mesophils with an optimum temperature of 37°C. The vast majority of pathogenic organisms are in this latter group.
- Thermophils: can grow at temperatures up to 70–90°C but have an optimum of 50–55°C and a minimum of 25–40°C.

Organisms kept below their minimum growth temperature will not divide but can remain viable. As a result, very low temperatures (–70°C) are used to preserve cultures of organisms for many years. Temperatures in excess of the maximum growth temperature have a much more injurious effect and will be dealt with in more detail later (see Chapter 41).

pH Most bacteria grow best at around neutrality in the pH range 6.8–7.6. There are, however, exceptions, such as the acidophilic organism lactobacillus, a contaminant of milk products, which grows best at pHs between 5.4 and 6.6. Yeasts and moulds prefer acid conditions with an optimum pH range of 4–6. The difference in pH optima between fungi and bacteria is used as a basis for the design of media permitting the growth of one group of organisms at the expense of others. Sabouraud medium, for example, has a pH of 5.6 and is a fungal medium, whereas nutrient broth, which is used routinely to cultivate bacteria, has a pH of 7.4. The adverse effect of extremes of pH has for many years been used as a means of preserving foods against microbial attack, for example pickling.

Osmotic pressure Bacteria tend to be more resistant to extremes of osmotic pressure than other cells owing to the presence of a very rigid cell wall. The concentration of intracellular solutes gives rise to an osmotic pressure equivalent to between 5 and 20 bar, and most bacteria will thrive in a medium containing around 0.75% w/v sodium chloride. Staphylococci have the ability to survive higher than normal salt concentrations, and this has enabled the formulation of selective media such as mannitol salt agar containing 7.5% w/v sodium chloride, which will support the growth of staphylococci but restrict other bacteria. Halophilic organisms can grow at much higher osmotic pressures, but these are all saprophytic and are non-pathogenic to humans. High osmotic pressures generated by either sodium chloride or sucrose have for a long time been used as preservatives. Syrup BP contains 66.7% w/w sucrose and is of sufficient osmotic pressure to resist microbial attack. This is used as a basis for many oral pharmaceutical preparations.

Handling and storage of microorganisms

Because microorganisms have such a diversity of nutritional requirements there has arisen a bewildering array of media for the cultivation of bacteria, yeasts and moulds. Media are produced either as liquids or solidified with agar. Agar is an extract of seaweed, which at concentrations of between 1 and 2% sets to form a firm gel below 45°C. Unlike gelatin, bacteria cannot use agar as a nutrient and so

even after growth the gel remains firm. Liquid media are stored routinely in test tubes or flasks, depending upon the volume, both secured with either loose-fitting caps or plugs of sterile cotton wool. Small amounts of solid media are stored in Petri dishes or slopes (also known as slants), whereas larger volumes may be incorporated in Roux bottles or Carrell flasks.

Bacteria may only be maintained on agar in Petri dishes for a short time (days) before the medium dries out. For longer storage periods the surface of an agar slope is inoculated, and after growth the culture may be stored at 4°C for several weeks. If even longer storage periods are required then the cultures may be stored at low temperatures (–70°C), usually in the presence of a cryoprotectant such as glycerol, or freeze-dried (lyophilized) before being stored at 4°C. Vegetative cells that survive this process may retain their viability for many years in this way.

When a single cell is placed on the surface of an overdried agar plate it becomes immobilized but can still draw nutrients from the substrate, and consequently grows and divides. Eventually the numbers of bacterial cells are high enough to become visible and a colony is formed. Each of the cells in that colony is a descendant from the initial single cell or group of cells, and so the colony is assumed to be a pure culture with each cell having identical characteristics. The formation of single colonies is one of the primary aims of surface inoculation of solid media and allows the isolation of pure cultures from specimens containing mixed flora.

Inoculation of agar surfaces by streaking The agar surface must be smooth and without moisture, which could cause the bacteria to become motile and the colonies to merge together. To dry the surface of the agar the plates are placed in an incubator or drying cabinet until the surface appears wrinkled. An inoculating loop is made of either platinum or nichrome wire twisted along its length to form a loop 2–3 mm in diameter at the end. Nichrome wire is cheaper than platinum but has similar thermal properties. The wire is held in a handle with an insulated grip and the entire length of the wire is heated in a Bunsen flame to red heat to sterilize it. The first few centimetres of the holder are also flamed before the loop is set aside in a rack to cool.

When cool the loop is used to remove a small portion of liquid from a bacterial suspension and this is then drawn across the agar surface from A to B, as indicated in Figure 39.7. The loop is then resterilized

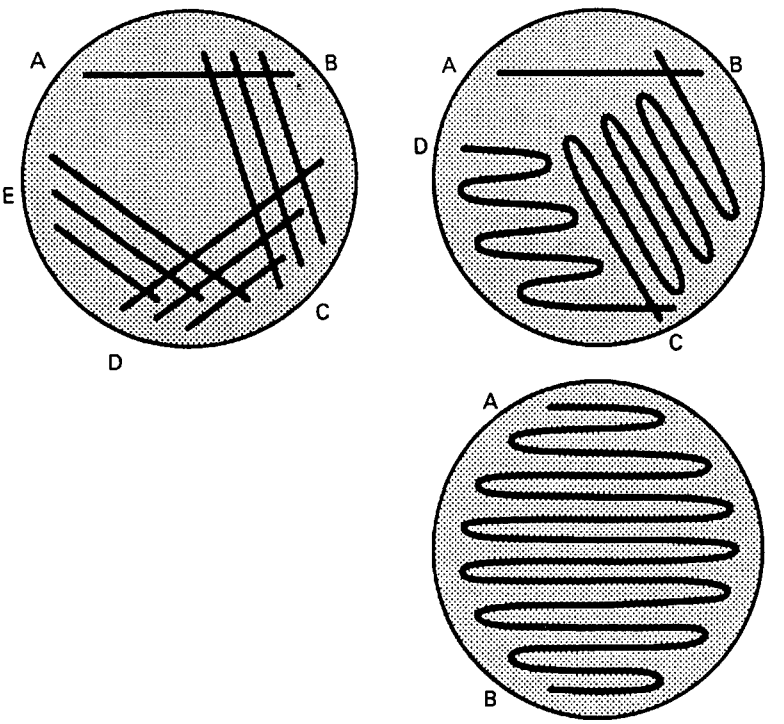


Fig. 39.7 Typical streaking methods for obtaining isolated colonies.

and allowed to cool. At this stage the loop is not reinoculated but streaked over the surface again, ensuring a small area of overlap with the previous streak line. The procedure is repeated as necessary. The pattern of streaking (see other examples in Fig. 39.7) is dictated largely by the concentration of the original bacterial suspension. The object of the exercise is to dilute the culture such that, after incubation, single colonies will arise in the later streak lines where the cells were sufficiently separated. All plates are incubated in an inverted position to prevent condensation from the lid falling on the surface of the medium and spreading the colonies.

Inoculation of slopes A wire needle may be used to transfer single colonies from agar surfaces to the surface of slopes for maintenance purposes. The needle is similar to the loop except that the wire is single and straight, not terminating in a closed end. This is flamed and cooled as before and a portion of a single colony picked off the agar surface. The needle is then drawn upwards along the surface of the slant. Before incubation the screw cap of the bottle should be loosened slightly to prevent oxygen starvation during growth. Some slopes are prepared with a shallower slope and a deeper butt to allow the needle to be stabbed into the agar when testing for gas production.

Transference of liquids Graduated pipettes and Pasteur pipettes may be used for this purpose, the latter being short glass tubes one end of which is drawn into a fine capillary. Both types should be plugged with sterile cotton wool and filled via pipette fillers of appropriate capacity. Mouth pipetting should *never* be permitted. Automatic pipettes have generally replaced glass graduated pipettes in most areas of science for the measurement of small volumes of liquid. Provided they are properly maintained and calibrated they have the advantage of being easy to use and reliable in performance.

Release of infectious aerosols During all of these manipulations two considerations must be borne in mind. First, the culture must be transferred with the minimum risk of contamination from outside sources. To this end all pipettes, tubes, media etc. are sterilized and the manipulations carried out under aseptic conditions. Second, the safety of the operator is paramount. During operations with microorganisms it must be assumed that all organisms are capable of causing disease and that any route of infection is possible. Most infections acquired in laboratories cannot be traced to a given incident but arise from the inadvertent release of infectious aerosols. Two types of aerosols may be produced. The first kind produces large droplets (>5 μm), containing many organisms, which settle locally and contaminate surfaces in the vicinity of the operator. These may initiate infections if personnel touch the surfaces and subsequently transfer the organisms to eyes, nose or mouth. The second type of aerosol contains droplets less than 5 μm in size, which dry instantly to form droplet nuclei that remain suspended in the air for considerable periods. This allows them to be carried on air currents to situations far removed from the site of initiation. These particles are so small that they are not trapped by the usual filter mechanisms and may be inhaled, giving rise to infections of the lungs. The aerosols just described may be produced by a variety of means, such as heating wire loops, placing hot loops into liquid cultures, splashing during pipetting, rattling loops and pipettes inside test tubes, opening screw-capped tubes and ampoules etc. All microbiologists should have an awareness of the dangers of aerosol production and learn the correct techniques to minimize them.

Cultivation of anaerobes

Anaerobic microbiology is a much-neglected subject owing principally to the practical difficulties involved in growing organisms in the absence of air. However, with the increasing implication of anaerobes in certain disease states and improved cultivation systems the number of workers in this field is growing.

The most common liquid medium for cultivation of anaerobes is thioglycollate medium. In addition to sodium thioglycollate the medium contains methylene blue as a redox indicator, and it permits the growth of aerobes, anaerobes and microaerophilic organisms. When in test tubes the medium may be used after sterilization until not more than one-third of the liquid is oxidized, as indicated by the colour of the methylene blue indicator. Boiling and cooling of the medium just prior to inoculation is recommended for maximum performance. In some cases the presence of methylene blue poses toxicity problems and under these circumstances the indicator may be removed.

Anaerobic jars have considerably improved in recent years, making the cultivation of even strict anaerobes now relatively simple. The most common ones consist of a clear polycarbonate jar with a lid housing a cold catalyst in a mesh container, and are designed to be used with disposable H_2/CO_2 generators. The agar plates, which may need to be prereduced prior to inoculation, are placed in the jar together with a gas generator and an anaerobic indi-

cator. A measured amount of water is added to the gas generator sachet and the lid sealed. Hydrogen and carbon dioxide are evolved and the hydrogen combines with any oxygen present under the action of the cold catalyst to form a light mist of water vapour. Carbon dioxide is produced in sufficient quantities to allow the growth of many fastidious anaerobes, which fail to grow in its absence. The absence of oxygen will be demonstrated by the action of the redox indicator, which in the case of methylene blue will be colourless.

Counting bacteria

Estimates of bacterial numbers in a suspension can be evaluated from a number of standpoints, each equally valid depending upon the circumstances and the information required. In some cases it may be necessary to know the total amount of biomass produced within a culture, irrespective of whether the cells are actively metabolizing. In other instances only an assessment of living bacteria may be required. Bacterial counts can be divided into total counts and viable counts.

Total counts These counts estimate the total number of bacteria present within a culture, both dead and living cells. A variety of methods are available for the determination of total counts and the one chosen will depend largely upon the characteristics of the cells being studied, i.e. whether they aggregate together.

Microscopic methods Microscopic methods employ a haemocytometer counting chamber (Fig. 39.8), which has a platform engraved with a grid of small squares each 0.0025 mm^2 in area. The platform is depressed 0.1 mm and a glass coverslip is placed over the platform, enclosing a space of known dimensions. The volume above each square is 0.00025 mm^3. For motile bacteria the culture is fixed by adding two to three drops of 40% formaldehyde solution per 10 mL of culture to prevent the bacteria from moving across the field of view. A drop of the suspension is then applied to the platform at the edge of the coverslip. The liquid is drawn into the space by capillary action. It is important to ensure that liquid does not enter a trench that surrounds the platform: the liquid must fill the whole space between the coverslip and the platform. This slide is examined using phase-contrast or dark-ground microscopy and, if necessary, the culture is diluted to give 2–10 bacteria per small square. A minimum of 300 bacterial cells should be counted to give statistically significant results.

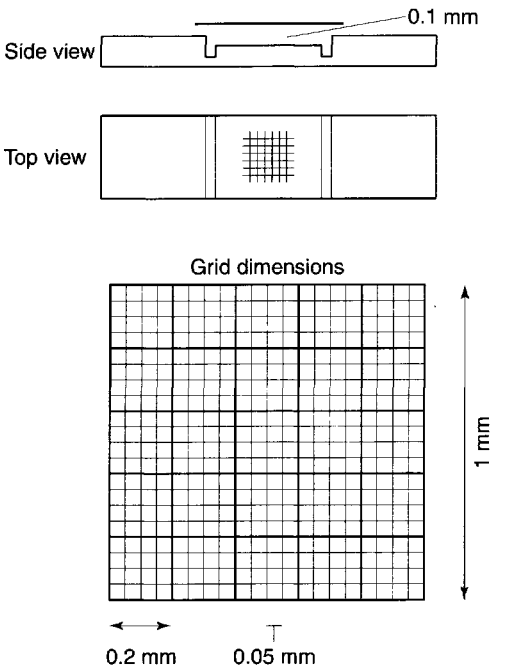


Fig. 39.8 Counting chamber for microscopic estimation of cell numbers.

Calculation

Assume the mean cell count per small square was 6.

Each small square

$$= 2.5 \times 10^{-4}\ mm^3$$
$$= 2.5 \times 10^{-7}\ cm^3$$

If the volume above each square contains 6 cells then

$$= \frac{6}{2.5 \times 10^{-7}}\ \text{cells/mL}$$
$$= 2.4 \times 10^{7}\ \text{cells/mL}.$$

Another microscopic technique is Breed's method. A microscope slide is marked with a square of known area (usually 1 cm^2), and 0.01 mL of bacterial suspension is spread evenly over the square. This is allowed to dry, fixed and stained. A squared-eyepiece micrometer is then used to determine the original count, knowing the dilution and the size of each square.

Spectroscopic methods These methods are simple to use and very rapid, but require careful calibration if meaningful results are to be obtained. Either opacity or light scattering may be used, but both methods may only be used for dilute, homogenous suspensions as at higher concentrations the cells obscure each other in the light path and the relationship between optical density and concentration is not linear. Simple colourimeters and nephelometers can

be used, but more accurate results are obtained using a spectrophotometer.

Electronic methods A variety of automated methods are available for bacterial cell counting, including electronic particle counting, micro-calorimetry, changes in impedance or conductivity, and radiometric and infrared systems for monitoring CO_2 production.

Other methods If an organism is prone to excessive clumping, or if a measure of biomass is needed rather than numbers, then estimates may be made by performing dry weight or total nitrogen determinations. For dry weight a sample of suspension is centrifuged and the pellet washed free of culture medium by further centrifugation in water. The pellet is collected and dried to a constant weight in a desiccator. Total nitrogen measures the total quantity of nitrogenous material within a cell population. A known volume of suspension is centrifuged and washed as before and the pellet digested using sulphuric acid in the presence of a $CuSO_4$–K_2SO_4–selenium catalyst. This produces ammonia, which is removed using boric acid and estimated either by titration or colourimetrically.

Viable counts These are counts to determine the number of bacteria in a suspension that are capable of division. In all these methods the assumption is made that a colony arises from a single cell, although clearly this is often not the case, as cells frequently clump or grow as aggregates, e.g. *Staphylococcus aureus*. In these cases the count is usually given as colony-forming units (c.f.u.) per mL rather than cells per mL.

Spread plates A known volume, usually 0.2 mL, of a suitably diluted culture is pipetted on to an over-dried agar plate and distributed evenly over the surface using a sterile spreader made of wire or glass capillary. The liquid must all be allowed to soak in before the plates are inverted. A series of tenfold dilutions should be made in a suitable sterile diluent and replicates plated out at each dilution, in order to ensure that countable numbers of colonies (30–300) are obtained per plate.

The viable count is calculated from the average colony count per plate, knowing the dilution and the volume pipetted onto the agar.

Example calculation

Serial dilution scheme

Stock bacterial suspension, 1 mL added to 99 mL of sterile diluent — *call dilution A* (the stock solution has therefore been diluted by a factor of 100 (10^2).

1 mL of dilution A added to 99 mL of sterile diluent — *call dilution B* (dilution B has been diluted by a factor of 10^4).

1 mL of dilution B added to 9 mL of sterile diluent — *call dilution C* (dilution C has been diluted by a factor of 10^5).

1 mL of dilution C added to 9 mL of sterile diluent — *call dilution D* (dilution D has been diluted by a factor of 10^6).

1 mL of dilution D added to 9 mL of sterile diluent — *call dilution E* (dilution E has been diluted by a factor of 10^7).

0.2 mL of each dilution plated in triplicate.

Mean colony counts for each dilution after incubation at 37°C:

Dilution A	too many to count
Dilution B	too many to count
Dilution C	400 colonies
Dilution D	45 colonies
Dilution E	5 colonies.

The result for dilution C is unreliable, as the count is too high. If the colony count exceeds 300 errors arise because the colonies become very small and some may be missed. This is why the colony count for dilution C does not exactly correspond to 10 × that found for dilution D. Similarly, the count for dilution E is unreliable because at counts below about 30 small variations introduce high percentage errors.

The result from dilution D is therefore taken for calculation, as the colony count lies between 30 and 300.

45 colonies in 0.2 mL, therefore
= 45 × 5 colonies per mL
= 225 cfu/mL in dilution D.

This was diluted by a factor of 10^6 (100 × 100 × 10 × 10) and so the count in the stock suspension was $225 \times 10^6 = 2.25 \times 10^8$ cfu/mL.

Pour plates A series of dilutions of original culture is prepared as before, ensuring that at least one is in the range 30–300 organisms/mL. One-millilitre quantities are placed into empty sterile Petri dishes and molten agar, cooled to 45°C, is poured on to the suspension and mixed by gentle swirling. After setting the plates are inverted and incubated. Because the colonies are embedded within the agar they do not exhibit the characteristic morphology seen with surface colonies. In general they assume a lens shape and are usually smaller. As the oxygen tension below the surface is reduced this method is not suitable for strict aerobes. Calculations are similar to that given above, except that no correction is necessary for volume placed upon the plate.

Membrane filtration This method is particularly useful when the level of contamination is very low,

such as in water supplies. A known volume of sample is passed through a membrane filter, typically made of cellulose acetate/nitrate, of sufficient pore size to retain bacteria (0.2–0.45 μm). The filtrate is discarded and the membrane placed bacteria-uppermost on the surface of an overdried agar plate, avoiding trapped air between membrane and surface. Upon incubation the bacteria draw nutrients through the membrane and form countable colonies.

ATP determination There are sometimes instances when viable counts are required for clumped cultures or for bacteria adhered to surfaces, for example in biofilms. Conventional plate count techniques are not appropriate here and ATP determinations can be used. The method assumes that viable bacteria contain a relatively constant level of ATP, but this falls to zero when the cells die. ATP is extracted from the cells using a strong acid such as trichloroacetic acid, and the extract is then neutralized by dilution with buffer. The ATP assay is based upon the quantitative measurement of a stable level of light produced as a result of an enzyme reaction catalysed by firefly luciferase.

$$\text{ATP} + \text{luciferin} + \text{O}_2 \xrightarrow{\text{luciferase}} \text{oxyluciferin} + \text{AMP} + \text{PPi} + \text{CO}_2 + \text{Light}$$

The amount of ATP is calculated by reference to light output from known ATP concentrations and the number of bacterial cells is calculated by reference to a previously constructed calibration plot.

Isolation of pure bacterial cultures

Mixed bacterial cultures from pathological specimens or other biological materials are isolated first on solid media to give single colonies, and the resultant pure cultures can then be subjected to identification procedures. The techniques used for isolation depend upon the proportion of the species of interest compared to the background contamination. Direct inoculation can only be used when an organism is found as a pure culture in nature. Examples include bacterial infections of normally sterile fluids such as blood or cerebrospinal fluid.

Streaking is the most common method employed, and if the proportions of bacteria in the mixed culture are roughly equal then streaking on an ordinary nutrient medium should yield single colonies of all microbial types. More usually the organism of interest is present only as a very small fraction of the total microbial population, necessitating the use of selective media.

A selective enrichment broth is initially inoculated with the mixed population of cells and this inhibits the growth of the majority of the background population. At the same time the growth of the organism of interest is encouraged. After incubation in these media the cultures are streaked out on to solid selective media, which frequently contain indicators to further differentiate species on the basis of fermentation of specific sugars.

Classification and identification

Taxonomy is the ordering of living organisms into groups on the basis of their similarities. In this way we can construct a hierarchy of interrelationships such that species with similar characteristics are grouped within the same genus, genera which have similarities are grouped within the same family, families grouped into orders, orders into classes and classes into divisions. The classification of bacteria does pose a problem because a species is defined as a group of closely related organisms that reproduce sexually to produce fertile offspring. Of course, bacteria do not reproduce sexually, and so a bacterial species is simply defined as a population of cells with similar characteristics.

Nomenclature Bergey's Manual of Determinative Bacteriology lists 562 bacterial genera and each of these contains many species. It is therefore extremely important to be sure there is no confusion when describing any one particular bacterial species. Although we are familiar with the use of trivial names in ornithology and botany (we understand what we mean when we describe a sparrow or a daffodil) such an approach could have disastrous consequences in clinical microbiology. For this reason the binomial system of nomenclature is used that Carolus Linnaeus developed in the 18th century. In this system every bacterium is given two names, the first being the genus name and the second the species name. By convention the name is italicized or underlined, and the genus name always begins with a capital letter whereas the species name begins in lower case.

Identification The organization of bacteria into groups of related microorganisms is based upon the similarity of their chromosomal DNA. Although this provides a very accurate indicator of genetic relatedness it is far too cumbersome a tool to use for the identification of an unknown bacterium isolated from a sample. In this instance a series of rapid and simple tests is required that probe the phenotypic characteristics of the microorganism. The tests are

conducted in a logical series of steps, the results from each providing information on the next stage of the investigation. An example of such a procedure is given below:

Morphology:	microscopical investigations using a wet mount to determine cell size, shape, formation of spores, aggregation, motility etc.
Staining reactions:	Gram stain, acid-fast stain, spore stain
Cultural reactions:	appearance on solid media (colony formation, shape, size, colour, texture, smell, pigments etc.); aerobic/anaerobic growth, temperature requirements, pH requirements
Biochemical reactions:	enzymatic activities are probed to distinguish between closely related bacteria. Can be performed in traditional mode or using kits.

Biochemical tests These are designed to examine the enzymatic capabilities of the organism. As there are a large number of biochemical tests that can be performed, the preliminary steps help to narrow down the range to those that will be most discriminatory. Given below are a few examples of commonly used biochemical tests.

Sugar fermentation is very frequently used and examines the ability of the organism to ferment a range of sugars. A number of tubes of peptone water are prepared, each containing a different sugar. An acid–base indicator is incorporated into the medium that also contains a Durham tube (a small inverted tube filled with medium) capable of collecting any gas produced during fermentation. After inoculation and incubation the tubes are examined for acid production, as indicated by a change in the colour of the indicator, and gas production as seen by a bubble of gas collected in the Durham tube.

Proteases are produced by a number of bacteria, e.g. *Bacillus* species and *Pseudomonas*, and they are responsible for the breakdown of protein into smaller units. Gelatin is a protein that can be added to liquid media to produce a stiff gel similar to agar. Unlike agar, which cannot be utilized by bacteria, those organisms producing proteases will destroy the gel structure and liquefy the medium. A medium made of nutrient broth solidified with gelatin is normally incorporated in boiling tubes or small bottles and inoculated by means of a stab wire. After incubation it is important to refrigerate the gelatin prior to examination, otherwise false positives may be produced. Proteases can also be detected using milk agar, which is opaque. Protease producers form colonies with clear haloes around them where the enzyme has diffused into the medium and digested the casein.

Oxidase is produced by *Neisseria* and *Pseudomonas* and can be detected using 1% tetramethylparaphenylene diamine. The enzyme catalyses the transport of electrons between electron donors in the bacteria and the redox dye. A positive reaction is indicated by a deep purple colour in the reduced dye. The test is carried out by placing the reagent directly on to an isolated colony on an agar surface. Alternatively, a filter paper strip impregnated with the dye is moistened with water and, using a platinum loop, a bacterial colony spread across the surface. If positive, a purple colour will appear within 10 seconds. Note that the use of iron loops may give false positive reactions.

