from the laboratory. The concern was particularly acute because *E. coli*, the bacterium that was and still is used for many experiments, is a normal inhabitant of the human intestinal tract.

The scientists doing the work were the first to be aware of its potential hazards and they took an unprecedented step. Meeting in 1974 under the aegis of the National Academy of Sciences of the USA they recommended that certain types of potentially hazardous recombinant DNA experiments not be done until an international meeting could be convened to draw up guidelines that would allow the research to proceed safely. The banned experiments included those in which genes for toxins or antibiotic resistance would be put into organisms that did not already have them.

The voluntary moratorium held until February 1975, when the international meeting was held at the Asilomar Conference Center in Pacific Grove, California. The participants recommended that recombinant DNA research be conducted under increasing degrees of physical or biological containment according to the severity of the risks that they might entail. Physical containment means the use of laboratory facilities that will minimize or, at the highest levels prevent, the escape of microorganisms. Biological containment refers to the use of organisms or vectors that have been altered so that they can only survive under specialised laboratory conditions.

Each nation represented at the meeting was to draw up its own guidelines for ranking the hazards of recombinant DNA experiments and defining the conditions under which they could be performed. In the United States this task fell to a committee convened by the National Institutes of Health in Bethesda, Maryland, which at the time supported most of the research in question. The NIH guidelines became available in June 1976. In the intervening years they have been greatly relaxed as increasing experience has failed to demonstrate any hazards of recombinant DNA research in the laboratory.

The NIH guidelines only applied in the first place to research supported by the institutes. The growth of the biotechnology industry has meant that an increasing amount of recombinant DNA work has not been subject to the guidelines, although the companies doing the work have generally followed them anyway. Current concerns centre largely on the safety of deliberately releasing into the environment the genetically altered organisms, including plants and microorganisms, that industry is beginning to produce for agricultural applications. The proposals to treat human genetic diseases by introducing good copies of the affected gene into the patients' cells have also raised concerns. The regulatory and ethical implications of the new biotechnology are considered in Chapters 8 and 16 of this book.

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2 Synthesis without cells

Ethan S. Simon, Alan Akiyama and George Whitesides

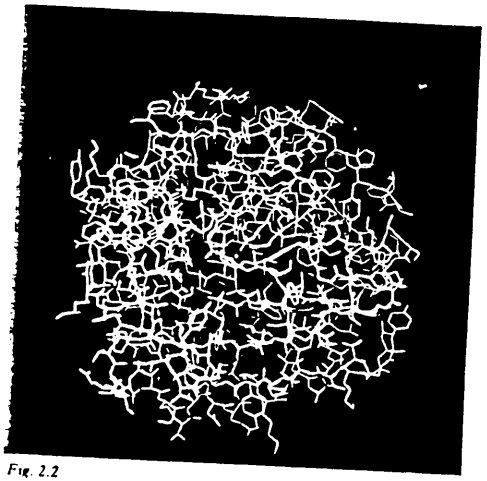


Fig. 2.2

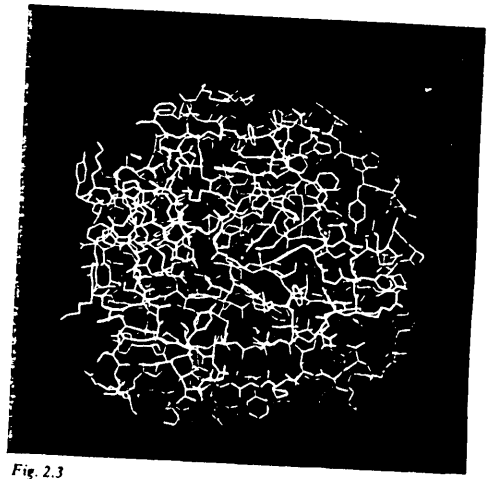


Fig. 2.3

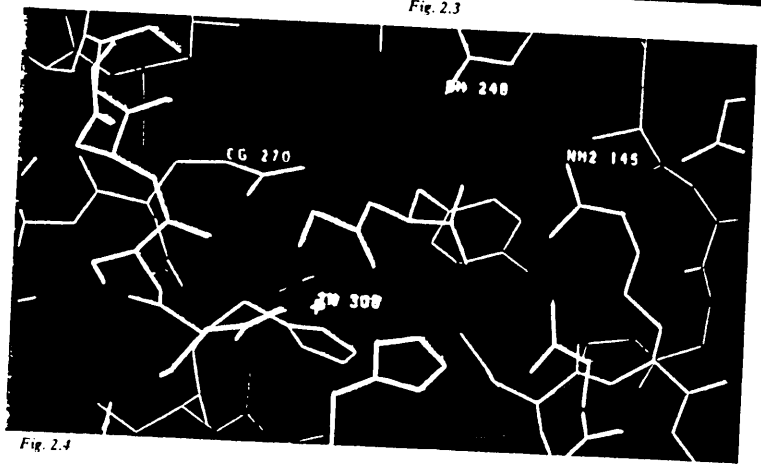


Fig. 2.4

Most of the chemical reactions that occur in living cells would take place too slowly to support life it it were not for the existence of the biological catalysts known as enzymes. These molecules accelerate the rates of the reactions, often by many orders of magnitude, thereby allowing cells to obtain the energy they need, synthesize the molecules of which they are composed, and break down and eliminate their waste products. For example, the first step of glycolysis, the metabolic pathway that converts the sugar glucose into chemical energy, is catalysed by the enzyme hexokinase. Hexokinase accelerates the reaction, the conversion of glucose to glucose-6-phosphate, by a factor of 1 000 000 000.

Enzymes not only make essential contributions to cellular activities, but also find wide application in biotechnology. They are used in the food industry for making cheese, beer, wine and sweeteners, among other things, and in the chemical and pharmaceutical industries for synthesizing amino acids and antibiotics. In addition, they have a small but growing role in medicine. One therapeutic application of increasing importance is the use of the enzyme called tissue plasminogen activator to dissolve blood clots in heart patients.

The biotechnological applications of enzymes depend on their unique properties. Enzymes are proteins that have molecular weights ranging from 10 000 to 500 000. For comparison, water has a molecular weight of 18, and common table salt (sodium chloride) has a molecular weight of 59. The large size of enzymes allows them to adopt well-defined three-dimensional shapes, stabilized by interactions between their constituent amino acids.

