Enzymes as Catalysts in Organic Synthesis

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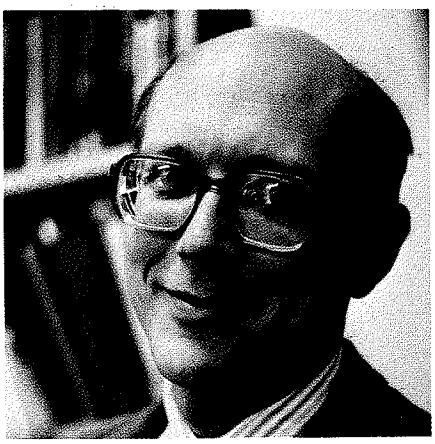
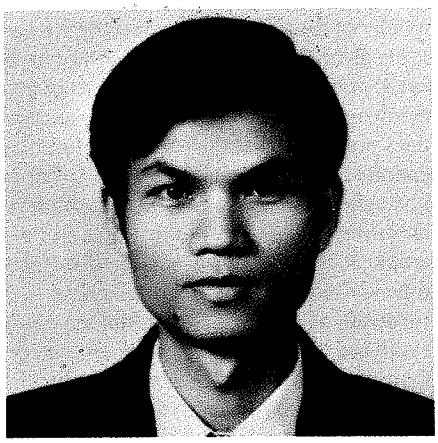




Enzymes are proteins having catalytic activity. They are involved in virtually every transformation which occurs *in vivo*, and thus catalyze large numbers of transformations of biologically important molecules. They also catalyze reactions of many substances which do not occur *in vivo*. Given this wide range of catalytic activity, one might expect enzymes to be widely used in organic synthesis *in vitro*. In fact, their use has been small compared with other classes of catalysts (acids and bases, metals, organometallic compounds).

Why are enzymes *not* more widely used as catalysts in organic synthesis? A number of factors contribute to a long-standing preference of organic chemists for non-enzymatic catalysis:

First, tradition, coupled with a certain lack of motivation. Organic synthesis, in recent years, has focused on terpenes, steroids, alkaloids, prostanoids, and other classes of water-insoluble substances. Its principal concerns have been the formation of carbon-carbon bonds and the regioselective functionalization of hydrocarbon skeletons. Enzymes which form carbon-carbon bonds are, in fact, neither the easiest to obtain nor the most straightforward to use. For the reactions involved in synthesis of complex carbon skeletons, conventional chemical methods are generally more efficient than biological methods.

Second, perceived technical difficulties. Organic chemists believe that enzymes are expensive and delicate. In fact some are and some are not, and even those which *are* expensive and delicate may still be very effective and inexpensive *as catalysts* by virtue of their high catalytic rate constants or their ability to simplify complex synthetic schemes. Many of the problems of instability and cost can be ameliorated by appropriate experimental technique.

Third, specificity. One of the strengths of conventional organic synthesis is that it produces methods which often have wide generality. Organic chemists suspect enzymes of being highly specific in their action. Again, some are and some are not. High specificity may be a disadvantage if one is trying to produce general synthetic methods; it can be a great advantage if one is interested in that particular transformation which the enzyme catalyzes.

Fourth, unfamiliarity. The techniques for isolating, manipulating, and assaying enzymes are unfamiliar to most organic chemists.[1,2] Many enzymes useful in synthesis are, of course, now commercially available, and others of use in new applications could be made available commercially if demand for them existed. The techniques for manipulating enzymes are, in general, relatively straightforward experimentally. Enzymatic *assays* represent a point of considerable and lingering dissatisfaction to many people trained in the relatively precise and easily understood analytical methods of organic chemistry. Enzyme assays seem intrinsically sloppy and ill-defined, relative to methods based on GLC or HPLC. It is nonetheless possible, by an excercise of will, to overcome one's sense of distrust and unease in using these methods, and to obtain reproducible and reliable results.

Enzymatic catalysis is, of course, no panacea. While enzymes have deficiencies as catalysts relative to metallic platinum, for example, the reverse is also true. The real strength of catalysis by enzymes lies in its selectivity. The real strength of catalysis by platinum lies in its generality. Which type of catalyst is best suited for a particular application? The answer depends upon the application. As chemistry turns more to the synthesis of complex substances which are derived from biology or related to biologically important materials (or, more accurately, as chemistry is *forced* to turn to these classes of materials by advances in other areas of science and by exhaustion of current problems in conventional chemistry), a number of new methods — enzymology, recombinant DNA technology, fermentation, tissue culture — will become an increasingly important part of the synthetic chemist's armamentarium.[3] Enzymology is the most fundamental of these biological techniques (the others simply represent methods of using enzymatic catalysts in preformed, packaged, cellular systems) and is certainly the most "chemical" of these techniques.

