ESSENTIAL Microbiology

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Preface

Every year, in UK universities alone, many hundreds of students study microbiology as part of an undergraduate course. For some, the subject will form the major part of their studies, leading to a BSc degree in Microbiology, or a related subject such as Bacteriology or Biotechnology. For the majority, however, the study of microbiology will be a brief encounter, forming only a minor part of their course content.

A number of excellent and well-established textbooks are available to support the study of microbiology; such titles are mostly over 1000 pages in length, beautifully illustrated in colour, and rather expensive. This book in no way seeks to replace or compete with such texts, which will serve specialist students well throughout their three years of study, and represent a sound investment. It is directed rather towards the second group of students, who require a text that is less detailed, less comprehensive, and less expensive! The majority of the students in my own classes are enrolled on BSc degrees in Biology, Human Biology and Forensic Science; I have felt increasingly uncomfortable about recommending that they invest a substantial sum of money on a book much of whose content is irrelevant to their needs. Alternative recommendations, however, are not thick on the ground. This, then, was my initial stimulus to write a book of 'microbiology for the non-microbiologist'.

The facts and principles you will find here are no different from those described elsewhere, but I have tried to select those topics that one might expect to encounter in years 1 and 2 of a typical non-specialist degree in the life sciences or related disciplines. Above all, I have tried to *explain* concepts or mechanisms; one thing my research for this book has taught me is that textbooks are *not* always right, and they certainly don't always explain things as clearly as they might. It is my wish that the present text will give the attentive reader a clear understanding of sometimes complex issues, whilst avoiding over-simplification.

The book is arranged into seven sections, the fourth of which, Microbial Genetics, acts as a pivot, leading from principles to applications of microbiology. Depending on their starting knowledge, readers may 'dip into' the book at specific topics, but those whose biological and chemical knowledge is limited are strongly recommended to read Chapters 2 and 3 for the foundation necessary for the understanding of later chapters. Occasional boxes are inserted into the text, which provide some further enlightenment on the topic being discussed, or offer supplementary information for the inquisitive reader. As far as possible, diagrams are limited to simple line drawings, most of which could be memorised for reproduction in an examination setting. Although a Glossary is provided at the end of the book, new words are also defined in the text at the point of

their first introduction, to facilitate uninterrupted reading. All chapters except the first are followed by a self-test section in which readers may review their knowledge and understanding by 'filling in the gaps' in incomplete sentences; the answers are all to be found in the text, and so are not provided separately. The only exceptions to this are two numerical questions, the solutions to which are to be found at the back of the book. By completing the self-test questions, the reader effectively provides a summary for the chapter.

A book such as this stands or falls by the reception it receives from its target readership. I should be pleased to receive any comments on the content and style of *Essential Microbiology* from students and their tutors, all of which will be given serious consideration for inclusion in any further editions.

Stuart Hogg
January 2005

Acknowledgements

I would like to thank those colleagues who took the time to read over individual chapters of this book, and those who reviewed the entire manuscript. Their comments have been gratefully received, and in some cases spared me from the embarrassment of seeing my mistakes perpetuated in print.

Thanks are also due to my editorial team at John Wiley, Rachael Ballard and Andy Slade, and production editor Robert Hambrook for ensuring smooth production of this book.

I am grateful to those publishers and individuals who have granted permission to reproduce diagrams. Every effort has been made to trace holders of copyright; any inadvertent omissions will gladly be rectified in any future editions of this book.

Finally, I would like to express my gratitude to my family for allowing me to devote so many weekends to 'the book'.

Part I

Introduction

1

Microbiology: What, Why and How?

As you begin to explore the world of microorganisms, one of the first things you'll notice is their extraordinary diversity – of structure, function, habitat and applications. Microorganisms (or microbes) inhabit every corner of the globe, are indispensable to life on Earth, are responsible for some of the most deadly human diseases and form the basis of many industrial processes. Yet until a few hundred years ago, nobody knew they existed!

In this opening chapter, we offer some answers to three questions:

- *What* is microbiology?
- *Why* is it such an important subject?
- *How* have we gained our present knowledge of microbiology?

What is microbiology?

Things aren't always the way they seem. On the face of it, 'microbiology' should be an easy word to define: the science (*logos*) of small (*micro*) life (*bios*), or to put it another way, the study of living things so small that they cannot be seen with the naked eye. Bacteria neatly fit this definition, but what about fungi and algae? These two groups each contain members that are far from microscopic. On the other hand, certain animals, such as nematode worms, can be microscopic, yet are not considered to be the domain of the microbiologist. Viruses represent another special case; they are most certainly microscopic (indeed, most are submicroscopic), but by most accepted definitions they are not living. Nevertheless, these too fall within the remit of the microbiologist.

In the central section of this book you can read about the thorny issue of microbial classification and gain some understanding of just what is and what is not regarded as a microorganism.

Why is microbiology important?

To the lay person, microbiology means the study of sinister, invisible 'bugs' that cause disease. As a subject, it generally only impinges on the popular consciousness in news

coverage of the latest 'health scare'. It may come as something of a surprise therefore to learn that the vast majority of microorganisms coexist alongside us without causing any harm. Indeed, many perform vital tasks such as the recycling of essential elements, without which life on our planet could not continue, as we will examine in Chapter 16. Other microorganisms have been exploited by humans for our own benefit, for instance in the manufacture of antibiotics (Chapter 14) and foodstuffs (Chapter 17). To get some idea of the importance of microbiology in the world today, just consider the following list of some of the general areas in which the expertise of a microbiologist might be used:

- medicine
- environmental science
- food and drink production
- fundamental research
- agriculture
- pharmaceutical industry
- genetic engineering.

The popular perception among the general public, however, remains one of infections and plagues. Think back to the first time you ever heard about microorganisms; almost certainly, it was when you were a child and your parents impressed on you the dangers of 'germs' from dirty hands or eating things after they'd been on the floor. In reality, only a couple of hundred out of the half million or so known bacterial species give rise to infections in humans; these are termed *pathogens*, and have tended to dominate our view of the microbial world.

A *pathogen* is an organism with the potential to cause disease.

In the next few pages we shall review some of the landmark developments in the history of microbiology, and see how the main driving force throughout this time, but particularly in the early days, has been the desire to understand the nature and cause of infectious diseases in humans.

How do we know? Microbiology in perspective: to the 'golden age' and beyond

We have learnt an astonishing amount about the invisible world of microorganisms, particularly over the last century and a half. How has this happened? The penetrating insights of brilliant individuals are rightly celebrated, but a great many 'breakthroughs' or 'discoveries' have only been made possible thanks to some (frequently unsung) development in microbiological methodology. For example, on the basis that 'seeing is believing', it was only when we had the means to *see* microorganisms under a microscope that we could prove their existence.

Microorganisms had been on the Earth for some 4000 million years, when Antoni van Leeuwenhoek started out on his pioneering microscope work in 1673. Leeuwenhoek was an amateur scientist who spent much of his spare time grinding glass lenses

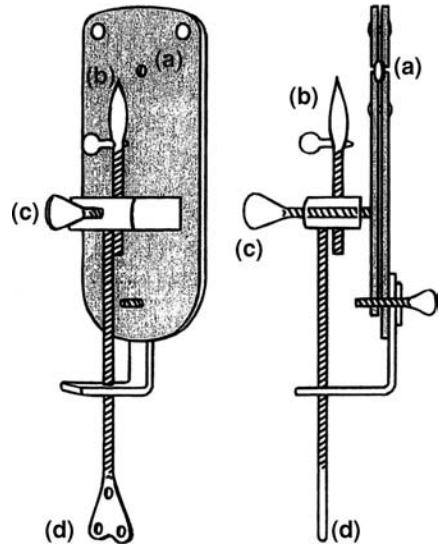


Figure 1.1 Leeuwenhoek's microscope. The lens (a) was held between two brass plates and used to view the specimen, which was placed on the mounting pin (b). Focusing was achieved by means of two screws (c) and (d). Some of Leeuwenhoek's microscopes could magnify up to 300 times. Original source: *Antony van Leeuwenhoek and his little animals* by CE Dobell (1932)

to produce simple microscopes (Figure 1.1). His detailed drawings make it clear that the 'animalcules' he observed from a variety of sources included representatives of what later became known as protozoa, bacteria and fungi. Where did these creatures come from? Arguments about the origin of living things revolved around the long held belief in spontaneous generation, the idea that living organisms could arise from non-living matter. In an elegant experiment, the Italian Francesco Redi (1626–1697) showed that the larvae found on putrefying meat arose from eggs deposited by flies, and not spontaneously as a result of the decay process. This can be seen as the beginning of the end for the spontaneous generation theory, but many still clung to the idea, claiming that while it may not have been true for larger organisms, it must surely be so for minute creatures such as those demonstrated by Leeuwenhoek. Despite mounting evidence against the theory, as late as 1859, fresh 'proof' was still being brought forward in its support. Enter onto the scene Louis Pasteur (1822–1895), still arguably the most famous figure in the history of microbiology. Pasteur trained as a chemist, and made a lasting contribution to the science of stereochemistry before turning his attention to spoilage problems in the wine industry. He noticed that when lactic acid was produced in wine instead of alcohol, rod-shaped bacteria were always present, as well as the expected yeast cells. This led him to believe that while the yeast produced the alcohol, the bacteria were responsible for the spoilage, and that both types of organism had originated in the environment. Exasperated by continued efforts to substantiate the theory of spontaneous generation, he set out to disprove it once and for all. In response to a call from the French Academy of Science, he carried out a series of experiments that led to the acceptance of *biogenesis*, the idea that life arises only from already existing life. Using his famous swan-necked flasks (Figure 1.2), he demonstrated in 1861 that as long as dust

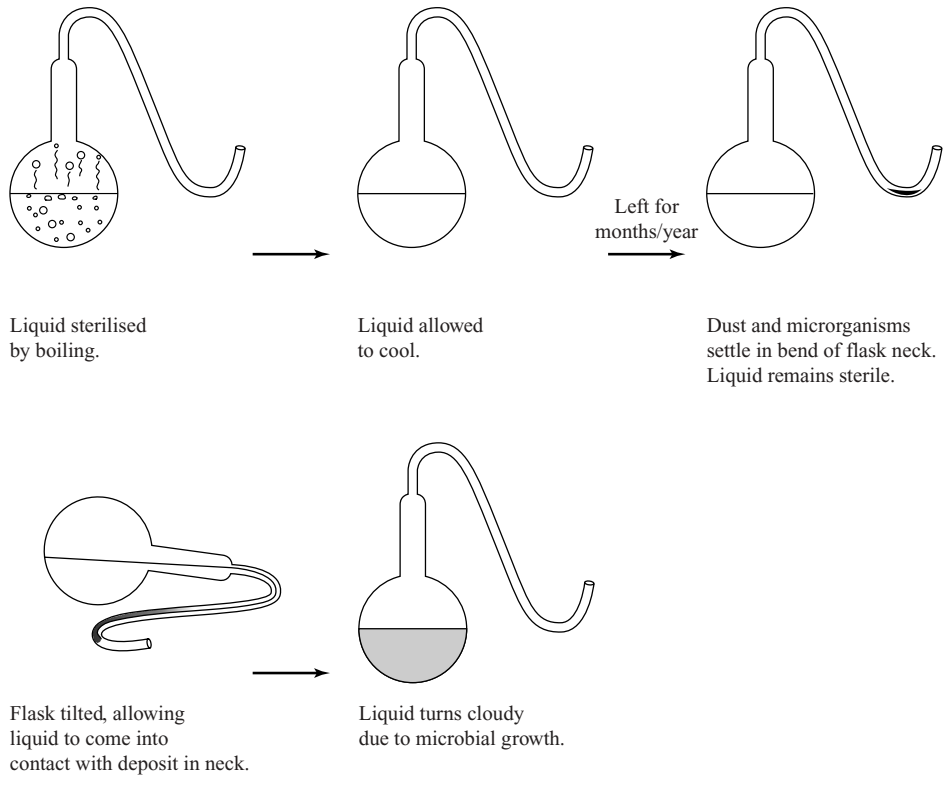


Figure 1.2 Pasteur's swan-necked flasks. Broth solutions rich in nutrients were placed in flasks and boiled. The necks of the flasks were heated and drawn out into a curve, but kept open to the atmosphere. Pasteur showed that the broth remained sterile because any contaminating dust and microorganisms remained trapped in the neck of the flask as long as it remained upright

particles (and the microorganisms carried on them) were excluded, the contents would remain sterile. This also disproved the idea held by many that there was some element in the air itself that was capable of initiating microbial growth. In Pasteur's words '. . . . the doctrine of spontaneous generation will never recover from this mortal blow. *There is no known circumstance in which it can be affirmed that microscopic beings came into the world without germs, without parents similar to themselves*.' Pasteur's findings on wine contamination led inevitably to the idea that microorganisms may be also be responsible for diseases in humans, animals and plants.

The notion that some invisible (and therefore, presumably, extremely small) living creatures were responsible for certain diseases was not a new one. Long before microorganisms had been shown to exist, the Roman philosopher Lucretius (~98–55 BC) and much later the physician Girolamo Fracastoro (1478–1553) had supported the idea. Fracastoro wrote 'Contagion is an infection that passes from one thing to another' and recognised three forms of transmission: by direct contact, through inanimate objects and via the air. We still class transmissibility of infectious disease in much the same way today. The prevailing belief at the time, however, was that an infectious disease was due

to something called a *miasma,* a poisonous vapour arising from dead or diseased bodies, or to an imbalance between the four humours of the body (blood, phlegm, yellow bile and black bile). During the 19th century, many diseases were shown, one by one, to be caused by microorganisms. In 1835, Agostino Bassi showed that a disease of silkworms was due to a fungal infection, and 10 years later, Miles Berkeley demonstrated that a fungus was also responsible for the great Irish potato blight. Joseph Lister's pioneering work on antiseptic surgery provided strong, albeit indirect, evidence of the involvement of microorganisms in infections of humans. The use of heat-treated instruments and of phenol both on dressings and actually sprayed in a mist over the surgical area, was found greatly to reduce the number of fatalities following surgery. Around the same time, in the 1860s, the indefatigable Pasteur had shown that a parasitic protozoan was the cause of another disease of silkworms called *pébrine*, which had devastated the French silk industry.

The first proof of the involvement of bacteria in disease and the definitive proof of the germ theory of disease came from the German Robert Koch. In 1876 Koch showed the relationship between the cattle disease *anthrax* and a bacillus which we now know as *Bacillus anthracis*. Koch infected healthy mice with blood from diseased cattle and sheep, and noted that the symptoms of the disease appeared in the mice, and that rod shaped bacteria could be isolated from their blood. These could be grown in culture, where they multiplied and produced spores. Injection of healthy mice with these spores (or more bacilli) led them too to develop anthrax and once again the bacteria were isolated from their blood. These results led Koch to formalise the criteria necessary to prove a causal relationship between a specific disease condition and a particular microorganism. These criteria became known as *Koch's postulates* (Box 1.1), and are still in use today.

A *bacillus* is a rod-shaped bacterium.

Box 1.1 Koch's postulates

1 The microorganism must be present in every instance of the disease and absent from healthy individuals.

2 The microorganism must be capable of being isolated and grown in pure culture.

3 When the microorganism is inoculated into a healthy host, the same disease condition must result.

4 The same microorganism must be re-isolated from the experimentally infected host.

Despite their value, it is now realised that Koch's postulates do have certain limitations. It is known for example that certain agents responsible for causing disease (e.g. viruses, prions: see Chapter 10) can't be grown *in vitro,* but only in host cells. Also, the healthy animal in Postulate 3 is seldom human, so a degree of extrapolation is necessary – if agent X does not cause disease in

The term *in vitro* (= 'in glass') is used to describe procedures performed outside of the living organism in test tubes, etc. (c.f *in vivo*).

Table 1.1 The discovery of some major human pathogens

Year	Disease	Causative agent	Discoverer
1876	Anthrax	*Bacillus anthracis*	Koch
1879	Gonorrhoea	*Neisseria gonorrhoeae*	Neisser
1880	Typhoid fever	*Salmonella typhi*	Gaffky
1880	Malaria	*Plasmodium* sp	Laveran
1882	Tuberculosis	*Mycobacterium tuberculosis*	Koch
1883	Cholera	*Vibrio cholerae*	Koch
1883/4	Diphtheria	*Corynebacterium diphtheriae*	Klebs & Loeffler
1885	Tetanus	*Clostridium tetani*	Nicoaier & Kitasato
1886	Pneumonia (bacterial)	*Streptococcus pneumoniae*	Fraenkel
1892	Gas gangrene	*Clostridium perfringens*	Welch & Nuttall
1894	Plague	*Yersinia pestis*	Kitasato & Yersin
1896	Botulism	*Clostridium botulinum*	Van Ermengem
1898	Dysentery	*Shigella dysenteriae*	Shiga
1901	Yellow fever	Flavivirus	Reed
1905	Syphilis	*Treponema pallidum*	Schaudinn & Hoffman
1906	Whooping cough	*Bordetella pertussis*	Bordet & Gengou
1909	Rocky Mountain spotted fever	*Rickettsia rickettsii*	Ricketts

a laboratory animal, can we be sure it won't in humans? Furthermore, some diseases are caused by more than one organism, and some organisms are responsible for more than one disease. On the other hand, the value of Koch's postulates goes beyond just defining the causative agent of a particular disease, and allows us to ascribe a specific effect (of whatever kind) to a given microorganism.

Critical to the development of Koch's postulates was the advance in culturing techniques, enabling the isolation and pure culture of specific microorganisms. These are discussed in more detail in Chapter 4. The development of pure cultures revolutionised microbiology, and within the next 30 years or so, the pathogens responsible for the majority of common human bacterial diseases had been isolated and identified. Not without just cause is this period known as the 'golden age' of microbiology! Table 1.1 summarises the discovery of some major human pathogens.

A *pure* or *axenic* culture contains one type of organism only, and is completely free from contaminants.

At around the same time, Charles Chamberland, a pupil of Pasteur's, invented the autoclave, contributing greatly to the development of pure cultures.

Koch's greatest achievement was in using the advances in methodology and the principles of his own postulates to demonstrate the identity of the causative agent of tuberculosis, which at the time was responsible for around one in every seven human deaths in Europe. Although it was believed by many to have a microbial cause, the causative agent had never been observed, either in culture or in the affected tissues. We now know that *Mycobacterium tuberculosis* (the tubercle bacillus) is very difficult to stain by conventional methods due to the high lipid content of the cell wall surface. Koch developed a staining technique that enabled it to be seen, but realised that in order to satisfy his own postulates, he must isolate the organism and grow it in culture. Again, there were

technical difficulties, since even under favourable conditions, *M. tuberculosis* grows slowly, but eventually Koch was able to demonstrate the infectivity of the cultured organisms towards guinea pigs. He was then able to isolate them again from the diseased animal and use them to cause disease in uninfected animals, thus satisfying the remainder of his postulates.

Although most bacterial diseases of humans and their aetiological agents have now been identified, important variants continue to evolve and emerge. Notable examples in recent times include Legionnaires' disease, an acute respiratory infection caused by the previously unrecognised genus, *Legionella*, and Lyme disease, a tickborne infection first described in Connecticut, USA in the mid-1970s. Also, a newly recognised pathogen, *Helicobacter pylori,* has been shown to play an important (and previously unsuspected) role in the development of peptic ulcers. There still remain a few diseases that some investigators suspect are caused by bacteria, but for which no pathogen has been identified.

Aetiology is the cause or origin of a disease.

Following the discovery of viruses during the last decade of the 19th century (see Chapter 10), it was soon established that many diseases of plants, animals and humans were caused by these minute, non-cellular agents.

The major achievement of the first half of the 20th century was the development of antibiotics and other antimicrobial agents, a topic discussed in some detail in Chapter 14. Infectious diseases that previously accounted for millions of deaths became treatable by a simple course of therapy, at least in the affluent West, where such medications were readily available.

If the decades either side of 1900 have become known as the golden age of microbiology, the second half of the twentieth century will surely be remembered as the golden age of molecular genetics. Following on from the achievements of others such as Griffith and Avery, the publication of Watson and Crick's structure for DNA in 1953 heralded an extraordinary 50 years of achievement in this area, culminating at the turn of the 21st century in the completion of the Human Genome Project.

The *Human Genome Project* is an international effort to map and sequence all the DNA in the human genome. The project has also involved sequencing the genomes of several other organisms.

What, you might ask, has this genetic revolution to do with microbiology? Well, all the early work in molecular genetics was carried out on bacteria and viruses, as you'll learn in Chapter 11, and microbial systems have also been absolutely central to the development of genetic engineering over the last three decades (Chapter 12). Also, as part of the Human Genome Project, the genomes of several microorganisms have been decoded, and it will become increasingly easy to do the same for others in the future, thanks to methodological advances made during the project. Having this information will help us to understand in greater detail the disease strategies of microorganisms, and to devise ways of countering them.

As we have seen, a recurring theme in the history of microbiology has been the way that advances in knowledge have followed on from methodological or technological developments, and we shall refer to a number of such developments during the course of this book. To conclude this introduction to microbiology, we shall return to the instrument that, in some respects, started it all. In any microbiology course, you are sure to spend some time looking down a microscope, and to get the most out of the instrument

it is essential that you understand the principles of how it works. The following pages attempt to explain these principles.

Light microscopy

Try this simple experiment. Fill a glass with water, then partly immerse a pencil and observe from one side; what do you see? The apparent 'bending' of the pencil is due to rays of light being slowed down as they enter the water, because air and water have different *refractive indices*. Light rays are similarly retarded as they enter glass and all optical instruments are based on this phenomenon. The compound light microscope consists of three sets of lenses (Figure 1.3):

- the *condenser* focuses light onto the specimen to give optimum illumination
- the *objective* provides a magnified and inverted image of the specimen
- the *eyepiece* adds further magnification

The *refractive index* of a substance is the ratio between the velocity of light as it passes through that substance and its velocity in a vacuum. It is a measure of how much the substance slows down and therefore refracts the light.

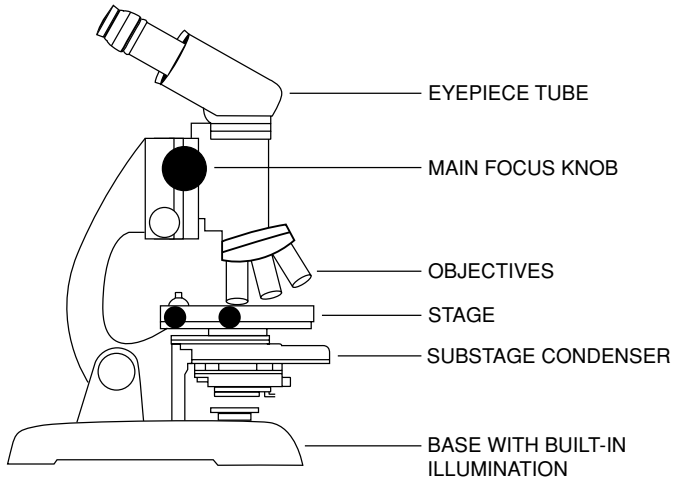


Figure 1.3 The compound light microscope. Modern microscopes have a built-in light source. The light is focused onto the specimen by the condenser lens, and then passes into the body of the microscope via the objective lens. Rotating the objective nosepiece allows different magnifications to be selected. The amount of light entering the microscope is controlled by an iris diaphragm. Light microscopy allows meaningful magnification of up to around 1000×

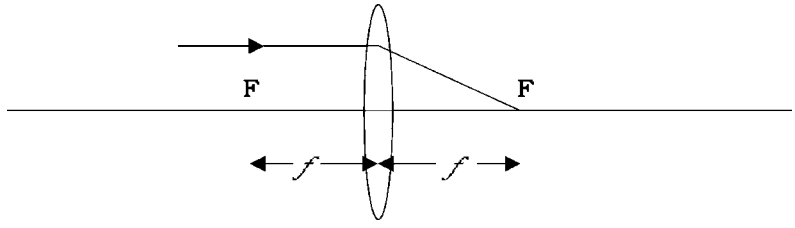


Figure 1.4 Light rays parallel to the axis of a convex lens pass through the focal point. The distance from the centre of the lens to the focal point is called the focal length (*f*) of the lens

Most microscopes have three or four different objectives, giving a range of magnifications, typically from 10× to 100×. The total magnification is obtained by multiplying this by the eyepiece value (usually 10×), thus giving a maximum magnification of 1000×.

In order to appreciate how this magnification is achieved, we need to understand the behaviour of light passing through a convex lens:

- rays parallel to the axis of the lens are brought to a focus at the *focal point* of the lens (Figure 1.4)
- similarly, rays entering the lens from the focal point emerge parallel to the axis
- rays passing through the centre of the lens from any angle are undeviated.

Because the condenser is not involved in magnification, it need not concern us here. Consider now what happens when light passes through an objective lens from an object AB situated slightly beyond its focal point (Figure 1.5a). Starting at the tip of the object, a ray parallel to the axis will leave the lens and pass through the focal point; a ray leaving the same point and passing through the centre of the lens will be undeviated. The point at which the two rays converge is an image of the original point formed by the lens. The same thing happens at an infinite number of points along the object's length, resulting in a primary image of the specimen, A′B′. What can we say about this image, compared to the original specimen AB? It is magnified and it is inverted (i.e. it appears upside down).

The primary image now serves as an object for a second lens, the eyepiece, and is magnified further (Figure 1.5b); this time the object is situated within the focal length. Using the same principles as before, we can construct a ray diagram, but this time we find that the two lines drawn from a point do not converge on the other side of the lens, but actually get further apart. The point at which the lines do eventually converge is actually 'further back' than the original object! What does this mean? The secondary image only *appears* to be coming from A″ B″, and isn't actually there. An image such as this is

A *real* image is one that can be projected onto a flat surface such as a screen. A virtual image does not exist in space and cannot be projected in this way. A familiar example is the image seen in a mirror.

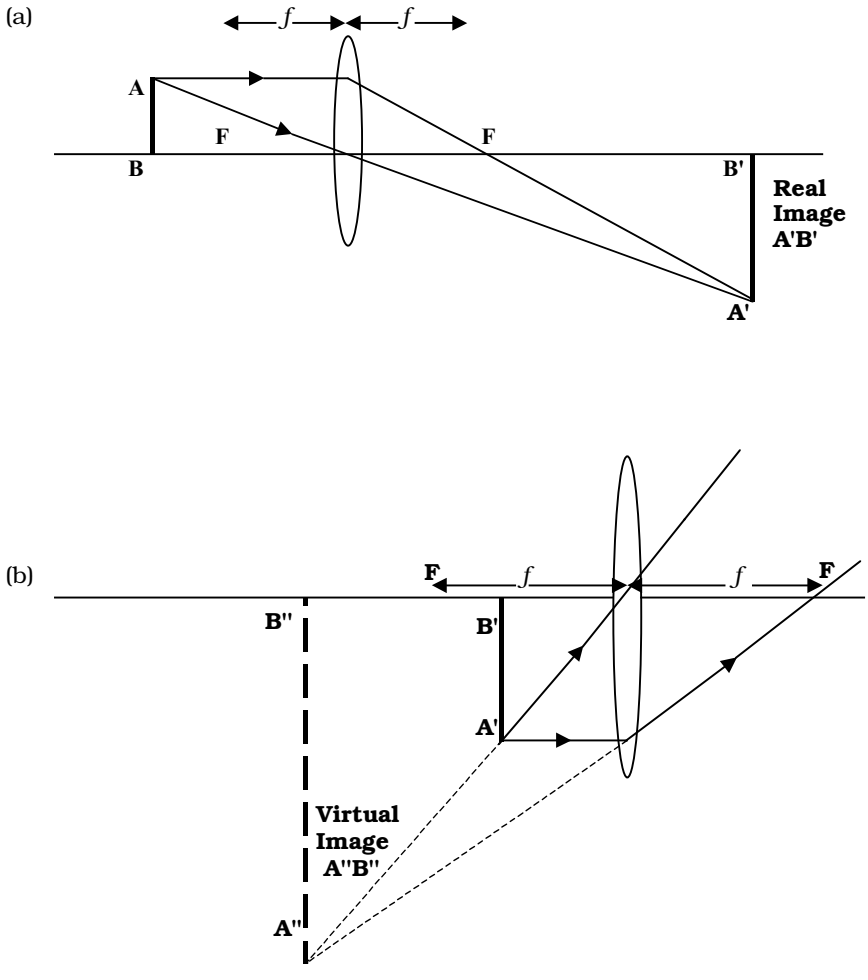


Figure 1.5 The objective lens and eyepiece lens combine to produce a magnified image of the specimen. (a) Light rays from the specimen AB pass through the objective lens to give a magnified, inverted and real primary image. (b) The eyepiece lens magnifies this further to produce a virtual image of the specimen

called a *virtual* image. Today's reader, familiar with the concept of virtual reality, will probably find it easier to come to terms with this than have students of earlier generations! The primary image A′B′, on the other hand, is a *real* image; if a screen was placed at that position, the image would be projected onto it. If we compare A″B″ with A′B′, we can see that it has been further magnified, but not further inverted, so it is still upside down compared with the original. One of the most difficult things to get used to when you first use a microscope is that everything appears 'wrong way around'. The rays of light emerging from the eyepiece lens are focussed by the lens of the observer's eye to form a real image on the retina of the viewer's eye.

