Nuffield Advanced Chemistry
BIOCHEMISTRY
Students' book

Contents

This students' book consists of the text of the Special Study with the experiments given separately at the end

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The examinable content of this Special Study is defined by the Edexcel specification. Clarification of the depth of treatment can be gauged by looking at these downloadable chapters and instructions for experiments, published in the Special Study section of the *Re:act* website http:/www.chemistry-react.org

The content of the comment and case study boxes does *not* have to be learnt.

Re:act provides study guides for each chapter, web-links, answers to frequently asked questions, and revision questions.

CHAPTER 1 INTRODUCING BIOCHEMISTRY

1.1 What is biochemistry?

Biochemistry, as its name suggests, is the area where the interests of biologists and chemists meet. Biochemists seek an understanding of the chemical structure of living organisms and the chemical processes which take place in them. They then hope to put this knowledge to good use.

Biochemistry has grown from the study of individual compounds, such as hormones, to the study of the networks of reactions involved in processes like photosynthesis. Today biochemists study how chemical processes take place in cells, how these processes are controlled, and how chemical compounds are used to pass on information from one generation of living organisms to the next.

Most of the biochemistry in this book dates from only the last 50 years, whereas the chemistry you are studying was developed over the last two centuries. An example is the work of the double Nobel prize winner Frederick Sanger who in 1955 described the very first sequencing of the amino acids in a protein, insulin, and then in 1977 published the full sequence of bases in the DNA of a virus. In 1997 he was the guest of honour at the opening of the Sanger Institute. One-third of the human genome project was carried out in the laboratories of this Institute.

1.2 Biochemistry and medicine

The relationship of biochemistry to medicine has always been close: advances in biochemistry result in new insights about our health, such as a better understanding of nutrition.

Although the causes are various, many diseases can be regarded as a breakdown in the normal chemical processes in our bodies. Doctors treated illnesses with plant extracts long before the active ingredients had been identified: an example is the treatment of a fever with willow bark, now replaced by aspirin. However, with an understanding of how a disease alters the chemistry of affected cells it becomes possible to ‘design’ molecules that are likely to correct a condition.

Another area where biochemistry has an important role is in the diagnosis of illness. Doctors can deduce a lot by taking your temperature, looking at your throat, and feeling for painful places under your skin. But nowadays you are often asked to provide samples of blood and urine which will be put through a set of biochemical tests to help decide what is wrong with you. Biochemical tests can reveal which chemical processes in your body are not working properly: doctors use test sticks to indicate the glucose level in urine as a preliminary test for diabetes, and diabetics can monitor their blood glucose levels in a similar way. For some babies the

importance of biochemical testing cannot be overestimated, because life-threatening conditions can be identified even before the symptoms appear – and for some conditions, such as phenylketonuria, that would be too late.

In the following chapters you will find some Case Studies which illustrate the relationship of biochemistry to medicine.

1.3 Molecular biology

The most recent benefits from biochemistry are related to the spectacular advances in our knowledge of DNA and the development of the branch of biochemistry called molecular biology. Molecular biology is concerned with the study of cells at the molecular level.

Inherited conditions

The origins of many inherited conditions have been traced to changes in the genes encoded in DNA, and this has lead to a much greater understanding of the nature of the conditions. However, understanding the cause does not automatically lead to a cure: people suffering from an inherited condition can often only be offered better treatment at present, not a cure.

DNA technologies

Whole new industries have sprung up based on DNA technologies. Useful characteristics can be transferred from one species to another by transferring pieces of DNA, creating what are known as transgenic organisms. The products obtained from such organisms can be as important as insulin, or as apparently frivolous as a naturally blue cotton for making jeans. When the products are proteins like insulin, the manufacturing processes require a new approach to the design of the equipment. If the wrong technique is used to stir the reaction mixture, the protein molecule may be affected and become inactive; rather like shaking milk can produce lumps of cream.

Understanding evolution

Another area where DNA techniques are making a contribution is in our understanding of evolution. The closer species are in their evolutionary history the more similar their DNA is; the further apart the more dissimilar their DNA. Some questions about evolution have been answered by DNA studies. In 1997 German scientists re-examined the bones of a Neanderthal man who lived in the Neander valley 30 000 to 100 000 years ago. They found that DNA extracted from the bones had so many differences from our DNA that we are most unlikely to have descended from the Neanderthals.

However, the advance in DNA technology raises important issues. There is concern about the impact on the way we live, about the dangers of genetic modification as well as the benefits. We shall return to this point in the last chapter after you have learnt more about biochemistry.

1.4 Experimental work in biochemistry

When you neutralize 25 cm^3 of 1.0 M hydrochloric acid with 1.0 M sodium hydroxide in the laboratory, the volume of sodium hydroxide required will always be 25 cm^3. Furthermore the mass of sodium chloride obtained, if the solution is allowed to evaporate to dryness, will always be the same. The same experiment can be repeated in any laboratory in the world and the same results obtained.

You might expect the same to be true for biochemistry experiments. However, there are often large differences between the results of a biochemistry experiment apparently carried out in identical ways. You will probably find during this Special Study that different groups doing the same experiment may get very different results.

Why should this be? The chemical reactions which a biochemist studies are no different from the reactions that an organic chemist studies. However, biochemical reactions are catalysed by enzymes, which are very sensitive to the smallest change in conditions. Furthermore biochemists often have to start their experiments by obtaining an enzyme from living cells. The amount of enzyme obtained will depend on how much was present in the original cells, and that will certainly vary from one source to another.

For example, the activity of your enzyme preparations in the experiments in Chapter 2 will depend on how effectively you carry out the extraction process. Insufficient breaking up of the cells will result in little enzyme being released and most of the enzymes will still be locked away within intact cells.

Furthermore, any delays in using your extract will result in the extract having reduced catalytic activity. So you must expect a range of results in your practical work and on occasions your experiment may give unexpected results.

COMMENT

Biochemists have to measure very small biological structures and they often use micrometres for sizes and microlitres for volumes. A micrometre is a millionth of a metre (1 µm = 10^{-6} m). Atoms of course are even smaller and usually measured in nanometres (1 nm = 10^{-9} m).

Example	**Diameter / µm**
Hydrogen atom	0.00007
Carbon atom	0.0002
Lysozyme enzyme	0.003
Polio virus	0.03
Cell nucleus	3
Human cell	50

But in contrast the total unravelled length of DNA in a human cell is 2 metres!

CHAPTER 2 ENZYMES

2.1 The structure of cells

The cell is the fundamental unit of life. Most living organisms are composed of cells containing nuclei in which the genetic material is stored. There are single-celled organisms, such as amoeba, but the familiar plants and animals we see every day are built up of many millions of cells. Within each such organism there are many types of cells, with different chemical compositions, structures, and functions. Muscle cells contain contractile fibres. Red blood cells contain haemoglobin to transport oxygen.

Similarities between cells

However, despite the apparent diversity, all plant and animal cells have a remarkably similar basic composition and structure, and many of the chemical changes which take place in the cells are essentially the same. For instance, the pathways by which glucose molecules are broken down to give energy are virtually the same in both a yeast cell and in a human muscle cell. Minor differences do exist, but they are not so striking as the similarities.

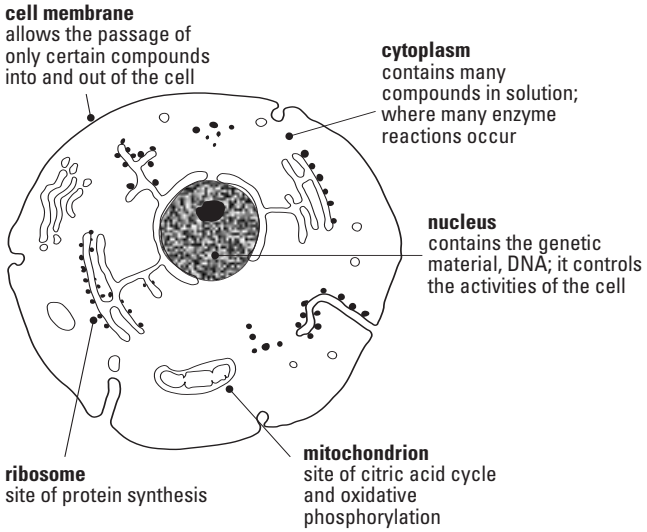


Figure 2.1 General features of animal cells.

Cell structure

Living cells have all been found to contain inorganic ions such as potassium, chloride and phosphate ions, and small organic molecules such as pyruvic acid and glycerol (see box).

A major component of the cell content, however, are the very large molecules with molar masses from tens of thousands to several million. Among the most important of these are the **enzymes**, nearly all of which are proteins, and the **nucleic acids** (see Chapter 4).

The enzymes are responsible for the catalysis of all chemical reactions in the cell. The nucleic acids contain the information which controls the synthesis of all proteins, and are the physical means of passing this information from one generation to the next.

Note about naming biochemicals

Pyruvic acid is the trivial name for the acid, 2-oxopropanoic acid, and glycerol is the trivial name for the alcohol, propane-1,2,3-triol. Biochemists prefer trivial names to systematic chemical names because of the complexity of many of the molecules met in biochemistry.

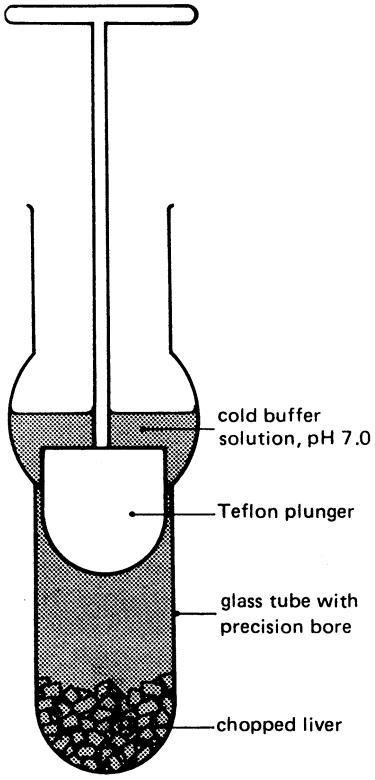
$CH_3–CO–CO_2H$	$CH_2OH–CHOH–CH_2OH$
2-oxopropanoic acid	propane-1,2,3-triol

Many biochemicals contain carboxylic acid groups. They are therefore weak acids but at the pH of our bodies they exist as their anions. For instance, in cells at a pH of about 7.2 pyruvic acid exists as the pyruvate anion and is called **pyruvate**.

$CH_3COCO_2H \rightleftharpoons CH_3COCO_2^- + H^+ \quad K_a = 3.2 \times 10^{-3}$ mol dm^{-3}

pyruvic acid	pyruvate anion
weak acid	conjugate base

The cells of both plants and animals are all bounded by a **membrane**, and they contain a variety of other structures which are surrounded by a fluid, the **cytoplasm**. Most of the structures inside a cell have definite functions which are the same for both animal and plant cells. Plant cells have, in addition, a cell wall surrounding the cell membrane and they often contain chloroplasts, the site of photosynthesis.



Extracting enzymes from cells

Biochemists depend on the techniques used in chemistry, for separating organic materials, for analysis, and for measuring rates of reaction. By studying the reactions in cells, biochemists are building an understanding of how cells function. But during biochemical experiments, the delicate nature of living material has to be constantly born in mind. Like any other scientist, a biochemist wants to produce experimental results that can be reproduced by other research groups. The following example highlights the considerable problems facing biochemists separating mitochondria to study an enzyme.

Selecting a suitable material to study

The activity of a particular enzyme depends on many factors; a biochemist must decide precisely which biological material will be used in every experiment. They must first select a suitable species of plant or animal for study, and they must then decide which tissue or organ to study. Animal material is only used when there is no other alternative. Samples must be kept cool in a buffer solution to stop the contents of the cells breaking down.

Disrupting the cell membranes

The biochemist must gently break open the cells to release their contents, in a process called homogenization (figure 2.2). This must be done without damaging the cells' contents. Cells differ greatly in the strength of their cell membranes and cell walls.

Yeast cells for instance have a hard cell wall which is very difficult to break. Plant cells also have strong outer walls; they may be broken open using a sharp rapidly rotating metal blade such as the liquidizer in a food processor.

Homogenization produces a mixture of undamaged cells, membranes, mitochondria, ribosomes, cytoplasm, and nuclei which is known as a **homogenate**, even though it is far from being homogeneous.

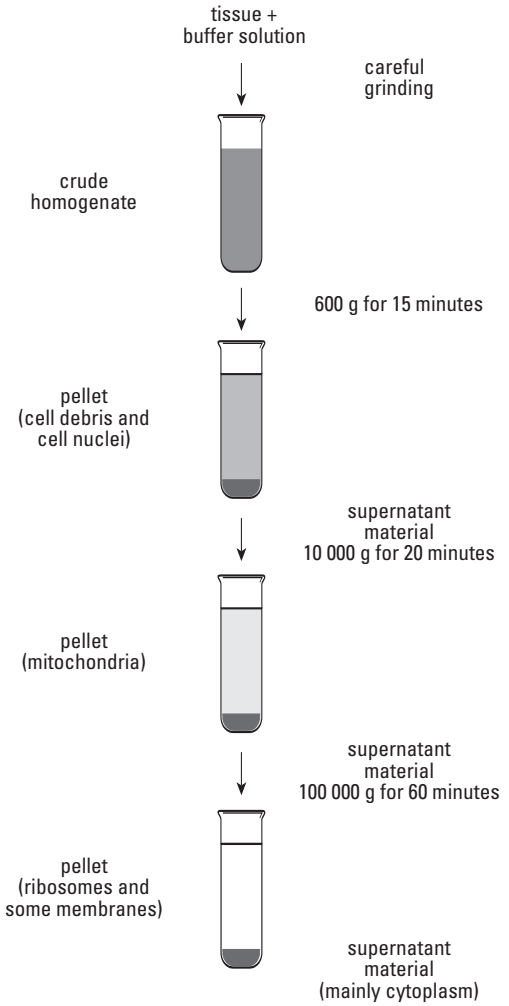


Figure 2.2 (top) The homogenization of cells. The diameter of the tube is only 0.025 cm larger than that of the plunger. Liver cells are broken open as they pass between the plunger and the glass wall but mitochondria are small enough to pass through undamaged.

Figure 2.3 (right) Stages in the separation of liver cells' constituents

Separating the cell contents

Now the homogenate is centrifuged in several stages (figure 2.3). Very large forces are needed to separate the smallest structures, but refrigerated centrifuges, capable of spinning large volumes of material at high speed for prolonged periods, are available in biochemistry laboratories.

Isolating a pure enzyme

While many enzyme studies may be done with crude homogenates, biochemists often wish to isolate pure enzymes for use in industry or medicine. Various chromatographic techniques can be used in the purification. An example is column chromatography on a gel, which separates proteins according to their size. Usually no one method on its own produces a pure protein; a combination of techniques is necessary if a protein is to be freed from all contaminating material.

2.2 Enzymes: proteins as catalysts

Proteins have a critical role to play in nearly all biochemical processes. They can form part of the cellular structure, for example collagen in bones, and they can act as a store of food, for example albumen in eggs. They can be used to transport small molecules around the body, as haemoglobin does in our blood. Complex processes, like muscle contraction, all depend on specific protein molecules. Most important of all, protein molecules act as enzymes, catalysing the thousands of chemical reactions that take place in a single cell.

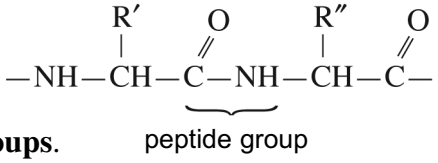
Enzyme activity

Biochemists often talk about **enzyme activity**. Enzymes are catalysts; they speed up otherwise slow reactions. Enzyme activity is simply the rate at which a chemical reaction takes place in the presence of an enzyme; it is therefore measured in terms of the rate at which the reactant disappears or product appears, often in mol min^{-1}.

The term **substrate** is used to describe any reactant in an enzyme catalysed reaction. The substrate for catalase (Experiment 2) is hydrogen peroxide.

The structure of enzymes

The structure for all proteins is described in terms of their primary, secondary and tertiary structure.



Primary structure

This is the order in which amino acids are linked together in a protein. You should be able to recall that amino acids are linked by **peptide groups**.

Secondary structure

This is the way the amino acid polymer is folded or coiled. Some of it may be in the form of an α-helix, in which the structure is maintained by hydrogen bonding between peptide groups.

Tertiary structure

The structure of the molecule: this is a description of the overall three-dimensional shape of the protein. Proteins have complex but precise shapes maintained by bonds within and between the protein chains.

Bonding within and between chains

Many amino acid side chains can form hydrogen bonds. But it is not just hydrogen bonds which maintain the structure of a protein. The carboxylic side chains of some amino acid side chains, and the amine groups of others, lose or gain protons at pH 7, forming negative and positive ions. These can form strong ionic bonds, although there are only relatively few of them in most proteins. Covalent bonds, known as disulphide bridges, —S—S—, can form when two cysteine amino acid side chains react. There are also van der Waals forces between the large non-polar side chains, which often contain benzene rings. Although the individual van der Waals forces are weak, they make the greatest overall contribution to the stability of the three-dimensional shape of proteins.

Amino acid	Side chain	Side chain	Amino acid	Bond between side chains
Alanine	Ⓐ—CH_3	CH_3>CH—Ⓐ	Valine	van der Waals
Serine	Ⓐ—CH_2OH	NH_2COCH_2—Ⓐ	Asparagine	Hydrogen
Aspartic acid	Ⓐ—$CH_2CO_2^-$	$^+NH_3(CH_2)_4$—Ⓐ	Lysine	Ionic
Cysteine	Ⓐ—CH_2—S—S—CH_2—Ⓐ		Cysteine	Covalent

Figure 2.4
The bonds that can occur between amino acid side chains.
Ⓐ represents the $NH_2—\underset{|}{CH}—CO_2H$ group

Lysozyme structure

Take lysozyme which has a typical enzyme structure. Lysozyme is a water-soluble enzyme present in tears, egg white, and nasal mucus. It has an important role in protecting us from bacteria.

The enzyme is a compact molecule, with dimensions 4.5 x 3 x 3 nanometres. Look at figure 2.5 on the next page and you will see that the protein chain starts with an amine group and finishes with a carboxylic acid group. You should also be able to identify three regions of α-helix where the protein chain is folded in a regular way; but most of the chain has an irregular arrangement.

In general, polar side groups lie on the surface of the molecule, where they can interact with water, while most of the non-polar side groups lie within the molecule. Some polar side groups do lie within the molecule and these seem to have a critical role to play in catalysis. Lysozyme acts as a catalyst for the hydrolysis of the carbohydrate cell walls of bacteria. The enzyme has an elongated cleft on the surface, into which a chain of six sugars can be fitted (shown in shadow outline in the diagram). This is known as the **active site**. This type of structure is typical of many enzymes.

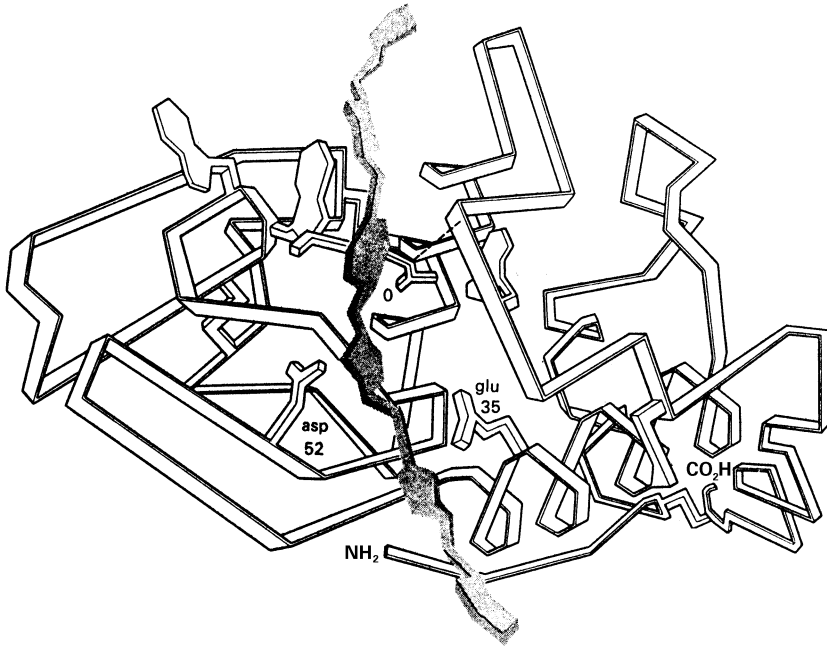


Figure 2.5
3-dimensional structure of lysozyme, with a carbohydrate molecule shown in the active site.

