Enzymes in organic synthesis

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The maturing petroleum-based chemical industry, the pressures of environmental constraints and the explosive development of biotechnology have increased the interest in catalysis in enzymology. Enzymes are now an attractive proposition as catalysts for new classes of reagents and products, especially sugars, chiral synthons, metabolites and food components.

Enzymes are catalytic proteins that control the rates of most biological reactions; they have been used *ex vivo* in small-scale applications both for the analysis[1] and the synthesis of research biochemicals,[2,3] and in a small but significant number of large-scale chemical processes.[4,5] Their use in the latter was limited because most enzymes were expensive and unstable, and they were not the best catalysts for the reactions of greatest interest in the chemical process industry or the pharmaceutical industry (see Table 1).

Several developments occurring simultaneously in both industries and in biology have changed the value of enzymes as catalysts. The revolution in molecular genetics has made enzymes available at dramatically lowered costs.[5,6] At the same time, the targets of central interest in the pharmaceutical industry have shifted away from low molecular weight hydrocarbons and heterocycles to complex biological substances—tissue plasminogen activator, lymphokines, cyclosporin A—or substances derived from them. The creation of chiral centres has become a major strategic concern in chemical synthesis, especially in drug synthesis. Moreover, public concern with environmental issues has increased the attractiveness of processes that operate with high selectivities—and thus minimise the problems of waste and byproduct disposal—in environmentally acceptable solvents.

Enzymes should not be considered as replacements for existing catalysts: the idea of replacing the chemical process for the conversion of propylene to propylene oxide based on organic peroxides and transition metal catalysts[7] by an oxidative enzymatic process[8] was never a very good one. Rather, they should be considered as a new class of catalysts for which new uses and new processes must be developed.

The processes that currently use enzymes have been extensively reviewed.[5,9] Already the use of enzymes as components in detergent formulations,[10] common in Europe and the US, has

proved economical. The proteases used in detergent formulations are a small part of the formulation, but they remove proteinaceous stains sufficiently well to justify their production on a large scale. The conversion of starch to glucose, and glucose to high fructose corn syrup is the largest scale chemical transformation effected by enzymes.[5,11] The quantity of high fructose corn syrup produced in the US is now in excess of 4.5×10^3 kg pa.

The Novo process for the production of semisynthetic insulin[12] provides the first example of what will probably be a very widespread use of enzymes that is to modify proteins and other biological macromolecules derived from recombinant DNA technology. Although the insulin used as a starting material in the Novo process is not derived by recombinant DNA methodology, the efficiency and economics of this process, and the high quality of the product establish the value of enzymatic catalysis in this transformation. The range of aminoacids now derived from enzymatic transformations illustrates the importance of enzymology as a technology for resolving and producing chiral centres.[2,4,13] For example, biological processes for synthesising L-DOPA (2-amino-3-(3,4-dihydroxyphenyl) propanoic acid) are now displacing technologies based on asymmetric hydrogenation using rhodium catalysts.