Nomenclature. One significant obstacle (not mentioned above) to the use of enzymes by organic chemists has been nomenclature. Certain enzymes are named accord-

ing to tradition ("old yellow enzyme") or according to their source (papain from papaya). These names give no hint of their catalytic activity, and make browsing for useful activities difficult for synthetic chemists not trained in biological chemistry. The systematic IUB (International Union of Biochemistry) nomenclature divides enzymes into six groups, and assigns a name and number to each based on its assumed function *in vivo* (Table 1). The numbers in this system are of archival use only. The names are often misleading: occasionally they do not correspond even to the principal function of the enzyme *in vivo*, and frequently do not indicate usefully the type of catalytic activity nor the specificity of the enzyme. For example, the enzyme glycerol kinase (EC 2.7.1.30) catalyzes the phosphorylation of glycerol to *sn*-glycerol-3-phosphate. The name of the enzyme does not imply that the phosphorylation is enantiospecific. More importantly, it does not indicate that this enzyme phosphorylates several other useful substrates (for example, it smoothly converts dihydroxyacetone to dihydroxyacetone phosphate — a substrate of great utility in aldol reactions catalyzed by aldolase). Unfortunately, from the vantage of a synthetic chemist searching for useful catalytic activity, there is presently no solution to the problem of recognizing the synthetically important features of the catalytic activity of a particular enzyme aside from simply knowing the appropriate literature (and often, even here, experimental research is required because the substrates of interest to biologists are not always those of interest to organic chemists).

We caution organic chemists that a uniform system of units for expressing catalytic activity is not used throughout biochemistry and enzymology. The standard unit of enzymatic activity is the International Unit (1 I.U. = 1 μmole of substrate transformed or product formed per min), but units such as nmol/min or hour and those based on optical absorbance are also common. Another system of units based on the katal (1 kat $\equiv$ 1 mol s^{-1} substrate transformed or product formed) has also been recommended, but has not been widely used. In searching the literature for characteristics of a new enzyme, it is essential to check explicitly the units in which catalytic activity is expressed. For reference, approximately 700 I.U. of enzymatic activity will catalyze the formation of one mole of product per day.

Rather than follow the IUB system of enzyme nomenclature, we have found it more useful to divide enzymes into five groups,

Table 1. International classification of enzymes (class names, code numbers, and types of reactions catalyzed) (partial listing)

1. Oxido-reductases (oxidation-reduction reactions)
   - 1.1 Acting on >CH—OH
   - 1.2 Acting on >C=O
   - 1.3 Acting on >C=CH—
   - 1.4 Acting on >CH—NH_2
   - 1.5 Acting on >CH—NH—
   - 1.6 Acting on NADH; NADPH
2. Transferases (transfer of functional groups)
   - 2.1 One-carbon groups
   - 2.2 Aldehydic or ketonic groups
   - 2.3 Acyl groups
   - 2.4 Glycosyl groups
   - 2.7 Phosphate groups
   - 2.8 S-containing groups
3. Hydrolases (hydrolysis reactions)
   - 3.1 Esters
   - 3.2 Glycosidic bonds
   - 3.4 Peptide bonds
   - 3.5 Other C—N bonds
   - 3.6 Acid anhydrides
4. Lyases (addition to double bonds)
   - 4.1 >C=C<
   - 4.2 >C=O
   - 4.3 >C=N—
5. Isomerases (isomerization reactions)
   - 5.1 Racemases
6. Ligases (formation of bonds with ATP cleavage)
   - 6.1 C—O
   - 6.2 C—S
   - 6.3 C—N
   - 6.4 C—C

in order of their increasing complexity of use in organic synthesis:

1) *Simple hydrolases and isomerases.*

2) *Enzymes requiring no added cofactors* (especially those using flavins and pyridoxal phosphate as cofactors).

3) *Enzymes requiring cofactor regeneration* [that is, those using ATP or other nucleoside triphosphates and NAD(P)(H)].

4) *Enzymes having particular problems of availability or stability*, or those requiring uncommon cofactors (S-adenosylmethionine or adenosine 3′-phosphate 5′-phosphosulfate).

5) *Complex multi-enzyme systems.*

This review will concentrate on the first three classes of enzymes. Its principal objective is to illustrate the types of synthetic reactions for which enzymes might be considered as catalysts.

GENERAL CONSIDERATIONS

Enzymes have three characteristics as catalysts:

i) they accelerate rates of reactions;

ii) they are often highly selective in their activity;

iii) their catalytic activity may be regulated, that is, strongly influenced by the concentration of reactants, products, cofactors or other species present in solution.