Generalized mechanism of enzyme action

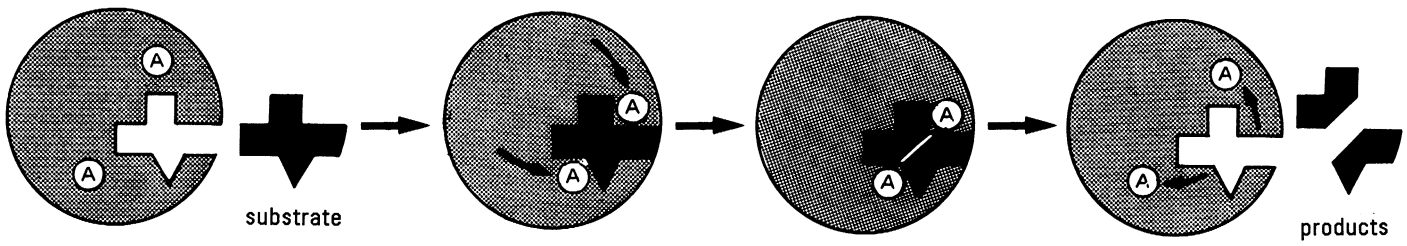
Enzymes are able to catalyse reactions in aqueous solutions under exceptionally mild conditions of temperature and pH. The range of reactions catalysed by enzymes is quite remarkable, for if the chemist confined experiments to such conditions the accomplishments would be very limited.

The complicated folding of the protein chain in enzymes gives rise to a binding site which is complementary in shape to the substrate. This region is described as the active site. It is generally a cleft or crevice on the surface of the enzyme. The site is lined with specific groups which can form weak interactions with particular atoms or groups of the substrate molecule, thus holding it correctly in place. In many cases the presence of the substrate in the binding site induces a small but significant change in the three-dimensional shape of the enzyme.

This change in shape results in specific amino acid side chains being brought up to the region of the substrate molecule where the reaction is to take place. Amino acids in the active site may act as proton donors or acceptors, or they may provide a suitable environment for the reaction, either polar or non-polar.

Once the reaction is complete, the products diffuse from the active site, and the enzyme returns to its original shape.

Figure 2.6 The mechanism of enzyme action. Ⓐ represents an amino acid required for catalysis. The binding of the substrate results in a change in the shape of the enzyme, and critical amino acids are brought close to the substrate.



2.3 Factors affecting enzyme activity

Effect of pH

Enzymes, in contrast to many other catalysts, are very sensitive to small changes in pH. Enzymes contain groups that can act as acids and bases, donating or accepting protons, when the pH changes. For example as the pH decreases, more and more of the negatively charged carboxylate groups, —CO_2^-, act as bases and accept protons. There are fewer and fewer negative ions in the enzyme.

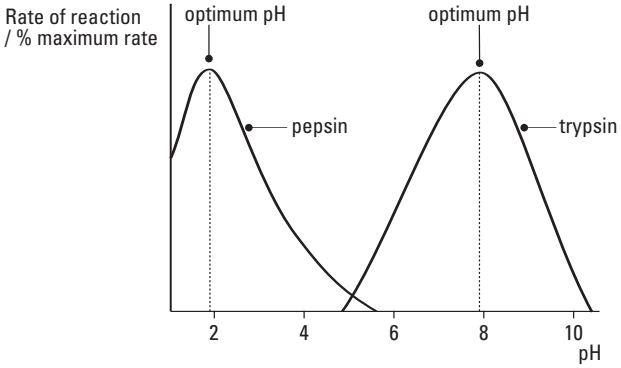


Figure 2.7 The effect of pH on the rate of two enzyme-catalysed reactions.

The change in the number of charged groups in the protein affects the number of ionic bonds and hydrogen bonds present. A precise shape is required for maximum enzyme activity, and any change in shape is likely to make the enzyme less active.

Sometimes acidic or basic groups are specifically required for catalysis. You should recall that acids can catalyse the hydrolysis of esters. Carboxylic acid side chains in an enzyme can catalyse reactions in a similar way by acting as proton donors. However, an increase in pH may result in the loss of the proton from the critical amino acid side chain. If the proton has been lost, the enzyme can no longer offer an alternative pathway for the reaction and it has become inactive.

The optimum pH value for most enzymes lies between pH 5 and pH 9, but there are some exceptions. For instance, pepsin, a enzyme secreted in the stomach, has a pH optimum of about 2. This ensures maximum activity in the acid conditions of the stomach.

Effect of temperature

Temperature has two effects on the rate of an enzyme-catalysed reaction. An increase in temperature always increases the number of collisions which have sufficient energy to bring about the reaction. Initially the rate of the reaction increases with increasing temperature until a maximum rate is achieved. However, beyond this temperature the rate of reaction decreases, often dramatically, and this loss of activity is often irreversible.

The activity of an enzyme depends on its precise shape. Many of the forces maintaining that structure are extremely weak. As the temperature rises, the weak bonds between the chains break as molecular movement increases. At quite moderate temperatures, the molecules start to unravel and become disordered. On cooling, the molecular chains are most unlikely to reform their original highly ordered three-dimensional shapes. When a protein loses its essential three-dimensional shape, it has been **denatured**.

Some enzymes are much more susceptible to temperature change than others; each enzyme is said to have a temperature optimum at which it works best.

There are microbes living in the hot springs of the Yellowstone National Park; one micro-organism has been isolated which grows best at about 105 °C and can survive in superheated water at 113 °C. Enzymes which have unusually high optimum temperatures are being used increasingly in industry. One example is Taq polymerase; this can be used at 60–90 °C to increase the amount of DNA available for DNA fingerprinting.

By contrast other bacteria live in Antarctic ocean water; one species grows best at only 4 °C, and cannot reproduce if the temperature is above 12 °C.

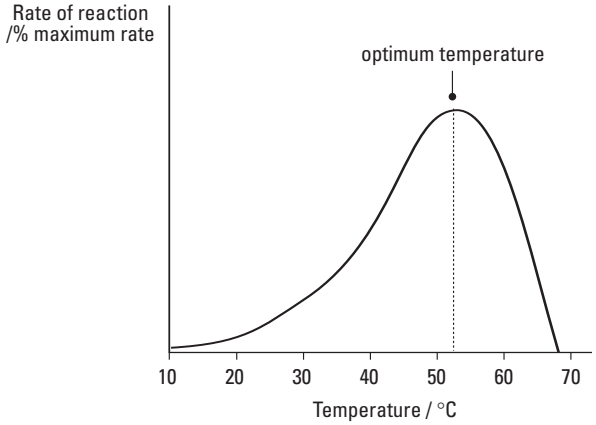
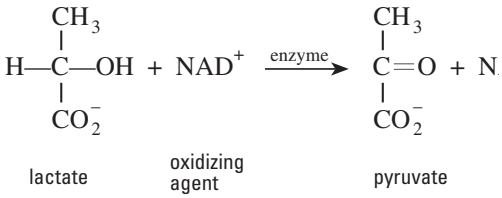


Figure 2.8 The effect of temperature on the rate of an enzyme catalysed reaction.

Effect of substrate concentration

You should use the results of Experiment 2 to write your own notes on the influence of substrate concentration on enzyme activity.

Specificity



Many enzymes will only act on one specific compound and will only catalyse one type of reaction. The active site has a shape which is complementary to that of the substrate. Furthermore specific bonds will hold the substrate in place. So only molecules of the correct shape and with the correct groups for binding can act as substrates. Activity may even be limited to one particular optical isomer: the enzyme which oxidizes lactate to pyruvate is completely specific for the L optical isomer.

Cofactors

Most enzymes have been found to contain non-protein groups, which are essential for catalytic activity. Catalase, an enzyme from liver, contains haem (see figure 3.7); in the absence of bound haem, the enzyme is unable to decompose hydrogen peroxide. A number of enzymes contain zinc(II), iron(II) or (III), or copper(II) ions.

Inhibition

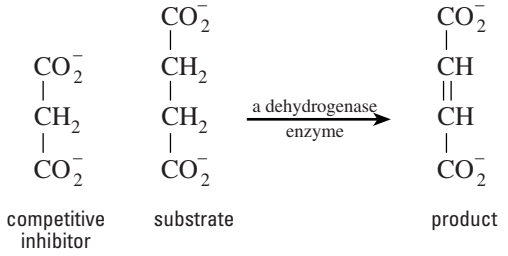
Enzymes are inactivated by any chemical which brings about the disruption of their three-dimensional shape. Strong acids and alkalis or detergents denature proteins and therefore affect enzyme activity. But the activity of many enzymes may also be reduced by specific compounds which have no denaturing effect on proteins. Such chemicals are described as **inhibitors**. The inhibition may be reversible or irreversible depending on whether or not the inhibitor becomes strongly bonded to the protein.

Irreversible inhibitors

Irreversible inhibitors often bind covalently to the enzyme. They disrupt the essential three-dimensional shape of the enzyme, or react with an amino acid side chain which is needed for catalysis. They can be considered as 'enzyme poisons' and include metal ions such as silver and mercury, and drugs designed to permanently block the action of an enzyme.

Competitive inhibitors

Competitive inhibitors are compounds which are similar in structure to an enzyme's substrate. They bind to the active site of the enzyme and they remain there unchanged. If, however, the concentration of the substrate is greatly increased, the inhibitor can be displaced and inhibition can be overcome. It is this property from which the term 'competitive' arises, in that there is competition between substrate and inhibitor for the active site. Malonate is an inhibitor which competes with the substrate succinate for a particular active site; furthermore it cannot react as it cannot be dehydrogenated to the product, fumarate.



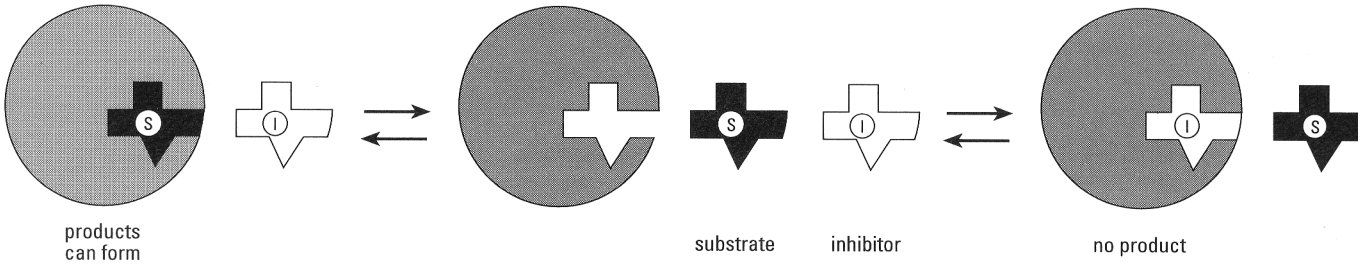


Figure 2.9 Competitive inhibition

Allosteric inhibitors

Finally, some enzymes have separate binding sites for a specific inhibitor. The presence of inhibitor in the inhibitor binding site is believed to alter the three-dimensional shape of the enzyme; the modified enzyme has reduced catalytic activity. However, since the inhibitor only binds weakly to the binding site, the inhibition is reversible. If the concentration of the inhibitor falls, the enzyme–inhibitor complex dissociates, the optimal three-dimensional shape required for catalysis is re-established, and full enzyme activity is restored. Such inhibition, which plays an important role in the regulation of biochemical processes, is known as **allosteric inhibition** (Greek: *allos stereos* other place).

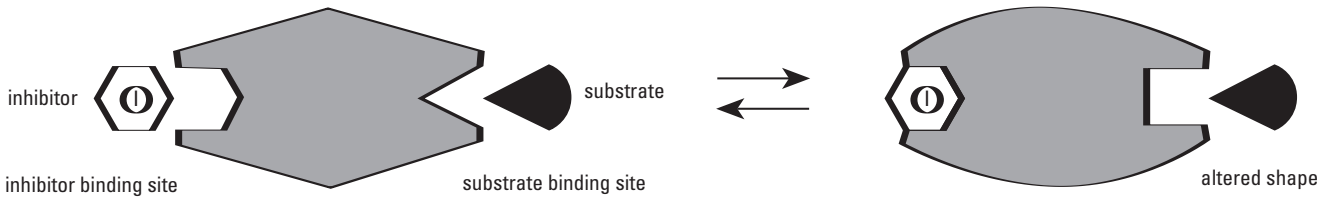


Figure 2.10 Allosteric inhibition. The inhibitor causes a change in the shape of the active site and the enzyme becomes inactive.

See Experiments 1 and 2 for investigating enzymes in action.

2.4 Immobilized enzymes in industry

The production of products like bread and beer predates our knowledge of even the existence of enzymes in the processes involved. However, the modern use of enzymes has revolutionized many industrial processes and the quality of their products. The benefits brought to industry by the use of enzymes are increased productivity, lowered costs, and reduced amounts of chemical waste for disposal.

Why immobilize enzymes?

Batch reactions using free, soluble enzymes are used in many industrial processes. The enzyme and the substrate are mixed at a suitable temperature in a reactor vessel and left until the required amount of product has been formed. Generally no attempt is made to recover the enzyme because it is too costly to do so. In many cases the enzyme preparations are expensive, and so only small quantities are used in each batch. Lengthy reaction times are therefore necessary, and this may result in the extensive formation of unwanted products due to side reactions.

For prolonged use in a continuous process or repeated use in a batch process, an enzyme needs to be immobilized, that is, attached to a solid surface so that it can be separated from the reaction mixture. Initially the cost of producing an immobilized enzyme system is greater than that of producing a soluble enzyme preparation for the same reaction. However, the insoluble enzyme may be packed into columns and used continuously for long periods.

Alternatively, the enzyme may be re-isolated simply by centrifugation or filtration after use in a batch reactor; it may then be re-used several times. Higher enzyme concentrations are possible; shorter reaction times reduce both the operational costs and the need for extensive purification of the product.

Methods of immobilization

Many methods of immobilizing soluble enzymes have been attempted, with varying degrees of success. The enzyme can be bound to a solid support, either by weak intermolecular forces or by strong covalent bonding.

Adsorption onto an insoluble support, such as porous glass

The protein is held in place by ionic interactions between charged amino acid side chains and charged groups on the support material, and van der Waals interactions between non-polar amino acid side chains and non-polar areas of the support. The immobilized enzyme is easily produced, but since the bonding between the enzyme and the support system is weak, there is a danger that the enzyme may be washed off the support.

Figure 2.11a
Enzyme immobilisation:
Adsorption onto an insoluble matrix, e.g. porous glass

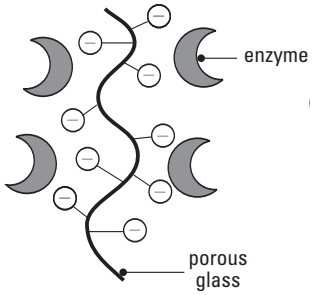
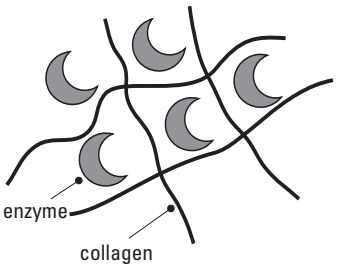


Figure 2.11 (and on the next page) Methods of enzyme immobiliation

Trapping the enzyme with a gel such as collagen

A solution containing the enzyme and a water-soluble monomer is prepared and the monomer is allowed to polymerize. The large enzyme molecules are trapped within a complex network of polymer molecules, but small molecules such as the substrate are able to penetrate the network to interact with the enzyme. Therefore enzymes which act on substrates of high molar mass cannot be used. This technique is inexpensive, and the immobilized enzymes can often be used for a long time.

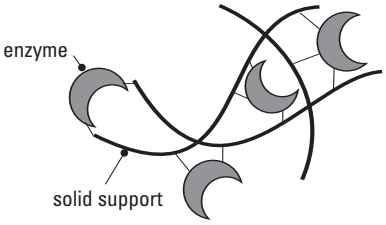
Figure 2.11b
Enzyme immobilisation:
Entrapment within a gel, e.g. collagen



Covalent binding onto a solid support such as celulose or nylon

One or more of the enzyme side chains, containing usually an —NH_2, —OH, or —CO_2H group, react covalently with the support material. The enzyme can show increased stability, and its active site remains readily accessible to the substrate. However, the production of a suitable solid support with reactive groups can be expensive.

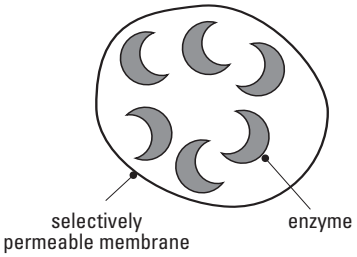
Figure 2.11c
Enzyme immobilisation:
Covalent binding onto a solid support, e.g. cellulose or nylon



Encapsulation behind a permeable membrane, for example a membrane of nylon

The enzyme is enclosed in a membrane which allows substrate and product molecules to pass freely but will retain large enzyme molecules. Production of the immobilized enzyme can be expensive, but this is a good method of recovering product free from enzyme.

Figure 2.11d
Enzyme immobilisation:
Encapsulation behind a selectively permeable membrane, e.g. nylon



The separation of the chiral forms of an amino acid

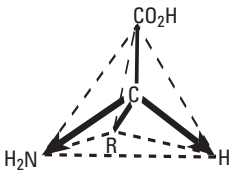
When an amino acid is prepared by chemical synthesis the result is usually a mixture of both chiral forms, the D-form and the L-form. An enzyme can be used to isolate the biologically useful L-amino acid from a mixture of the acyl D- and L-amino acids.

The first industrial use of an immobilized enzyme has been to produce L-amino acids from a synthetic racemic mixture of the D- and L-forms. The enzyme used is extracted from a fungus. The enzyme is absorbed onto a chemically treated polysaccharide where it is held in place by ionic bonding. The immobilised enzyme is packed in columns and can be used continuously for several weeks. Immobilisation does not affect the specificity of the enzyme, but it does reduce its activity.

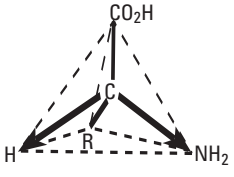
The amino acids are acylated before being passed through the columns. The enzyme selectively de-acylates the L-form. The free L-amino acid can then easily be separated from the D-form which is still acylated.

The acylated D-from is recycled by being treated to turn it into a racemic mixture of the two forms ready put through the process again.

The process based on an immobilized enzyme produces L-amino acids continuously at a much lower cost than using a batch process with free, soluble enzyme.



L-amino acid
(occurs naturally)



D-amino acid
(made in the laboratory)

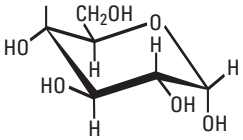
Figure 2.12 Chiral forms of an amino acid

The production of high fructose syrup

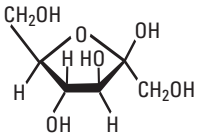
Sucrose, ordinary sugar, is our most commonly used sweetener but for use in the food industry starch is a cheaper source than from beet or sugar cane. However, the hydrolysis of starch by acids produces glucose, which has only about 40% of the sweetness of sucrose. But glucose can be converted to fructose which is considerably sweeter than sucrose.

In the production of high fructose syrup, starch from maize (corn) is hydrolysed to glucose, which is then partially converted to fructose using an enzyme. The result is a mixture of glucose and fructose which is similar in sweetness to sugar.

Enzymes which convert glucose to fructose are readily available from bacteria and the enzyme glucose isomerase has proved easy to immobilize. The immobilized enzyme is used in continuous reactors at 60–65 °C and pH 7 for over 1000 hours. The process has dramatically reduced the cost of producing high fructose syrup.



glucose



fructose

Figure 2.13
Structures of two sugars

CHAPTER 3 METABOLISM

The chemical processes which take place in living organisms are given the general term metabolism. Many of these processes are enzyme-catalysed reactions involving the hydrolysis and oxidation of the compounds in food to simpler molecules. Other processes are concerned with the production of complex molecules from the simple molecules obtained from food. Living organisms have to achieve a difficult energy balance. The digestion of food to simpler molecules is likely to have a favourable total entropy change (ΔS_{total} positive), while the building of complex molecules will have an unfavourable entropy change (ΔS_{total} negative).

Metabolic pathways

The movement of molecules through the chemical processes in living organisms is summarized in **metabolic pathways**. Each metabolic pathway consists of a sequence of chemical reactions, and each reaction is catalysed by a specific enzyme. There are four important aspects.

- Digestion and absorption: food is broken down into simpler molecules which are small enough to pass into the bloodstream.
- Synthesis of biochemical compounds: some of the simpler molecules produced by digestion are converted to other compounds, such as acetyl-CoA which plays a central role in metabolism.
- Reactions which are energetically favourable: these reactions provide the energy required for other metabolic processes: the overall result is often the oxidation of organic compounds to carbon dioxide and water.
- Reactions which are energetically unfavourable: these reactions are usually involved in the synthesis of compounds with complex structures, such as proteins.