Before deciding to use enzymes as catalysts in a process a number of issues need to be considered.
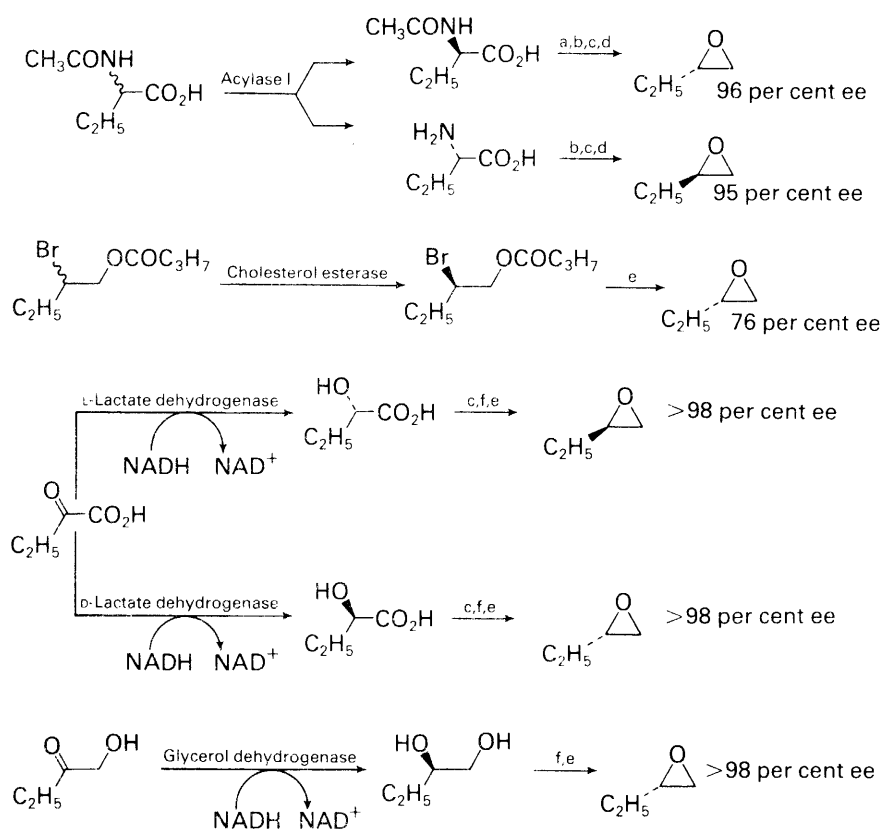
● *Enzyme production.* Enzymes used in chemical processes are mainly derived by extraction from natural sources (especially microorganisms). It is usually satisfactory, in synthetic applications, to work with relatively crude preparations, and immobilised whole cells are often the most convenient and economical form in which to use an enzyme. Immobilisation facilitates recovery, and improves the stability of the enzyme. A range of techniques for immobilising enzymes is available.[4,13,14,15,16] The most commonly used in large-scale preparations is that based on cross-linking reactions with glutaraldehyde.[4,14,17] This procedure works well with relatively stable enzymes, but often fails with the more delicate enzymes required for complex synthesis. For the latter group reactive organic polymers, eg PAN (polyacrylonitrile),[18] are more effective.

● *Cofactors.* Most of the enzymatic reactions used in synthesis require cofactors. Essentially all of the common cofactors (ATP, NAD, NADH, CoASH) are too expensive to use in stoichiometric quantities. The development of *in situ* cofactor regeneration schemes has progressed so that it is now easy to regenerate ATP from ADP or AMP by using either acetyl phosphate[19] or phosphoenol pyruvate[20] as the phosphate donor. The best method for regenerating NADH from NAD is based on formate as the hydride donor.[16,21] The regeneration of NAD remains slightly complicated,[22,23] but the best regeneration scheme is

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Table 1. Perceived characteristics of enzymes.

Advantages	Disadvantages
Highly selective for substrates	Expensive
Highly enantioselective	Unstable
Environmentally acceptable	Restricted to aqueous environments
Applicable to products relevant in pharmaceuticals, food, biotechnology, and agriculture	Inapplicable to substrates not occurring in Nature
Large numbers of catalytic activities available	Inapplicable to many important types of reactions
Cost can be lowered and properties improved by using biotechnology	Difficult to manipulate

Scheme 1. Four synthetic routes to optically enriched butene oxide by using enzymes.

Reaction conditions

(a) 2 N HCl, 100 °C, 2 h; (b) 6 N HCl, NaNO$_2$; (c) BH$_3$, THF, 0°C, 20 h; (d) KOH; (e) C$_5$H$_{11}$OK, C$_5$H$_{11}$OH, room temperature; (f) 30 per cent HBr·AcOH, −15°C →25°C, 3 h.

probably one based on α-ketoglutarate as the hydride acceptor.[22] The use of stoichiometric amounts of α-ketoglutarate is expensive, and thus limits this type of regeneration.

● *Enzyme purification*. In certain cases, especially those involving cofactors or delicate substrates, it is important to consider highly purified enzymes. For example, it can be particularly advantageous to exclude proteases—which may shorten the lifetime of the enzyme of interest by degrading it proteolytically—ATPases—which can decrease the efficiency of ATP usage in a cofactor recycling scheme—and certain types of contaminating enzyme activities. Contaminating activities are especially troublesome in chiral transformations where the required substrate may be accepted by more than one enzyme in a crude enzyme preparation.[24] If these competing enzymes have different enantioselectivities, the result can be an unacceptably low enantiomeric excess (ee <90 per cent) in the product. Establishing that a crude preparation of enzymes has the purity and reproducibility required for a stable and useful process is an important step in designing an enzyme-based reaction sequence.

