Enzymes in organic synthesis

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Maturing of the petroleum-based chemical industry, pressures of environmental constraints, and the explosive development of biotechnology have increased interest 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 for both the analysis (*1*) and the synthesis of research biochemicals (*2*, *3*) and in a small but significant number of large-scale chemical processes (*4–6*). 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 (Table 1).

Several developments in industry and biology have changed the value of enzymes as catalysts. The revolution in molecular genetics has made enzymes available at dramatically lowered costs (*5*, *7*). 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—for example, tissue plasminogen activator (TPA), lymphokines, and cyclosporin A—or substances derived from them. The creation of chiral centers 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 minimize 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 (*8*) by an oxidative enzymatic process (*9*) was never a 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*, *10*). Already the use of enzymes as components in detergent formulations (*11*) common in Europe and the United States has proved economical. Enzymes are a small part of detergent formulations, 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*, *12*). The yearly quantity of high-fructose corn syrup production in the United States is now more than a billion pounds.

Table 1. **Perceived characteristics of enzymes**

Advantages	Disadvantages
Highly selective for substrates	Expensive
Highly enantioselective	Unstable
Environmentally acceptable	Restricted to predominantly aqueous environments
Applicable to products relevant in pharmaceuticals, food, biotechnology, 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 using biotechnology	Difficult to manipulate

The Novo process for the production of semisynthetic insulin (*13*) provides the first example of what will probably be a widespread use of enzymes, that is, to modify proteins and other biological macromolecules derived from recombinant DNA technology. The efficiency and economics of this process and the high quality of the product establish the value of enzymatic transformations. The range of amino acids now derived from enzymatic transformations illustrates the importance of enzymology as a technology for resolving and producing chiral centers (*2*, *4*, *14*). For example, biological processes for synthesizing L-dopa (2-amino-3[3,4-di-hydroxyphenyl] propanoic acid) are now displacing technologies based on asymmetric hydrogenation using rhodium catalysts.

Before discussing reactions and processes now under development, let us summarize some issues related to the use of enzymes as catalysts.

Enzyme production. Enzymes used in chemical processes are mainly derived by extraction from natural sources, especially microorganisms. In synthetic applications, it is usually satisfactory to work with

relatively crude preparations, and immobilized whole cells are often the most convenient and economical form in which to use an enzyme. Immobilization facilitates recovery and improves the stability of the enzyme. A range of techniques for immobilizing enzymes is available (*4*, *14–17*). The method most commonly used in large-scale preparations is based on crosslinking reactions with glutaraldehyde (*4*, *15*, *18*). 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, such as polyacrylamide (PAN) (*19*), are more effective.

Cofactors. Most of the enzymatic reactions used in synthesis require cofactors. Essentially all of the common cofactors—ATP, NAD, NADH, and CoASH—are too expensive to use in stoichiometric quantities. We have developed in situ cofactor regeneration schemes that allow us to easily regenerate ATP from ADP or AMP by using either acetyl phosphate (*20*) or phosphoenol pyruvate (*21*) as the phosphate donor. The best method for regenerating NADH from NAD is based on formate as the hydride donor (*17*, *22*). Regeneration of NAD remains slightly complicated (*23*, *24*), but the best regeneration scheme is probably one based on α-ketoglutarate as the hydride acceptor (*23*). Using stoichiometric amounts of α-ketoglutarate is expensive and 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 and ATPases because proteases can shorten the lifetime of the enzyme of interest by degrading it proteolytically, and ATPases can decrease the efficiency of ATP usage in a cofactor recycling scheme. In addition, certain contaminating enzyme activities are especially troublesome in chiral transformations where the required substrate may be accepted by more than one enzyme in a crude enzyme preparation (*25*). If these competing enzymes have different enantioselectivities, the result can be unacceptably low enantiomeric excess (*ee*) 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 product 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 vapor-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 analyzed. In addition, enzymes sometimes suffer from intrinsic kinetic limitations (*26*)—for example, product inhibition (*24*)—that are part of the enzyme regulation system in vivo but are a nuisance in synthesis.

We have explored a number of strategies to avoid product (and sometimes reactant) inhibition (*24*), but convenient general strategies are not available.

Reactor design. Relatively little work has been 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 (*27*) and hollow fibers (*28*) are now beginning to attract interest.

The following sections illustrate some applications of enzymes to current problems in organic synthesis. First we'll look at the generation of a low molecular weight chiral synthon.