3.1 The oxidation of food

The main components of the diet of mammals such as ourselves are carbohydrate, protein and fat. The carbohydrates in our diet are the main source of energy for all our living cells (and the same is true for all plants and animals). The carbohydrate glucose can be oxidized in almost all cells, in reactions involving the use of enzymes as catalysts. When glucose is fully oxidized, a large amount of energy is liberated (data at 298 K).

$$C_6H_{12}O_6(s) + 6O_2(g) \rightarrow 6CO_2(g) + 6H_2O(l)$$

$$\Delta H = -2800 \text{ kJ mol}^{-1}$$

$$\Delta S_{total} = +9600 \text{ J mol}^{-1} \text{ K}^{-1}$$

Metabolic pathways in cells allow this energy to be released in a controlled and useable manner.

In mammals and birds, some of the energy from the catalytic oxidation of glucose in cells is used to maintain the body temperature. Some of the available energy is needed for doing mechanical work, for instance, in muscle cells. During growth and development, much energy is required for the synthesis of cell constituents. In mature cells the chemical composition remains relatively constant, but there is still a constant turnover of many components with energy needed for their synthesis.

This, and the next, section considers how the energy from the oxidation of food is made available for synthetic reactions.

The oxidation of food can be divided into three main stages:

1 the hydrolysis of food into smaller molecules, such as glucose

2 the further breakdown of these molecules within individual cells

3 the oxidation of compounds in the citric acid cycle to carbon dioxide.

These three stages will be considered in turn.

1 The hydrolysis of food: digestion

The main bulk of food is in polymer form as polysaccharides (carbohydrate), protein, or triglycerides (fats). These large molecules must be split into smaller molecules by enzymes in the stomach and small intestine before they can be absorbed.

Proteins are split to peptides and eventually to amino acids by proteases.

Polysaccharides are hydrolysed with the help of the enzyme amylase to disaccharides, and thence to monosaccharides (mainly glucose).

Fats are digested by the enzyme lipase, which produces fatty acids (carboxylic acids) and glycerol (propane-1,2,3-triol).

Most of these products are soluble in water, whereas many of the original food polymers were insoluble.

2 The production of acetyl-CoA from glucose

First glucose is converted to pyruvic acid. This whole process, the conversion of glucose to pyruvic acid in cells, is known as **glycolysis**.

There are ten steps in the pathway, each of which is catalysed by a specific enzyme, but only one of the steps is an oxidation reaction.

$$\text{glucose } (C_6H_{12}O_6) \xrightarrow{\text{10 steps}} 2 \text{ pyruvate } (2C_3H_3O_3^-)$$

In cells at a pH of about 7.2, pyruvic acid exists as the pyruvate anion and is called ‘pyruvate’.

Next the pyruvate formed from glucose reacts to form acetyl-CoA. Acetyl-CoA is the name given to a compound formed when a complex organic molecule, coenzyme A, combines with an ethanoyl group, $CH_3CO—$, sometimes called the acetyl group. The formation of acetyl-CoA from pyruvate is an oxidation reaction. Carbon dioxide is also produced.

So from each mole of glucose:

2 pyruvate + 2 coenzyme-A → 2 acetyl-CoA + 2 carbon dioxide
($2C_3H_3O_3^-$) (2CoA) ($2CH_3CO–CoA$) ($2CO_2$)

(Remember 1 mole of glucose produced 2 moles of pyruvate):

3 The oxidation of acetyl-CoA in the citric acid cycle in mitochrondria

Acetyl-CoA can be used as the building block for many useful biochemicals, particularly fatty acids, but most acetyl-CoA enters the citric acid cycle. The remaining 4 carbons from the glucose are now oxidized to carbon dioxide, and the coenzyme A is regenerated for reuse in a cycle involving eight steps. From each molecule of glucose:

$$2CH_3CO–CoA \xrightarrow{8\ \text{steps}} 4CO_2 + 2CoA$$

The overall result of the glycolysis pathway and the citric acid cycle is the complete oxidation of glucose to carbon dioxide and water (see figure 3.1).

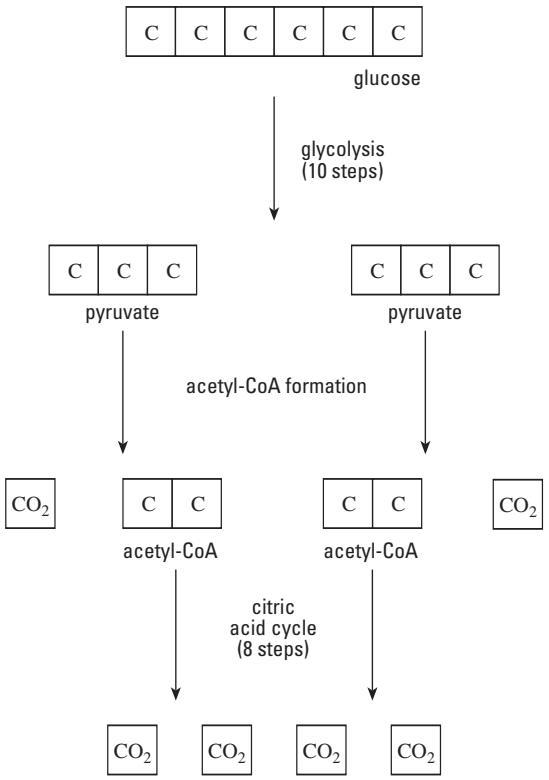


Figure 3.1 The complete oxidation of glucose via the glycolysis pathway and the citric acid cycle.

The role of NAD in metabolic oxidation reactions

The oxidation reactions which occur during the breakdown of glucose from foods are mostly catalysed by enzymes known as dehydrogenases. These are enzymes which catalyse the removal of two hydrogen atoms from a substrate molecule by an oxidizing agent.

Although the air is the ultimate source of oxygen for metabolic oxidation reactions, the actual oxidizing agent involved in most of these reactions is a nucleotide called NAD (nicotinamide adenine dinucleotide). NAD is the general name of the compound, with NAD^+ used for the oxidized form and NADH for the reduced form of the molecule. NAD^+ was the oxidizing agent in the oxidation of pyruvate to acetyl-CoA and carbon dioxide that was mentioned above.

$$C_3H_3O_3^- + CoA + NAD^+ \rightarrow CH_3CO–CoA + CO_2 + NADH$$

The complete oxidation of 1 mole of glucose uses 10 moles of NAD^+ (and 2 moles of another oxidizing agent). So the products include 10 moles of the reducing agent NADH as well as carbon dioxide and some energy.
This is an important advantage of the metabolic pathway compared with simple combustion, which would have produced carbon dioxide, water, 2800 kilojoules and nothing else.

3.2 Energy and the role of ATP

In all living organisms a particular compound, ATP (adenosine triphosphate), has a critical role to play in making energy available for metabolic reactions. ATP is a nucleotide and its structure is shown in figure 3.2. It consists of three phosphate groups linked in a short chain and covalently bonded to the hydroxyl group of a sugar, ribose. The last part of the molecule is adenine, an organic base.

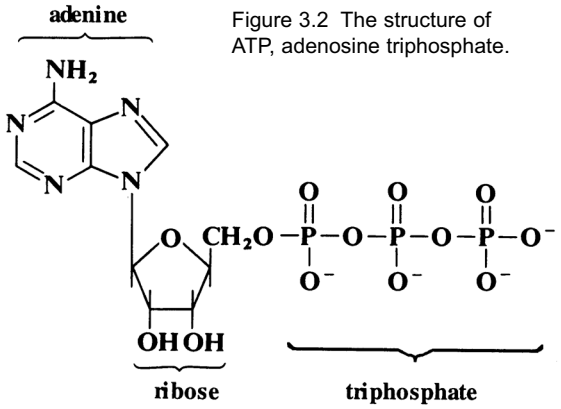
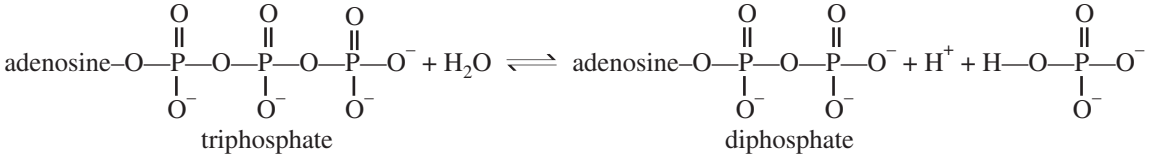


Figure 3.2 The structure of ATP, adenosine triphosphate.

ATP is hydrolysed to ADP (adenosine diphosphate) and an inorganic phosphate ion in a reaction that is energetically favourable. Energy is required to break bonds between phosphate groups, and in water, but there is a net gain of energy when the products form.

ATP and ADP are anions but the negative charges are not normally shown, so their equations appear to be unbalanced. The equation can also be simplified by writing Ⓟ for PO_3 groups.



$$ATP + H_2O \rightleftharpoons ADP + H^+ + HPO_4^{2-} \quad \Delta S_{total} = +107 \text{ J mol}^{-1}\text{K}^{-1}$$

In many chemical transformations and synthetic reactions involving enzymes, ATP plays an essential role. Metabolic pathways use large amounts of ATP all the time, so its synthesis is an important process. Ultimately each ATP-dependent process produces ADP, and a key step in metabolism is the regeneration of ATP from this. This reaction is of course energetically unfavourable, and is where the energy available from the oxidation of food is needed.

ATP synthesis

The synthesis of ATP involves the reaction

This process is called **phosphorylation** because it involves adding a phosphate group. Under standard conditions the reaction is energetically unfavourable, but in the cell it is linked to the oxidation of NADH back to NAD^+ which is favourable. The two processes are said to be **coupled** in a process called **oxidative phosphorylation** and are illustrated in figure 3.3.

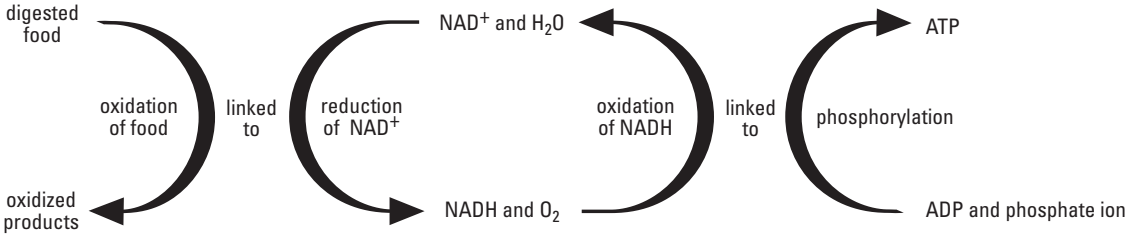


Figure 3.3 The synthesis of ATP and NAD^+ in coupled reactions, by the process called oxidative phosphorylation.

ATP production from the oxidation of glucose

The main function of glycolysis and the citric acid cycle is therefore to produce ATP for use in energetically unfavourable processes. The complete oxidation of 1 mole of glucose produces 10 moles of NADH which can be converted to 30 moles of ATP through their coupled reactions. In addition, other processes yield a further 8 moles of ATP. Thus the maximum amount of ATP that can be produced from the oxidation of 1 mole of glucose is 30 + 8 = 38 moles.

Plants also make ATP from ADP and phosphate ions but the energy can also come from sunlight; whereas in animals the energy comes solely from the oxidation of food.

The role of ATP in metabolism is summarized below.

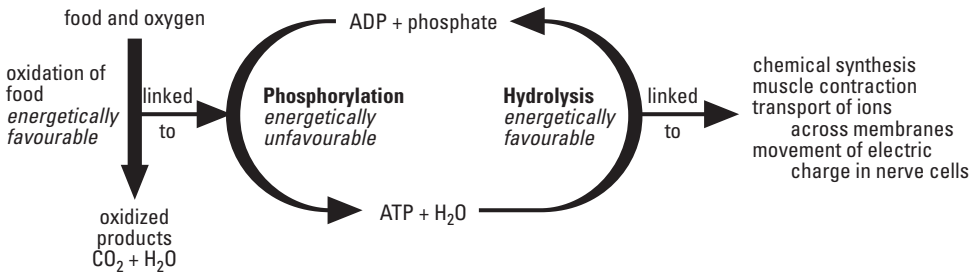


Figure 3.5 The role of ATP in metabolism.

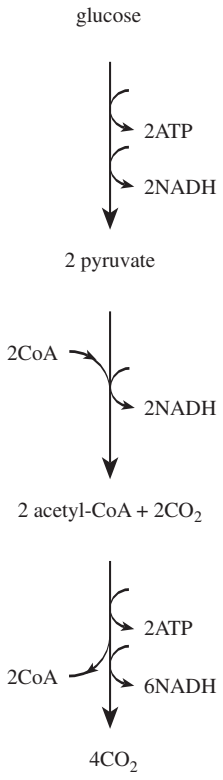


Figure 3.4 The involvement of NADH/ATP in the oxidation of glucose.

If cellular activities are to be controlled, spontaneous reactions must not occur in the absence of enzymes. The intermediates formed during metabolism must be kinetically inert under physiological conditions. For instance, the hydrolysis of ATP to ADP and phosphate is energetically favourable (ΔS_{total} = +107 J mol^{-1}K^{-1}), but the rate of reaction is very slow. Because of the high activation energy of the reaction, spontaneous hydrolysis does not occur. This ensures that the ATP produced during the oxidation of food is available for cellular processes.

3.3 Metabolic pathways

The links between some metabolic pathways for the main components of our food are illustrated in figure 3.6.

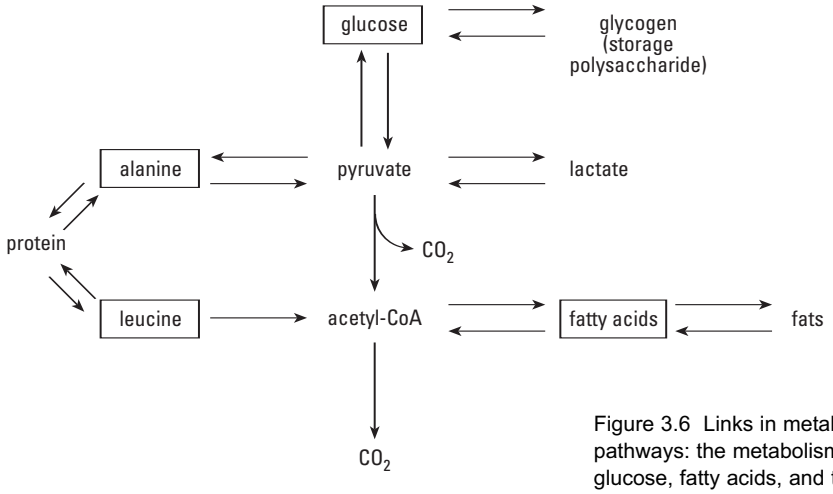


Figure 3.6 Links in metabolic pathways: the metabolism of glucose, fatty acids, and two amino acids.

Amino acids

The protein in our food is hydrolysed to amino acids. Some amino acids can be synthesized in metabolic processes, but others cannot and we can only obtain them from our food. Amino acids which must be supplied in our food are called **essential amino acids.**

Amino acids are needed for the synthesis of proteins, for example, enzymes to catalyse reactions and structural proteins such as hair and nails. They are also used to make the nitrogenous organic bases which are needed for the synthesis of DNA and RNA. Amino acids, once the amine group has been removed, can enter metabolic pathways at a number of different points: for the sake of simplicity only two examples, leucine and alanine, are shown.

Fats

Fats, after hydrolysis to fatty acids and glycerol, can be oxidized to acetyl-CoA. This process releases considerable amounts of energy. The acetyl-CoA can either be used for synthetic reactions, or further oxidized in the citric acid cycle to produce more energy. Fats release more energy per gram than either proteins or carbohydrates; if fatty acids and glycerol are not needed when they are first absorbed, they can be stored as an energy reserve by reforming fats.

Notice that acetyl-CoA can be formed in three ways: from glucose, from fatty acids, and from some amino acids, following the loss of their amine groups.

Interconversion

You should also notice that many reactions and processes are reversible but some are not. This has important implications. For instance pyruvate cannot be made from acetyl-CoA; this means that carbohydrates cannot be produced from fatty acids or from some amino acids, such as leucine. Similarly leucine cannot be made from acetyl-CoA and for healthy growth must be available from our food. In contrast other amino acids, like alanine, can be synthesized from glucose and are therefore not classified as essential amino acids.

The interconversion of sugars, amino acids, and fats through the links between metabolic pathways is of considerable importance to cells with very specific requirements, particularly when the variety of food is limited.

The human brain, for example, needs about 120 g of glucose per day. The total readily available in the body fluids and stored as glycogen does not amount to much more than 200 g. Thus during periods of starvation or low carbohydrate intake the production of glucose from non-carbohydrate sources is essential.

CASE STUDY Phenylketonuria

Phenylketonuria is a disorder of amino acid metabolism. It results from the reduced activity of the enzyme which converts phenylalanine to tyrosine in the liver. It is an inherited condition and 1 in 7000 children in the UK are affected. The condition causes severe mental retardation, and the children may have a characteristic mousy odour due to the presence of phenylethanoic acid.

Even though the condition is quite rare, prompt treatment at birth can prevent the damage to the nervous system. This justifies routine screening of all babies at the end of the first week of life for phenylalanine in their blood.

Babies with the condition must be put on a diet low in phenylalanine as soon as possible to protect the brain. However, sufficient phenylalanine must be provided for growth as it is an essential amino acid. Unfortunately the diet tastes awful.

After the age of eight the diet is less important as the nervous system is less vulnerable. If a woman with phenylketonuria is pregnant even tighter dietary control is necessary if the foetus is not to suffer.

3.4 Metabolic control

The DNA within the simplest cell contains enough information to direct the synthesis of many enzymes, each of which can catalyse a reaction or, exceptionally, more than one. Some of these enzymes are involved in the oxidation of food. Others catalyse the synthesis of the thousands of different proteins, carbohydrates, fats, and smaller organic molecules needed for the healthy survival of an organism. The exact requirements of a particular cell, both for energy and for the products of synthetic reactions, will be continually changing. Therefore, the flow of material through the intricate network of chemical reactions must be carefully controlled if the individual needs of a cell are to be supplied at all times.

Metabolic control may be exerted in several ways, but one possible method involves the control of enzyme activities within a cell.

The control of a biosynthetic pathway

As an example of metabolic control through enzyme activity, consider the synthesis of haem. The structure of haem is shown in figure 3.7. Haem molecules are normally bound to specific protein molecules forming haemproteins. Haemproteins have an important role to play in many life processes. For example, haemoglobin is involved in oxygen transport, so a sufficient supply of haem is essential for the survival of an organism.

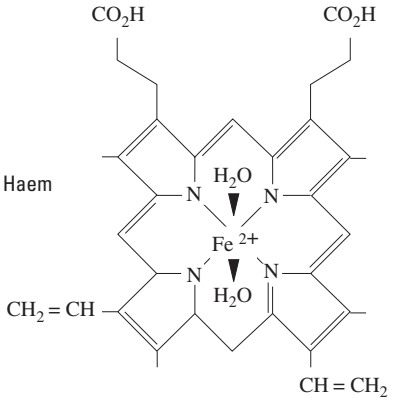


Figure 3.7 The structure of haem. Haem is an example of a transition metal complex involving iron(II).

Haem synthesis

The cells of nearly all organisms are found to contain appreciable quantities of several haemproteins, although the concentration of free haem is generally maintained at a very low level. The net synthesis of haem needed in any tissue is extremely small; it has been estimated that the total hourly requirement for haem synthesis per gram of rat liver is less than 2×10^{-8} moles. Lower production of haem has serious consequences, reducing the ability of the animal to withstand infection. Conversely, overproduction may be equally undesirable; high concentrations of unwanted haem disrupts cell function seriously, and the excretion of the unwanted haem from the liver can affect tissues all over the body.

In the liver, haem is synthesized in six enzymic steps from two readily available substrates. These are succinyl-CoA, an intermediate in the citric acid cycle, and glycine, an amino acid available from the hydrolysis of protein.

The production of the first intermediate, **ALA** (5-amino laevulinic acid), is catalysed by the **enzyme ALA synthetase**. This enzyme has a number of interesting properties.

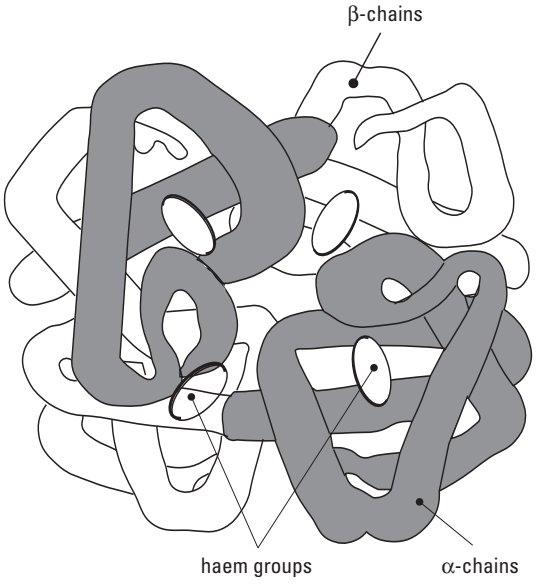


Figure 3.8 The structure of haemoglobin

Enzyme ALA synthetase properties

1 The activity of the enzyme is much lower than any of the subsequent enzymes in the pathway. Thus this first step in the pathway is the rate-determining step.