● *Specific activity*. The specific activity of an enzyme is a measure of its catalytic activity, usually given in micromoles of

substrate generated per minute per milligram of enzyme. It is important to establish this value early in any consideration of an enzyme-based process because it determines the upper limit to the productivity of an enzymatic reactor. It is currently not practical to increase the specific activity of an enzyme significantly beyond its intrinsic value. If the specific activity is too low to form the basis for an acceptable process, other enzymes should be examined for higher activity.

● *Kinetic considerations*. Enzymatic reactions take place in fluid phases, and are thus slower than many of the vapour-phase processes widely used in large-scale chemical synthesis. A number of enzymatic processes—for example, those involving lipases (enzymes that require a water–hydrocarbon interface for activity) or other enzymes in two-phase water–hydrocarbon systems—introduce problems in interfacial mass transport that have not yet been thoroughly analysed. In addition, enzymes sometimes suffer from intrinsic kinetic limitations,[25] for example product inhibition,[23] which is part of the enzyme regulation system *in vivo*, but is a nuisance in synthesis.

A number of strategies are being explored to avoid product (and sometimes reactant) inhibition,[23] but convenient general strategies are not available.

● *Reactor design*. There has been rel-

atively little work done on the development of reactors engineered specifically to take advantage of the catalytic characteristics of enzymes. Many existing processes use column reactors adapted more or less directly from those used with heterogeneous catalysts in classical chemical synthesis. New reactors based on membranes[26] and hollow fibres[27] are now beginning to attract interest.

Chiral epoxy alcohols

These substances are versatile intermediates, widely used in pharmaceutical synthesis. They have been studied extensively by using asymmetric epoxidising systems—usually based on an organic peroxide and a complex between a titanium salt and a chiral ligand such as tartaric acid.[28] By examining routes to this compound we can compare the characteristics of enzymatic and non-enzymatic processes for the synthesis of enantiomerically enriched compounds.

Most of our work has focused on the kinetic resolution of epoxy alcohols by enantioselective hydrolysis of the derived esters with lipases. To produce chiral synthons we need to generate products with a high enantiomeric excess. Currently, an ee of less than 95 per cent is only marginally useful, and values of greater than 98 per cent are preferred. Studies of the hydrolysis of glycidyl butyrate either with pancreatic lipase or cholesterol esterase show that it is possible to achieve very high values of ee.[29] A typical process involves treating a racemic mixture of glycidyl butyrate with a lipase until about 60 per cent of the ester has hydrolysed; the remaining ester is then recovered and purified. This procedure works very smoothly for glycidyl butyrate and for several structurally related compounds, and is now the basis for a commercial process for producing glycidyl butyrate.

For substrates other than glycidyl butyrate, available lipases work with variable enantioselectivities. Certain hydrolyses are as enantioselective as those of glycidyl butyrate; others are appreciably less so. We have considered the modification and improvement of enantioselectivity of an enzyme-catalysed hydrolysis by changing the reaction conditions. We have identified three strategies that can lead to an improvement in the enantiomeric excess.

1. Lowering the reaction temperature to 0°C or below is often successful. Changing (especially lowering) pH or adding small (10–20 per cent) quantities of organic cosolvents can also be helpful. In our experience, there is no single set of reaction conditions that is best for every substrate, and the combination of substrate and enzyme must be examined on a case by case basis. Physiological conditions are not necessarily those that produce the best results in applications of enzymes in organic synthesis.

2. We have developed procedures for improving the enantioselectivity of crude enzyme mixtures without elaborate column chromatographies. Useful strategies

involve partial denaturation of a crude mixture of enzymes by heating, shifting the pH, or by adding denaturants. With luck, the impurities will be less stable to some of these conditions than the important enzyme, and a relatively simple process can then be developed which selectively destroys unwanted impurity activities while retaining the necessary activity.