The indole test distinguishes those bacteria capable of decomposing the amino acid tryptophan to indole. Any indole produced can be tested by a colourimetric reaction with *p*-dimethylaminobenzaldehyde. After incubation in peptone water 0.5 mL Kovacs reagent is placed on the surface of the culture, shaken, and a positive reaction is indicated by a red colour. Organisms giving positive indole reactions include *E. coli* and *Proteus vulgaris*.

Catalase is responsible for the breakdown of hydrogen peroxide into oxygen and water. The test may be performed by adding 1 mL of 10-vol hydrogen peroxide directly to the surface of colonies growing on an agar slope. A vigorous frothing of the surface liquid indicates the presence of catalase. *Staphylococcus* and *Micrococcus* are catalase positive, whereas *Streptococcus* is catalase negative.

Urease production enables certain bacteria to break down urea to ammonia and carbon dioxide:

$$NH_2\text{–}CO\text{–}NH_2 + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2$$

This test is readily carried out by growing the bacteria on a medium containing urea and an acid–base indicator. After incubation the production of ammonia will be shown by the alkaline reaction of the indicator. Examples of urease-negative bacteria include *E. coli* and *Enterococcus faecalis*.

Simmons citrate agar was developed to test for the presence of organisms that could utilize citrate as the sole source of carbon and energy and ammonia as the main source of nitrogen. It is used to differenti-

ate members of the Enterobacteriaceae. The medium, containing bromothymol blue as indicator, is surface inoculated on slopes and citrate utilization demonstrated by an alkaline reaction and a change in the indicator colour from a dull green to a bright blue. *E. coli, Shigella, Edwardsiella* and *Yersinia* do not utilize citrate, whereas *Serratia, Enterobacter, Klebsiella* and *Proteus* do and so give a positive result.

The methyl red test is used to distinguish organisms that produce and maintain a high level of acidity from those that initially produce acid but restore neutral conditions with further metabolism. The organism is grown on glucose phosphate medium and, after incubation, a few drops of methyl red are added and the colour immediately recorded. A red colour indicates acid production (positive), whereas a yellow colour indicates alkali (negative).

Some organisms can convert carbohydrates to acetyl methyl carbinol (CH_3–CO–CHOH–CH_3). This may be oxidized to diacetyl (CH_3–CO–CO–CH_3), which will react with guanidine residues in the medium under alkaline conditions to produce a colour. This is the basis of the Voges Proskauer test, which is usually carried out at the same time as the methyl red test. The organism is again grown in glucose phosphate medium and, after incubation, 40% KOH is added together with 5% α-naphthol in ethanol. After mixing, a positive reaction is indicated by a pink colour in 2–5 minutes gradually becoming darker red up to 30 minutes. Organisms giving positive Voges Proskauer reactions usually give negative methyl red reactions, as the production of acetylmethyl carbinol is accompanied by low acid production. *Klebsiella* species typically give a positive Voges Proskauer reaction.

Rapid identification systems With the increasing demand for quick and accurate identification of bacteria a number of micromethods have been developed combining a variety of biochemical tests selected for their rapidity of reading and high discrimination. The API bacterial identification system is an example of such a micromethod and comprises a plastic tray containing dehydrated substrates in a number of wells. Culture is added to the wells, dissolving the substrate and allowing the fermentation of carbohydrates, or the presence of enzymes similar to those just described, to be demonstrated. In some cases incubation times of 2 hours are sufficient for accurate identification. Kits are available with different reagents, permitting the identification of Enterobacteriaceae, Streptococcaceae, staphylococci, anaerobes, yeasts and moulds. Accurate identification is made by reference to a table of results.

The tests described so far will enable differentiation of an unknown bacterium to species level. However, it is apparent that not all isolates of the same species behave in an identical manner. For example, *E. coli* isolated from the intestines of a healthy person is relatively harmless compared to the well publicized *E. coli* O157.H7, which causes intense food poisoning and haemolytic uraemic syndrome. On occasions it is therefore, necessary to distinguish further between isolates from the same species. This can be performed using, among other things, serological tests and phage typing.

Serological tests Bacteria have antigens associated with their cell envelopes (O-antigens), with their flagella (H-antigens) and with their capsules (K-antigens). When injected into an animal, antibodies will be produced directed specifically towards those antigens and able to react with them. Specific antisera are prepared by immunizing an animal with a killed or attenuated bacterial suspension and taking blood samples. Serum containing the antibodies can then be separated. If a sample of bacterial suspension is placed on a glass slide and mixed with a small amount of specific antiserum, then the bacteria will be seen to clump when examined under the microscope. The test can be made more quantitative by using the tube dilution technique, where a given amount of antigen is mixed with a series of dilutions of specific antisera. The highest dilution at which agglutination occurs is called the agglutination titre.

Phage typing Many bacteria are susceptible to lytic bacteriophages whose action is very specific. Identification may be based on the susceptibility of a culture to a set of such type-specific lytic bacteriophages. This method enables very detailed identification of the organisms to be made, e.g. one serotype of *Salmonella typhi* has been further subdivided into 80 phage types using this technique.

FUNGI

Fungus is a general term used to describe all yeasts and moulds, whereas a mould is a filamentous fungus exhibiting a mycelial form of growth. The study of fungi is called mycology. Yeasts and moulds are eukaryotic microorganisms possessing organized demonstrable nuclei enclosed within an outer membrane, a nucleolus, and chromatin strands that become organized into chromosomes during cell division. Fungal cell walls are composed predominantly of polysaccharide, and in most cases this is chitin mixed with cellulose, glucan and mannan.

Proteins and glycoproteins are also present, but peptidoglycan is absent. The polysaccharide polymers are crosslinked to provide a structure of considerable strength which gives the cell osmotic stability. The fungal membrane contains sterols such as ergosterol and zymosterol not found in mammalian cells, and this provides a useful target for antifungal antibiotics. The role of fungi in nature is predominantly a scavenging one and in this respect they are vital for the decomposition and recycling of organic materials. Of the more than 100 000 species of known fungi fewer than 100 are human pathogens, and most of these are facultative and not obligate parasites.

Fungal morphology

The fungi can be divided into five broad groups on the basis of their morphology.

Yeasts

These are spherical or ovoid unicellular bodies 2–4 μm in diameter which typically reproduce by budding. In liquid cultures and on agar they behave very much like bacteria. Examples include *Saccharomyces cerevisiae*, strains of which are used in baking and in the production of beers and wines. *Cryptococcus neoformans* is the only significant pathogen and this gives rise to a respiratory tract disease called cryptococcosis, which in most cases is relatively mild. However, the microorganism may disseminate, leading to multiorgan disease, including meningitis. Cryptococcosis is of particular significance in immune-compromised patients. If left untreated, 80% of patients with disseminated cryptococcosis will die within 1 year.

Yeast-like fungi

These organisms normally behave like a typical budding yeast but under certain circumstances the buds do not separate and become elongated. The resulting structure resembles a filament and is called a pseudomycelium. It differs from a true mycelium in that there are no interconnecting pores between the cellular compartments comprising the hyphae.

The most important member of this group is *Candida albicans*, which is usually resident in the mouth, intestines and vagina. Under normal conditions *Candida* does not cause problems but if the environmental balance is disturbed then problems can arise. These include vaginal thrush (vaginitis) and oral thrush. Overgrowth of *Candida albicans* within the gut can lead to symptoms of inexplicable fatigue and malaise, which is difficult to diagnose. Predisposing factors may include poor diet, diabetes, alcoholism and long-term treatment with steroids.

Dimorphic fungi

These grow as yeasts or as filaments depending upon the cultural conditions. At 22°C, either in the soil or in culture media, filamentous mycelial forms and reproductive spores are produced, whereas at 37°C in the body the microorganisms assume a yeast-like appearance. *Histoplasma capsulatum* is an important pathogen that gives rise to respiratory illness. The infectious form is the spore, which is borne on the wind and is inhaled. It has been postulated that a single spore can elicit an infection. On entering the body the spores germinate to give rise to the yeast form. Primary infections are often mild, but progressive disseminated histoplasmosis is a very severe disease that can affect many organs of the body.

Filamentous fungi

This group comprises those multicellular moulds that grow in the form of long, slender filaments 2–10 μm in diameter called hyphae. The branching hyphae, which constitute the vegetative or somatic structure of the mould, intertwine and gradually spread over the entire surface of the available substrate, extracting nutrients and forming a dense mat or mycelium. The hyphae may be non-septate (coenocytic) or septate, but in each case the nutrients and cellular components are freely diffusible along the length of the filament. This is facilitated by the presence of pores within the septa.

Mushrooms and toadstools

This group is characterized by the production of large reproductive fruiting bodies of complex structure. They also possess elaborate propagation mechanisms. Some of these fungi are edible and are used in cooking, but others, such as *Amanita phalloides* (Death Angel), produce potent mycotoxins that may result in death if eaten.

Reproduction of fungi

In the somatic portion of most fungi the nuclei are very small and the mechanism of nuclear division is uncertain. Under the correct environmental conditions the organisms will switch from the somatic or vegetative growth phase to a reproductive form, so

that the fungus may propagate the species by producing new mycelia on fresh food substrates. Two types of reproduction are found, asexual and sexual.

Asexual reproduction

Asexual reproduction is in general more important for the propagation of the species and mechanisms include binary fission, budding, hyphal fragmentation and spore formation. Each progeny is an exact replica of the parent and no species variation can occur.

Some yeasts (e.g. *Schizosaccharomyces rouxii*) reproduce by binary fission in the same way as bacteria. The parent cell enlarges, its nucleus divides and, when a cross-wall is produced across the cell, two identical daughter cells form.

Budding occurs in the majority of yeasts and is the production of a small outgrowth or bud from the parent cell. As the bud increases in size the nucleus divides and one of the pair migrates into the bud. The bud eventually breaks off from the parent to form a new individual. A scar is left behind on the parent cell and each parent can produce up to 24 buds.

Fungi growing in a filamentous form may employ hyphal fragmentation as a means of asexual propagation. The hyphal tips break up into component segments (called arthroconidia or arthrospores), each of which can disperse on the wind to other environments and fresh food substrates.

The formation of specialized spore-bearing structures containing reproductive spores is the most common method of asexual reproduction (Fig. 39.9). The spores can be borne in a sporangium, supported on a sporangiophore. A limiting membrane surrounds the sporangium and the spores contained within it are called sporangiospores. The spores are released when the sporangium ruptures. This type of reproduction is found in the lower fungi possessing non-septate hyphae (e.g. *Mucor* and *Rhizopus*). Separate spores produced at the tips of specialized conidiophores are called conidiospores, and a diverse range of structures is found in nature.

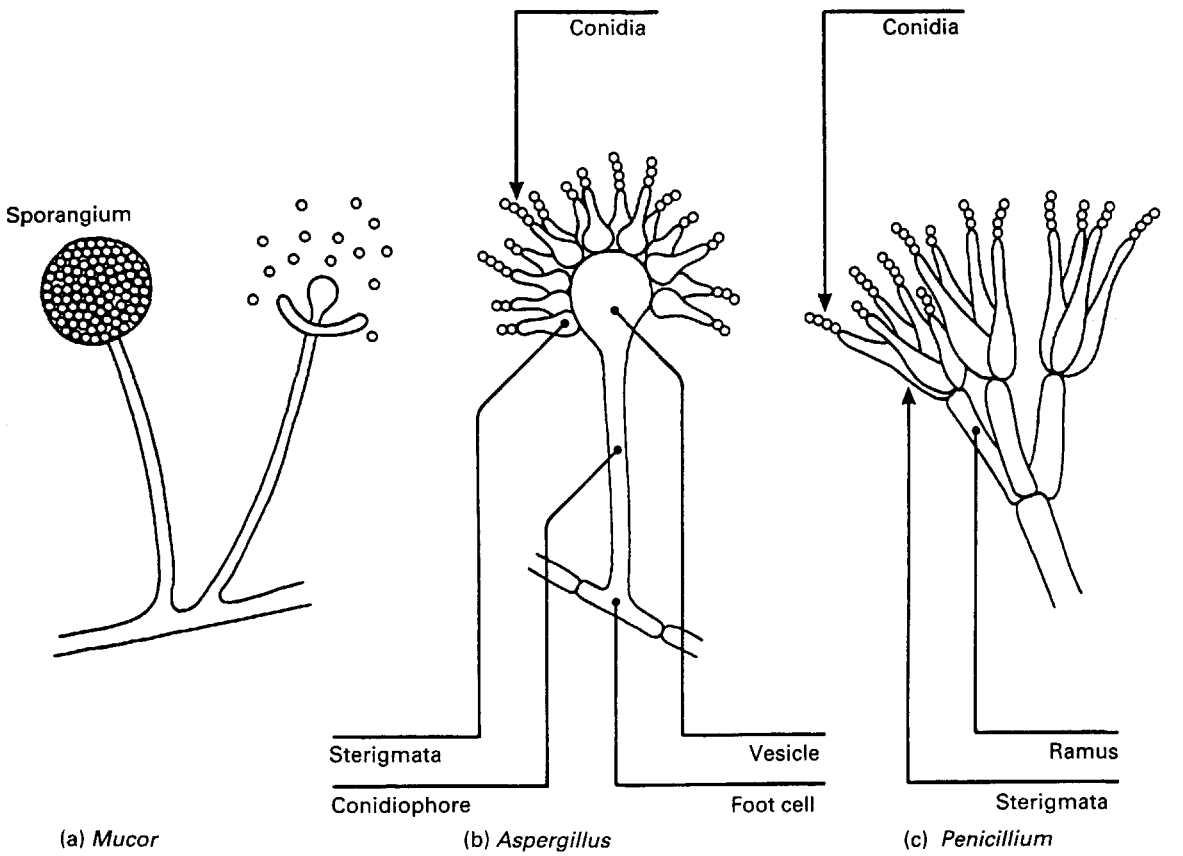


Fig. 39.9 Spore-bearing structures of selected fungi.

Figure 39.9 illustrates some of the different types of asexual spores found in fungi.

Sexual reproduction

Sexual reproduction involves the union of two compatible nuclei and allows variation of the species. Mycology is made much more complex because individual fungi are given different names depending upon whether they are in the sexual or the asexual stage. Not all fungi have been observed to carry out sexual reproduction. Some species produce distinguishable male and female sex organs on the same mycelium and are therefore hermaphroditic, i.e. a single colony can reproduce sexually by itself. Others produce mycelia which are either male or female (called dioecious), and can therefore only reproduce when two dissimilar organisms come together.

Fungal classification

The pharmaceutically important fungi can be found within four main taxonomic classes.

Zygomycetes

These are terrestrial saprophytes possessing non-septate hyphae and are sometimes referred to as the lower fungi. Apart from their hyphae they can be distinguished from other filamentous fungi by the presence of sporangia. Examples are *Mucor* and *Rhizopus*, which are important in the manufacture of organic acids and the biotransformation of steroids. They are also common spoilage organisms.

Ascomycetes

Ascomycetes possess septate hyphae and the sexual or perfect stage is characterized by the presence of a sac-like reproductive structure called an ascus. This typically contains eight ascospores. The asexual or imperfect stage involves conidiospores. An example is *Claviceps purpurea*, which is a parasite of rye and is important as a source of ergot alkaloids used to control haemorrhage and in treating migraine. A subclass of the Ascomycetes is the Hemiascomycetes, and this includes the yeasts such as *Saccharomyces* and *Cryptococcus*, together with *Torulopsis* and *Candida*.

Deuteromycetes

Sometimes called the Fungi Imperfecti this group includes those fungi in which the sexual stage of reproduction has not been observed. *Penicillium* and *Aspergillus* are Ascomycetes but classified among the Deuteromycetes as the perfect stage is apparently absent. *Penicillium chrysogenum* is important in the production of the antibiotic penicillin, whereas *Aspergillus* species have found widespread industrial usage owing to their extensive enzymic capabilities. Some *Aspergillus* species also produce mycotoxins and can cause serious infections in humans. The Deuteromycetes contains most of the human pathogens, such as *Blastomyces*, *Coccidioides*, and some of the dermatophyte fungi.

Basidiomycetes

This is the most advanced group, containing the mushrooms and toadstools. Sexual reproduction is by basidiospores. The group also includes the rusts (cereal parasites) and smuts.

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Pharmaceutical applications of microbiological techniques

Norman Hodges

CHAPTER CONTENTS

The purpose of this chapter is to bring together those microbiological methods and procedures that are relevant to the design and production of medicines and medical devices. These are methods used (a) to determine the potency or activity of antimicrobial chemicals, e.g. antibiotics, preservatives and disinfectants; and (b) as part of the microbiological quality control of manufactured sterile and non-sterile products.

There are several areas of biotechnology in which microorganisms or their enzymes are used in the production of medicines. These include antibiotic biosynthesis and 'fermentation'; the production of dextran, asparaginase, streptokinase and other microbial metabolites which have a medicinal application; interconversion of steroid molecules; the detection of mutagenicity by the Ames test; and the toxicity screening of pharmaceutical materials using mammalian cell cultures. Human genes have also been inserted into microorganisms which, as a consequence, are used to produce such materials as insulin, growth hormone and interferons. All of these biotechnological aspects of microbiology are, however, beyond the scope of this book, although they are included in the bibliography.

This chapter describes the experimental procedures which are unique or particularly relevant to pharmacy, rather than those that are common to microbiology as a whole. In the latter category, for example, are procedures used to identify and enumerate microorganisms, and these, together with staining and microscopical techniques, are described in Chapter 39.

Several of the methods and tests discussed in this chapter are the subject of monographs or appendices in the British Pharmacopoeia, or they are described in British Standards or other recognized reference works. It is not the intention to reproduce these official testing procedures in detail here, but rather to explain the principles of the tests, to draw attention to difficult or important aspects, and to indicate the advantages, problems or shortcomings of the various methods.

MEASUREMENT OF ANTIMICROBIAL ACTIVITY

In most of the methods used to assess the activity of antimicrobial chemicals an inoculum of the test organism is added to a solution of the chemical under test, samples are removed over a period of time, the chemical is inactivated and the proportion of cells surviving determined. Alternatively, culture medium is present together with the chemical, and the degree of inhibition of growth of the test organism is measured. In each case it is necessary to standardize and control such factors as the concentration of the test organism, its origin, i.e. the species and strain employed, together with the culture medium in which it was grown, the phase of growth from which the cells were taken, and the temperature and time of incubation of the cells after exposure to the chemical. Because such considerations are common to several of the procedures described here, e.g. antibiotic assays, preservative efficacy (challenge) tests and determinations of minimum inhibitory concentration (MIC), it is appropriate that they should be considered first, both to emphasize their importance and to avoid repetition.

Factors to be controlled in the measurement of antimicrobial activity

Origin of the test organism

Although two cultures may bear the same generic and specific name, i.e. they may both be called *Escherichia coli*, this does not mean that they are identical. Certainly they would normally be similar in many respects, e.g. morphology (appearance), cultural requirements and biochemical characteristics, but they may exhibit slight variations in some of these properties. Such variants are described as strains of *E. coli*, and it is desirable in experimental work to name or describe the strain being used. A variety of strains of a single species may normally be obtained from a culture collection, e.g. the National Collection of Industrial and Marine Bacteria or the National Collection of Type Cultures. Different strains may also occur in hospital pathology laboratories by isolation from swabs taken from infected patients, or by isolation from contaminated food, cosmetic or pharmaceutical products, and from many other sources. Strains obtained in these ways are likely to exhibit variations in resistance to antimicrobial chemicals. Strains from human or animal infections are frequently more resistant to antimicrobial chemicals, particularly antibiotics, than those from other sources. Similarly, strains derived from contaminated medicines may be more resistant to preservative chemicals than those obtained from culture collections. Therefore, to achieve results that are reproducible by a variety of laboratories it is necessary to specify the strain of the organism used for the determination.

It is becoming increasingly common, too, for official testing methods to limit the number of times the culture collection specimen may be regrown in fresh medium (called the number of subcultures or passages) before it must be replaced. This is because the characteristics of the organism (including its resistance to antimicrobial chemicals) may progressively change as a result of mutation and natural selection through the many generations that might arise during months or years of laboratory cultivation.

Composition and pH of the culture medium

There are several methods of assessing antimicrobial activity which all have in common the measurement of inhibition of growth of a test organism when the antimicrobial chemical is added to the culture medium. In such cases the composition and pH of the medium may influence the result. The medium may contain substances that antagonize the action of the test compound, e.g. high concentrations of thymidine or para-aminobenzoic acid will interfere with sulphonamide activity.

The antimicrobial activities of several groups of chemical are influenced by the ease with which they cross the cell membrane and interfere with the metabolism of the cell. This, in turn, is influenced by the lipid solubility of the substance, as the membrane contains a high proportion of lipid and tends to permit the passage of lipid-soluble substances. Many antimicrobial chemicals are weak acids or weak bases, which are more lipid soluble in the unionized form. pH therefore affects their degree of ionization, hence their lipid solubility and so, ultimately, their antimicrobial effect. Benzoic acid, for example, is a preservative used in several oral mixtures which has a much greater activity in liquids buffered to an acid pH value than those which are neutral or alkaline. Conversely, weak bases such as the aminoglycoside antibiotics, e.g. streptomycin, neomycin and gentamicin, are more active at slightly alkaline pH values. The presence of organic matter, e.g. blood, pus or serum, is likely to have a marked protective effect on the test organism, and so antimicrobial chemicals may appear less active in the presence of such material.

The activity of several antibiotics, notably tetracyclines and aminoglycosides, is reduced by the presence of high concentrations of di- or trivalent cations in the medium.

Exposure and incubation conditions

The temperature, duration and redox conditions of exposure to the antimicrobial chemical (or incubation of survivors after exposure) may all have a significant effect on its measured activity. Increasing the temperature of exposure of the test organism to the chemical increases the antimicrobial activity by a factor which is quantified by the temperature coefficient (Q_{10} value: the number of -fold increase in activity for a 10°C rise in temperature). Alcohols and phenols, for example, may respectively exhibit Q_{10} values of 3–5 and >10, and so a variation of 5°C in the temperature of exposure (which is permitted by pharmacopoeial preservative efficacy tests, for example) may lead to a markedly different rate of kill of the organism in question.

The period of time for which the test organism is exposed to the antimicrobial chemical may influence the recorded result because it is possible for the organism to adapt and become resistant to the presence of the chemical. In preservative efficacy tests the exposure period is normally 28 days, which is sufficient time for any cells that are not killed during the first 24–48 hours to recover and start to reproduce, so that the final bacterial concentration may be much higher than that at the start. This is illustrated in Figure 40.1, which shows the effect of the quaternary ammonium preservative benzethonium bromide on *Pseudomonas aeruginosa*.

The concentration of bacteria was reduced to approximately 0.01 % of the initial value during the first 6 hours, but the bacteria that survived this early period recovered to the original level within 2 days. There is the potential for a similar phenomenon to arise in other situations, e.g. in minimum inhibitory concentration (MIC) determinations of bacteristatic agents (those that do not kill, but merely inhibit the growth of the test organism), although it is not common in MICs because the exposure (incubation) time is much shorter than that in preservative testing.

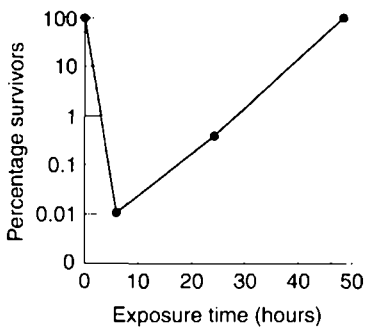

Fig. 40.1 The survival and recovery of *Pseudomonas aeruginosa* exposed to benzethonium chloride during a preservative efficacy test

The effect of some antibiotics may be influenced by the redox conditions during their period of contact with the test organism. Aminoglycosides, for example, are far less active, and metronidazole is far more active, under conditions of low oxygen availability. Such effects may even be seen during agar-diffusion antibiotic assays, in which the antibiotic diffuses from a well into an agar gel inoculated with the test organism; the diameter of the zone of growth inhibition that surrounds a well filled with neomycin solution, for example, may be significantly greater at the surface of the agar (where there is abundant oxygen) than at its base, where the oxygen concentration is limited by its poor diffusion through the gel.

Inoculum concentration and physiological state

It is perhaps not surprising that the concentration of the inoculum can markedly affect antimicrobial action, with high inoculum levels tending to result in reduced activity. There are two main reasons for this. First, there is the phenomenon of drug adsorption on to the cell surface or absorption into the interior of the cell. If the number of drug molecules in the test tube is fixed yet the number of cells present is increased, this obviously results in fewer molecules available per cell and consequently the possibility of a diminished effect. In addition to this there is the second, more specialized case, again concerning antibiotics, where it is frequently observed that certain species of bacteria can synthesize antibiotic-inactivating enzymes, the most common of which are the various types of β-lactamase (those destroying penicillin, cephalosporin and related antibiotics). Thus a high inoculum means a high carryover of enzyme with the inoculum cells, or at least a greater potential synthetic capacity.