Chiral epoxy alcohols

These versatile synthetic intermediates, which are widely useful in pharmaceutical synthesis, have been studied extensively by Sharpless and co-workers (*29*, *30*). Sharpless's chemistry uses asymmetric epoxidizing systems, usually based on an organic peroxide in combination with a complex between a titanium salt and a chiral ligand such as tartaric acid. Thus we have an opportunity to compare the characteristics of enzymatic and nonenzymatic processes for the synthesis of these enantiomerically enriched compounds.

Most of our work has focused on the kinetic resolution of epoxy alcohols by enantioselective hydrolysis of the derived esters using lipases. Chiral synthons with an *ee* <95% are only marginally useful, and values of >98% are strongly preferred. Hydrolysis of glycidyl butyrate using either pancreatic lipase or cholesterol esterase gives very high values of *ee* (*31*). A typical process involves treating racemic glycidyl butyrate with a lipase until ~60% of the ester has hydrolyzed; the remaining ester is then recovered and purified. This procedure works smoothly for glycidyl butyrate and for a number of structurally related compounds, and it is now used for commercial production of glycidyl butyrate.

For substrates other than glycidyl butyrate, the presently available lipases work with variable enantioselectivities. Why are some hydrolyses appreciably less enantioselective than those of glycidyl butyrate? Is it possible to modify and improve the enantioselectivity of an enzyme-catalyzed hydrolysis by changing the reaction conditions? We have identified three strategies that often lead to improvement in enantiomeric excess.

- **Changing reaction conditions alters and sometimes improves enantioselectivity.** Lowering the reaction temperature to 0 °C or below is the most commonly successful strategy. Changing (especially lowering) pH or adding 10–20% organic cosolvents can also be helpful. There is, in our experience, 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. The important point is that physiological conditions don't necessarily produce the best results, and altering reaction conditions to nonphysiological values can be accepted without serious loss in activity or lifetime by certain enzymes (*32*).