The first and second are the bases for the utility of enzymes as catalysts; the third is most often the cause of problems since it is the basis for product inhibition, that is, the (not infrequent) decrease in catalytic activity of the enzyme as relatively low concentrations of products accumulate.

Enzymes are normally most soluble and stable in water or in water containing relatively small quantities of polar co-solvents (especially polyhydric alcohols, dimethyl sulfoxide, and related species). They normally function best with substrates which are soluble in these media, although it is often possible to carry out reactions using substrates which are only partially soluble in water. Two-phase systems comprising water and an insoluble organic phase as well as miscible aqueous/organic solvent systems are being explored extensively as media in which to reverse hydrolytic reactions, *i.e.*, dehydrations.[4-6] Presently, these types of reactions are primarily of use in protein chemistry,[7-8] but their applications will probably be extended to other areas.

Enzymes are most often immobilized on insoluble supports.[9,10] There are two reasons for immobilizing enzymes: to make it possible to recover and reuse them at the conclusion of a reaction, and to enhance their stability under the conditions of the reaction. The second is usually the more important. In practice, we almost always use enzymes in immobilized form, because the stability enhancement more than compensates for the activity lost during immo-

bilization. We have developed a method (based on a water-soluble polyacrylamide derivative containing active ester functionalities — polyacrylamide-co-*N*-acryloxysuccinamide, PAN) which has very wide applicability to the relatively delicate enzymes useful in complex organic synthesis (Scheme I).[11] Many other methods are available,[12] but in our experience, procedures based on PAN have shown the widest generality and have given the highest retention of enzymatic activity on immobilization. This procedure is particularly useful in enzyme-catalyzed synthesis of complex organic compounds on scales of 1g to several kilos.

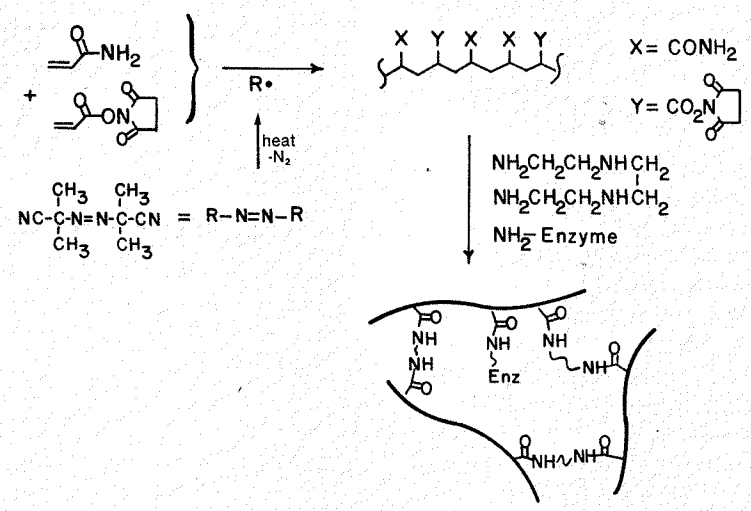
Enzymes used as catalysts in organic synthesis (as opposed to enzymes for mechanistic enzymology studies) need not be particularly pure. The major considerations are that the cost of a unit of activity (in this context "cost" means either the purchase price or the expenditure of effort in a biochemical preparation) and the specific activity — the number of units of activity per milligram of protein — be acceptable. The second parameter is of practical importance in immobilization: an enzyme having a low specific activity may require a very large volume of polymer gel for immobilization, and may therefore be difficult or impractical to handle in immobilized form.

The stability of an enzyme determines its lifetime under operating conditions, and can be quite high. The major factors leading to stability are immobilization, exclusion of dioxygen and other oxidizing agents (when working with enzymes having oxidation-sensitive groups — especially cysteine SH groups[13] — close to the active site), and exclusion of proteases (which might degrade the enzyme) from the reaction medium. The stability of many enzymes is improved by the addition of substrates or products to their solutions so that their active sites are always occupied; this strategy is usually absolutely necessary for obtaining high yields during immobilization.[11]