Perhaps less predictable than the inoculum concentration effect is the possibility of the inoculum history influencing the result. There is a substantial amount of evidence to show that the manner in which the inoculum of the test organism has been grown and prepared can significantly influence its susceptibility to toxic chemicals. Features such as the nature of the culture medium, e.g. nutrient broth or a defined glucose-salts medium, the metal ion

composition of the medium and hence of the cells themselves and the physiological state of the cells, i.e. 'young' actively growing cells from the logarithmic growth phase or 'old' non-dividing cells from the stationary phase, all have the potential to influence the observed experimental values.

Antibiotic assays

Methods of assaying antibiotics may be broadly divided into three groups:

- Conventional chemical assays, e.g. titrations, spectrophotometry and high-performance liquid chromatography (HPLC);
- Enzyme-based and immunoassays, where the antibiotic is, respectively, the substrate for a specific enzyme or the antigen with which a specific antibody combines;
- Biological assays in which biological activity – in this case bacterial growth inhibition of the 'test' solution is compared with that of a reference standard.

Biological methods offer the advantage that the parameter being measured in the assay (growth inhibition) is the property for which the drug is used, and so inactive impurities or degradation products will not interfere and lead to an inaccurate result. Biological methods also offer other advantages (Table 40.1), but they have several significant limitations and non-biological methods are now generally preferred.

Enzyme-based and immunoassay kits are used in hospitals, notably for therapeutic monitoring of toxic antibiotics (e.g. aminoglycosides and vancomycin), whereas HPLC tends to be preferred in the pharmaceutical industry, particularly for quality assurance applications. Biological assays are most likely to be used when the alternatives are inappropriate, especially when the active antibiotic cannot readily be separated from inactive impurities, degradation products or interfering substances, or it cannot easily be assayed by HPLC without derivatization to enhance ultraviolet absorption (e.g. aminoglycosides). These situations may arise:

1. when the antibiotic is present in a solution containing a wide variety of complex substances that would interfere with a chemical assay, e.g. fermentation broth, serum or urine;
2. when the antibiotic is present together with significant concentrations of its breakdown products, e.g. during stability studies as part of product development;
3. when it has been extracted from a formulated medicine, for example a cream or linctus, when excipients might cause interference;
4. where the commercially available product is a mixture of isomers that have inherently different antimicrobial activities, which cannot easily be distinguished chemically and which may differ in proportion from batch to batch (e.g. neomycin and gentamicin).

Biological antibiotic assays, or bioassays as they are frequently known, may be of two main types, agar diffusion and turbidimetric. The European Pharmacopoeia (1997) section 2.7.2 describes experimental details for both methods, e.g. test microorganisms, solvents, buffers, culture media and incubation conditions. In each case a reference material of known activity must be available. When antibiotics were in their infancy few could be produced in the pure state free from contaminating material, and specific chemical assays were rarely available. Thus the potency or activity of reference standards was expressed in terms of (international)

Table 40.1 Relative merits of alternative antibiotic assay methods

Assay method	Advantages	Disadvantages
Biological methods	Inactive impurities or degradation properties do not interfere	Slow, usually requiring overnight incubation
	Easily scaled up for multiple samples	Relatively labour-intensive
	Do not require expensive equipment	Relatively inaccurate and imprecise, particularly with inexperienced operators
Non-biological methods	Usually rapid, accurate and precise	May require expensive equipment (e.g. HPLC) or expensive reagents or assay kits (enzyme and immunological methods)
	May be more sensitive than biological assays	
	Enzyme and immunological methods are usually assay kits, which give reliable results with inexperienced operators	HPLC can only assay samples sequentially, so unusually large sample numbers may cause problems

units of activity. There are few antibiotics for which dosage is still normally expressed in units: nystatin and polymyxin are two of the remaining examples. More commonly, potencies are recorded in terms of $\mu g\ mL^{-1}$ of solution or μg antibiotic mg^{-1} of salt, with dosages expressed in mg. Antibiotic assay results are usually in the form of a potency ratio of the activity of the unknown or test solution divided by that of the standard.

Agar diffusion assays

In this technique the agar medium in a Petri dish or a larger assay plate is inoculated with the test organism, wells are created in it by removing circular plugs of agar, and these wells are filled with a solution of the chemical under test (Fig. 40.2).

The chemical diffuses through the gel from A towards B and the concentration falls steadily in that direction. The concentration in the region A to X is sufficiently high to prevent growth, i.e. it is an inhibitory concentration. Between X and B the concentration is subinhibitory and growth occurs. The concentration at X at the time the zone edge is formed is known as the critical inhibitory concentration (CIC). After incubation the gel between A and X is clear and that between X and B is opaque as a result of microbial growth which, with the common test organisms, is usually profuse. A zone of inhibition is therefore created, the diameter of which will increase as the concentration of chemical in the well increases.

A graph may be constructed which relates zone diameter to the logarithm of the concentration of the solution in the well (Fig. 40.3). It is normally found to be linear over a small concentration range, but the square of the diameter must be plotted to achieve linearity over a wide range. A plot such as that in Figure 40.3 may, quite correctly, be used to calculate the concentration of a test solution of antibiotic. In practice, however, it is found to be more convenient to obtain reliable mean zone diameters for the standard at just two or three concentrations, rather than somewhat less reliable values for six or seven concentrations. There is no reason why an assay should not be based upon a two- or three-point line, provided that those points are reliable and that preliminary experiments have shown that the plotted relationship over the concentration range in question is linear.

It is not common to conduct antibiotic assays in Petri dishes because too few zones may be accommodated on a standard-sized dish to permit the replication necessary to obtain the required accuracy and precision. Antibiotic assays, when performed on a large scale, are more often conducted using large assay plates 300 mm or more square. The wells are created in a square design and the number that may be accommodated will depend upon the anticipated

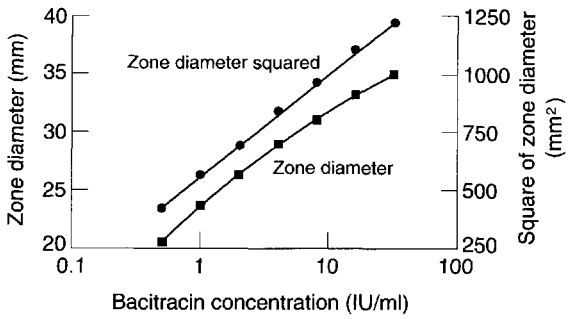

Fig. 40.3 Calibration plots for agar diffusion assays.

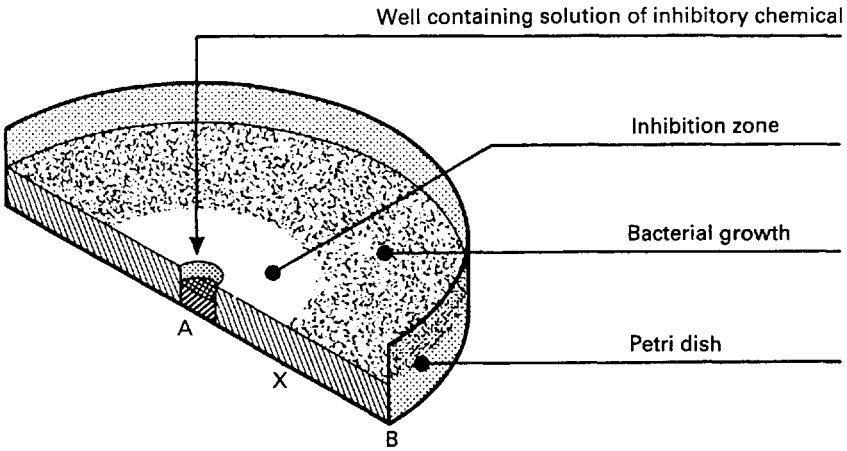

Fig. 40.2 Assessment of antimicrobial activity by agar diffusion.

zone diameters: 36 or 64 wells are common (6 × 6 or 8 × 8, respectively). The antibiotic standard material may be used in solution at three known concentrations (frequently referred to as 'doses') and the antibiotic solution of unknown concentration treated likewise; alternatively, each may be employed at two concentrations. A randomization pattern known as a Latin square is used to ensure that there is a suitable distribution of the solutions over the plate, thereby minimizing any errors due to uneven agar thickness.

In the case of an assay based upon standard solutions used at two concentrations, the potency ratio may be calculated directly from the graph (as shown in Fig. 40.4) or by using the formula below:

$$\log X = \text{LDR} \times \frac{(\text{UH} + \text{UL}) - (\text{SH} + \text{SL})}{(\text{SH} - \text{SL}) + (\text{UH} - \text{UL})}$$

where X is the potency ratio, LDR is the logarithm of the dose ratio (i.e. ratio of concentrations of standard solutions), UH, UL, SH and SL are the mean zone diameters for the unknown and standard high and low doses. The derivation of this is described in detail by Wardlaw (1999), who deals extensively with the subject of antibiotic assays. The tests for acceptable limits of parallelism between the line joining the standards and that joining the test points, together with confidence limits applicable to the calculated potency ratios, are described in the current EP.

In calculating the potency ratio directly from Figure 40.4 the zone diameters for the standard and unknown high concentrations are plotted at the same abscissa values, and those for the low concentrations similarly. Two zone diameters are considered which are as widely separated on the ordinate as possible while still being covered by the standard and the test lines. The ratio of the concentrations required to achieve the selected diameter is thus an estimate of the potency ratio. The mean of the two estimates taken at the extremes of the range of common zone diameters should be identical to the value by calculation from the formula. Thus, in Figure 40.4, at a zone diameter of 23.75 mm the first estimate of potency ratio is 0.557 (antilog of 0.445 divided by antilog of 0.699); the second is 0.507 (antilog of zero divided by the antilog of 0.295). The mean value of 0.53 indicates the unknown solution to have approximately half the activity of the standard.

Practical aspects of the conduct of agar diffusion assays

The agar may be surface inoculated, or inoculated throughout while in the molten state prior to pouring. In the latter case zones may arise which are different in diameter at the agar surface than at the base of the Petri dish; this may complicate the recording of zone diameters. Zones which are not perfectly circular may be disregarded, although it may be appropriate to record the mean of the long and short axes. Such zones may result from non-circular wells, careless filling, or uneven drying of the agar gel owing to a poorly fitting plate cover. The zones may be read directly with callipers or, more conveniently, after enlargement by projection on to a screen. Automatic zone readers incorporating a series of photocells that detect opacity changes at the zone edge are available, and may be linked to a personal computer which rapidly calculates the result together with the appropriate statistical analyses. The size of the zone is determined by the relative rates of diffusion of the drug molecule and growth of the test organism. If the assay plates are left at room temperature for 1–4 hours prior to incubation, growth is retarded whereas diffusion proceeds. This may result in larger zones and improved precision.

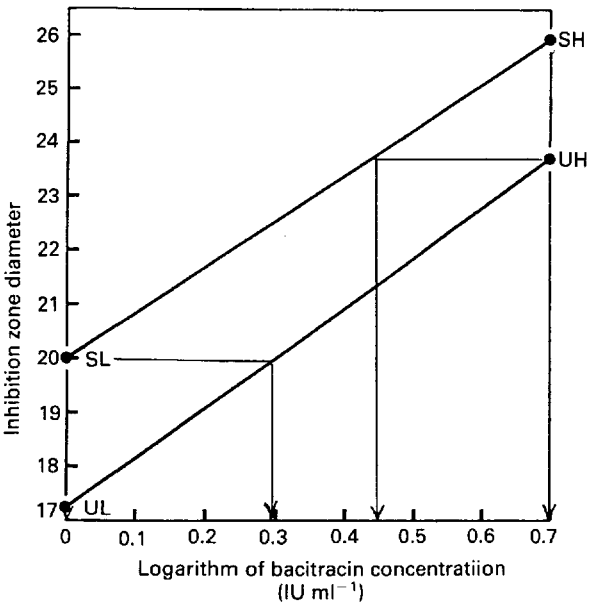

Fig. 40.4 Four-point agar diffusion assay of bacitracin.

The zone diameter is affected by most of the factors previously stated to influence antimicrobial activity and, in addition, gel strength and the presence of other solutes in the antibiotic solution, e.g. buffer salts. If the antibiotic has been extracted from a formulated medicine, e.g. cream, lotion or mixture, excipients may be simultaneously removed and influence the diffusion of the antibiotic in the gel; sugars are known to have this effect. Because antibiotic assays involve a comparison of two solutions which are similarly affected by changes in experimental conditions, day to day variations in, for example, inoculum concentration will not have a

great effect on the accuracy of the potency ratio obtained, but the precision may be affected. The volume of liquid in the well is of minimal importance; it is usually of the order of 0.1 mL and is delivered by semiautomatic pipette. As an alternative to wells the antibiotic may be introduced on to the agar using absorbent paper discs, metal cylinders or 'fish spine' beads (beads having a hole drilled in them which contains the liquid).

For many antibiotics, the test organism is a *Bacillus* species and the inoculum is in the form of a spore suspension, which is easy to prepare, standardize and store. Alternatively, frozen inocula from liquid nitrogen may be used as a means of improving reproducibility.

Careful storage and preparation of the reference standards are essential. The reference antibiotic is usually stored at low temperature in a freeze-dried condition.

Turbidimetric assays

In this case antibiotic standards at several concentrations are incorporated into liquid media and the extent of growth inhibition of the test organism is measured turbidimetrically using a nephelometer or spectrophotometer. The unknown or test antibiotic preparation is run simultaneously, again at several concentrations, and the degree of growth inhibition compared. Such assays are less commonly used than agar diffusion methods because their precision is rather inferior, but they do have the advantage of speed: the result may be available after an incubation period as short as 3–4 hours. They are also more sensitive than diffusion assays and consequently may be applied to low-activity preparations.

The shape and slope of the dose–response plot for a turbidimetric assay may be more variable than that for agar diffusion, and non-linear plots are common. Typical dose–response plots are shown in Hewitt and Vincent (1989). The plotted points are usually the mean turbidity values obtained from replicate tubes, and the assay may be conducted using a Latin square arrangement of tubes incubated in a shaker, which is necessary to ensure adequate aeration and uniform growth throughout the tube.

Practical aspects of the conduct of turbidimetric assays
Incubation time is critical in two respects. First, it is necessary to ensure that the culture in each of the many tubes in the incubator has exactly the same incubation period, because errors of a few minutes become significant in a total of only 3–4 hours' incubation. Care must therefore be taken to ensure that the tubes are inoculated in a precise order, and that growth is stopped in the same order by the addition of formalin, heating or other means.

The incubation period must be appropriate to the inoculum level so that the cultures do not achieve maximal growth. At the concentrations used for such assays the antibiotics usually reduce growth rate but do not limit total growth. Therefore, if the incubation period is sufficiently long, all of the cultures may achieve the same cell density regardless of the antibiotic concentration.

There are certain other limitations to the use of turbidimetric assays. Because it is the 'cloudiness' of the culture that is measured, standard and test solutions in which the organisms are suspended should, ideally, be clear before inoculation. Cloudy or hazy solutions which may result from the extraction of the antibiotic from a cream, for example, can only be determined after similarly compensating the standards or otherwise eliminating the error. Test organisms that produce pigments during the course of the incubation period should be avoided; so too should those that normally clump in suspension.

The rate of growth of the test organism may vary significantly from one batch of medium to another. Thus it is important to ensure that all the tubes in the assay contain medium from the same batch, and were prepared and sterilized at the same time. Many liquid media become darker brown on prolonged heating, and so samples from the same batch may differ in colour if the sterilizing time is not strictly controlled.

Minimum inhibitory concentration determinations (MICs)

The MIC is the lowest concentration of an antimicrobial chemical found to inhibit the growth of a particular test organism. It is therefore a fundamental measure of the intrinsic antimicrobial activity (potency) of a chemical, which may be an antiseptic, disinfectant, preservative or antibiotic. MIC determinations are applied to chemicals in the pure state, i.e. they are particularly relevant to raw materials rather than to the final formulated medicines; the latter are usually subject to preservative efficacy (challenge) tests to assess their antimicrobial activity. MIC values are usually expressed in terms of $\mu g\ mL^{-1}$ or, less commonly, as in the case of some antibiotics, units mL^{-1}. It is important to recognize that the test organism is not necessarily killed at the MIC. Whether or not the cells die or merely cease growing depends upon the mode of action of the antimicrobial agent in question.

An MIC is an absolute value which is not based upon a comparison with a standard/reference prepa-

ration, as in the case of antibiotic assays and certain disinfectant tests. For this reason inadequate control of experimental conditions is particularly likely to have an adverse effect on results. Discrepancies in MIC values measured in different laboratories are often attributable to slight variations in such conditions, and care must be taken to standardize all the factors previously stated to influence the result. It is important also to state the experimental details concerning an MIC determination. A statement such as 'the MIC for phenol against *E. coli* is 0.1% w/v' is not, by itself, very useful. It has far more value if the strain of *E. coli*, the inoculum concentration and the culture medium etc. are also stated.

MIC test methods

The most common way to conduct MIC determinations is to incorporate the antimicrobial chemical at a range of concentrations into a liquid medium, the containers of which are then inoculated, incubated and examined for growth.

Test tubes may be used, but microtitre plates (small rectangular plastic trays with, usually, 96 wells each holding approximately 0.2 mL liquid) and other miniaturized systems are common. It is possible to incorporate the chemical into molten agar, which is then poured into Petri dishes and allowed to set. Two advantages of using a series of agar plates are that several organisms can be tested at the same time using a multipoint inoculator, and there is a greater chance of detecting contaminating organisms (as uncharacteristic colonies) on the agar surface than in liquid media. Usually the presence or absence of growth is easier to distinguish on the surface of agar than in liquid media. In tubes showing only faint turbidity it is often difficult to decide whether growth has occurred or not. Regardless of the method used the principle is the same, and the MIC is the lowest concentration at which growth is inhibited.

In addition to the other experimental details that should be described in order to make the measured result meaningful, it is necessary to specify the increment by which the concentration of test chemical changes from one container to the next. The operator could, for example, change the concentration 10-fold from one tube to the next in the rare circumstance where even the likely order of magnitude of the MIC is not known. Far more commonly, however, the concentration changes by a factor of 2, and this is almost invariably the case when antibiotic MIC values are determined; thus, reference is made to 'doubling dilutions' of the antibiotic. If, for example, an MIC was to be measured using test tubes, an aqueous solution of the chemical would normally be mixed with an equal volume of ***double***-strength growth medium in the first tube in the series, then half the contents of the first tube added to an equal volume of ***single***-strength medium in the second, and so on. In this case half the contents of the last tube in the series would have to be discarded prior to inoculation in order to maintain the same volume in each tube. Control tubes may be included to demonstrate (a) that the inoculum culture was viable and that the medium was suitable for its growth (a tube containing medium and inoculum but no test chemical), and (b) that the operator was not contaminating the tubes with other organisms during preparation (a tube with no test chemical or added inoculum). It is possible to use an arithmetic series of concentrations of test chemical, e.g. 0.1, 0.2, 0.3, 0.4 ... rather than 0.1, 0.2, 0.4, 0.8 ... μg mL^{-1}. The potential problem with this approach is that there may be merely a gradation in growth inhibition, rather a sharp point of demarcation, with obvious growth in one tube in the series and no growth in the next.

All the solutions used must be sterilized; it must not be assumed that the test chemical is self-sterilizing. Most disinfectants, antiseptic and preservative chemicals are bactericidal, but they are unlikely to kill bacterial spores. Also, several antibiotics act by inhibiting growth, and so would not necessarily kill vegetative cells with which they might be contaminated. If the experiment is conducted in tubes, all the tube contents must be mixed before inoculation as well as after, otherwise there is the possibility of the inoculum cells being killed by an artificially high concentration of the test chemical towards the top of the tube. If there is any risk of precipitation of the test chemical or the medium components during incubation a turbidity comparison must be available for each concentration (same tube contents without inoculum); alternatively, in the case of bactericidal chemicals the liquid in each tube may be subcultured into pure medium to see whether the inoculum has survived. Each of the tubes in the series may be prepared in duplicate or triplicate if it is considered desirable. This is the case where the incremental change in concentration is small.

Preservative efficacy tests (challenge tests)

These are tests applied to the formulated medicine in its final container to determine whether it is adequately protected against microbial spoilage. Preservative efficacy tests are used for this purpose (rather than chemical assays of preservatives) because

it is not normally possible to predict how the activity of a preservative chemical will be influenced by the active ingredients, the excipients and the container itself.

Certain products may contain no added preservative, either because the active ingredients have sufficient antimicrobial activity themselves or because they already contain high concentrations of sugar or salts which restrict the growth of microorganisms. However, such products are rare, and multidose injections or eye drops, the majority of oral mixtures, linctuses and similar preparations, together with creams and lotions, all contain preservatives. They are not normally required in anhydrous products, e.g. ointments, or in single-dose injections.

Again it must not be assumed that products containing antimicrobial agents as the active ingredients are self-sterilizing, It is quite possible for an antibiotic cream, for example, to be active against certain bacteria yet fail to restrict the growth of contaminating yeasts or moulds in the cream itself.

The basic principle of a preservative test is to inoculate separate containers of the product with known concentrations of a variety of test organisms, then remove samples from each container over a period of time and determine the proportion of the inoculum that has survived. When first introduced into national pharmacopoeias, preservative efficacy tests differed to some extent in experimental detail and differed markedly in the required performance criteria for preservatives to be used in different product categories. In the late 1990s moves towards international harmonization of preservative testing procedures in the European, United States and Japanese pharmacopoeias (EP, USP and JP, respectively) meant that many (but not all) of the discrepancies in experimental detail were eliminated. The differences in performance criteria remain, however, with the EP generally requiring a greater degree of microbial inactivation for the preservative to be considered satisfactory than the USP and JP which, in this respect, are very similar.

The EP (2000) recommends the routine use of four test organisms, each at a final concentration of 10^5–10^6 cells mL^{-1} or g^{-1} in the product. Counts are performed on samples removed at 0 h, 6 h, 24 h, 48 h, 7 days, 14 days and 28 days. Various aspects of the test are considered in more detail below.

Choice of test organisms and inoculum concentration

The test organisms used are the bacteria *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *E. coli* (which is used for testing all product types in the USP test but for oral products only in the EP test), together with the yeasts/moulds *Candida albicans* and *Aspergillus niger* (plus the osmophilic *Zygosaccharomyces rouxii* in the EP test for oral syrups). The current EP recommends that the designated organisms be supplemented, where appropriate, by other strains or species that may represent likely contaminants to the preparation. A similar recommendation was contained in all previous versions of the USP preservative test, but this has been deleted from the current test (USP 2000). One problem with adding other organisms (such as those isolated from the manufacturing environment) is that they are not universally available, and so a particular product could be tested at different manufacturing sites of the same company and pass in one location yet fail in another simply because the organisms used locally were not the same. The possibility of using resistant strains isolated from previous batches of spoilt product has been advocated, but this too may pose problems, in that organisms may rapidly lose their preservative resistance unless routinely grown on media supplemented with the preservative in question.

Previous versions of the British Pharmacopoeial test have recommended consideration of extending the sampling period beyond 28-days, and reinoculating the product after the first 28-day sampling period is complete. Both of these practices, however, militate against the development of an international standardized test which is capable of providing reproducible results in different laboratories; consequently, both procedures are no longer part of the current EP or USP tests.

The inoculum concentration of 10^5–10^6 microorganisms mL^{-1} or g^{-1} of the preparation under test has been criticized as being unrealistic, as it is much higher than that which would be acceptable in a freshly manufactured product. It is adopted, however, in order for the 1000-fold fall in microbial concentration that would be required from an effective parenteral or ophthalmic preservative to be easily measured. The test organisms are added separately to different containers rather than as a mixed inoculum.

Inactivation of preservative

It is quite possible for sufficient of the preservative to be contained in, and carried over with, the sample removed from the container to prevent or retard growth of colonies on the Petri dishes. If the inoculum level of the test organism initially is about 10^6 cells mL^{-1} or g^{-1} of product, the problem of carry-

over may not arise because a dilution factor of 10^3 or 10^4 would be required to achieve a countable number of colonies on a plate; at this dilution most preservatives would no longer be active. When a high proportion of the cells in the product have died, however, little or no such dilution is required, so preservative carryover is a real problem which may artificially depress the count even more. To avoid this, preservative inhibitors or antagonists may be used. There are several of these, common examples being glycine for aldehydes, thioglycollate or cysteine for heavy metals, and mixtures of lecithin and polysorbate-80 with or without Lubrol W for quaternary ammonium compounds, chlorhexidine and parabens. The use of these and other inactivators has been reviewed by Russell (1981).