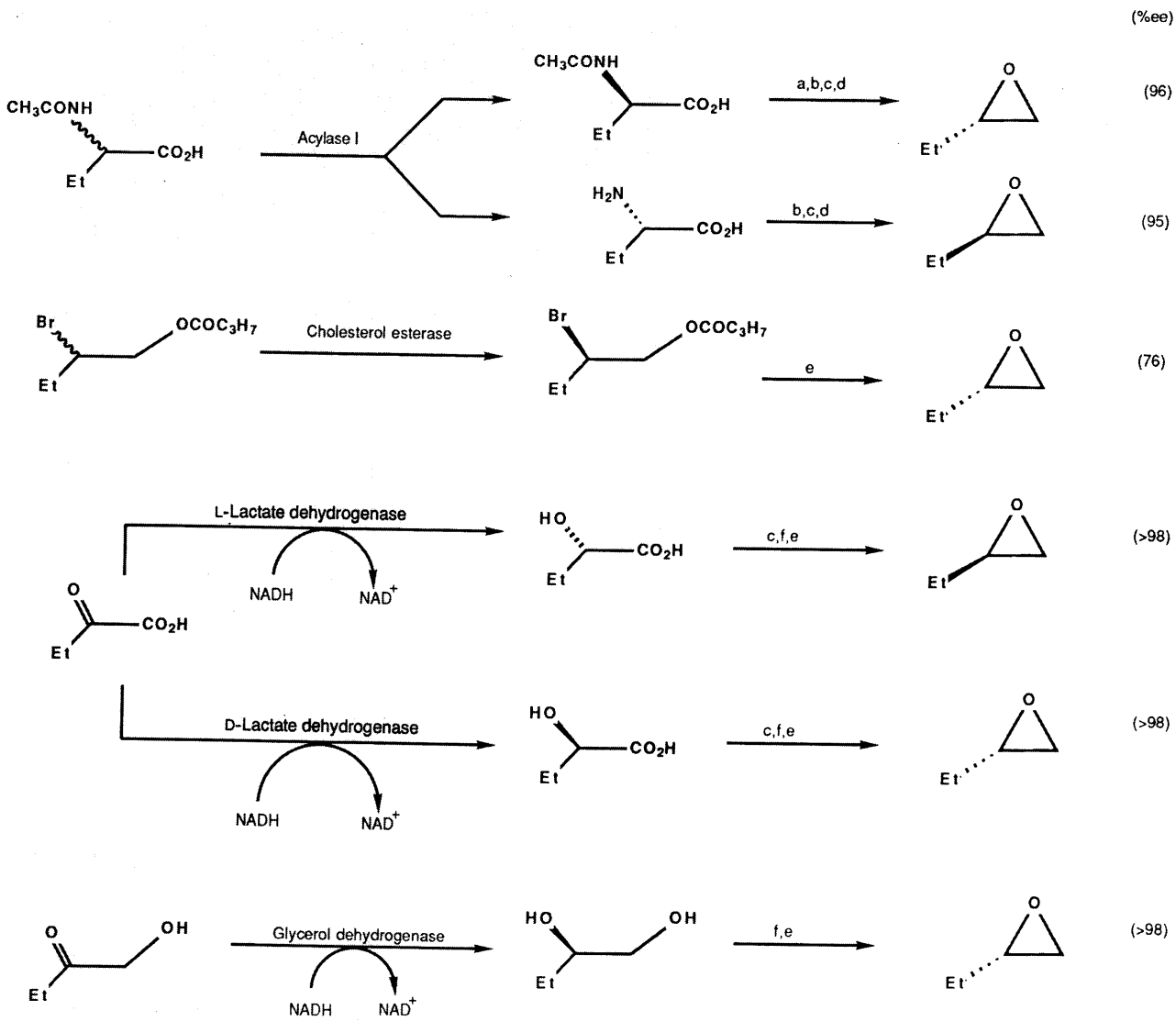

Figure 1. **Four synthetic routes to optically enriched butene oxide using enzymes. Reaction conditions: a, 2 N HCl, 100 °C, 2 h; b, 6 N HCl, $NaNO_2$; c, BH_3, THF, 0 °C, 20 h; d, KOH; e, $C_5H_{11}OK$, $C_5H_{11}OH$, room temperature; f, 30% HBr·AcOH, −15 °C → 25 °C, 3 h**

- **Low enantiomeric selectivity in a kinetic hydrolysis is sometimes intrinsic to the enzyme being used, and sometimes reflects competing activities by other enzymes present as impurities.** We have improved the enantioselectivity of crude enzyme mixtures without carrying out elaborate column chromatographies. Useful strategies involve partial denaturation of a crude mixture of enzymes by heat treatment, by shifting pH, or by adding denaturants. With luck, the undesired impurities will prove less stable to some of these conditions than will the desired majority enzyme, and a relatively simple process can then be developed that selectively destroys unwanted impurity activities while retaining a large part of the desired activity.
- **Analysis.** One issue that is often slighted in its importance in the development of processes based on enantioselective hydrolysis is analysis of enantioselectivity. The procedure commonly used to analyze enantioselectivity is based on a conversion-independent measure of enantioselectivity proposed by Sih and co-workers (33), the so-called E value.

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

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

where c is extent of conversion, ee_s is enantiomeric excess of remaining starting material, and ee_p is enantiomeric excess of product.

E values are useful parameters but are sensitive to the measured extent of conversion. One must look carefully at the accuracy of measurements of both enantiomeric excess and conversion in order to realistically assess whether changes in methods of manipulating the enzymes and reaction conditions significantly improve the enantioselectivity of an enzymatic reaction.

Are enzymatic methods based on enantioselective hydrolysis better than the Sharpless reaction for synthesis of chiral epoxy alcohols? We can't give a general answer. For certain compounds, enzymatic methods work well. They are simple and easy but are not universally applicable. Because they are kinetic resolutions, however,

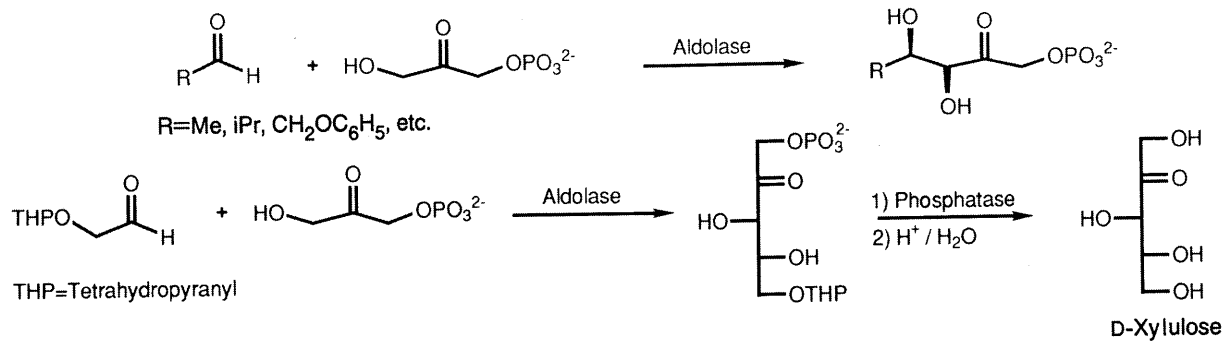

Figure 2. **Fructose-1,6-diphosphate aldolase catalyzes a stereospecific aldol condensation between dihydroxyacetone phosphate (DHAP) and a variety of aldehydes. The method is especially useful in the preparation of rare carbohydrates as exemplified above by the synthesis of D-xylulose**