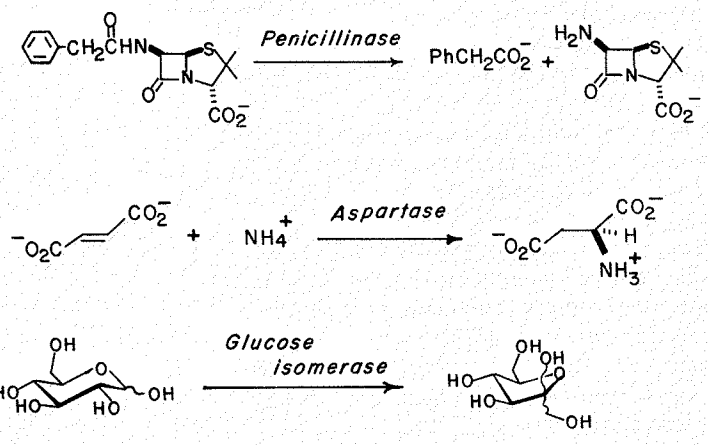
SPECIFIC CHARACTERISTICS

Simple hydrolases and isomerases. The first group of enzymes is that most widely used in industrial enzymology: the production of 6-aminopenicillanic acid[16] and aspartic acid,[17] the isomerization of glucose to fructose,[18] and the various applications of proteases and glycosidases in detergents and food processing all depend upon this type of enzyme (Scheme II).[9,10,12] Most of these processes have been developed for specific large-volume applications, *e.g.*, the interconversion of glucose and fructose is carried out on a scale of more than 10^9 lbs

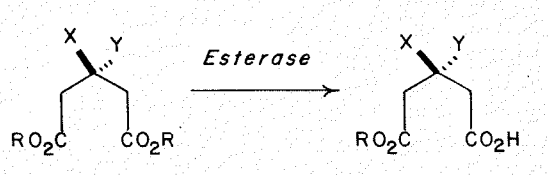
Scheme I



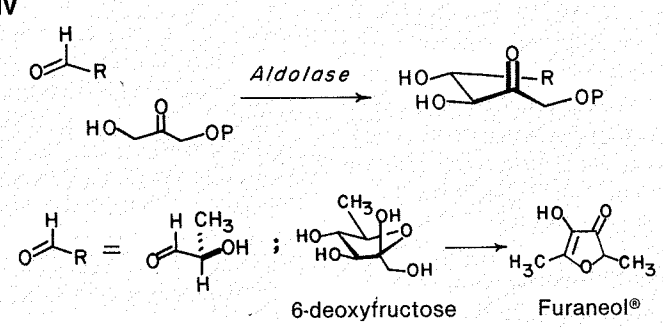
Scheme II



Scheme III



Scheme IV



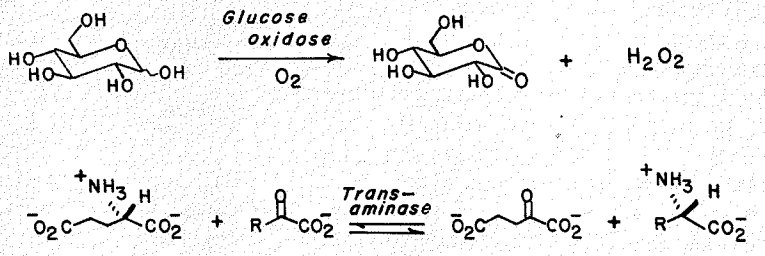
per year in the United States. These industrial processes are interesting in that they establish the practicality of enzymatic processes for large-scale synthesis, but they are not of wide generality. They also establish the fact that these enzymes can be manipulated by chemical engineers.

Applications of these enzymes to research should be more widespread than they are, because, as a class, they are readily available and easily handled. One important example is provided by the asymmetric synthesis based on the hydrolysis of diesters by esterase (Scheme III).[19] A second is the generation of unusual sugars by the regiospecific aldol condensation catalyzed by aldolase (Scheme IV).[20] The preparation of 6-deoxyfructose (used as a starting material for the flavor principle Furaneol®) provides an illustration of the application of this type of reaction.