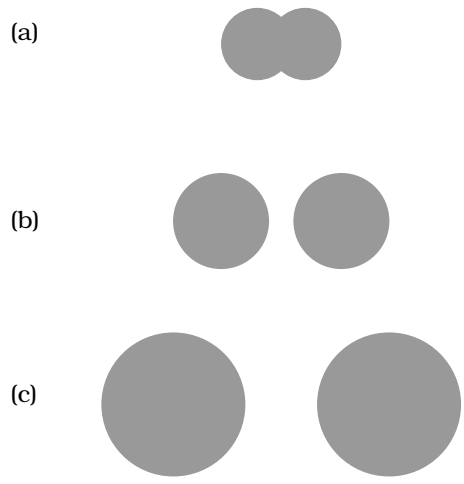


Figure 1.6 Magnification must be accompanied by improved resolution. Compared to (a), the image in (b) is magnified, but also provides improved detail; there are two objects, not just one. Further magnification, as seen in (c), provides no further information (empty magnification)

So, a combination of two lens systems allows us to see a considerably magnified image of our specimen. To continue magnifying an image beyond a certain point, however, serves little purpose, if it is not accompanied by an increase in detail (Figure 1.6). This is termed *empty magnification*. The *resolution* (resolving power, d) of a microscope is its capacity for discerning detail. More specifically, it is the ability to distinguish between two points a short distance apart, and is determined by the equation:

$$d = \frac{0.61\lambda}{n \sin\theta}$$

where λ is the wavelength of the light source, n is the refractive index of the air or liquid between the objective lens and the specimen and θ is the aperture angle (a measure of the light-gathering ability of the lens).

Immersion oil is used to improve the resolution of a light microscope at high power. It has the same refractive index as glass and is placed between the high power objective and the glass slide. With no layer of air, more light from the specimen enters the objective lens instead of being refracted outside of it, resulting in a sharper image.

The expression $n \sin\theta$ is called the *numerical aperture* and for high quality lenses has a value of around 1.4. The lowest wavelength of light visible to the human eye is approximately 400 nm, so the maximum resolving power for a light microscope is approximately

$$d = \frac{0.61 \times 400}{1.4} = 0.17\mu\text{m}$$

that is, it cannot distinguish between two points closer together than about 0.2 μm. For comparison, the naked eye is unable to resolve two points more than about 0.2 mm apart.

For us to be able to discern detail in a specimen, it must have contrast; most biological specimens, however, are more or less colourless, so unless a structure is appreciably denser than its surroundings, it will not stand out. This is why preparations are commonly subjected to staining procedures prior to viewing. The introduction of coloured dyes, which bind to certain structures, enables the viewer to discern more detail.

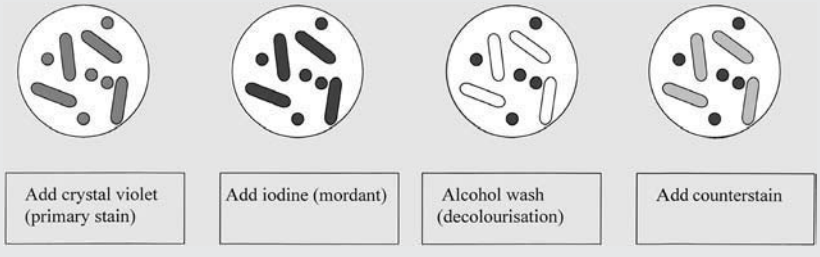
A nanometre (nm) is 1 millionth of a millimetre. There are 1000 nm in one micron (μm), which is therefore one thousandth of a millimetre.
1 mm = 10^{-3} m
1 μm = 10^{-6} m
1 nm = 10^{-9} m

Since staining procedures involve the addition and washing off of liquid stains, the sample must clearly be immobilised or fixed to the slide if it is not to end up down the sink. The commonest way of doing this is to make a heat-fixed smear; this kills and attaches the cells to the glass microscope slide. A thin aqueous suspension of the cells is spread across the slide, allowed to dry, then passed (sample side up!) through a flame a few times. Excessive heating must be avoided, as it would distort the natural structure of the cells.

Using simple stains, such as methylene blue, we can see the size and shape of bacterial cells, for example, and their arrangement, while the binding properties of differential stains react with specific structures, helping us to differentiate between bacterial types. Probably the most widely used bacterial stain is the Gram stain (see Box 1.2), which for more than 100 years has been an invaluable first step in the identification of unknown bacteria.

Box 1.2 The Gram stain

The Gram stain involves the sequential use of two stains (see below). The critical stage is step 3; some cells will resist the alcohol treatment and retain the crystal violet, while others become decolorised. The counterstain (safranin or neutral red) is weaker than the crystal violet, and will only be apparent in those cells that have been decolorised.



The Gram stain is a differential stain, which only takes a few minutes to carry out, and which enables us to place a bacterial specimen into one of two groups, Gram-positive or Gram-negative. The reason for this differential reaction to the stain was not understood for many years, but is now seen to be a reflection of differences in cell wall structure, discussed in more detail in Chapter 3.

Specialised forms of microscopy have been developed to allow the viewer to discern detail in living, unstained specimens; these include phase contrast and dark-field microscopy. We can also gain an estimate of the number of microorganisms in a sample by directly counting them under the microscope. This is discussed along with other enumeration methods in Chapter 5.

Phase contrast microscopy exploits differences in thickness and refractive index of transparent objects such as living cells to give improved contrast.

Dark field microscopy employs a modified condenser. It works by blocking out direct light, and viewing the object only by the light it diffracts.

Electron microscopy

From the equation shown above, you can see that if it were possible to use a shorter wavelength of light, we could improve the resolving power of a microscope. However, because we are limited by the wavelength of light visible to the human eye, we are not able to do this with the light microscope. The electron microscope is able to achieve greater magnification and resolution because it uses a high voltage beam of electrons, whose wavelength is very much shorter than that of visible light. Consequently we are able to resolve points that are much closer together than is possible even with the very best light microscope. The resolving power of an electron microscope may be as low as 1–2 nm, enabling us to see viruses, for example, and the internal structure of cells. The greatly improved resolution means that specimens can be meaningfully magnified over 100 000×.

Electron microscopes, which were first developed in the 1930s and 1940s, use ring-shaped electromagnets as 'lenses' to focus the beam of electrons onto the specimen. Because the electrons would collide with, and be deflected by, molecules in the air, electron microscopes require a pump to maintain a vacuum in the column of the instrument. There are two principal types of electron microscope, the transmission electron microscope (TEM) and the scanning electron microscope (SEM).

Figure 1.7 shows the main features of a TEM. As the name suggests, the electron beam passes *through* the specimen and is scattered according to the density of the different parts. Due to the limited penetrating power of the electrons, extremely thin sections (<100 nm, or less than one-tenth of the diameter of a bacterial cell) must be cut, using a diamond knife. To allow this, the specimen must be fixed and dehydrated, a process that can introduce shrinkage and distortion to its structure if not correctly performed.

After being magnified by an objective 'lens', an image of the specimen is projected onto a fluorescent screen or photographic plate. More dense areas, which scatter the beam, appear dark, and those where it has passed through are light. It is often necessary to enhance contrast artificially, by means of 'staining' techniques that involve coating the specimen with a thin layer of a compound containing a heavy metal, such as osmium or palladium. It will be evident from the foregoing description of sample preparation and use of a vacuum that electron microscopy cannot be used to study living specimens.

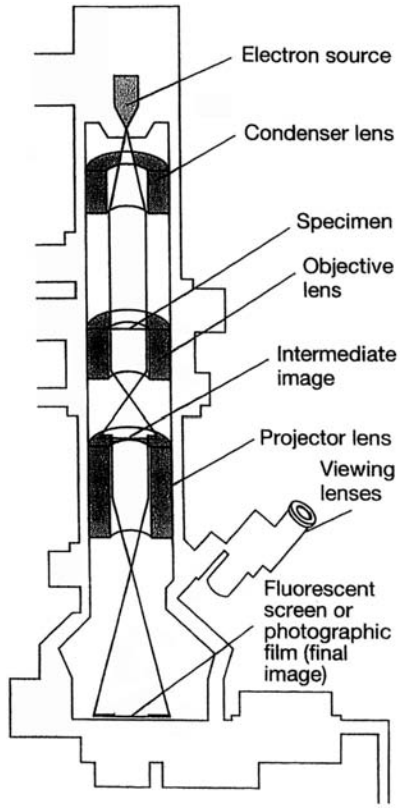


Figure 1.7 The transmission electron microscope. Electrons from a tungsten filament pass through a vacuum chamber and are focused by powerful electromagnets. Passage through the specimen causes a scattering of the electrons to form an image that is captured on a fluorescent screen. From Black, JG: Microbiology: Principles and Explorations, 4th edn, John Wiley & Sons Inc., 1999. Reproduced by permission of the publishers

The TEM has been invaluable in advancing our knowledge of the fine structure of cells, microbial or otherwise. The resulting image is, however, a flat, two-dimensional one, and of limited use if we wish to learn about the surface of a cell or a virus. For this, we turn to SEM. The scanning electron microscope was developed in the 1960s and provides vivid, sometimes startling, three-dimensional images of surface structure. Samples are dehydrated and coated with gold to give a layer a few nanometres thick. A fine beam of electrons probes back and forth across the surface of the specimen and causes secondary electrons to be given off. The number of these, and the angle at which they are emitted, depends on the topography of the specimen's surface. SEM does not have quite the resolving power of the TEM, and therefore does not operate at such high magnifications. Between them, SEM and TEM have opened up a whole new world to microbiologists, allowing us to put advances in our knowledge of microbial biochemistry and genetics into a structural context.

2
Biochemical Principles

All matter, whether living or non-living, is made up of *atoms*; the atom is the smallest unit of matter capable of entering into a chemical reaction. Atoms can combine together by *bonding*, to form *molecules*, which range from the small and simple to the large and complex. The latter are known as *macromolecules*; major cellular constituents such as carbohydrates and proteins belong to this group and it is with these that this chapter is mainly concerned (Table 2.1). In order to appreciate how these macromolecules operate in the structure and function of microbial cells however, we need to review the basic principles of how atoms are constructed and how they interact with one other.

Atomic structure

All atoms have a central, positively charged *nucleus*, which is very dense, and makes up most of the mass of the atom. The nucleus is made up of two types of particle, *protons* and *neutrons*. Protons carry a positive charge, and neutrons are uncharged, hence the nucleus overall is positively charged. It is surrounded by much lighter, and rapidly orbiting, *electrons* (Figure 2.1). These are negatively charged, the charge being equal (but of course opposite) to that of the protons, but they have only 1/1840 of the mass of either protons or neutrons. The attractive force between the positively charged protons and the negatively charged electrons holds the atom together.

The number of protons in the nucleus is called the *atomic number*, and ranges from 1 to over 100. The combined total of protons and neutrons is known as the *mass number*. All atoms have an equal number of protons and electrons, so regardless of the atomic number, the overall charge on the atom will always be zero.

Atoms having the same atomic number have the same chemical properties; such atoms all belong to the same *element*. An element is made up of one type of atom only and cannot be chemically broken down into simpler substances; thus pure copper for example is made up entirely of copper atoms. There are 92 of these elements occurring naturally, 26 of which commonly occur in living things. Each element has been given a universally agreed symbol; examples which we shall encounter in biological macromolecules include carbon (C), hydrogen (H) and oxygen (O). The atomic numbers of selected elements are shown in Table 2.2.

The relationship between neutrons, protons, atomic number, and mass number is illustrated in Table 2.3, using carbon as an example, since all living matter is based

Table 2.1 Biological macromolecules

Proteins	Carbohydrates	Lipids	Nucleic acids
Enzymes	Sugars	Triacylglycerols	DNA
Receptors	Cellulose	(fats)	RNA
Antibodies	Starch	Phospholipids	
Structural		Waxes	
proteins		Sterols	

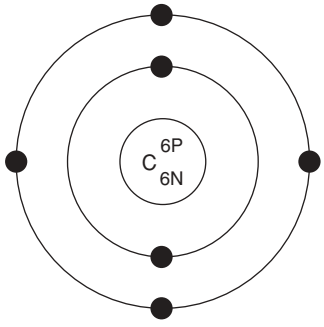


Figure 2.1 Atomic structure. The nucleus of a carbon atom contains six protons and six neutrons, surrounded by six electrons. Note how these are distributed between inner (2) and outer (4) electron shells

Table 2.2 Symbols and atomic numbers of some elements occurring in living systems

Element	Symbol	Atomic no.
Hydrogen	H	1
Carbon	C	6
Nitrogen	N	7
Oxygen	O	8
Sodium	Na	11
Magnesium	Mg	12
Phosphorus	P	15
Sulphur	S	16
Chlorine	Cl	17
Potassium	K	19
Iron	Fe	26

Table 2.3 The vital statistics of carbon

No. of Protons	No. of Neutrons	Atomic number	Mass number	Atomic mass
6	6	6	12	12.011

upon this element. The carbon represented can be expressed in the form:

$$\begin{matrix} 12 & \text{(mass number)} \\ C = & \text{(Element symbol)} \\ 6 & \text{(atomic number)} \end{matrix}$$

The number of neutrons in an atom can be deduced by subtracting the atomic number from the mass number. In the case of carbon, this is the same as the number of protons (6), but this is not always so. Phosphorus for example has 15 protons and 16 neutrons, giving it an atomic number of 15 and a mass number of 31.

Isotopes

Although the number of protons in the nucleus of a given element is always the same, the number of neutrons can vary, to give different forms or *isotopes* of that element. Carbon-14 (^{14}C) is a naturally occurring but rare isotope of carbon that has eight neutrons instead of six, hence the atomic mass of 14. Carbon-13 (^{13}C) is a rather more common isotope, making up around 1 per cent of naturally occurring carbon; it has seven neutrons per atomic nucleus. The *atomic mass* (or atomic weight) of an element is the average of the mass numbers of an element's different isotopes, taking into account the proportions in which they occur. (Box 2.1 shows how atomic weight is used to quantify amounts of compounds using moles.) Carbon-12 is by far the predominant form of the element in nature, but the existence of small amounts of the other forms means that the atomic mass is 12.011. Some isotopes are stable, while others decay spontaneously, with the release of subatomic particles. The latter are called *radioisotopes*; ^{14}C is a radioisotope, while the other two forms of carbon are stable isotopes. Radioisotopes have been an extremely useful research tool in a number of areas of molecular biology.

The electrons that orbit around the nucleus do not do so randomly, but are arranged in a series of electron shells, radiating out from the nucleus (Figure 2.1). These layers correspond to different energy levels, with the highest energy levels being located furthest away from the nucleus. Each shell can accommodate a maximum number of electrons, and electrons always fill up the shells starting at the innermost one, that is, the one with the lowest energy level. In our example, carbon has filled the first shell with two electrons, and occupied four of the eight available spaces on the second.

The chemical properties of atoms are determined by the number of electrons in the outermost occupied shell. Neon, one of the 'noble' gases, has an atomic number of 10, completely filling the first two shells, and is chemically unreactive or *inert*. Atoms that do not achieve a similar configuration are unstable, or *reactive*. Reactions take place between atoms that attempt to achieve stability by attaining a full outer shell. These reactions may involve atoms of the same element or ones of different elements; the result in either case is a *molecule* or *ion* (see below). Figure 2.2 shows how atoms combine to form a molecule. A substance made up of molecules containing two or more different elements is called a *compound*. In each example, the product of the reaction has a full outer electron shell; note that some atoms are donating electrons, while others are accepting them.

The number of unfilled spaces in the outermost electron shell determines the reactivity of an atom. If most of the spaces in the outermost shell are full, or if most are empty, atoms tend to strive for stability by gaining or losing electrons, as shown in Figure 2.3.

Box 2.1 How heavy is a mole?

When you work in a laboratory, something you'll need to come to grips with sooner or later is the matter of quantifying the amounts and concentrations of substances used. Central to this is the *mole*, so before we go any further, let's define this:

A mole is the molecular mass of a compound expressed in grams.

(The *molecular mass* is simply the sum of the atomic mass of all the atoms in a compound.)

So, to take sodium chloride as an example:

Molecular formula	=	NaCl (one atom each of sodium and chlorine)
Atomic mass of sodium	=	22.99
Atomic mass of chlorine	=	35.45
∴ Molecular mass	=	58.44

Thus *one mole of sodium chloride equals 58.44 grams (58.44 g)*

Concentrations are expressed in terms of mass per volume, so here we introduce the idea of the *molar solution*. This is a solution containing one mole dissolved in a final volume of 1 litre of an appropriate solvent (usually water).

Molar solution = one mole per litre

A one molar (1 M) solution of sodium chloride therefore contains 58.44 g dissolved in water and made up to 1 litre. A 2 M solution would contain 116.88 g in a litre, and so on.

In biological systems, a molar solution of anything is actually rather concentrated, so we tend to deal in solutions which are so many millimolar (mM, one thousandth of a mole per litre) or micromolar (μM, one millionth of a mole per litre).

Why bother with moles?

So far, so good, but why can't we just deal in grams, or grams per litre? Consider the following example. You've been let loose in the laboratory, and been asked to compare the effects of supplementing the growth medium of a bacterial culture with several different amino acids. 'Easy', you think. 'Add X milligrams of each to the normal growth medium, and see which stimulates growth the most'. The problem is that although you may be adding the same *weight* of each amino acid, you're not adding the same number of *molecules*, because each has a different molecular mass. If you add the same number of moles (or millimoles or micromoles) of each instead, you would be comparing the effect of the same number of molecules of each, and thus obtain a much more meaningful comparison. This is because *1 mole of one compound contains the same number of molecules as a mole of any other compound*. This number is called *Avogadro's Number*, and is 6.023×10^{23} molecules per mole.

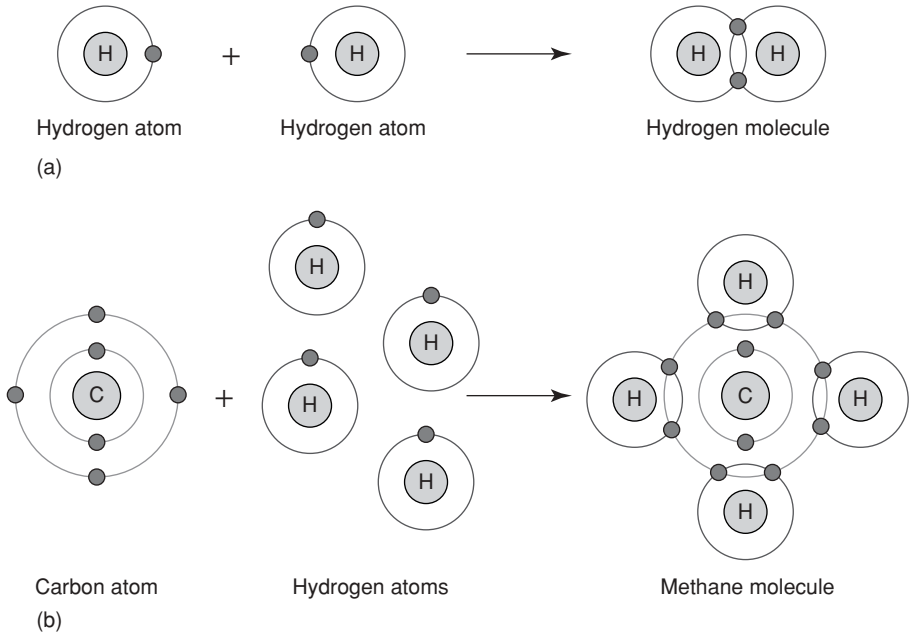


Figure 2.2 The formation of molecules of (a) hydrogen and (b) methane by covalent bonding. Each atom achieves a full set of electrons in its outer shell by sharing with another atom. A shared pair of electrons constitutes a covalent bond

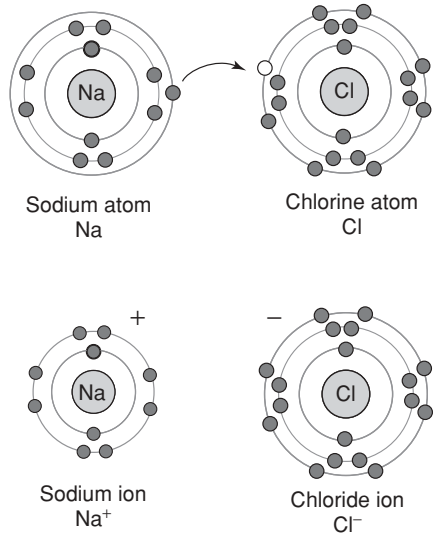


Figure 2.3 Ion formation. Sodium achieves stability by losing the lone electron from its outermost shell. The resulting sodium ion Na^+ has 11 protons and 10 electrons, hence it carries a single positive charge. Chlorine becomes ionised to chloride (Cl^-) when it gains an electron to complete its outer shell

When this happens, an ion is formed, which carries either a positive or negative charge. Positively charged ions are called *cations* and negatively charged ones *anions*. The sodium atom for example has 11 electrons, meaning that the inner two electron shells are filled and a lone electron occupies the third shell. If it were to lose this last electron, it would have more protons than electrons, and therefore have a net positive charge of one; if this happened, it would become a sodium ion, Na^+ (Figure 2.3).

Chemical bonds

The force that causes two or more atoms to join together is known as a *chemical bond*, and several types are found in biological systems. The interaction between sodium and chloride ions shown in Figure 2.4 is an example of *ionic* bonding, where the transfer of an electron from one party to another means that both achieve a complete outer electron shell. There is an attractive force between positively and negatively charged ions, called an *ionic bond*. Certain elements form ions with more than a single charge, by gaining or losing two or more electrons in order to achieve a full outer electron shell; thus calcium ions (Ca^{2+}) are formed by the loss of two electrons from a calcium atom.

The goal of stability through a full complement of outer shell electrons may also be achieved by means of sharing one or more pairs of electrons. Consider the formation of water (Figure 2.2); an oxygen atom, which has two spaces in its outer shell, can achieve a full complement by sharing electrons from two separate hydrogen atoms. This type of bond is a *covalent bond*.

Sometimes, a pair of atoms share not one but two pairs of electrons (see Figure 2.5). This involves the formation of a double bond. Triple bonding, through the sharing of three pairs of electrons, is also possible, but rare.

In the examples of covalent bonding we've looked at so far, the sharing of the electrons has been equal, but this is not always the case because sometimes the electrons may be drawn closer to one atom than another (Figure 2.6a). This has the effect of making one atom slightly negative and another slightly positive. Molecules like this are called *polar* molecules and the bonds are polar bonds. Sometimes a large molecule may have both polar and non-polar areas.

Polar molecules are attracted to each other, the negative areas of one molecule and the positive areas of another acting as magnets for one another (Figure 2.6b). In water,

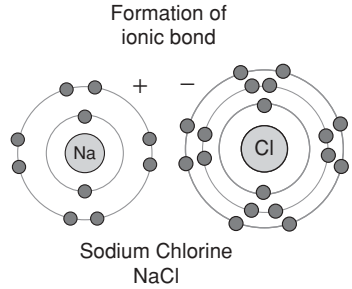


Figure 2.4 A positively charged Na^+ and negatively charged Cl^- attract each other, and an ionic bond is formed. The result is a molecule of sodium chloride

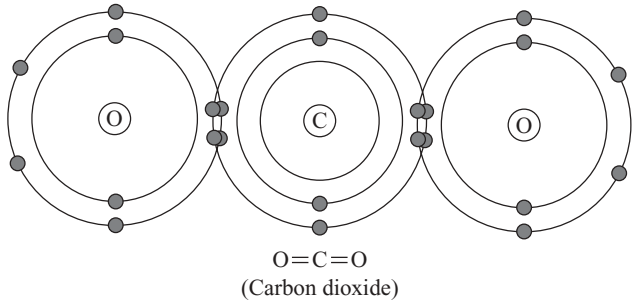


Figure 2.5 Double bond formation. In the formation of carbon dioxide, the carbon atom shares *two* pairs of electrons with each oxygen atom

hydrogen atoms bearing a positive charge are drawn to the negatively charged oxygens. You only have to look at raindrops on a window pane fusing together to see how this bonding is reflected in the physical properties of the compound.

This attraction between polar atoms is called *hydrogen bonding*, and can take place between covalently bonded hydrogen and any electronegative atom, most commonly oxygen or nitrogen. Hydrogen bonds are much weaker than either ionic or covalent

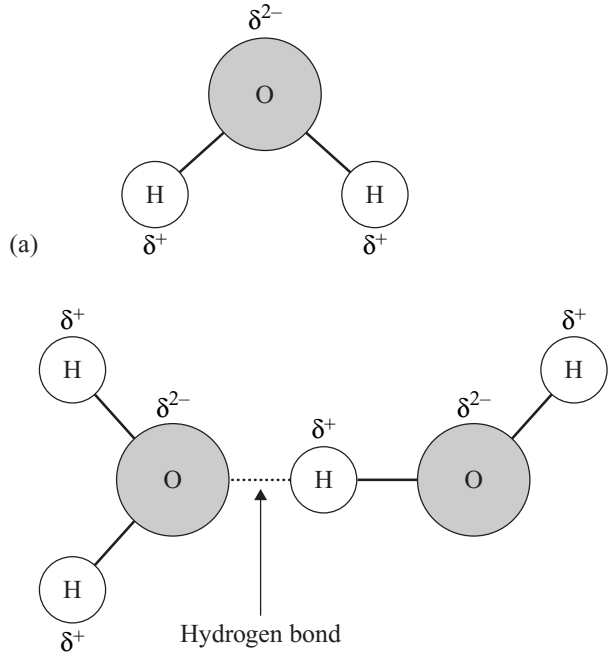


Figure 2.6 (a) The electrons of the hydrogen atoms are strongly attracted to the oxygen atom, causing this part of the water molecule to carry a slightly negative charge, and the hydrogen part a slightly positive one. (b) Because of their polar nature, water molecules are attracted to each other by hydrogen bonding. Hydrogen bonding is much weaker than ionic or covalent bonding, but plays an important role in the structure of macromolecules such as proteins and nucleic acids

bonds; however, if sufficient of them form in a compound the overall bonding force can be appreciable. Each water molecule can form hydrogen bonds with others of its kind in four places (Figure 2.6b). In order to break all these bonds, a large input of energy is required, explaining why water has such a relatively high boiling point, and why most of the water on the planet is in liquid form.

Another weak form of interaction is brought about by Van der Waals forces, which occur briefly when two non-polar molecules (or parts of molecules) come into very close contact with one another. Although transient, and generally even weaker than hydrogen bonds, they occur in great numbers in certain macromolecules and play an important role in holding proteins together (see below).

Water is essential for living things, both in the composition of their cells and in the environment surrounding them. Organisms are made up of between 60 and 95 per cent water by weight, and even inert, dormant forms like spores and seeds have a significant water component. This dependence on water is a function of its unique properties, which in turn derive from its polar nature.

Water is the medium in which most biochemical reactions take place; it is a highly efficient *solvent*, indeed more substances will dissolve in water than in any other solvent. Substances held together by ionic bonds tend to dissociate into anions and cations in water, because as individual solute molecules become surrounded by molecules of water, *hydration shells* are formed, in which the negatively charged parts of the solute attract the positive region of the water molecule, and the positive parts the negative region (Figure 2.7). The attractive forces that allow the solute to dissolve are called *hydrophilic* forces, and substances which are water-soluble are hydrophilic (water-loving). Other polar substances such as sugars and proteins are also soluble in water by forming hydrophilic interactions.