the ultimate yield is intrinsically limited. The Sharpless reaction is a chiral synthesis and thus, in principle, capable of giving product in higher yield. Workup of reaction mixtures, though, 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. Enzymatic methods are not limited to epoxy alcohols. Thus evaluation of the relative applicability of enzymes and organometallic catalysts for production of chiral epoxy alcohols and derivatives must also be conducted on a case-by-case basis.

Chiral butene oxide

We also have examined a number of strategies for the preparation of chiral epoxides that are *not* epoxy alcohols. Both the Sharpless procedure and lipase-catalyzed kinetic hydrolyses are restricted to epoxy alcohols and related species. Chiral epoxides are, however, generally useful chiral synthons, and we wished to evaluate routes to them based on enzymatic procedures. We have chosen butene oxide as a representative structure. Figure 1 summarizes four synthetic strategies for its preparation. We emphasize 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%—but in which both enantiomers may be produced—from those based on asymmetric syntheses (in which a single enantiomer can, in principle, be generated in 100% yield).

Lipase. The enantioselectivity of lipase-catalyzed hydrolysis of bromohydrin and chlorohydrin esters depends markedly on the structures involved. Pancreatic lipase is only moderately enantioselective in catalyzing the hydrolysis of the bromohydrin ester derived from butene, although it works considerably more selectively with other bromohydrin and chlorohydrin esters. To produce butene oxide with high enantioselectivity following this route, one must carry the extent of conversion of the racemic starting mixture relatively far. It would be desirable to find more enantioselective lipases to catalyze the hydrolysis, or to improve the enantioselectivity of the porcine lipase by changing reaction conditions.

Acylase I. The kinetic resolution of amino acids by acylase I is broadly applicable and highly enantioselective. However, the route to butene oxide based on acylase I has four reaction steps, with one exotic reaction: conversion of the α-amino acid 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 reduction of prochiral α-ketobutyric acid are chiral syntheses. In the particular case of 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 does the D enzyme. 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.

Glycerol dehydrogenase. Reduction of 1-hydroxy-2-butanone with glycerol dehydrogenase is another asymmetric synthesis that requires a cofactor. It is an efficient reaction but it requires low molecular weight substances because of the limited substrate specificity of glycerol dehydrogenase (*24*).

Unnatural sugars

We have explored the usefulness of aldolases as catalysts for the preparation of sugars. Many different enzymes have aldolase activity (*2*). The most readily available, rabbit muscle aldolase, catalyzes the condensation of dihydroxyacetone phosphate with a variety of aldehydes (*34*). Past work has seen the application of this system to the synthesis of naturally occurring monosaccharides. Recent efforts have demonstrated that this system is useful for transferring the chirality present in hexoses and pentoses into C_8 and C_9 sugars (*35*). These compounds are interesting as precursors for and analogues of sialic acids (amino sugars containing nine or more carbon atoms) and related species (Figures 2 and 3).

Another useful class of enzymes include those that condense pyruvate and pyruvate derivatives with aldehydes. For example, the use of ketodeoxyoctanoic acid (KDO) synthetase to synthesize KDO-8-phosphate from arabinose-5-phosphate and phosphoenol pyruvate (PEP) illustrates one such enzymatic activity (*36*). We are now examining the ability of this enzyme to accept analogues of arabinose phosphate and PEP and are developing a practical large-scale synthesis of KDO.

Metabolic intermediates

Enzymatic synthesis is ideal for preparing many classes of metabolic intermediates and their analogues, such as glycerol-3-phosphate (*37*) and phosphoribosyl pyrophosphate (*38*). The former compound is optically