An alternative method of removing residual preservative is to pass the sample of inoculated product through a bacteria-proof membrane, so that surviving organisms are retained and washed on the surface of the membrane and the preservative is thus physically separated from them. After washing, the membrane is transferred to the surface of a suitable agar medium and colonies of microorganisms develop on it in the normal way. It is necessary to incorporate controls (validate the method) to demonstrate both that the inactivator really works and that it is not, itself, toxic. The former usually involves mixing the inactivator with the concentrations of preservative likely to be carried over, then inoculating and demonstrating no viability loss. Details of these validation procedures and other aspects of the test are described more fully elsewhere (Hodges, 1999).

One further control is a viable count of the inoculum performed by dilution in peptone water to check the actual number of cells introduced into the product. This is necessary because even a 'zero time sample' of the product will contain cells that have been exposed to the preservative for a short period, as it usually takes 15–45 seconds or more to mix the inoculum with the product and then remove the sample. Some of the cells may be killed even in such a short time, and so a viable count of the inoculum culture will reflect this.

Interpretation of results

The extent of microbial killing required at the various sampling times for a preservative to be considered acceptable for use in parenteral or ophthalmic products is greater than that required for a preservative to be used in topical products, which in turn exceeds that for an oral product preservative (Table 40.2).

In the case of the first two product categories the EP specifies two alternative performance criteria, designated A and B. The A criteria express the recommended efficacy to be achieved, whereas the B criteria must be satisfied in justified cases where the A criteria cannot be attained, for example because of an increased risk of adverse reactions. The baseline used as the reference point to assess the extent of killing is the concentration of microorganisms expected to arise in the product after addition and mixing of the inoculum, ***as calculated from a viable count performed on the concentrated inoculum suspension prior to its addition to the product.*** The viable count on the time-zero sample removed from the inoculated product is not the baseline.

Disinfectant evaluation

A variety of tests have been described over many years for the assessment of disinfectant activity. Those developed during the early part of the 20th century, e.g. the Rideal–Walker and Chick–Martin tests, were primarily intended for testing phenolic disinfectants against pathogenic organisms such as *Salmonella typhi*. Such phenol coefficient tests are now rather outmoded because *S. typhi* is no longer endemic in Britain and phenolics are no longer preeminent; in a recent survey, phenolics represented only 26% of the total biocides used for floor disinfection in aseptic dispensing areas in British hospital pharmacies (Murtough et al 2000).

In the second half of the 20th century several other testing procedures were described for use in the UK which reduced the sampling or other problems associated with the early phenol coefficient tests; these included the Berry and Bean method, the British Standard 3286 test for quaternary ammonium compounds and the Kelsey Sykes test. Other countries adopted procedures that were similar in concept but which differed in experimental detail, e.g. the American Association of Official Analytical Chemists (1990) described a collection of methods applicable to a variety of situations. These and other tests used in the UK, Europe and the USA are described by Reybrouck (1999). At present there is no internationally applicable and officially recommended disinfectant testing procedure, although a measure of uniformity has emerged in Europe with the establishment by the European Committee for Standardization in 1990 of Technical Committee (TC) 216, which has a responsibility for chemical

Table 40.2 Log reductions required in viable counts of microorganisms used in EP (2000) preservative efficacy tests

Product type	Microorganism	Criteria	6 h	24 h	48 h	7 d	14 d	28 d
Parenteral and ophthalmic	Bacteria	A	2	3				NR
	Pseudomonas aeruginosa *Staphylococcus aureus* *Escherichia coli**	B		1		3		NI
	Fungi	A				2		NI
	Aspergillus niger *Candida albicans*	B					1	NI
Topical	Bacteria	A			2	3		NI
		B					3	NI
	Fungi	A					2	NI
		B					1	NI
Oral	Bacteria						3	NI
	Fungi						1	NI
Ear Preparations	Bacteria		2	3				NR
BP (1998) only	Fungi					2		NI

* In oral products only.
NR, no recovery;
NI, no increase (see text).

disinfectants and antiseptics. The European Standard BS EN 1276 (1997) was the first result of the work of TC 216; this deals with assessment of bactericidal activity of disinfectants on bacteria in aqueous suspension. Other procedures applicable to more specialized situations, e.g. disinfection of solid surfaces, are currently under development by TC 216.

A confusing variety of methods for describing and categorizing test procedures are in use. Thus, some schemes classify tests according to the organisms to be killed (bactericidal, fungicidal, virucidal etc.), but classification based upon test design is more common, e.g. suspension tests; capacity tests which measure the extent to which the disinfectant can withstand repeated additions of test organisms; carrier tests, where the organism is loaded or dried on to a carrier; and in-use tests, which are intended to simulate actual conditions of use as closely as possible.

Although most suspension tests of disinfectants have in common the addition of a defined concentration of test organism to the disinfectant solution at a specified temperature, followed by assessment of viability in samples removed after suitable time periods, there are four aspects of disinfectant testing that merit special note:

1. Because disinfectants are normally used in circumstances where there is a significant amount of organic 'dirt' present, modern testing procedures invariably attempt to take this into consideration. Thus, yeast, albumin or other material is added in known concentration to the disinfectant/microorganism mixture.
2. Regardless of the method by which the antimicrobial activity is assessed (see below), it is a fundamental principle of disinfectant testing, just as it is with preservative efficacy tests, that the antimicrobial activity of the disinfectant must be halted (also referred to as neutralized, inactivated or quenched) in the sample when it is removed from the disinfectant/organism mixture. Clearly, meaningful results cannot be obtained if it is impossible to distinguish what fraction of the microbial killing occurred during the timed period of exposure to the disinfectant from that arising due to carryover of disinfectant into the incubation step that follows exposure. Verification that the disinfectant inactivation method is effective and that any chemical neutralizers used are, themselves, non-toxic to the test organisms, is an integral part of the test.
3. It is in viability assessment that there is a fundamental difference of approach between recently developed tests (exemplified by BS EN 1276) and many of the tests that originated before the 1980s. The simplest method of viability assessment, which was employed in the

Rideal–Walker and Kelsey–Sykes tests, for example, is to transfer the sample from the disinfectant/microorganism mixture to a known volume of neutralizing broth, incubate and examine for growth (manifest as turbidity). This procedure contains the inherent defect that any growth in the tubes of broth may result from the transfer of very few surviving cells, or from many. Thus, it is possible for the disinfectant to kill a high proportion of the inoculum within a short period yet fail to kill a small fraction of the cells, possibly mutants, which have atypically high resistance. In this case there is the risk that the disinfectant may be dismissed as insufficiently active despite the fact that it achieved a rapid and extensive initial kill. For this reason it has become common for disinfectant and preservative efficacy tests to be very similar in design, in that both employ viable counting methods to assess microorganism survival, but the former utilize a sampling period of minutes or hours, whereas the latter use a 28-day period.

4. When viable counting is used to assess the survival of test organisms the adoption of disinfection performance criteria based upon a required reduction in the number of surviving organisms is a logical strategy, just as it is in preservative testing. Thus, the so-called 5-5-5 testing principle has found much favour. Here, five test organisms are (separately) exposed for 5 minutes to the disinfectant, which is considered satisfactory if a 5-log reduction in viable numbers (a 10^5 fall in viable cells mL^{-1}) is recorded in each case. This principle is adopted in the BS EN 1276, although only four bacterial strains are recommended for routine use; there is, however, the option to supplement the standard organisms with others more relevant to the intended use of the disinfectant in question.

MICROBIOLOGICAL QUALITY OF PHARMACEUTICAL MATERIALS

Non-sterile products

Non-sterile pharmaceutical products obviously differ from sterile products in that they are permitted to contain some microorganisms, but the European Pharmacopoeia (1997) specifies in section 5.1.4 the maximum concentrations acceptable in different types of product and the species of organism that are not permitted at all (Table 40.3). Similar specifications arise in the U S and other pharmacopoeias.

The required microbiological quality of the manufactured medicine cannot be achieved by the application of an antimicrobial process (heating, radiation etc.) as the final production step for two reasons: first, an approach that uses poor-quality raw materials and manufacturing procedures and then attempts to 'clean up' the product at the end is not acceptable to the licensing authorities; and secondly, some products would not withstand such antimicrobial treatment, e.g. heating an emulsion may cause cracking or creaming. Thus, the most reliable approach to ensure that the manufactured medicine complies with the pharmacopoeial specification is to ensure that the raw materials are of good quality and that the manufacturing procedures conform to the standards laid down in the *Rules and Guidance for Pharmaceutical Manufacturers and Distributors (1997)*.

Implicit in these standards is the principle that the extent of product contamination originating from the manufacturing environment and production personnel should be subject to regular monitoring and control.

Environmental monitoring

Environmental monitoring is normally taken to mean regular monitoring of the levels of microbial contamination of the atmosphere, of solid surfaces and, less frequently, of the personnel in the production areas. Water used to clean floors, benches and equipment (as distinct from water incorporated in the product) may be considered as part of environmental monitoring, but will not be considered here as the procedures for counting microorganisms in water are described below.

Atmospheric monitoring is most commonly undertaken by means of settle plates, which are simply Petri dishes containing media suitable for the growth of bacteria and/or yeasts and moulds, e.g. tryptone soya agar, which are exposed to the atmosphere for periods of, typically, 1–4 hours. Microorganisms in the air may exist as single cells, e.g. mould spores, but more commonly they are attached to dust particles, so that any organisms in the latter category (for which the culture medium is suitable) will grow into visible colonies during incubation after dust particles have settled on the agar surface. The colony counts recorded on the plates are obviously influenced by:

- the duration of exposure;
- the degree of air turbulence, which determines the volume of air passing over the plate;

Table 40.3 European Pharmacopoeia (2000) specifications for the microbiological quality of pharmaceutical products*

Product Category	Quantitative specification	Organisms which must be absent
Topical and non-sterile respiratory products	• Not more than 10^2 aerobic bacteria and fungi per g or mL • Not more than 10^1 enterobacteria and certain other Gram-negative bacteria per g or mL	• *Pseudomonas aeruginosa* • *Staphylococcus aureus*
Oral and rectal products	• Not more than 10^3 aerobic bacteria per g or mL • Not more than 10^2 fungi per g or mL	• *Escherichia coli*
Oral products containing raw materials of natural origin for which antimicrobial pretreatment is not feasible	• Not more than 10^4 aerobic bacteria and not more than 10^2 fungi per g or mL • Not more than 10^2 enterobacteria and certain other Gram-negative bacteria per g or mL	• *Salmonella* • *Escherichia coli* • *Staphylococcus aureus*
Herbal remedies made with boiling water	• Not more than 10^7 aerobic bacteria and not more than 10^5 fungi per g or mL • Not more than 10^2 *Escherichia coli* per g or mL	
Other herbal remedies	• Not more than 10^5 aerobic bacteria and not more than 10^4 fungi per g or mL • Not more than 10^3 enterobacteria and certain other Gram-negative bacteria per g or mL	• *Escherichia coli* • *Salmonella*

* Excluding transdermal patches.

the intrinsic level of atmospheric contamination (microorganisms per litre of air), which in turn is often a reflection of the number and activity level of the operating personnel, as skin scales shed by the operators are usually the most potent source of atmospheric contaminants.

The disadvantage of settle plates is that it is not possible to relate colony counts directly to air volume. This limitation is overcome in active sampling methods, whereby a known volume of air is drawn over, or caused to impact upon, the agar surface. These methods and the equipment available for active sampling have been reviewed recently by Baird (2000).

Surface and equipment sampling is most frequently undertaken by swabbing or the use of contact plates (also known as RODAC – replicate organism detection and counting – plates). Swabbing a known area of bench, floor or equipment with a culture medium-soaked swab is convenient for irregular surfaces. The organisms on the swab may be counted after they have been dispersed, by agitation, into a fixed volume of suspending medium, but it is not easy to quantify either the proportion of total organisms removed from the swabbed surface or the proportion dispersed in the diluent. This second limitation is overcome using contact plates, which are simply specially designed Petri dishes slightly overfilled with molten agar which, on setting, presents a convex surface that projects above the rim of the plate. When the plate is inverted on to the surface to be sampled, microorganisms are transferred directly on to the agar.

Sampling of manufacturing personnel usually consists of sampling clothing, face masks or, more commonly, gloves. 'Finger dabs' is the phrase used to describe the process whereby an operator rolls the gloved surface of each finger over a suitable solid medium in a manner similar to that in which fingerprints are taken. Operator sampling by any means other than finger dabs is rare, particularly outside aseptic manufacturing areas.

Counting of microorganisms in pharmaceutical products

Most pharmaceutical raw materials are contaminated with microorganisms. The levels of contamination are often a reflection of the source of the raw material in question, with 'natural' products derived from vegetable or animal sources, or mined minerals such as kaolin and talc, being more heavily contaminated than synthetic materials whose microbial burden has been reduced by heat, extremes of pH or organic solvents during the course of manufacture. Determining the bioburden in these materials is often straightforward, utilizing without modification the viable counting procedures described in Chapter 41. Occasionally the physical nature of the raw material makes this

difficult or impossible, and this is often found to be the case with the finished manufactured medicine, where problems of dispersibility, sedimentation or viscosity cause complications. As a consequence, modifications to the standard viable counting procedures are necessary to reduce errors. Some of modifications and the circumstances that necessitate them are considered below.

Very low concentrations of microorganisms in aqueous solutions The reliability of calculated viable cell concentrations becomes much reduced when they are based upon colony counts much lower than about 10–15 per Petri dish. Using a surface-spread method it is rarely possible to place more than about 0.5 mL of liquid on to the agar surface in a standard Petri dish because it will not easily soak in. By a pour-plate method 1 mL or more may be used, but a point is reached where the volume of sample significantly dilutes the agar and nutrients. Thus using a conventional plating technique the lowest concentration conveniently detectable is of the order of 10–50 cells mL^{-1}. When the cell concentration is below this value it is necessary to pass a known quantity of the liquid – 10–100 mL or more – through a filter membrane having a pore size sufficiently small to retain bacteria. The membrane is then placed with the organisms uppermost on to the agar surface in a Petri dish, which is incubated without inversion. As a result of diffusion of nutrients through the membrane colonies grow on the surface in the normal way. Diffusion may be assisted by the inclusion of a medium-soaked pad between the membrane and the agar. It is important to ensure that all of the membrane is in contact with the pad or agar, otherwise elevated areas may become dry and no colonies will appear upon them.

Insoluble solids It is necessary to suspend an insoluble solid in a medium that will permit uniform dispersion and adequate wetting of the suspended material. Nutrient broth, peptone water or a buffered salt solution are frequently used and a low concentration of a surfactant incorporated to promote wetting, e.g. polysorbate 80 (0.01–0.05%). Suspension in distilled water alone carries the risk of osmotic damage to sensitive cells, with a consequently low count; for this reason it is best avoided. Having obtained the suspension, there are two options available depending upon the nature and concentration of the suspended material.

The first is to remove a sample of the mixed suspension, dilute if necessary, and plate in or on a suitable medium using a pour- or spread-plate method. If the concentration of suspended material is low it may still be possible to see clearly the developing colonies. High concentrations may obscure the colonies and make counting impossible. The alternative is to dislodge the microbial cells from the solid to which they are attached, allow the solid to sediment out and then sample the supernatant. Methods of removal include vigorous manual shaking, use of a vortex mixer, or instruments designed for the purpose, e.g. the Colworth 'stomacher', in which the aqueous suspension is placed in a sealed sterile bag which is repeatedly agitated by reciprocating paddles. The use of ultrasonics to dislodge the cells carries the risk of damage to or lysis of the cells themselves.

Assuming the suspended material has no antimicrobial activity, plating the 'whole suspension' is probably the easiest and most reliable method. The alternative strategy of sampling the supernatant involves the assumption that all the cells have been removed from the solid, but this would have to be confirmed by control (validation) experiments in which a known quantity of similar organisms were artificially dried on to sterile samples of the material. The second method also relies upon the solid sedimenting sufficiently rapidly for it to be separated from the bacteria in aqueous suspension above. If all or part of the sample has a particle size similar to that of bacteria, yeasts or mould spores, i.e. approximately 1–5 μm, then a separation cannot easily be achieved.

Oils and hydrophobic ointments These materials are usually not heavily contaminated because they are anhydrous and microorganisms will not multiply without water. Thus the microorganisms contained in oily products have usually arisen by contamination from the atmosphere, equipment used for manufacture, and from storage vessels. To perform a viable count the oil sample must be emulsified or solubilized without the aid of excessive heat or any other agent that might kill the cells.

An oil-in-water emulsion must be produced using a suitable surfactant; non-ionic emulsifiers generally have little antimicrobial activity. The proportion of surfactant to use must be determined experimentally and validation experiments conducted to confirm that the surfactant is not, itself, toxic to the species that typically arise as contaminants of the sample in question; Millar (2000) has described the use of up to 5.0 g of polysorbate-80 added to a 10.0 g sample. Such an emulsion may be diluted in water or buffered salts solution if necessary, and aliquots placed on or in the agar medium in the usual way. Alternatively, the oil may be dissolved in a sterile non-toxic solvent and passed through a membrane filter. Isopropyl myristate, for example, is recom-

mended in pharmacopoeial sterility testing procedures as a solvent for anhydrous materials, but it may kill a significant fraction of the cells of some sensitive species, even during an exposure period of only a few minutes.

Creams and lotions Oil-in-water emulsions do not usually represent a problem because they are miscible with water and thus easily diluted. Water-in-oil creams, however, are not miscible and cannot be plated directly because bacteria may remain trapped in a water droplet suspended in a layer of oil on the agar surface. Such bacteria are unlikely to form colonies because the diffusion of nutrients through the oil would be inadequate. These creams are best diluted, dispersed in an aqueous medium and membrane filtered, or converted to an oil-in-water type and then counted by normal plating methods.

Dilution and emulsification of the cream in broth containing Lubrol W, polysorbate-80 or Triton X 100 is probably the best procedure, although the addition of approximately 0.1 g of the w/o emulsion sample to 25 g of isopropyl myristate followed by membrane filtration may be satisfactory.

Detection of specific hazardous organisms

In addition to placing limits on the maximum concentration of microorganisms that is acceptable in different materials, pharmacopoeias usually specify certain organisms that must not be present at all. In practice, this means that detection methods which are described in the pharmacopoeia must be applied to a known weight of material (typically 1–50 g), and the sample passes the test if no organisms arise on the culture plates that conform to the standard textbook descriptions of those to be excluded. Typically the pharmacopoeial methods involve preliminary stages using selective liquid culture media; these are designed to increase the concentration of the organism that is the subject of the test ('target' organism) and so render it more readily detectable. There are, too, supplementary biochemical tests used to ***confirm*** the identity of any isolates having the typical appearance of the target organisms.

Both the EP (2000) and the USP (2000) describe detection tests for *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and salmonellae; in addition, the EP describes a test for clostridia, but this is unlikely to be applied to any material other than mined minerals, e.g. talc and bentonite. The four organisms common to both pharmacopoeias are the subject of these tests primarily because of their potential to cause infections, but they may also represent common contaminants of the products to which the tests are applied, or their presence may be indicative of the quality of the raw material or finished manufactured product. *E. coli*, for example, is a natural inhabitant of mammalian intestines and so its presence in a material such as gelatin (which originates in the slaughterhouse) would indicate unacceptable quality. The most likely source of *Staphylococcus aureus* in a manufactured medicine is the production personnel, so that if this origin were confirmed it would indicate the need for higher manufacturing standards. In general the tests are applied to pharmaceutical raw materials of 'natural' origin, e.g. carbohydrates, cellulose derivatives, gums and vegetable drugs. In addition, there is a requirement that topical products should be free of both *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Table 40.4 summarises the EP (2000) testing schemes for the four principal organisms of interest. These schemes are described in more detail elsewhere, together with photographs of the typical appearance of the organisms in question (Hodges 2000).

Microbiological assays of B-group vitamins

Microbiological assays of B-group vitamins employ similar techniques to turbidimetric assays of antibiotics (see earlier in this chapter). A culture medium is used which is suitable for the assay organism, except for the omission of the vitamin in question. The extent of bacterial growth in the medium is thus directly proportional to the amount of reference standard or test vitamin added. It is important to select an assay organism that has an absolute requirement for the substance in question and is unable to obtain it by metabolism of other medium components; species of *Lactobacillus* are often used for this purpose. 'Carryover' of the vitamin with the inoculum culture must be avoided because this results in some growth even when none of the test material has been added. Growth may be determined turbidimetrically or by acid production from sugars.

Just as HPLC has become the favoured method of antibiotic assay, so too has it become the method of choice for assaying B-group vitamins. Turbidimetric assays *are* still used, however, for example when insurmountable problems arise in resolving the many peaks that might arise on an HPLC chromatogram from a multivitamin product (which might contain 10 or more active ingredients plus excipients, all of which may cause assay interference). Further details of vitamin assays are provided by Hewitt and Vincent (1989).

Table 40.4 Procedures recommended by the EP (2000) in tests for specified microorganisms

Medium	Organism				
	Escherichia coli	Salmonellae		*Pseudomonas aeruginosa*	*Staphylococcus aureus*
Liquid enrichment	MacConkey broth	Tetrathionate bile brilliant green broth		Casein soya bean digest broth (tryptone soya broth)	Casein soya bean digest broth (tryptone soya broth)
			Appearance		
Agar media (primary test)	MacConkey agar (appearance: pink colonies with precipitation of bile due to acid production)	Deoxycholate citrate agar, Xylose lysine deoxycholate (XLD) agar and	Yellow colonies with grey or black centre Red colonies with black centres	Growth on cetrimide agar	Baird-Parker agar (black colonies immediately surrounded by zones of opacity beyond which are zones of clearing)
		Brilliant green agar	Pink colonies		
Result(s) of secondary tests which confirm the presence of organism in question	Production of indole at 44°C	Reactions characteristic of *Salmonella* on triple sugar iron agar and other biochemical or serological tests	Black precipitate of iron sulphide Yellow (acid) butt (i.e. subsurface), with pink (alkaline) slope (i.e. surface)	Absence of growth at 41–43°C	Positive coagulase or deoxyribonuclease tests

Sterile products

Sterile products must, by definition, be free of microorganisms, and it is important to understand that this is an absolute requirement. Thus, the presence of one single surviving microbial cell is sufficient to render the product non-sterile; there is not a level of survivors which is so small as to be regarded as negligible and therefore acceptable.

The principal component of microbiological quality assurance which has traditionally been applied to sterile products is, of course, the test for sterility itself. In essence, this is quite simple: a sample of the material to be tested is added to culture medium which is incubated and then examined for signs of microbial growth. If growth occurs the assumption is made that the contamination arose from the sample, which consequently fails the test. However, the limitations of this simplistic approach became more widely recognized throughout the second half of the 20th century, and there was an increasing awareness of the fact that contaminated products could pass the test and sterile ones apparently fail it (because of contamination introduced during the testing procedure itself). For these reasons the sterility test alone could no longer be relied upon to provide an assurance of sterility, and that assurance is now derived from a strict adherence to high-quality standards throughout the manufacturing process. These encompass:

1. The adoption of the highest possible specifications for the microbiological quality of the raw materials. The rationale here is that sterilization processes are more likely to be effective when the levels of microorganisms to be killed or removed (bioburdens) are as low as possible to begin with. Procedures used to determine bioburdens are described in Chapter 39 and earlier in this chapter.
2. The rigorous application of environmental monitoring procedures (as described above) during the course of manufacture, with more stringent limits for acceptable levels of microorganisms than those applicable during the manufacture of non-sterile products.
3. Comprehensive validation procedures when sterilization processes are designed, together with regular in-process monitoring when those processes are in operation for product manufacture. Initial validation seeks to demonstrate that adequate sterilizing conditions are achieved throughout the load, and entails extensive testing with thermocouples, radiation dosimeters and biological indicators (see below) as appropriate.

The pharmacopoeias and regulatory authorities require a sterility assurance level for terminally sterilized products of 10^{-6} or better. This means that the

probability of non-sterility in an item selected at random from a batch should be no more than 1 in 1 million. This sterility assurance level (SAL) may be demonstrated in the case of some terminally sterilized products simply by reference to data derived from bioburdens, environmental monitoring and in-process monitoring of the sterilization procedure itself. In this case the sterility test is unnecessary and omitted; the term 'parametric release' is used to describe the release of products for sale or use under these circumstances.