3. We have also considered the analysis of enantioselectivity. The most common technique used is based on a conversion-independent measure of enantioselectivity proposed by Sih and coworkers, the so-called E value:[30]

$$E = \frac{\ln[(1 - c)(1 - ee_s)]}{\ln[(1 - c)(1 + ee_s)]}$$

$$E = \frac{\ln[1 - c(1 + ee_p)]}{\ln[1 - c(1 - ee_p)]}$$

where c = extent of conversion, ee_s = enantiomeric excess of the remaining starting material and ee_p = enantiomeric excess of the product. The E values are useful parameters but are sensitive to the measured extent of conversion. It is important to look carefully at the accuracy of measurements both of enantiomeric excess and conversion to get a realistic assessment of whether changes in methods of manipulating the enzymes and reaction conditions significantly improve the enantioselectivity of an enzymatic reaction.

There is no general answer to the question of whether or not enzymatic methods based on enantioselective hydrolysis are better than the Sharpless reaction for the synthesis of chiral epoxy alcohols. For certain compounds, enzymatic methods work very well. They are simple and easy, but they are not universally applicable. They have the disadvantage that they are kinetic resolutions, and thus intrinsically limited in ultimate yield. The Sharpless reaction is a chiral synthesis, and, in principle, is capable of giving the optically pure product in a higher yield. However, workup of reaction mixtures can be a problem. Certain classes of compounds are accepted well by enzymes and perform poorly in the Sharpless reaction; the reverse is also true.

Chiral butene oxide

We have examined a number of strategies for the preparation of chiral epoxides that are not epoxy alcohols. Both the Sharpless procedure and lipase-catalysed kinetic hydrolyses are restricted to epoxy alcohols and related species. Chiral epoxides are, however, generally useful chiral synthons, and we wanted to evaluate routes to them based on enzymatic procedures. For example, Scheme 1 summarises four synthetic strategies for the preparation of butene oxide. We emphasise the distinction between strategies based on kinetic resolutions, in which the yield of one enantiomer from a racemic mixture is, in principle, limited to 50 per cent (but in which both enantiomers may be produced) from

Scheme 2. Fructose-1,6-diphosphate aldolase catalyses a stereospecific aldol condensation between dihydroxyacetone phosphate and a variety of aldehydes, useful in the preparation of rare carbohydrates.