2 The enzyme is inhibited by haem. This is an example of allosteric inhibition (see section 2.3).

3 The enzyme is very unstable. It has been shown that the half-life of ALA synthetase is about 70 minutes. The other enzymes in the pathway have half-lives of several days.

4 The rate of synthesis of the enzyme from amino acids is reduced by the presence of free haem, an example of 'self-inhibition'.

5 The rate of synthesis of the enzyme in the liver is increased by a variety of drugs (particularly barbiturates).

Rate of haem production

The production of ALA seems to be the step that controls the rate of production of haem. Any ALA produced is rapidly converted to haem with no accumulation of intermediates.

Figure 3.9 The synthesis of haem.

There would seem to be two possible mechanisms by which the pathway might be controlled: either by modifying the activity of the ALA synthetase molecules, or by altering their concentration.

The experimental evidence suggests that both possibilities occur in mammalian tissue. Haem, the end product of this particular pathway, both inhibits ALA synthetase activity and represses the formation of new enzyme. In this way it directly controls the rate of its own synthesis.

The short half-life of the enzyme ensures a rapid response to new situations. ALA synthetase levels can rise rapidly in the presence of drugs. Conversely, accumulation of haem can lead to a significant reduction in the concentration of the enzyme within hours.

The action of hormones

A second level of control is needed in higher organisms because the activities of different cells must be co-ordinated. This second level of control is exerted through the action of hormones. Although many hormones do not enter cells but interact with the cell membrane, they can affect the concentration or activity of enzymes within 'target' cells. Hormones can alter the behaviour of a cell wall, the rate of synthesis of a particular enzyme, or the activity of an enzyme.

Insulin

Insulin is an example of a hormone; it is released from the pancreas when blood sugar levels are high. The presence of insulin in the bloodstream increases the passage of glucose through certain cell membranes. In muscles it stimulates glycogen formation, while in the liver it stimulates glycolysis, fatty acid synthesis, and glycogen formation. Also the synthesis in the liver of glucose from pyruvate is reduced. All these processes (see figure 3.6) accelerate the metabolism of glucose and encourage the removal of sugar from the blood. Diabetes is often caused by a failure of the pancreas to produce sufficient active insulin.

Control of metabolism

The following general principles seem to apply.

- The rate-controlling step is generally the first step of a pathway.
- The activity of the enzyme catalysing this step is increased or decreased by a variety of activators or inhibitors: often inhibition is brought about by the end product.
- In some cases, the enzyme catalysing the rate-determining step has a short half-life, so the concentration of the enzyme may be affected by a change in the rate of production.
- Hormones co-ordinate the activities of different cells.

COMMENT Poisoning by cyanide

Hydrogen cyanide gas and cyanide ions in solution are very dangerous rapid-acting poisons. Some fruit stones contain compounds which are converted to cyanide during digestion and can cause accidental cyanide poisoning. Headache and nausea are followed by convulsions and coma. Death may occur in hours, but treatment is generally effective if an antidote is given quickly.

Cyanide forms stable complexes with iron(III) ions and will rapidly bond to the iron(III) of a haem group in a vital protein (cytochrome). Metabolic reactions involving oxidation cease and cells soon stop functioning.

Administration of oxygen is the only safe first aid treatment. In hospital, possible treatments include the injection of nitrites, which convert oxyhaemoglobin containing iron(II), to a form of haemoglobin containing iron(III). The iron(III) form of haemoglobin forms a more stable cyanide complex, and the cyanide is therefore not able to disrupt essential metabolic reactions. Patients can also be treated with thiosulphate, $S_2O_3^{2-}$, which reacts with cyanide to form thiocyanate, CNS^-, which is relatively non-toxic and readily excreted.

3.5 Applications of metabolic control in agriculture and medicine

Metabolic control in agriculture and medicine is widely achieved by the use of enzyme inhibitors which affect metabolism within one particular type of cell or a limited range of plants or animals. The doctor prescribing an antibiotic to treat an infection, the farmer using poison to control rats, or the gardener using an insecticide to kill greenfly on roses are all likely to be using a selective inhibitor.

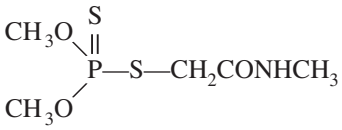
The biochemist designing a drug or pesticide may achieve selectivity in three ways:

- selectivity due to enzyme differences between organisms
- selectivity due to structural differences between the cells of organisms
- selectivity due to differences in distribution between different types of cell.

Insecticides

All cultivated crops are attacked by insects, ranging from the highly specific carrot fly to the all-devouring locust. Chemical methods of insect control have been sought for centuries, but the discovery of organophosphorus compounds was a major breakthrough in insect pest control. These compounds are toxic because they inhibit an enzyme, acetylcholinesterase. Lack of the enzyme disrupts the transfer of impulses from one nerve cell to another. The active site of the enzyme contains an amino acid which is essential for the enzyme's activity, and which reacts covalently with organophosphorus compounds. When this happens, the enzyme ceases to function and the resulting disruption of the nervous system kills the insect.

The problem is to find compounds which are toxic to insects but harmless to birds and mammals. Dimethoate is readily absorbed by plant cells, and so is particularly effective at killing sucking and biting insects. Other insects, which are essential for pollination, are unaffected provided they do not damage plants. Dimethoate is also relatively non-toxic to birds and mammals as they have enzyme systems which rapidly convert it into a harmless compound.



dimethoate

Antibiotics

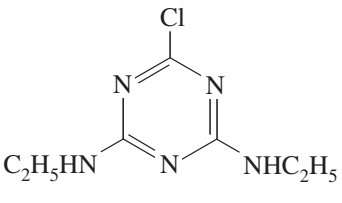
The antibiotic penicillin was discovered by Alexander Fleming in 1928 but not used until World War II. With Chain and Florey, who developed methods for large scale production, Fleming was awarded the Nobel prize in 1945.

Many bacteria have a strong outer cell wall as well as an inner membrane. An example is *Streptococcus*, which can cause tonsillitis and some forms of meningitis. Penicillin can prevent the formation of the outer wall of bacteria by inhibiting an enzyme involved in the synthesis of the outer wall. Without the protection of their outer wall, they burst when exposed to the conditions present in an animal cell. Human cells do not have this type of cell wall outside their cell membrane and are therefore unaffected by penicillin.

An increasing number of bacteria have become resistant to the range of antibiotics currently available. It is becoming more of a challenge to develop new drugs which will selectively kill bacteria but have minimal side-effects in humans and animals. Exposure of bacteria to antibiotics tends to select for resistant types.

Herbicides

Plants depend on photosynthesis for survival whereas animals obtain their energy from food. Triazine herbicides, when transported to a plant's leaves, disrupt the production of ATP from ADP in sunlight; without ATP the plant dies. Examples of triazines are simazine and atrazine.



simazine

Some crops, such as sugar cane, are tolerant to triazines because they have an enzyme that can convert the herbicides to non-toxic compounds. The gene for this enzyme has now been transferred to other crops so that weeds can be killed while the crop is growing. But at least 40 species of weed have become resistant to these herbicides.

CHAPTER 4 NUCLEIC ACIDS

So far we have considered the action of enzymes and looked at metabolism, but much of the most exciting research in biochemistry over the past 50 years has involved genes and genetics.

Every living organism carries genetic material. It directs the development of the organism and is passed on from generation to generation. It must be duplicated precisely every time a cell divides. In this chapter we will seek answers to the following questions.

- What is the structure of the genetic material?
- How is the genetic material copied during cell division?
- How is the genetic information stored, and how is the information used to control the activities of cells?
- What happens when there are faults in genetic material?

4.1 DNA, the information molecule

The discovery of DNA

DNA, or deoxyribonucleic acid, was first isolated over a hundred years ago by a Swiss biochemist, Friedrich Miescher. He was studying white blood cells obtained from the pus on the bandages of patients recovering after operations.

A white precipitate was obtained and found to contain the elements carbon, hydrogen, oxygen, nitrogen and phosphorus. It came from the nucleus of the cells, and subsequent experiments showed it to be acidic; so it was given the name ‘nucleic acid’.

By 1900 chemical analysis had shown that nucleic acids have very large molecules containing just six components, a sugar, a phosphate group, and four different organic bases. However, because of their apparently simple composition, nucleic acids were largely discounted as a possible genetic material. Protein seemed to be a much more likely candidate, since it was assumed that the genetic material must be exceptionally complex.

Further research revealed that nucleic acids are polymers made up of monomers called nucleotides. Nucleotides contain a sugar, an organic base containing nitrogen, and up to three phosphate groups (as with ATP – see figure 3.3 on page 21).

The composition of DNA

The sugar component in DNA is always deoxyribose. Deoxyribose is a pentose, which is a five-carbon sugar. Most pentoses have five oxygen atoms but deoxyribose has only four (see figure 4.1).

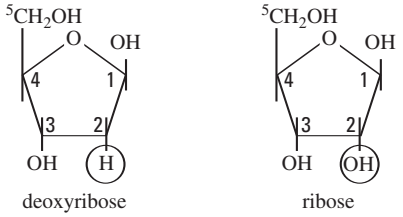


Figure 4.1 Deoxyribose, the sugar found in DNA and the related sugar ribose. Ribose is a component of RNA (see section 4.3).

There are four bases. Two of the bases, **adenine (A)** and **guanine (G)**, are purines; they both contain a planar double ring system. The other two bases, **cytosine (C)** and **thymine (T)**, are pyrimidines; they too contain a planar ring system, but with only six atoms in the ring. Sugar molecules link to the N atoms (see figure 4.2).

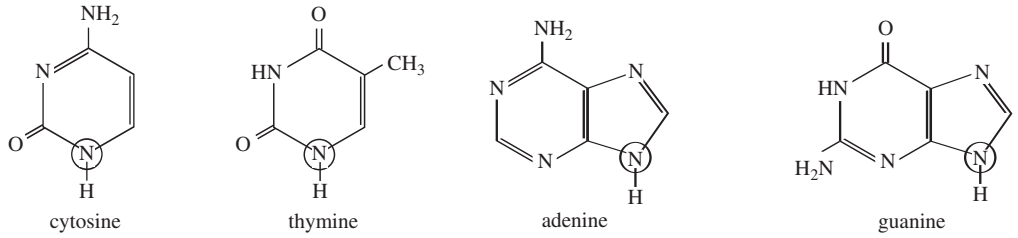


Figure 4.2 The bases found in nucleic acids. In DNA sugar molecules link to the ringed N atoms.

A complete nucleotide consists of the three components linked together by covalent bonds. In the diagram of a nucleotide (figure 4.3) notice that the phosphate group is linked to carbon 5′ of the sugar and the organic base to carbon 1′.

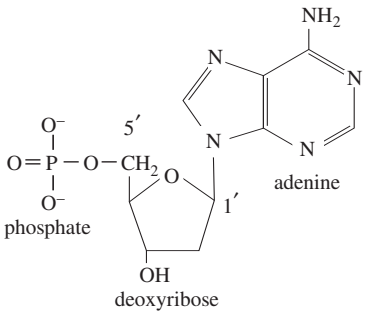


Figure 4.3 A nucleotide (deoxyadenosine monophosphate). The phosphate group is linked to carbon 5′ of the sugar and the organic base to carbon 1′.

In nucleic acids, the nucleotides are linked together in very long chains. The phosphate groups are linked to carbon 3′ and carbon 5′ of each sugar molecule. This gives rise to a long polymer chain of alternate phosphate and deoxyribose units, with organic bases attached to each of the sugar molecules at carbon 1′ (see figure 4.4).

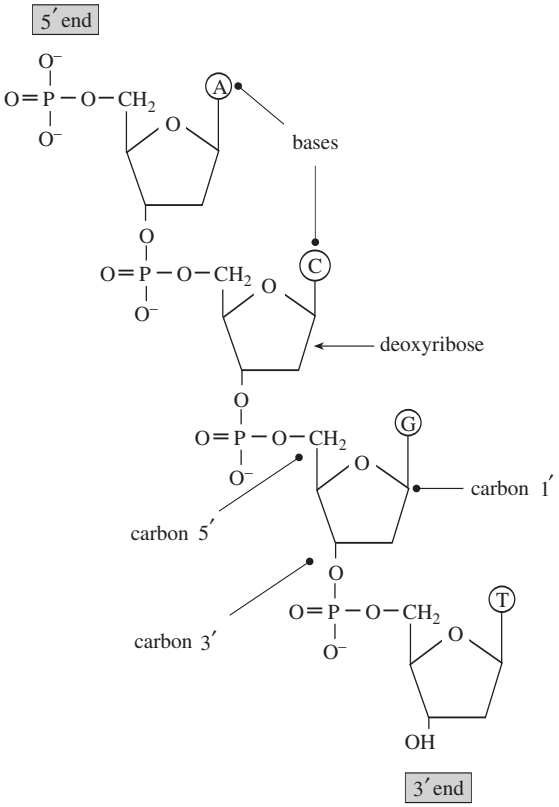


Figure 4.4 The structure of a single DNA chain. The phosphate groups link to carbons 3′ and 5′ in the sugar.

The structure of DNA

Important evidence about the composition of DNA was published in 1951 by Chargaff, an Austrian chemist working in the USA. He had determined the relative molar amounts of the four bases in the DNA from many different species, and a remarkable feature became apparent. In bacteria, in plants and in animals, he found that the number of moles of adenine in the DNA always equalled the number of moles of thymine, while the number of moles of cytosine was always the same as the moles of guanine.

Source of DNA	Adenine /molar %	Thymine /molar %	Cytosine /molar %	Guanine /molar %
Bacteria	15.1	14.6	35.4	34.9
Wheat	27.3	27.1	22.8	22.7
Salmon	29.7	29.1	20.4	20.8
Human	30.9	29.4	19.8	19.9

In 1952 Alfred Hersey and Martha Chase, two Americans, carried out experiments which showed for certain that DNA, and not protein, was the genetic material. They did so by studying a virus that is made of only DNA and protein.

In parallel with the biochemical research, X-ray crystallographers were learning how to obtain good photographs of fibre specimens of DNA. Among the most successful were Maurice Wilkins and Rosalind Franklin, working at King's College, London. It was Franklin's diffraction photographs which generated the more useful data related to the three-dimensional shape of the DNA molecule.

DNA in three dimensions

By now all the evidence needed to solve the problem was available, and scientists in America and Britain were racing to be the first people to publish a paper describing the three-dimensional structure of DNA. Francis Crick and James Watson, working at the Cavendish Laboratory at Cambridge, used model-building in an attempt to find a structure which was consistent with the covalent structure of DNA, Chargaff's work on the bases, and the information from X-ray analysis.

On 25 April 1953, Watson and Crick published a paper in *Nature* on the structure of DNA. The article began with a considerable understatement:

'This structure has novel features which are of considerable biological interest.'

• DNA contains two polynucleotide strands
• the strands run anti-parallel
• the deoxyribose-phosphate chains are on the outside
• the bases link the two strands
• the bases are linked by hydrogen bonds
• hydrogen bonds always link adenine with thymine, cytosine with guanine
• the two strands are twisted to give a helix.

Complementary base pairs	
Purines	**Pyrimidines**
Adenine (A) –	Thymine (T)
Guanine (G) –	Cytosine (C)

The structure of DNA is illustrated in figure 4.5.
The two strands run in opposite directions (anti-parallel). Look at the deoxyribose molecules at the bottom of the diagram. One has a free OH group on carbon 3′; and the chain runs from 3′ to 5′ up the diagram. On the other strand there is a phosphate group on carbon 5′, and the chain runs from 5′ to 3′ up the diagram.

The bases, in the centre of figure 4.5, are linked by hydrogen bonds: **A** and **T** by two bonds, and **C** and **G** by three bonds. **A** always pairs with **T**, while **C** always pairs with **G**, and they are called **base pairs**. You can see this more clearly in figure 4.6 on the next page. Because the bases on one strand always link to their specific matching base on the other strand, the two strands of the helix are known as **complementary**.

In the helical structure, at the top of figure 4.5, the planar base molecules stack one above another like a pile of plates. This maximizes the van der Waals interactions between bases. Indeed the van der Waals interactions actually make a greater contribution to the stability of the DNA structure than the hydrogen bonds between base pairs.

We are now so familiar with the structure of DNA, that we easily underestimate the importance of the discovery. It was perhaps one of the most significant moments in the development of biochemistry.

Since the sequence of bases on one strand determines the order of bases on the other, the Watson and Crick structure offered an immediate and elegant answer to the second question posed at the beginning of this chapter: how is the genetic material copied during cell division?

See Experiments 5 and 6 (alternatives) for the extraction of DNA plant material.

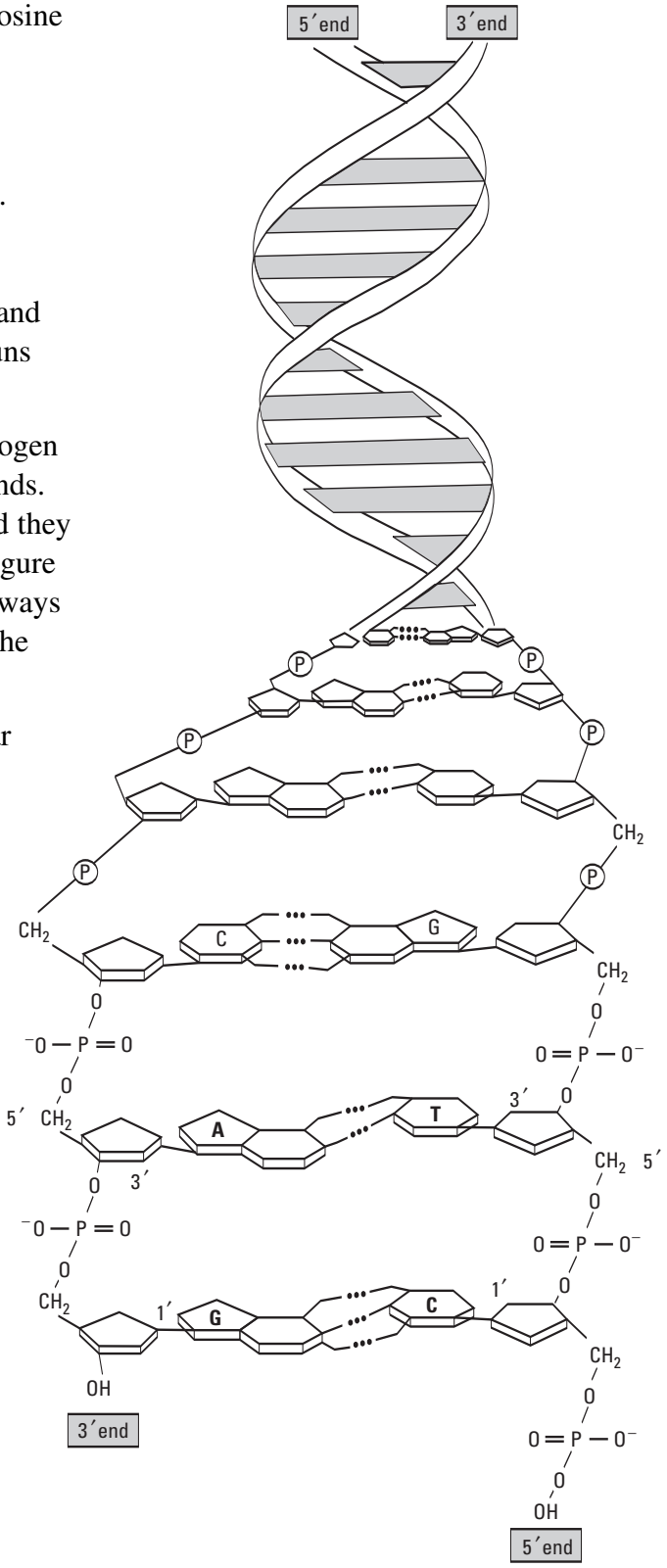


Figure 4.5 The structure of DNA

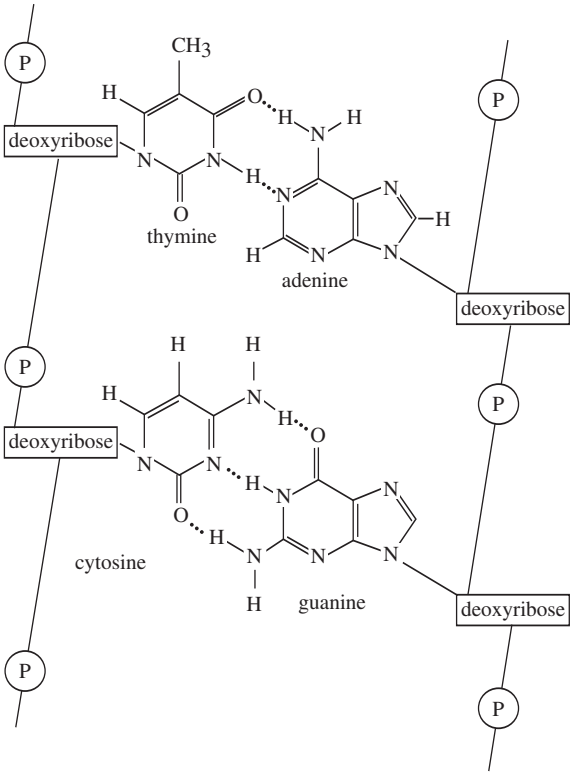


Figure 4.6 Hydrogen bonding between base pairs.