Sterilization monitoring

Sterilization processes may be monitored physically, chemically or biologically. Physical methods are exemplified by thermocouples, which are routinely incorporated at different locations within an autoclave load, whereas chemical indicators usually exhibit a colour change after exposure to a heat sterilization process. Biological indicators consist of preparations of spores of the *Bacillus* species that exhibits the greatest degree of resistance to the sterilizing agent in question. The principle of their use is simply that if such spores are exposed to the sterilization process and fail to survive it can be assumed that all other common organisms will also have been killed and the process is safe. Spores of *Bacillus stearothermophilus* are used to monitor autoclaves and gaseous hydrogen peroxide or peracetic acid sterilization processes, whereas *Bacillus subtilis* var *niger* is the organism normally employed for dry heat, ethylene oxide and low-temperature steam–formaldehyde methods; *Bacillus pumilus* is used in radiation sterilization procedures.

Such biological indicators are regularly employed for validation of a sterilization process which is under development for a new product, or when a new autoclave is being commissioned; they are less commonly used for routine monitoring during product manufacture. Spores possess the advantage that they are relatively easy to produce, purify and dry on to an inert carrier, which is frequently an absorbent paper strip or disc, or a plastic or metal support. Spore resistance to the sterilizing agent must be carefully controlled, and so rigorous standardization of production processes followed by observance of correct storage conditions and expiry dates is essential.

Tests for sterility

It is sufficient here to repeat that the test is really one of the absence of gross contamination with readily grown microorganisms, and is not capable of affording a guarantee of sterility in any sample that passes.

The experimental details of these procedures are described in the European Pharmacopoeia (2000). This section is therefore restricted to an account of the major features of the test and a more detailed consideration of those practical aspects that are important or problematical.

It is obviously important that materials to be tested for sterility are not subject to contamination from the operator or the environment during the course of the test. For this reason it is essential that sterility tests are conducted in adequate laboratory facilities by competent and experienced personnel. Clearly, the consequences of recording an incorrect sterility result may be very severe. If a material which was *really* sterile were to fail the test it would need to be resterilized or, more probably, discarded, with significant cost implications. If, on the other hand, a contaminated batch were to pass a test for sterility and be released for use this would obviously represent a significant health hazard. For these reasons sterility testing procedures have improved significantly in recent years and failures are now viewed very seriously by the regulatory authorities. If a product does fail, it means either that the item in question was *really* contaminated, in which case the manufacturing procedures are seriously inadequate, or it means that the item was in fact sterile but the testing procedure was at fault. Either way, it is not possible to dismiss a failure lightly.

Sterility tests may be conducted in clean rooms or laminar flow cabinets which provide a grade A atmosphere as defined by the *Rules and Guidance for Pharmaceutical Manufacturers and Distributors* (1997). However, it is becoming increasingly common for testing to be undertaken in an isolator, which physically separates the operator from the test materials and so reduces the incidence of false positive test results due to extraneous contamination introduced during the test itself. Such isolators are similar in principle to a glove box, and typically consist of a cabinet (supported on legs or a frame) which is sufficiently large for the operator, who is covered by a transparent hood of moulded flexible plastic forming the cabinet base, to sit or stand within it.

A sterility test may be conducted in two ways. The direct inoculation method involves the removal of samples from the product under test and their transfer to a range of culture media that might be expected to support the growth of contaminating organisms. After incubation the media are examined for evidence of growth which, if present, is taken to

indicate that the product may not be sterile. It is not certain that the product is contaminated because the organisms responsible for the growth may have arisen from the operator or have been already present in the media to which the samples were transferred, i.e. the media used for the test were not themselves sterile. Thus, in conducting a sterility test it is necessary to include controls that indicate the likelihood of the contaminants arising from these sources. The size and number of the samples to be taken are described in the EP (2000).

Again it is necessary to inactivate any antimicrobial substances contained in the sample. These may be the active drug, e.g. antibiotic, or a preservative in an eye drop or multidose injection. Suitable in activators may be added to the liquid test media to neutralize any antimicrobial substances, but in the case of antibiotics particularly, no such specific inactivators are available (with the exception of β-lactamases which hydrolyse penicillins and cephalosporins). This problem may be overcome using a membrane filtration technique. This alternative method of conducting sterility tests is obviously only applicable to aqueous or oily solutions that will pass through a membrane having a pore size sufficiently small to retain bacteria. The membrane, and hence the bacteria retained on it, is washed with isotonic salts solution, which should remove any last traces of antimicrobial substances. It is then placed in a suitable liquid culture medium. This method is certainly to be preferred to direct inoculation because there is a greater chance of effective neutralization of antimicrobial substances.

Solids may be dissolved in an appropriate solvent; almost invariably water is used because most other common solvents have antimicrobial activity. If no suitable solvent can be found the broth dilution method is the only one available. If there is no specific inactivator available for antimicrobial substances that may be present in the solid then their dilution to an ineffective concentration by use of a large volume of medium is the only course remaining.

The controls associated with a sterility test are particularly important because incomplete control of the test may lead to erroneous results. Failure to neutralize a preservative completely may lead to contaminants in the batch going undetected and subsequently initiating an infection when the product is introduced into the body.

The EP (2000) recommends four controls be incorporated. The so-called growth promotion test simply involves the addition of low inocula (10–100 cells or spores per container) of suitable test organisms into the media used in the test to show that they do support the growth of the common contaminants for which they are intended. *Staphylococcus aureus, Bacillus subtilis* and *Pseudomonas aeruginosa* are the three aerobic bacteria used, *Clostridium sporogenes* the anaerobic bacterium and *Candida albicans* and *Aspergillus niger* the fungi. Organisms having particular nutritional requirements, such as blood, milk or serum, are not included; therefore they, in addition to the more obvious omissions such as viruses, may not be detected in a routine sterility test because suitable cultural conditions are not provided. On the other hand, it is impossible to design an all-purpose medium, and sterilization processes that kill the spore-forming bacteria and other common contaminants are likely also to eradicate the more fastidious pathogens such as streptococci and *Haemophilus* species, which would be more readily detected on blood-containing media. This argument does not, however, cover the possibility of such pathogens entering the product, perhaps via defective seals or packaging, after the sterilization process itself and then going undetected in the sterility test.

The second control (validation test) is intended to demonstrate that any preservative or antimicrobial substance has been effectively neutralized. This requires the addition of test organisms to containers of the various media as before, but in addition, samples of the material under test must also be added to give the same concentrations as those arising in the test itself. For the sterility test as a whole to be valid growth must occur in each of the containers in these controls.

It is necessary also to incubate several tubes of the various media just as they are received by the operator. If the tubes are not opened but show signs of growth after incubation this is a clear indication that the medium is, itself, contaminated. This should be an extremely rare occurrence, but in view of the small additional cost or effort the inclusion of such a control is worthwhile.

A control to check the likelihood of contamination being introduced during the test may be included in the programme of regular monitoring of test facilities. The European Pharmacopoeia 2000 recommends the use of 'preparations known to be sterile', which may be employed to check the adequacy of facilities and operator technique. These items, identical to the sample to be tested, are manipulated in exactly the same way as the test samples. If, after incubation, there are signs of microbial growth in the media containing these 'known steriles', the conclusion is drawn that the contamination arose during the testing process itself.

Some items present particular difficulties in sterility testing because of their shape or size, e.g. surgical dressings and medical devices. These problems are most conveniently overcome simply by testing the whole sample rather than attempting to withdraw a portion of it. So, for example, large clear plastic bags which have been radiation sterilized may be used to hold the entire medical device or complete roll or pack of dressings, which would then be totally immersed in culture medium. This method would only be valid if the culture medium gained access to the entire sample; otherwise the possibility exists, for example, of aerobic bacterial spores trapped within it failing to grow owing to insufficient diffusion of oxygen. This approach has the advantage of imposing a more rigorous test because a much larger sample is used. In the case of dressings, it may also reduce the risk of operator-induced contamination compared to the alternative approach, which would require the withdrawal of representative samples for testing from different areas of the roll or pack.

The final aspect of the test which is worthy of comment is the interpretation of results. If there is evidence that any of the test samples is contaminated the batch fails the test. If, however, there is convincing evidence that the test was invalid because the testing facility, procedure or media were inadequate, a single retest is permitted; this contrasts with earlier pharmacopoeial protocols, which under certain circumstances permitted two retests.

Endotoxin and pyrogen testing

This is an aspect of microbial contamination of medicines which is not normally considered part of microbiology but is discussed here because pyrogens are normally the products of microbial growth. A pyrogen is a material which when injected into a patient will cause a rise in body temperature (pyrexia). The lipopolysaccharides that comprise a major part of the cell wall of Gram-negative bacteria are called endotoxins, and it is these that are the most commonly encountered pyrogens (although any other substance that causes a rise in body temperature may be classified under the same heading). Bacterial cells may be pyrogenic even when they are dead and when they are fragmented, and so a solution or material that passes a test for sterility will not necessarily pass a pyrogen test. It follows from this that the more heavily contaminated with bacteria an aqueous injection becomes during manufacture, the more pyrogenic it is likely to be at the end of the process.

Two main procedures are used for the detection of pyrogens. The traditional method requires the administration of the sample to laboratory rabbits, whose body temperature is monitored for a period of time thereafter. The alternative procedure, which is now by far the most common, is to use the Limulus Amoebocyte Lysate Test (LAL), in which the pyrogen-containing sample causes gel formation in the lysis product of amoebocyte cells of the giant horseshoe crab *Limulus polyphemus*. A detailed account of endotoxin testing is outside the scope of this chapter but the review by Weary (1996) describes both LAL and rabbit testing.

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The action of physical and chemical agents on microorganisms

Geoff Hanlon, Norman Hodges

CHAPTER CONTENTS

The subject of this chapter is of importance to pharmaceutical scientists because they have a responsibility for:

1. the production of medicines having as their prime function the destruction of microorganisms, e.g. antiseptic liquids and antibiotic formulations;
2. the production of sterile medicaments having no living microorganisms, e.g. injections and eye drops;
3. the production of a wide range of medicines which must be effectively protected against microbial spoilage.

Thus the major pharmaceutical interest in microorganisms is that of killing them, or at least preventing their growth. Consequently it is necessary to have both an understanding of the physical processes, e.g. heating and irradiation, that are used to kill microorganisms and a knowledge of the more diverse subject of antimicrobial chemicals.

This background knowledge must therefore include an understanding of the kinetics of cell inactivation, the calculation of parameters by which microbial destruction or growth inhibition are measured, and an appreciation of the factors that influence the efficiency of the physical and chemical processes used. These aspects, together with a synopsis of the major groups of antimicrobial chemicals, are the subject of this chapter.

THE KINETICS OF CELL INACTIVATION

The death of a population of cells exposed to heat or radiation is often found to follow or approximate to first-order kinetics. In this sense it is similar to bacterial growth during the logarithmic phase of the cycle, the graphs representing these processes being similar but of opposite slope. Assuming first-order

kinetics (the exceptions will be considered later), an initial population of N_0 cells per mL will, after a time t minutes, be reduced to N_t cells per mL, according to the following equations in which k is the inactivation rate constant;

$$N_t = N_0 e^{-kt} \quad (41.1)$$

$$\ln N_t = \ln N_0 - kt \quad (41.2)$$

$$\log_{10} N_t = \log_{10} N_0 \frac{-kt}{2.303} \quad (41.3)$$

Thus the data in Table 41.1 may be used to produce a plot of logarithm of cell concentration against exposure time (Fig. 41.1), where the intercept is log N_0 and the slope is $-k/2.303$. This may be plotted with the logarithm of the percentage of survivors as the ordinate; thus the largest numerical value on this axis is 2.0. An important feature of Figure 41.1 is the fact that there is no lower end-point to the ordinate scale – it continues indefinitely. If the initial population was 1000 cells mL^{-1} the logarithmic value would be 3.0; at 100 cells mL^{-1} the value would be 2.0; at 10 cells mL^{-1} 1.0, and at 1 cell mL^{-1} zero. The next incremental point on the logarithmic scale would be –1, which corresponds to 0.1 cells mL^{-1}. It is clearly nonsense to talk of a fraction of a viable cell per mL, but this value corresponds to one whole cell in 10 mL of liquid. The next point, –2.0, corresponds to one cell in 100 mL, and so on. Sterility is the complete absence of life, i.e. zero cells mL^{-1}, which has a log value of $-\infty$. ***Guaranteed*** sterility would therefore require an infinite exposure time.

Table 41.1 Death of *B. megaterium* spores in pH 7.0 buffer at 95°C

Time (minutes)	Viable cell concentration mL^{-1}	Percent survivors	Log_{10} % survivors
0	2.50×10^6	100	2.000
5	5.20×10^5	20.8	1.318
10	1.23×10^5	4.92	0.692
15	1.95×10^4	0.78	– 0.108
20	4.60×10^3	0.18	– 0.745
25	1.21×10^3	0.048	– 1.319
30	1.68×10^2	0.0067	– 2.174

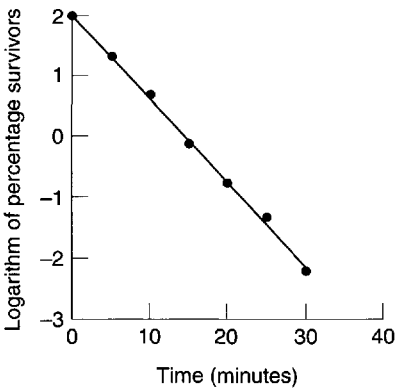


Fig. 41.1 Heat inactivation of *B. megaterium* spores at 95 °C.

D value, or decimal reduction time

It is characteristic of first-order kinetics that the same percentage change in concentration occurs in successive time intervals. Thus in Figure 41.1 it can be seen that the viable population falls to 10% of its initial value after 7.5 minutes; in the next 7.5-minute period the population again falls to 10% of its value at the start of that period. This time period for a 90% reduction in count is related to the slope of the line and is one of the more useful parameters by which the death rate may be indicated. It is known as the decimal reduction time, or D value, and usually has a subscript showing the temperature at which it was measured, e.g. D_{121} or D_{134}. It is quite possible to indicate the rate of destruction by the inactivation rate constant calculated from the slope of the line, but the significance of this value cannot be as readily appreciated during conversation as that of a D value, and so the former is rarely used.

Z values

When designing steam sterilization processes it is necessary to know both the D value, which is a measure of the effectiveness of heat at any given temperature, and the extent to which a particular increase in temperature will reduce the D value, i.e. it is necessary to have a measure of the effect of temperature change on death rate. One such measure is the Z value, which is defined as the number of degrees temperature change required to achieve a tenfold change in D value, e.g. if the D value for *Bacillus stearothermophilus* spores at 110°C is 20 minutes and they have a Z value of 9°C, this means that at 119°C the D value would be 2.0 minutes and at 128°C the D value would be 0.20 minutes. The relationship between D and Z values is shown in Figure 41.2. The Z value is one of several parameters that relate change in temperature to change in death rate, and is probably the most commonly used and readily understood. The activation energy obtained from an Arrhenius plot (see Chapter 7) or a temperature coefficient, a Q_{10} value (change in rate for a 10°C change in temperature), does the same but is less commonly used.

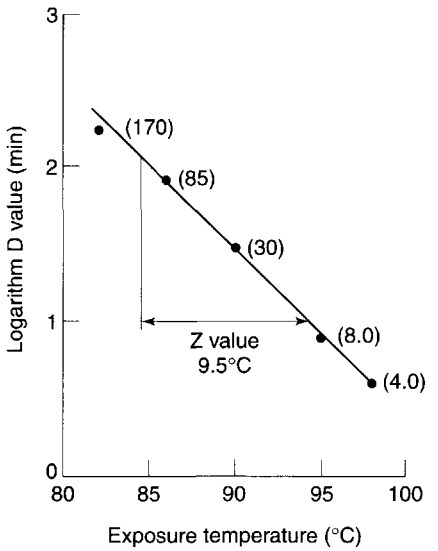


Fig. 41.2 Relationship between logarithm of *D* value and exposure temperature for heated *B. megaterium* spores. Individual *D* values (minutes) are shown in parentheses.

Alternative survivor plots

It was stated earlier that bacterial death often approximates to first-order kinetics, although exceptions do arise. Some of the more common are illustrated in Figure 41.3. The plot labelled A is that conforming to first-order kinetics, which has already been described. A shoulder on the curve, as in case B, is not uncommon and various explanations have been offered. Cell aggregation or clumping may be responsible for such a shoulder, because it would be necessary to apply sufficient heat to kill all of the cells in the clump, not merely the most sensitive, before a fall is observed in the number of colonies appearing on the agar. Under normal circumstances one single colony could arise both from one cell alone or, say, from 100 cells aggregated together. In the latter case, if sufficient heat was applied to kill only the 99 most sensitive cells in the clump the colony count would be unaltered. Clumping is not the only explanation, because substantial shoulders may arise using suspensions where the vast majority of cells exist individually.

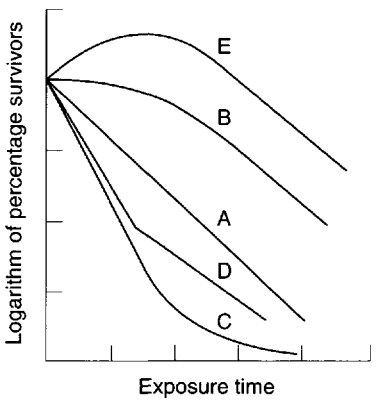


Fig. 41.3 Alternative survivor plots for cells exposed to lethal agents.

Tailing of survivor curves, as in plot C, is often observed if the initial cell concentration is high. This has been attributed to the presence of mutants that are exceptionally resistant to the lethal agent. If the proportion of mutants was 1 in 10^6 cells and the initial concentration only 10^5 cells mL^{-1} the mutant would not be detected, but an initial population of 10^9 cells mL^{-1} would permit easy detection if the inactivation plot were continued down to low levels of survivors. Again there are alternative explanations, one of the most common being that the cells dying during the early exposure period release chemicals which help to protect those that are still alive.

A sharp break in the line, as in D, usually indicates that there are two distinct populations of cells present which have markedly different resistances. Contamination of a cell suspension or culture is a possible explanation, or it may be that a mutant has arisen naturally and the cultural conditions are such that it has a selective advantage and its numbers have increased until it is a substantial proportion of the population.

Plot E is uncommon and is usually only seen as a result of 'heat activation' of bacterial spores. This is a situation in which a significant proportion of a population of spores (usually a thermophil) remain dormant and fail to germinate and produce colonies under 'normal' conditions. If the suspension receives a heat stimulus or shock which is insufficient to kill the spores, some or all of those that would otherwise remain dormant become activated, germinate, and thus produce a rise in the colony count.

Killing of microorganisms by chemicals results in first-order kinetics less commonly than heat- or radiation-induced killing. This is because the chemical must interact with a target molecule within the cell, and the concentration of both the chemical and the intracellular target might influence death rate and so result in second-order kinetics. In practice, however, the antimicrobial chemical is often present in such a high concentration that the proportion of it that is 'used up' by interaction with the cell is negligible; this means its concentration is effectively constant, and pseudo-first order kinetics result.

ANTIMICROBIAL EFFECTS OF MOIST AND DRY HEAT

Moist heat (steam) and dry heat (hot air) both have the potential to kill microorganisms but their efficiencies and their mechanisms of action differ. In autoclaves dry, saturated steam, i.e. 100% water vapour with no liquid water present, is used at temperatures between 121 and 135°C, at which it rapidly kills microorganisms. An advantage of using steam is that it possesses latent heat of vaporization, which it transfers to any object upon which it condenses (see Chapter 38). It is essential to use dry saturated steam if maximal autoclaving efficiency is to be achieved. If the steam is wet, i.e. contains liquid water, penetration of vapour-phase steam into dressings may be retarded. If the steam is superheated, i.e. its temperature has been raised while the pressure remained constant, or the pressure fell while the temperature remained constant, it contains less moisture and latent heat than dry saturated steam at the same temperature. In this case the effect is similar to using a steam–air mixture at that temperature. The process by which steam kills cells is hydrolysis of essential proteins (enzymes) and nucleic acids. In contrast, dry heat causes cell death by oxidative processes, although again it is the proteins and nucleic acids that are the vulnerable targets. Dry heat is much less effective at killing microorganisms than steam at the same temperature. Exposures of not less than 2 hours at 160°C (or an equivalent temperature/time combination) are recommended in the BP for sterilization by dry heat methods. The state of hydration of a cell is thus an important factor determining its resistance to heat.

Resistance of microorganisms to moist and dry heat

Numerous factors influence the observed heat resistance of microbial cells and it is difficult to make comparisons between populations unless these factors are controlled. Not surprisingly, marked differences in resistance exist between different genera, species and strains, and between the spore and vegetative cell forms of the same organism. The resistance may be influenced, sometimes extensively, by the age of the cell, i.e. lag, exponential or stationary phase; its chemical composition, which in turn is influenced by the medium in which the cell is grown; and by the composition and pH of the fluid in which the cell is heated. It is difficult to obtain strictly comparable heat resistance data for grossly dissimilar organisms, but the values quoted in Table 41.2 indicate the relative order of heat resistance of the

Table 41.2 A 'league table' of heat resistances of different microorganisms and infectious agents

Organism or agent	Heat resistance (values are for fully hydrated organisms unless otherwise stated)
Prions	The most heat-resistant infectious agent. May survive steam sterilization at 134–138°C for 1 hour
Bacterial spores (endospores)	Little or no inactivation at <80°C. Some species survive boiling for several hours
Fungal spores	Ascospores of *Byssochlamys* species may survive 88°C for 60 minutes but most fungal spores are less resistant
Actinomycete spores	Spores of *Nocardia sebivorans* reported to survive for 10 minutes at 90°C, but the majority of species are less resistant
Mycobacterium tuberculosis	May survive for 30 minutes at 100°C in the dry state but when hydrated is killed by pasteurization (63°C for 30 minutes or 72°C for 15 seconds)
Yeasts	Ascospores and vegetative cells show little difference in resistance. Survival for 20 minutes at 60°C is typical
Most non-sporing bacteria of pharmaceutical or medical importance	D_{60} of 1–5 minutes is typical of staphylococci and many Gram-negative enteric organisms. Enterococci may be more resistant, and pneumococci may survive for 30 minutes at 110°C when dry
Fungi and actinomycetes	Vegetative mycelia exhibit similar resistance to that of non-sporing bacteria described above
Viruses	Rarely survive for > 30 minutes at 55–60°C except perhaps in blood or tissues, but papovaviruses and hepatitis viruses are more resistant
Protozoa and algae	Most are no more resistant than mammalian cells and survive only a few hours at 40–45°C, but cysts of *Acanthamoeba* species are more resistant

various microbial groups. Tabulation of *D* values at a designated temperature is perhaps the most convenient way of comparing resistance, but this is only suitable for first-order kinetics. Alternative methods of comparison include the time to achieve a particular percentage kill or the time required to achieve no survivors; the latter is, of course, dependent upon the initial population level.

The most heat-resistant infectious agents (as distinct from microbial cells) are prions, which are proteins rather than living cells and the cause of spongiform encephalopathies e.g. Creutzfeldt–Jakob disease (CJD) and bovine spongiform encephalopathy (BSE, or 'mad cow disease'). Prion proteins are so resistant to heat inactivation that an autoclave cycle of 134–138°C has been recommended for the decontamination of prion-contaminated materials, and the efficacy of even this extreme heat treatment has been questioned.

Bacterial endospores are invariably found to be the most heat resistant ***cell*** type, and those of certain species may survive boiling water for many hours. (The term endospores refers to the spores produced by *Bacillus* and *Clostridium* species and is not to be confused with the spores produced by other bacteria, such as actinomycetes, which do not develop within the vegetative cell.) The majority of *Bacillus* and *Clostridium* species normally form spores which survive in water for 15–30 minutes at 80°C without significant damage or loss of viability. Because endospores are more resistant than other cells they have been the subject of a considerable amount of research in the food and pharmaceutical industry over the last 50 years, and much of the earlier work has been reviewed by Russell (1999).

Mould spores and those of yeasts and actinomycetes usually exhibit a degree of moist heat resistance intermediate between endospores and vegetative cell forms; survival at 60°C for several hours but death at 80°C or higher would be typical of such cells. Bacterial and yeast vegetative cells and mould mycelia all vary significantly in heat resistance: mycobacteria, which possess a high proportion of lipid in their cell wall, tend to be more resistant than others. Protozoa and algae are, by comparison, susceptible to heat and, when in the vegetative (uncysted) state, like mammalian cells they rapidly die at temperatures much in excess of 40°C. Information on the heat resistance of viruses is limited, but the available data suggest that they may vary significantly between types. The majority of viruses are no more heat resistant than vegetative bacteria, but hepatitis viruses have been reported to be less susceptible than others.