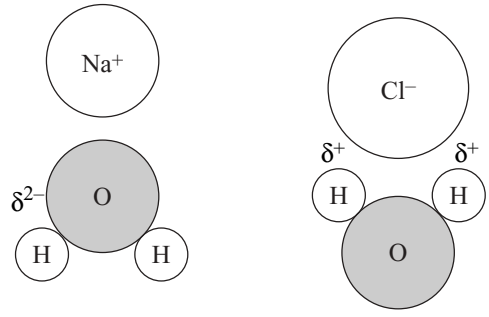


Figure 2.7 An ionic compound such as sodium chloride dissociates in water to its constituent ions. Water molecules form hydration shells around both Na^+ and Cl^- ions

Molecules such as oils and fats are non-polar, and because of their non-reactivity with water are termed *hydrophobic* ('water-fearing'). If such a molecule is mixed with water, it will be excluded, as water molecules 'stick together'. This very exclusion by water can act as a cohesive force among hydrophobic molecules (or hydrophobic areas of large molecules). This is often called hydrophobic bonding, but is not really bonding as such, rather a shared avoidance of water. All living cells have a hydrophilic interior surrounded by a hydrophobic membrane, as we will see in Chapter 3.

An *amphipathic* substance is one which is part polar and part non-polar. When such a substance is mixed with water, *micelles* are formed (Figure 2.8); the non-polar

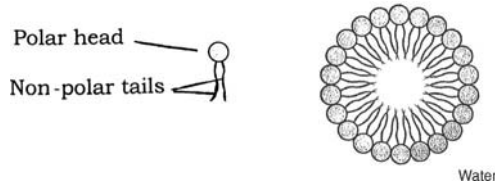


Figure 2.8 In an aqueous environment, amphipathic substances align their molecules so that the non-polar parts are hidden away from the water. From Black, JG: Microbiology: Principles and Explorations, 4th edn, John Wiley & Sons Inc., 1999. Reproduced by permission of the publishers

parts are excluded by the water and group together as described above, leaving the polar groups pointing outwards into the water, where they are attracted by hydrophilic forces. Detergents exert their action by trapping insoluble grease inside the centre of a micelle, while interaction with water allows them to be rinsed away.

Water takes part in many essential metabolic reactions, and its polar nature allows for the breakdown to hydrogen and hydroxyl ions (H^+ and OH^-), and re-synthesis as water. Water acts as a reactant in hydrolysis reactions such as:

$$A—B + H_2O \rightarrow A—H + B—OH$$

and as a product in certain synthetic reactions, such as:

$$A—H + B—OH \rightarrow A—B + H_2O$$

Acids, bases, and pH

Only a minute proportion of water molecules, something like one in every 5×10^8, is present in its dissociated form, but as we have already seen, the H^+ and OH^- ions play an important part in cellular reactions. A solution becomes acid or alkaline if there is an imbalance in the amount of these ions present. If there is an excess of H^+, the solution becomes *acid*, whilst if OH^- predominates, it becomes *alkaline*. The *pH* of a solution is an expression of the molar concentration of hydrogen ions:

$$pH = -\log_{10}[H^+]$$

In pure water, hydrogen ions are present at a concentration of 10^{-7}M, thus the pH is 7.0. This is called neutrality, where the solution is neither acid or alkaline. At higher concentrations of H^+, such as 10^{-3} M (1 millimolar), the pH value is lower, in this case 3.0, so acid solutions have a value below 7. Conversely, alkaline solutions have a pH above 7. You will see from this example that an increase of 10^4 (10 000)-fold in the $[H^+]$ leads to a change of only four points on the pH scale. This is because it is a logarithmic scale; thus a solution of pH 10 is 10 times more alkaline than one of pH 9, and 100 times more than one of pH 8. Figure 2.9 shows the pH value of a number of familiar substances.

Most microorganisms live in an aqueous environment, and the pH of this is very important. Most will only tolerate a small range of pH, and the majority occupy a range around neutrality, although as we shall see later on in this book, there are some

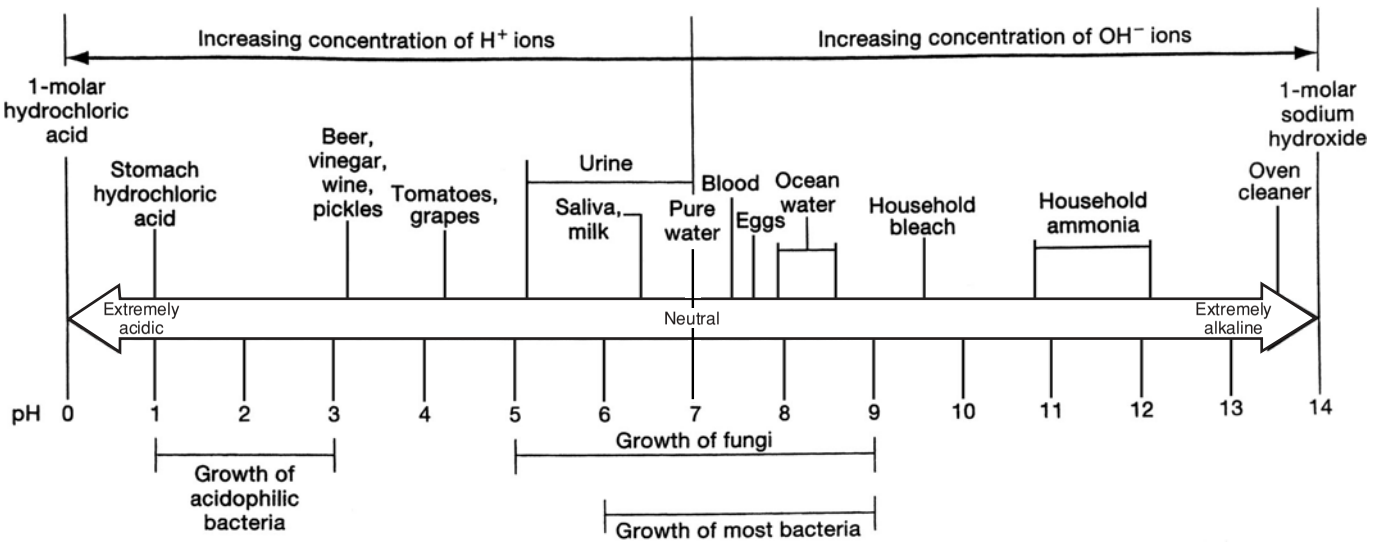
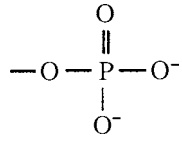


Figure 2.9 The pH value of some common substances. Most microorganisms exist at pH values around neutrality, but representatives are found at extremes of both acidity and alkality. From Black, JG: Microbiology: Principles and Explorations, 4th edn, John Wiley & Sons Inc., 1999. Reproduced by permission of the publishers

Table 2.4 Occurrence and characteristics of some functional groups

Functional Group	Formula	Type of molecule	Found in:	Remarks
Hydroxyl	-OH	Alcohols	Sugars	Polar group, making organic molecules more water soluble
Carbonyl		Aldehydes	Sugars	Carbonyl at end of chain
		Ketones	Sugars	Carbonyl elsewhere in chain
Carboxyl	-COOH	Carboxylic acids	Sugars, fats, amino acids	
Amino	$-NH_2$	Amines	Amino acids, proteins	Can gain H^+ to become NH_3^+
Sulphhydryl	-SH	Thiols	Amino acids, proteins	Oxidises to give S=S bonds
Phosphate			Phospholipids nucleic acids	Involved in energy transfer

startling exceptions to this. Most of the important molecules involved in the chemistry of living cells are organic, that is, they are based on a skeleton of covalently linked carbon atoms. Biological molecules have one or more *functional groups* attached to this skeleton; these are groupings of atoms with distinctive reactive properties, and are responsible for many of the chemical properties of the organic molecule. The possession of a functional group(s) frequently makes an organic molecule more polar and therefore more soluble in water.

Some of the most common functional groups are shown in Table 2.4. It can be seen that the functional groups occur in simpler organic molecules as well as in the macromolecules we consider below.

Biomacromolecules

Many of the most important molecules in biological systems are *polymers*, that is, large molecules made up of smaller subunits joined together by covalent bonds, and in some cases in a specific order.

Carbohydrates

Carbohydrates are made up of just three different elements, carbon, hydrogen and oxygen. The simplest carbohydrates are *monosaccharides*, or simple sugars; these

The suffix -ose always denotes a carbohydrate.

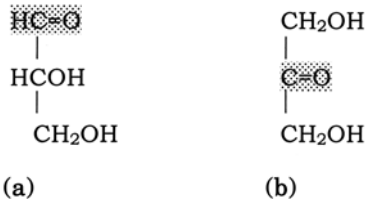


Figure 2.10 Monosaccharides may be aldoses or ketoses. The three carbon sugars (a) glyceraldehyde and (b) dihydroxyacetone share the same molecular formula, but have different functional groups. The two molecules are isomers (see Box 2.2)

have the general formula $(CH_2O)_n$. They are classed as either aldoses or ketoses, according to whether they contain an aldehyde group or a ketone group (Figure 2.10). Monosaccharides can further be classified on the basis of the number of carbon atoms they contain. The simplest are trioses (three carbons) and the most important biologically are hexoses (six carbons) (see Boxes 2.2 and 2.3).

Monosaccharides are generally crystalline solids which are soluble in water and have a sweet taste. They all reducing sugars, so called because they are able to reduce alkaline solutions of cupric ions (Cu^{2+}) to cuprous ions (Cu^{+}).

A *disaccharide* is formed when two monosaccharides (which may be of the same type or different), join together with a concomitant loss of a water molecule (Figure 2.11). Further monosaccharides can be added, giving chains of three, four, five

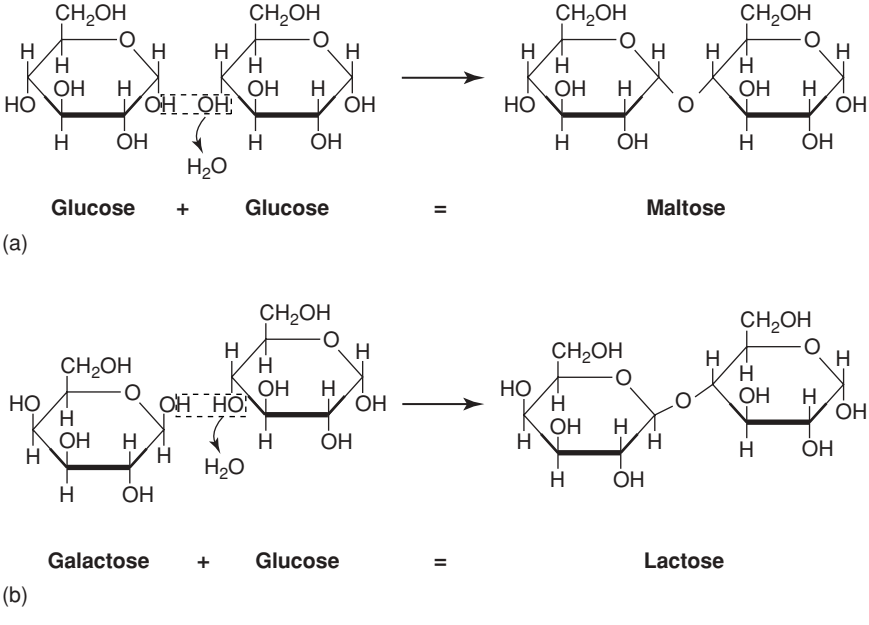
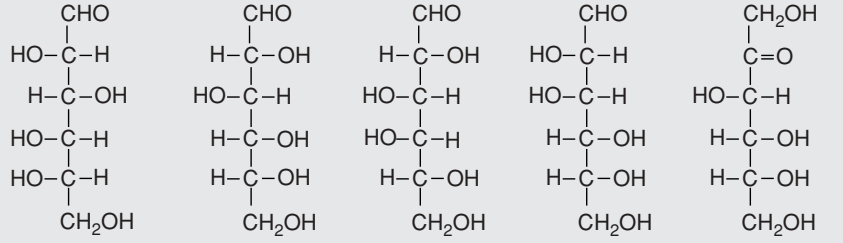


Figure 2.11 Monosaccharides such as two glucose molecules can be joined by a glycosidic linkage to form a disaccharide. The reaction is a condensation reaction, in which a molecule of water is lost. α- (a) and β-linkages (b) result in different orientations in space

Box 2.2 Isomers: same formula, different structure

The simplest monosaccharides are the trioses glyceraldehyde and dihydroxyacetone (Figure 2.10). Look carefully at the structures, and you will see that although they both share the same number of carbons (3), hydrogens(6) and oxygens (3), the way in which these atoms are arranged is different in the two sugars. Molecules such as these, which have the same chemical formula but different structural formulas, are said to be structural isomers. The different groupings of atoms lead to structural isomers having different chemical properties. When we come to look at the hexoses (six carbon sugars), we see that there are many structural possibilities for the general formula $C_6H_{12}O_6$; some of these are shown below.



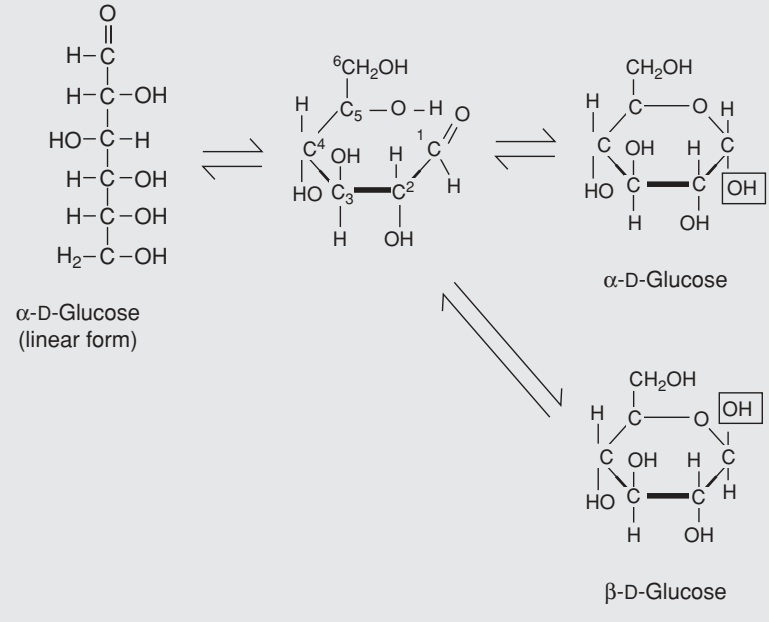
Note that some of these structures are identical apart from the orientation of groups around the central axis; D-glucose and L-glucose for example differ only in the way H atoms and -OH groups are arranged to the right or left. They are said to be *stereoisomers* or optical isomers, and are mirror images of each other, just like your right and left hands. (D- and L- are short for *dextro-* and *laevo*-rotatory, meaning that the plane of polarised light is turned to the right and left respectively when passed through a solution of these substances). Generally, living cells will only synthesise one or other stereoisomer, and not both.

or more units. These are termed *oligosaccharides* (*oligo*, a few), and chains with many units are *polysaccharides*. The chemical bond joining the monosaccharide units together is called a *glycosidic linkage*. The bond between the two glucose molecules that make up maltose is called an α-glycosidic linkage; in lactose, formed from one glucose and one galactose, we have a β-glycosidic linkage. The two bonds are formed in the same way, with the elimination of water, but they have a different orientation in space. Thus disaccharides bound together by α- and β-glycosidic linkages have a different overall shape and as a result the molecules behave differently in cellular metabolism.

Biologically important molecules such as starch, cellulose and glycogen are all polysaccharides. Another is dextran, a sticky substance produced by some bacteria to aid their adhesion. They differ from monosaccharides in being generally insoluble in water, not tasting sweet and not being able to reduce cupric ions. Most polysaccharides

Box 2.3 Sugars are more accurately shown as ring structures

When dissolved in water, the aldehyde or ketone group reacts with a hydroxyl group on the fifth carbon to give a cyclic form. D-Glucose is shown in both forms below. The cyclic form of the molecule is shown below as a *Haworth projection*. The idea is that the ring is orientated at 90° to the page, with the edge which is shown thicker towards you, and the top edge away from you. Notice that there are even two forms of D-Glucose! Depending on whether the -OH on carbon-1 is below or above the plane of the ring, we have α- or β-D-Glucose.



are made up from either pentose or hexose sugars, and, like di- and oligosaccharides, can be broken down into their constituent subunits by hydrolysis reactions.

Proteins

Of the macromolecules commonly found in living systems, proteins are the most versatile, having a wide range of biological functions and this fact is reflected in their structural diversity.

The five elements found in most naturally occurring proteins are carbon, hydrogen, oxygen, nitrogen and sulphur. In addition, other elements may be essential components of certain specialised proteins such as haemoglobin (iron) and casein (phosphorus).

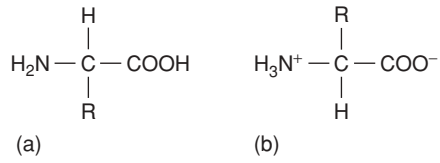


Figure 2.12 Amino acid structure. (a) The basic structure of an amino acid. (b) In solution, the amino and carboxyl groups become ionised, giving rise to a *zwitterion* (a molecule with spatially separated positive and negative charges). All the 20 amino acids commonly found in proteins are based on a common structure, differing only in the nature of their 'R' group (see Figure 2.13)

Proteins can be very large molecules, with molecular weights of tens or hundreds of thousands. Whatever their size, and in spite of the diversity referred to above, all proteins are made up of a collection of 'building bricks' called *amino acids* joined together. Amino acids are thought to have been among the first organic molecules formed in the early history of the Earth, and many different types exist in nature. All these, including the 20 commonly found occurring in proteins, are based on a common structure, shown in Figure 2.12. It comprises a central carbon atom (known as the α-carbon) covalently bonded to an amino (NH_2) group, a carboxyl (COOH) group and a hydrogen atom. It is the group attached to the final valency bond of the α-carbon which varies from one amino acid to another; this is known as the *'R'-group*.

The 20 amino acids found in proteins can be conveniently divided into five groups, on the basis of the chemical nature of their 'R'-group. These range from a single hydrogen atom to a variety of quite complex side chains (Figure 2.13). It is unlikely nowadays that you would need to memorise the precise structure of all 20, as the author was asked to do in days gone by, but it would be advisable to familiarise yourself with the groupings and examples from each of them. The groups differentiate on the basis of a polar/non-polar nature and on the presence or absence of an ionisable 'R'-group. Box 2.4 shows how we normally refer to proteins in shorthand.

Note that one amino acid, proline, falls outside the main groups. This differs from the others in that it has one of its N—H linkages replaced by an N—C, which forms part of a cyclic structure (Figure 2.13). This puts certain conformational constraints upon proteins containing proline residues.

As can be seen from Figure 2.13, the simplest amino acid is glycine, whose R-group is simply a hydrogen atom. This means that the glycine molecule is symmetrical, with a hydrogen atom on opposite valency bonds. All the other amino acids however, are asymmetrical. The α-carbon acts as what is known as a chiral centre, giving the molecule right or left 'handedness'. Thus two stereoisomers known as the D- and L-forms are possible for each of the amino acids except glycine. All the amino acids found in naturally occurring proteins have the L-form; the D-form also occurs in nature but only in certain specific, non-protein contexts.

Proteins, as we've seen, are polymers of amino acids. Amino acids are joined together by means of a *peptide bond*. This involves the -NH_2 group of one amino acid and the -COOH group of another. The formation of a peptide bind is a form of condensation reaction in which water is lost (Figure 2.14). The resulting structure of two linked amino

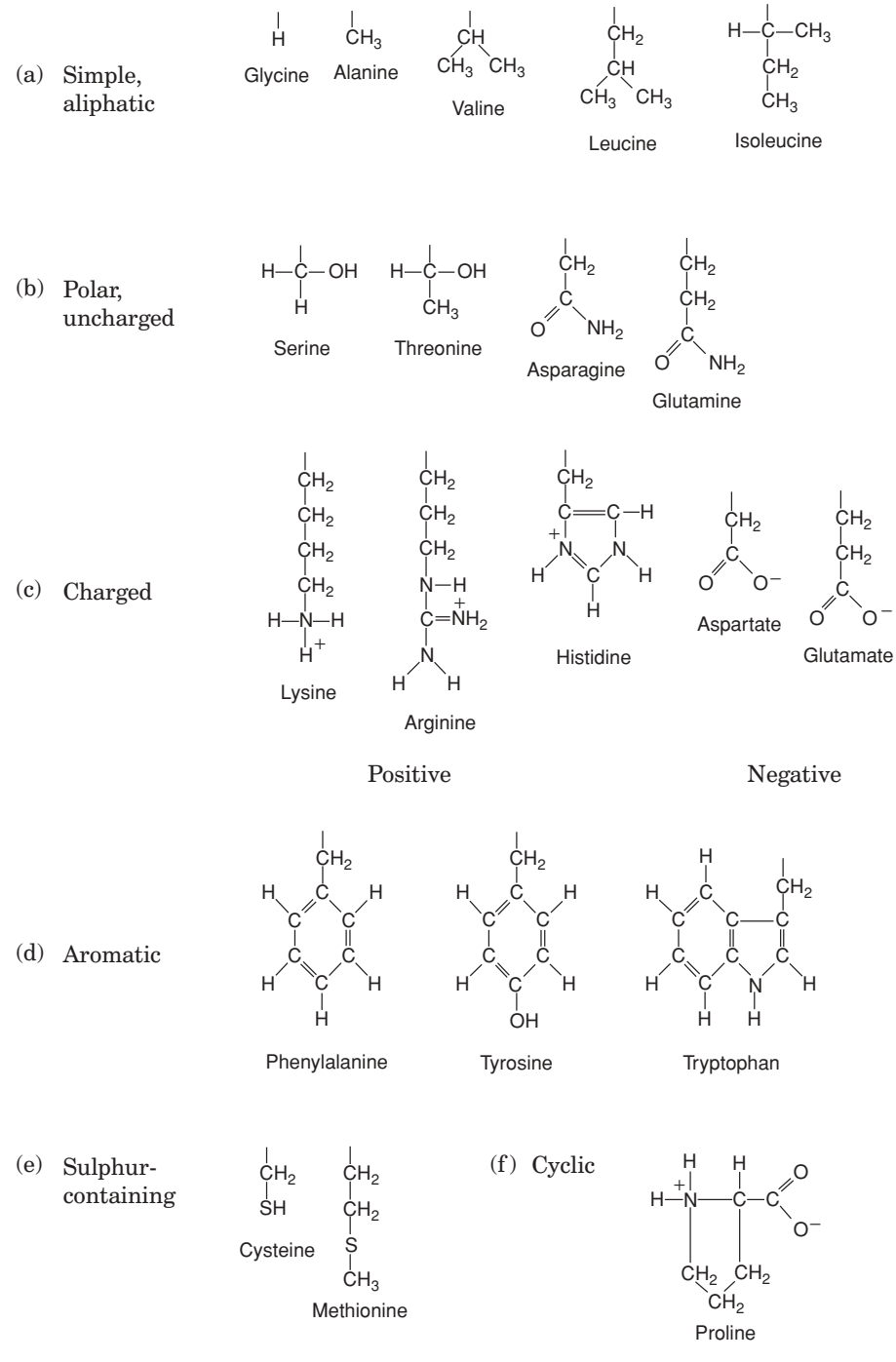


Figure 2.13 The 20 amino acids found in proteins. The 'R' group of each amino acid is shown. These range from the simplest, glycine, to more complex representatives such as tryptophan

Box 2.4 Amino acid shorthand

It is sometimes necessary to express in print the sequence of amino acids which make up the primary structure of a particular protein; clearly it would be desperately tedious to express a sequence of hundreds of bases in the form 'glycine, phenylalanine, tryptophan, methionine… etc', so a system of abbreviations for each amino acid has been agreed. Each amino acid can be reduced to a three letter code, thus you might see something like:

1	2	3	4	5	6	7	8	9	10	11
Gly	Phe	Try	Met	His	Lys	Gly	Ala	His	Val	Glu….and so on.

Note that each residue has a number; this *numbering always begins at the N-terminus*.

Each amino acid can also be represented by a single letter. The abbreviations using the two systems are shown below.

A	Ala	Alanine	M	Met	Methionine
B	Asx	Asparagine/aspartic acid	N	Asn	Asparagine
C	Cys	Cysteine	P	Pro	Proline
D	Asp	Aspartic acid	Q	Gln	Glutamine
E	Glu	Glutamic acid	R	Arg	Arginine
F	Phe	Phenylalanine	S	Ser	Serine
G	Gly	Glycine	T	Thr	Threonine
H	His	Histidine	V	Val	Valine
I	Ile	Isoleucine	W	Trp	Tryptophan
K	Lys	Lysine	Y	Tyr	Tyrosine
L	Leu	Leucine	Z	Glx	Glutamine/glutamic acid

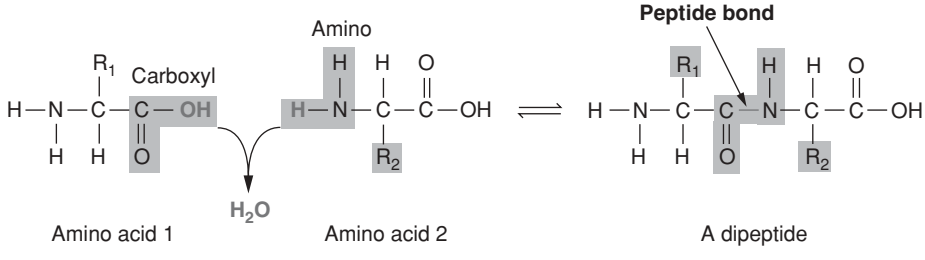


Figure 2.14 The carboxyl group of one amino acid is joined to the amino group of another. This is another example of a condensation reaction (c.f. Figure 2.11). No matter how many amino acids are added, the resulting structure always has a free carboxyl group at one end and a free amino group at the other

acids is called a *dipeptide*; note that this structure still retains an $-NH_2$ at one end and a -COOH at the other. If we were to add on another amino acid to form a tripeptide, this would still be so, and if we kept on adding them until we had a *polypeptide*, we would still have the same two groupings at the extremities of the molecule. These are referred to as the N-terminus and the C-terminus of the polypeptide. Since a water molecule has been removed at the formation of each peptide bond, we refer to the chain so formed as being composed of amino acid residues, rather than amino acids. The actual distinction between a protein and a polypeptide based on the number of amino acid residues is not clear-cut; generally, with over 100, we refer to proteins, but some naturally occurring proteins are a lot smaller than this.

So far, we can think of proteins as long chains of many amino acid residues, rather like a string of beads. This is called the *primary structure* of the protein; it is determined by the relative proportions of each of the 20 amino acids, and the order in which they are joined together. It is the basis of all the remaining levels of structural complexity, and it ultimately determines the properties of a particular protein. It is also what makes one protein different from another. Since the 20 types of amino acid can be linked together in any order, the number of possible sequences is astronomical, and it is this great variety of structural possibilities that gives proteins such diverse structures and functions.

In theory, there are 20^{100} or some 10^{130} different ways in which 20 different amino acids could combine to give a protein 100 amino acid residues in length!

Some parts of the primary sequence are more important than others. If we took a protein of, say, 200 amino acid residues in length, took it apart and reassembled the amino acids in a different order, we would almost certainly alter (and probably lose completely) the properties of that protein. If we look at the primary sequence of a protein molecule which serves essentially the same function in several species, we find that nature has allowed slight alterations to occur during evolution, but these are often conservative substitutions, where an amino acid has been replaced by a similar one (one from the same group in Figure 2.13), and thus have little effect on the protein's properties. In certain parts of the primary sequence, such substitutions are less well tolerated, for example the few residues that make up the active site of an enzyme (see Chapter 6). In cases such as the one above, alterations have not been allowed at these points in the primary sequence, and the sequence is the same, or almost so, in all species possessing that protein. The sequence in question is said to have been conserved.

Higher levels of protein structure

The structure of proteins is a good deal more complicated than a just a linear chain of amino acids. A long thin chain is unlikely to be very stable; proteins therefore undergo a process of folding which makes the molecule more stable and compact. The results of this folding are the secondary and tertiary structures of a protein.

The *secondary structure* is due to hydrogen bonding between a carbonyl (-CO) group and an amido (-NH) group of amino acid residues on the peptide backbone (Figure 2.15). The 'R' group plays no part in secondary protein structure. Two regular patterns of folding result from this; the *α-helix* and the *β-pleated sheet*.

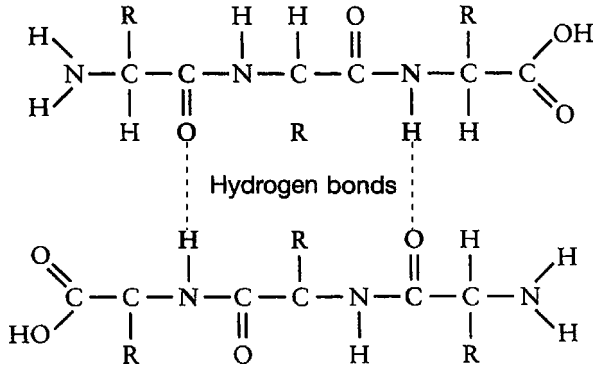


Figure 2.15 Secondary structure in proteins. Hydrogen bonding occurs between the -CO and -NH groups of amino acids on the backbone of a polypeptide chain. The two amino acids may be on the same or different chains

Very small distances within molecules are measured in Angstrom units (A). One Angstrom unit is equal to one tenbillionth (10^{-10}) of a metre.