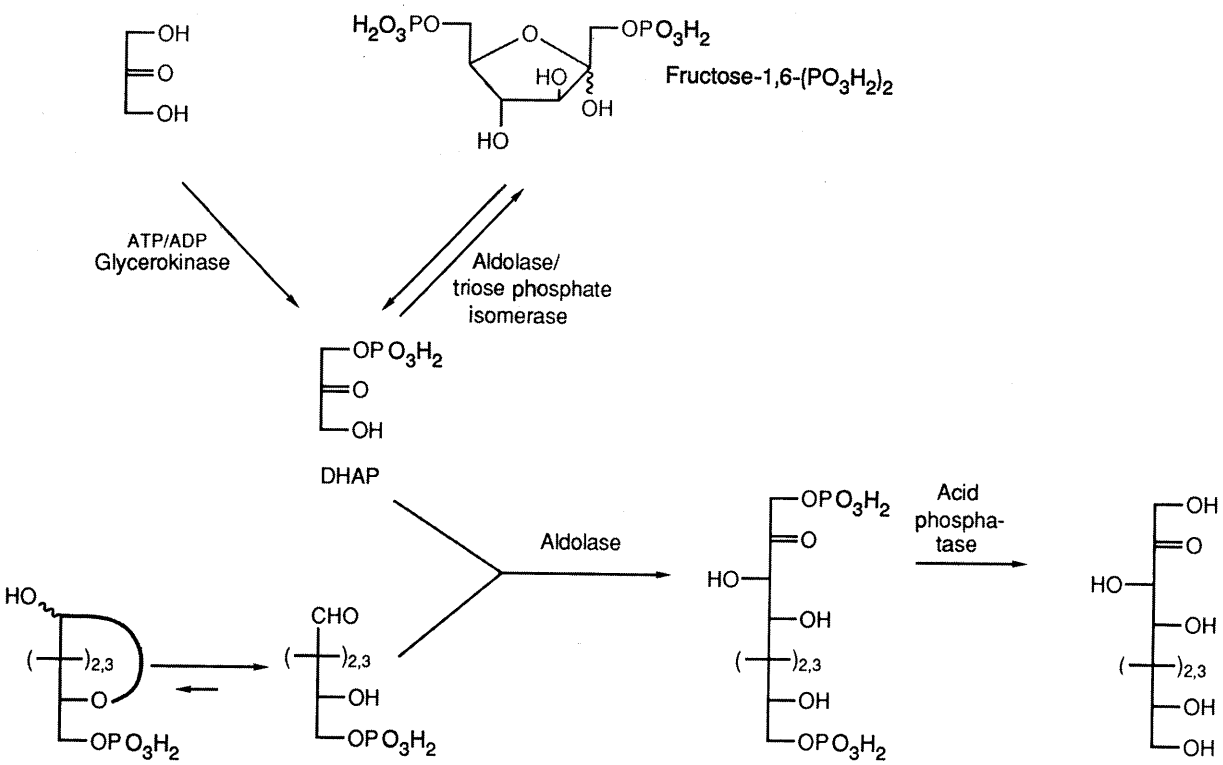

Figure 3. **The elaboration of simple C_5 and C_6 monosaccharides into octuloses and nanuloses using fructose-1,6-diphosphate aldolase as a catalyst**

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 valuable as a branch point intermediate in the synthesis of many nucleosides and nucleotides. For both compounds classical chemical synthesis cannot compete with enzymatic methods for ease and specificity.

Oligosaccharides and polysaccharides

Oligosaccharides are important as cell surface markers and are potentially useful in drug delivery systems. They are difficult or impossible to make in large quantities by using classical synthetic techniques, although they have been the object of elegant and highly successful small-scale syntheses (*39*). Polysaccharides are important for their influence on rheology of aqueous solutions and for certain specific biological activities. Enzymatic methods are applicable to both. The biosynthesis of these classes of substances follows two biosynthetic pathways. One, the Leloir pathway, which uses sugar moieties activated as nucleoside di- or monophosphate sugars, has been demonstrated by preparations of di-, tri-, and tetrasaccharides (*40*). These syntheses, which can be used to prepare 10-g quantities of oligosaccharides, are relatively complex because they require regeneration of the nucleoside phosphates. The principal limitation of Leloir pathway syntheses is the availability of the required glycosyl transferases. The second pathway uses sugar phosphates per se. It is not broadly applicable but is especially good for the preparation of dextrans (*41*) and laminarins (*42*) (Figure 4).

Multienzyme systems

One of the features that distinguishes enzymatic from nonenzymatic catalysts is the broad mutual compatibility of enzymatic catalysts. Most enzymes operate satisfactorily in aqueous solution at pH 7 and room temperature; relatively few broadly used nonenzymatic catalysts operate under conditions that are compatible with one another. It is thus possible almost uniquely with enzymes to consider the construction of complicated serial and parallel catalytic sequences involving the combination of different enzymes to provide complementary catalytic activities. This area of catalyst development—the development of multienzyme systems—is still in the research stage. Many of the examples cited above have involved groups of three to five cooperating enzymes. Systems of this level of complexity are easily controlled and assembled using commonly available enzymes. A more complex example is that illustrated in Figure 5. The complexities of working with this system are such that, although it provides a useful system with which to study the mechanisms of metabolic regulation, it seems unlikely to be synthetically useful. Our experience to date suggests that the limit in complexity that can be tolerated in most synthetic applications is between 5 and 10 cooperating enzymes. Numbers larger than 10 become too difficult to be synthetically practical; numbers smaller than this can be handled with more or less facility.