Resistance to dry heat by different groups of infectious agents and microorganisms usually follows a pattern similar to that in aqueous environments. Again, prions head the 'league table' by exhibiting extreme heat resistance, and endospores are substantially more resilient than other cell types, with those of *B. stearothermophilus* and *B. subtilis* usually more resistant than other species. Exposures of 2 hours at 160°C are required by the European Pharmacopoeia (1997) to achieve an acceptable level of sterility assurance for materials sterilized by dry heat.

Cells of pneumococci have been reported to survive dry heat at 110°C for 30 minutes, but this represents exceptional resistance for vegetative cells, most of which may be expected to die after a few minutes, heating at 100°C or less.

Valid comparisons of dry heat resistance among dissimilar organisms are even less common than those for aqueous environments because there is the additional problem of distinguishing the effects of drying from those of heat. For many cells desiccation is itself a potentially lethal process, even at room temperature, so that experiments in which the moisture content of the cells is uncontrolled may produce results that are misleading or difficult to interpret. This is particularly so when the cells are heated under conditions where their moisture content is changing and they become progressively drier during the experiment.

Factors affecting heat resistance and its measurement

The major factors affecting heat resistance are listed in the previous section and will be considered in some detail here. The subject has been extensively studied, and again many of the experimental data and consequently many of the examples quoted in this section come from the field of spore research.

The measurement of heat resistance in fully hydrated cells, i.e. those suspended in aqueous solutions or exposed to dry saturated steam, does not normally represent a problem when conducted at temperatures less than 100°C, but errors may occasionally arise when spore heat resistance is measured at higher temperatures. In these circumstances it is necessary to heat suspensions sealed in glass ampoules immersed in glycerol or oil baths or to expose the spores to steam in a modified autoclave. Monitoring and control of heat-up and cool-down times become important, and failure to pay adequate attention to these aspects may lead to apparent differences in resistance, which may be due simply to factors such as variations in the thickness of glass in two batches of ampoules.

Species and strain differences

Variations in heat resistance between the species within a genus are very common, although it is difficult to identify from the published reports the precise magnitude of these differences because different species may require different growth media and incubation conditions which, together with other factors, might influence the results. Murrell and Warth (1965), for example, described a 700-fold variation in spore heat resistance within 13 *Bacillus* species, but to produce the spore crops for testing they necessarily had to use eight culture media, three incubation temperatures and six procedures for cleaning the spores. Differences between strains of a single species are, not surprisingly, more limited; D_{90} values ranging from 4.5 to 120 minutes have been reported for five strains of *Clostridium perfringens* spores.

Cell form

Whether or not the heated cells exist in the vegetative or the spore form may in some cases be related to the age of the culture or the cell population being heated. In cultures of *Bacillus* and *Clostridium* species the proportion of spores usually increases as the incubation period is extended and the culture ages. This may be due to more and more of the vegetative cells producing spores, in which case the spore count increases. Alternatively, the spore count may remain unchanged but the vegetative cell count falls as a result of the action of lytic enzymes produced by the cells themselves. Among the common mesophilic *Bacillus* species spore formation is largely complete 6–10 hours after the end of exponential growth under optimal cultural conditions. The degree of heat resistance and the concentration of spores would not be expected to rise much after this time. Conducting heat-resistance studies on a mixture of spores and vegetative cells is undesirable because the likely result is a rapid initial fall in count due to killing of the vegetative cells, and a subsequent slower rate due to death of spores. If necessary the vegetative cells can usually be removed by addition of the enzymes lysozyme and trypsin.

The degree of heat resistance shown by vegetative cells may also be influenced by the stage of growth from which the cells were taken. It is normally found that stationary-phase cells are more heat resistant than those taken from the logarithmic phase of growth, although several exceptions have been reported.

Cultural conditions

The conditions under which the cells are grown is another factor that can markedly affect heat resistance. Insufficient attention has been paid to this potential source of variation in a substantial part of the research conducted during the first half of the 20th century, and the same criticism might even be made of some of that reported more recently. Not infrequently, insufficient details of the cultivation procedures are described in the scientific reports, or materials of variable composition, e.g. tap water or soil extracts, were used in media without regard to the possible differences that might have arisen between successive batches or populations of cells.

Factors such as growth temperature, medium pH and buffering capacity, oxygen availability and concentrations of culture medium components may all affect resistance.

Thermophilic organisms are generally more heat resistant than mesophils, which in turn tend to be more resistant than psychrophils. If a 'league table' of spore heat resistance were constructed it is probable that *B. stearothermophilus*, *B. coagulans* and *Cl. thermosaccharolyticum* would head the list; all three have growth optima of 50–60°C. Variable results have arisen when single species have been grown at a variety of temperatures. *Escherichia coli* and *Streptococcus faecalis* have both been the subject of conflicting reports on the influence of growth temperature on heat resistance, whereas spores of *B. cereus* produced at temperatures between 20 and 41°C showed maximal resistance at 30°C.

The effects of medium pH, buffering capacity, oxygen availability and the concentrations of culture medium components are often complex and interrelated. An unsuitable pH, inadequate buffer or insufficient aeration may all limit the extent of growth, with the result that the cells that do grow each have available to them a higher concentration of nutrients than would be the case if a higher cell density had been achieved. The levels of intracellular storage materials and metal ions may therefore differ and so influence resistance to heat and other lethal agents. Cells existing in or recently isolated from their 'natural' environment, e.g. water, soil, dust or pharmaceutical raw materials, have often been reported to have a greater heat resistance than their progeny, which have been repeatedly subcultured in the laboratory and then tested under similar conditions.

pH and composition of heating menstruum

It is frequently found that cells survive heating more readily when they are at neutrality (or their optimum pH for growth, if this differs from neutrality). The combination of heat and an unfavourable pH may be additive or even synergistic in killing effects; thus

B. stearothermophilus spores survive better at 110°C in dilute pH 7.0 phosphate buffer than at 85°C in pH 4.0 acetate buffer. Differences in heat resistance may also result merely from the presence of the buffer, regardless of the pH it confers. Usually an apparent increase in resistance occurs when cells are heated in buffer rather than in water alone. A similar increase is often found to occur on the addition of other dissolved or suspended solids, particularly those of a colloidal or proteinaceous nature, e.g. milk, nutrient broth, serum. Because dissolved solids can have such a marked effect on heat resistance, great care must be taken in attempting to use experimental data from simple solutions to predict the likely heat treatment required to kill the same cells in a complex formulated medicine or food material. An extreme case of protection of cells from a lethal agent is the occlusion of cells within crystals. When spores of *B. subtilis* var. *niger* were occluded within crystals of calcium carbonate their resistances to inactivation were approximately 900 times and 9 times higher than for unoccluded spores when subjected to steam and dry heat, respectively; an exposure period of 2.5 hours at 121°C (moist heat) was required to eliminate survivors within the crystals. It is to minimize the risk of such situations arising that there is a requirement in the *Guide to Good Manufacturing Practice* that medicines be prepared in clean conditions.

The solute concentrations normally encountered in dilute buffer solutions used as suspending media for heat-resistance experiments cause no significant reduction in the vapour pressure of the solution relative to that of pure water, i.e. they do not reduce the water activity, A_w, of the solution (which has a value of 1.0 for water). If high solute concentrations are used or the cells are heated in a 'semi-dry' state the A_w is significantly lower and the resistance is increased, e.g. a 1000-fold increase in D value has been reported for *B. megaterium* spores when the water activity was reduced from 1.0 to between 0.2 and 0.4.

Recovery of heat-treated cells

The recovery conditions available to cells after exposure to heat may influence the proportion of cells that produce colonies. A heat-damaged cell may require an incubation time longer than normal to achieve a colony of any given size, and the optimum incubation temperature may be several degrees lower. The composition of the medium may also affect the colony count, with nutritionally rich media giving a greater percentage survival than a 'standard' medium, whereas little or no difference can be detected between the two when unheated cells are used. Adsorbents such as charcoal and starch have been found to have beneficial effects in this context.

IONIZING RADIATIONS

Ionizing radiations can be divided into electromagnetic and particulate (corpuscular) types. Electromagnetic radiations include γ-rays and X-rays, whereas particulate radiation includes α and β particles, positrons and neutrons.

Particulate radiation

The nuclear disintegration of radioactive elements results in the production of charged particles. α particles are heavy and positively charged, being equivalent to the nuclei of helium atoms. They travel relatively slowly in air, and although they cause a great deal of ionization along their paths they have very little penetrating power, their range being just a few centimetres in air. α particles have no application in this aspect of pharmacy and will not be considered further. β particles are negatively charged and have the same mass as an electron. In air the penetrating power of these particles is a few metres, but they will be stopped by a thin sheet of aluminium. β particles resulting from radioactive decay are therefore not sufficiently penetrative for use in sterilization processes, but the production of accelerated electrons from man made machines (cathode rays) results in particles of great energy with enhanced penetrating power.

Electromagnetic radiation

γ radiation results when the nucleus still has too much energy even after the emission of α or β particles. This energy is dissipated in the form of very short wavelength radiation which, as it has no mass or charge, travels with the speed of light, penetrating even sheets of lead. Although travelling in a wave form γ radiation behaves as if composed of discrete packets of energy called quanta (photons). A ^{60}Co source emits γ-rays with photons of 1.17 and 1.33 MeV, and the source has a half-life of 5.2 years. X-rays are generated when a heavy metal target is bombarded with fast electrons, and they have similar properties to γ-rays despite originating from a shift in electron energy rather than from the nucleus.

Units of radioactivity

The unit of activity is the becquerel (Bq), which is equal to one nuclear transformation per second. This replaces the term curie (Ci), and 3.7×10^{10} becquerels = 1 curie. The unit of absorbed dose according to the SI system is the gray (Gy), which is equal to one joule per kilogram. However, the term rad is still widely used and is equivalent to 100 ergs per gram of irradiated material.

1 gray = 100 rads

The energy of radiation is measured in electron volts (eV) or millions of electron volts (MeV). An electron volt is the energy acquired by an electron falling through a potential difference of 1 volt.

Effect of ionizing radiations on materials

Ionizing radiations are absorbed by materials in a variety of ways, depending upon the energy of the incident photons.

1. *Photoelectric effect*: low-energy radiation (< 0.1 MeV) is absorbed by the atom of the material, resulting in the ejection or excitation of an electron.
2. *Compton effect*: incident photons of medium energy 'collide' with atoms and a portion of the energy is absorbed with the ejection of an electron. The remaining energy carries on impacting with further atoms and emitting further electrons until all the energy is scattered.
3. *Pair production*: radiations of very high energy are converted on impact into negatively charged electrons and positively charged particles called positrons. The positron has an extremely short life and quickly annihilates itself by colliding with an orbital electron.

The ionization caused by the primary radiation results in the formation of free radicals, excited atoms etc. along a discrete track through the material. However, if secondary electrons contain sufficient energy they may cause excitation and ionization of adjacent atoms, thereby effectively widening the track. (Accelerated electrons used in electron beam sterilizers are essentially equivalent to the secondary electrons arising from γ irradiation – they cause direct ionization of molecules within materials.) Temperature rise during irradiation is very small and even high-energy radiation resulting in pair production is only accompanied by an increase of approximately 2°C, but nevertheless the chemical changes that occur in irradiated materials are very widespread. Of particular significance here are the deleterious changes that may occur in packaging materials at normal dosage levels. Such effects may include changes in tensile strength, colour, odour and gas formation. Polymers most affected include acetal, FEP, PTFE and PVA. Total absorbed energy determines the extent of physical and chemical reactions that occur, and so damage is cumulative. For sterilization purposes exposure times can be long, but the process is predictable and delivers a reproducible level of lethality.

The lethal effect of irradiation on microorganisms can occur in two ways.

1. *Direct effect* In this case the ionizing radiation is directly responsible for the damage by causing a direct hit on a sensitive target molecule. It is generally accepted that cellular DNA is the principal target for inactivation, and that the ability to survive irradiation is attributable to the organism's ability to repair damaged DNA rather than to any intrinsic resistance of the structure. Further damage may be caused by free radicals produced within the cell but not directly associated with DNA. These radicals can diffuse to a sensitive site and react with it, causing damage.
2. *Indirect effect* The passage of ionizing radiation through water causes ionization along and immediately next to the track and the formation of free radicals and peroxides. These peroxides and free radicals are highly reactive and destructive and are responsible for both killing capability and the ability to modify the properties of polymers.
   Some of the possible reactions are as follows:

$$\xrightarrow{\text{radiation}} H_2O \rightarrow H_2O^+ + e^-$$

$$H_2O^+ \rightarrow \cdot OH + H^+$$

$$e^- + H_2O \rightarrow OH^- + H\cdot$$

$$2H\cdot \rightarrow H_2$$

$$2\cdot OH \rightarrow H_2O_2$$

The presence of oxygen has a significant effect on the destructive properties of ionizing radiation owing to the formation of hydroperoxyl radicals.

$$H\cdot + O_2 \rightarrow \cdot HO_2$$

Peroxides and free radicals can act as both oxidizing and reducing agents according to the conditions.

Factors affecting the radiation resistance of microorganisms

Across the spectrum of microorganisms viruses are the most resistant forms to the effects of radiation, followed by bacterial endospores, then Gram-positive cells and finally, Gram-negative cells. Resistance to radiation is genetically determined and a particularly resistant bacterium called *Deinococcus radiodurans* can withstand a radiation dose up to three times that which would kill a normal bacterium. Fortunately, this organism does not have any clinical significance. It is worth noting that microbial products such as endotoxins will not be inactivated by normal doses of ionizing radiations, and so it is important to ensure that initial bioburden levels are low.

Oxygen has already been mentioned as having a significant effect on radiation resistance as the increased levels of hydroperoxyl radicals lead to marked increases in sensitivity. Vegetative cells such as *E. coli* and *Pseudomonas aeruginosa* are three to four times more sensitive in the presence of oxygen than in its absence. The presence of moisture will influence sensitivity, and dehydration increases resistance owing to an indirect effect on the formation and mobility of free radicals. Freezing also increases radiation resistance owing to the reduction of mobility of free radicals in the menstruum, preventing them from diffusing to sites of action at the cell membrane. Above the freezing point there is very little effect of temperature.

A variety of organic materials provide a protective environment for microorganisms, and comparison of radiation resistance is greatly complicated by different complexities of the media used. Sulphydryl groups, such as may be found in amino acids and proteins, have a protective effect on microorganisms owing to their interaction with free radicals. In contrast, compounds that combine with -SH groups, such as halogenated acetates, tend to increase sensitivity. Some naturally occurring materials, particularly foods, may have a profound protective effect on contaminant bacteria, and this is of concern to the food processing industry.

ULTRAVIOLET RADIATION

Although UV radiation has a range of wavelength from approximately 15 nm to 330 nm its range of maximum bactericidal activity is much narrower (220–280 nm), with an optimum of about 265 nm. Whereas ionizing radiations cause electrons to be ejected from atoms in their path, UV radiation does not possess sufficient energy for this and merely causes the electrons to become excited. It has much less penetrating power than ionizing radiations and tends to be used for the destruction of microorganisms in air and on surfaces.

The bactericidal effect of UV light is due to the formation of linkages between adjacent pyrimidine bases in the DNA molecule to form dimers. These are usually thymine dimers, although other types have been identified. The presence of thymine dimers alters the structural integrity of the DNA chain, thereby hindering chromosome replication. Certain cells can repair damaged DNA in a variety of ways, enhancing their radiation resistance.

Exposure of UV-damaged cells to visible light (photoreactivation) enables a light-dependent photoreactivating enzyme to split the thymine dimers into monomers. A second mechanism is not light dependent and is called dark recovery. In this case the thymine dimers are removed by a specific endonuclease enzyme that nicks the damaged DNA strand either side of the dimer. DNA polymerase then replaces the missing nucleotides and the ends are joined by a ligase enzyme.

Factors affecting resistance to UV light

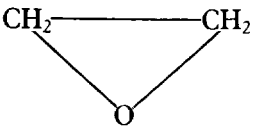
As already mentioned, UV light has very little penetrating power and anything that acts as a shield around the cells will afford a degree of protection. The formation of aggregates of cells will result in those cells at the centre of the aggregate surviving an otherwise lethal dose of radiation. Similarly, microorganisms suspended in water withstand considerably higher doses of radiation than in the dry state owing to lack of penetration of the radiation. Suspension of bacteria in broth containing organic matter such as proteins increases the resistance of the cells still further. The stage of growth of the culture will affect the sensitivity of the cells, with maximum sensitivity being shown during the logarithmic phase.

Other factors shown to influence radiation resistance include pH, temperature and humidity, although the effect of the last parameter is still somewhat confused.

GASES

The use of gases as antimicrobial agents has been documented for centuries, although it is only recently that their mechanisms of action and factors affecting activity have been elucidated. A wide variety of gaseous agents have been used for their antimicrobial properties, but owing to the constraints of space only a few of the major ones will be dealt with here.

Ethylene oxide



Ethylene oxide is a gas at room temperature (with a boiling point at 10.7°C) which readily permeates a variety of materials (plastics, cardboard, cloth etc.) but not crystals. Its odour is reported as being rather pleasant, although the levels at which it is detected in the atmosphere (700 ppm) greatly exceed the 5 ppm maximum safety limit for humans. Toxicity problems include burns and blistering if the material comes into contact with the skin, whereas inhalation results in lachrymation, headache, dizziness and vomiting. Great care must be taken to ensure the removal of ethylene oxide from treated products (e.g. rubber gloves) to avoid the risk of skin reactions. Explosive mixtures are formed when ethylene oxide is mixed with air at any concentration above 3%, and this is especially dangerous if the gas mixture is confined. The addition of carbon dioxide or fluorinated hydrocarbons will eliminate this risk, and for sterilization purposes gas mixtures of 10% ethylene oxide/90% carbon dioxide are typically used.

Ethylene oxide is extremely effective at killing microorganisms and its activity is related to its action as an alkylating agent. Reactive hydrogen atoms on hydroxyl, carboxyl, sulphydryl and amino groups can all be replaced with hydroxyethyl groups, thereby interfering with a wide range of metabolic activities. Ethylene oxide inactivates the complete spectrum of microorganisms, including endospores and viruses. The difference in resistance between endospore-forming bacteria and vegetative cells is only of the order of five to ten times, compared to several thousandfold differences with other physical and chemical processes. In addition, no microorganism of genetically determined high resistance has been found. Spores of *B. subtilis* var. *niger* are among the most resistant to the effect of ethylene oxide. The moist heat-resistant spore-former *B. stearothermophilus* and spores of *Clos. sporogenes* are no more resistant than a number of vegetative organisms, such as *Staph. aureus* and *Micrococcus luteus*. Fungal spores exhibit the same order of resistance as vegetative cells.

Factors affecting the activity of ethylene oxide

The bactericidal activity of ethylene oxide is proportional to the partial pressure of gas in the reaction chamber, time of exposure, temperature of treatment and level and type of contamination. At room temperature the time taken to reduce the initial concentration of cells by 90% can be very slow, and for this reason elevated temperatures of 50–60°C are recommended, which results in greatly increased rates of kill. Concentrations of ethylene oxide between 500 and 1000 mg L^{-1} are usually employed. Relative humidity has a most pronounced effect, as at very high humidities ethylene oxide may be hydrolysed to the much less active ethylene glycol, and this is borne out by the observation that the gas is 10 times more active at 30% RH than at 97% RH. The optimum value for activity appears to be between 28 and 33%. Below 28% the alkylating action of ethylene oxide is inhibited due to lack of water. The degree of dehydration of cells greatly influences activity and it may not be possible to rehydrate very dry organisms simply by exposure to increased RH. In practice the RH value chosen is usually between 40 and 70%.

Microorganisms may be protected from the action of ethylene oxide by occlusion within crystalline material or when coated with organic matter or salts. *B. subtilis* var. *niger* spores dried from salt-water solutions are much more resistant to the gas than are suspensions dried from distilled water.

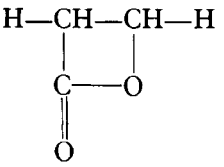
Biological indicators used to test the efficacy of ethylene oxide treatment employ spores of *B. subtilis* dried on to suitable carriers, such as pieces of aluminium foil.

Formaldehyde

Formaldehyde (H.CHO) in its pure form is a gas at room temperature, with a boiling point of −19°C, but readily polymerizes at temperatures below 80°C to form a white solid. The vapour, which is extremely irritating to the eyes, nose and throat, can be generated either from solid polymers such as paraformaldehyde or from a solution of 37% formaldehyde in water (formalin). Formalin usually contains about 10% methanol to prevent polymerization. As with ethylene oxide, formaldehyde is a very reactive molecule and there is only a small differential in resistance between bacterial spores and vegetative cells. Its bactericidal powers are superior to those of ethylene oxide (concentrations of 3–10 mg L^{-1} are effective) but it has weak penetrating power and is really only a surface bactericide. It is also more readily inactivated by organic matter. Adsorbed gas is very difficult to remove and long airing times are required. Its mechanism of action is thought to involve the production of intramolecular crosslinks between proteins, together with interactions with RNA and DNA. It acts as a mutagenic

agent and an alkylating agent, reacting with carbonyl, thiol and hydroxyl groups. In order to be effective the gas must dissolve in a film of moisture surrounding the bacteria, and for this reason relative humidities in the order of 75% are required. Formaldehyde used in conjunction with low-temperature steam is a very effective sterilization medium.

β-Propiolactone



This is a colourless liquid at room temperature, with a pungent odour and a boiling point of 162°C. It has a wide spectrum of activity, the vapour being bactericidal to both Gram-positive and Gram-negative bacteria, viruses, rickettsiae, fungi and also *Mycobacterium* species. It is less active against bacterial spores. It is faster acting than formaldehyde and does not give problems with polymerization, but it has feeble penetrating powers. In order to sterilize enclosed spaces concentrations between 2 and 5 mg L^{-1} of air are necessary at a relative humidity of 80%, and these conditions must be maintained for 2 hours at 24°C.

Unfortunately, the use of β-propiolactone has been limited by doubts about its safety, as there is evidence showing it to be carcinogenic in animals. Acute short-term inhalation exposure causes severe irritation of the eyes, nose, throat and respiratory tract. Acute dermal exposure may cause irritation of the skin, blistering or burns. It has been used in liquid form for the sterilization of rabies vaccine and of various graft tissues.

Propylene oxide

Propylene oxide is a liquid (boiling point = 34°C) at room temperature which requires heating to volatilize. It is inflammable between 2.1 and 21.5% by volume in air, but this can be reduced by mixing with CO_2. Its mechanism of action is similar to that of ethylene oxide and involves the esterification of carbonyl, hydroxyl, amino and sulphydryl groups present on protein molecules. It is, however, less effective than ethylene oxide in terms of its antimicrobial activity and its ability to penetrate materials. Whereas ethylene oxide breaks down to give ethylene glycol or ethylene chlorohydrin, both of which are toxic, propylene oxide breaks down to propylene glycol, which is much less so.

Methyl bromide

Methyl bromide boils at 3.46°C and is a gas at room temperature. It is used as a disinfectant and a fumigant at concentrations of 3.5 mg L^{-1} with a relative humidity between 30 and 60%. It has inferior antimicrobial properties compared to the previous compounds but has good penetrating power.

Gas plasmas

A plasma is formed by applying energy to a gas or vapour under vacuum. Natural examples are lightning and sunlight, but plasmas can also be generated under low energy such as in fluorescent strip lights. Within a plasma, positive and negative ions, electrons and neutral molecules collide to produce free radicals. The destructive power of these entities has already been described, and so plasmas can be used as biocidal agents in a variety of applications. This type of system can be produced at temperatures below 50°C using vapours generated from hydrogen peroxide or peracetic acid.

ANTIMICROBIAL EFFECTS OF CHEMICAL AGENTS

Chemical agents have been used since very early times to combat such effects of microbial proliferation as spoilage of foods and materials, infection of wounds and decay of bodies. Thus, long before the role of microorganisms in disease and decay was recognized salt and sugar were used in food preservation, a variety of oils and resins were applied to wounds and employed for embalming, and sulphur was burned to fumigate sick rooms.