The α-helix occurs when hydrogen bonding takes place between amino acids close together in the primary structure. A stable helix is formed by the -NH group of an amino acid bonding to the -CO group of the amino acid four residues further along the chain (Figure 2.16a). This causes the chain to twist into the characteristic helical shape. One turn of the helix occurs every 3.6 amino acid residues, and results in a rise of 5.4 Å (0.54 nm); this is called the pitch height of the helix. The ability to form a helix like this is dependent on the component amino acids; if there are too many with large R-groups, or R-groups carrying the same charge, a stable helix will not be formed. Because of its rigid structure, proline (Figure 2.13) cannot be accommodated in an α-helix. Naturally occurring α-helices are always right-handed, that is, the chain of amino acids coils round the central axis in a clockwise direction. This is a much more stable configuration than a left-handed helix, due to the fact that there is less steric hindrance (overlapping of electron clouds) between the R-groups and the C=O group on the peptide backbone. Note that if proteins were made up of the D-form of amino acids, we would have the reverse situation, with a left-handed form favoured. In the β-pleated sheet, the hydrogen bonding occurs between amino acids either on separate polypeptide chains or on residues far apart in the primary structure (Figure 2.16b). The chains in a β-pleated sheet are fully extended, with 3.5 Å (0.35 nm) between adjacent amino acid residues (c.f. α-helix, 1.5 Å). When two or more of these chains lie next to each other, extensive hydrogen bonding occurs between the chains. Adjacent strands in a β-pleated sheet can either run in the same direction (e.g. N→C), giving rise to a parallel β-pleated sheet, or in opposite directions (antiparallel β-pleated sheet, as shown in Figure 2.16b).

A common structural element in the secondary structure of proteins is the β-turn. This occurs when a chain doubles back on itself, such as in an antiparallel β-pleated sheet. The -CO group of one amino acid is hydrogen bonded to the -NH group of the

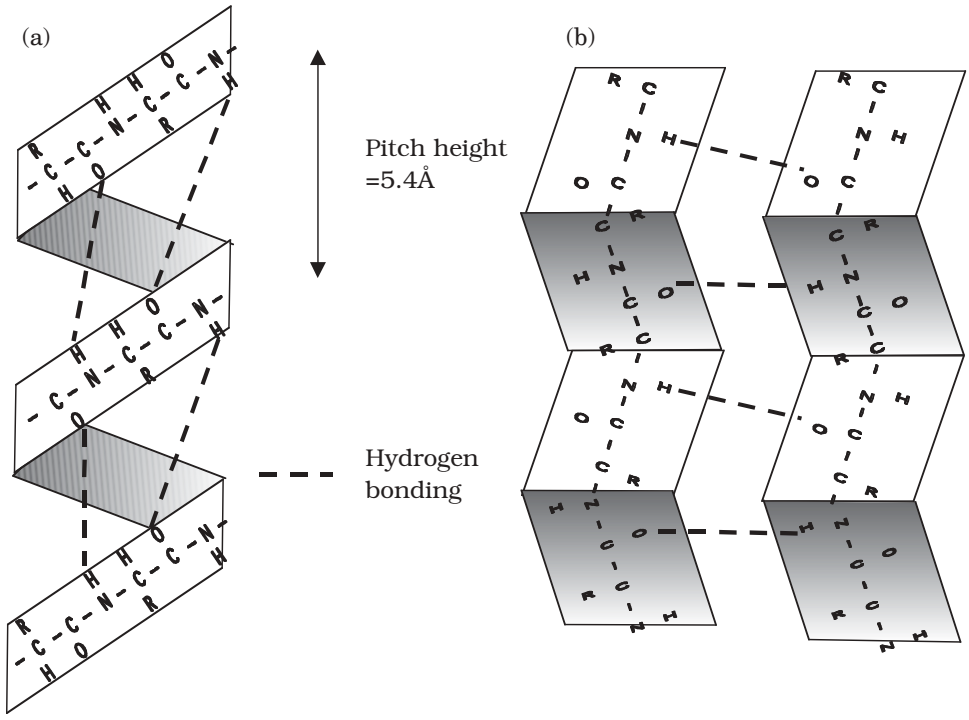


Figure 2.16 Secondary structure in proteins: the α-helix and β-pleated sheet. (a) Hydrogen bonding between amino acids four residues apart in the primary sequence results in the formation of an α-helix. (b) In the β-pleated sheet hydrogen bonding joins adjacent chains. Note how each chain is more fully extended than in the α-helix. In the example shown, the chains run in the same direction (parallel)

residue three further along the chain. Frequently, it is called a hairpin turn, for obvious reasons (Figure 2.17). Numerous changes in direction of the polypeptide chains result in a compact, globular shape to the molecule.

Typically about 50 per cent of a protein's secondary structure will have an irregular form. Although this is often referred to as *random coiling*, it is only random in the sense that there is no regular pattern; it still contributes towards the stability of the molecule. The proportions and combinations in which α-helix, β-pleated sheet and random coiling occur varies from one protein to another. Keratin, a structural protein found in skin, horn and feathers, is an example of a protein entirely made up of α-helix, whilst the lectin (sugar-binding protein) concanavalin A is mostly made up of β-pleated sheets.

The *tertiary structure* of a protein is due to interactions between side chains, that is, R-groups of amino acid residues, resulting in the folding of the molecule to produce a thermodynamically more favourable structure. The structure is formed by a variety of weak, non-covalent forces; these include hydrogen bonding, ionic bonds, hydrophobic interactions, and Van der Waals forces. The strength of these forces diminishes with distance, therefore the formation of a compact structure is encouraged. In addition, the -SH groups on separate cysteine residues can form a covalent -S—S- linkage. This is

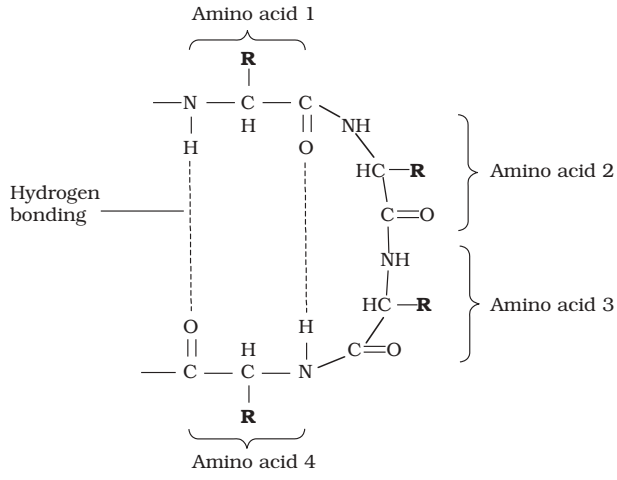


Figure 2.17 The β-turn. The compact folding of many globular proteins is achieved by the polypeptide chain reversing its direction in one or more places. A common way of doing this is with the β-turn. Hydrogen bonding between amino acid residues on the same polypeptide stabilizes the structure

known as a *disulphide bridge* and may have the effect of bringing together two cysteine residues that were far apart in the primary sequence (Figure 2.18).

In globular proteins, the R-groups are distributed according to their polarities; non-polar residues such as valine and leucine nearly always occur on the inside, away from the aqueous phase, while charged, polar residues including glutamic acid and histidine generally occur at the surface, in contact with the water.

The protein can be *denatured* by heating or treatment with certain chemicals; this causes the tertiary structure to break down and the molecule to unfold, resulting in a loss of the protein's biological properties. Cooling, or removal of the chemical agents, will lead to a restoration of both the tertiary structure and biological activity, showing that both are entirely dependent on the primary sequence of amino acids.

Complex molecules such as globular proteins become denatured when their three-dimensional structure is disrupted, leading to a loss of biological function.

Even the tertiary structure is not always the last level of organisation of a protein, because some are made up of two or more polypeptide chains, each with its own secondary and tertiary structure, combined together to give the *quaternary structure* (Figure 2.19). These chains may be identical or different, depending on the protein. Like the tertiary structure, non-covalent forces between R-groups are responsible, the difference being that this time they link amino acid residues on separate chains rather than on the same one.

Such proteins lose their functional properties if dissociated into their constituent units; the quaternary joining is essential for their activity. Phosphorylase A, an enzyme involved in carbohydrate metabolism, is an example of a protein with a quaternary structure. It has four subunits, which have no catalytic activity unless joined together as a tetramer.

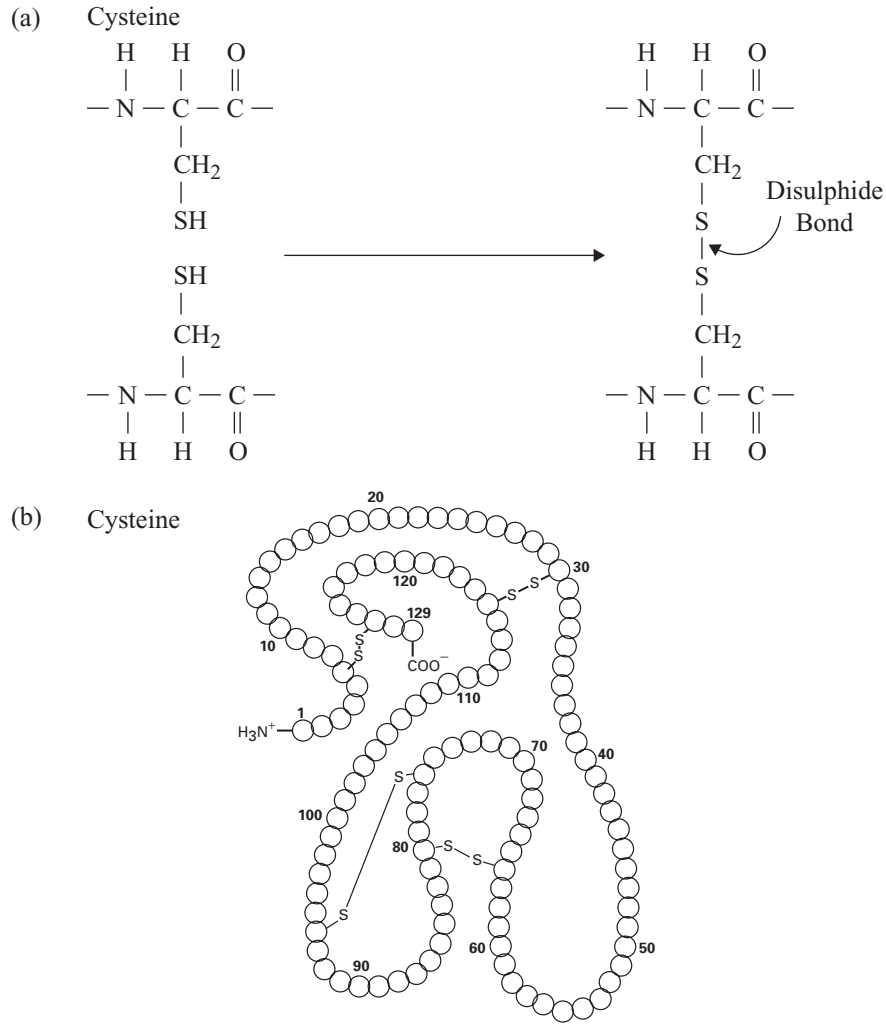


Figure 2.18 Disulphide bond formation. (a) Disulphide bonds formed by the oxidation of cysteine residues result in cross-linking of a polypeptide chain. (b) This can have the effect of bringing together residues that lie far apart in the primary amino acid sequence. Disulphide bonds are often found in proteins that are exported from the cell, but rarely in intracellular proteins

Although all proteins are polymers of amino acids existing in various levels of structural complexity as we have seen above, some have additional, non-amino acid components. They may be organic, such as sugars (glycoproteins) or lipids (lipoproteins) or inorganic, including metals (metalloproteins) or phosphate groups (phosphoproteins). These components, which form an integral part of the protein's structure, are called *prosthetic groups*.

A prosthetic group is a non-polypeptide component of a protein, such as a metal ion or a carbohydrate

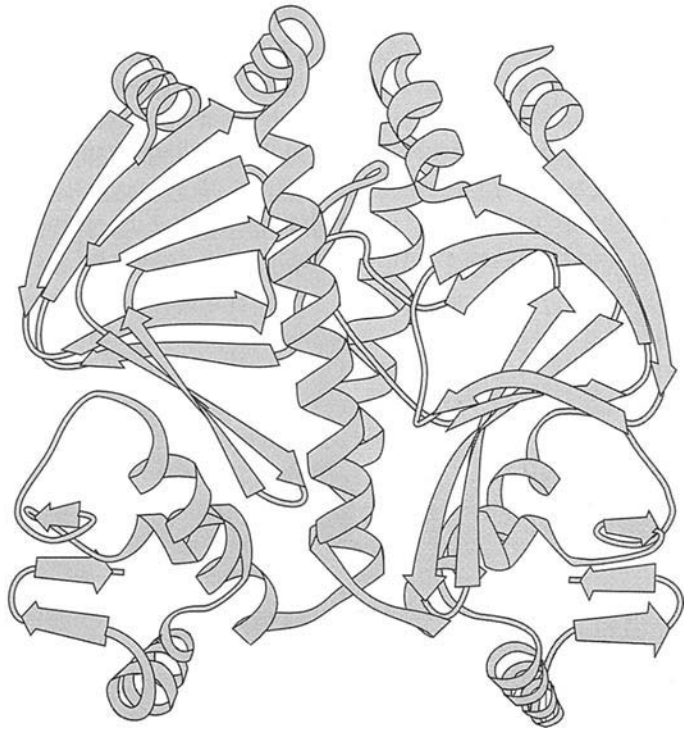


Figure 2.19 Polypeptide chains may join to form quaternary structure. The example shown comprises two identical polypeptide subunits. Coils indicate α-helical sequences, arrows are β-pleated sheets. From Bolsover, SR , Hyams, JS, Jones, S, Shepherd, EA & White, HA: From Genes to Cells, John Wiley & Sons, 1997. Reproduced by permission of the publishers

Nucleic acids

The third class of polymeric macromolecules are the nucleic acids. These are deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), and both are polymers of smaller molecules called nucleotides. As we shall see, there are important differences both in the overall structures of RNA and DNA and in the nucleotides they contain, so we shall consider each of them in turn.

The structure of DNA

The composition of a DNA nucleotide is shown in Figure 2.20(a). It has three parts, a five-carbon sugar called deoxyribose, a phosphate group and a base. This base can be any one of four molecules; as can be seen in Figure 2.21, these are all based on a cyclic structure containing nitrogen. Two of the bases, cytosine and thymine, have a single ring and are called pyrimidines. The other two, guanine and adenine, have a double ring structure; these are the purines. The four bases are often referred to by their initial letter only, thus we have A, C, G and T.

One nucleotide differs from another by the identity of the base it contains; the rest of the molecule (sugar and phosphate) is identical. You will recall from the previous section that the properties of a protein depend on the order in which its constituent amino acids

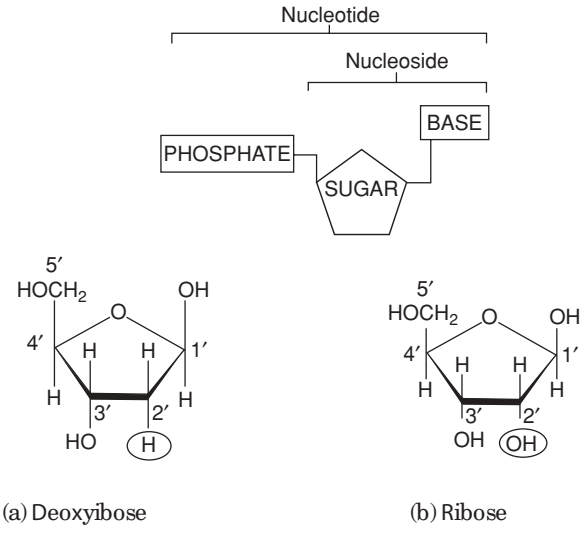


Figure 2.20 A nucleotide comprises a pentose sugar, a phosphate group and a nitrogenous base (see Figure 2.21). Note the difference between the sugars (a) deoxyribose (DNA) and (b) ribose (RNA)

are linked together; we have exactly the same situation with nucleic acids, except that instead of an 'alphabet' of 20 'letters', here we have one of only four. Nevertheless, because nucleic acid molecules are extremely long, and the bases can occur in almost any order, an astronomically large number of different sequences is possible.

The nucleotides join together by means of a *phosphodiester bond*. This links the phosphate group of one base to an -OH group on the 3-carbon of the deoxyribose sugar of another (Figure 2.22). The chain of nucleotides therefore has a free -OH group attached to the 3-carbon (the 3′ end) and a free phosphate group attached to the 5-carbon (the 5′ end). This remains the case however long the chain becomes.

The structure of DNA however is not just a single chain of linked nucleotides, but *two* chains wound around each other to give the *double helix* form made famous by the model of James Watson and Francis Crick in 1953 (Figure 2.23, see also Chapter 11). If we compare this to an open spiral staircase, alternate sugar and phosphate groups make up the 'skeleton' of the staircase, while the inward-facing bases pair up by hydrogen bonding to form the steps. Notice that each nucleotide pair always comprises three rings, resulting from a combination of one purine and one pyrimidine base. This means that the two strands of the helix are always evenly spaced. The way in which the bases pair is further governed by the phenomenon of *complementary base pairing*. A nucleotide containing thymine will only pair with one containing adenine, and likewise guanine always pairs with cytosine (Figure 2.24). Thus, the sequence of

Erwin Chargaff measured the proportions of the different nucleotides in a range of DNA samples. He found that T always = A and C always = G. Watson and Crick interpreted this as meaning that the bases always paired up in this way.

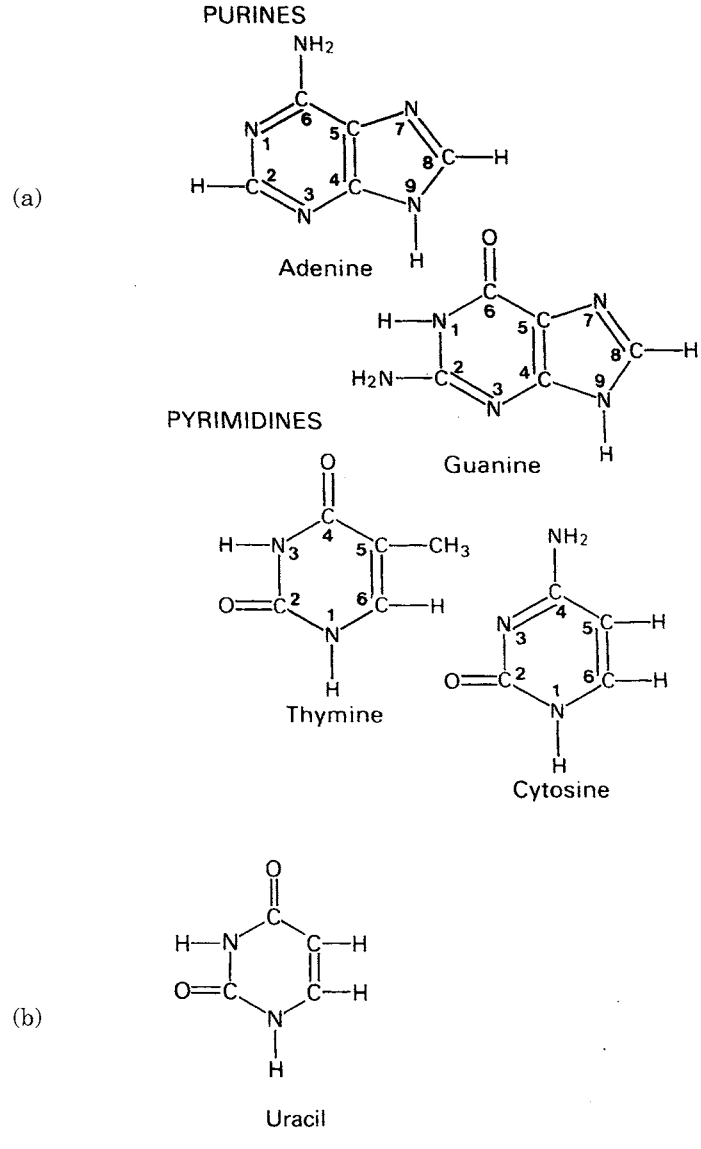


Figure 2.21 Bases belong to two classes. Nucleotides differ from each other in the identity of the nitrogenous base. (a) In DNA these are adenine (A), cytosine (C), guanine (G) or thymine (T). The purines (A and G) have a two-ring structure, while the pyrimidines (C and T) have only one ring. (b) In RNA, thymine is replaced by a similar molecule, uracil (U)

nucleotides on one strand of the double helix determines that of the other, as it has a complementary structure. Figure 2.23 shows how the two strands of the double helix are *antiparallel*, that is they run in opposite directions, one $5' \rightarrow 3'$ and the other $3' \rightarrow 5'$. In Chapter 12 we shall look at how this structure was used to propose a mechanism for the way in which DNA replicates and genetic material is copied.

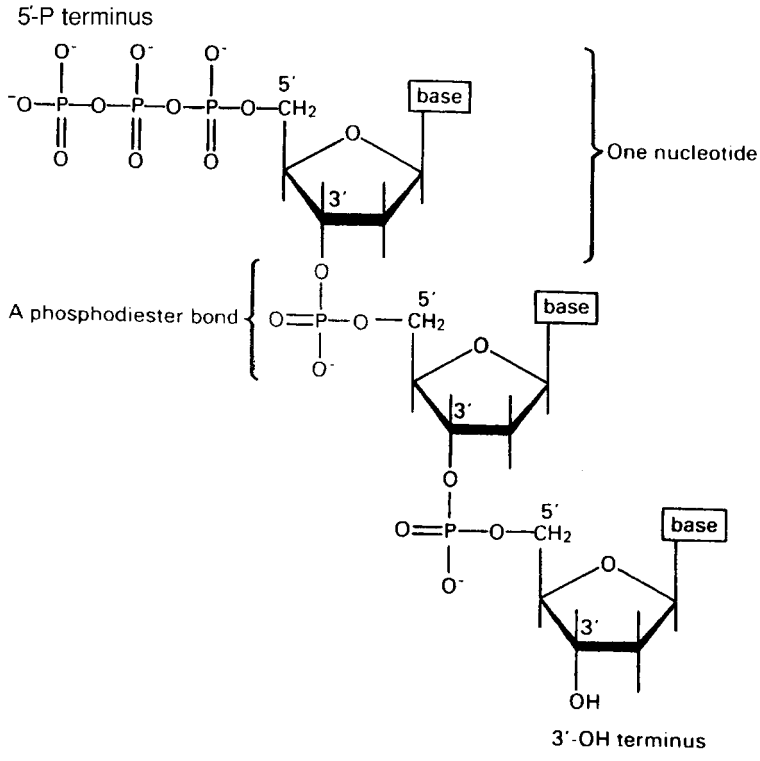
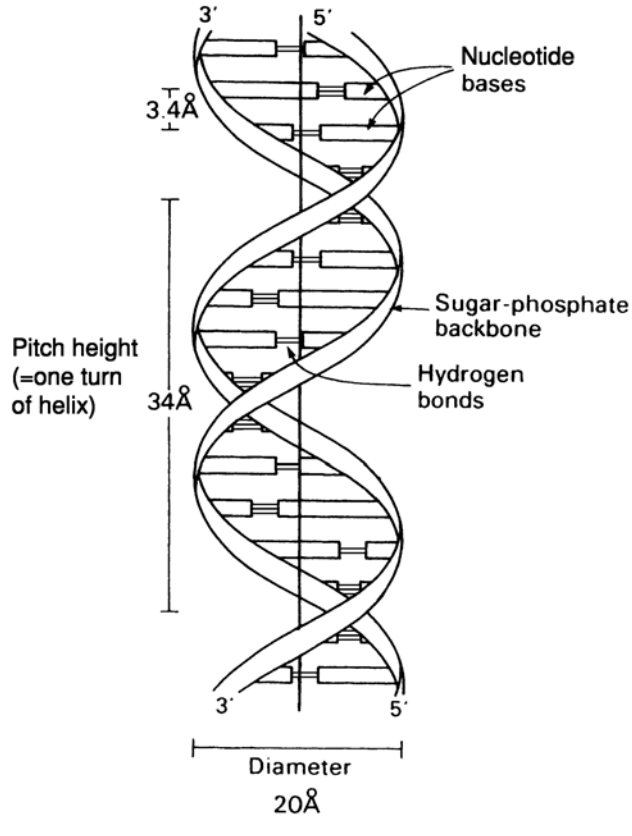


Figure 2.22 The phosphodiester bond. A chain of DNA is made longer by the addition of nucleotides containing not one but three phosphate groups; on joining the chain, two of these phosphates are removed. Nucleotides are joined to each other by a phosphodiester bond, linking the phosphate group on the 5-carbon of one deoxyribose to the -OH group on the 3-carbon of another. (These carbons are known as 5′ and 3′ to distinguish them from the 5- and 3-carbon on the nitrogenous base). Note that the resulting chain, however many nucleotides it may comprise, always has a 5′(PO_4) group at one end and a 3′(OH) group at the other

The structure of RNA

In view of the similarities in the structure of DNA and RNA, we shall confine ourselves here to a consideration of the major differences. There are two important differences in the composition of nucleotides of RNA and DNA. The central sugar molecule is not deoxyribose, but ribose; as shown in Figure 2.20, these differ only in the possession of an -H atom or an -OH group attached to carbon-2. Second, although RNA shares three of DNA's nitrogenous bases (A, C and G), instead of thymine it has uracil. Like thymine, this pairs specifically with adenine.

The final main difference between RNA and DNA is the fact that RNA generally comprises only a single polynucleotide chain, although this may be subject to secondary and tertiary folding as a result of complementary base pairing within the same strand. The roles of the three different forms of RNA will be discussed in Chapter 11.



(Å = Angstrom unit = 10^{-10} metres)

Figure 2.23 The model of DNA proposed by Watson and Crick has two chains of nucleotides joined together by hydrogen-bonded base pairs pointing inwards towards the centre of the helix. The rules of complementary base pairing means that the sequence of one chain can be predicted from the sequence of the other. Note how the chains run in opposite directions (antiparallel)

Lipids

Although lipids can be large molecules, they are not regarded as macromolecules because unlike proteins, polysaccharides and nucleic acids, they are not polymers of a basic subunit. Moreover, lipids do not share any single structural characteristic; they are a diverse group structurally, but have in common the fact that they are *insoluble in water*, but soluble in a range of organic solvents. This non-polar nature is due to the predominance of covalent bonding, mainly between atoms of carbon and hydrogen.

Fats are simple lipids, whose structure is based on *fatty acids* (see Box 2.5). Fatty acids are long hydrocarbon chains ending in a carboxyl (-COOH) group. They have the

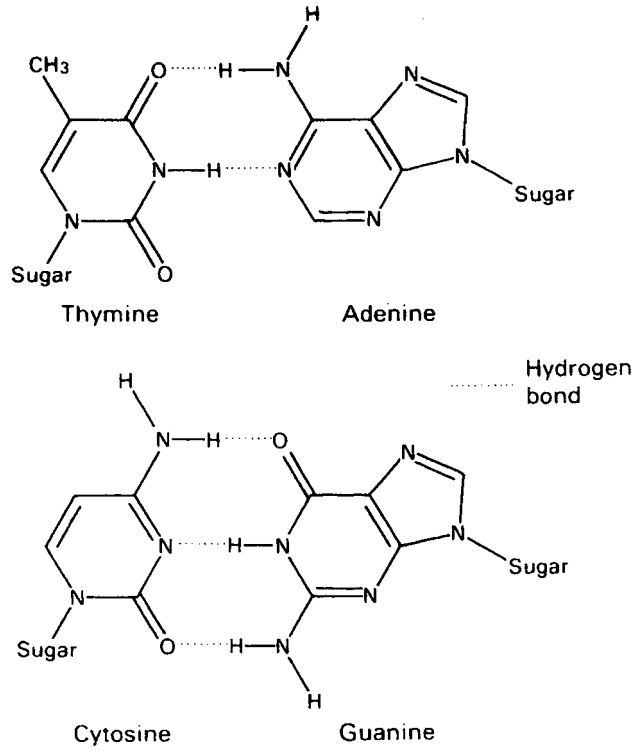


Figure 2.24 Adenine pairs only with thymine, and guanine with cytosine, thus if the sequence of bases in one strand of a DNA molecule is known, that of the other can be predicted. This critical feature of Watson and Crick's model offers an explanation for how DNA is able to replicate itself. Note that GC pairs are held together by three hydrogen bonds, while AT pairs only have two

general formula:

$$CH_3—(CH_2)_n—COOH$$

where *n* is usually an even number. They combine with glycerol according to the basic reaction:

$$\text{Alcohol} + \text{Acid} \rightarrow \text{Ester}$$

The bond so formed is called an ester linkage, and the result is an acylglycerol (Figure 2.25). One, two or all three of the -OH groups may be esterified with a fatty acid, to give respectively mono-, di- and *triacylglycerols*. Natural fats generally contain a mixture of two or three different fatty acids substituted at the three positions; consequently, a considerable diversity is possible among fats. Fats serve as energy stores; a higher proportion of C—C and C—H bonds in comparison with proteins or carbohydrates results in a higher energy-storing capacity.