4.2 The replication of DNA

When Watson and Crick published their model of DNA in *Nature* they wrote

'It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material.'

In cell nuclei, the DNA is packed into relatively large structures called **chromosomes**.

Whenever a cell divides, both of the new cells need a complete copy of the DNA in the chromosomes – it contains the information needed for all the activities of the cell. The process of copying the DNA in a cell when it divides is called **replication**.

Nucleic acid replication is a remarkable process: each molecule acts as a template for the synthesis of copies of itself.

During replication, the hydrogen bonds and van der Waals forces between the base pairs in the double helix are broken, and two new strands are formed using the original strands as templates for the synthesis. A new strand contains a complementary sequence of bases – this is dictated by the order of bases in the original strand. Hydrogen bonds and van der Waals forces then form between original and new strands, creating the stable helical structure (see figure 4.7 on the next page).

We are now ready to answer our third question.

How is information stored in the genetic material, and how can it direct protein synthesis?

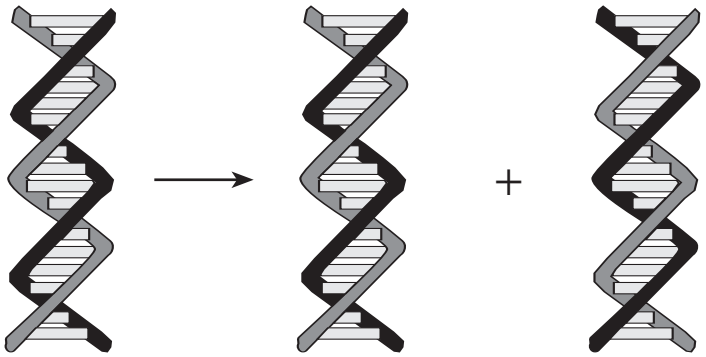


Figure 4.7 The replication of DNA produces two molecules, each containing one strand from the original molecule

COMMENT Chromosomes

We have 23 pairs of chromosomes in each of our cells, and a single chromosome may have as many as 2000 genes. The complete genetic material is known as the human genome. Our DNA is thought to contain about 3×10^9 base pairs, and the mass of this DNA is about three picograms, 10^{-12} g. This may not seem very much, but if this DNA was unravelled it would be some 2 metres long. Since the nucleus of a cell is about
5×10^{-6} metres in diameter, it is clear that the DNA must be folded extremely compactly in the chromosomes.

The DNA in other species varies from a few thousand base pairs (bp) for a virus to billions in a flowering plant.

4.3 The synthesis of proteins

A cell might be likened to a computer-controlled machine tool. To make anything useful, the machine tool needs access to the computer-coded instructions which guide its operations. DNA contains within its base sequence the digital coded instructions which control protein synthesis and so direct the activities of the cell. But how can a sequence of bases along a strand of DNA direct protein synthesis?

Coded instructions

A coded instruction is needed for each of the 20 amino acids that occur in proteins. The four bases on their own could only be used to code for four amino acids, one each. A pair of bases is still not sufficient as it can only generate 16 codes – AG, GA, AT, TA, AC, CA and so on. A sequence of three bases has $4 \times 4 \times 4 = 64$ possible combinations. By using sequences of three bases there are sufficient codes for all the amino acids. Therefore, protein might be coded for by successive sets of three bases along the DNA chain, each triplet coding for an amino acid, with the sequence of triplets determining the order in which the amino acids are to be joined together.

Combinations of the four bases	Number of codes
individual bases	$4^1 = 4$
pairs of bases	$4^2 = 16$
triplets of base	$4^3 = 64$
quartets of bases	$4^4 = 256$

Research has indeed shown that the genetic code is based on triplets. All 64 possible codes are used in DNA. Three of the triplets indicate the termination of a protein sequence, another triplet is a start code (although confusingly the same triplet also codes for an amino acid). The remaining 60 triplets each code for amino acids. This results in nearly all the amino acids having more than one code; for example there are four DNA triplets which code for valine, CAA, CAG, CAT, CAC. Look at these codes carefully and you will see that in this case the first two bases identify the amino acid and the third base just completes the triplet.

A universal code

Most remarkably the same code is used by virtually all organisms; the DNA code is universal. Whether the DNA is from plants or animals or bacteria, the base–amino acid code does not seem to change. Many biologists regard this as the strongest available evidence for the theory that all forms of life have evolved from a common beginning.

The length of DNA which carries the instructions for a particular protein (or part of a protein) is a gene. The DNA in a human cell contains sufficient base pairs to code for about three million proteins. However, only about 60 000 proteins are produced from genes. Some DNA is required for control regions, so that proteins are only synthesized where and when required. Other regions of DNA are needed for the production of special RNA molecules. However, much of the DNA has no known use; it may be ‘junk’, or it may have uses of which, as yet, we know nothing.

Transcription

Protein synthesis does not take place in the nucleus of the cells where DNA is found but on the ribosomes (see figure 2.1). Part of the DNA double helix unravels and a complementary copy of a gene is synthesized (see figure 4.8). The copy diffuses out of the nucleus and moves to the ribosome where protein synthesis takes place. This molecule is called messenger RNA (mRNA). It has three of the four bases present in DNA but thymine (T) is replaced by a different base, uracil (U). The sugar is ribose instead of deoxyribose.

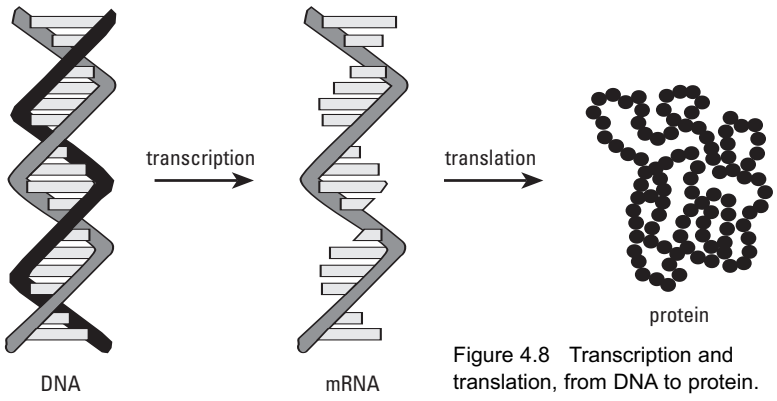


Figure 4.8 Transcription and translation, from DNA to protein.

The mRNA will carry a start code, and from that point each triplet of bases along the chain codes for one specific amino acid. A triplet of three bases on the mRNA is known as a codon. The complete genetic code is listed in figure 4.10 on page 36.

Translation

Codon base on mRNA	Anticodon base on tRNA
A	U
C	G
G	C
U	A

The mRNA at the ribosome directs the bringing together of amino acids in the correct order to produce proteins; this is translation. The language of DNA, the order of bases along a strand, is being translated into the language of proteins, an order of amino acids along a chain.

Amino acids on their own cannot bind to mRNA. However, in the cytoplasm, there are small RNA molecules, known as transfer RNAs (tRNA). Each tRNA has a specific triplet of bases, an anticodon, at one end of the molecule and it can bind the amino acid, corresponding to the complementary codon, at the other end.

In the translation process, one codon of the mRNA is paired to its anticodon on a tRNA (see figure 4.15). The correct amino acids are therefore lined up in the correct order. Enzymes link the new amino acids to the growing protein chain through peptide bonds. The tRNA molecules then diffuse away without their amino acids. When a STOP codon is reached, synthesis is complete and the protein is released. The protein chain will have folded spontaneously, but at this stage not always to its final tertiary shape.

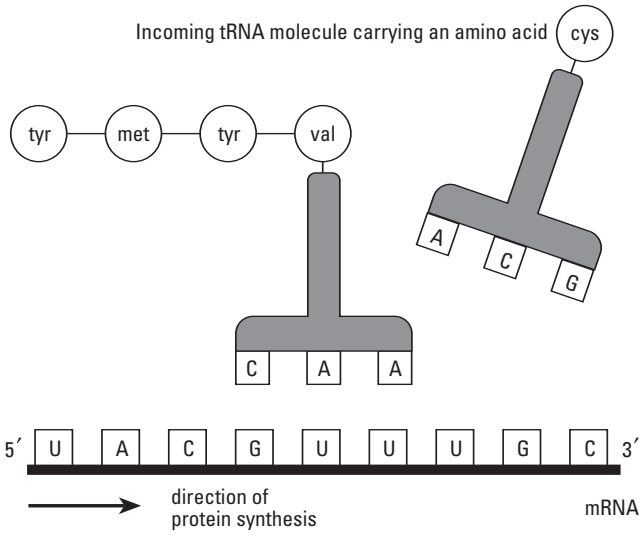


Figure 4.9 Translation. In this simplified diagram the ribosome is not shown.

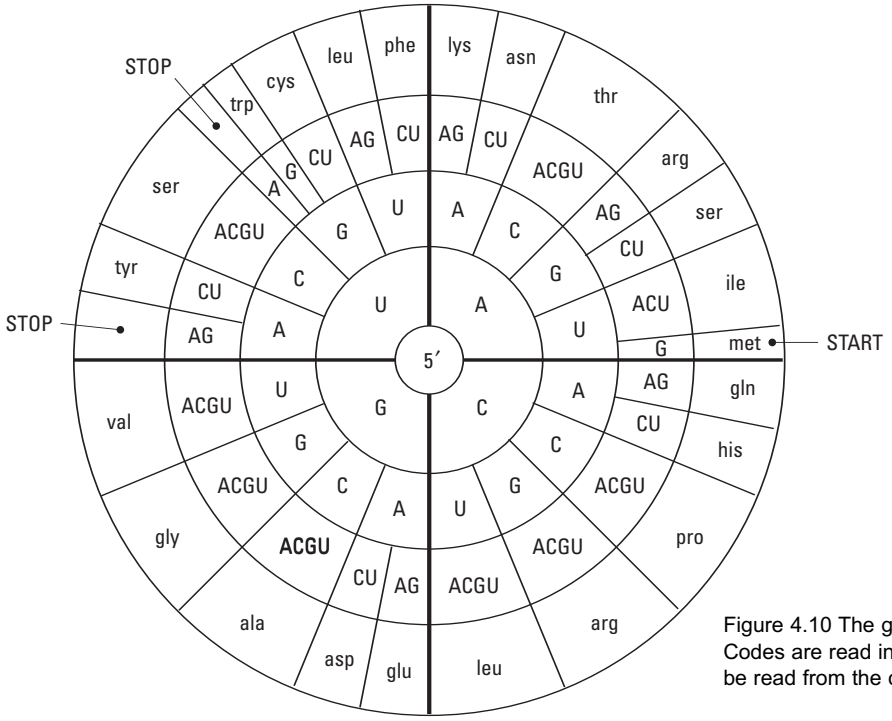


Figure 4.10 The genetic code.
Codes are read in the 5′–3′ direction on mRNA, and should be read from the centre of the diagram.

4.4 Mutations

Errors can happen during DNA replication, but it is only on very rare occasions that an error is not corrected by the cell's own mechanisms. Changes from the original DNA are known as mutations. Apart from errors in the replication process, mutations can be caused by any process that damages DNA. UV light, cigarette smoke and many other chemical compounds can cause mutations. The chemicals do not have to be synthetic; there are naturally occurring chemicals in many foods, even potatoes, that can cause mutations.

In some cases the change in the DNA may be very small; a single base may be miscopied, and a single base pair may then be altered in the DNA molecule in future generations. Such mutations are not uncommon.

Since changes in the sequence of base pairs alter the amino acid coding, the ultimate result may be a change in the structure and behaviour of a protein. In many cases a single change in the base sequence has no effect on the protein that is being produced. You will remember that most amino acids have several codes, and a change in the DNA from a CAA sequence to a CAG for instance will still produce a protein containing a valine in the correct place. Mutations which produce or remove a start or stop codon, on the other hand, may have serious consequences as a critical protein may not be produced or may be so changed that it is unable to fulfil its function.

Sickle cell anaemia

There are several kinds of sickle cell disease. They get their name from the shape of red blood cells in people affected by the diseases. The cells have a cresent moon (or sickle) shape. One example of this type of disease is sickle cell anaemia.

The disorder affects the red blood cells which contain haemoglobin (Hb for short). People with sickle cell anaemia have sickle haemoglobin (HbS) which is different from the normal haemoglobin (HbA).

The disease arises from a single mutation. The result of the mutation is an abnormal amino acid sequence in one of the protein chains in haemoglobin. The abnormality is limited to a single amino acid at the sixth position of the 146 amino acid chain:

Normal β-chain	Val	His	Leu	Thr	Pro	Glu	Glu	…
Sickle cell β-chain	Val	His	Leu	Thr	Pro	Val	Glu	…

When sickle haemoglobin gives up its oxygen to the tissues, it sticks together to form long rods inside the red blood cells, making these cells rigid and sickle-shaped. Normal red blood cells can bend and flex easily.

Because of their shape, sickle-shaped red blood cells cannot squeeze through small blood vessels as easily as the almost doughnut-shaped normal cells. This can lead to these small blood vessels getting blocked, which then stops the oxygen from getting through to where it is needed. This in turn can lead to severe pain and damage to organs.

Everyone has two copies of the gene for haemoglobin: one from their mother and one from their father. If one of these genes carries the instructions to make sickle haemoglobin (HbS) and the other carries the instructions to make normal haemoglobin (HbA) then the person has sickle cell trait and is a carrier of the sickle haemoglobin gene. This means that this person has enough normal haemoglobin in their red blood cells to keep the cells flexible and they don't have the symptoms of the sickle cell disorders.

If both copies of the haemoglobin gene carry instructions to make sickle haemoglobin then this will be the only type of haemoglobin they can make and sickle cells can occur. These people have sickle cell anaemia and can suffer from anaemia and severe pain.

The different kinds of sickle cell disease and the different traits are found mainly in people whose families come from Africa, the Caribbean, the Eastern Mediterranean, Middle East and Asia. In Britain the disease is most common in people of African and Caribbean descent (at least 1 in 10 to 40 have sickle cell trait and 1 in 60 to 200 have sickle cell disease). Estimates suggest that there are over 6000 adults and children with sickle cell disease in Britain.

Cystic fibrosis

Cystic fibrosis is the commonest genetic disorder in the UK. It occurs in 1 in 2000 live births, and 1 in 22 Caucasians are carriers of the gene. The disease affects the lungs, pancreas, gut and sweat glands. Instead of the normal fluid secretions a thick sticky mucous forms. This viscous mucous blocks and damages the intestines and lungs. Because the supply of digestive enzymes from the pancreas is blocked, nutrients cannot be absorbed and babies fail to thrive; they have repeated chest infections and in particular can get intestinal obstruction. The malfunctioning of the sweat glands results in abnormally salty sweat, which is used as part of the diagnosis of the condition.

Thanks to better understanding of the disease and its treatment, people with cystic fibrosis are living longer than ever before. Until the 1930s, the life expectancy of a baby with cystic fibrosis was only a few months. Today the average life expectancy for someone with cystic fibrosis is around 31 years. There is no cure for cystic fibrosis, but the faulty gene has been identified and doctors and scientists are working to find ways of repairing or replacing it.

Cystic fibrosis affects the cells that line the cavities and tubes inside organs such as the lungs. The membranes of these cells have a mechanism for pumping chloride ions into the cells from the blood supply. In lungs the chloride ions normally diffuse out of the cells through channels in the cell membrane lining the airways. This is part of the process for keeping a runny layer of watery mucus on the surface of the cells.

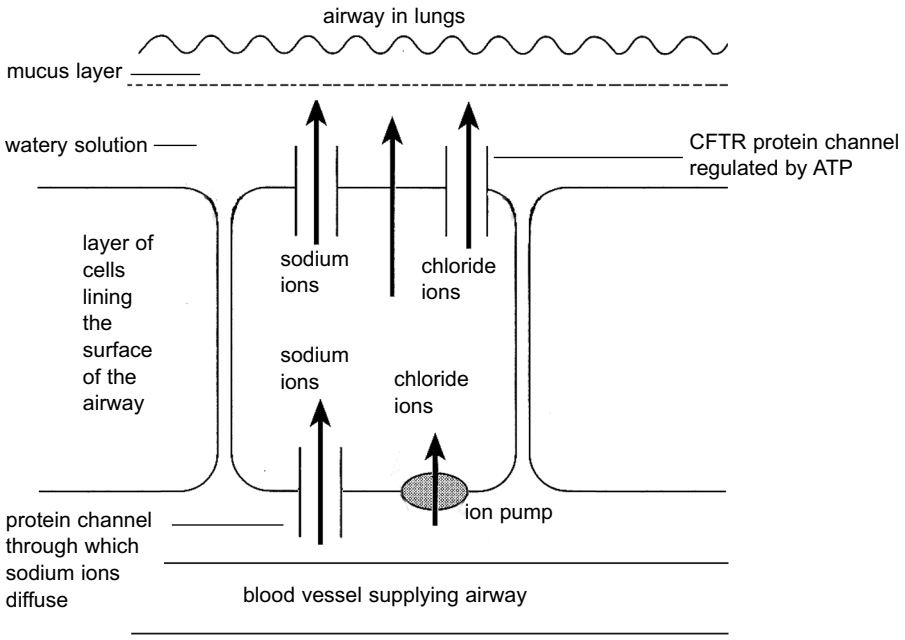


Figure 4.11 Movement of ions and molecules across the cell membranes of cells lining an airway in the lungs

The chloride ions diffuse out of the cell through a channel created by a protein. The protein channel is only open in the presence of ATP.

The name of this membrane protein is CFTR protein (short for cystic fibrosis transmembrane regulatory protein). In a person with cystic fibrosis the CFTR protein may be missing or, if present, it does not work properly. It does not allow chloride ions which are being pumped into the cell to leave. The chloride ion concentration in the cell builds up. The high solute concentration in the cell causes water to move into the cell instead of out of it by osmosis. As a result the mucus covering the cells lining the airways becomes thick and sticky.

The genetics of cystic fibrosis is not as simple as that of sickle cell disease. Hundreds of different mutations have been identified that can give rise to the disease. The various mutations affect the CFTR protein in different ways. In some cases ATP is unable to bind to it so the channel cannot open.
In other cases the channel opens but in a way that does not let the chloride ions escape.

The gene is on chromosome 7. The commonest mutation is the deletion of three nucleotides which result in the loss of phenylalanine, the 508th amino acid in the structure of the protein.

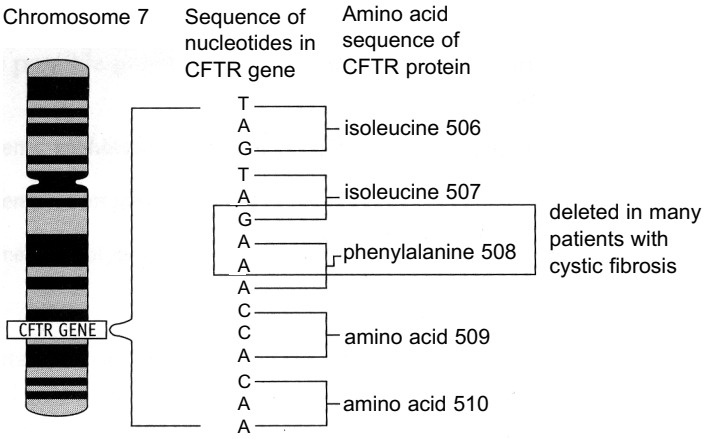


Figure 4.12 *(right)* A common mutation that causes cystic fibrosis (from Salters-Nuffield Advanced Biology, Topic 2, Heinemann, 2005).

4.5 DNA fingerprinting

A DNA 'fingerprint' requires a small sample of your blood, as a source of DNA, and not an impression of the pattern on your fingertips. But it is becoming a more useful and reliable method of identification. It has been possible to confirm the identity of bones found in a mine shaft at Ekaterinburg in Siberia as those of Tsar Nicholas II and his family 80 years after their deaths during the Russian revolution.

The technique was developed by Professor Alec Jeffreys at Leicester University in 1984 and has been publicly available since 1987.