These shapes are critical to one of their most important characteristics – the ability to recognize and act specifically on one or a few substances, which are called substrates, even if the substrates are present in a complex mixture. Hexokinase has recognition sites for glucose and adenosine triphosphate (ATP), which bind to the enzyme in close proximity to one another (Fig. 2.1). After binding, a phosphate group is transferred from ATP to the glucose, thereby producing adenosine diphosphate (ADP) and glucose-6-phosphate. The enzyme then releases the two products and is able to catalyse another cycle of reaction between glucose and ATP.

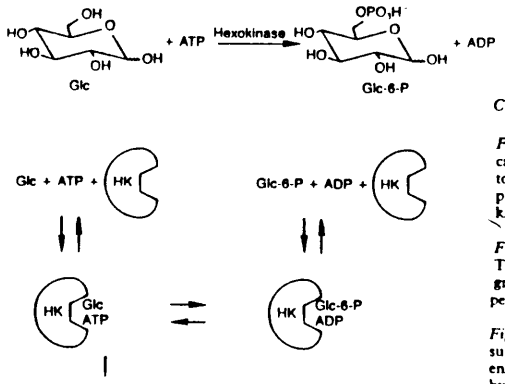


Fig. 2.1 The enzyme hexokinase (HK) accelerates the reaction of glucose (Glc) and adenosine triphosphate (ATP) to form glucose-6-phosphate (Glc-6-P) and adenosine diphosphate (ADP). The hexokinase itself is not consumed in the reaction.

Captions to previous page

Fig. 2.2 Computer-generated model of the active site of the enzyme carboxypeptidase A. The active site, which is the region of the protein to which the substrate binds and where the catalytic activity takes place, forms a small cleft in the surface of the enzyme protein. (By kind permission of David W. Christianson, Harvard University.)

Fig. 2.3 Substrate binding to the active site of carboxypeptidase A. The substrate, here a derivative of benzylpropionic acid (shown in green), fits nearly into the cleft in the enzyme surface. (By kind permission of David W. Christianson, Harvard University.)

Fig. 2.4 Close-up of the active site of carboxypeptidase A. The substrate here is the dipeptide glycyltyrosine. The amino acids of the enzyme that are important for binding are denoted in red. As indicated by the numbers designating their positions in the protein chain, they may be located far apart in the linear sequence of the protein, but are brought close together when the protein folds into its three-dimensional structure. (By kind permission of David W. Christianson, Harvard University.)

A substrate fits into a specific recognition site on an enzyme much as a key fits into a lock (Figs. 2.2 and 2.3), although enzymes are more dynamic than locks in that the shape of the enzyme may change somewhat in response to substrate binding. Only a few of the amino acids of the enzyme are actually in contact with the bound substrate (Fig. 2.4). These amino acids may be widely separated from one another along the linear sequence of the enzyme protein. If the three-dimensional structure that holds them in the correct alignment for substrate binding is disrupted, the enzyme may lose its activity.

The high specificity of enzymes in substrate recognition and catalytic action is the basis for many of their applications. One important type of selectivity that is exhibited by many enzymes is the ability to discriminate between enantiomeric, or mirror-image, molecules (Fig. 2.5). Enantiomers are stereoisomers that have the same chemical composition, but have the atoms arranged differently in space. Enantiomers can be formed by any molecule that has an asymmetric carbon atom; that is, a carbon with four different groups attached to it. Enantiomers are indistinguishable in most of their physical properties and are therefore difficult to separate.

However, enzymes can usually discriminate between them. Exposure of a mixture of the enantiomers *N*-acetyl-L-alanine and *N*-acetyl-D-alanine to the enzyme acylase I results in removal of the acetyl group from the L-enantiomer only. The reaction releases L-alanine, which is the naturally occurring enantiomer of this amino acid. The free amino acid can be easily separated from the unchanged *N*-acetyl-D-alanine. Enantioselective enzymatic hydrolysis thus provides a convenient and widely used technique for separating enantiomers.

The importance of such separation techniques is very great. Enantiomers often behave very differently in biological systems. One may be a useful drug, whereas the other may be highly toxic. The drug thalidomide provides a case in point. The pure R(+) enantiomer is a relatively safe tranquilizer. The serious birth defects that were produced when thalidomide was given to pregnant women resulted from the very small quantities of the S(-) enantiomer that were present as an impurity. Increasing sophistication in drug synthesis and increasing pressure from health regulatory agencies now provide important stimuli for the development of methods for making pharmaceutical agents that are free of contamination by unwanted enantiomers.

Enzyme catalysts have several other important characteristics in addition to their substrate specificity. They are subject to regulation; that is other molecules can modulate their activities – a feature that is especially important for controlling biochemical pathways in the cell. Moreover, enzymes work under mild conditions. Most operate best in water at a neutral pH and at room temperature. For industrial applications, these conditions are attractive with regard to both saving energy and preserving the environment. Accomplishing comparable chemical reactions without enzymes often requires harsh conditions, including high temperatures and very acidic or alkaline pHs. Perhaps most important from an industrial point of view, enzymes can carry out reactions unachievable by any other method. In particular, they may be used for the synthesis of complex biological molecules that cannot be made by other means.

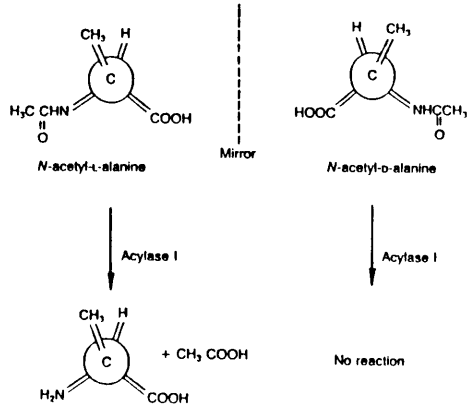


Fig. 2.5 The amino acid alanine has an asymmetric centre. It exists in two forms that are identical except that they are mirror-image isomers of one another. Mirror-image structures such as these are called enantiomers. The L-enantiomer of alanine is the one that occurs in proteins and most other naturally occurring alanine derivatives. D-alanine rarely occurs in nature.

A major disadvantage of enzyme catalysts is that they are relatively fragile in comparison with non-biological catalysts such as platinum or sulphuric acid. Enzymes are rapidly deactivated by temperatures above room temperature, which disrupt their three-dimensional structures. They may also lose activity if exposed to air, to organic solvents, or to acidic or basic conditions. The fragility of enzymes is particularly disadvantageous because they remain expensive relative to most non-enzymatic catalysts, even though methods for producing enzymes are improving.