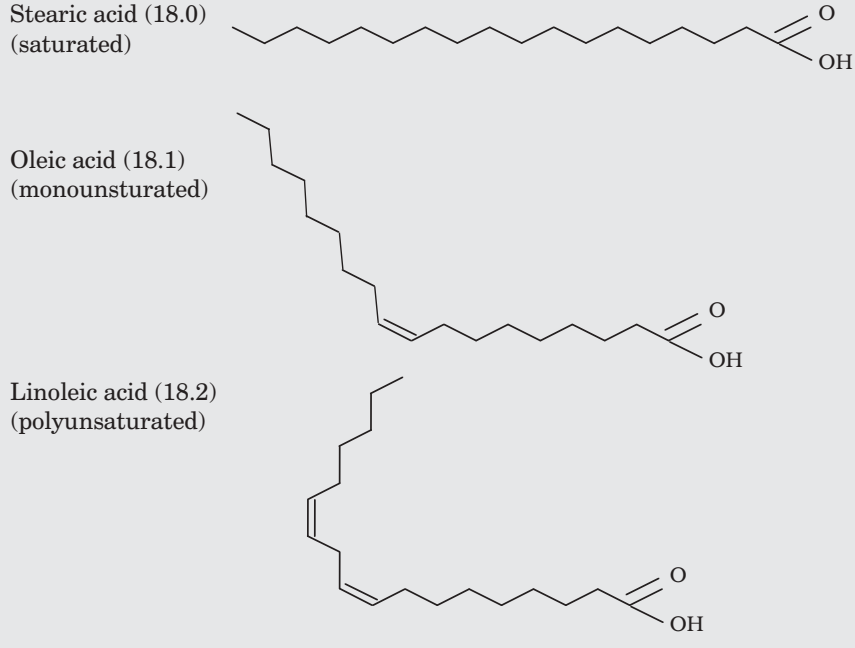
Box 2.5 Saturated or unsaturated?

You may well have heard of saturated and unsaturated fats in the context of the sorts of foods we should and shouldn't be eating. This terminology derives from the type of fatty acids which make up the different types of fat.

Each carbon atom in the hydrocarbon chain of a *saturated* fatty acid such as stearic acid is bonded to the maximum possible number of hydrogen atoms (i.e. it is saturated with them).

Fatty acids containing one or more double bonds have fewer hydrogen atoms and are said to be *unsaturated*.

Compare the structures of stearic acid and oleic acid below. Both have identical structures except that oleic acid has two fewer hydrogen atoms and in their place a C═C double bond. A kink or bend is introduced into the chain at the point of the double bond; this means that adjacent fatty acids do not pack together so neatly, leading to a drop in the melting point. The presence of unsaturated fatty acids in membrane phospholipids makes the membrane more fluid.



The second main group of lipids to be found in living cells are *phospholipids*. These have a similar structure to triacylglycerols, except that instead of a third fatty acid chain, they have a phosphate group joined to the glycerol (Figure 2.26), introducing a hydrophilic element to an otherwise hydrophobic molecule. Thus, phospholipids are an example of an *amphipathic* molecule, with a polar region at one end of the

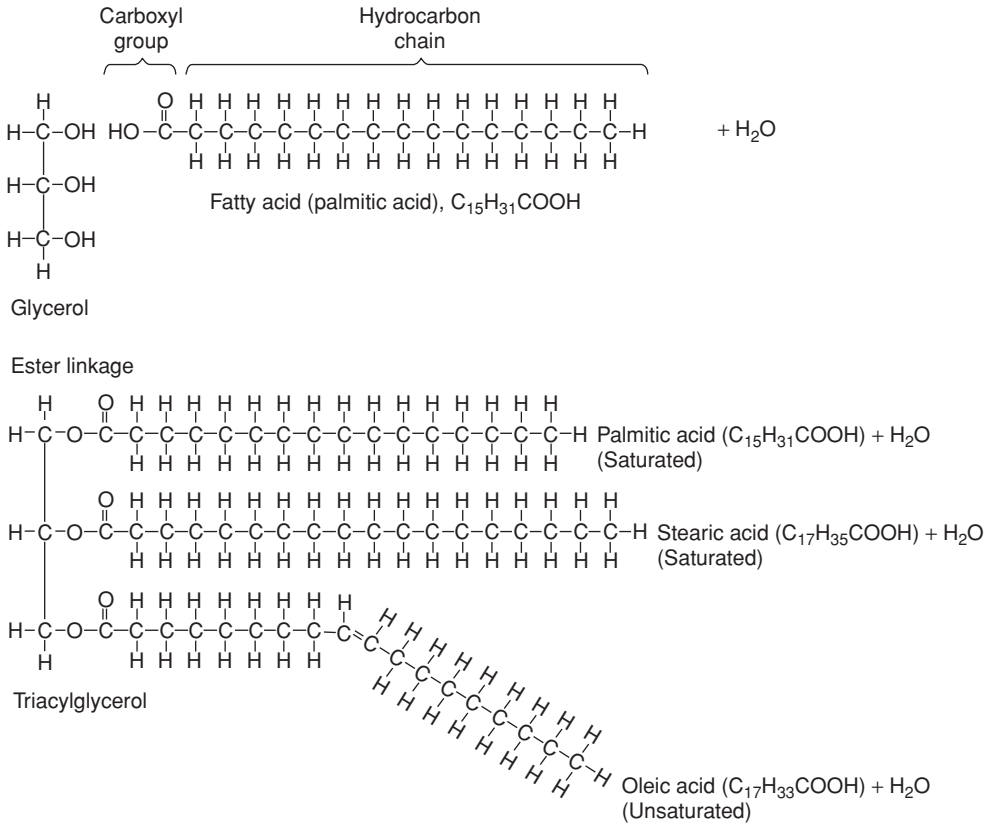


Figure 2.25 Fatty acids are linked to glycerol to form an acylglycerol. When all three -OH groups on the glycerol are esterified, the result is a triacylglycerol or triglyceride. The three fatty acids may or may not be the same. In the example shown, one of the fatty acids is unsaturated (see Box 2.5)

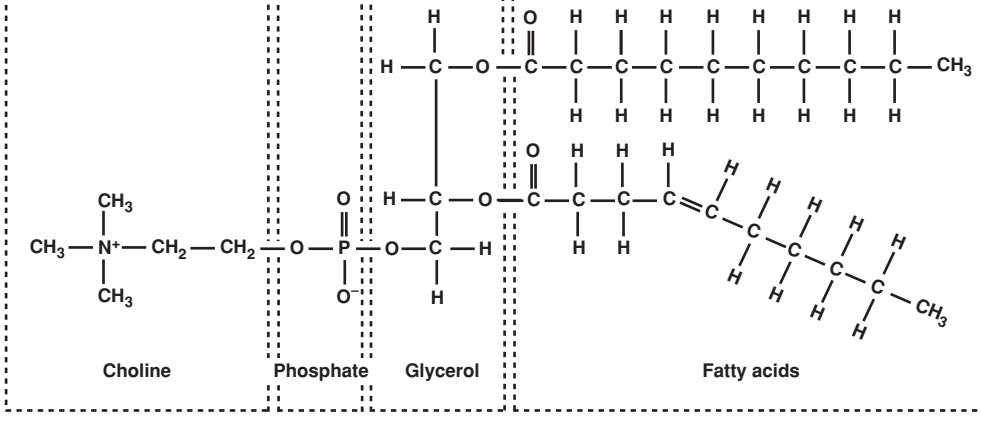


Figure 2.26 Phospholipids introduce a polar element to acylglycerols by substituting a phosphate at one of the glycerol -OH groups. A second charged group may attach to the phosphate group; the phospholipid shown is phosphatidylcholine

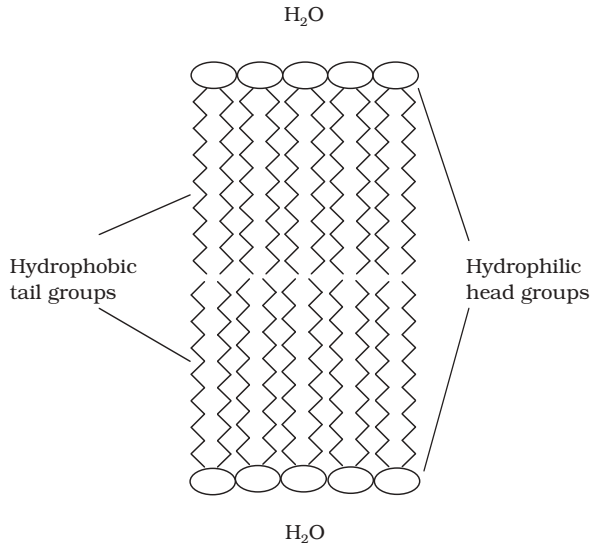


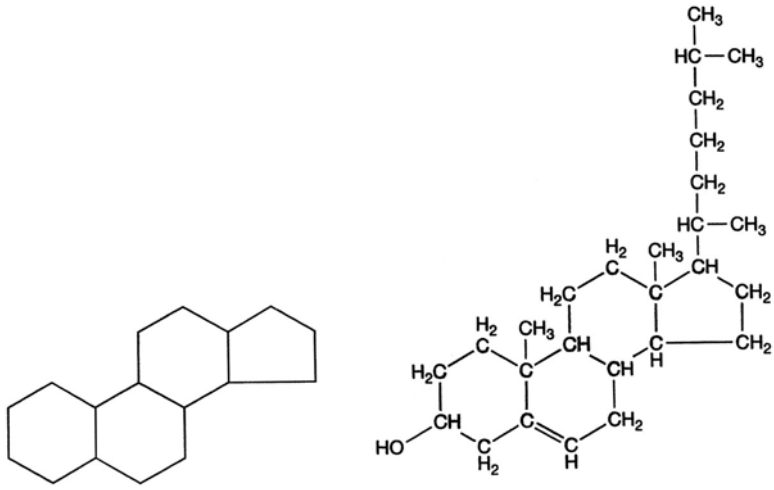
Figure 2.27 Phospholipids can form a bilayer in aqueous surroundings. A 'sandwich' arrangement is achieved by the polar phosphate groups facing outwards and burying the fatty acid chains within. Water is thus excluded from the hydrophobic region, a key property of biological membranes (see Figure 3.5)

molecule and a non-polar region at the other. This fact is essential for the formation of a bilayer when the phospholipid is introduced into an aqueous environment; the hydrophilic phosphate groups point outwards towards the water, while the hydrophobic hydrocarbon chains 'hide' inside (Figure 2.27, and c.f. Figure 2.8, micelle formation).

This bilayer structure forms the basis of all biological membranes (see Chapter 3), forming a barrier around cells and certain organelles. Phospholipids generally have another polar group attached to the phosphate; Figure 2.25 shows the effect of substituting serine.

The structural diversity of lipids can be illustrated by comparing fats and phospholipids with the final group of lipids we need to consider, the steroids. As can be seen from Figure 2.28, these have a completely different form, but still share in common the property of hydrophobicity. The four ring planar structure is common to all steroids, with the substitution of different side groups producing great differences in function. Cholesterol is an important component of many membranes.

It would be wrong to gain the impression that living cells contain only molecules of the four groups outlined above. Smaller organic molecules play important roles as precursors or intermediates in metabolic pathways (see Chapter 6), and several inorganic ions such as potassium, sodium and chloride play essential roles in maintaining the living cell. Finally, some macromolecules comprise elements of more than one group, for example, lipopolysaccharides (carbohydrate and lipid) and glycoproteins (protein and carbohydrate).



General steroid structure

Cholesterol

Figure 2.28 All steroids are based on a four-ringed structure. The presence of an –OH group on the lower left ring makes the molecule a *sterol*. Cholesterol plays an important role in the fluidity of animal membranes by interposing itself among the fatty acid tails of phospholipids. The only bacterial group to contain sterols are the mycoplasma; however some other groups contain *hopanoids*, which have a similar structure and are thought to play a comparable role in membrane stability

Test yourself

1 The number of protons in an atom of an element is called the _____ _____ of that element.

2 The sum of the protons and neutrons in an atom is the _____ _____ of the element.

3 The transfer of an electron from one atom to another so that both achieve a full outer electron shell is called _____ bonding.

4 _____ bonding involves the sharing of one or more pairs of electrons.

5 A solution with a pH of 3.0 is _____ times more acidic than one with a pH of 6.0.

6 Possession of a functional group such as phosphate or aldehyde makes a molecule more _____ and therefore more readily _____ in water.

7 Simple carbohydrates may be classed on the basis of whether they have a _____ or _____ group, or according to how many _____ atoms they possess.

8 Sugars are joined together by _____ linkages.

9 No matter how long a peptide chain grows, it always has an _____ group at one end and a _____ group at the other.

10 An example of a negatively charged amino acid is _____.

11 Disulphide bonds are formed between residues of the amino acid _____.

12 Secondary protein structure is brought about by the formation of _____ bonds between a _____ group and a _____ group.

13 Naturally occurring amino acids are all the _____-isomer. This results in an α-helix taking on a _____ configuration for maximum stability.

14 Heating or treatment with certain chemicals cause proteins to lose their three-dimensional structure and become _____.

15 A short stretch of double stranded DNA has 52 adenine residues and 61 guanine residues. There are therefore _____ cytosine residues and a total of _____ hydrogen bonds joining the two strands.

16 The arrangement of the two strands of a DNA molecule is described as _____.

17 The nucleotides of RNA contain _____ instead of thymine.

18 Lipids are a diverse group of molecules, sharing the common property of _____ in _____.

19 Phospholipids are described as _____ because their molecules have both polar and non-polar regions.

20 In fats, fatty acids are joined to glycerol via _____ linkages.

3

Cell Structure and Organisation

The basic unit of all living things is the cell. The *cell theory* is one of the fundamental concepts of biology; it states that:

- all organisms are made up of cells,

  and that

- all cells derive from other, pre-existing cells.

As we shall see in this chapter, there may exist within a cell many smaller, subcellular structures, each with its own characteristics and function, but these are not capable of independent life.

An organism may comprise just a single cell (*unicellular*), a collection of cells that are not morphologically or functionally differentiated (*colonial*), or several distinct cell types with specialised functions (*multicellular*). Among microorganisms, all bacteria and protozoans are unicellular; fungi may be unicellular or multicellular, while algae may exist in all three forms. There is, however, one way that organisms can be differentiated from each other that is even more fundamental than whether they are uni- or multicellular. It is a difference that is greater than that between a lion and a mushroom or between an earthworm and an oak tree, and it exists at the level of the individual cell. All organisms are made up of one or other (definitely not both!) of two very distinct cell types, which we call *procaryotic* and *eucaryotic* cells, both of which exist in the microbial world. These differ from each other in many ways, including size, structural complexity and organisation of genetic material (Table 3.1).

The names given to the two cell types derive from Greek words:
Procaryotic = 'before nucleus'
Eucaryotic = 'true nucleus'

The most fundamental difference between procaryotic and eucaryotic cells is reflected in their names; eucaryotic cells possess a true nucleus, and several other distinct subcellular organelles that are bounded by a membrane. Procaryotes have no such organelles. Most of these differences only became apparent after the development of electron microscopy techniques.

As can be seen from Table 3.2, the procaryotes comprise the simpler and more primitive types of microorganisms; they are generally single celled, and arose much earlier in evolutionary history than the eucaryotes. Indeed, as discussed later in this chapter, it

Table 3.1 Similarities and differences between procaryotic and eucaryotic cell structure

Similarities	
Cell contents bounded by a plasma membrane	
Genetic information encoded on DNA	
Ribosomes act as site of protein synthesis	
Differences	
Procaryotic	**Eucaryotic**
Size	
Typically 1–5 μm	Typically 10–100 μm
Genetic material	
Free in cytoplasm	Contained within a membrane-bound nucleus
Single circular chromosome or nucleoid	Multiple chromosomes, generally in pairs
Histones absent.	DNA complexed with histone proteins
Internal features	
Membrane-bound organelles absent	Several membrane-bound organelles present, including mitochondria, Golgi body, endoplasmic reticulum and (in plants & algae) chloroplasts
Ribosomes smaller (70S), free in cytoplasm	Ribosomes larger (80S), free in cytoplasm or attached to membranes
Respiratory enzymes bound to plasma membrane	Respiratory enzymes located in mitochondria
Cell wall	
Usually based on peptidoglycan (not Archaea)	When present, based on cellulose or chitin
External features	
Cilia absent	Cilia may be present
Flagella, if present, composed of flagellin. Provide rotating motility	Flagella, if present, have complex (9 + 2) structure. Provide 'whiplash' motility
Pili may be present	Pili absent
Outside layer (slime layer, capsule, glycocalyx) present in some types	Pellicle or test present in some types

is widely accepted that eucaryotic cells actually arose from their more primitive counterparts. Note that the viruses do not appear in Table 3.2, because they do not have a cellular structure at all, and are not therefore considered to be living organisms. (See Chapter 10 for further discussion of the viruses).

The use of DNA sequencing methods to determine *phylogenetic* relationships between organisms has revealed that within the procaryotes there is another fundamental division. One group of bacteria were shown to differ greatly from all the others; we now call these the *Archaea*, to differentiate them from the true *Bacteria*.

Phylogenetic: pertaining to the evolutionary relationship between organisms.

Table 3.2 Principal groups of procaryotic and eucaryotic organisms

Procaryotes	Eucaryotes
Bacteria	Fungi
Blue-green 'algae'*	Algae
	Protozoa
	Plants
	Animals

*An old-fashioned term: this group are in fact a specialized form of bacteria, and are known more correctly as the Cyanobacteria, or simply the blue-greens. They are discussed in more detail in Chapter 7. Animals and plants fall outside the scope of this book.

These two groups, together with the eucaryotes, are thought to have evolved from a common ancestor, and represent the three *domains* of life (Figure 3.1). The Archaea comprise a wide range of mostly anaerobic bacteria, including many of those that inhabit extreme environments such as hot springs. In this book we shall largely confine our discussions to the Bacteria, however in Chapter 7 there is a discussion of the principal features of the Archaea and their main taxonomic groupings.

Despite their differences, *Archaea* and *Bacteria* are both procaryotes.

Taxonomy is the science of classifying living (and once-living) organisms.

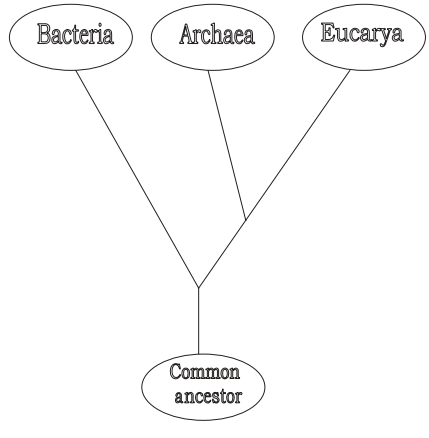


Figure 3.1 The three domains of life. All life forms can be assigned to one of three domains on the basis of their ribosomal RNA sequences. The Archaea are quite distinct from the true bacteria and are thought to have diverged from a common ancestral line at a very early stage, before the evolution of eucaryotic organisms. The scheme above is the one most widely accepted by microbiologists, but alternative models have been proposed

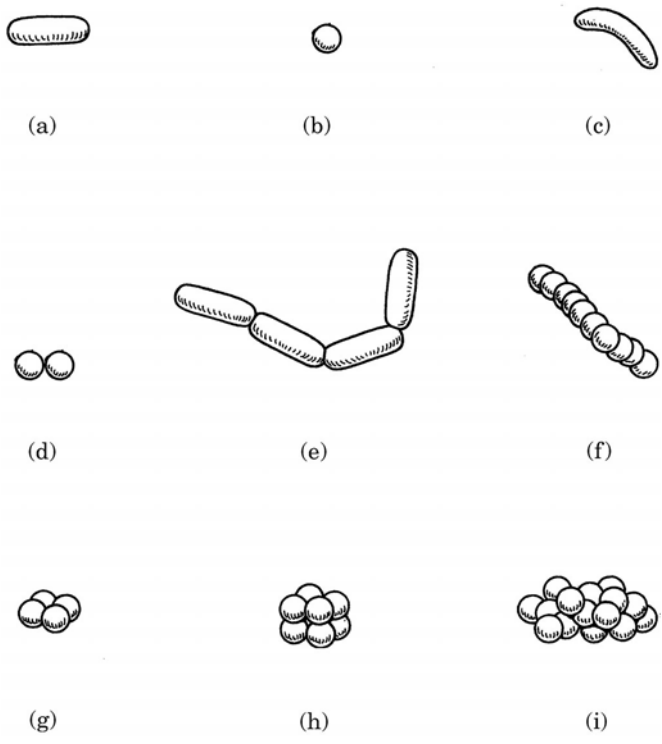


Figure 3.2 Bacterial shapes. Most bacteria are (a) rod shaped, (b) spherical or (c) curved. These basic shapes may join to form (d) pairs, (e and f) chains, (g) sheets, (h) packets or (i) irregular aggregates

The procaryotic cell

Bacteria are much smaller than eucaryotic cells; most fall into a size range of about 1–5μm, although some may be larger than this. Some of the smallest bacteria, such as the mycoplasma measure less than 1μm, and are too small to be resolved clearly by an ordinary light microscope.

Because of their extremely small size, it was only with the advent of the electron microscope that we were able to learn about the detailed structure of bacterial cells. Using the light microscope however, it is possible to recognise differences in the shape and arrangement of bacteria. Although a good deal of variation is possible, most have one of three basic shapes (Figure 3.2):

In recent years, square, triangular and star-shaped bacteria have all been discovered!

- rod shaped (*bacillus*)
- spherical (*coccus*)
- curved: these range from comma-shaped (*vibrio*) to corkscrew-shaped (*spirochaete*)

All these shapes confer certain advantages to their owners; rods, with a large surface area are better able to take up nutrients from the environment, while the cocci are less prone to drying out. The spiral forms are usually motile; their shape aids their movement through an aqueous medium.

As well as these characteristic cell shapes, bacteria may also be found grouped together in particular formations. When they divide, they may remain attached to one another, and the shape the groups of cells assume reflects the way the cell divides. Cocci, for example, are frequently found as chains of cells, a reflection of repeated division in one plane (Figure 3.2(f)). Other cocci may form regular sheets or packets of cells, as a result of division in two or three planes. Yet others, such as the staphylococci, divide in several planes, producing the irregular and characteristic 'bunch of grapes' appearance. Rod-shaped bacteria only divide in a single plane and may therefore be found in chains, while spiral forms also divide in one plane, but tend not to stick together. Blue–greens form filaments; these are regarded as truly multicellular rather than as a loose association of individuals.

Procaryotic cell structure

When compared with the profusion of elaborate organelles encountered inside a typical eucaryotic cell, the interior of a typical bacterium looks rather empty. The only internal structural features are:

- a bacterial chromosome or *nucleoid*, comprising a closed loop of double stranded, supercoiled DNA. In addition, there may be additional DNA in the form of a *plasmid*
- thousands of granular *ribosomes*
- a variety of granular *inclusions* associated with nutrient storage.

All of these are contained in a thick aqueous soup of carbohydrates, proteins, lipids and inorganic salts known as the *cytoplasm*, which is surrounded by a *plasma membrane*. This in turn is wrapped in a *cell wall*, whose rigidity gives the bacterial cell its characteristic shape. Depending on the type of bacterium, there may be a further surrounding layer such as a *capsule* or *slime layer* and/or structures external to the cell associated with motility (*flagella*) or attachment (*pili/fimbriae*). Figure 3.3 shows these features in a generalised bacterial cell. In the following pages we shall examine these features in a little more detail, noting how each has a crucial role to play in the survival or reproduction of the cell.

Genetic material

Although it occupies a well defined area within the cell, the genetic material of procaryotes is not present as a true nucleus, as it lacks a surrounding nuclear membrane (c.f. the eucaryotic nucleus, Figure 3.12). The nucleoid or bacterial chromosome comprises a closed circle of double stranded DNA, many times the length of the cell and highly folded and compacted. (The common laboratory

Not all bacteria conform to the model of a single circular chromosome; some have been shown to possess two with genes shared between them, while examples of linear chromosomes are also known.

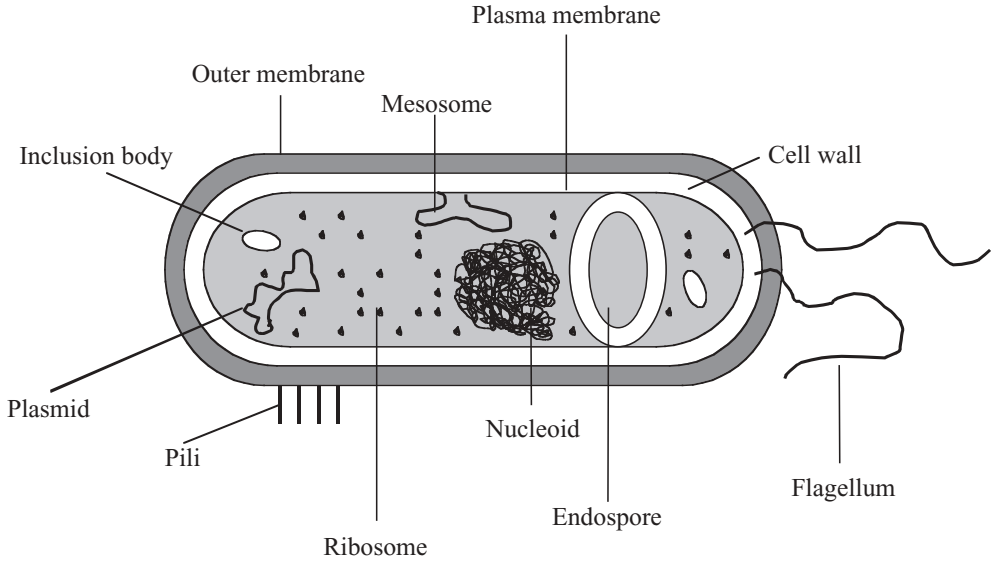


Figure 3.3 Structure of a generalised procaryotic cell. Note the lack of complex internal organelles (c.f. Figure 3.12). Gram-positive and Gram-negative bacteria differ in the details of their cell wall structure (see Figures 3.7 & 3.8)

bacterium *Escherichia coli* is around 3–4 μm in length, but contains a DNA molecule some 1400 μm in length!) The DNA may be associated with certain bacterial proteins, but these are not the same as the histones found in eucaryotic chromosomes. Some bacteria contain additional DNA in the form of small, self-replicating extrachromosomal elements called plasmids. These do not carry any genes essential for growth and reproduction, and thus the cell may survive without them. They can be very important however, as they may include genes encoding toxins or resistance to antibiotics, and can be passed from cell to cell (see Chapter 12).

Plasmids are small loops of DNA independent of the chromosome. They are capable of directing their own replication.

Ribosomes

Apart from the nucleoid, the principal internal structures of procaryotic cells are the ribosomes. These are the site of protein synthesis, and there may be many thousands of these in an active cell, lending a speckled appearance to the cytoplasm. Ribosomes are composed of a complex of protein and RNA, and are the site of protein synthesis in the cell.

Although they carry out a similar function, the ribosomes of procaryotic cells are smaller and lighter than their eucaryotic counterparts. Ribosomes are measured in *Svedberg units* (*S*), a function of their size and shape, and determined by their rate of sedimentation in a centrifuge; procaryotic ribosomes are 70S, while those of eucaryotes are 80S. Some types of antibiotic exploit this difference by

Table 3.3 Comparison of procaryotic and eucaryotic ribosomes

	Procaryotic	Eucaryotic
Overall size	70S	80S
Large subunit size	50S	60S
Large subunit RNA	23S & 5S	28S, 5.8S & 5S
Small subunit size	30S	40S
Small subunit RNA	16S	18S

targeting the procaryotic form and selectively disrupting bacterial protein synthesis (see Chapter 14).