Technology

Enzymes are clearly attractive and practical as catalysts for a number of types of synthesis. Where will they be used in practice? What are the problems currently limiting their use?

- *Chiral synthesis.* The now-established capability of enzymes to produce chiral synthons will be widely used in the pharmaceutical industry for the synthesis of complex drugs. Among the major problems to be overcome in this area are the identification of classes of

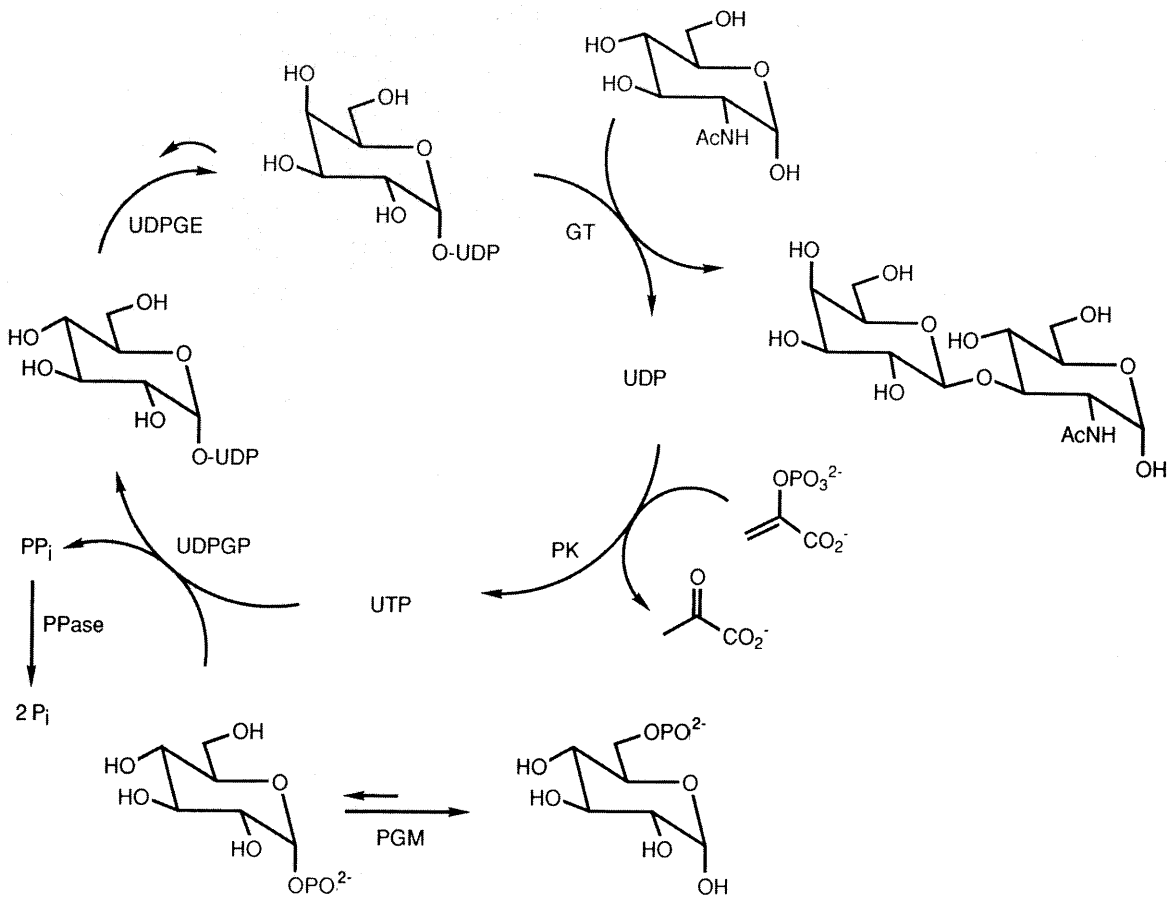

Figure 4. **Enzymatic synthesis of lactosamine. GT, galactosyltransferase; PK, pyruvate kinase; PGM, phosphoglucomutase; UDPGP, UDP-glucose pyrophosphorylase; UDPGE, UDP-glucose epimerase; PPase, inorganic pyrophosphatase; PP_i, inorganic pyrophosphate; UTP, uridine 5′-triphosphate**