Nevertheless, this handicap may be overcome by the development of methods for enzyme stabilization. Providing an environment that prolongs the life of an enzyme is a matter of trial and error, but enzyme immobilization is one approach that often works and, at the same time, lowers the cost of using enzymes.

An enzyme can be immobilized by fixing it to, or enclosing it in, a solid support, a procedure which offers a number of practical advantages over using the enzyme in soluble form. Immobilization stabilises enzymes, in part by making them

more resistant to shear forces and to attack by proteases, a class of enzymes that destroys other proteins. In addition, an immobilized enzyme can easily be separated from the reaction mixture at the end of the reaction and then re-used. This recovery makes product isolation easier and the reaction less expensive.

Enzymes can be immobilized either as pure entities or as components of whole, but usually dead, cells. Immobilization methods include covalently attaching the enzyme to a solid support, entrapping it in a gel, cross-linking the enzyme molecules to one another, or encapsulating them in small artificial cells (Fig. 2.6).

Once immobilized, an enzyme can be used in any of several types of reactors, each of which has its own advantages and disadvantages. A batch reactor – essentially a tank containing the enzyme and substrate (Fig. 2.7) – is simple to set up, but the mixture requires continuous stirring, which sometimes degrades the enzyme. The most common reactor employed in

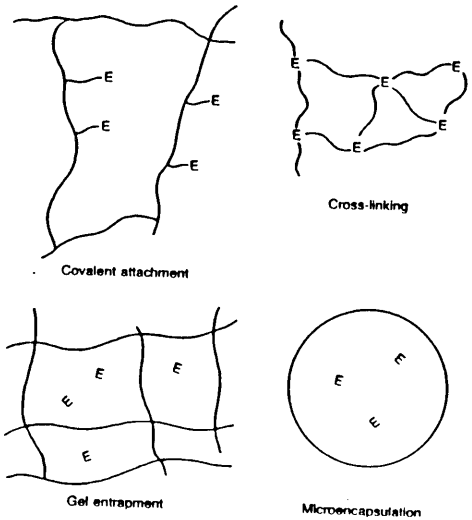
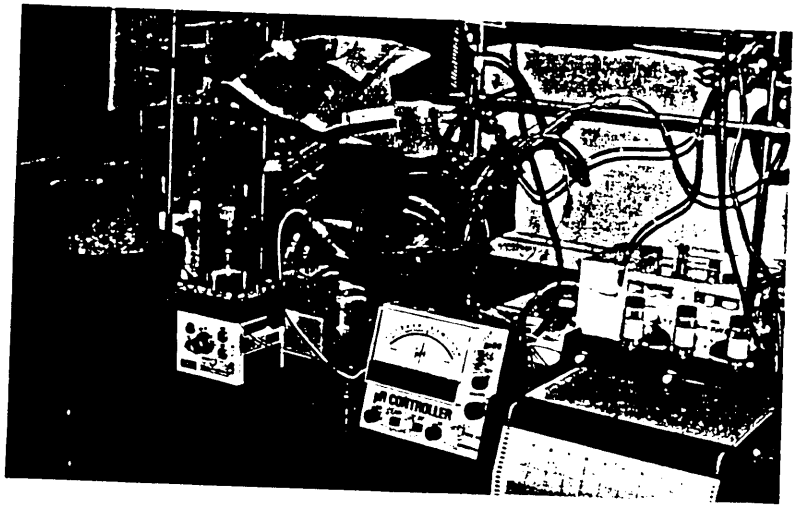


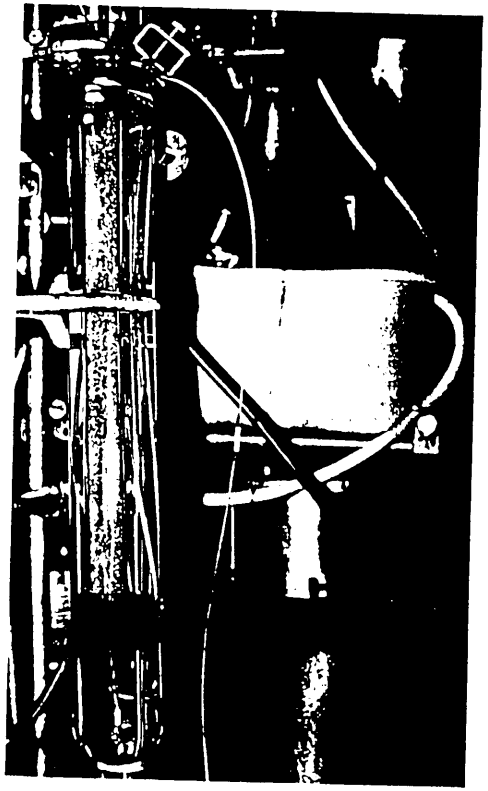
Fig. 2.6 Common methods of immobilizing enzymes (E) include binding them to surfaces (covalent attachment), trapping them in gels, cross-linking the enzyme molecules to one another, and sequestering the enzyme in small artificial cells.

Fig. 2.7 A laboratory-scale reactor that contains an immobilized enzyme.



industrial applications, such as the production of high-fructose corn syrup, is the fixed bed reactor. It consists of a column that is packed with an immobilized enzyme through which the substrate flows (Fig. 2.8). The method is efficient and amenable to automation, although plugging of the column may occur with some types of immobilization. The fluidized bed-reactor, in which an upward flow of substrate causes the immobilized enzyme to act like a fluid, solves this problem; however, this procedure is more complicated and costly than other methods.

Fig. 2.8 A laboratory-scale column reactor containing an immobilized enzyme.



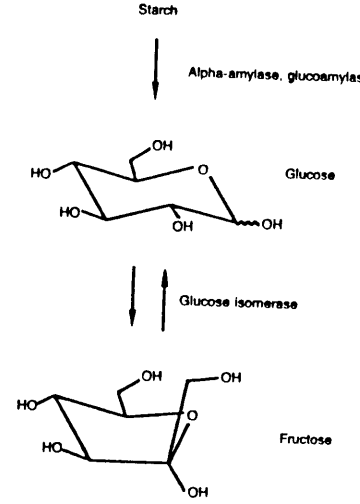
Current uses of enzymes

Food and beverages

Enzymes have long been used in the manufacture of cheese, beer and wine, and more recently the commercial application of enzymes for other purposes has grown. In the United States high-fructose corn syrup is the largest volume product made by immobilized enzyme technology. The syrup, which is produced by the enzymatic digestion of cornstarch, is sweeter than the sugar sucrose and is used in the beverage industry to flavour soft drinks and in the commercial baking industry to sweeten biscuits and cakes. A large commercial operation can convert 2 million pounds of cornstarch to high-fructose corn syrup in one day.