$$R = CH_3, Pr^i, CH_2OPh, \text{etc}$$

THP = Tetrahydropyranyl

D-Xylulose
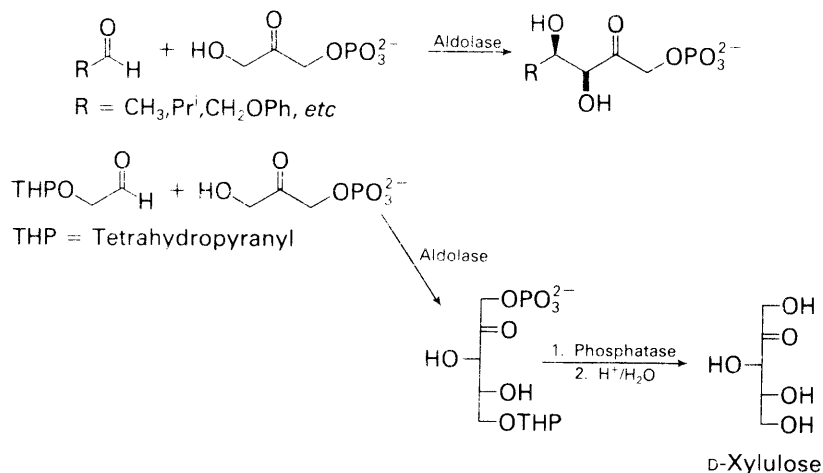
Scheme 3. The elaboration of simple C_5 and C_6 monosaccharides into octuloses and nanuloses by using fructose-1,6-diphosphate aldolase as a catalyst.
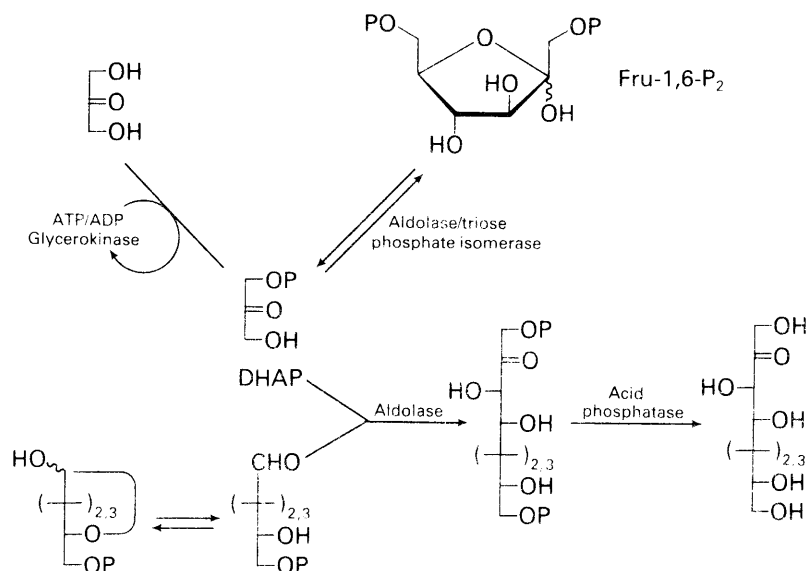
those based on asymmetric syntheses (in which a single enantiomer can, in principle, be generated in 100 per cent yield).
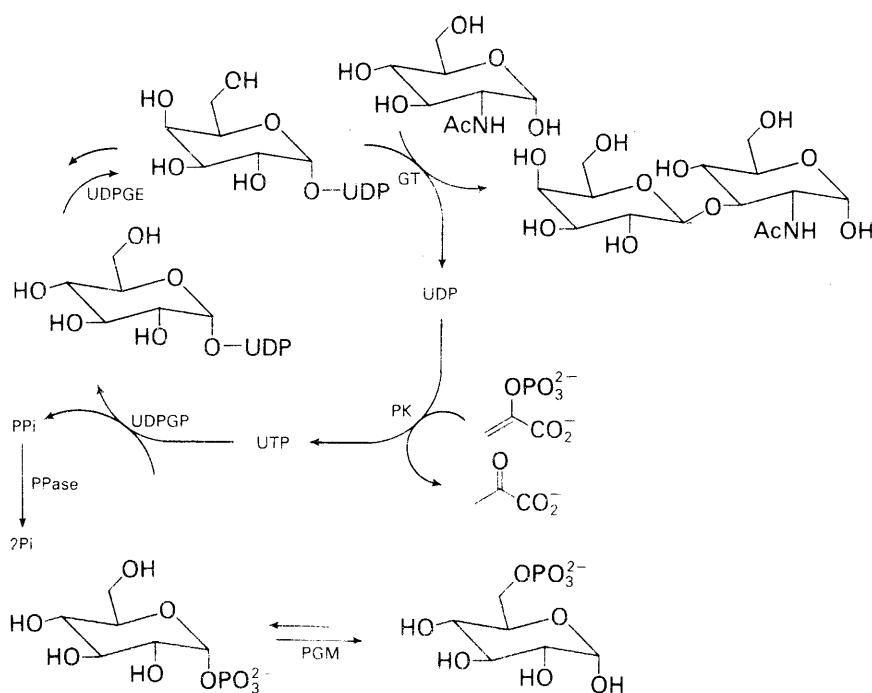
● *Lipase*. Lipase catalyses the hydrolysis of a number of bromohydrin and chlorohydrin esters. The enantioselectivity of these hydrolyses depends on the structures involved. Pancreatic lipase is moderately enantioselective in catalysing the hydrolysis of the bromohydrin ester derived from butene, but it works considerably more selectively with other bromohydrin and chlorohydrin esters. It would be better to find more enantioselective lipases to catalyse the hydrolysis, or to improve the enantioselectivity of the porcine lipase by changing reaction conditions.

● *Acylase I*. The kinetic resolution of aminoacids by acylase I is a broadly applicable and highly enantioselective reaction. The route to butene oxide based

on acylase I has the disadvantage of four reaction steps, with one exotic reaction—conversion of the α-aminoacid to an α-chloroacid by nitrosation. It has the advantage that it generates other useful intermediates (especially the chiral α-chloroacid).