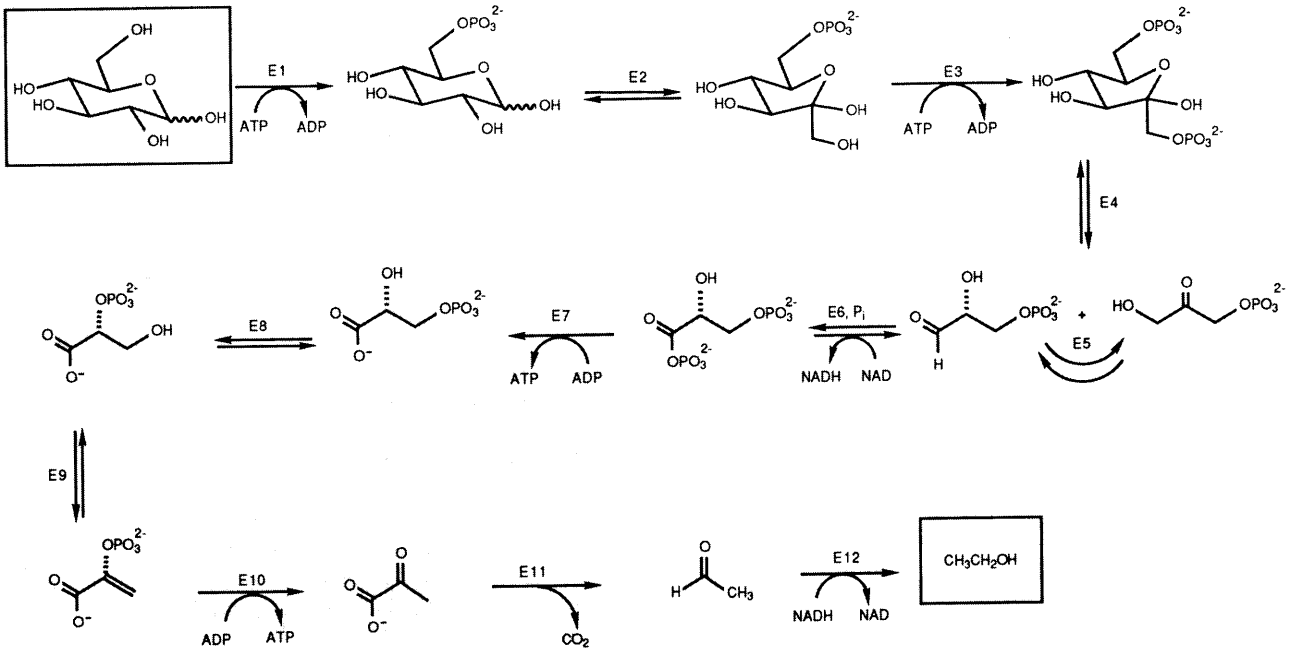

Figure 5. **Multienzyme system to convert glucose to ethanol. E1, hexokinase; E2, phosphoglucomutase; E3, phosphofructokinase; E4, aldolase; E5, triosephosphate isomerase; E6, 3-phosphoglycerate dehydrogenase; E7, 3-phosphoglycerate kinase; E8, phosphoglycerate mutase; E9, enolase; E10, pyruvate kinase;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

enzymes with useful enantioselectivity, the development of conditions for their use, and the identification of chiral products sufficiently valuable to warrant process optimization.