The transformation of cornstarch into the syrup requires three enzymes (Fig. 2.9). Treatment with alpha-amylase and glucoamylase first converts the starch to a glucose-containing syrup. Then immobilized glucose isomerase acts on the glucose, producing a mixture of glucose and fructose, which is sweeter, and thus more valuable, than the syrup containing glucose by itself.

Fig. 2.9 High-fructose corn syrup, an important commercial sweetener, is prepared from cornstarch with the aid of the enzymes alpha-amylase, glucoamylase and glucose isomerase. The syrup is an equilibrium mixture of glucose and fructose.



In the dairy industry, enzymes are essential for the production of cheese. Milk contains a group of proteins called caseins. One of the proteins, kappa-casein, prevents milk from coagulating in the presence of calcium ions. The enzyme rennin, which has traditionally been obtained from the lining of calves' stomachs, breaks down casein into the smaller protein called para-casein. Once the kappa-casein is destroyed, coagulation occurs to form a soft solid curd that can be separated from the liquid portion of the milk. The curd is the starting material for the production of cheese.

The fluid remaining after the curd is separated is 'whey'. It contains, among other components, proteins and a non-sweet sugar, lactose. Although these materials are potentially valuable nutrients, no good uses have yet been developed for whey because many individuals cannot digest lactose. If the sugar is present in substantial quantities in food and is not broken down during digestion, the microorganisms in the intestine feed on the lactose, producing gases and causing gastrointestinal distress. Whey has in the past presented a disposal problem.

Current research is exploring methods of using enzymes to convert whey into a material that can be used as an additive to foods. When whey is treated with the enzyme lactase, the lactose is broken down into two sweet, digestible sugars: glucose and galactose. The resulting sweet, protein-rich syrup can be added to certain food products, such as ice-cream.

Because the demand by the cheese industry for rennet, a dry rennin-containing powder, is so large, substitutes for the calf product have been developed. A rennet derived from microorganisms is widely used, but produces cheese of slightly inferior quality because the microbial enzyme is harder to inactivate than the calf product when its action is no longer desired. Scientists at Genentech Inc., South San Francisco, California, are now investigating ways of generating improved microbial rennet. One possibility is to use recombinant DNA techniques to modify the microbial enzyme genetically so that its stability is decreased and it can be deactivated by brief heating at the appropriate time.

In the beverage industry, enzymes are used to chill-proof juices, wines and beer. Juices and wines contain a polysaccharide called pectin that is soluble at room temperature but, when cooled, may form a colloidal suspension that gives the liquid a cloudy appearance. In Europe this cloudiness is considered a sign of high quality in bottled fruit juices, but in the United States it is unacceptable to most consumers. To prevent the haze from forming, enzymes called pectinases are added to the juice or wine. The enzymes degrade the pectin to lower molecular weight, and therefore more soluble, fragments that do not precipitate on cooling.

A similar problem of haze formation occurs during beer manufacture, although in beer the compounds that cause the trouble are proteins and tannins rather than polysaccharides. The proteins and tannins become suspended in the brew and precipitate when the liquid is cooled, thereby making it cloudy. To chill-proof beer, proteases such as pepsin or papain are added to break down the proteins and limit formation of the 'chillhaze' as it is called.

Another application of enzymes is the manufacture of soft-centred sweets. The confections are first made with solid sucrose centres that contain the enzyme invertase. Over a period of three to four weeks, the invertase transforms the sucrose into a liquid mixture of fructose and glucose. In addition to being sweeter than sucrose, fructose has the advantage of retaining more moisture, so that its presence also prevents the sweet from drying out and tasting stale.

Enzymes are also important in packaging foods. For example, the plastic wrapper used for cheese may be coated with glucose and two enzymes, glucose oxidase and peroxidase, to prevent spoilage. The coating slows the development of the rancid flavours that can be produced when oxygen reacts with components of the cheese. Under the influence of the glucose oxidase, the oxygen reacts instead with the glucose in the plastic wrapper to form gluconic acid and hydrogen peroxide, which is in turn converted to water by peroxidase. The products generated in this way have no effect on the taste of the cheese. Similarly, glucose oxidase may be added to mayonnaise to prevent spoilage by oxidation. The amount of enzyme added in these applications is very small.

Large volume chemicals

Although the largest volume transformations carried out by enzymes are in the food industry, as in the production of high-fructose corn syrup, enzymes are also used for synthesizing chemicals. The most important of these chemicals are probably amino acids, which are needed for protein synthesis, as nutritional supplements for hospital patients who are unable to eat normally, as supplements for animal feed, and as chemical intermediates.

Moreover, certain transformations in pharmaceutical synthesis depend on enzymes. For example, the microbially produced antibiotic penicillin G is converted by the enzyme penicillin acylase to 6-aminopenicillinic acid, which is the starting material for production of semi-synthetic penicillins (Fig. 2.10).

Detergents

The best agents available for removing proteinaceous stains such as egg, blood or grass from cloth are the protein-degrading protease enzymes. The first enzyme-containing laundry detergent was introduced in the United States in 1966, but in the early 1970s concerns that the enzymes in detergents might cause adverse effects, such as lung irritation from breathing enzyme dust, on the people who make and use the products caused the removal of the detergents from the market. They remained in use in Europe, however, and proved safe and effective when properly formulated and handled. Enzyme-containing detergents are being reintroduced in the United States where approximately 15 per cent of detergents now have protease additives.

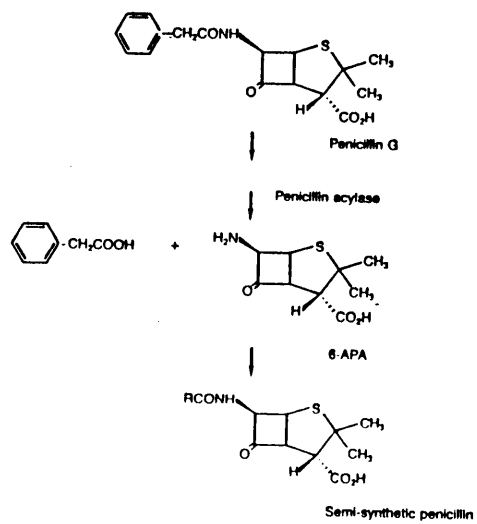


Fig. 2.10 Penicillin acylase selectively hydrolyses the side-chain amide bond in the readily available penicillin G and forms 6-aminopenicillinic acid (6-APA), which is a starting material for synthesizing a variety of semi-synthetic penicillins.