● *Lactate dehydrogenase*. Routes to butene oxide based on the reduction of prochiral α-ketobutyric acid are chiral syntheses. With lactate dehydrogenase, both enantiomers of α-hydroxybutyrate are available with high enantiomeric excess, because both D- and L-lactate dehydrogenase are available and inexpensive. The L-lactate dehydrogenase has, however, much broader substrate specificity than the D-enzyme,[31] and for larger α-ketoacids, the availability of both enantiomers is more restricted. Lactate dehydrogenase requires NADH as a cofactor, and its use is more complex and expensive than that of simple hydrolases.

Scheme 4. Enzymatic synthesis of lactosamine.

Key
GT, galactosyltransferase; PK, pyruvate kinase; PGM, phosphogluco-mutase; UDPGP, UDP-glucosepyrophosphorylase; UDPGE, UDP-glucose epimerase; PPase, inorganic pyrophosphatase.

● *Glycerol dehydrogenase.* The reduction of 1-hydroxy-2-butanone with glycerol dehydrogenase is again a cofactor-requiring asymmetric synthesis. It is an efficient reaction, but is limited to low molecular weight substances by the limited substrate specificity of glycerol dehydrogenase.[22]

These reactions illustrate the range of enzymatic processes that can generate a low molecular weight chiral synthon.

Unnatural sugars
We have explored the use of aldolases as catalysts for the preparation of sugars. A variety of enzymes have aldolase activity.[2] The most readily available—rabbit muscle aldolase—catalyses the condensation of dihydroxyacetone phosphate with a variety of aldehydes.[32] This system has been applied to the synthesis of naturally occurring monosaccharides and recently to the transfer of chirality present in hexoses and pentoses into C_8 and C_9 sugars.[33] These compounds are interesting as precursors for and analogues of sialic acids and related species (Schemes 2 and 3).

Another useful class of enzymes are those that condense pyruvate and pyruvate derivatives with aldehydes. For example, the use of 3-deoxy-D-manno-octulosonate-8-phosphate synthetase to synthesise KDO-8-phosphate from arabinose-5-phosphate and PEP illustrates such enzymatic activity.[34] We are now examining the ability of this enzyme to accept analogues of arabinose phosphate

and PEP and developing a practical large-scale synthesis of KDO.

Metabolic intermediates
Enzymatic synthesis is ideal for the preparation of many classes of metabolic intermediates and of analogues of these substances, for example sn-glycerol-3-phosphate[35] and phosphoribosyl pyrophosphate[36]. The former is optically active, and has the correct stereochemistry to be a precursor for lipids for possible use as surfactants in liposomes and related drug delivery systems; the latter is a valuable intermediate in the synthesis of a wide range of nucleosides and nucleotides *in vivo*. Both are compounds in which classical chemical synthesis cannot compete with enzymatic methodology for ease and specificity.

Oligosaccharides and polysaccharides
Oligosaccharides are important as cell-surface markers, and in drug delivery systems. They are difficult or impossible to make in large quantities, although they have been the object of elegant and highly successful small-scale syntheses.[37] Polysaccharides are important for their influence on the flow properties of aqueous solutions, and for certain specific biological activities. They are virtually impossible to make by classical synthetic methods. However, there are two types of biosynthetic pathways followed in the biosynthesis of these classes of substances. One—the Leloir

pathway—uses sugar moieties activated as nucleoside di- or monophosphate sugars. The second uses sugar phosphates *per se*. Leloir pathway syntheses have been demonstrated by preparations of di-, tri-, and tetrasaccharides.[38] They are relatively complex because they need to regenerate nucleoside phosphates, but can easily be applied to the synthesis of oligosaccharides in 10 g quantities. The main limitation of the Leloir pathway syntheses is the limited availability of the required glycosyl transferases. The non-Leloir pathway processes are not broadly applicable, but are especially good for the preparation of certain classes of polymers such as dextrans[39] and laminarins[40] (Scheme 4).