The classic researches of Pasteur, which established microorganisms as causative agents of disease and spoilage, paved the way for the development and rational use of chemical agents in their control. Traditionally, two definitions have been established describing the antimicrobial use of chemical agents. Those used to destroy microorganisms on inanimate objects are described as ***disinfectants***, and those used to treat living tissues, as in wound irrigation, cleansing of burns or eye washes, are called ***antiseptics***. Other definitions have been introduced to give more precise limits of meaning, namely, ***bactericide*** and ***fungicide*** for chemical agents that kill bacteria and fungi, respectively; and ***bacteriostat*** and ***fungistat*** for those that prevent the growth of a bacterial or fungal population. The validity of drawing a rigid demarcation line between those compounds that

kill and those that inhibit growth without killing is doubtful. In many instances concentration and time of contact are the critical factors. The term ***preservative*** describes those antimicrobial agents used to protect medicines, pharmaceutical formulations, cosmetics, foods and general materials against microbial spoilage, and ***biocide*** is a general term for antimicrobial chemicals, but it excludes antibiotics and other agents used for systemic treatment of infections.

The mechanisms whereby biocides exert their effects have been intensively investigated and the principal sites of their attack upon microbial cells identified. These are the cell wall, the cytoplasmic membrane and the cytoplasm. Chemical agents may weaken the cell wall, thereby allowing the extrusion of cell contents, distortion of cell shape, filament formation or complete lysis. The cytoplasmic membrane, controlling as it does permeability, and being a site of vital enzyme activity, is vulnerable to a wide range of substances that interfere with reactive groups or can disrupt its phospholipid layers. Chemical and electrical gradients exist across the cell membrane and these represent a proton-motive force which drives such essential processes as oxidative phosphorylation, adenosine triphosphate (ATP) synthesis and active transport; several agents act by reducing the proton-motive force. The cytoplasm, site of genetic control and protein synthesis, presents a target for those chemical agents that disrupt ribosomes, react with nucleic acids or generally coagulate protoplasm.

Principal factors affecting activity

The factors most easily quantified are temperature and concentration. In general an increase in temperature increases the rate of kill for a given concentration of agent and inoculum size. The commonly used nomenclature is Q_{10} (temperature coefficient), which is the change in activity of the agent per 10°C rise in temperature (e.g. Q_{10} phenol = 4).

The effect of change in concentration of a chemical agent upon the rate of kill can be expressed as:

$$\eta = \frac{\log t_2 - \log t_1}{\log C_1 - \log C_2}$$

where C_1 and C_2 represent the concentrations of agent required to kill a standard inoculum in times t_1 and t_2. The concentration exponent η represents the slope of the line when log death time (t) is plotted against log concentration (C).

When values of η are greater than 1, changes of concentration will have a pronounced effect. Thus, in the case of phenol, when η = 6, halving the concentration will decrease its activity by a factor of 2^6 (64-fold), whereas for a mercurial compound, η = 1, the same dilution would only reduce activity twofold (2^1). Further details and tabulations of both temperature coefficients and concentration exponents may be found in Denyer and Wallhaeusser (1990).

The range of chemical agents

The broad categories of antibacterial chemical compounds have remained surprisingly constant over the years, with phenolics and hypochlorites comprising the major disinfectants and quaternary ammonium compounds widely used as antiseptics. The compounds capable of use as preservatives in preparations for oral, parenteral or ophthalmic administration are obviously strictly limited by toxicity requirements. As concerns over toxicity have intensified the range of available preservatives has diminished: mercury-containing compounds, for example, are now very little used for the preservation of parenteral and ophthalmic products, and the high cost of research and testing coupled with the poor prospects for an adequate financial return militate against the introduction of new agents. For this reason there is a tendency towards the use of existing preservatives in combination, with a view to achieving one or more of the following benefits: synergy; a broader antimicrobial spectrum; or reduced human toxicity resulting from the use of lower concentrations. The subjects of preservative toxicity and their potentiation and synergy are reviewed by Denyer and Wallhaeusser (1990), and Hugo and Russell (1999) have described in detail characteristics of the commonly used biocides.

Phenolics

Various phenolic compounds are shown in Figure 41.4.

Various distillation fractions of coal tar yield phenolic compounds, including cresols, xylenols and phenol itself, all of which are toxic and caustic to skin and tissues. Disinfectant formulations traditionally described as 'black fluids' and 'white fluids' are prepared from higher-boiling coal tar fractions. The former make use of soaps to solubilize the tar fractions in the form of stable homogenous solutions, whereas the latter are emulsions of the tar products and unstable on dilution.

Remarkable success has been achieved in modifying the phenol molecule by the introduction of chlorine and methyl groups, as in chlorocresol and chloroxylenol. This has the dual effect of eliminating toxic and corrosive properties while at the same time enhancing and prolonging antimicrobial activity.

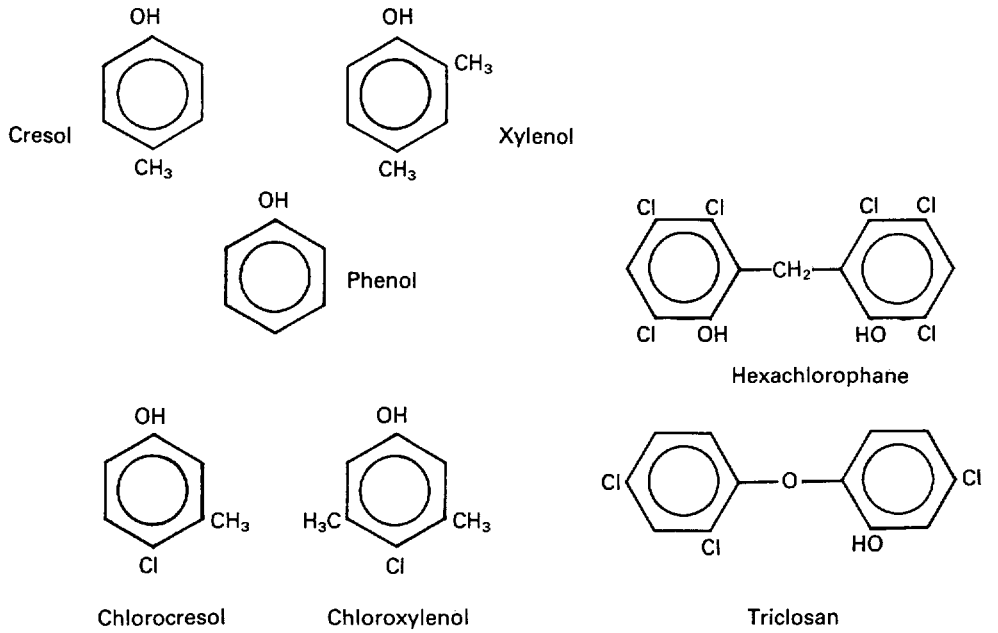


Fig. 41.4 Chemical structures of a range of phenols.

Thus, chlorocresol is used as a bactericide in injections and to preserve oil-in-water creams, whereas chloroxylenol is employed as a household and hospital antiseptic. Phenol may itself be rendered less caustic by dilution to 1% w/v or less for lotions and gargles, or by dissolving in glycerol for use as ear drops. Bisphenols, such as hexachlorophane and triclosan (Irgasan), share the low solubility and enhanced activity of the other phenol derivatives described, but have a substantive effect which makes them particularly useful as skin antiseptics. Formulated as creams, cleansing lotions or soaps, they have proved valuable in reducing postoperative and cross-infection. Again toxicity concerns have emerged, and so, for example, hexachlorophane is restricted in the UK both in respect of the concentrations that may be employed and the type of product in which it may be used.

Phenols generally are active against vegetative bacteria and fungi, are readily inactivated by dilution and organic matter, and are most effective in acid conditions. Depending on concentration, phenols may cause cell lysis at low concentrations, or general coagulation of cell contents at higher concentrations.

Alcohols, aldehydes, acids and esters

Ethyl alcohol has long been used, usually as 'surgical spirit' for rapid cleansing of preoperative areas of skin before injection, and is most effective at concentrations of 60–70%. It is rapidly lethal to bacterial vegetative cells and fungi, but has no activity against bacterial endospores and little effect on viruses. The effect of aromatic substitution is to produce a range of compounds which are less volatile and less rapidly active and find general use as preservatives, e.g. phenylethanol for eye drops and contact lens solutions, benzyl alcohol in injections, Bronopol (2-bromo-2-nitropropane-1,3-diol) in shampoos and other toiletries. Phenoxyethanol, which has good activity against *Ps. aeruginosa*, has been used as an antiseptic. In general the alcohols act by disrupting the bacterial cytoplasmic membrane and can also interfere with the functioning of specific enzyme systems contained within the membrane.

Formaldehyde and glutaraldehyde are both powerful disinfectants, denaturing protein and destroying vegetative cells and spores. Formaldehyde is used in sterilization procedures both as a gas and as a solution in ethyl alcohol. Glutaraldehyde solutions are also used to sterilize surgical instruments.

The organic acids, sorbic and benzoic and their esters, because of their low toxicity, are well established as preservatives for food products and medicines (see Chapter 42). The exact mode of action of these agents on microorganisms is still uncertain, but they have been shown to influence the pH gradient

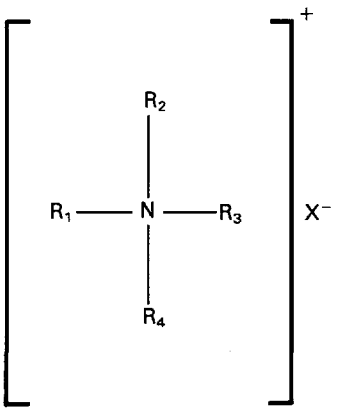
across the cell membrane, and at higher concentrations the parabens induce leakage of intracellular constituents.

Quaternary ammonium compounds

The chemical formula for quaternary ammonium compounds is shown in Figure 41.5.

These cationic surface-active compounds are, as their name implies, derivatives of an ammonium halide in which the hydrogen atoms are substituted by at least one lipophilic group, a long-chain alkyl or aryl-alkyl radical containing C_8–C_{18} carbon atoms. In marked contrast to phenol and the cresols these compounds are mild in use and active at such high dilutions as to be virtually non-toxic. Their surface-active properties make them powerful cleansing agents, a useful adjunct to their common use as skin antiseptics and preservatives in contact lens cleansing and soaking solutions. They are also safe for formulating into eye drops and injections, and are widely used in gynaecology and general surgery. Active as cations, ambient pH is important, as is interference caused by anions. Thus, alkaline conditions promote activity and it is important that all traces of soap, which is anion active, are removed from the skin prior to treatment with a quaternary ammonium compound. Foreign organic matter and grease also cause inactivation.

One effect of the detergent properties of these compounds is to interfere with cell permeability such that susceptible bacteria – mainly the Gram-positive groups – leak their contents and eventually undergo lysis. Gram-negative bacteria are less susceptible and, to widen the spectrum to include these, mixtures of quaternary ammonium compounds with other antimicrobial agents such as phenoxyethanol or chlorhexidine are used.



Cetrimide BP
$R_1R_2R_3$—CH_3
R_4—mainly $C_{14}H_{29}$
X—Br
Benzalkonium Chloride BP
R_1R_2 — CH_3
R_3 — $C_6H_5CH_2$
R_4 — mainly $C_{13}H_{27}$
X — Cl

Fig. 41.5 Chemical structure of centrimide and benzalkonium chloride.

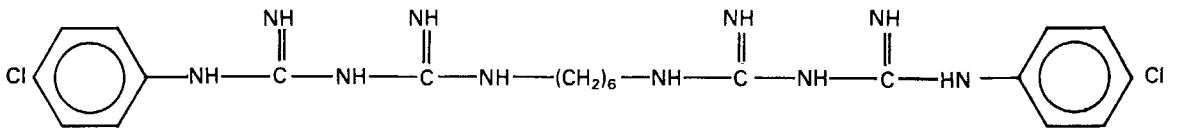
Biguanides and amidines

Chlorhexidine is a widely used biocide which has activity against Gram-positive and Gram-negative bacteria but has little activity against endospores or viruses (Fig. 41.6). It is widely used in general surgery, both alone and in combination with cetrimide, and can also be used as a preservative in eye drops. Polyhexamethylene biguanide (PHMB) is a polymeric biguanide used widely in the food, brewing and dairy industries, and has also found application as a disinfectant in contact lens cleaning solutions. The biguanides act on the cytoplasmic membrane, causing leakage of intracellular constituents.

The aromatic diamidines, propamidine and dibromopropamidine, are non-toxic antiseptics mainly active against Gram-positive bacteria and fungi. However, resistance to these agents can develop quickly during use.

The halogens and their compounds

Chlorine gas is a powerful disinfectant used in the municipal treatment of drinking water and in swimming baths. Solutions of chlorine in water may be made powerful enough for use as general household bleach, and disinfectant and dilute solutions are



Chlorhexidine

Fig. 41.6 Chemical structure of chlorhexidine.

used for domestic hygiene. Unionized hypochlorous acid (HOCl) is an extremely potent and widely used bactericidal agent which acts as a non-selective oxidant, reacting readily with a variety of cellular targets. Well-known pharmaceutical formulations containing chlorine are Eusol (Chlorinated Lime and Boric Acid Solution BPC 1973) and Dakin's Solution (Surgical Chlorinated Soda Solution BPC 1973), both of which are designed to provide slow release of chlorine.

An alternative method of obtaining more prolonged release of chlorine is by the use of organic chlorine compounds such as Chloramine T (sodium *p*-toluene-sulphonchloramide) and Halazone BPC 1973 (*p*-sulphondichloramide benzoic acid), the former used as a skin antiseptic and the latter for treating contaminated drinking water. The high chemical reactivity of chlorine renders it lethal to bacteria, fungi and viruses, and to some extent spores and this activity is optimal at acid pH levels around 5.0.

Iodine, like chlorine a highly reactive element, denatures cell proteins and essential enzymes by its powerful oxidative effects. Traditionally it has been used in alcoholic solutions such as Tincture of Iodine (BP 1973) or complexed with potassium iodide to form an aqueous solution (Lugol's Iodine BP 1973). The staining and irritant properties of iodine have resulted in the development of iodophores, mixtures of iodine with surface-active agents, which hold the iodine in micellar combination from which it is released slowly. Such a preparation is Betadine (polyvinylpyrrolidone-iodine), used as a non-staining, non-irritant antiseptic.

Metals

Many metallic ions are toxic to essential enzyme systems, particularly those utilizing thiol (–SH) groups, but those used medically are restricted to mercury, silver and aluminium. The extreme toxicity of mercury has rendered its use obsolete apart from in organic combination. The organic compounds that still have a limited use in pharmacy are phenylmercuric nitrate (and acetate) as a bactericide in eye drops and injections, and thiomersal (sodium ethylmercurithiosalicylate) as a preservative in biological products and certain eye drops.

Silver, in the form of the nitrate, has been used to treat infections of the eyes, as have silver protein solutions. Aluminium foil has been used as a wound covering in the treatment of burns and venous ulcers, and has been shown to adsorb micro-organisms and inhibit their growth.

The acridines

This group of compounds interferes specifically with nucleic acid function and has some ideal antiseptic properties, thus aminacrine hydrochloride is non-toxic, non-irritant, non-staining and active against Gram-positive and Gram-negative bacteria even in the presence of serum.

This brief survey has given some indication of the variety of antimicrobial compounds available. Each of these has a defined spectrum of utility and in the correct conditions of use can substantially contribute to the control of microbial proliferation and infection.

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42

Microbiological contamination and preservation of pharmaceutical products

Malcolm Parker (updated by Norman Hodges)

CHAPTER CONTENTS

SOURCES AND INCIDENCE OF CONTAMINATION

Microorganisms form an integral part of our environment. They are present in the air that we breathe, the food that we eat and the water that we drink. Some microorganisms indigenous to the body are present in considerable numbers: they constitute up to one-third of the dry weight of faeces. In this situation it is apparent that both raw materials and final medicines will contain microorganisms unless specific measures are adopted to exclude them. The preparation of sterile medicaments is a skilled and expensive procedure demanding sophisticated equipment, skilled personnel and a controlled working environment. To produce all medicines to such a standard would require clear arguments to justify the considerable costs involved and the consequent expense to consumers. Factors to be considered are: the sources and incidence of microorganisms in drugs and medical preparations, the consequences of such contamination both for the stability of medicines and for the health of the patient and, arising from these, the levels and types of microorganism that might be tolerated.

As indicated in Table 42.1 and Fig. 42.1, many factors potentially contribute to the microbial load carried by a pharmaceutical preparation at every stage of manufacture, from assembling the raw materials to packaging the final product.

There have been many reports, particularly in the 1960s and 1970s, of pathogenic organisms isolated, sometimes in large numbers, from pharmaceutical raw materials and manufactured products. These reports, reviewed by Bloomfield (1990) and, more recently, by Spooner (1996), drew attention to the need for better control of the microbiological quality of both raw materials and manufacturing processes. As a consequence, the frequency with which high concentrations or hazardous species are isolated has been reduced in recent years, although the problem has by no means

Table 42.1 Sources of microbial contaminants

Water	Low-demand Gram-negative groups: *Pseudomonas, Xanthamonas, Flavobacterium, Achromobacter*
Air	Mould spores: *Penicillium, Mucor, Aspergillus* Bacterial spores: *Bacillus* spp. Yeasts Micrococci
Raw materials	
Earths	Anaerobic spore formers: *Clostridium* spp.
Pigments	*Salmonella*
Starches	Coliforms
Gums	*Achnomyces*
Animal products	*Salmonella* Coliforms
Personnel	Coliforms Staphylococci Streptococci Corynebacteria

been eliminated. It is well established that major sources of microbial contamination of medicines are water – the most common raw material – and raw materials of natural origin, including vegetable drugs and mined minerals such as talc, kaolin and bentonite. The European Pharmacopoeia (2000) specifies limits for microorganisms in different product types (see Table 40.3) and the relatively high levels of contamination of natural products is reflected in these limits.

The usual waterborne organisms found are the *Pseudomonas–Achromobacter–Alcaligenes* types, including occasionally *Ps. aeruginosa*. Purified water has proved to be a typical source of microorganisms in that, during use, the ion-exchange column may become contaminated from the water passing through and the entrapped organisms rapidly multiply to produce high counts in the outflowing water.

Water produced by reverse osmosis might also represent a problem if the osmosis membrane is not disinfected at regular intervals. Even distilled water, which is free of microorganisms on leaving the still, may be a significant source of contamination after storage. This is because the chlorine that protects tap water is lost on distillation, and Gram-negative bacteria may grow to concentrations as high as 10^5–10^6 mL^{-1} within a few days at ambient temperature. These bacteria usually gain access to distilled water by way of poor-quality rubber or plastic connection tubing or inadequate closure of storage containers.

Some manufactured medicines possess antimicrobial activity, by virtue of unfavourable pH, for example, so it is not inevitable that medicines made from contaminated raw materials will, themselves, be contaminated, although this is normally the case. Further, it is important to realize that in some cases the initial bacterial count in a freshly prepared

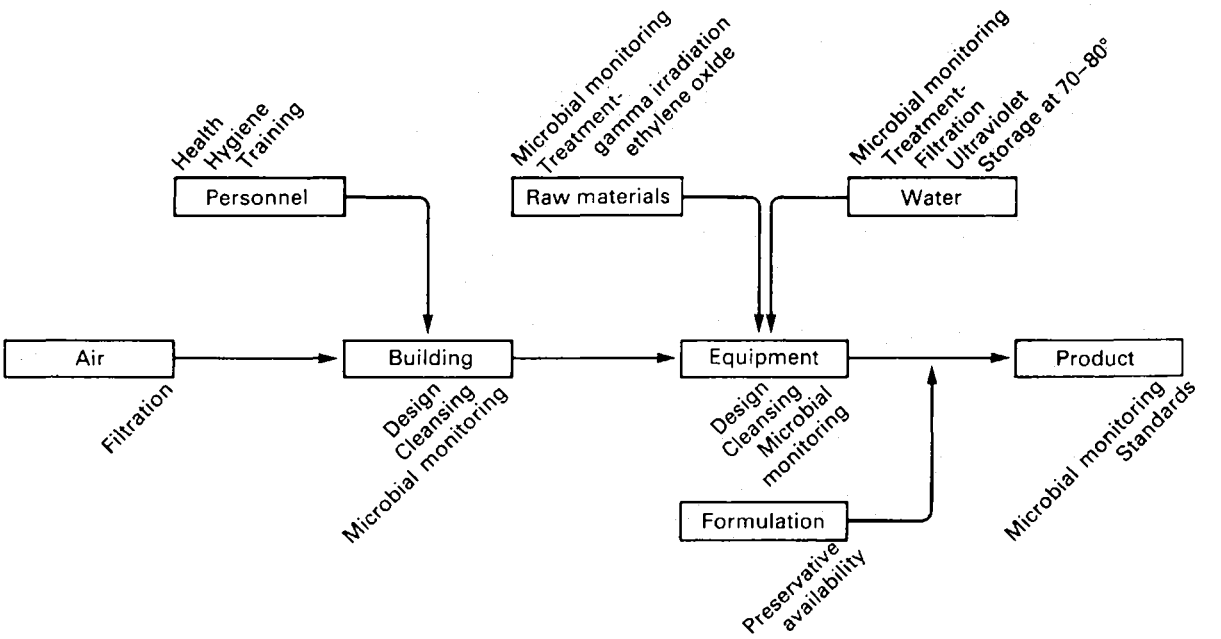

Fig. 42.1 Factors in hygienic manufacture

mixture can increase substantially during storage, particularly in those cases in which the formulation contains no preservative.

A major potential source of microbial contamination is that represented by the personnel preparing medicines and the patients using them (Table 42.1). In the environment generally it is people who generate most of the airborne bacteria. Body movements, exhaling, speaking and, of course, coughing and sneezing, represent significant sources of contamination. The microorganisms that can be disseminated in this way include staphylococci, present on the skin and in the nostrils of healthy persons, streptococci, sometimes present in the throat, and a variety of *Enterobacteriaceae*, including salmonellae and coliforms present in the intestines. Other microflora that may be found as contaminants of medicines, and which are not normally associated with humans, are the airborne spores of both bacteria and moulds, including several wild yeasts, anaerobic inhabitants of soil and earths such as the clostridia and low-demand water-borne bacteria, usually Gram-negative forms.

There is considerable potential for microorganisms to enter medicines during both manufacture and use, and in this situation it is not surprising that whenever non-sterile preparations and their ingredients are screened for microbial contamination, organisms are detected. As has been discussed, the incidence of microflora in medicines as issued from the dispensary or manufacturers is dictated very much by the nature of the ingredients, i.e. whether natural or synthetic, the quality of the vehicle, and the care and attitude of personnel involved.

The situation is very different for sterile preparations in that detection of any microorganisms represents an unacceptable situation usually indicative of a breakdown in the sterilization process. Thus when in 1972 infusion fluids used in a Plymouth hospital were found to be contaminated, the Secretary of State required the Medicines Commission to 'review measures which should be taken in the course of the production, distribution, storage and use of medicinal products to prevent their becoming vehicles of infection'. It is indicative of the rarity of such incidents that an inquiry was deemed to be necessary.

GROWTH AND MULTIPLICATION OF MICROORGANISMS IN PHARMACEUTICAL PRODUCTS

Most raw materials, and consequently the pharmaceutical preparations containing them, will support some form of microorganism. Although this ability varies considerably according to the nutritive properties and moisture content of the materials concerned, it is unwise to assume that, for instance, a dry powder or a tablet is safe from microbial spoilage. The problem can be appreciated by considering the range of habitats of microorganisms, encompassing as they do volcanic regions to the Antarctic wastes and nutrient sources as unlikely as glass and concrete. The majority of medicines present a ready source of nutrients and moisture and there are many reports of the effects of microbial proliferation within them, with attendant odours and visible spoilage. Troublesome and expensive as this obvious deterioration may be, a more serious problem is the development of microorganisms without obvious signs or involving delayed effects. For this reason it is important to have a knowledge of the microbial content of all drugs and medicines, rather than restrict attention to those required to be sterile and those shown to be particularly spoilage prone. A study of the interaction of microorganisms in foods such as milk or meat products has shown how pioneer forms can prepare the way for later invaders by degrading complex nutrients, altering pH levels, or making more moisture available until the final spoilage population is established. The initial invaders, in either foods or medicines, can reach high levels without visible effects, and furthermore, when the finally spoiled product is investigated, they may have been completely displaced by a final spoilage form. Unless this chain reaction of spoilage is appreciated harmful effects of apparently stable medicines can be unresolved and the importance of some contaminants not realized. Thus, syrup or mixtures rich in sugar may become initially contaminated by osmophilic yeasts which can thrive at high sugar concentrations and, by utilizing the sugars create conditions that allow secondary, less specialized organisms to become established. When such syrups are examined there may be little evidence of the yeasts that initiated the spoilage process, and so their role may be overlooked.