Medicinal applications

Enzymes control and carry out the myriad of chemical reactions occurring in the body. They mediate the digestion of food, build the components of cells, and generate and respond to intracellular messengers such as hormones and the chemical neurotransmitters that carry nerve signals. Enzymes are valuable both for studying these complicated systems and, occasionally, for medical therapies.

In one simple therapeutic application, a crude mixture of pancreatic enzymes called 'pancreatin' is given orally as a digestive aid to people who are deficient in digestive enzymes as a result of genetic disorders, surgical removal of the gall bladder, or advancing age.

A more complex medical application involves the use of the enzyme heparinase for controlling blood clotting. Patients who are undergoing kidney dialysis or certain forms of surgery or who have had heart attacks or strokes are often treated with a polysaccharide called heparin, which acts to decrease the ability of the blood to clot. Although heparin treatment is usually uneventful, it may sometimes be necessary to reverse its action rapidly – if the patient bleeds excessively, for example.

Heparinase selectively cuts heparin apart, thereby destroying its activity. Medical researchers are attempting to use the enzyme, which is immobilised on a filter, to achieve reversal of the anti-clotting effects of heparin. When this becomes necessary, the patient's blood is circulated outside the body through a filter with the immobilized heparinase and then returned to the patient.

A therapeutic enzyme use with possibly enormous potential is treatment with tissue plasminogen activator (TPA) to promote the dissolution of the blood clots that form during heart attacks. If a clot can be dissolved sufficiently early, permanent damage to the heart muscle may be prevented or at least minimized.

In the past, TPA could only be isolated with great difficulty from human uterine tissue and was in too short supply for widespread application. Now, however, it can be produced commercially by recombinant DNA technology and is expected to be the first major new product of the technology to enter the human health care market.

The enzyme has the advantage of being highly selective. It acts only inside the clot and causes little damage elsewhere in the patient. Initial tests of TPA to reopen the heart arteries that have been blocked by clots have proved very promising, provided that the treatment is started soon after the clot forms. Several other enzymes, including urokinase, pro-urokinase and streptokinase, have clot-dissolving activities and may have therapeutic benefits for heart attack victims when administered either alone or in conjunction with TPA.

Enzymes are beginning to be used to modify proteins, an application that will grow in importance as more proteins that have medical or other uses are made available by recombinant DNA technology. Enzymes are the catalysts of choice for this purpose because they provide the selectivity required in this technically demanding area.

The first commercial example of enzyme modification of a protein for human use is the conversion of porcine insulin to human insulin. Diabetes patients who must take insulin to control their disease sometimes become allergic to the bovine or porcine versions of the protein, but may have better tolerance for the human variant, which has been in short supply until recently.

Although porcine and human insulins differ by only one amino acid, chemical methods for converting one to the other are not available. This can be done enzymatically, however. First, an enzyme clips the protein backbone of porcine insulin next to the unwanted amino acid, thus removing it on a short peptide fragment. Then, under different reaction conditions, another enzyme replaces the fragment that has been removed by attaching the corresponding human peptide to the truncated porcine molecule. The human peptide is short enough to be chemically synthesized. Much of the 'human' insulin sold for treating diabetes in Europe is porcine insulin that has been converted to the human amino acid sequence in this way.

A number of efforts have been made to treat cancer with enzymes, but so far there have been no major successes. For

example, asparaginase, which converts the amino acid L-asparagine to aspartic acid, has been used to treat leukaemia. Some leukaemic cells seem to have a higher requirement for L-asparagine than normal cells and it was hoped that injection of the enzyme into the blood might reduce the amount of L-asparagine available to the cancer cells and cause them to starve for want of a required nutrient. The treatment proved of little benefit because the tumors often develop resistance to it and the L-asparaginase can have toxic side effects.

Analytical chemistry

Enzymes are extremely valuable analytic tools, for both medical and non-medical applications. Because of their specificity they can be used to assay the amount of a substance, even of another enzyme, in a complex mixture such as blood, urine or other biological fluids. Often a number of enzyme reactions are coupled together in solution so that the sequence of enzyme-catalysed reactions culminates in the conversion of NAD (nicotinamide adenine dinucleotide) to NADH, the reduced form of the molecule. This conversion results in a change in the ability of the sample to absorb light, which can easily be measured by a spectrophotometer. Many of the diagnostic tests carried out by physicians depend on enzymes. Enzymes are routinely used to measure the concentrations of glucose, urea, amino acids, ethanol and lactic acid in biological fluids and to identify proteins and nucleic acids.

Home diagnostic procedures also often depend on enzymes. For example, diabetics must monitor the glucose content of their urine as an indicator of their need for insulin. The glucose analysis is done simply by dipping an analytical test stick in a urine sample. The stick contains the enzymes glucose oxidase and peroxidase as well as a reagent that registers the reaction with glucose by changing colour.

Potential new uses of enzymes

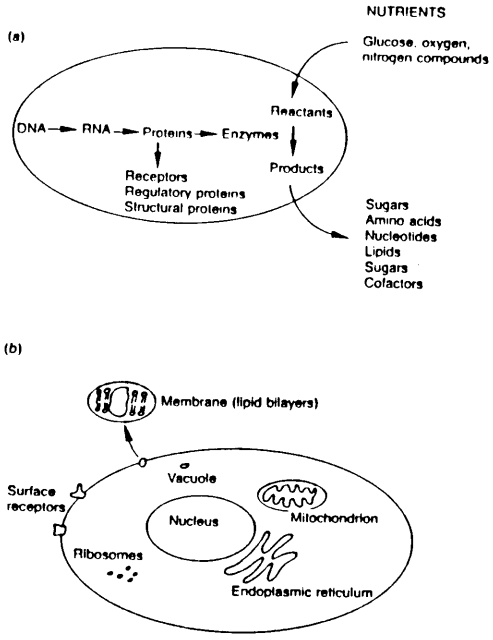
Most of the current applications of enzymes in industry and research involve relatively simple processes, such as the conversion of starch to high-fructose corn syrup. In the future, enzymes may be used as catalysts in more complicated systems. Many biologically important molecules, including polysaccharides, nucleic acids and proteins, are too complex to be easily synthesized by the standard methods of organic chemistry. Because enzymes function well in transformations involving these substances, current research efforts seek to develop enzymatic methods for their synthesis.