All ribosomes comprise two unequal subunits (in procaryotes, these are 50S and 30S, in eucaryotes 60S and 40S: Table 3.3)). Each subunit contains its own RNA and a number of proteins (Figure 3.4). Many ribosomes may simultaneously be attached to a single mRNA molecule, forming a threadlike *polysome*. The role of ribosomes in bacterial protein synthesis is discussed in Chapter 11.

A *polyribosome* (polysome) is a chain of ribosomes attached to the same molecule of mRNA.

Inclusion bodies

Within the cytoplasm of certain bacteria may be found granular structures known as inclusion bodies. These act as food reserves, and may contain organic compounds such as starch, glycogen or lipid. In addition, sulphur and polyphosphate can be stored as inclusion bodies, the latter being known as volutin or metachromatic granules. Two special types of inclusion body are worthy of mention. Magnetosomes, which contain a form of iron oxide, help some types of bacteria to orientate themselves downwards into favourable conditions, whilst gas vacuoles maintain bouyancy of the cell in blue greens and some halobacteria.

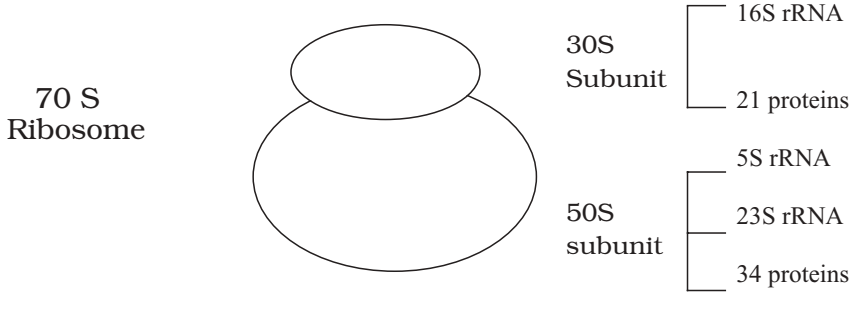


Figure 3.4 The bacterial ribosome. Each subunit comprises rRNA and proteins. The nucleotide sequence of small subunit (16S) rRNA is widely used in determining the phylogenetic (evolutionary) relationship between bacteria (see Chapter 7)

Endospores

Certain bacteria such as *Bacillus* and *Clostridium* produce endospores. They are dormant forms of the cell that are highly resistant to extremes of temperature, pH and other environmental factors, and germinate into new bacterial cells when conditions become more favourable. The spore's resistance is due to the thick coat that surrounds it.

Endospores of pathogens such as *Clostridium botulinum* can resist boiling for several hours. It is this resistance that makes it necessary to autoclave at 121°C in order to ensure complete sterility.

The plasma membrane

The cytoplasm and its contents are surrounded by a plasma membrane, which can be thought of as a bilayer of phospholipid arranged like a sandwich, together with associated proteins (Figure 3.5). The function of the plasma membrane is to keep the contents in, while at the same time allowing the selective passage of certain substances in and out of the cell (it is a semipermeable membrane).

Phospholipids comprise a compact, hydrophilic (= water-loving) head and a long hydrophobic tail region (Figure 2.27); this results in a highly ordered structure when the membrane is surrounded by water. The tails 'hide' from the water to form the inside of the membrane, while the heads project outwards. Also included in the membrane are a variety of proteins; these may pass right through the bilayer or be associated with the inner (cytoplasmic) or outer surface only. These proteins may play structural or functional roles in the life of the cell. Many enzymes associated with the metabolism of nutrients and the production of energy are associated with the plasma membrane in procaryotes. As we will see later in this chapter, this is fundamentally different from

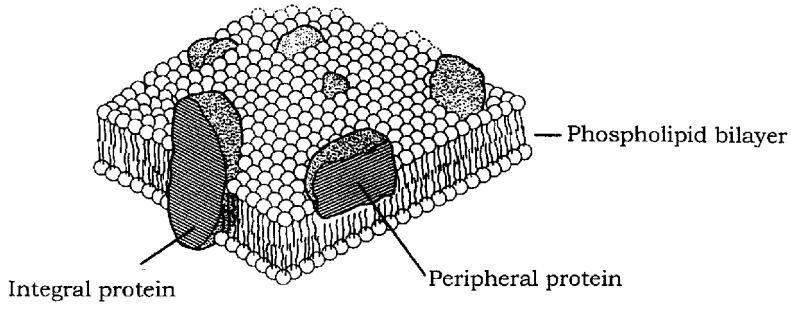


Figure 3.5 The plasma membrane. Phospholipid molecules form a bilayer, with the hydrophobic hydrocarbon chains pointing in towards each other, leaving the hydrophilic phosphate groups to face outwards. Proteins embedded in the membrane are known as integral proteins, and may pass part of the way or all of the way through the phospholipid bilayer. The amino acid composition of such proteins reflects their location; the part actually embedded among the lipid component of the membrane comprises non-polar (hydrophobic) amino acids, while polar ones are found in the aqueous environment at either side. Singleton, P: Bacteria in Biology, Biotechnology and Medicine, 5th edn, John Wiley & Sons, 1999. Reproduced by permission of the publishers

eucaryotic cells, where these reactions are carried out on specialised internal organelles. Proteins involved in the active transport of nutrients (see Chapter 4) are also to be found associated with the plasma membrane. The model of membrane structure as depicted in Figure 3.5 must not be thought of as static; in the widely accepted *fluid mosaic model*, the lipid is seen as a fluid state, in which proteins float around, rather like icebergs in an ocean.

The majority of bacterial membranes do not contain sterols (c.f. eucaryotes: see below), however many do contain molecules called hopanoids that are derive from the same precursors. Like sterols, they are thought to assist in maintaining membrane stability. A comparison of the lipid components of plasma membranes reveals a distinct difference between members of the Archaea and the Bacteria.

The bacterial cell wall

Bacteria have a thick, rigid cell wall, which maintains the integrity of the cell, and determines its characteristic shape. Since the cytoplasm of bacteria contains high concentrations of dissolved substances, they generally live in a hypotonic environment (i.e. one that is more dilute than their own cytoplasm). There is therefore a natural tendency for water to flow into the cell, and without the cell wall the cell would fill and burst (you can demonstrate this by using enzymes to strip off the cell wall, leaving the naked *protoplast*).

A protoplast is a cell that has had its cell wall removed.

The major component of the cell wall, which is responsible for its rigidity, is a substance unique to bacteria, called *peptidoglycan* (murein). This is a high molecular weight polymer whose basic subunit is made up of three parts: *N*-acetylglucosamine, *N*-acetylmuramic acid and a short peptide chain (Figure 3.6). The latter comprises the amino acids L-alanine, D-alanine, D-glutamic acid and either L-lysine or diaminopimelic acid (DAP). DAP is a rare amino acid, only found in the cell walls of procaryotes. Note that some of the amino acids of peptidoglycan are found in the D-configuration. This is contrary to the situation in proteins, as you may recall from Chapter 2, and confers protection against proteases specifically directed against L-amino acids.

Proteases are enzymes that digest proteins.

Precursor molecules for peptidoglycan are synthesised inside the cell, and transported across the plasma membrane by a carrier called bactoprenol phosphate before being incorporated into the cell wall structure. Enzymes called *transpeptidases* then covalently bond the tetrapeptide chains to one another, giving rise to a complex network (Figure 3.7); it is this cross-linking that gives the wall its mechanical strength. A number of antimicrobial agents exert their effect by inhibiting cell wall synthesis; β-lactam antibiotics such as penicillin inhibit the transpeptidases, thereby weakening the cell wall, whilst bacitracin prevents transport of peptidoglycan precursors out of the cell. The action of antibiotics will be discussed further in Chapter 14. Although all bacteria (with a few exceptions) have a cell wall containing peptidoglycan, there are two distinct structural types. These are known as *Gram-positive* and *Gram-negative*. The names derive from the Danish scientist Christian Gram, who, in the 1880s developed a rapid staining technique that could differentiate bacteria as belonging to one of two basic types (see Box 1.2). Although the usefulness of the Gram stain was recognised for many years, it

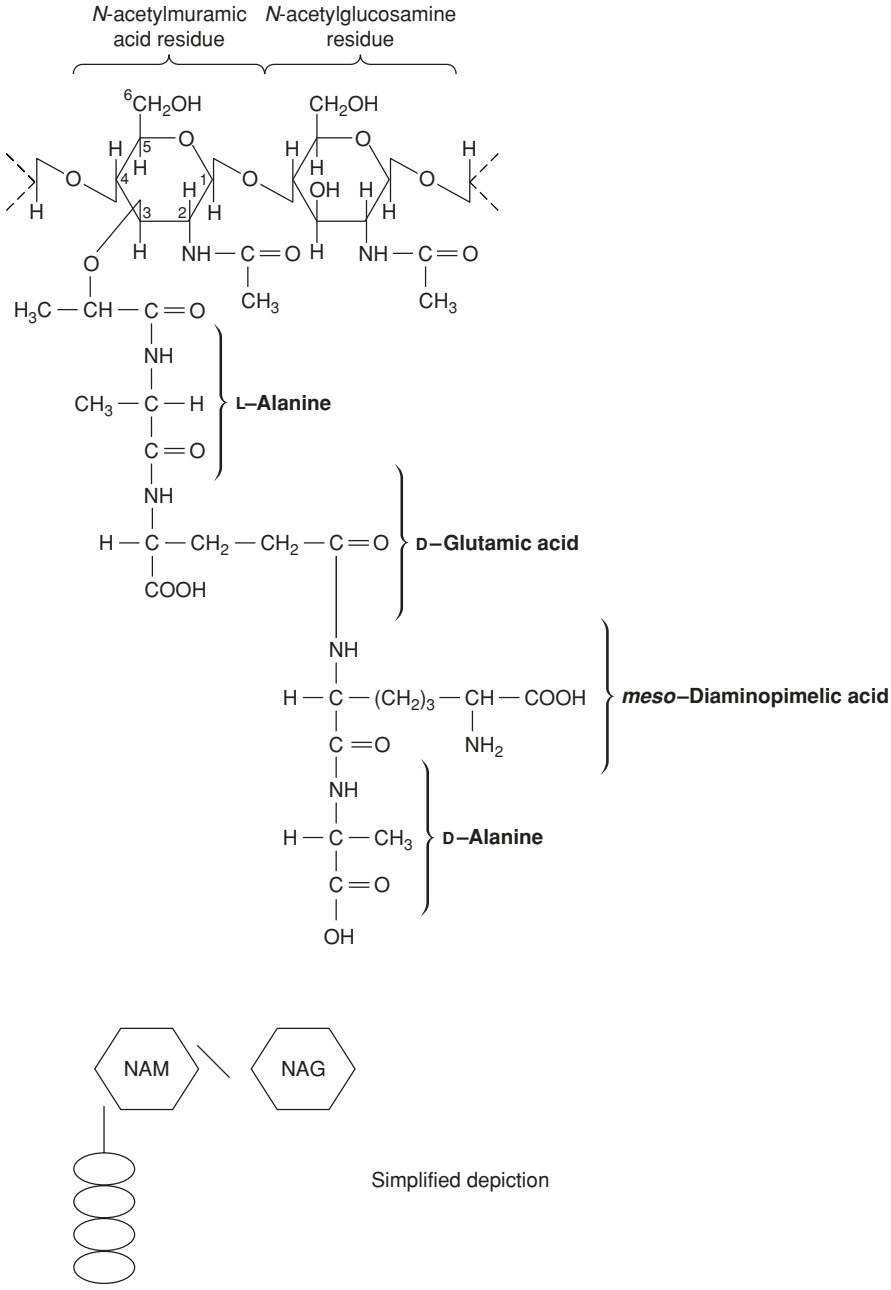


Figure 3.6 Peptidoglycan structure. Peptidoglycan is a polymer made up of alternating molecules of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM). A short peptide chain is linked to the NAM residues (see text for details). This is important in the cross-linking of the straight chain polymers to form a rigid network (Figure 3.6). The composition of *E. coli* peptidoglycan is shown; the peptide chain may contain different amino acids in other bacteria. Partly from Hardy, SP: Human Microbiology, Taylor and Francis, 2002. Reproduced by permission of Thomson Publishing Services

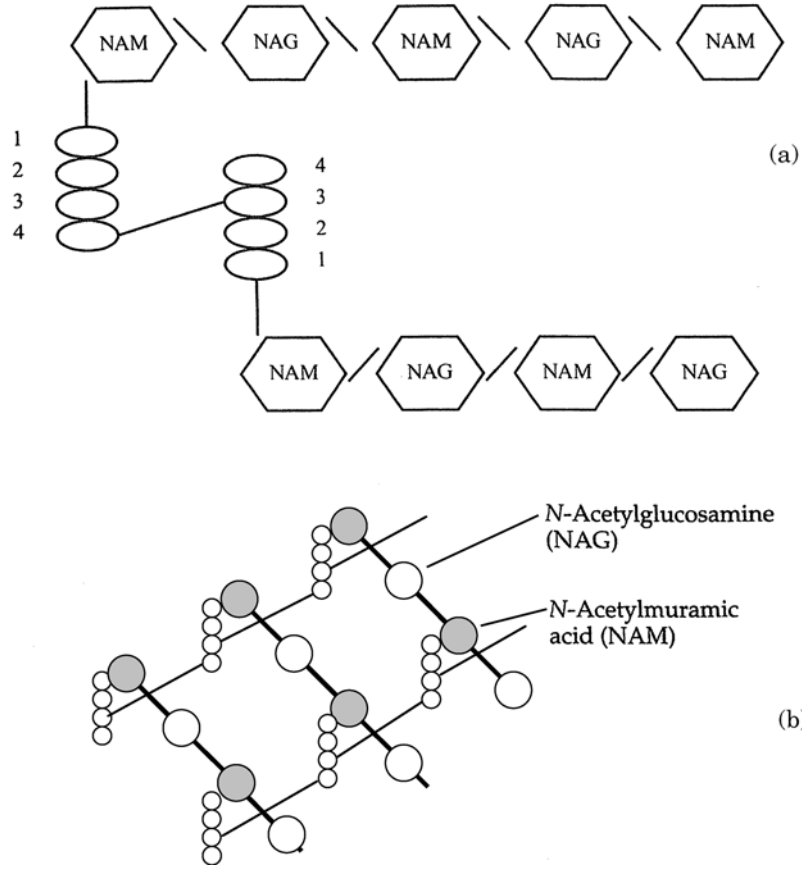


Figure 3.7 Cross-linking of peptidoglycan chains in *E. coli*. (a) The D-alanine on the short peptide chain attached to the *N*-acetylmuramic acid cross-links to a diaminopimelic acid residue on another chain. In other bacteria, the precise nature of the cross-linking may differ. From Hardy, SP: Human Microbiology, Taylor and Francis, 2002. Reproduced by permission of Thomson Publishing Services. (b) Further cross-linking produces a rigid network of peptidoglycan. The antibiotic penicillin acts by inhibiting the transpeptidase enzymes responsible for the cross-linking reaction (see Chapter 15)

was only with the age of electron microscopy that the underlying molecular basis of the test could be explained, in terms of cell wall structure.

Gram-positive cell walls are relatively simple in structure, comprising several layers of peptidoglycan connected to each other by cross-linkages to form a strong, rigid scaffolding. In addition, they contain acidic polysaccharides called *teichoic acids*; these contain phosphate groups that impart an overall negative charge to the cell surface. A diagram of the gram-positive cell wall is shown in Figure 3.8.

Gram-negative cells have a much thinner layer of peptidoglycan, making the wall less sturdy, however the structure is made more complex by the presence of a layer of lipoprotein, polysaccharide and phospholipid known as the *outer membrane*

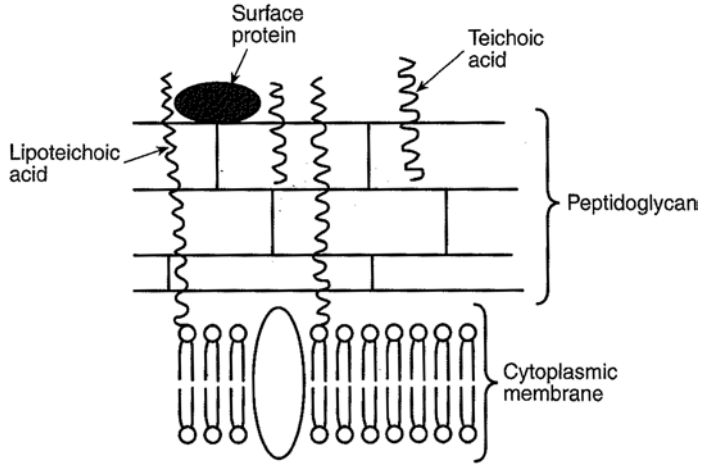


Figure 3.8 The Gram-positive cell wall. Peptidoglycan is many layers thick in the Gram-positive cell wall and may account for 30–70% of its dry weight. Teichoic acids are negatively charged polysaccharides; they are polymers of ribitol phosphate and cross-link to peptidoglycan. Lipoteichoic acids are teichoic acids found in association with glycolipids. From Henderson, B, Wilson, M, McNab, R & Lax, AJ: Cellular Microbiology: Bacteria-Host Interactions in Health and Disease, John Wiley & Sons Inc., 1999. Reproduced by permission of the publishers

(Figure 3.9). This misleading name derives from the fact that it superficially resembles the bilayer of the plasma membrane; however, instead of two layers of phospholipid, it has only one, the outer layer being made up of *lipopolysaccharide*. This has three parts: lipid A, core polysaccharide and an O-specific side chain. The lipid A component may act as an *endotoxin*, which, if released into the bloodstream, can lead to serious conditions such as fever and toxic shock. The O-specific antigens are carbohydrate chains whose composition often varies between strains of the same species. Serological methods can distinguish between these, a valuable tool in the investigation, for example, of the origin of an outbreak of an infectious disease. Proteins incorporated into the outer membrane and penetrating its entire thickness form channels that allow the passage of water and small molecules to enter the cell. Unlike the plasma membrane, the outer membrane plays no part in cellular respiration.

Box 3.1 Mesosomes – the structures that never were?

When looked at under the electron microscope, Gram-positive bacteria often contained localised in-foldings of the plasma membrane. These were given the name *mesosomes*, and were thought by some to act as attachment points for DNA during cell division, or to play a role in the formation of cross-walls. Others thought they were nothing more than artefacts produced by the rather elaborate sample preparation procedures necessary for electron microscopy. Nowadays, most microbiologists support the latter view.

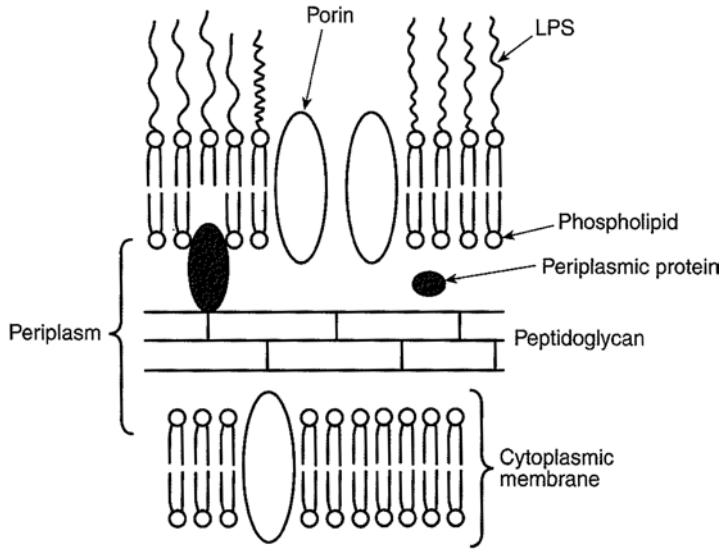


Figure 3.9 The Gram-negative cell wall. Note the thinner layer of peptidoglycan compared to the gram-positive cell wall (Figure 3.8). It accounts for <10% of the dry weight. Beyond this lies the outer membrane, with its high lipopolysaccharide content. Channels made of *porins* allow the passage of certain solutes into the cell. From Henderson, B, Wilson, M, McNab, R & Lax, AJ: Cellular Microbiology: Bacteria-Host Interactions in Health and Disease, John Wiley & Sons Inc., 1999. Reproduced by permission of the publishers

Members of the Archaea have a cell wall chemistry quite different to that described above (see Chapter 7). Instead of being based on peptidoglycan, they have other complex polysaccharides, although a distinction between gram-positive and gram-negative types still occurs.

Beyond the cell wall

A number of structural features are to be found on the outer surface of the cell wall; these are mainly involved either with locomotion of the cell or its attachment to a suitable surface.

Perhaps the most obvious extracellular structures are *flagella* (sing: flagellum), thin hair-like structures often much longer than the cell itself, and used for locomotion in many bacteria. There may be a single flagellum, one at each end, or many, depending on the bacteria concerned (Figure 3.10). Each flagellum is a hollow but rigid cylindrical filament made of the protein flagellin, attached via a hook to a basal body, which secures it to the cell wall and plasma membrane (Figure 3.11). The basal body comprises a series of rings, and is more complex in Gram-negative than Gram-positive bacteria. Rotation of the flagellum is an energy-dependent process driven by the basal body, and the direction of rotation determines the nature of the resulting cellular movement. Clockwise rotation of a single flagellum results in a directionless 'tumbling',

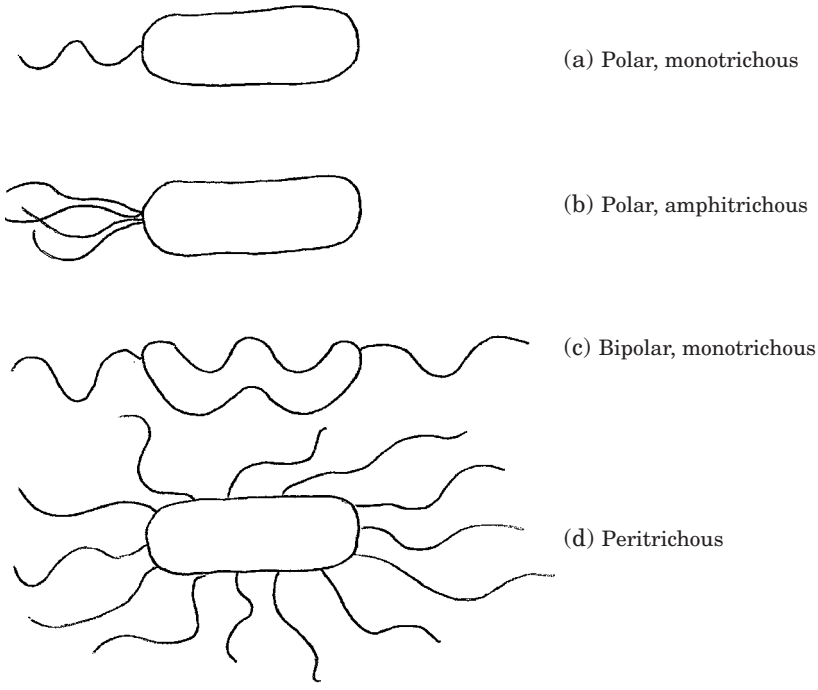


Figure 3.10 Flagella may be situated at one end (a & b), at both ends (c) or all over the cell surface (d)

but if it rotates anticlockwise, the bacterium will 'run' in a straight line (Figure 3.12). Likewise, anticlockwise rotation causes bunched flagella to 'run' by winding around each other and acting as a single structure, whilst spinning in the opposite direction gives rise to multiple independent rotations and tumbling results once more.

Pili (sing: pilus) are structures that superficially resemble short flagella. They differ from flagella, however, in that they do not penetrate to the plasma membrane, and they are not associated with motility. Their function, rather, is to anchor the bacterium to an appropriate surface. Pathogenic (disease-causing) bacteria have proteins called adhesins on their pili, which adhere to specific receptors on host tissues. Attachment pili are sometimes called *fimbriae*, to distinguish them from another distinct type of pilus, the *sex pilus*, which as its name suggests, is involved in the transfer of genetic information by conjugation. This is discussed in more detail in Chapter 12.

Outside the cell wall, most bacteria have a polysaccharide layer called a glycocalyx. This may be a diffuse and loosely bound slime layer or a better defined, and generally thicker capsule. The slime layer helps protect against desiccation, and is instrumental in the attachment of certain bacteria to a substratum (the bacteria that stick to your teeth are a good example of this). Capsules offer protection to certain pathogenic bacteria against the phagocytic cells of the immune system. Both capsules and slime layers are key components of biofilms, which form at liquid/solid interfaces, and can be highly significant in such varied settings as wastewater treatment systems, indwelling catheters and the inside of your mouth!

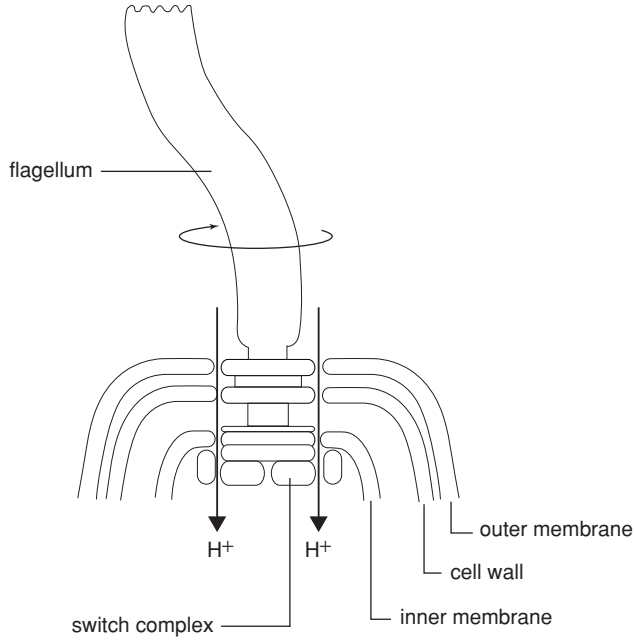


Figure 3.11 Bacterial flagella are anchored in the cell wall and plasma membrane. The filament of the flagellum is anchored by a basal body. In Gram-positive organisms, this comprises two rings inserted in the plasma membrane. In Gram-negative organisms (as shown), there are additional rings associated with the outer membrane and the peptidoglycan layer. Some modern interpretations of flagellar structure view the M and S rings as a single structure. Energy for rotation of the flagellum is derived from the proton motive force generated by the movement of protons across a membrane (see Chapter 6). From Bolsover, SR, Hyams, JS, Jones, S, Shepherd, EA & White, HA: From Genes to Cells, John Wiley & Sons, 1997. Reproduced by permission of the publishers

The eucaryotic cell

We have already seen that eucaryotic cells are, for the most part, larger and much more complex than procaryotes, containing a range of specialised subcellular organelles (Figure 3.13). Within the microbial world, the major groups of eucaryotes are the fungi and the protists (protozoans and algae); all of these groups have single-celled representatives, and there are multicellular forms in the algae and fungi.

Our survey of eucaryotic cell structure begins once more with the genetic material, and works outwards. However, since many internal structures in eucaryotes are enclosed in a membrane, it is appropriate to preface our description by briefly considering eucaryotic membranes. These are, in fact, very similar to the fluid mosaic structure we described earlier in this chapter, as depicted in Figure 3.5. The main difference is that eucaryotic membranes contain lipids called sterols, which enhances their rigidity. We shall consider the significance of this when we discuss the plasma membrane of eucaryotes below. Cholesterol, which we usually hear about in a very negative context, is a very important sterol found in the membranes of many eucaryotes.