Multienzyme systems
One of the features that distinguishes enzymatic from non-enzymatic catalysts is the mutual compatibility of enzymatic catalysts. Most enzymes operate satisfactorily in aqueous solution at pH 7 and room temperature; relatively few non-enzymatic catalysts operate under conditions that are compatible with one another. It is thus possible to consider the construction of complicated serial and parallel catalytic sequences involving the combination of different enzymes to provide complementary catalytic activities. The development of these multienzyme systems is still in the stage of research.

Many of the examples we have cited involve groups of three to five cooperating enzymes. Systems of this level of complexity are easily controlled and assembled by using commonly available enzymes. An example is the glycolytic system (from glucose to ethanol) modified by adding several subsystems to control the concentrations of ATP and NADH (Scheme 5). Although it provides a useful system for studying the mechanisms of metabolic regulation, it is too complex to be synthetically useful. Our experience suggests that the limit in complexity that can be tolerated in most synthetic applications is between five and 10 cooperating enzymes.

Technology
Enzymes are clearly attractive, practical catalysts for a number of types of synthesis. Where will they, in practice, be used? What are the problems currently limiting their use?
1. Chiral synthesis. The capability of enzymes to produce chiral synthons is now well established, and it will be widely used in the pharmaceutical industry for the synthesis of complex drugs. The major problems are identifying classes of enzymes with useful enantioselectivity, developing conditions for their use, and identifying chiral products sufficiently valuable for process optimisation.
2. Metabolic intermediates and analogues: aminoacids, sugars, oligosaccharides, polypeptides. Again, the value of enzymes in manipulating these classes of substances is clear. A number of questions remain, however, particularly

concerning the use of enzymes as catalysts relative to other types of catalysts. Enzymes can, for example, be used as dehydrating agents to catalyse the formation of peptide bonds.[20,41] Classical methods of peptide bond formation are, however, very highly developed, and it remains to be seen how broadly enzymes will be used. They have, in principle, the advantages of high enantioselectivity, and they do not need activating reagents. They are, however, more limited in their applicability.

3. *Process aids in biotechnology.* Recombinant DNA technology has made available a broad range of proteins. Enzymes could be used to manipulate these proteins: that is to attach sugar moieties, to do selective proteolytic clipping or stitching of polypeptide fragments, to destroy unwanted impurities *etc.* The attention of the emerging recombinant industry has, until recently, been focused on the problems of producing the proteins: genetics, microbiology, and cell culture. These problems are by no means completely solved, but the attention is now shifting to problems of isolation, modification and purification.

4. *Large-scale synthesis.* It is unclear how useful enzymes will be in large-scale synthesis. Hopes of replacing or improving commodity hydrocarbon transformations—especially those based on oxidative functionalisation—do not seem realistic. The most plausible application for enzymes is to reactions involving hydrations or dehydrations. Thus, the formation processes of esters or amides are possible targets for enzymology, as are reactions such as the hydrolysis of nitriles to amides or acids.[42]

5. *Waste treatment.* Enzymes work well as catalysts in water, and one of the most pressing problems facing the chemical process industry is waste disposal and water purification at plant sites. One of the major concerns for wastewater treatment—digestion of impurities in bioponds—is already an enzyme-based procedure in the sense that the microorganisms responsible for water purification are based on enzymatic activities. We can speculate that the isolation and concentration of desirable enzymatic activities might be useful for the treatment of certain types of waste streams: for example, removal of cyanide[43] or phenol[44] from dilute aqueous solution.

6. *Food applications.* Here, a number of problems in improvement of flavour and control of rheology depend upon chemical transformations whose nature either is or will soon be understood at the molecular level. In the food industry, enzymes can be used as catalysts when many classical organic reagents are not permitted by regulatory agencies.

A number of areas are now receiving active attention. First is the development of improved processes. The use of enzymes as catalysts in two-phase organic aqueous systems is an attractive method of avoiding some of the problems in solubilities that have limited the applicability to water-soluble substrates.