Success in these endeavours will represent a large step towards two goals. First, when these complex biological compounds are readily obtainable scientists will be in a better position to understand the molecular basis of life. Second, this new form of biotechnology will provide the tools for making useful products, not only for biology but also for medicine and agriculture.

A chemist interested in adapting the unique catalytic properties of enzymes views a cell differently from a traditional biologist (Fig. 2.11). The biologist is concerned with the structure and function of the cell and its components and with understanding how the parts work together. The synthetic chemist considers the cell as a bag of enzyme catalysts into which he can place reactants and out of which will come valuable products. Nevertheless, the chemist needs the information garnered by the biologist.

The development of the new enzyme technology requires the design of schemes that rival the intricacy of some of the cells' major metabolic pathways. Enzymes, substrates, plus additional essential molecules called cofactors, will have to be

Fig. 2.11 Chemists and biologists view the cell differently. Chemists concern themselves with molecular details (*a*), whereas biologists study the overall function of the cell (*b*).



combined in complex cycles. At this stage, no one knows exactly how complicated these systems of artificial metabolism can be while still remaining practical.

Current enzyme research is aimed at developing and improving methods for cofactor regeneration, exploring and extending the range of structures accepted as substrates, synthesizing the larger molecules unattainable by classic synthetic methods, developing systems of artificial metabolism, and genetically engineering new or altered enzymes.

Cofactors and enzyme-catalysed syntheses

Enzymes can be classified in several ways, one of which is by their requirement for cofactors, low molecular weight substances that also participate in the enzyme-catalysed reaction. The cofactor NAD, for example, acts as an oxidizing agent in some enzymatic reactions, whereas its reduced form, NADH, acts as a reducing agent. Another cofactor, ATP, serves as a donor of phosphate groups.

Enzymes that require an added cofactor are in general more difficult to use in large-scale commercial operations than those not requiring added cofactors. Because the cost of cofactors is high – 98% pure NADH costs about $11 000 per pound – their use would be prohibitively expensive in an enzyme-catalysed reaction in which one molecule of cofactor is consumed for every molecule of product formed.

This problem can be solved by coupling a reaction for regenerating the cofactor to the reaction in which it is consumed, a strategy which is similar to that used in nature. Another enzyme and inexpensive starting materials convert the product form of the cofactor back to its original reactant form. A method for the reduction of NAD by formate and the enzyme formate dehydrogenase, which was developed by Zeev Shaked and George Whitesides of Harvard University in Cambridge, Massachusetts, is an example of such a coupled cofactor regeneration system (Fig. 2.12).

To make cofactor regeneration schemes practical, it is necessary to be able to synthesize the ultimate regenerating reagents in an economical way. Practical regeneration schemes are becoming available for the three most important cofactors: ATP, NAD and NADH. Although the schemes can currently provide many kilograms of product, none is yet used on an industrial scale.

Some enzymes have cofactors that are effectively built-in. They are tightly attached to the enzyme protein and regenerate as part of the normal enzyme action. Because no external cofactor regeneration schemes are needed for these enzymes, they are easier to use than those requiring added cofactor. Tyrosinase, which catalyses the formation of the amino acid tyrosine, and tryptophanase, which produces the amino acid tryptophan, are examples of enzymes with built-in cofactors. Different amino acids can be made by varying the substrates fed to these enzymes.

The simplest enzymes to use in organic synthesis do not require any added cofactors at all. Lipase and aldolase belong to this category. Lipase catalyses the splitting of ester bonds to produce the free acid and alcohol. Its natural substrates are phospholipids, fatty acid esters of glycerol, esters of cholesterol, and related substances. A wide range of esters that do not exist in nature can also serve as substrates for this enzyme.

Lipase is another enzyme that can aid in the isolation of individual enantiomers (Fig. 2.13). If either the acid or alcohol portion of an ester substrate has an asymmetric centre, the enzyme will often split the ester of one enantiomer more rapidly than the ester of the other enantiomer. In this circumstance the lipase-catalysed reaction will yield an alcohol or acid product that is enriched with regard to the more quickly released enantiomer. Such products may be useful in synthetic reactions.

Aldolase catalyses the formation of a carbon – carbon bond

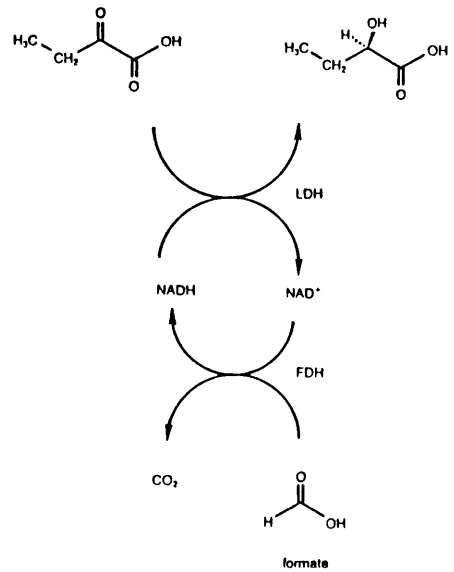


Fig. 2.12 A coupled cofactor regeneration system converts NAD back to its reduced form (NADH). As the enzyme lactate dehydrogenase (LDH) reduces alpha-ketobutyric acid to alpha-hydroxybutyric acid, it uses NADH. The enzyme formate dehydrogenase (FDH) then regenerates the NADH by oxidizing formic acid to carbon dioxide.

between dihydroxyacetone phosphate and any of a wide variety of aldehydes (Fig. 2.14). The enzyme's specificity towards different aldehydes and the stereospecificity of the reaction are currently under investigation.

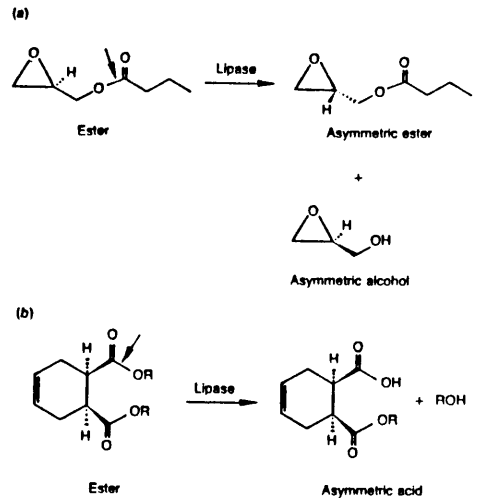
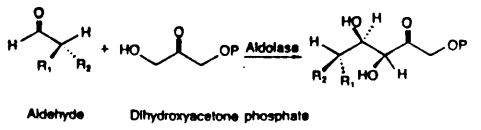


Fig. 2.13 The enzyme lipase catalyses the cleavage of esters, releasing the alcohol and acid that were originally joined in the ester molecule. The bonds split by the enzyme are marked with arrows. The alcohol of the ester shown in reaction (*a*) has an asymmetric carbon and can therefore exist in either of two enantiomeric forms. In such circumstances lipase may split the ester containing one enantiomer either slowly or not at all and can be used to separate the enantiomers. The ester shown in (b) does not contain an asymmetric centre, but lipase creates one in the acid produced by removing one of the two alcohols.