How DNA finger-printing works

The method depends on some of the 'junk' lengths of DNA between genes. These lengths can vary dramatically between individuals. There are core sequences of about 16 bases that are repeated many times over and are so different that only identical twins will have the same 'genetic markers'.

DNA from a blood sample is treated in a strictly controlled procedure using gel electrophoresis to produce a pattern of DNA fragments. This is based on either a probe which binds only to a specific base sequence or a 'multi-locus' probe which binds to many different types of fragments, producing what is known as a DNA fingerprint. The multi-locus probe produces a much more detailed pattern but the single-locus probe is more sensitive, needing as little as 10 nanograms of DNA.

Uses of DNA finger-printing

The procedure is used for the identification of individuals or establishing if there is a family relationship between individuals. It was DNA fingerprinting that established that we are not related to Neanderthals (see Chapter 1).

DNA from a blood sample of a child and his/her alleged parents will confirm whether they are the mother and father with a probability stated to be 99.99%.

The method is widely used by the police: the first conviction solely on the evidence of a DNA fingerprint being a Bristol man in November 1987. As the sensitivity of the method has improved the Bristol police have re-examined evidence from 17 cases, going back to 1970 before the method was available.

One method by which the sensitivity of the method has been improved is the use of the polymerase chain reaction (PCR). In PCR the reaction conditions are arranged so that very small amounts of DNA replicate in a cycle repeated 20–30 times, with the potential to double the amount of DNA at each cycle.

DNA fingerprinting can be used to match an alleged stolen bird to feathers left behind in its nest or cage; badger blood has been used to convict four Yorkshire men accused of badger baiting; and a man, who wrote a letter trying to blackmail a supermarket, was convicted as a result of a DNA test on saliva left on the back of the postage stamp he licked.

Another use of gel electrophoresis is in screening for inherited genetic disorders. This is discussed on page 42.

4.6 Gel electrophoresis

The experimental study of samples of DNA and their sequence of base-pairs is based on a technique called gel electrophoresis. But first the DNA has to be cut up into fragments of manageable length. This is done by using one of a group of enzymes that catalyse the digestion of DNA. They are found in bacteria and are called **restriction enzymes** because they are able to restrict the growth and replication of an invading virus by destroying the DNA of the virus.

They cut DNA between two particular bases within a definite sequence of bases. This is illustrated by the enzymes BamHI and Apa I which cut DNA at the points indicated by the asterisks.

5′-G * GATCC-3′	5′-GGGCC * C-3′
BamHI	Apa I

After digestion of the DNA sample with a restriction enzyme, **gel electrophoresis** is used to separate the DNA fragments produced. The technique depends on the movement of negatively charged DNA fragments through an electrically conducting gel.

Gels are usually made from **agarose**, a polysaccharide seaweed extract dissolved in an alkaline buffer. The gel consists of a sponge-like network of polymer molecules stabilized by hydrogen bonds. When a voltage is applied, smaller molecules, or fragments, move faster through the gel than the larger ones so they are separated according to their different sizes.

Small wells in the gel act as reservoirs for the samples of DNA. In order to observe the progress of the electrophoresis a dye, the loading dye, is usually added to each sample.

When electrophoresis is complete, another dye, the staining dye, has to be added to reveal the positions of the invisible DNA fragments.

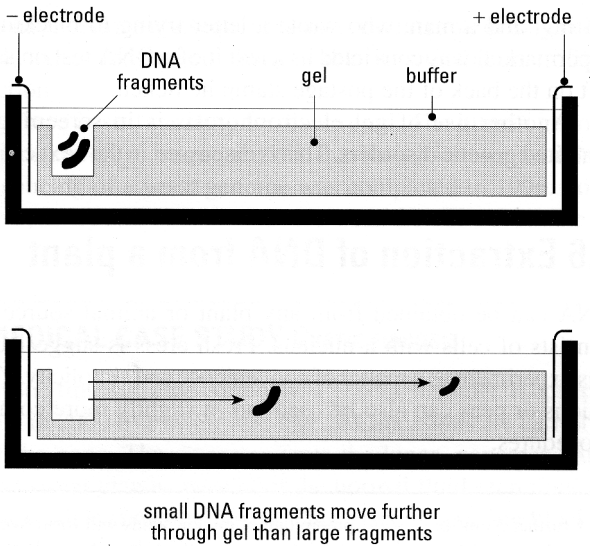


Figure 4.13

The diagram shows some typical results.

- The strong bands near the wells are uncut DNA.
- The smear down the gel is typical of plant DNA.
- A set of bands is characteristic of a low molar mass DNA.
- The loading dye usually moves further than all the DNA fragments.

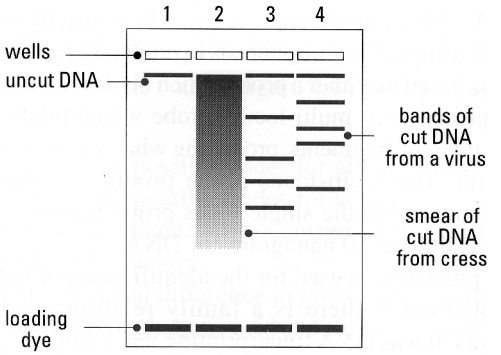


Figure 4.14

Screening for genetic disorders

Genetic screening in adults can be achieved by taking a small sample of blood, using restriction enzymes to cut up the DNA, and gel electrophoresis to separate the fragments by size (as described above). Chemiluminescent or radioactive probes which bind to very specific fragments of DNA, are then used to look for the DNA carrying the mutations responsible for the disorder. Doctors are already able to test for hundreds of genetic disorders.

Genetic screening can also be used in early pregnancy to find out if the foetus has inherited a disorder from its parents, such as sickle-cell anaemia but taking samples from a foetus increases the risk of a miscarriage.

There are some conditions such as anencephaly (absence of a brain) and Tay-Sachs disease (resulting in blindness, loss of mobility and death within five years) where parents usually elect for a legal abortion when they are told of the problem. Huntington's disease is an example of a more difficult situation: people with the condition develop involuntary muscular movements and find it more and more difficult to look after themselves. However, Huntington's disease usually only appears in people over the age of 35.

The moral issue for parents is whether to seek an abortion or let a child be born knowing that a distressing condition will develop. The ethical issue for doctors and society is the extent to which genetic screening should be available. New knowledge brings new issues to challenge our sense of moral responsibility.

CHAPTER 5 OPPORTUNITIES AND CHALLENGES

This chapter briefly examines some uses of genetic modification and indicates some of the opportunities and concerns these developments create.

5.1 Genetically-modified micro-organisms

Micro-organisms have been used for thousands of years in the production of fermented food and drink such as cheese, bread, beer and wine. The success of traditional fermentations was, however, often a matter of luck. The microbes drifting into the brewery or dairy vat sometimes turned wine to vinegar, or prevented yoghurt from setting.

In the mid-19th century aseptic techniques were developed, making it possible to select and grow pure cultures of individual species and strains of microbes. These could be used as 'starter' cultures to seed fermentations, greatly improving the reliability of the process and to some extent the quality of the finished products.

Selected strains of microorganisms are today used to manufacture a wide range of food, medicines and industrial products. For example, special strains of *Aspergillus niger* are used to make citric acid, *Penicillium* is used in the manufacture of antibiotics, and many species of bacteria and fungi are cultivated to produce a wide variety of valuable enzymes.

Until the second half of the 20th century all of the microbes used for fermentation were selected from nature. In the 1950s and '60s crude attempts were made to modify the DNA of microbes and plants by exposing them to radiation or mutagenic chemicals. This sometimes resulted in improved microbial production strains or crops, but the method was unpredictable and success was limited.

Spurred on by the discovery of the structure of DNA in 1953, our understanding of genetics has improved dramatically. A gene is a stretch of DNA that contains the instructions for making all or part of a protein, or for regulating protein production.

By 1973 techniques were developed that allowed individual genes to be transferred between microorganisms with precision: this process is called genetic modification (GM). Later it also became possible to modify plants and animals, including humans, in a similar way (we tend to call genetic modification of humans 'gene therapy', as it is currently only used to treat diseases). In genetic modification, a selected gene is isolated from a microorganism, plant or animal. It is then introduced into another organism, creating a **transgenic** or genetically-modified organism (GMO) – see figure 5.1 on the next page. Genes encoding desirable proteins can be transferred into microbes, for example, so that the modified organisms can be grown in large quantities to produce useful substances.

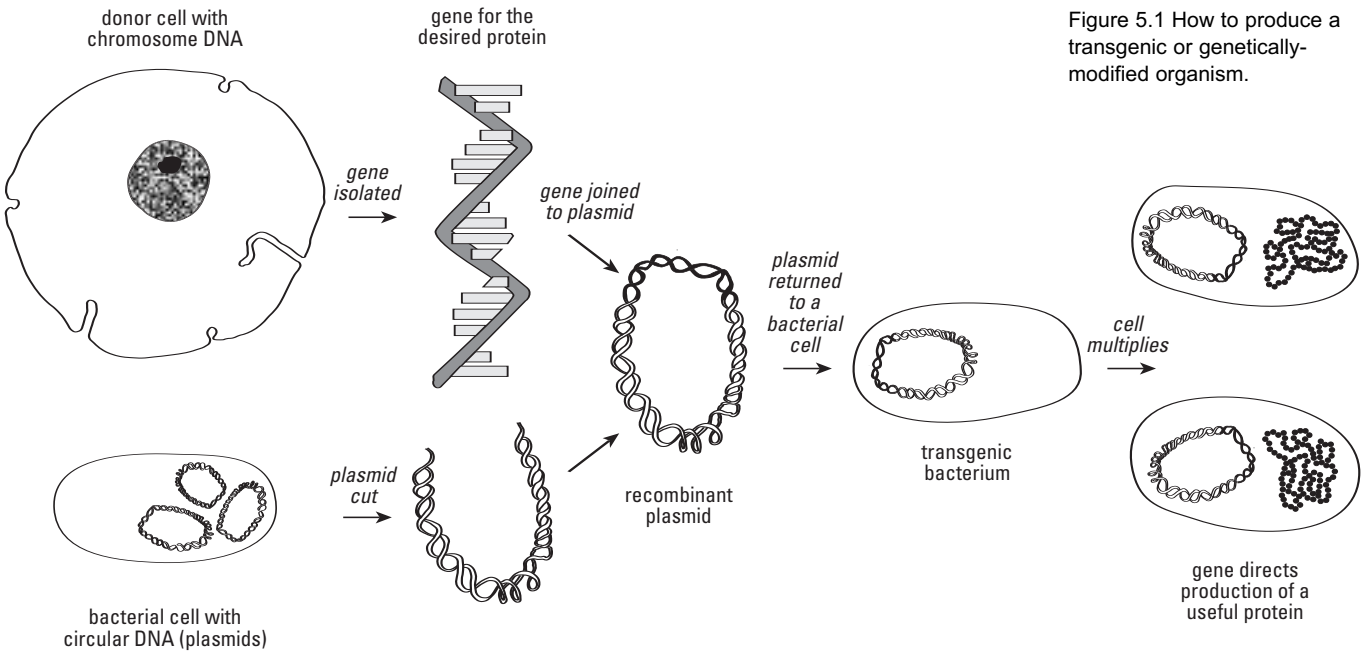


Figure 5.1 How to produce a transgenic or genetically-modified organism.

Human insulin from GMOs has been available in Europe since 1982. Before this development, people with diabetes had to inject themselves with pig or cow insulin, which differs by a few amino acids from the human form, and consequently produces undesirable side-effects with long-term use. Human insulin can be made by modified strains of either the bacterium *Escherichia coli* or a yeast.

The gene for human insulin, in a slightly modified form, is transferred into the microorganism. The GMOs are grown in nutrient broth in large fermenter vessels. A form of insulin is synthesized at the ribosomes in the microbial cells and then folds into the correct tertiary structure.

Human growth hormone

Children with growth hormone deficiency do not grow to a normal adult height. Injections of human growth hormone (hGH) during adolescence can remedy this. The hormone was once obtained from the pituitary glands of human cadavers, but the supply was very limited and costly. Furthermore, several people receiving contaminated hGH died from a degenerative brain disorder, Creutzfeldt-Jakob disease (CJD) – this is similar to 'mad cow disease' or variant CJD. The use of hormone from dead bodies was therefore banned in the 1980s.

Since then, growth hormone has been produced by genetically-modified bacteria and experience has shown it to be a safe and effective treatment. This is a valuable advance but it has created problems. Firstly, it is difficult to decide who requires treatment, and doctors have sometimes come under pressure to administer the drug to children who produce normal levels of hGH. Secondly, like several similar hormones, hGH has been abused by athletes and body-builders wishing to increase their muscle mass and reduce fat levels.

An empty phial that had contained growth hormone was found in a changing room at the Barcelona Olympics in 1992. This and similar finds have fuelled speculation that hGH may well be the drug of choice across a range of sports, although the benefits to the athletes remain unproven. Hormone abuse has many undesirable side-effects, including joint pain, arthritis, abnormal heart growth, muscle weakness, increased blood fats, impaired glucose regulation, diabetes and impotence. Tests for detecting hGH from GMOs have recently been developed, and may help to reduce the incidence of such drug abuse.

5.2 Plant breeding

During the last 50 years, conventional plant breeding and improvements in agricultural methods have more than doubled the yield per hectare of crops such as wheat, barley, maize and rice. Traditional plant breeding is a relatively slow and labour-intensive process: if two parent plants are crossed, the seeds from them must be collected, planted and the resulting plants cultivated, before the results of the cross can be seen. Furthermore, plant breeders must work with whole sets of inherited characteristics. Consequently a cross to introduce a desirable characteristic is likely to introduce one or more undesirable characteristics as well — and these must then be painstakingly 'bred out'. Genetic modification can be used to speed up and improve the precision of plant breeding compared with conventional methods.

Techniques of plant biotechnology

Plants can be genetically-modified using several different techniques. The best-established method uses a soil bacterium as a go-between. This organism, *Agrobacterium*, has a natural ability to alter the genetic material of plant cells so that small swellings (or galls) are formed on the plant. Molecular biologists have adapted the mechanism used by *Agrobacterium* so that desirable genetic information rather than that which promotes the formation of galls is transferred into plants. The *Agrobacterium* method has been used successfully with a wide variety of plants and has proved particularly useful for the modification of tree species which, because they are large and slow-growing, are difficult to alter by conventional breeding. However, the most important cereal crops are not affected by *Agrobacterium*, so other mechanisms have to be used for them.

Ballistic impregnation is an unlikely-sounding method that has achieved some success with cereals and other crops. It involves sticking the DNA to be introduced into the plant onto minute gold or tungsten particles, then firing these (like bullets) into the plant tissue. A proportion of the plant cells treated in this way take up the DNA from the metal pellets. Whole plants are then re-grown from the cells by tissue culture.

Another method is electroporation. Short pulses of a strong electric field cause minute pores to appear momentarily in the plant cells, allowing DNA to enter from a surrounding solution. This works best with plant tissues that have no cell walls (such as the tubes which develop from pollen grains).

Antisense technology can be used to 'neutralise' the action of specific undesirable genes (such as those involved in the excessive softening of fruit). The same technique can be utilised to combat the activity of plant viruses, providing a means of controlling viral infection, for which there is currently no effective treatment. Antisense technology lies behind many of the current applications of plant biotechnology.

Although genetic modification receives much attention, of as much significance is the application of genetic mapping to plant breeding. By determining the location and likely action of many plant genes, conventional plant breeding is being conducted with greater precision, as it becomes possible to detect quickly those plants which carry desirable characteristics.

Marker genes

With all current gene transfer techniques, only a small proportion of the treated cells successfully incorporate the novel DNA. Therefore, so-called 'marker genes' are usually linked to the DNA fragments before they are transferred. These markers can be detected easily, enabling scientists to select the organisms that have taken up the desired DNA. To date, the most frequently-used markers have been genes for proteins that allow the GMOs to grow in the presence of a specific antibiotic or herbicide.

Concern has been voiced about these, as it is feared that genes conferring resistance to the effects of antibiotics may spread into bacterial populations (such as microbes in our intestines), or that herbicide resistance may transfer to weeds growing alongside fields of modified crops. Among the other questions that regulatory authorities have asked are whether the marker genes permit their recipient to make a new protein and, if so, what levels of that protein (if any) would be expected in the food. Could that protein trigger any unwanted effects such as allergic reactions?

The Food Safety Unit of the World Health Organisation and a working party of the Organisation for Economic Co-operation and Development have looked specifically at the safety issues associated with marker genes in plants that are to be consumed as foods. The presence of marker genes (the DNA itself) in food is not thought to constitute a safety concern. There is DNA in abundance in almost all the food we eat, but no recorded evidence for the transfer of whole functional genes from plants to microorganisms in the gut or to any other living things (including humans).

Both the possibility of DNA transfer and the production of proteins from marker genes, and their possible effects, are considered on a case-by-case basis by the regulatory authorities in the USA and Europe. As a precaution, the use of antibiotic and herbicide-resistance markers in new GM crops is being phased out.

Current applications of plant genetic modification

The majority of current plant biotechnology is directed towards the improvement of food plants: the remaining work is concerned with non-food

crops such as cotton, tobacco, ornamental plants and medicinal compounds. The initial emphasis has generally been on the improvement or introduction of qualities of value to the farmer rather than those that directly benefit the consumer.

Many thousands of field trials of genetically-modified plants have been carried out world-wide. Although several different modified crops are grown extensively in the USA, China and elsewhere, public opposition to the technology means that few are so far in commercial production in the European Union (EU).

Only a handful of GM-derived products have been approved for food use in the EU: processed soya derivatives such as lecithin; oil from oil seed rape; processed tomato purée and maize. No fresh GM products (such as tomatoes, potatoes or unprocessed soya beans) have been approved for human consumption in the EU. The only GM crop currently grown to any extent in the EU is maize, which is used for animal feed. Two examples show today's principal commercial uses of GM technology in agriculture: herbicide-resistant soya and insect-resistant maize.

Herbicide-resistant soya

The first herbicide-resistant soya plants were produced in the USA using Agrobacterium technology. Novel genes allow the plants to withstand the broad-spectrum herbicide glyphosate, which is sold under the trade name 'Roundup'. Glyphosate can therefore be sprayed onto the fields in which the soya is grown, where it kills any weeds but leaves the soya plants unharmed. This boosts the growth of the soya plants, as they do not face competition for water, nutrients and light from surrounding weeds.

The US firm Monsanto manufactures the herbicide and sells the soya beans. 'Roundup' is considered by some to be an environmentally-acceptable herbicide because it breaks down rapidly on contact with soil. Others argue that such a wide-spectrum herbicide could reduce the food supplies for animal species and lead to a reduction in biodiversity.

Nutritionally there is no difference between modified and unmodified beans. More than 60% of processed foods contain soya or soya products. The GM soya beans were approved for marketing in the EU — as processed beans only — in April 1996, although public opposition has led most European food producers to seek non-GM alternatives to the imported GM product. Monsanto's 'Roundup Ready' soya has been the subject of considerable international opposition from the environmental campaigning organisation, Greenpeace.

Insect-resistant maize

Several varieties of maize have been genetically-modified (using ballistic impregnation) to be resistant to an insect pest, the European Corn Borer. This creature bores through the stem and ear of the plant causing it to topple over. On average, the Corn Borer destroys 4% of the world's annual maize crop and up to 20% in severely-infested regions.

The European Corn Borer is traditionally controlled using chemical or biological insecticide sprays which are applied to the outside of the plant. These insecticides are only effective during the first three days in the corn borer's life cycle (before it burrows into the stem, where it is protected). The new varieties of maize contain a gene that encodes a protein (Bt toxin) which kills the corn borer as it tries to eat the plant. The gene is derived from a soil bacterium *Bacillus thuringiensis*, which is widely used as a biological insecticide by organic farmers.

Maize kernels are processed to make starch and glucose syrups, and the remaining protein is used as animal feed. Several varieties of GM maize include copies of a marker gene encoding ampicillin resistance. Ampicillin is an antibiotic that is widely-used in medical treatment. Numerous authorities have voiced concern over the presence of the ampicillin marker gene and the possibility that it could be transferred into gut bacteria, particularly in animals fed on large quantities of maize or in people for whom maize forms a significant part of the diet. The proportion of the US harvest which is from genetically-modified varieties is not separated from the rest of the crop; the importation into the EU of processed forms of this maize for use in food products was approved in January 1997. Public concern has led the majority of European food producers to buy maize products from non-GM sources, which is not too difficult as Europe is largely self-sufficient in maize and grows very little of the GM varieties.

Herbicide-tolerant maize

A herbicide-tolerant GM fodder maize (Chardon LL, developed by German firm Bayer CropScience) was given the green light for commercial cultivation in the European Union in 1999. Limited regulatory approval in the UK took many more years to obtain, however. Although in March 2004 the English and Scottish parliaments finally approved the commercial cultivation of a Chardon LL, Bayer CropScience claimed that the strict regulations imposed made cultivation of the crop uneconomic, and withdrew the product. Consequently the earliest we are now likely to see any commercial growing of GM crops in the UK is 2008.