- *Metabolic intermediates and analogues—amino acids, sugars, oligosaccharides, and polypeptides.* Again, the value of enzymes in manipulating these classes of substances is clear. A number of substantial questions remain in these areas, however, particularly questions that concern the usefulness of enzymes as catalytic entities relative to other types of catalysts. Enzymes can, for example, be used as dehydrating agents to catalyze the formation of peptide bonds (*21*, *43*). Classical methods of peptide bond formation are highly developed, and it remains to be seen how broadly enzymes will be used. In principle, they have the advantages of high enantioselectivity, and they do not need activating reagents. On the other hand, they are more limited in their applicability.
- *Process aids in biotechnology.* Recombinant DNA technology has made available a broad range of proteins. Enzymes could be used to manipulate these proteins, for example, to attach sugar moieties, to do selective proteolytic clipping or stitching of polypeptide fragments, or to destroy unwanted impurities. Until recently attention of the emerging recombinant industry has 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 over to the problems of isolation, modification, and purification.
- *Large-scale synthesis.* It is presently unclear how useful enzymes will be in large-scale synthesis. The production of high-fructose corn syrup represents a special case in that it is a one-step conversion of a sugar, the type of process in which enzymes have special utility. Hopes of replacing or improving commodity hydrocarbon transformations—especially those based on oxidative functionalization—do not seem realistic. The most plausible applications for enzymes are hydrations or dehydrations. Thus formation of esters or amides is a plausible target for enzymology, as are reactions such as the hydrolysis of nitriles to amides or acids (*44*). After initial and unrealistic expectations for the application of enzymes in large-scale process chemistry, proposals in this area fell into a certain disrepute. As the characteristics of enzymes have become clearer, they are now being reexamined in a more realistic light.
- *Waste treatment.* Enzymes work well as catalysts in water, and two of the most pressing problems facing the chemical process industry are waste disposal and water purification at plant sites. One of the major strategies for waste-water 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. It is plausible to speculate that isolation and concentration of desirable enzymatic activities might be useful for the treatment of certain types of waste streams: for example, removal of cyanide (*45*) or phenol (*46*) from dilute aqueous solution. These types of applications are now being actively explored.
- *Food processing.* A number of problems in improvement of flavor and control of rheology depend on chemical transformations whose nature either is or will soon be understood at the molecular level. Enzymes are attractive in that they can be used as catalysts in situations where many classical organic reagents are not permitted by regulatory agencies.

Future

Several 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 circumventing some of the problems in solubilities that have limited the applicability to water-soluble substrates. Supercritical fluids may provide a way of improving mass transport in enzymatic reactors and of continuously extracting products (*47*). Continuous extraction and related schemes provide strategies for circumventing the problems of noncompetitive product inhibition and facilitating purification.

Second, exploratory work in enzymology continues—that is, examining a range of enzymes to identify those that combine the activity, stability, and breadth of applicability required to be useful as catalysts in organic synthesis.

Third, genetic engineering is increasingly being applied to improve the catalytic characteristics of enzymes. The initial efforts in this area have been focused on relatively small modifications in the amino acid sequences of existing enzymes, with the intent of altering kinetics or changing stabilities (*48*). These initial 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 broader problem of large changes in amino acid 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 and will not be solved soon.

The most important issue is, however, a basic change in philosophy in large-scale synthesis. In the past, enzymes have been (probably correctly) regarded less useful as catalysts than the more commonly used inorganic and organic substances for the classes of problems important in practical-scale chemistry. The maturing of the petroleum-based chemical process industry, the pressures of environmental constraints, and the explosive development of biotechnology have now shifted the focus of problems in catalysis such that enzymology has become much more attractive. The initial considerations of enzymes as potential catalysts for the chemical process industry were based on the hypothesis that enzymes might be useful in providing more economic processes for *existing* petrochemically derived products. This hypothesis may be correct in certain specialized cases—especially those involving reactions that add or remove water from reactants or products—but probably is not generally valid. The use of enzymes as catalysts for *new* classes of reagents and products—especially sugars, water-soluble materials, chiral synthons, biopolymers, metabolites, food components, and related substances—seems entirely realistic, and to the extent that chemistry will be concerned with these types of problems, enzymes will play an important role.

Acknowledgments

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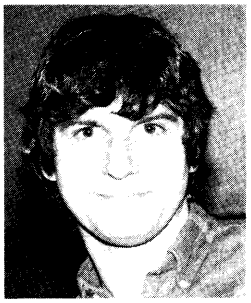

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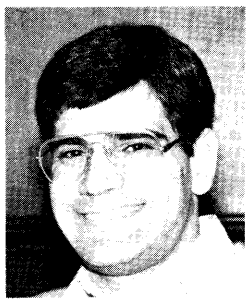

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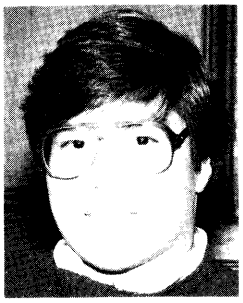

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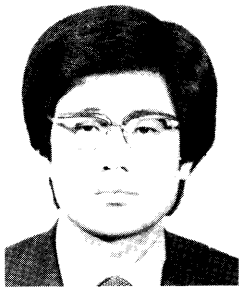

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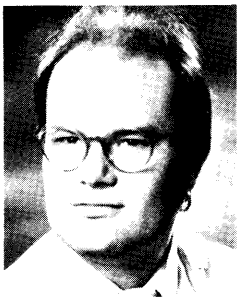

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