Fig. 2.14 Aldolase catalyses the reaction of an aldehyde and dihydroxyacetone phosphate to yield an aldol product of known stereochemistry.



Extending the substrate ranges of enzymes

As already mentioned, the specificity of enzymes for particular substrates is the basis for most of their applications in organic chemistry. The specificities of different enzymes vary, however. Some will accept only one substrate, whereas others accept many. Hexokinase, for example, can act on more than 30 compounds that are structurally related to glucose. Enzymes often accept 'unnatural' substrates that do not occur in nature, in addition to the natural variety.

A typical research programme in enzyme-catalysed synthesis will often be aimed at making a particular molecule, either because it is valuable in itself or because it can serve as the starting point for the synthesis of other compounds. If an enzyme that produces similar molecules is known, the programme can begin by surveying the range of substrates that it recognizes. The survey provides an understanding of how the enzyme works and may allow predictions of other potential substrates. Even if the enzyme does not work as well with the unnatural substrates as with its natural one, the transformation may still be useful – especially if the product cannot be made any other way.

In the next phase, research on a small scale determines the best conditions of temperature, pH, and salt and substrate concentrations for the enzyme-catalysed reaction. If immobilization of the enzyme is desirable and if cofactor regeneration is necessary, appropriate systems are devised. Finally, enough information is collected so that the reaction can be run on the large scale demanded by commercial operations. Such 'scaling-up' can be difficult if problems in plant engineering are not carefully considered.

Modification of enzymes

Known enzyme catalysts do not exist for many of the reactions of interest to synthetic chemists. Sometimes, random screening of previously unexplored enzymes or enzyme sources uncovers the desired catalytic activity. This process is inefficient and very labour-intensive, however. One of the exciting prospects for the future is the modification of existing enzymes to alter or improve their specificity. Even more exciting, is the possibility of designing new enzymes and producing them by recombinant DNA technology, although this goal belongs to the distant future. Accomplishing this objective requires a detailed knowledge of the relations between amino acid sequence, three-dimensional protein structure, and catalytic recognition and action. Virtually none of this information is available now, although researchers are beginning to acquire it.

Nevertheless, it will be some time before chemists can synthesize new enzymes from scratch. Chemical synthesis of an entire enzyme by the stepwise construction of the amino acid chain is technically possible, but not yet practical. One difficulty is in predicting how a particular linear sequence of amino acids will fold. The amino acids of an enzyme's active site, which is the portion of the molecule that contacts the substrates and

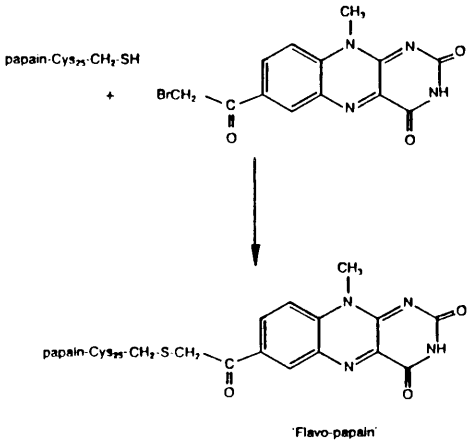
effects their conversion to products, are frequently distant from one another in the linear protein chain and are only brought into conjunction when the enzyme folds into the correct three-dimensional structure.

Another difficulty concerns determining which amino acids to incorporate in the active site. Computer models can estimate the contribution of particular amino acids to substrate binding and catalysis, but the reliability of these estimates has been checked experimentally in only a few instances and much remains to be learned before the models have firm predictive value.

For the present then, research efforts are aimed at modifying existing enzymes. Once the amino acids in or near the active site have been identified, they can be modified either by direct chemical alteration or by site-specific mutagenesis. Chemical transformations allow one amino acid to be changed into another; or reactive groups may be attached to some amino acids to alter the reactivity of the active site. For example, papain, a proteinase, has been converted by D. Lawrence and E. Kaiser of Rockefeller University into a protein active in oxidation and reduction reactions. They did this by attaching a flavin group to the sulphur of a cysteine residue (Fig. 2.15). Flavins are cofactors for the oxidation-reduction reactions catalysed by a number of enzymes.

Site-specific mutagenesis modifies the enzyme by incorporating a specific mutation in the corresponding gene. The linear

Fig. 2.15 The enzyme papain is chemically modified by attachment of a flavin group that alters the reactivity of the enzyme.



sequence of amino acids in the enzyme protein is determined by the linear sequence of nucleotides in the gene DNA, with three nucleotides constituting a codon for each amino acid (see Chapter 1). If the gene sequence that codes for the amino acids of the enzyme's active site is known, one amino acid can often be replaced by another by changing just one nucleotide in the appropriate codon.

A reliable and predictable method for modifying the gene coding for a protein is 'oligonucleotide-directed mutagenesis'. This method involves synthesizing a short section of DNA (the oligonucleotide) that corresponds to the gene sequence to be mutated except for a change in a single, specific base. When introduced into appropriate cells, this oligonucleotide serves as a primer for DNA replication with the result that the altered sequence becomes incorporated into the gene. Further manipulations permit the generation, identification and isolation of mutant clones that produce the modified enzyme, often with great efficiency.

Alan Fersht of the Imperial College of Science and Technology in London, performed this type of genetic engineering on the gene for tyrosyl-tRNA synthetase, thereby changing a cysteine residue into a serine residue at position 35 of the enzyme. The only difference between the two amino acids is that serine has a hydroxyl (–OH) group whereas cysteine has a sulphydryl (–SH) group. The mutation resulted in a change in the enzyme's affinity for its substrates because the affected amino acid participates in substrate binding and serine forms stronger hydrogen bonds than cysteine. A number of other amino acids have also been changed in tyrosyl-tRNA synthetase and used to explore the mechanism of action of the enzyme.