GM plants: the concerns

Environmental safety

Two main concerns about the effects of genetically-modified food plants on the environment are that the new plants will become pernicious weeds, or that they will transfer their new genes to wild relatives or similar crops growing nearby with unforeseen effects. A great deal of research has been carried out by ecologists to determine whether or not these worries are likely to be substantiated. This is one of the major reasons for carrying out field trials of GM crops.

Evidence from thousands of field trials suggests that, from the farmer's point of view, the new plants will behave just like the varieties currently in

cultivation. There is evidence to suggest that the transfer of genetic material from modified crops to their wild relatives or unmodified plants does occur, although the frequency of such transfer and its significance is still debated.

A further concern is whether plants with introduced genes that enable them to resist insect attack will quickly lead to the establishment of populations of resistant pests. This might happen because plants that have been modified to produce insecticides will kill all the susceptible insects where they grow, leaving alive only those insects that are naturally resistant to the toxin. These will multiply rapidly, rendering the GM plants' defence mechanism useless.

One method of hindering the emergence of resistant insect populations is to plant 'refuges' of susceptible plants alongside the modified crops so that the susceptible insects can thrive in a limited area and the resistant insects therefore do not take over. This has been a legal or voluntary requirement in the USA and Australia where GM insect-resistant cotton has been grown. To date, there have been no confirmed cases of resistant populations developing, but it is generally accepted that without measures such as the use of 'refuges', resistant pest populations will certainly emerge. With this in mind, in January 1999, four major producers of Bt maize plants proposed that 20% of farmland should be set aside for non-transgenic crops when Bt maize is grown.

English Nature, the RSPB and others fear that the cultivation of herbicide-tolerant plants coupled with the use of broad-spectrum herbicides will reduce food supplies for wildlife and lead to a dramatic reduction in biodiversity.

Field trials

In November 2004 a UK study of several GM crops grown over four years found no evidence that they are more harmful to the environment than conventional varieties. The Bright Link project studied sugar beet and winter oil-seed rape which had been modified to make them tolerant of specific herbicides. The novel crops were grown in rotation with non-GM cereals, and compared with similar rotations involving non-GM beet and rape. This study apparently contradicted the findings of another major GM investigation, called Farm-Scale Evaluations.

The Farm-Scale Evaluations found that two GM varieties, a sugar beet and a spring rape, were more damaging to biodiversity than conventional crops. There were fewer insect groups, such as bees and butterflies, recorded among the plants. A GM maize, on the other hand, appeared to do better than its conventional cousin. There were more weeds in and around the modified maize crops, more butterflies and bees around at certain times of the year, and more weed seeds. The contradiction seems to be explained by the sorts of herbicides used in the two sets of trials and frequency with which they were applied. Such studies have emphasised the need for caution and the importance of case-by-case analysis.

Changes in farming structure

Biotechnology has the potential to affect world agriculture dramatically. Although great benefits may come, it has been suggested that there might also be accompanying disadvantages. Several of these disadvantages are no different from existing trends in world agriculture, such as the shift towards larger farms and more capital-intensive farming systems. This tends to favour, for example, wealthy farmers in the Northern hemisphere who can invest in new technologies rather than those in the impoverished South. In the developed world, there are concerns about over-production of food, although these worries are unlikely to be shared by those countries where the growth in population far outstrips the capacity of farmers to provide sufficient food. According to an independent study conducted by The Royal Society and other international scientific bodies, biotechnology, alongside other changes and technologies offers a realistic prospect of long-term sustainable agriculture to farmers in the Third World.

Consumer choice

Numerous surveys show that most people in Western Europe wish to choose whether they consume GM food. The first GM product in Europe (tomato purée) presented no problem in this respect, as the cans were clearly labelled and always offered alongside a similar non-GM product. The problem arose when GM food ingredients that are traded as bulk commodities entered the market. First GM soya, then maize started to be grown in the USA and traded internationally. This presented UK retailers, who had planned carefully for the introduction of the tomato purée, with an unexpected problem.

Initially, the amount of GM maize and soya grown was very small, forming just a fraction of the total US harvest. It was impossible, with GM and non-GM material being mixed after harvesting, to devise a sampling regime that would reliably permit the detection of the GM material in bulk shipments. UK retailers therefore assumed that GM material *would* be present in any maize or soya obtained from the USA, even if in only very small amounts and voluntarily labelled their products accordingly. The major retailers prepared leaflets that were available to shoppers, explaining this decision and the reasons for it. This fed right into the hands of anti-GM campaigners, who claimed that up to 65% of processed food sold in the UK was made with GM ingredients — and the food packets in the shops seemed to bear this out. Consumers were apparently being denied a choice. Newspaper articles and even entire books were devoted to lists of GM-containing and GM-free products, although they were often misleading.

As the proportion of GM soya and maize on the world market increased, food producers and retailers tried to obtain certified non-GM material. In August 1999, a major UK firm estimated that the cost of obtaining crops that were segregated at source added 10–15% to the cost of the food sold in their shops.

Within a few months, however, all of the major UK food producers and retailers had obtained GM-free material, aided by lists of suppliers prepared by the Government. The GM-free material was routinely tested using the

sensitive polymerase chain reaction method which is, in theory, able to detect as little as one molecule of DNA. The very sensitivity of technique caught out some producers who had unwittingly incorporated GM material into their products, but in fact the levels detected were usually below those (1%) which would at that time have triggered the necessity under UK law to label the products.

Today the situation is rather different. Almost all food in UK shops is non-GM and it seems very likely that producers and retailers will continue to obtain certified non-GM material to meet consumer demand. In October 2003 the EU labelling regulations became even stricter. They require that, for example, oil from GM rape is labelled along with other products such as glucose syrup produced using starch from GM maize, even though they contain no trace of GM material and are identical to the non-GM product. The amount of approved GM material permitted before labelling is required is now 0.9%. Just 0.5% contamination by non-approved GM crops is permitted, and only then if the inclusion of this material is 'technically unavoidable'. Also, for the first time, animal feed derived from GM sources has to be labelled as such. Because the presence of material from GM plants will, in many cases, not be detectable, each food ingredient has to be accompanied by paperwork confirming its origin.

Regulation of genetic modification

New safety regulations often follow in the wake of a problem that was not anticipated. An example is the restrictions on the processing and sale of beef once the nature of BSE was appreciated. However, the regulations covering genetic modification are an exception, as they were developed in advance of the occurrence of any hazards to health or the environment.

Just over thirty years ago, when the possibility of combining genetic information from different organisms was first proposed, the international scientific community voluntarily imposed a world-wide moratorium on the use of this technology. Later these restrictions were eased, as safe working practices and regulatory frameworks to control their use were developed. In 1978, the United Kingdom was the first country in the world to pass legislation controlling genetic modification. Similar regulations were also introduced in the USA and elsewhere in Europe, the most stringent being in Germany and Denmark.

European Directives

In October 1990 all European Union nations adopted two European Council Directives governing the use of genetically-modified organisms. These Directives harmonised existing laws in the different member states, so that a single set of regulations applies across national borders.

The two Directives cover genetically-modified organisms used in 'containment', such as in a laboratory or an enclosed factory or brewery and those 'deliberately released' into the environment through field trials or crops, commercial production and (potentially) the marketing a living GMO (such as a whole fruit) which may have been grown outside the EU and imported.

Deliberate releases and field trials

'Deliberate releases' of GMOs into the environment include field trials of crops. The European Council Directive requires that proposed field trials should be considered individually. People intending to test new varieties must first seek permission from the appropriate national authority. In the UK the responsible authority is currently the Department for the Environment, Food and Rural Affairs (DEFRA). The DEFRA has an Advisory Committee on Releases to the Environment (ACRE). ACRE is the independent body which reviews applications for field trials of organisms. Any application to ACRE must contain a technical dossier including information about the personnel concerned and their training, and a full environmental risk assessment. The DEFRA also consults the Food Standards Agency and other relevant government departments and advisory bodies (such as the Department of Health).

In certain cases, interest groups or the public may be consulted about a proposed release. It is up to individual EU countries to decide whether and how this should be done. Action has been taken in the several countries, including the UK, to ensure that there is 'public interest' representation on ACRE, and that the public should have access to information about proposed tests – all ACRE's deliberations and advice to Ministers are available from the ACRE website. Companies or others may ask for some information to be kept confidential on grounds of commercial competitiveness, but in such cases they must give verifiable justification.

A general principle is that tests of new crops should be conducted a step at a time, and that the scale of use should be increased gradually only as it becomes apparent that it is safe to do this. Once a field test has been carried out a report on it must be sent to the appropriate authorities. The European Council Directive makes provisions for the exchange of information on releases between member states, and for the widespread publication of details.

The Novel Foods Regulation

In 1997, a third European regulation was approved after several years of discussion. The Regulation for Novel Foods and Novel Food Ingredients replaced some older rules and also introduced requirements for safety assessment, environmental risk assessment (for GMOs) and labelling of products. These rules have since been altered several times as new products have come onto the market or new concerns have arisen.

Before it can be placed on the market, the Novel Foods Regulation demands that a novel food or ingredient:

- is safe for the consumer when eaten at the foreseeable levels of use;
- is not presented in such a way as to mislead the consumer; and
- does not differ from a food or ingredient that it replaces in such a way that its foreseeable consumption is nutritionally disadvantageous to the consumer.

Under the Regulation, when a company wishes to place a novel food or ingredient on the market, it must first apply to the responsible authority (such as the Food Standards Agency) in the country where the product is to be

marketed for the first time. In the UK, the Food Standards Agency will consult with the Advisory Committee on Novel Foods and Processes, an independent group of specialists which advises the Government on novel foods. The Food Standards Agency will also consult with the DEFRA, the Department of Health and other relevant departments. The UK advice will then be forwarded to other EU states who need to agree before approval can be given.

5.3 Animal breeding

The genetic modification of animals is still in its infancy. The majority of GM animals are flies, nematode worms and mice used in medical research. The majority of features in livestock for example are controlled by many genes, each with a small effect. Just which genes should be altered to improve animal productivity or health is therefore difficult to predict. This area requires very careful consideration: developments that compromise animal welfare are increasingly unlikely to be accepted by regulatory authorities or the public. Although there were early successes with modifying sheep to produce useful proteins in their milk, such as alpha-1-antitrypsin, which is used to treat emphysema, such developments remain at the research stage. In the immediate future, most benefit is likely to come from the development of new diagnostic agents, vaccines and therapeutic agents for veterinary medicine.

Agriculture in Europe and North America already produces sufficient food for the indigenous population. In the future, the real benefits from improved animal production might be seen in the Third World. For example, it may one day be possible to introduce disease resistance into otherwise vulnerable animals. There are well-advanced animal genome projects which parallel the successful mapping of the entire human genome. The Bovine Genome Project could result in, for instance, resistance to trypanosomiasis being introduced into more productive breeds of cattle from their naturally-resistant African counterparts.

GLOSSARY

ACTIVE SITE. The place in an enzyme where the catalytic reaction occurs.

ADP. (adenosine diphosphate) A nucleotide with two phosphate groups; it forms when ATP is hydrolysed in an energetically favourable reaction.

AGAROSE GEL. A network of carbohydrate polymers in water that restricts the passage of protein and DNA molecules, depending on their size.

ALA. (amino laevulinic acid) A precursor to haem in a metabolic pathway.

Allosteric inhibitor. A compound that reduces the activity of an enzyme by binding at a specific site, that is not the active site.

AMPLIFICATION. A reaction process that increases the number of copies of a sequence of DNA (see PCR).

AMYLASE. An enzyme in saliva that can catalyse the hydrolysis of starch.

ANTIBIOTICS. Compounds that kill or prevent the growth of micro-organisms, such as bacteria

ANTICODON. The sequence of bases on tRNA that is complementary to a codon on mRNA.

ATP. (adenosine triphosphate) A nucleotide with three phosphate groups; formed from ADP in an energetically unfavourable reaction.

BACTERIA. Single-cell micro-organisms which can reproduce themselves; they commonly causes disease.

BARBITURATES. Drugs which have a sedative effect on the human central nervous system.

BASE PAIR. A pair of bases that bond together in DNA by hydrogen bonds; also link tRNA to mRNA. Cytosine pairs to Guanine, Adenine pairs to Thymine or Uracil.

BIOTECHNOLOGY. The scientific use of living organisms to produce useful products, on a laboratory or industrial scale.

CATALASE. An enzyme in liver that catalyses the decomposition of hydrogen peroxide.

CHROMOSOME. A DNA molecule folded into a compact structure; found in the nuclei of cells.

CITRIC ACID CYCLE. The pathway by which ethanoyl groups are oxidized to carbon dioxide and water.

CLONES. Cells, or organisms, with identical sets of chromosomes; from a single parent cell.

CoA. (coenzyme A) An essential substrate in a number of biochemical reactions.

CODON. The sequence of three bases on mRNA that codes for one of 20 amino acids; there are also start and stop codons.

COFACTOR. A compound or ion that is required for an enzyme to be active.

COMPETITIVE INHIBITOR. Binds reversibly to an enzyme, blocking the active site and reducing enzyme activity.

COMPLEMENTARY DNA. A matching strand of DNA, with a complementary set of base pairs.

DEHYDROGENASES. Enzymes that remove two hydrogens from a molecule.

DENATURE. The unravelling of the tertiary shape of a molecule; this is usually reversible in DNA but not in proteins.

DNA (deoxyribonucleic acid). An organic polymer with a chain of phosphate groups and pentose sugars, with side chains of four organic bases; usually found as a double-stranded helix.

DNA FINGERPRINT. A unique pattern of DNA fragments obtained by gel electrophoresis; everyone has their own unique pattern.

DNA POLYMERASE. The enzyme that catalyses the formation of two double stranded DNA molecules using single strands of DNA as templates.

ELECTROPHORESIS. A method of separating charged molecules in a gel or on paper by the application of a voltage.

ENZYME ACTIVITY. The rate at which a reaction takes place in the presence of a particular amount of enzyme.

ENZYMES. Proteins that catalyse specific biochemical reactions by lowering the activation energy of the reaction.

ESSENTIAL AMINO ACID. An amino acid which is needed in the diet because it cannot be synthesized by a particular mammal.

GENES. The long sequences of bases in DNA that code for proteins.

GENETIC CODE. The complete set of the possible sequences of three bases (codons) that can occur in mRNA and their translation into amino acids or stop and start codes.

GENETIC ENGINEERING. The ability to alter the genetic material of an organism in a deliberate manner, in particular moving genes from one species to another.

GENOME. The complete DNA in the chromosomes of an organism.

GLYCOGEN. A glucose polymer that acts as an energy store in the human body.

GLYCOLYSIS. The pathway in which glucose is oxidized to pyruvate.

HALF-LIFE. The time for the concentration of a compound to halve from an initial concentration; for biochemical reactions half-lives are usually constant (independent of the initial concentration).

HERBICIDE. A compound designed to kill unwanted plants, such as weeds.

HOMOGENIZATION. The process of breaking the cell membrane to release the content of the cell.

HORMONES. Compounds helping to co-ordinate the activities of different cells.

IMMOBILIZED ENZYMES. Enzymes that are bonded to insoluble support materials or trapped inside an insoluble material.

INHIBITION. The reduction of the activity of an enzyme by a specific chemical compound.

INSECTICIDE. A compound designed to kill harmful insects.

INSULIN. One of the hormones that regulate the concentration of glucose in the blood.

IRREVERSIBLE INHIBITION. The irreversible binding of a compound to the active site of an enzyme that permanently reduces its activity.

LACTATE. The anion of lactic acid, 2-hydroxypropanoic acid.

LIPASE. An enzyme which hydrolyses the ester group in fats (lipids).

LYSOZYME. An enzyme that can destroy the cell walls of bacteria.

METABOLIC PATHWAY. A sequence of reactions in cells.

METABOLISM. The reactions that maintain cells and living organisms.

MICROBE. A micro-organism that can replicate.

MITOCHONDRIA. The part of a cell where the citric acid cycle, oxidative phosphorylation and haem synthesis take place.

mRNA. Synthesized using DNA as a template; moves to the ribosome to direct protein synthesis.

MUTATION. A change in the sequence of the bases on DNA

NAD^+. (nicotinamide adenine dinucleotide) A compound that is an oxidizing agent in many biochemical reactions.

NUCLEIC ACID. A biochemical acid formed from a sugar, organic bases and phosphate groups; see DNA and RNA.

NUCLEOTIDES. The monomer building blocks for DNA and RNA; they consist of phosphate groups, a pentose sugar and an organic base.

NUCLEUS. The part of a cell where chromosomes (DNA) are found.

OXIDATIVE PHOSPHORYLATION. The formation of ATP by addition of a phosphate group to ADP; by a reaction coupled to the oxidation of NADH.

PCR (POLYMERASE CHAIN REACTION). A laboratory procedure for obtaining multiple copies of a target length of DNA.

PEPSIN. An enzyme secreted in the stomach.

PHOSPHATE. The anion of phosphoric acid, H_3PO_4.

PHOSPHORYLATION. A reaction that adds a phosphate group to a molecule, such as ADP.

PLASMIDS. DNA molecules in which the strands have joined in a circle; capable of self-replication.

PRIMARY STRUCTURE. The amino acid sequence of a protein.

PRIMERS. Short lengths of DNA (or RNA) that mark the site on large DNA strands where replication will start; used in PCR.

PROBES. Short lengths of DNA that will bind to specific sequences of bases in DNA, enabling them to be detected; used in DNA fingerprinting.

PROTEASE. An enzyme which hydrolyses the peptide group in proteins.

PURINES. Organic bases with two rings, both with nitrogen atoms; adenine and guanine are purines.

PYRIMIDINES. Organic bases with two nitrogen atoms in a six-membered ring; cytosine, thymine and uracil are pyrimidines.

PYRUVATE. The anion of pyruvic acid, 2-oxopropanoic acid.

RECOMBINANT DNA. Technology process of cutting and then rejoining DNA molecules to alter their genetic make-up.

REPLICATION. The production of two identical double-stranded DNA molecules from one DNA molecule.

RESTRICTION ENZYME. Cuts DNA at a specific sequence of bases.

RIBOSOME. The part of a cell where protein synthesis occurs.

RNA (RIBONUCLEIC ACID). A nucleotide polymer that differs from DNA having uracil instead of thymine, and ribose instead of deoxyribose.

SECONDARY STRUCTURE. Regular folding in the amino acid chain of a protein.

SUBSTRATE. A compound involved in a chemical reaction, catalysed by an enzyme.

SUBSTRATE LEVEL PHOSPHORYLATION. Is the formation of ATP during one of the reactions by which food molecules are broken down, by the transfer of a phosphate group from a substrate to ADP.

TBE BUFFER. (tris-borate-EDTA) A buffer that provides the right conditions for the gel electrophoresis of DNA.

TE BUFFER. (tris-EDTA) A buffer used when cutting DNA with restriction enzymes.

TERTIARY STRUCTURE. The complete three dimensional structure of a protein.

TRANSCRIPTION. The formation of mRNA that is a complementary copy of a gene, but with thymine replaced by uracil.

TRANSGENIC ORGANISMS. Organisms that have a gene in their DNA from another species, put there by laboratory methods (genetic engineering).

TRANSLATION The production of a protein molecule using the information on a mRNA molecule.

tRNA The group of compounds that carry amino acids to the ribosome for protein synthesis.

VIRUS An infectious agent that can only replicate in a host cell; not usually regarded as a living organism.

Nuffield Advanced Chemistry
BIOCHEMISTRY
Experiments

Contents

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Health and Safety

See the safety notes given with each experiment.

Health and safety in school and college science affects all concerned: teachers and technicians, their employers, students, their parents or guardians, as well as authors and publishers.

As part of the reviewing process, these publications have been checked for health and safety. In particular, we have attempted to ensure that:
• all recognized hazards have been identified,
• suitable precautions are suggested,
• where possible, the procedures are in accordance with commonly adopted model (general) risk assessments,
• if a special risk assessment is likely to be necessary this has been pointed out
• where model (general) risk assessments are not available, we have done our best to judge the procedures to be satisfactory and of an equivalent standard.

It is assumed that:
• practical work is conducted in a properly equipped and maintained laboratory,
• rules for student behaviour are strictly enforced,
• mains-operated equipment is regularly inspected, properly maintained and appropriate records are kept,
• care is taken with normal laboratory operations such as heating substances and handling heavy objects,
• good laboratory practice is observed when chemicals are handled,
• eye protection is worn whenever risk assessments require it,
• any fume cupboard required operates at least to the standard of Building Bulletin 88,
• students are taught safe techniques for such activities as heating chemicals, smelling them, or pouring from bottles,
• hand-washing facilities are readily available in the laboratory.