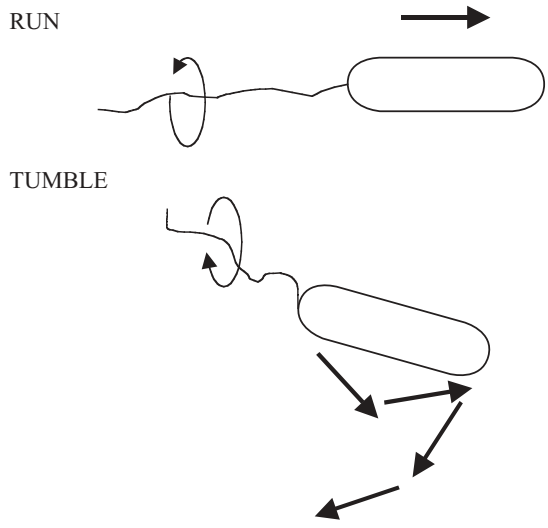


Figure 3.12 Running and tumbling. Anticlockwise rotation of the flagellum gives rise to 'running' in a set direction. Reversing the direction of rotation causes 'tumbling', and allows the bacterial cell to change direction

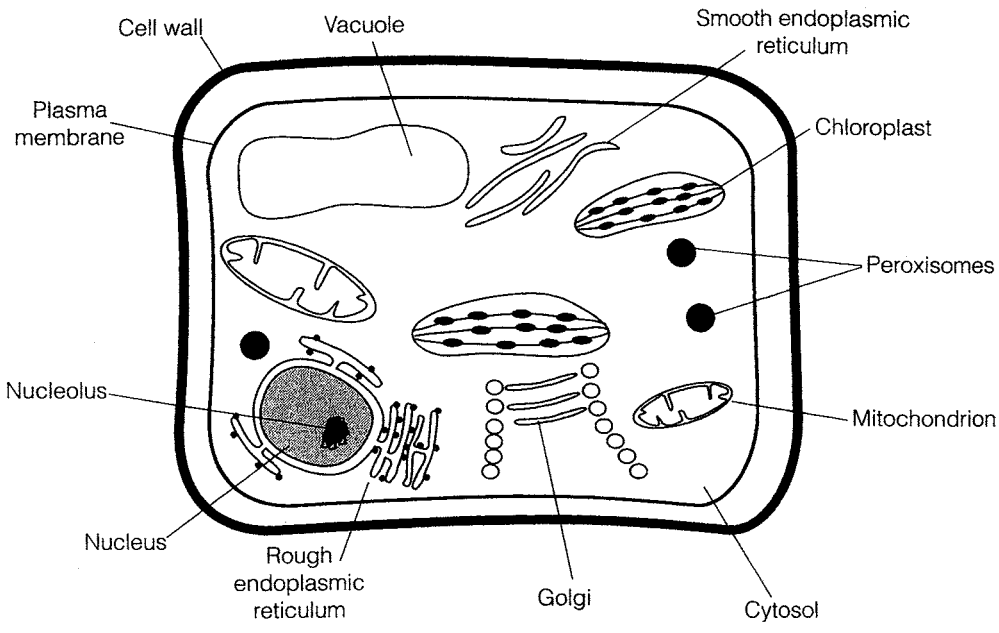


Figure 3.13 This example of eucaryotic cell structure shows a plant cell. Other eucaryotic cells may differ with respect to the cell wall and the possession of choroplasts. Note the much more elaborate internal structure compared to a typical procaryotic cell (Figure 3.3), in particular the presence of membrane-bounded organelles such as mitochondria, chloroplasts, endoplasmic reticulum and a true nucleus. From Nicklin, J, Graeme-Cook, K & Killington, R: Instant Notes in Microbiology, 2nd edn, Bios Scientific Publishers, 2002. Reproduced by permission of Thomson Publishing Services

The nucleus

The principal difference between procaryotic and eucaryotic cells, and the one that gives the two forms their names, lies in the accommodation of their genetic material. Eucaryotic cells have a true nucleus, surrounded by a nuclear membrane. This is in fact a double membrane; it contains pores, through which messenger RNA leaves the nucleus on its way to the ribosomes during protein synthesis (see Chapter 11).

The organisation of genetic material in eucaryotes is very different from that in procaryotes. Instead of existing as a single closed loop, the DNA of eucaryotes is organised into one or more pairs of *chromosomes*. The fact that they occur in pairs highlights another important difference from procaryotes: eucaryotes are genetically *diploid* in at least some part of their life cycle, while procaryotes are *haploid*. The DNA of eucaryotic chromosomes is linear in the sense that it has free ends; however, because there is so much of it, it is highly condensed and wound around proteins called *histones*. These carry a strong positive charge and associate with the negatively charged phosphate groups on the DNA.

A cell containing only one copy of each chromosome is said to be haploid. The term is also applied to organisms made up of such cells The haploid state is often denoted as N. (c.f. diploid (2N): containing two copies of each chromosome)

As well as the chromosomes, the nucleus also contains the *nucleolus*, a discrete structure rich in RNA, where ribosomes are assembled. The ribosomes themselves have the same function as their procaryotic counterparts; the differences in size have already been discussed (see Table 3.3). They may be found free in the cytoplasm or associated with the endoplasmic reticulum (see below), depending on the type of protein they synthesise.

A histone is a basic protein found associated with DNA in eucaryotic chromosomes.

Endoplasmic reticulum

Running throughout the cell and taking up much of its volume, the endoplasmic reticulum (ER) is a complex membrane system of tubes and flattened sacs. The presence of numerous ribosomes on their surface gives those parts of the ER involved in protein synthesis a granular appearance when seen under the electron microscope, giving rise to the name *rough ER*. Areas of the ER not associated with ribosomes are known as *smooth ER*; this is where the synthesis of membrane lipids takes place. The ER also serves as a communications network, allowing the transport of materials between different parts of the cell.

Golgi apparatus

The Golgi apparatus is another membranous organelle, comprising a set of flattened vesicles, usually arranged in a stack called a *dictyosome*. The function of the Golgi

apparatus is to package newly synthesised substances such as proteins and assist in their transport away from the cell. The substances are contained in vesicles that are released from the main part of the complex, and fuse with the cytoplasmic membrane. The Golgi apparatus is poorly defined in certain fungi and protozoans.

Lysosomes

Another function of the Golgi apparatus is to package certain hydrolytic (digestive) enzymes into membrane-bound packets called *lysosomes*. The enzymes are needed to digest nutrient molecules that enter the cell by *endocytosis* (Figure 3.14), and would break down the fabric of the cell itself if they were not contained within the lysosomes. *Peroxisomes* are similar to lysosomes, but smaller, and also contain degradative enzymes. They contain the enzyme catalase, which breaks down the potentially toxic hydrogen peroxide generated by other breakdown reactions within the peroxisome.

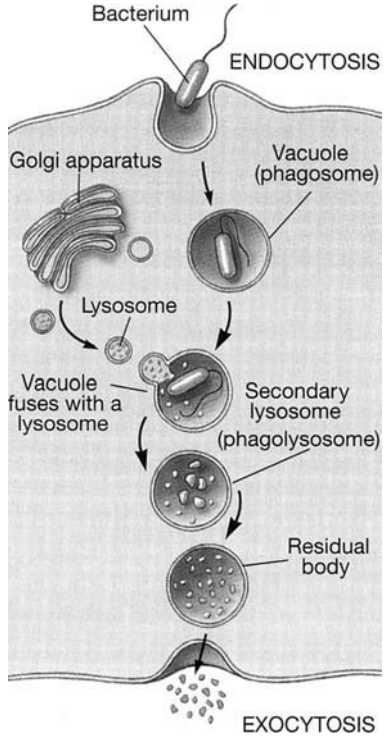


Figure 3.14 Endocytosis. Membrane-bound vacuoles surround a food particle and internalise it in the form of a *phagosome*. This fuses with a lysosome, which releases digestive enzymes, resulting in the breakdown of the contents. The process of endocytosis is unique to eucaryotic cells. From Black, JG: Microbiology: Principles and Explorations, 4th edn, John Wiley & Sons Inc., 1999. Reproduced by permission of the publishers

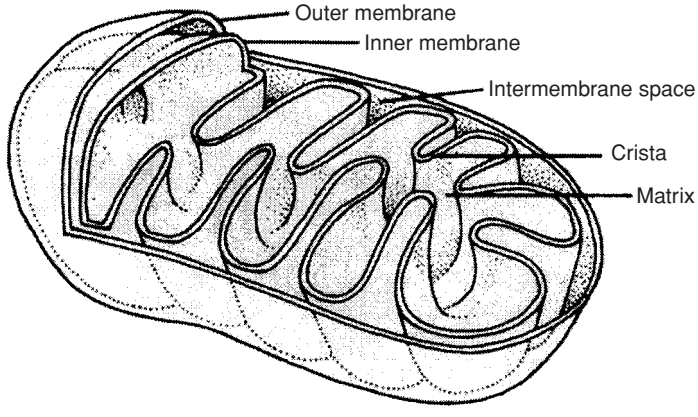


Figure 3.15 Mitochondrial structure. The inner membrane, the location of the electron transport chain in aerobic respiration, is formed by the invagination of the more permeable outer membrane. Mitochondria have similar dimensions to many bacteria (approx. 1–3μm), but may vary in shape due to the plasticity of their membranes

Mitochondria

Whereas in procaryotes the enzymes involved in adenosine triphosphate generation (see Chapter 6) are associated with the plasma membrane, in eucaryotes they are found in specialised organelles called mitochondria. These are generally rod-shaped and may be present in large numbers. They are enclosed by a double membrane, the inner surface of which is folded into finger-like projections called *cristae*. Respiratory enzymes are located on the increased surface area this provides, while other metabolic reactions take place in the semi-fluid matrix (Figure 3.15) (see also Chapter 6).

The mitochondrial cristae of algae, fungi and protozoans each have their own characteristic shapes. Until very recently, a few primitive protozoans, such as *Giardia*, appeared to lack mitochondria completely, and were thought to represent an intermediate stage in the evolution of the eucaryotic condition. Recent research, however, has shown them to possess highly reduced remnants of mitochondria, which have been given the name *mitosomes*. It seems that such organisms did, after all, once possess mitochondria, but have subsequently lost much of their function – an example of so-called reductive evolution.

Chloroplasts

Chloroplasts are specialised organelles involved in the process of *photosynthesis*, the conversion of light into cellular energy. As such, they are characteristic of green plants and algae. Like mitochondria, chloroplasts are surrounded by a double membrane, and serve as the location for energy-generating reactions. Inside the chloroplast are

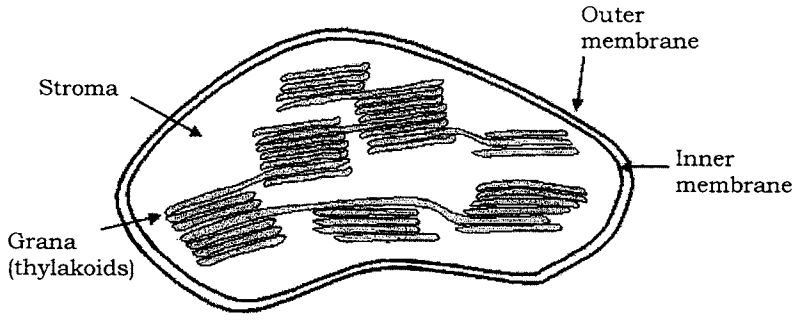


Figure 3.16 Chloroplast structure. Adenosine triphosphate generation from photosynthesis occurs on the thylakoid membranes. In green algae these take the form of discrete structures called grana. The enzyme ribulose bisphosphate carboxylase, responsible for fixing carbon dioxide via the Calvin cycle (see Chapter 6) is located in the stroma. The outer membrane of chloroplasts is relatively permeable, allowing the diffusion of the products of photosynthesis into the surrounding cytoplasm. Reproduced by permission of Dr Lance Gibson, Iowa State University

flattened membranous sacs known as *thylakoids*, which contain the photosynthetic pigment *chlorophyll*. Thylakoids are arranged in stacks called *grana* (Figure 3.16).

Mitochondria and chloroplasts both contain 70S ribosomes (similar to those found in procaryotes), a limited amount of circular DNA and the means to replicate themselves. This is seen as key evidence for the *endosymbiotic theory* of eucaryotic evolution, which envisages that specialised organelles within eucaryotic cells arose from the ingestion of small procaryotes, which over a long period of time lost their independent existence.

Vacuoles

Vacuoles are membrane-covered spaces within cells, and derive from the Golgi apparatus. They act as stores for various nutrients, and also for waste products. Some types of vacuole are important in regulating the water content of the cell.

Plasma membrane

Many eucaryotes do not have cell walls, so the plasma membrane represents the outermost layer of the cell. The sterols mentioned earlier are important in helping these cells to resist the effects of osmotic pressure. The only procaryotes to contain sterols are the mycoplasma, which are unusual in not possessing the typical bacterial cell wall. Although the eucaryotic plasma membrane does not have the role in cellular respiration associated with its procaryotic counterpart, it does have additional functions. The process of endocytosis (and its reverse, *exocytosis*), by which particles or large soluble molecules are enveloped and brought into the cell, is carried out at the plasma membrane. Also, carbohydrate residues in the membrane act as receptors for cell-to-cell recognition, and may be involved in cell adhesion.

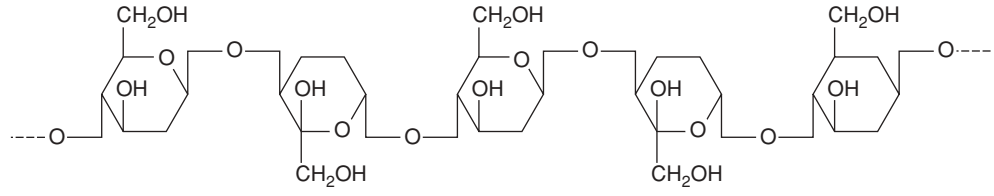


Figure 3.17 The structures of (a) cellulose and (b) chitin. Cellulose is composed of repeating glucose units joined by β-1,4 linkages, and chitin is a polymer of *N*-acetylglucosamine

Cell wall

As we have just noted, not all eucaryotes possess a cell wall; among those that do are fungi, algae and plants. Whilst the function, like that of procaryotes, is to give strength to the cell, the chemical composition is very different, generally being a good deal simpler. The cell walls of plants, algae and lower members of the fungi are based on *cellulose* (Figure 3.17a), a repeating chain of glucose molecules joined by β-1,4 linkages, and may also include pectin and hemicellulose, both also polymers of simple sugars. Most fungi such as yeasts and mushrooms contain *chitin*, a polymer of *N*-acetylglucosamine (Figure 3.17b: we have encountered *N*-acetylglucosamine before, as a component of peptidoglycan in bacterial walls.) Chitin is also to be found as the major component of insect and crustacean exoskeletons, where the function is also to provide strength and rigidity. As in procaryotes, the cell wall plays little part in the exchange of materials between the cell and its environment, a role fulfilled by the plasma membrane.

Some protozoans and unicellular algae are surrounded by a flexible *pellicle* made of protein.

Flagella and cilia

Motility in eucaryotic cells may be achieved by means of flagella or *cilia*; cilia can be thought of as, essentially, short flagella. Both are enclosed within the plasma membrane and anchored by means of a basal body. Flagellated cells generally have a single flagellum, whereas cilia are often present in very large numbers on each cell. In the microbial world, flagella are found in protozoans and motile algal forms, whilst cilia are mostly found in a class of protozoans called the Ciliophora. Flagella and cilia are not found in members of the Fungi. Although they share the same thread-like gross morphology,

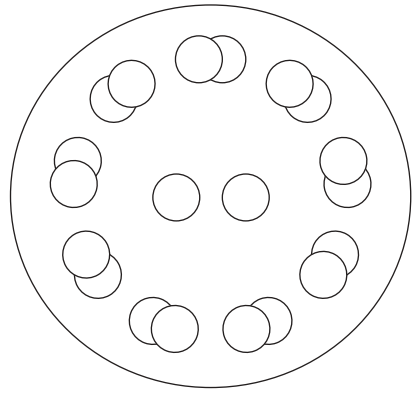


Figure 3.18 Eucaryotic flagella have a characteristic '9 + 2' structure. Although functionally analogous to their procaryotic counterparts, eucaryotic flagella differ appreciably in their fine structure. A membrane surrounds an arrangement of proteinaceous microtubules, in which nine pairs surround a single central pair. Movement of eucaryotic flagella is by means of an adenosine triphosphate-driven whiplike motion

eucaryotic flagella differ dramatically in their ultrastructure from those of procaryotes. Seen in cross-section, they have a very characteristic appearance, made up of two central *microtubules*, surrounded by a further nine pairs arranged in a circle (Figure 3.18). The microtubules are made of a protein called *tubulin*. Flagella in eucaryotes beat in waves, rather than rotating; cilia, present in large numbers, beat in a coordinated fashion so that some are in forward motion while others are in the recovery stroke (rather like a 'Mexican wave'!). In animals, ciliary motion has been adapted to move particulate matter across a tissue surface; ciliated cells of the respiratory tract, for example, act as a first line of defence in the removal of inhaled particles, such as bacteria from the airways.

Cell division in procaryotes and eucaryotes

In, unicellular procaryotes, cell division by *binary fission* leads to the creation of a new individual. Growth occurs in individual cells until a maximum size is achieved and a cross-wall forms. Before cell division takes place, the genetic material must replicate itself (see Chapter 11), and one copy pass to each new daughter cell (Figure 3.19).

Cell division in eucaryotes also results in two identical daughter cells. In the case of unicellular eucaryotes, this results in two individual organisms (asexual reproduction), while in multicellular forms there is an increase in overall size. Cell division is preceded by a process of nuclear division called *mitosis*, which ensures that both daughter cells receive a full complement of chromosomes. The principal phases of mitosis are summarised in Figure 3.20(a). In *interphase*, the chromosomes are not clearly visible under the microscope; DNA replication takes place during this period. The duplicated chromosomes, held together as sister *chromatids* by the centromere, move towards the centre of the cell during *prophase*. A series of microtubules form a spindle between

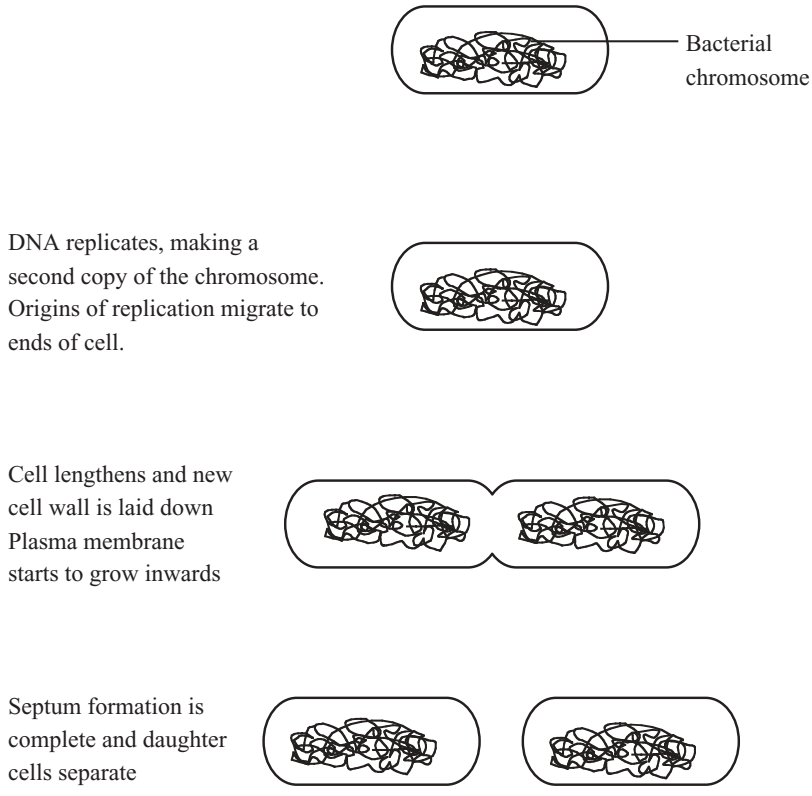


Figure 3.19 Binary fission in *E. coli*. Replication of the single circular chromosome is accompanied by an increase in cell size. The plasma membrane invaginates, and a new cross-wall is synthesised, resulting in two new daughter cells

the centrioles, and the chromosomes line up along this during *metaphase*. Also, during this phase the nuclear membrane breaks down, and each centromere duplicates. One chromosome from each pair then migrates away from the centre to opposite ends of the spindle. This stage is called *anaphase*. Finally, in *telophase*, new nuclear membranes surround the two sets of chromosomes, to form two nuclei. Mitosis is followed by cell division. Overall, the process of mitosis results in two identical nuclei containing the original (diploid) chromosome number.

At various stages of eucaryotic life cycles, a process of *meiosis* may occur, which halves the total number of chromosomes, so that each nucleus only contains one copy of each. In sexual reproduction, the haploid gametes are formed in this way, and the diploid condition is restored when two different gametes fuse. In some eucaryotes, not just the gametes but a substantial part of the life cycle may occur in the haploid form (see Chapters 8 & 9). Meiosis (Figure 3.20b) comprises two nuclear divisions, the second of which is very similar to the process of mitosis just described. In the first meiotic division, homologous chromosomes (i.e. the two members of a pair) line up on the spindle together and eventually migrate to opposite poles. While they are together, it

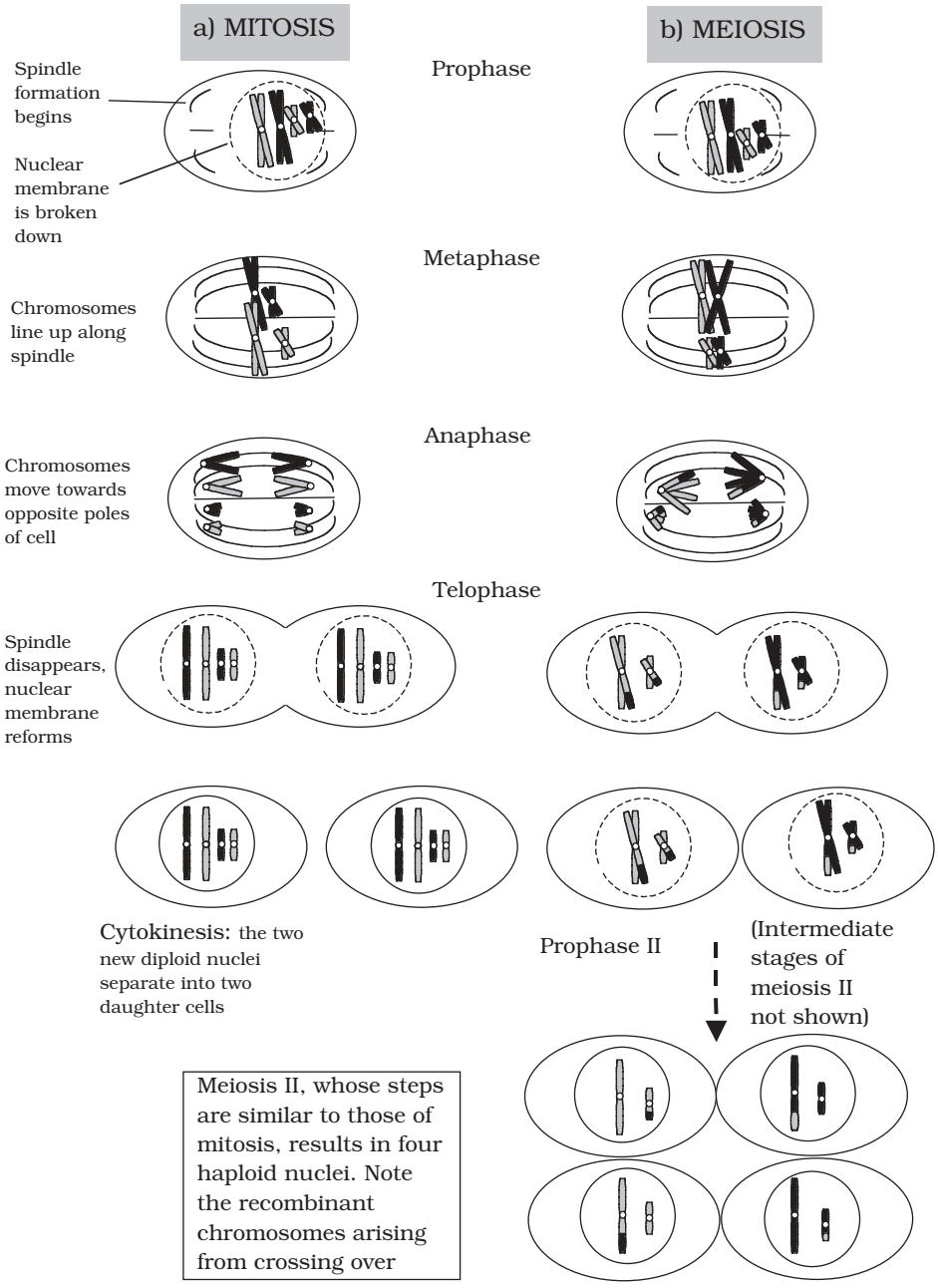


Figure 3.20 The main steps of (a) mitosis and (b) meiosis in an organism whose diploid number (2n) =4. Mitosis results in two cells identical to the parent. Meiosis results in a reduction in the chromosome number and introduces genetic variation by means of crossing over. For details see the text

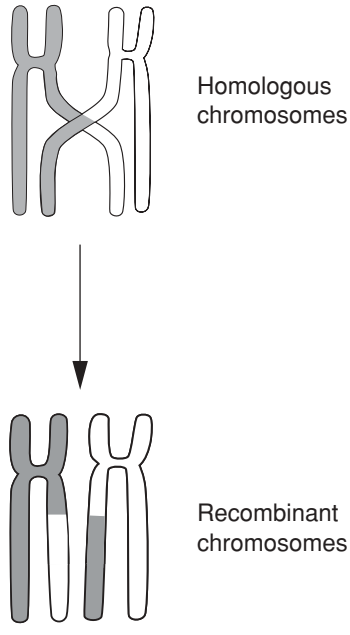


Figure 3.21 Crossing over leads to recombination of genetic material. During crossing over, portions of homologous chromosomes are exchanged. This forms the basis of genetic recombination in eucaryotes, and ensures that offspring contain new combinations of genetic material

is possible for *crossing over* to occur, a process by which the two chromosomes swap homologous stretches of DNA (Figure 3.21). Since these may not be identical, crossing over serves to introduce genetic variation into the daughter nuclei. In the second meiotic division, sister chromatids separate as before, resulting in four haploid nuclei.

Test yourself

1 Procaryotic cells have a much simpler structure than eucaryotes, lacking internal _____ and a true _____.

2 Most bacterial cells are rod-shaped (_____), spherical (_____) or curved (_____).

3 Many bacteria commonly carry extrachromosomal pieces of DNA called _____, which are able to _____ independently of the bacterial chromosome.

4 Protein synthesis takes place at _____.

5 The main components of cell membranes are _____ and _____.

6 Gram-positive cell walls contain a higher percentage of _____ than those of Gram-negative cells.

7 Many bacteria have long, hair-like structures called _____ projecting from the cell wall. These are used for _____.

8 The DNA of eucaryotes is organized into chromosomes and associated with proteins called _____.

9 In eucaryotic cells, extranuclear DNA is also found in _____ and _____.

10 Eucaryotic ribosomes may be found associated with the _____ _____ or free in the cytoplasm.

11 The Golgi apparatus _____ and _____ newly synthesised substances.

12 _____ are the site of energy generation in eucaryotic cells. In procaryotic cells, some of these reactions take place at the _____ _____.

13 The photosynthetic membranes of chloroplasts are called _____.

14 The cell walls of algae are mostly made up of _____.

15 The structure of eucaryotic flagella is more complex than that of procaryotes, comprising an arrangement of _____ made of _____.

Part II

Microbial Nutrition, Growth and Metabolism

4

Microbial Nutrition and Cultivation

In Chapter 2 we introduced the major groups of macromolecules found in living cells; the raw materials from which these are synthesised are ultimately derived from the organism's environment in the form of nutrients (Table 4.1). These can be conveniently divided into those required in large quantities* (macronutrients) and those which are needed only in trace amounts (micronutrients or trace elements).

You will recall that carbon forms the central component of proteins, carbohydrates, nucleic acids and lipids; indeed, the living world is based on carbon, so it should come as no surprise that this is the most abundant element in all living cells, microbial or otherwise. Of the other macronutrients, nitrogen, oxygen, hydrogen, sulphur and phosphorus are also constituents of biological macromolecules, while the remainder (magnesium, potassium, sodium, calcium and iron in their ionised forms) are required in lesser quantities for a range of functions that will be described in due course. Micronutrients are all metal ions, and frequently serve as cofactors for enzymes.

All microorganisms must have a supply of the nutrients described above, but they show great versatility in the means they use to satisfy these requirements.

The metabolic processes by which microorganisms assimilate nutrients to make cellular material and derive energy will be reviewed in Chapter 6. In the following section we briefly describe the role of each element, and the form in which it may be acquired.

Carbon is the central component of the biological macromolecules we discussed in Chapter 2. Carbon incorporated into biosynthetic pathways may be derived from organic or inorganic sources (see below); some organisms can derive it from CO_2, while others require their carbon in 'ready-made', organic form.

Hydrogen is also a key component of macromolecules, and participates in energy generation processes in most microorganisms. In autotrophs (see 'Nutritional categories' below), hydrogen is required to reduce carbon dioxide in the synthesis of macromolecules.