Scheme 5. Multienzyme system to convert glucose to ethanol.
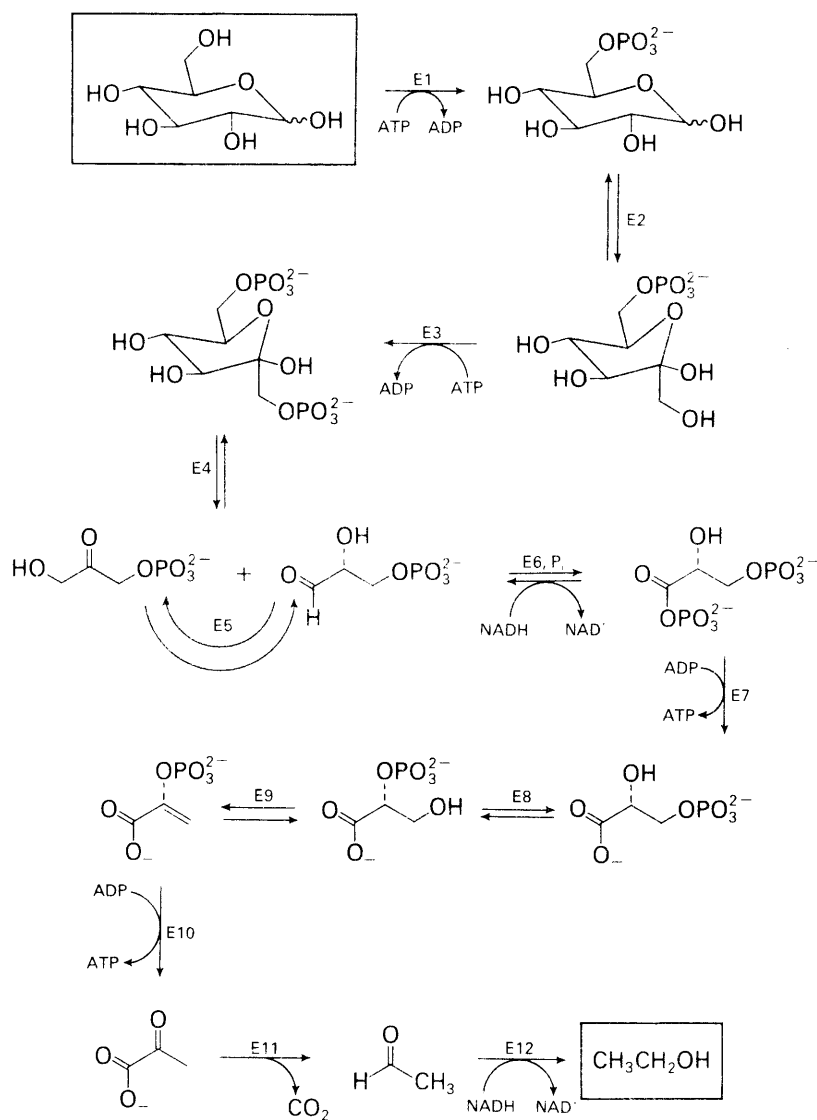
Key
E1, hexokinase; E2, phosphoglucomutase; E3, phosphofructokinase; E4, aldolase; E5, triosephosphate isomerase; E6, 3-phosphoglycerate dehydrogenase; E7, 3-phosphoglycerate kinase; E8, phosphoglycerate mutase; E9, enolase; E10, pyruvate kinase; E11, pyruvate decarboxylase; E12, alcohol dehydrogenase.

Supercritical fluids may provide a way of improving mass transport in enzymatic reactors and of continuously extracting products.[45] Continuous extraction and related schemes provide strategies for circumventing the problems of noncompetitive product inhibition, and for facilitating purification. Second, exploratory work in enzymology continues: that is, examining a range of enzymes to identify those that have the combination of activity, stability and breadth of applicability required to be useful as catalysts in organic synthesis. Finally, genetic engineering is increasingly being applied to improving the catalytic characteristics of enzymes. The initial efforts in this area concentrated on the relatively small modifications in the aminoacid sequences of existing enzymes, with the intention of altering Michaelis constants or changing stabilities.[46] These efforts have been remarkably successful, and suggest that site-specific mutagenesis will be useful in the rational improvement of existing enzymatic activities in the relatively immediate future. The problem of large changes in aminoacid sequence to produce major changes in catalytic properties, or of *de novo* design and construction of new catalytic activities, require substantial advances in basic science.

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