Under the COSSH and the Management of Health and Safety at Work regulations, employers are responsible for carrying out risk assessments before hazardous procedures are undertaken or hazardous chemicals used or made. Teachers are required to co-operate with their employers by complying with such risk assessments.

However, teachers should be aware that mistakes can be made and, in any case, different employers adopt different standards. Therefore, before carrying out any practical activity, teachers should always check that what they are proposing is compatible with their employer's risk assessments and does not need modification for their particular circumstances. Any local rules issued by the employer must always be followed, whatever is recommended here.

Model (general) risk assessments have been taken from, or are compatible with:
CLEAPSS Hazcards (see annually updated CD-ROM)
CLEAPSS Laboratory handbook (see annually updated CD-ROM)
CLEAPSS Recipe cards (see annually updated CD-ROM)
ASE Safeguards in the school laboratory 10th edition 1996
ASE Topics in Safety 3rd edition, 2001
ASE Safety reprints, 2000 or later

Clearly, teachers must follow whatever procedures for risk assessment their employers have laid down. As far as we know, all the practical work and demonstrations in this course are covered by the model (general) risk assessments detailed in the above publications, and so, in most schools and colleges, you will not need to take further action.

If teachers or students decide to try some procedure with hazardous substances beyond what is in this course, and you cannot find it in these or other model (general) assessments, then the teachers' employer will have to make a special risk assessment. If the employer is a member, then CLEAPSS will act for them. Otherwise the ASE may be able to help.

Only the teacher can know when the school or college needs a special risk assessment. But thereafter, the responsibility for taking all the steps demanded by the regulations lies with the employer.

Investigations will involve independent action by the student. Our notes on investigations warn students to carry out a risk assessment; students should be responsible for safety in the first instance and credited in any assessment for making safe plans. Nevertheless, proposals must be seen by the teacher and who must make an appropriate check, particularly with respect to safety, on what will go on. The teacher will need to take particular care if students consult library books published before modern safety standards came into force or get ideas from the internet.

Experiment 1 The effect of pH on enzyme activity

(See Chapter 2)

Starch is a mixture of carbohydrate polymers consisting of long chains of glucose units, some with a branched-chain structure. Both types of structure react with iodine to give a characteristic blue–black colour.

Amylase enzyme in our saliva hydrolyses starch, producing a complex mixture containing mainly maltose. This mixture does not affect the colour of iodine solution although other carbohydrate molecules, produced during the course of the hydrolysis, give rise to violet, brown, and orange colours.

/!\ SAFETY
Saliva can transmit certain infectious diseases.
The procedure should be followed carefully.

Mouth pipettes must not be used. Handle only your own saliva. At the end of the experiment, rinse all the apparatus contaminated by your saliva yourself.

Then leave the apparatus to soak in freshly-diluted 1% sodium chlorate(I) solution.

Procedure

Isolation of enzyme

Rinse your mouth out with about 20 cm^3 of pure water into a small beaker. Make sure your saliva and the rinse water are well mixed.

Preliminary measurement to find the approximate level of enzyme activity

a Put 5 cm^3 of 1% starch, 1 cm^3 of 0.2 M sodium chloride, and 2 cm^3 of 0.067 M phosphate buffer solution, pH 7.0, into a test-tube.

b Using a dropping pipette, fill each of the hollows in a spotting tile with 5 drops of 0.001 M iodine solution.

c Using a graduated pipette, fitted with a pipette filler, add 1 cm^3 of the saliva solution to the test-tube. Start the clock, stopper the test-tube and shake it vigorously. Quickly remove a small portion of the mixture, using a dropping pipette. Add 3 drops of the mixture to one of the iodine samples and stir with a glass rod.

- At 2-minute intervals, shake the test-tube, withdraw a further 3 drops, and add them to a fresh sample of iodine.
- Continue until no positive starch test is obtained.

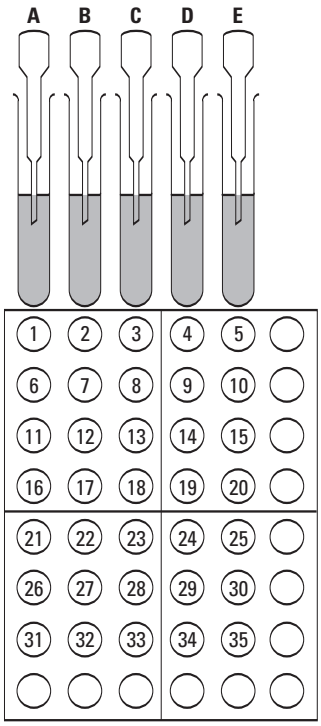
d Calculate the volume of saliva solution required to hydrolyse most of the starch in about 8 minutes. This volume will be used in subsequent experiments. If necessary, concentrate the solution by adding more saliva, or dilute with distilled water.

Continued

Experiment 1 The effect of pH on enzyme activity *continued*

(See Chapter 2)

The effect of pH on amylase activity



a Set up five test-tubes each containing 5 cm^3 of 1% starch, 1 cm^3 of 0.2 M sodium chloride, and 2 cm^3 of buffer solution of different pH values, as below.

Tube	A	B	C	D	E
pH of buffer solution	4.5	5.9	7.0	8.0	8.8

b Arrange four spotting tiles as shown. Fill each cavity with 5 drops of iodine.

c Add the calculated volume of saliva solution to test-tube A. Start the clock, shake the test-tube, and immediately transfer 3 drops of the mixture to cavity 1, using a dropping pipette. Stir with a glass rod.

d At 30 seconds exactly, add a similar volume of saliva solution to test-tube B. Shake and immediately transfer 3 drops to cavity 2, using a clean dropping pipette.

d At 60, 90, and 120 seconds, add saliva to tubes C, D, and E. Test the mixtures by adding 3 drops to cavities 3, 4, and 5.

e At 150 seconds, shake test-tube A and transfer a further 3 drops to cavity 6.

f At 180 seconds, shake test-tube B and transfer a further 3 drops to cavity 7.

g Remove samples sequentially, every 30 seconds, until 6 or 7 samples have been removed from each test-tube.

h Devise a suitable way of recording your results. Remember that the addition of saliva to tubes A–E at regular intervals allows the direct comparison of the colours in each row of cavities. For example, the colour in each cavity of row 2 reflects the extent of the reaction in each tube 150 seconds after the addition of enzyme.

Questions

1 After the initial iodine-starch colour develops in a cavity, why does it not alter with time?

2 What effect does a change in pH have on the overall charge of a protein molecule? Why do such changes affect enzyme activity?

Experiment 2 How does the rate of an enzyme-catalysed reaction vary with the concentration of the substrate?

(See Chapter 2)

The enzyme catalase is found in bacterial and animal tissue. In the liver of mammals it catalyses the following reaction:

$2H_2O_2(aq) \rightarrow 2H_2O(l) + O_2(g)$

⚠ SAFETY
20 volume hydrogen peroxide is irritant. Wear eye protection during this experiment.

The role of catalase in cells is uncertain, but it may prevent the accumulation of poisonous hydrogen peroxide in cells. In this experiment, we find out how the rate of a reaction catalysed by an enzyme varies with the concentration of its substrate, hydrogen peroxide. The rate of decomposition of hydrogen peroxide is determined by measuring the volume of oxygen produced in a given time.

Procedure

Extraction of catalase

a Weigh out 10 g of liver and cut it into small pieces with scissors. Put the pieces in a mortar and add 3 g of sand. Grind the liver while slowly adding 10 cm^3 of 0.067 M phosphate buffer solution, pH 7. Continue grinding until there are no lumps of liver left.

b Scrape the homogenate into a depression made in a muslin cloth placed over a 100 cm^3 beaker, and extract as much liquid as possible by squeezing.

c Centrifuge this liquid for 3 minutes. Decant the supernatant into a 10-cm^3 measuring cylinder. Pour half of this supernatant into a 250-cm^3 beaker, make the volume up to 200 cm^3 with phosphate buffer solution, and stir. This is the enzyme extract to be used in runs number 1 to 8 on the next page.

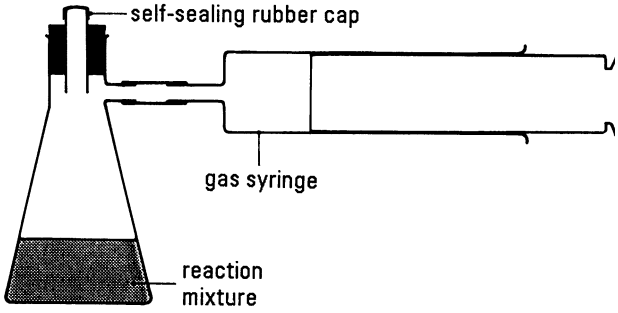
d Finally take approximately 4 cm^3 of the diluted extract, boil it for 1 minute, then cool it to room temperature. Use this boiled extract for run number 9.

Continued

Experiment 2 How does the rate of an enzyme-catalysed reaction vary with the concentration of the substrate? *continued*

Testing for catalase activity

Set up the apparatus as shown. The contents of the flasks for each run are shown at the bottom of the page. Pour the hydrogen peroxide into the flasks, using a burette.



a The same flask may be used for the nine separate runs, provided it is thoroughly washed with distilled water and excess drops of water are removed by shaking.

b Before starting the experiment, disconnect the self-sealing cap and push the plunger of the gas syringe to zero. Replace the self-sealing cap. Fit a hypodermic needle to a 2-cm^3 syringe. Draw 2 cm^3 of the enzyme solution into the hypodermic syringe, making sure that all air is expelled from the syringe.

c Start the reaction by piercing the self-sealing cap with the hypodermic needle and rapidly injecting the enzyme solution into the flask. After all the enzyme has been injected, immediately remove the needle, start the stop clock, and shake the flask vigorously.

d Measure the volume of oxygen produced after 15 seconds, keeping the flask vigorously swirled all the time. If the gas-syringe plunger appears to stick, ease the plunger out slightly by hand. It may be necessary to adjust the concentration of enzyme in run number 1 so that a suitable volume of oxygen is produced in 15 seconds. This volume should be between 40 cm^3 and 60 cm^3.

e Repeat this procedure for runs 2 to 8, washing the flasks thoroughly with distilled water between experiments and shaking well to get rid of the water. Lastly, using a fresh hypodermic needle and syringe, repeat the procedure (run number 9), but in this case inject 2 cm^3 of the boiled enzyme solution.

Run number	1	2	3	4	5	6	7	8	9
Volume of 0.067 M phosphate buffer solution, pH7 /cm^3	10	10	10	10	10	10	10	10	10
Volume of '20 volume' H_2O_2 /cm^3	15	10	7	5	3.5	2	1	0	15
Volume of distilled water /cm^3	3	8	11	13	14.5	16	17	18	3

Continued

Experiment 2 How does the rate of an enzyme-catalysed reaction vary with the concentration of the substrate? *continued*

Treatment of results

f Subtract a volume of 2 cm^3 from each reading of the gas syringe. After this correction has been made, subtract the readings of the control run (8) from the gas syringe readings.

g Calculate the initial molarity of hydrogen peroxide in each experiment. '20 volume' H_2O_2 has a concentration of 1.74 moles per dm^3, assuming that no decomposition has occurred.

h Plot a graph of volume of oxygen released (proportional to the rate of reaction) against the concentration of substrate (hydrogen peroxide).

Questions

1 Why was a volume of 2 cm^3 subtracted from each reading of the gas syringe?

2 What was the purpose of run number 8?

3 What was the effect of boiling the catalase? Using your knowledge of protein structure, give an explanation of this effect.

4 How does the rate of reaction depend on substrate concentration at
i relatively low substrate concentrations,
ii high substrate concentrations?

5 What prevents the enzyme from working at its highest catalytic activity at low substrate concentrations?

6 At high substrate concentrations, why does a further increase in substrate concentration have little effect on the rate of the enzyme-catalysed reaction?

Experiment 3 Making whey syrup using an immobilized enzyme

(see Chapter 2)

Whey is a by-product from cheese-making, but the sugar it contains, lactose, is not very sweet. The hydrolysis of lactose, using enzyme, produces glucose and galactose. These are much sweeter and make the product, whey syrup, useful in the confectionary trade.

Procedure

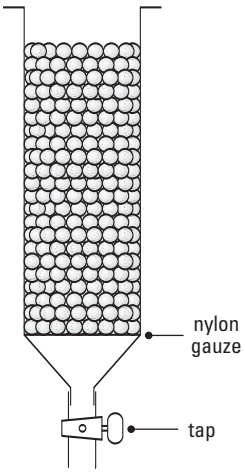
a Add 2 cm^3 of lactase enzyme solution to 8 cm^3 of a 2% sodium alginate solution. Mix well and draw up into a plastic syringe (without the needle).

b Allow drops of the mixture to fall into 100 cm^3 of 0.1 M calcium chloride solution so that small beads are formed. Let the beads set for a few minutes. Collect the beads in a small strainer, and wash free of calcium chloride solution.

c Put a circle of nylon gauze at the bottom of a 10-cm^3 syringe, fitted with a stop-tap to prevent the beads from blocking the nozzle. Suspend the beads in water and pour into the syringe.

d Pass 50 cm^3 of an 8% whey solution through the column of enzyme beads. Test the effluent at regular intervals with semi-quantitative diabetic glucose test strips. Finish by washing the column free of whey solution.

e Plot a graph of your results.



Questions

1 What can you deduce from your graph? Make an estimate of the concentration of glucose in your whey syrup.

2 Can the column be used again?

3 What type of immobilization was involved in this experiment?

Experiment 4 Investigating the synthesis of starch by a metabolic reaction

(See Chapter 3)

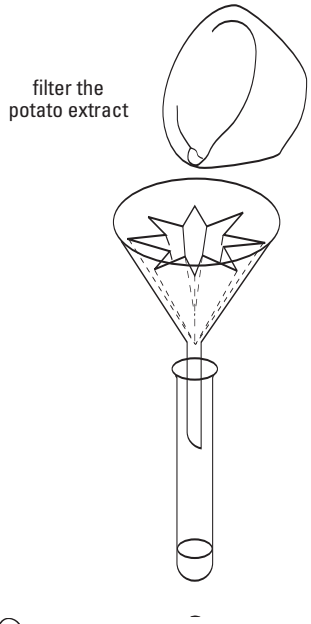
You are going to use the enzyme **starch phosphorylase** to try to prepare starch from glucose and some closely related compounds. Starch is easily detected by its reaction with aqueous iodine to give a blue–black colour.

The enzyme used in this experiment is obtained from potato cells. However, potatoes already contain starch, so the enzyme extract will have to be tested to check that it contains no starch.

/!\ CARE
The glucose-1-phosphate solution you are using is quickly hydrolysed. Keep the solution in an ice bath.

Procedure

a Place a 2-cm cube of potato in a mortar with 10 cm^3 of pure water and cut into small pieces. Add a little clean fine sand and grind to a fine pulp. Filter the pulp through a fluted filter paper of qualitative grade which has been moistened with water. Discard the first two drops of filtrate.



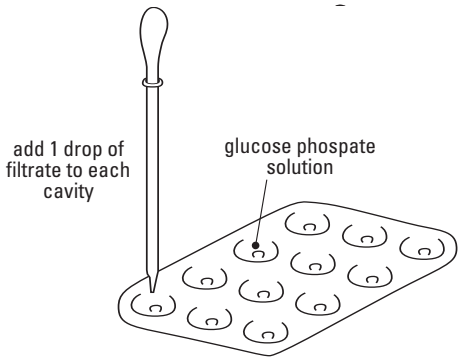
b Test the filtrate for the absence of starch, by mixing 1 drop with 1 drop of 0.005 M iodine solution. If the mixture turns blue, filter the extract a second time.

c Put 1 drop of a 0.05 M solution of glucose in each cavity (five is sufficient) of a row on a spotting tile; repeat using sucrose and glucose-1-phosphate solutions in two other rows.

d Add 1 drop of filtered and starch-free potato extract to each cavity on your spotting tile.

e At 5-minute intervals, add iodine solution to one cavity in each row. Record any colour changes.

f Carry out a blank test (a 'control') by adding iodine solution to drops of the solutions of glucose, sucrose and glucose-1-phosphate. At the end of the experiment, repeat the test on the potato extract.



Questions

1 Which of the three possible substrates you tested were used by an enzyme to produce starch?

2 Why were the blank tests necessary?

3 Suggest why starch phosphorylase is so named.

Experiment 5 Isolation of DNA from cress

(See Chapter 4; this is an alternative to the peas in Experiment 6)

The aim is to isolate nucleic acids from cress tissue. Nucleic acids prepared in this method are sufficiently pure to cut up with restriction enzymes (see *Biochemistry* section 4.6 'Gel electrophoresis').

The method uses the detergent **SDS** (**s**odium **d**odecyl **s**ulphate) to disrupt cell membranes and the tertiary structure of proteins, and thus liberate nucleic acids – DNA and RNA – from plant tissue. The detergent solution is pH buffered to minimize degradation of the nucleic acids present. The cells are broken up by gentle grinding. Centrifugation is used to separate cell debris from the nucleic acids in solution. Finally, the nucleic acids are precipitated by adding ethanol.

Procedure

a Use scissors to harvest about a mortar-full of green cress leaves (about 3–5 g of fresh material). Immediately grind the plant tissue to a smooth purée using a little silver sand and 5 cm^3 of SDS extraction buffer, pre-warmed to 65 °C.

b Pour the ground tissue into a small conical flask, rinsing any material off the pestle and mortar into the flask with a further 5 cm^3 of buffer solution. Loosely seal the top of the flask with aluminium foil and maintain at 65 °C for 30–60 minutes in a water bath.

Heat will tend to denature enzymes which would degrade DNA. From time to time, swirl the contents of the flask. Because of the presence of plant pigments, the extract may change from green to a brown or purple colour.

c At the end of the heating period, remove the plant debris by centrifuging at about 1000 g for 5 minutes, or until a pellet of plant material forms at the bottom of the tube. Take care to balance the centrifuge. The pellet is not needed.

Note that, with slower centrifuges, you may need to extend the time spent centrifuging two- or even three-fold (2500 rpm with a rotor arm of 15 cm corresponds to 1000 g).

d Without disturbing the pellet, carefully pipette or pour off the supernatant liquid into two fresh tubes. Precipitate the nucleic acids (DNA and RNA) by pouring an equal volume of ethanol onto the surface of the liquid extract.

A cottonwool-like precipitate of nucleic acids will form over about 10 minutes. Rock the tube gently but thoroughly to mix the two liquids to precipitate the DNA from the plant extract.

Continued

Experiment 5 Isolation of DNA from cress *continued*

Procedure *continued*

e Collect the nucleic acids by centrifuging at 1000 g for 5 minutes, or whatever is necessary. Discard the supernatant liquid and leave the pellet to drain by inverting the tube above a paper towel for 2 minutes.

f Stopper and label your test-tube. Your sample of DNA is suitable for gel electrophoresis if the necessary apparatus is available.

Questions

1 What other water-soluble compounds are likely to be present in the supernatant liquid after centrifuging?

2 Why is the effect of detergents on proteins helpful in obtaining a good yield of nucleic acids?

3 Suggest a reason why ethanol precipitates nucleic acids from aqueous solution.

Experiment 6 Isolation of DNA from peas

(See Chapter 4; this is an alternative to the cress in Experiment 5)

Procedure

a Dissolve the salt in 90 cm^3 of distilled water. Add the washing-up liquid and mix gently.

b Mash the peas using a glass rod or a spoon. Add the pea pulp to a beaker with the salty washing-up liquid solution.

c Stand the beaker in a water bath at 60 °C for exactly 15 minutes. This treatment causes the pea cell membranes to break down. The detergent forms complexes surrounding the membrane phospholipids and proteins, causing them to precipitate. In addition, the sodium ions from the salt shield the negatively-charged phosphate groups of the DNA molecules, causing them to coalesce. At 60 °C, the enzymes, which would otherwise start to cut the DNA into fragments, are partially denatured.

d Cool the mixture by placing the beaker in an ice water bath for 5 minutes, stirring frequently. This slows the breakdown of the DNA which would occur if a high temperature was maintained.

e Filter the mixture into a second beaker. Ensure that any foam on top of the liquid does not contaminate the filtrate. The filtrate contains the DNA.

f OPTIONAL: Add 2 or 3 drops of protease to about 10 cm^3 of the pea extract in a boiling tube and mix well. The protease will degrade some of the proteins in the preparation.

g Very carefully pour ice-cold ethanol or industrial methylated spirit down the side of the boiling tube, to form a layer on top of the pea extract.

i Leave the tube, undisturbed, for a few minutes. Nucleic acids (DNA and RNA) will precipitate into the upper (ethanol) layer. The DNA is the white material in the clear alcohol layer above the pea extract.

Questions

1 What other water soluble compounds are likely to be present in the supernatant liquid after filtering in step **e**?

2 Why is the effect of detergents on proteins helpful in obtaining a good yield of nucleic acids?

3 Suggest a reason why ethanol precipitates nucleic acids from aqueous solution.