Oxygen is of central importance to the respiration of many microorganisms, but in its molecular form (O_2), it can be toxic to some forms (see Chapter 5). These obtain the oxygen they need for the synthesis of macromolecules from water.

* Everything is relative in the microbial world; a typical bacterial cell weighs around three tenmillion millionths (3×10^{-13}) of a gram!

Table 4.1 Elements found in living organisms

Element	Form in which usually supplied	Occurrence in biological systems
Macronutrients		
Carbon (C)	CO_2, organic compounds	Component of all organic molecules, CO_2
Hydrogen (H)	H_2O, organic compounds	Component of biological molecules, H^+ released by acids
Oxygen (O)	O_2, H_2O, organic compounds	Component of biological molecules; required for aerobic metabolism
Nitrogen (N)	NH_3, NO_3^-, N_2, organic N compounds	Component of proteins, nucleic acids
Sulphur (S)	H_2S, SO_4^{2-}, organic S compounds	Component of proteins; energy source for some bacteria
Phosphorus (P)	PO_4^{3-}	Found in nucleic acids, ATP, phospholipids
Potassium (K)	In solution as K^+	Important intracellular ion
Sodium (Na)	In solution as Na^+	Important extracellular ion
Chlorine (Cl)	In solution as Cl^-	Important extracellular ion
Calcium (Ca)	In solution as Ca^{2+}	Regulator of cellular processes
Magnesium (Mg)	In solution as Mg^{2+}	Coenzyme for many enzymes
Iron (Fe)	In solution as Fe^{2+} or Fe^{3+} or as FeS, $Fe(OH_3)$ etc	Carries oxygen; energy source for some bacteria
Micronutrients	Present as contaminants at very low concentrations	
Copper (Cu)	In solution as Cu^+, Cu^{2+}	Coenzyme; microbial growth inhibitor
Manganese (Mn)	In solution as Mn^{2+}	Coenzyme
Cobalt (Co)	In solution as Co^{2+}	Vitamin B_{12}
Zinc (Zn)	In solution as Zn^{2+}	Coenzyme; microbial growth inhibitor
Molybdenum (Mo)	In solution as Mo^{2+}	Coenzyme
Nickel (Ni)	In solution as Ni^{2+}	Coenzyme

Nitrogen is needed for the synthesis of proteins and nucleic acids, as well as for important molecules such as ATP (you will learn more about ATP and its role in the cell's energy relations in Chapter 6). Microorganisms range in their demands for nitrogen from those that are able to assimilate ('fix') gaseous nitrogen (N_2) to those that require all 20 amino acids to be provided preformed. Between these two extremes come species that are able to assimilate nitrogen from an inorganic source such as nitrate, and those that utilise ammonium salts or urea as a nitrogen source.

Table 4.2 Selected microbial growth factors

Growth factor	Function
Amino acids	Components of proteins
p-Aminobenzoic acid	Precursor of folic acid, involved in nucleic acid synthesis
Niacin (nicotinic acid)	Precursor of NAD^+ and $NADP^+$
Purines & pyrimidines	Components of nucleic acids
Pyridoxine (vitamin B_6)	Amino acid synthesis
Riboflavin (vitamin B_2)	Precursor of FAD

Sulphur is required for the synthesis of proteins and vitamins, and in some types is involved in cellular respiration and photosynthesis. It may be derived from sulphur-containing amino acids (methionine, cysteine), sulphates and sulphides.

Phosphorus is taken up as inorganic phosphate, and is incorporated in this form into nucleic acids and phospholipids, as well as other molecules such as ATP.

Metals such as copper, iron and magnesium are required as *cofactors* in enzyme reactions.

A cofactor is a non-protein component of an enzyme (often a metal ion) essential for its normal functioning.

Many microorganisms are unable to synthesise certain organic compounds necessary for growth and must therefore be provided with them in their growth medium. These are termed *growth factors* (Table 4.2), of which three main groups can be identified: amino acids, purines and pyrimidines (required for nucleic acid synthesis) and vitamins. You will already have read about the first two of these groups in Chapter 2. Vitamins are complex organic compounds required in very small amounts for the cell's normal functioning. They are often either *coenzymes* or their precursors (see Chapter 6). Microorganisms vary greatly in their vitamin requirements. Many bacteria are completely self-sufficient, while protozoans, for example, generally need to be supplied with a wide range of these dietary supplements. A vitamin requirement may be absolute or partial; an organism may be able, for example, to synthesise enough of a vitamin to survive, but grow more vigorously if an additional supply is made available to it.

Nutritional categories

Microorganisms can be categorised according to how they obtain their carbon and energy. As we have seen, carbon is the most abundant component of the microbial cell, and most microorganisms obtain their carbon in the form of organic molecules, derived directly or indirectly from other organisms. This mode of nutrition is the one that is familiar to us as humans (and all other animals); all the food we eat is derived as complex organic molecules from plants and other animals (and even some representatives of the microbial world such as mushrooms!). Microorganisms which obtain their carbon in

this way are described as *heterotrophs*, and include all the fungi and protozoans as well as most types of bacteria. Microorganisms as a group are able to incorporate the carbon from an incredibly wide range of organic compounds into cellular material. In fact there is hardly any such compound occurring in nature that cannot be metabolised by some microorganism or other, explaining in part why microbial life is to be found thriving in the most unlikely habitats. Many synthetic materials can also serve as carbon sources for some microorganisms, which can have considerable economic significance.

A heterotroph must use one or more organic compounds as its source of carbon.

A significant number of bacteria and all of the algae do not, however, take up their carbon preformed as organic molecules in this way, but derive it instead from carbon dioxide. These organisms are called *autotrophs*, and again we can draw a parallel with higher organisms, where all members of the plant kingdom obtain their carbon in a similar fashion.

An autotroph can derive its carbon from carbon dioxide.

We can also categorise microorganisms nutritionally by the way they derive the energy they require to carry out essential cellular reactions. Autotrophs thus fall into two categories. *Chemoautotrophs* obtain their energy as well as their carbon from inorganic sources; they do this by the oxidation of inorganic molecules such as sulphur or nitrite. *Photoautotrophs* have photosynthetic pigments enabling them to convert light energy into chemical energy. The mechanisms by which this is achieved will be discussed in Chapter 6.

A chemotroph obtains its energy from chemical compounds. A phototroph uses light as its source of energy.

The great majority of heterotrophs obtain energy as well as carbon from the same organic source. Such organisms release energy by the chemical oxidation of organic nutrient molecules, and are therefore termed *chemoheterotrophs*. Those few heterotrophs which do not follow this mode of nutrition include the green and purple non-sulphur bacteria. These are able to carry out photosynthesis and are known as *photoheterotrophs*.

There is one final subdivision of nutritional categories in microorganisms! Whether organisms are chemotrophs or phototrophs, they need a molecule to act as a source of electrons (reducing power) to drive their energy-generating systems (see Chapter 6). Those able to use an inorganic electron donor such as H_2O, H_2S or ammonia are called *lithotrophs*, while those requiring an organic molecule to fulfil the role are *organotrophs*. Most (but not all) microorganisms are either photolithotrophic autotrophs (algae, blue-greens) or chemo-organotrophic heterotrophs (most bacteria). For the latter category, a single organic compound can often act as the provider of carbon, energy and reducing power. The substance used by chemotrophs as an energy source may be organic (chemoorganotrophs) or inorganic (chemolithotrophs).

A lithotroph is an organism that uses inorganic molecules as a source of electrons. An organotroph uses organic molecules for the same purpose.

How do nutrients get into the microbial cell?

Having found a source of a given nutrient, a microorganism must:

- have some means of taking it up from the environment
- possess the appropriate enzyme systems to utilise it.

The plasma membrane represents a selective barrier, allowing into the cell only those substances it is able to utilise. This selectivity is due in large part to the hydrophobic nature of the lipid bilayer. A substance can be transported across the cell membrane in one of three ways, known as simple diffusion, facilitated diffusion and active transport.

In *simple* diffusion, small molecules move across the membrane in response to a concentration gradient (from high to low), until concentrations on either side of the membrane are in equilibrium. The ability to do this depends on being small (H_2O, Na^+, Cl^-) or soluble in the lipid component of the membrane (non-polar gases such as O_2 and CO_2).

Larger polar molecules such as glucose and amino acids are unable to enter the cell unless assisted by membrane-spanning *transport proteins* by the process of *facilitated* diffusion (Figure 4.1). Like enzymes, these proteins are specific for a single/small number of related solutes; another parallel is that they too can become saturated by too much 'substrate'. As with simple diffusion, there is no expenditure of cellular energy, and an inward concentration gradient is required. The transported substance tends to be metabolised rapidly once inside the cell, thus maintaining the concentration gradient from outside to inside.

Diffusion is only an effective method of internalising substances when their concentrations are greater outside the cell than inside. Generally, however, microorganisms find themselves in very dilute environments; hence the concentration gradient runs in the other direction, and diffusion into the cell is not possible. *Active transport* enables the cell to overcome this unfavourable gradient. Here, regardless of the direction of the gradient, transport takes place in one direction only, *into* the cell. Energy, derived from

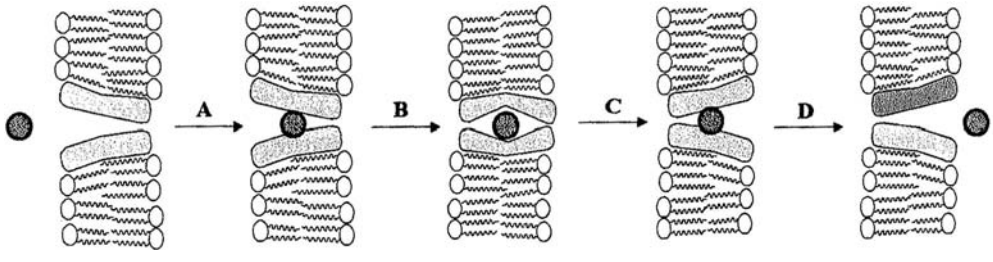


Figure 4.1 In facilitated diffusion, substances can move across the plasma membrane by binding to an embedded transport protein (shown shaded). No energy input is required, but the diffusion can only occur from an area of high concentration to one of low concentration. From Thomas, G: Medicinal Chemistry, an Introduction, John Wiley & Sons Inc., 2000. Reproduced by permission of the publishers

hydrolysis of adenosine triphosphate (see Chapter 6) is required to achieve this, and again specific transmembrane proteins are involved. They bind the solute molecule with high affinity outside of the cell, and then undergo a conformational change that causes them to be released into the interior. Procaryotic cells can carry out a specialised form of active transport called *group translocation*, whereby the solute is chemically modified as it crosses the membrane, preventing its escape. A well-studied example of this is the phosphorylation of glucose in *E. coli* by the phosphotransferase system. Glucose present in very low concentrations outside the cell can be concentrated within it by this mechanism. Glucose is unable to pass back across the membrane in its phosphorylated form (glucose-6-phosphate), however it can be utilised in metabolic pathways in this form.

Often it may be necessary to employ extracellular enzymes to break down large molecules before any of these mechanisms can be used to transport nutrients into the cell.

Laboratory cultivation of microorganisms

Critical to the development of microbiology during its 'golden age' was the advance in culturing techniques, enabling the isolation and pure culture of specific microorganisms. The study of pure cultures made it possible to determine the properties of a specific organism such as its metabolic characteristics or its ability to cause a particular disease. It also opened up the possibility of classifying microorganisms, on the basis of the characteristics they display in pure culture.

The artificial culture of any organism requires a supply of the necessary nutrients, together with the provision of appropriate conditions such as temperature, pH and oxygen concentration. The nutrients and conditions provided in the laboratory are usually a reflection of those found in the organism's natural habitat. It is also essential that appropriate steps are taken to avoid contamination (Box 4.1). In the next section we shall describe the techniques used to isolate and propagate microorganisms in the laboratory. The section refers specifically to the culture of bacteria; laboratory propagation of algae, fungi and viruses will be referred to in the chapters devoted to those groups.

Box 4.1 Aseptic technique

Most commonly used culture media will support the growth of a number of different bacteria. It is therefore essential when working in the microbiology laboratory that suitable precautions are taken to prevent the growth of unwanted contaminants in our cultures. These simple practical measures are termed *aseptic technique*, and it is essential to master them if reliable experimental results are to be obtained. Any glassware and equipment used is sterilised before work begins. Containers such as tubes, flasks and plates are kept open for the minimum amount of time, and the necks of bottles and tubes are passed through a flame to maintain their sterility. The wire loops and needles used to transfer small volumes of microbial cultures are sterilised by heating them to redness in a flame. Your instructor will normally demonstrate aseptic technique to you in an early practical session.

Obtaining a pure culture

Bacteria may be cultured using either liquid or solid media. Solid media are particularly useful in the isolation of bacteria; they are also used for their long-term storage. Liquid (broth) cultures are used for rapid and large-scale production of bacteria.

Microorganisms in the natural world do not live in pure cultures; they exist as part of complex ecosystems comprising numerous other organisms. The first step in the cultivation of microorganisms is therefore the creation of a pure culture. A key development for the production of pure cultures was the ability to grow microorganisms on a solid medium. Koch had noticed that when a nutrient surface such as cut potato was exposed to air, individual microbial *colonies* grew up, and he inferred from this that these had each arisen from the numerous divisions of single cells.

It soon became apparent that a number of organisms would not grow on potatoes, so Koch and his colleagues turned to gelatin as a means of solidifying a synthetic nutrient growth medium. Horizontal slabs were cut, and covered to help keep them free from atmospheric contaminants. Gelatin was a convenient means of solidifying media, as it could be boiled and then allowed to set in the desired vessel. There were two main drawbacks to its use, however; many organisms needed to be incubated at around body temperature (37 °C), and gelatin melted before this temperature was reached. Also, it was found that a number of bacteria were capable of utilising gelatin as a nutrient source, resulting in the liquefaction of the gel.

A culture consisting entirely of one strain of organism is called a *pure* or *axenic* culture. In theory, such a culture represents the descendants of a single cell.

A more suitable alternative was soon found in the form of *agar*. This is a complex polysaccharide derived from seaweeds, and was suggested by the wife of one of Koch's colleagues, who had used it as a setting agent in jam making. Agar does not melt until near boiling point; this means that cultures can be incubated at 37 °C or above without the medium melting. Moreover, when it cools, agar remains molten until just over 40 °C, allowing heat-sensitive media components such as blood to be added. In addition, most bacteria can tolerate a short exposure to temperatures in this range, so they too can be inoculated into molten agar (see pour plate method below). Crucially, agar is *more or less inert nutritionally*; only a very few organisms are known that are able to use agar as a food source; consequently, it is the near ideal setting agent, resisting both thermal and microbial breakdown. Agar soon became the setting agent of choice, and has remained so ever since; shortly afterwards, Richard Petri developed the two-part culture dish that was named after him, and which could be sterilised separately from the medium and provided protection from contamination by means of its lid,. This again is still standard equipment today, although the original glass has been largely replaced by presterilised, disposable plastic.

A petri dish is the standard vessel for short-term growth of solid medium cultures in the laboratory. It comprises a circular dish with an overlapping lid.

The standard method of obtaining a pure bacterial culture is the creation of a *streak plate* (Figure 4.2). A wire inoculating loop is used to spread out a drop of bacterial suspension on an agar plate in such a way that it becomes progressively more dilute;

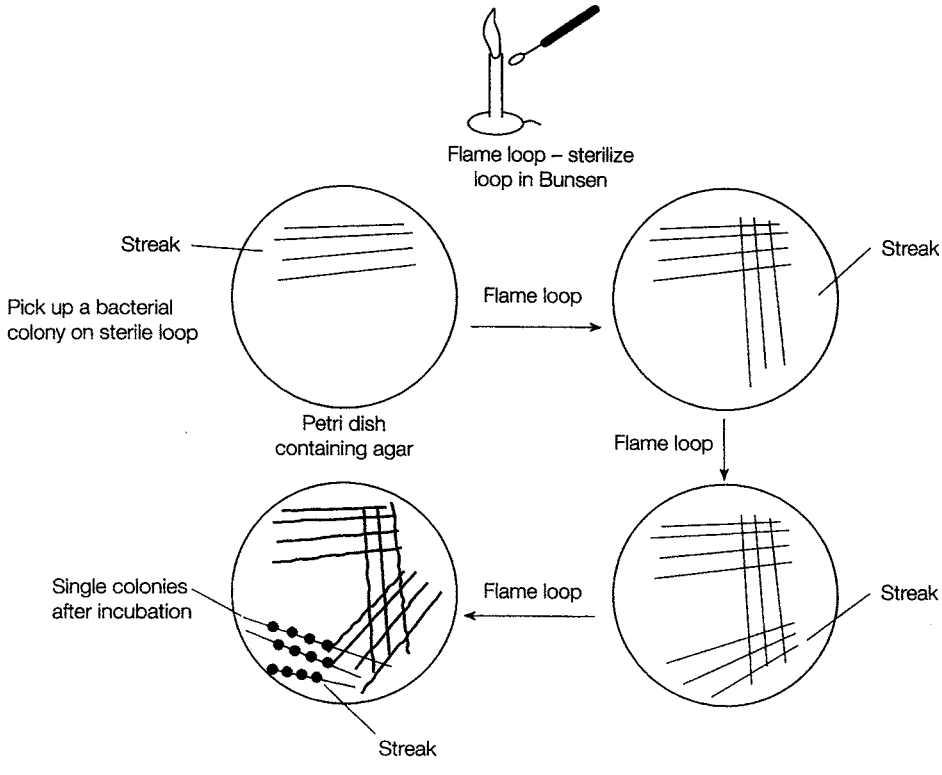


Figure 4.2 The streak plate. Streaking the sample across the agar surface eventually results in individual cells being deposited. Repeated cycles of cell division lead to the production of visible, isolated colonies. From Nicklin, J, Graeme-Cook, K & Killington, R: Instant Notes in Microbiology, 2nd edn, Bios Scientific Publishers, 2002. Reproduced by permission of Thomson Publishing Services

eventually, individual cells will be deposited on the agar surface. Following incubation at an appropriate temperature, a succession of cell divisions occurs, resulting in the formation of a bacterial *colony*, visible to the naked eye. Colonies arise because movement is not possible on the solid surface and all the progeny stay in the same place. A colony represents, in theory at least, the offspring of a single cell and its members are therefore genetically identical. (In reality, a clump of cells may be deposited together and give rise to a colony; this problem can be overcome by repeated isolation and restreaking of single colonies.)

An alternative method for the isolation of pure cultures is the *pour plate* (Figure 4.3). In this method, a dilute suspension of bacteria is mixed with warm molten agar, and poured into an empty petri plate. As the agar sets, cells are immobilised, and once again their progeny are all kept together, often within, as well as on, the agar. This method is especially useful for the isolation of bacteria that are unable to tolerate atmospheric levels of oxygen.

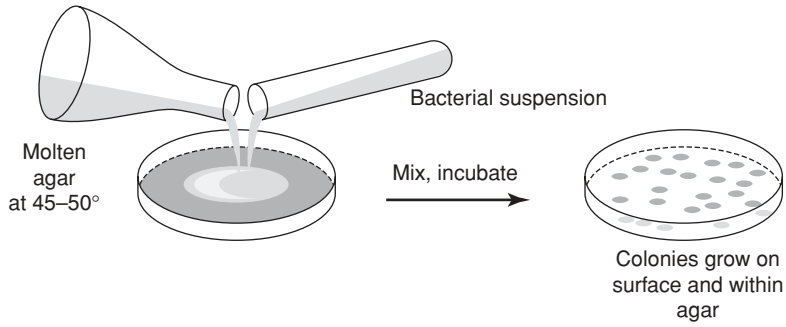


Figure 4.3 The pour plate. A sample of diluted bacterial suspension is mixed with molten agar and poured into a petri dish. Most bacteria can tolerate a short exposure to the agar, which is held at a temperature just above its setting point

Growth media for the cultivation of bacteria

A synthetic growth medium may be *defined*, that is, its exact chemical composition is known, or *undefined*. A defined growth medium may have few or many constituents, depending on the nutritional requirements of the organism in question. Examples of each are given in Table 4.3. An undefined or *complex* medium may have a variable composition due to the inclusion of a component such as blood, yeast extract or tap water (Table 4.4). Peptones are also commonly found in complex media; these are the products of partially digesting protein sources such as beef or casein. The exact composition of a complex medium is neither known nor critically important. A medium of this type would generally be chosen for the cultivation of *fastidious* bacteria such as *Neisseria gonorrhoeae* (the causative agent of gonorrhoea); it is easier and less expensive to supply the many nutrients required by such an organism in this form rather than supplying them all individually. Bacteria whose specific nutrient requirements are not known are also grown on complex media.

A defined medium is one whose precise chemical composition is known.

An undefined or complex medium is one whose precise chemical composition is not known.

A fastidious organism is unable to synthesise a range of nutrients and therefore has complex requirements in culture.

Whilst media such as nutrient agar are used to support the growth of a wide range of organisms, others are specifically designed for the isolation and identification of particular types. *Selective* media such as bismuth sulphite medium preferentially support the growth of particular bacteria. The bismuth ion inhibits the growth of Gram-positive organisms as well as many Gram-negative types; this medium is used for the isolation of the

A selective medium is one that favours the growth of a particular organism or group of organisms, often by suppressing the growth of others.

Table 4.3 Defined growth media

(a) Medium for *Acidithiobacillus ferrooxidans*	
$FeSO_4.7H_2O$	40 g
$(NH_4)_2SO_4$	2 g
KH_2PO_4	0.5 g
$MgSO_4.7H_2O$	0.5 g
KCl	0.1 g
$Ca(NO_3)_2$	0.01 g
Distilled H_2O (pH 3.0) to	1 litre

(b) Medium for *Leuconostoc mesenteroides*			
Glucose	25 g	Phenylalanine	100 mg
Sodium acetate	20 g	Proline	100 mg
NH_4Cl	3 g	Serine	50 mg
KH_2PO_4	0.6 g	Threonine	200 mg
K_2HPO_4	0.6 g	Tryptophan	40 mg
NaCl	3 g	Tyrosine	100 mg
$MgSO_4 \cdot 7H_2O$	0.2 g	Valine	250 mg
$MnSO_4 \cdot 4H_2O$	20 mg	Adenine	10 mg
$FeSO_4.7H_2O$	10 mg	Cytosine	10 mg
Alanine	200 mg	Guanine	10 mg
Arginine	242 mg	Uracil	10 mg
Aspartic acid	100 mg	Nicotinic acid	1 mg
Asparagine	400 mg	Pyridoxine	1 mg
Cysteine	50 mg	Riboflavin	0.5 mg
Glutamic acid	300 mg	Thiamine	0.5 mg
Glycine	100 mg	Ca pantothenate	0.5 mg
Histidine	62 mg	Pyridoxamine	0.3 mg
Isoleucine	250 mg	Pyridoxal	0.3 mg
Leucine	250 mg	*p*-Aminobenzoic acid	0.1 mg
Lysine	250 mg	Biotin	1 μg
Methionine	100 mg	Folic acid	10 μg
Distilled H_2O to	1 litre		

Examples of defined (synthetic) media for (a) the iron-oxidising bacterium *Acidithiobacillus ferrooxidans* and (b) the lactic acid bacterium *Leuconostoc mesenteroides*. Note how *L. mesenteroides* must be provided with numerous amino acids, nucleotides and vitamins as well as glucose as a carbon source, whereas *A. ferrooxidans* requires only mineral salts, including reduced iron to act as an energy source.

Table 4.4 Composition of an undefined growth medium

Calf brain infusion	200 g
Beef heart infusion	250 g
Proteose peptone	10 g
Glucose	2 g
NaCl	5 g
Na_2HPO_4	2.5 g
H_2O (pH 7.4)	To 1 litre

Brain heart infusion broth contains three undefined components. It is used for the culture of a wide variety of fastidious species, both bacterial and fungal.

pathogenic bacterium *Salmonella typhi*, one of the few organisms that can tolerate the bismuth. Specific media called *differential* media can be used to distinguish between organisms whose growth they support, usually by means of a coloured indicator. MacConkey agar contains lactose and a pH indicator, allowing the differentiation between lactose fermenters (red colonies) and non-lactose fermenters (white/pale pink colonies). Many media act both selectively and differentially; MacConkey agar, for example, also contains bile salts and the dye crystal violet, both of which serve to inhibit the growth of unwanted Gram-positive bacteria. Mannitol salt agar is also both selective and differential. The high (7.5 per cent) salt content suppresses growth of most bacteria, whilst a combination of mannitol and an indicator permits the detection of mannitol fermenters in a similar fashion to that just described. Sometimes, it is desirable to isolate an organism that is present in small numbers in a large mixed population (e.g. faeces or soil). *Enrichment* media provide conditions that selectively encourage the growth of these organisms; the use of blood agar in the isolation of streptococci provides an example of such a medium. Blood agar can act as a differential medium, in allowing the user to distinguish between haemolytic and non-haemolytic bacteria (see Chapter 7).

A differential medium allows colonies of a particular organism to be differentiated from others growing in the same culture.

An enrichment culture uses a selective medium to encourage the growth of an organism present in low numbers.

If we are to culture microorganisms successfully in the laboratory, we must provide appropriate physical conditions as well as providing an appropriate nutrient medium. In the next chapter, we shall examine how physical factors such as pH and temperature influence the growth of microorganisms, and describe how these conditions are provided in the laboratory.

Preservation of microbial cultures

Microbial cultures are preserved by storage at low temperatures, in order to suspend growth processes. For short periods, most organisms can be kept at refrigerator temperature (around 4 °C), but for longer-term storage, more specialised treatment is necessary. Using deep freezing or freeze-drying, cultures can be kept for many years, and then resurrected and re-cultured. Deep freezing requires rapid freezing to −70 °C to −95 °C, while freeze-drying (lyophilisation) involves freezing at slightly less extreme temperatures and removing the water content under vacuum. Long-term storage may be desirable to avoid the development of mutations or loss of cell viability.

Test yourself

1 Heterotrophic organisms acquire their carbon in an _____ form, whilst autotrophic organisms acquire theirs in an _____ form.

2 Some autotrophs can derive energy from the Sun; these are termed _____.

3 The passage of solutes into a cell across a concentration gradient is known as _____.

4 Agar has a high _____ point and a relatively low _____ point.

5 The use of agar as a setting agent was a crucial step in the development of _____ _____ techniques.

6 Successive divisions of a single cell lead to the formation of a _____ on a solid medium.

7 The chemical composition of an undefined medium is _____.

8 _____ organisms must have a wide range of organic nutrients supplied.

9 _____ media encourage the growth of chosen species, whilst _____ media prevent the growth of unwanted forms.

10 _____ (removal of the water content at low temperatures) is used in the preservation of microbial cultures.

5
Microbial Growth

When we consider growth as applied to a multicellular organism such as a tree, a fish or a human being, we think in terms of an ordered increase in the size of an individual. Growth in unicellular microorganisms such as bacteria, yeasts and protozoans, however, is more properly defined in terms of an increase in the size of a given *population*. This may be expressed as an increase in either the number of individuals or the total amount of *biomass*. Methods employed in the measurement of growth of unicellular microorganisms may be based on either of these. In this chapter we shall describe some of these methods, before considering the dynamics of microbial growth and some of the factors that affect it.

Biomass is the total amount of cellular material in a system.

Estimation of microbial numbers

Several methods exist for the measurement of bacterial numbers, most of which are also applicable to the enumeration of other unicellular forms such as yeasts. Such methods fall into two main categories: those that count total cell numbers, and those that count viable cells only.

Total cell counts are generally done by direct microscopic examination. A specialised glass slide is employed, which carries an etched grid of known area (Figure 5.1). The depth of the liquid sample is also known, so by counting the number of cells visible in the field of view, the number of cells per unit volume can be determined. The method may be made more accurate by the use of a fluorescent dye such as acridine orange, which binds to DNA, and hence avoids confusion with non-cellular debris. However, such methods cannot differentiate between living and non-living cells. Their usefulness is further limited by the fact that the smallest bacteria are difficult to resolve as individual cells by light microscopy. Other total cell count methods use cell-sorting devices, originally developed for separating blood cells in medical research. These pass the cell suspension through an extremely fine nozzle, and a detector registers the conductivity change each time a particle passes it. Again, no distinction can be made between viable and non-viable cells.

A *viable cell count*, on the other hand, is a measure of the number of *living* cells in a sample, or more specifically those capable of multiplying and producing a visible colony of cells. It is most commonly estimated by spreading a known volume of cell suspension onto an agar plate, and counting the number of colonies that arise after a period of