Multi-enzyme systems

An important potential use of enzymes is in multi-enzyme systems. Because most enzymes are active under the same conditions – in water at room temperature and a pH of approximately 7 – different enzymes will all exhibit catalytic activity when mixed in the same solution. Moreover, the substrate specificities of the enzymes will allow each to act on its own substrates without interfering with the action of the other enzymes. If the product of each enzymatic reaction is the substrate for another enzyme, the reactions will become coupled into a chain, in much the same way that the metabolic pathways of the cell are composed of sequences of coupled reactions leading from initial reactants to final products.

Laboratory-designed systems of complex sets of coupled enzymatic reactions may be termed 'artificial metabolism'. Such systems are complicated to design and operate, however. Careful control of the enzyme concentrations relative to one another to give optimum concentrations of the intermediates in the sequence and recycling of any necessary enzyme cofactors are required to prevent the build-up of inhibitory intermediates or the depletion of cofactors. Either occurrence could shut the system down.

At this time, the use of multi-enzyme systems to carry out the total synthesis of complex molecules remains at the stage of laboratory demonstration. One example of such a system, which was developed by Chi-Huey Wong in Whitesides' laboratory at Harvard, uses six cooperating enzymes to convert glucose-6-phosphate and N-acetylglucosamine to lactosamine, a disaccharide occurring as part of the carbohydrate groups that are attached to the surfaces of many proteins.

Multi-enzyme systems for synthesizing complex carbohydrates may eventually be a useful adjunct to recombinant DNA technology. The mammalian proteins made in *Escherichia coli* and other bacteria by recombinant DNA methods do not contain the carbohydrate that they normally would because the bacteria are not capable of adding the material to the proteins. If the carbohydrate turns out to be necessary for the normal function of the proteins, a way to add it will have to be found. Artificial metabolism may help in this regard.

Economic considerations: the future

Economics

Although thousands of enzymes have been identified, a relative few are of commercial importance. Twenty enzymes account for most of the worldwide market. The US Office of Technology Assessment estimated that in 1985 this market produced some 75 metric tons of enzymes, worth about $600 million. In the United States glucose isomerase and the two amylases used for producing high-fructose corn syrup from cornstarch account for about 50% of the market for enzymes. Production of the syrup saved the United States about $1.3 billion in foreign exchange for imported cane and beet sugar in 1980. Other enzymes produced in large volume are rennets for cheese-making, papain for chill-proofing beer, and proteases for detergents.

The short-term potential for growth in the industrial enzyme market is modest. A substantial number of new applications for enzymes are being explored, but the number of processes being actively developed for large-scale use is small. The major opportunities are probably in reactions involving water. The use of a nitrile hydratase to make acrylonitrile and acrylamide for the plastics industry is being actively explored in Japan. Many applications of lipases in the production of fine chemicals, especially for enantiomeric intermediates for the pharmaceutical industry, are very promising. In addition, enzymes are becoming useful for dealing with the important problem of toxic wastes. For example, a commercial enzyme system for destroying cyanide ions in aqueous wastes from chemical plants has recently been developed. Water treatment is an application driven by regulatory and societal pressures.

In general, however, the development of new, enzyme-based technologies for the chemical industry is inhibited by the slow growth and modest profitability of this area. Moreover, the majority of the current products of the chemical industry are water-insoluble and thus not ideal targets for enzymatic transformation.

What then is the future of enzyme-based technologies? In the longer term, it is extremely bright for several reasons.

Recombinant DNA technology provides a new method for lowering production costs for scarce enzymes. The enzymes used until recently have been those that could be prepared inexpensively by classic microbiological methods. Research aimed at developing commercial applications focused on these simply because they were the only ones with which an economical process might be built. The removal of this economic constraint allows current research to explore possible applications, such as chemical synthesis, for enzymes once considered only as biological research tools. Any of these enzymes that are identified as having promise in synthesis will be produced economically by recombinant DNA technology.

Environmental issues – from waste water treatment and industrial waste disposal to the development of chemical manufacturing methods that are perceived by society as safe – are now a major and unavoidable concern of all industries. An increasing awareness of this reality provides a reason for developing enzyme-based technologies for waste treatment and chemical synthesis. Even if these technologies are not quite competitive with traditional technologies, the enzymatic methods may be preferred because they operate in water at low temperatures and often produce few by-products.

The pharmaceutical industry, in particular, requires an entirely new group of technologies to deal with the opportunities afforded by biotechnology. Many of the products emerging from biotechnology are of types with which the pharmaceutical industry has had relatively little experience. Examples are proteins such as the clot-dissolving TPA and the immune-system regulator interleukin-2; polysaccharides such as hyaluronic acid, which is used in eye surgery; and the nucleic acid segments that are used for analysing DNA. All of these classes of compounds are exactly those to which enzymes are most productively applied, and enzymatic synthesis and modification of the substances will clearly be important. The value of the enzymes used for these applications may not be large, but the value added to the product by appropriate enzyme treatment may be very large.

Even for existing classes of pharmaceutical products, enzymes will play an increasingly important role in providing enantiomerically pure intermediates, and in carrying out difficult transformations with high selectivity. Enzymes will always be reserved for 'difficult' problems – those resistant to solution by conventional methods. The sophistication of current activities in drug development is such that difficult problems are commonplace.

The specificity of enzymes will continue to be useful in analytical methods. Continued development and marketing of electrodes that contain immobilized enzymes will make the assay of many aqueous biological substances routine. The specificity of enzymes often allows accurate measurement of one substance in a complex mixture without any sample preparation.

Enzymes will play an increasing role in food processing. The major driving forces in this area are safety and cost reduction. Enzymes provide both in certain areas of application. The food industry is technologically conservative, however, and change will come slowly.

Most of the chemical industry is based on declining petroleum feedstocks. In the long term, and especially in developing nations, there will be economic reasons to use biologically-derived starting materials, including cellulose, starch, lignin and plant proteins, instead of petroleum products. Enzymes will certainly play a role in processing the biological materials.

The pace of the research on the relation between the structure of an enzyme and its catalytic activity is very rapid. Although this problem is a very difficult one, it will eventually – perhaps in 20 to 50 years – be possible to design proteins that will catalyse new types of reactions and produce these unnatural enzymes economically by recombinant DNA technology. The first applications of this type of activity are now appearing from modest programmes that use site-specific mutagenesis to modify the properties of existing enzymes. These programmes will grow more ambitious as time goes on.

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