CONSEQUENCES OF CONTAMINATION

It is now realized that the presence of microorganisms in a pharmaceutical preparation may have a variety of consequences, ranging from the negligible to the very serious. For example, spores of the mould *Mucor* may be present in a dormant form and never produce spoilage or harm the patient who takes the medicine. In complete contrast to this would be the

presence of *Salmonella* in a medicine which, although causing little or no visible spoilage, would represent a serious health hazard.

The instances in which there have been serious consequences attending contaminations have been, in the main, concerned with those preparations which are required to be supplied sterile. This might be anticipated, as sterile preparations are usually administered parenterally or into the eyes, and in these circumstances extraneous microorganisms present a particular danger. Intravenous infusion fluids are recognized as a potential area for concern, particularly because of their implication in the case previously mentioned, where contaminated fluid resulted in the death of several patients. The established practice of adding drugs to infusions, often at patient level in hospital wards, can present an additional microbiological hazard unless closely supervised by skilled staff. Preparations for ophthalmic use, including contact lens solutions, have been responsible for serious infections of the eye, some resulting in blindness, as a result of microbial contamination.

Considered against the background of the high volume of medicinal products used annually by the public, the serious consequences of contamination are very few. Where they do occur, however, the public is justifiably worried and the implications for the profession are grave.

Apart from possible infection of patients the other important effect of contamination of medicines is that of general spoilage. This may result in obvious changes, such as discolouration, breakdown of emulsions and the production of gas and various odours. Such comparatively dramatic effects of deterioration do have the virtue of directing the consumer's attention to the problem and, hopefully, discouraging their use of the medicine. In other cases, however, active ingredients may be utilized by invading microorganisms without overt visible signs of spoilage. Thus, salicylates (including aspirin), paracetamol, atropine, chloramphenicol, prednisolone and hydrocortisone can be degraded to a variety of therapeutically inactive products. Preservatives, intended to protect formulations against microorganisms, can themselves present a ready source of microbial nutrition, particularly if their levels become depleted and if they are aromatic in structure.

Bacteria can produce various toxic substances which are a potential danger in contaminated products, even when a sterilization procedure has been applied and only dead cells or their residues remain. In parenteral preparations endotoxins, which are lipopolysaccharide components of Gram-negative bacteria, may cause fever following injection (see Chapter 40). Moulds produce mycotoxins, which from early times have been implicated in illnesses such as ergotism, and more recently with gastroenteritis, both caused by using contaminated grain. The involvement of mycotoxins, particularly aflatoxin, in cancer has added an urgency to our study of them.

SCREENING FOR CONTAMINATION

A consideration of Figure 42.1 will show that if contamination is to be minimized then a knowledge of the microbial levels associated with all aspects of the production of a medicine is required. Thus, examining prepared medicines for their contamination will not, in itself, further our efforts to reduce this unless parallel screening is done upon the manufacturing process and environment, including air, equipment, personnel and raw materials.

Methods for the detection, enumeration and identification of microorganisms have been described in Chapters 39 and 40. Some of these can be applied to determine the number and type of microorganisms present at any stage in the preparation or manufacture of a medicine. The most relevant are discussed below and are considered also in Chapter 40).

Air sampling

The usual methods for sampling from air are by free fall or settling, forced air flow and filtration.

Free fall or settling

As the name implies, this involves the sampling of organisms deposited naturally from the atmosphere. This is typically carried out by exposing plates of suitable nutrient medium for selected periods in the locations to be sampled. The procedure is empirical, with the counts obtained depending upon time of exposure, the nature of any activity in the area and the siting of the plates in relation to such activity.

Forced air flow

Forced air flow samplers allow a measured volume of air to be examined by directing it on to a solid agar surface or drawing it through sterile saline or nutrient broth from which plate counts can be made. A variety of commercially available apparatus may be used to apply this method.

Filtration

Filtration involves the drawing of air through a membrane filter which is then aseptically removed, placed upon suitable nutrient agar and incubated.

Care must be taken in interpreting results obtained by these various methods and thought given to location and timing of sampling, period of exposure or volume of air examined and movement of personnel. In general, if contamination problems are likely to be due to particles settling from the air, then free-fall methods are adequate. If, however, contaminating particles tend to remain airborne some form of forced air sampling is essential. The actual timing of sampling will provide information on conditions during typical busy working periods and the efficiency of any air filtration system in coping at peak times.

Sampling of surfaces and equipment

Simple techniques can be used to assess the level of contamination of surfaces, such as swabbing with sterile cotton-tipped sticks, which can be transferred to suitable recovery media, or by placing a sterile agar surface in contact with the area to be sampled and, after removal, incubating. It is easier to use swabs for flexible and uneven surfaces and agar contact methods for firm flat surfaces.

Measuring contamination levels in raw materials and final preparations

The nature of raw material and final preparations will determine the method used to detect microbial contamination. Water, water-miscible liquids or soluble solids present no difficulties for conventional plate counting or filter membrane methods. Insoluble or oily materials and preparations need to be suitably dispersed and homogenized if contaminating microorganisms are to be isolated. In every instance any antimicrobial activity of the sample under examination must be neutralized. Methods of counting microorganisms and the inherent errors are described briefly in Chapter 40 and in more detail in standard bacteriological textbooks.

There is increasing interest in methods for the rapid detection of microorganisms in food and drugs which can be automated and should remove much of the drudgery of traditional counting techniques. These methods are based upon the measurement of some metabolic activity or other feature of organisms, and include adenosine triphosphate (ATP) assays, direct epifluorescence techniques and impedance techniques; the pharmaceutical applications of these and other methods are reviewed by Newby (2000), and the instrumentation is described by Collins et al (1995). In the case of sterile production, the entire system can be monitored by processing a sterile nutrient medium (for liquid preparations) or soluble sterile powder (for solids) and examining final preparations for contamination, together with samples taken at various intermediate stages.

CONTROL OF MICROBIAL CONTAMINATION

There are essentially two strategies to be adopted in the preparation of microbiologically acceptable pharmaceutical preparations. The first and most important is to minimize the access of microorganisms from the sources summarized in Figure 42.1 and Table 42.1, and the second is to formulate the final product so as to be hostile to microorganisms, normally by the addition of preservatives.

For sterile preparations there is either a terminal sterilization process or a closely controlled aseptic manufacturing procedure. In every case the final pack should be designed to protect the product during storage and minimize in-use contamination. The *Rules and Guidance for Pharmaceutical Manufacturers and Distributors (1997)* or 'Orange Guide' provides guidance on premises, equipment, raw materials, packaging, storage and the training of personnel.

Premises and equipment

Premises need to be purpose-built to provide a logical workflow, with separation of areas of different grades of cleanliness, appropriate air supplies, and construction materials which are resistant to dirt and easy to keep clean. The equipment should be as simple as possible for the purposes required, with a minimum of junctions, valves and pumps to allow cleaning in place by the circulation of detergents or other chemical antimicrobial agents such as hypochlorites, followed by steam or hot-water flushing. The degree of air treatment required will depend upon the type of product involved, with aseptic manipulation demanding filtration efficient enough to remove particles down to 0.1 μm. For aseptic preparation and filling a grade A clean room is required; this requires an atmosphere containing no more than 3500 particles m^{-3} in the 0.5–5μm range when the room is not in operation. The

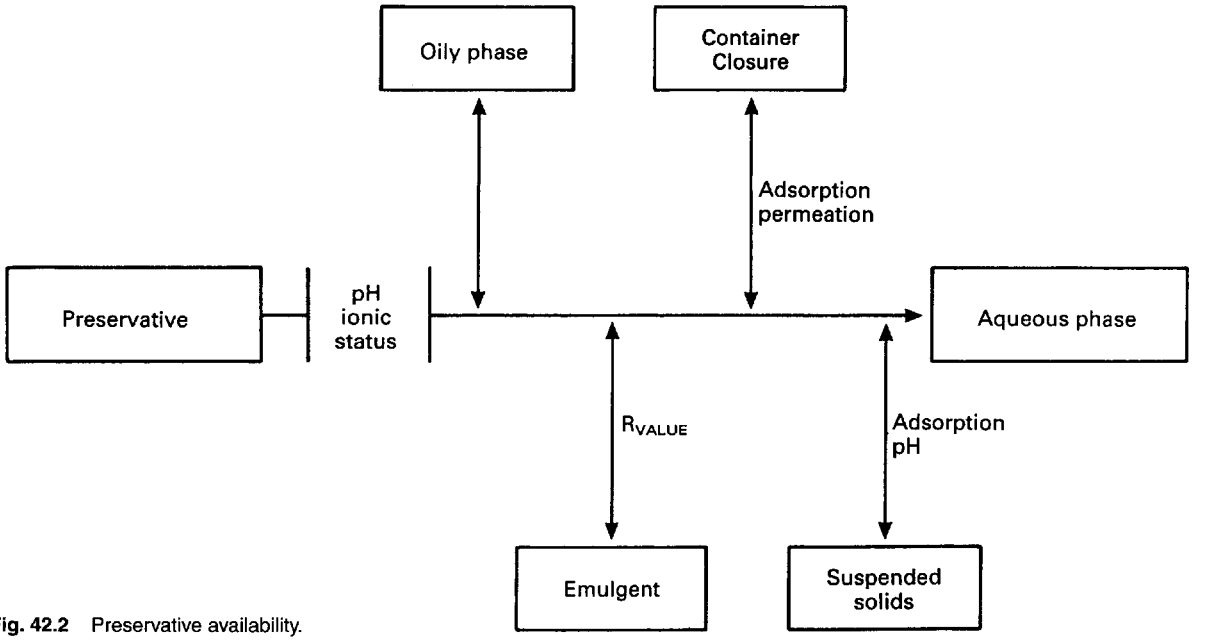

Fig. 42.2 Preservative availability.

Table 42.2 Examples of preservatives commonly used in pharmaceutical formulations

Product type	Preservative	Concentration (%w/v)	% of USP formulations in which preservative used*
Parenteral	Benzyl alcohol	0.1–3.0	31.0
	Methyl/propyl paraben	0.08–0.1/0.001–0.023	13.8
	Phenol	0.2–0.5	7.9
	Methyl paraben (alone)	0.1	6.6
	Chlorbutanol	0.25–0.5	5.3
	Sodium metabisulphite	0.025–0.66	5.3
	Sodium bisulphite	0.13–0.2	2.6
			Total 69.9
Ophthalmic	Benzalkonium chloride	0.0025–0.0133	50.0
	Thiomersal	0.001–0.5	19.8
	Methyl/propyl paraben	0.05/0.01	6.6
	Benzalkonium chloride plus EDTA	0.01/0.1	3.3
			Total 79.7
Oral	Sodium benzoate	NA	34.4
	Methyl/propyl paraben	NA	18.3
	Methyl paraben (alone)	0.1	9.7
	Methyl paraben plus sodium benzoate	NA	7.5
			Total 69.9
Creams	Benzyl alcohol	1.0–2.0	25.4
	Methyl/propyl paraben	NA	18.6
	Methyl paraben (alone)	0.1–0.3	11.9
	Benzoic acid	0.2	8.5
	Sorbic acid	0.1	8.5
	Chlorocresol	0.05	6.8
			Total 79.7

NA, not available.
* Only the most commonly used agents are listed, so the percentages in each product category do not total 100%.
After Dabbah (1996)

'Orange Guide' gives advice upon basic environmental standards, with allowance made for people present in working areas.

Raw materials

Raw materials, particularly those of natural origin, and water are a potentially rich source of microorganisms and may require treatment to reduce or eliminate them. Potable (tap) water may be used for the manufacture of some pharmaceuticals, but as the microbial load in potable water may vary with geographical region and season of the year it is more common to use deionized water. Provided the deionizing bed (ion-exchange resin) is regenerated on a regular basis, an acceptable microbiological standard can be maintained for deionized water. Water may be treated by UV treatment units or filtration and stored at elevated temperature (65–80°C) to discourage microbial growth.

For raw materials generally, any treatment applied to remove or reduce the microbial load must be such that the materials are not adversely affected. A variety of processing procedures are available, including ionizing radiations, microwaves, gassing and, of course, heat. In every case close monitoring for possible deleterious effects is essential.

Personnel

There is little doubt that however comprehensive the procedures adopted to control contamination, they will be of little avail unless the personnel involved understand and appreciate the problems and significance of microbial contamination. This requires education in hygiene to minimize the introduction of microorganisms by staff and to underline the importance of appropriate protective measures. The approach will, of course, differ depending upon the level of education of the people involved, but it cannot be overemphasized that all personnel and visitors, however senior, must be required to comply with regimens of hygiene and protective clothing. A full range of appropriate clothing is commercially available which, for the manufacturer of sterile preparations, completely shields the product from body surfaces with, for instance, hood, mask, overall, protective gloves and boots. For other manufacturing areas good-quality overalls, overshoes and head cover represent a minimal requirement. General dispensing requires careful attention to hygiene, the provision of suitable overalls and a ban on food consumption and smoking in the dispensary.

THE PRESERVATION OF PHARMACEUTICAL PREPARATIONS

With an understanding of the many factors involved in the microbial contamination of medicines and application of the procedures described, a range of products can be obtained which, if required, are sterile or have an acceptable level of organisms present. This in itself is not sufficient without further steps being taken to minimize contamination and spoilage of the medicines during use. Well designed containers, usually single-dose in the case of sterile preparations, and sensible storage contribute a great deal to this end, but whenever acceptable an added safeguard is to incorporate an antimicrobial substance or preservative into the formulation.

The correct approach to preservation has as its foundation two important principles. The first is that a preservative must not be added to a product to mask any deficiencies in the manufacturing procedures, and the second is that the preservative should be an integral part of the formulation, chosen to afford protection in that particular environment. The increasing care now being given to the hygienic preparation of pharmaceutical products has removed the need for preservatives to cope with high initial microbial loads, but the problem remains of protecting against spoilage resulting from in-use contamination. If a preservative is to prevent such spoilage the factors affecting its efficacy must be appreciated.

Factors in preservative efficacy

The range of antimicrobial agents available is apparently very wide (see Chapter 41 and Table 42.2) until the particular requirements of a preservative for a formulated medicine are considered. A review of the United States Pharmacopoeia formulae (Dabbah 1996) showed that 38 different preservatives were in use, together with 42 different preservative combinations. However, an analysis of the most commonly used agents revealed that the same few were regularly employed in 70% or more of the products in each of the various categories (Table 42.2). When this table is compared with the corresponding one in the first edition of this book it is apparent that no new preservatives have entered into common use during the intervening period; indeed some, e.g. the phenylmercury salts, have become less popular because of toxicity concerns, so the range of available preservatives is diminishing not expanding.

To state that the preservative must be non-toxic, odourless, stable and compatible with other formulation components while exerting its effect against the wide range of potential microbial contaminants is an oversimplification. Toxicity alone debars many antimicrobial compounds from use in preparations for parenteral, ophthalmic or oral use, and the increased sophistication of some modern formulations makes it difficult to avoid preservative–ingredient interactions. Any organisms that do enter a preparation will multiply in the aqueous phase or immediate interface, and so it is the prime function of the preservative to attain a protective concentration in this phase. As shown in Figure 42.2, the major reasons for a preservative not attaining an effective concentration in the aqueous phase are its possible solubility in oil, interaction with emulgents, hydrocolloids and suspended solids, its interaction with the container or its volatility. In addition, the ambient pH of the formulation can have a marked influence upon preservative effectiveness.

Oil/water partition

In a simple two-phase system of oil and water a preservative will partition until:

$$\frac{C_o}{C_w} = K_w^o$$

where C_o is the concentration of preservative in oil at equilibrium, C_w is the concentration of preservative in water at equilibrium and K_w^o is the oil/water partition coefficient at the given temperature. Oil solubility is an important parameter in preserving formulations containing vegetable oils such as arachis or soya; and for such preparations the esters of parahydroxybenzoic acid (parabens) are unsuitable owing to their high oil/water partition coefficients (K^o = 0.02 for methyl paraben in mineral oil and 7.5 in vegetable oil: for propyl paraben K^o = 0.5 in mineral oil and 80.0 in vegetable oil). Faced with this problem it is necessary to change the preservative or alter the formulation and, given the restricted choice of preservatives, the formulation becomes all important. Thus, it was found that by substituting liquid paraffin (mineral oil) for arachis oil (vegetable oil) in formulations of Calamine Cream, a preservative such as chlorocresol would afford better protection (K_w^o = 117 (arachis oil): 1.5 (liquid paraffin)). The further step of using a less oil-soluble preservative such as phenoxyethanol (K_w^o = 0.12 liquid paraffin) further improved the system.

Emulsions

Many emulgents are used in pharmaceutical preparations to produce elegant applications or palatable medicines (see Chapter 23). A variety of interactions will occur between preservatives and the emulsified oil phase and with emulgent molecules or micelles. Attempts have been made to quantify such interactions by measuring the proportion of free preservative in emulsions in a variety of ways, including dialysis and dissolution techniques. The use of predictive data can greatly assist in the formulation, provided that the different behaviour of different emulgents, effects of temperature and influence of oil:water ratio are appreciated. A simple mathematical model has been developed:

$$C_w = C\frac{\phi + 1}{K_w^o\phi + 1}$$

where C_w is the concentration of preservative in the aqueous phase, C is the total concentration of preservative and ϕ is the oil:water ratio. In the presence of an emulgent the amount of free preservative can be measured and the ratio expressed as the factor R:

$$\text{where } R = \frac{\text{total preservative}}{\text{free preservative}}$$

The equation then becomes:

$$C_w = C\frac{\phi + 1}{K_w^o\ \phi + R}$$

where C_w is now the free concentration in the water.

Using this model it can be calculated, for instance, that to attain the required protective concentration of 0.2% w/v methyl paraben in a liquid paraffin/water emulsion containing 5% polysorbate-80 and 50% oil, a total concentration of some 0.5% must be used. Alterations in the type and concentration of emulgent, nature of oil and oil:water ratio will all influence the concentration of preservative needed to protect the system.

Interaction with other formulation components

Many of the hydrocolloids used as dispersants or thickeners, such as methylcellulose, alginates, polyvinylpyrrolidone and tragacanth, can interact to some degree with preservatives and diminish their activity. In some cases this is a direct incompatibility, as between alginates, which are anionic, and cation-active preservatives, whereas in other cases a variety of physicochemical interactions operate.

Therapeutically active ingredients in the form of suspended solids in mixtures, such as magnesium trisilicate and kaolin, have been shown to deplete preservative concentrations, probably by adsorption. Similarly, fillers and disintegrants cause problems in tablet preservation owing to their interaction with added preservatives such as methylhydroxybenzoate.

Effects of containers

Preparations packed in traditional glass containers can be expected to retain their preservative content provided the closure is suitably airtight. The greatly increased use of plastics in packaging has brought with it a number of difficulties, ranging from permeation of preservatives through the container to interaction with it. There is a great deal of published work describing the losses of preservative to plastic medicine bottles, contact lenses and their containers and plastic syringes. Given the complexity of modern plastics, with their differences in thickness, surface characteristics, filler and plasticizer content, it is necessary to choose the material for the pack of a preserved formulation on the basis of adequate trials.

Although rubber reacts with many preservatives it still finds use for teats and closures. These are required to be pretreated with the preservatives they are to be in contact with, in order to minimize subsequent uptake during storage.

Influence of pH

An appreciation of the many obstacles that may prevent a preservative attaining an adequate protective concentration in a given preparation must be complemented by some understanding of the interaction between that preservative and any microorganisms present as contaminants. Thus, not only must free preservative be available in a formulation, but it must be present in an active form. This is particularly important when activity is related to degree of ionization, as is the case with both anion- and cation-active antimicrobial agents. An example is that of a weak acid preservative such as benzoic acid, which requires to be predominantly in the undissociated form in order to exert antimicrobial activity. Since this acid has a pK_a value of 4.2, an ambient pH below this is needed for efficient preservative activity.

The relationship between degree of dissociation and pH is given by:

$$\text{Fraction of undissociated preservative} = \frac{1}{1 + \text{antilog}\left(\text{pH} - \text{p}K_a\right)}$$

In most cases more than one factor is involved, as for example when benzoic acid is used to preserve kaolin mixtures. The dual problem here is that the adsorption of the benzoic acid by the light kaolin diminishes at pH values above 5, whereas the acid preservative is most efficient at lower values. In this situation the formulator faces opposing choices, opting for an alternative preservative or using a mixture of preservatives. Kaolin mixture has the added interest that in order to render it attractive to children, raspberry syrup flavouring is used and so the pH is reduced to 3.5, at which value about 83% of benzoic acid is present in the biologically active undissociated form but adsorption on to the kaolin is favoured.

The majority of preservatives are less dependent upon pH, although cationic active quaternary ammonium compounds are more active at high pH values.

Preservatives in combination

The use of a single preservative to protect a pharmaceutical preparation may be unrealistic. Increasing attention has been focused on the use of mixtures of preservatives and the addition of various potentiators to achieve better results. The justification for using mixtures of antimicrobial compounds must reside in an increased spectrum of antimicrobial activity, a synergistic effect enabling decreased levels of component preservatives to be used, an attendant decrease in toxicity, and a reduction in the emergence of resistant forms. One of the oldest examples of a preservative mixture used in pharmacy is the former vehicle for eye drops, 'Solution for Eyedrops', which contained a mixture of methyl and propyl esters of *p*-hydroxybenzoic acid designed to exert antibacterial and antifungal effects. Modern formulations for eye drops and contact lens solutions include phenylethyl alcohol and phenoxetol, in conjuction with benzalkonium chloride to widen the antimicrobial spectrum and aid access to susceptible sites on the microorganisms. The chelating agent ethylenediamine tetra-acetate (EDTA) has also been used with preservatives other than those yielding metallic ions, to enhance activity by interfering with vital metal ion balance and associated permeability. A rather different mechanism is to increase the availability of lipophilic preservatives by reducing their loss to emulgents in the formulation, as with the addition of propylene glycol to emulsions preserved with parabens to reduce loss to micelles.

A correctly designed preservative system is the appropriate complement to hygienic manufacture. Both demand a rational approach based on an appre-

ciation of interacting factors. Thus, just as there is little justification in providing a high-quality manufacturing environment and introducing poor-quality raw materials, there is equally little point in an ad hoc addition of preservatives to preparations without investigation of formulation interactions involved.

MICROBIAL STANDARDS FOR PHARMACEUTICAL PREPARATIONS

The design of microbial standards for pharmaceutical preparations must be realistic in that they relate to the intended use of the preparation and can be applied without ambiguity. The types of standard used to monitor microbial content are twofold, namely, an absolute exclusion of all microorganisms or named organisms, and a numerical limit upon all organisms or named organisms. The first type, or exclusion standard in its most severe form, is that requiring sterility, and is applied to solutions for injection, ophthalmic preparations and certain other fluids for body irrigation. In these cases no organisms can be tolerated and the role of any preservative is to maintain the sterility of the sterilized preparation during use. Although such an absolute standard is not required for medicines for oral or topical use, nevertheless certain bacteria can represent a hazard and be indicative of poor manufacturing practice and should be excluded. The US Pharmacopoeia suggests an exclusion standard for *E. coli* to all solutions for oral use and for *Staph. aureus* and *Ps. aeruginosa* in topical preparations. In addition, both the British and US pharmacopoeias apply exclusion standards for named organisms from certain raw materials and final preparations (Table 40.3).

Compliance with these standards can only be assured by strict control of manufacture from raw materials to final preparation (Fig. 42.1). The incorporation of a product-designed preservative system is an additional safeguard.

The application of the various standards described depends in the last analysis upon the reliability of the techniques available for detection and enumeration of microorganisms in raw materials and final preparations. The established methods of the microbiologist are time-consuming and have the errors of any biological measurements. As indicated earlier, a range of methods of detection and quantification based upon other physiological or metabolic characteristics are well established in the food industry and their adoption in the pharmaceutical industry should add a new dimension to the use of microbial standards.

Challenge testing

The methods and philosophy of challenge testing are dealt with in Chapter 40. In the particular case of preserved preparations the test must be designed to provide a realistic measure of the ability of the formulation to cope with normal use. Many arguments have been made for the choice of challenge organisms, their use at various concentrations, the number of challenges made and so on. The current BP test specifies all of these parameters for parenteral, ophthalmic, topical and oral liquid preparations, together with the end-point required. In this respect it provides general guidelines, but in addition most manufacturers apply their own challenge tests based on their experience with the particular product.

CONCLUSION

As with many areas of study that of the microbial contamination of pharmaceuticals began with an awareness that a problem existed. The consequences of this problem extended both to economy of production and to the safety of the patient. The skills of the microbiologist, chemist, engineer and pharmacist have been combined to enable medicines to be prepared which are microbiologically safe. The maintaining of this situation depends upon constant vigilance over every aspect of manufacture and formulation.

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