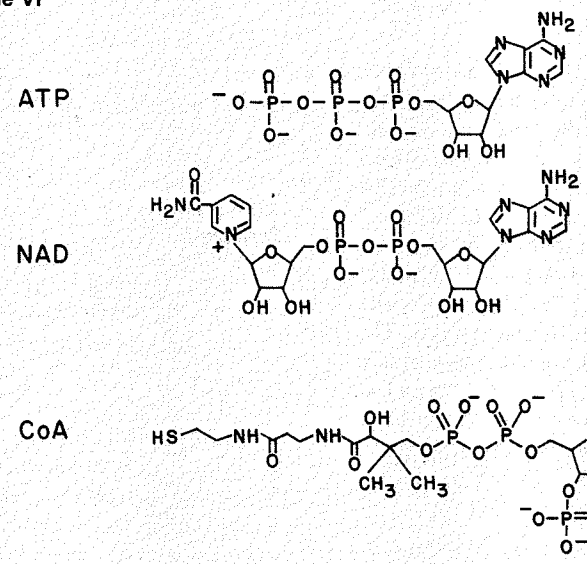
More complex enzymes requiring no added cofactors. The second group involves enzymes which require cofactors which do not themselves require an added regeneration system. Flavins, pyridoxal phosphate, lipoic acid, biotin, metal porphyrin complexes, and related species bind tightly to their respective enzymes, and in general, regenerate automatically during the course of the enzyme-catalyzed reactions. This group includes oxygenases and hydroxylases (flavoenzymes), transaminases (pyridoxal-phosphate-containing enzymes), carboxylases and decarboxylases (lipoic-acid- and biotin-containing enzymes), monooxygenases, peroxidases and mutases (metal-porphyrin-containing enzymes).[21] With the exception of glucose oxidase (primarily to remove dioxygen from foods)[22] and transaminases (occasionally for analysis),[23] this group has had relatively few applications in organic synthesis (Scheme V). Potential uses of these enzymes are amino acid synthesis (transaminases), selective hydroxylation and peroxidation (peroxidases,[24] hydroxylases, prostaglandin cyclooxygenases, lipoxygenase[25]), Baeyer-Villiger oxidation and asymmetric epoxidation (ketone monooxygenases), selective elimination and addition of water (dehydrases), epimerization (epimerases), and carbon-skeleton rearrangement (B_{12}-containing mutases).[21]

Enzymes requiring nucleoside triphosphate or nicotinamide cofactors. The third group of enzymes — those requiring nucleoside triphosphates (especially ATP) or nicotinamide cofactors [NAD(P)(H)] — is probably the group of greatest interest to academic synthetic organic chemists and to others concerned with syntheses of fine chemicals.[26] Most enzymatic synthetic reactions — the transformation of smaller molecules to larger, more complex ones — involve the nucleoside triphosphates; the nicotinamide cofactors are utilized in most enzymatic redox reactions (Scheme VI). It has been estimated that approximately 70% of all enzymes use XTP, NAD(P)(H) or CoA as a cofactor. In general, these are the enzymes which seem to hold the key to the enzyme-catalyzed synthesis of complex substances.

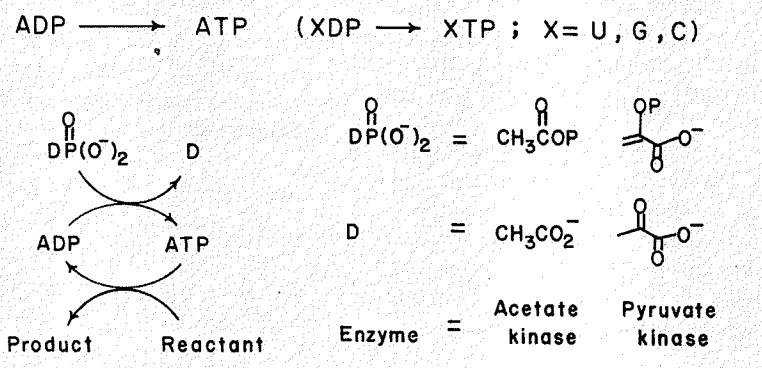
Scheme V



Scheme VI



Scheme VII



The principal barrier to the use of these enzymes has been the cost of the cofactors. These costs range from approximately \$1,000/mol for NAD^+ to several hundred thousand dollars/mol for the more expensive cofactors; these prices are sufficiently high that they exclude stoichiometric use of the cofactors. Instead, it has been necessary to develop schemes for the *in situ* regeneration of the cofactors. The problems of cofactor regeneration have been essen-

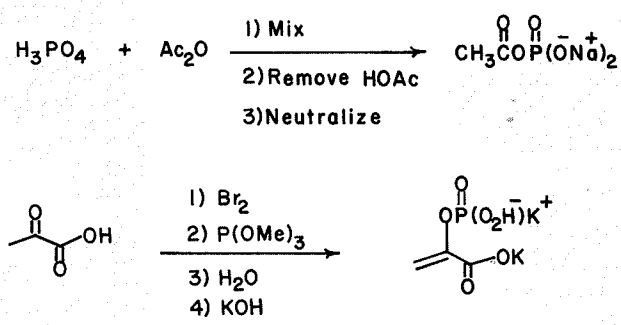
tially solved (at least at the level required for synthesis of fine chemicals) for ATP[27–30] and NAD(P)(H);[31–39] the regeneration of XTP (X = G,C, and U) from XDP[40] is also straightforward. Scheme VII shows the two methods preferred for the *in situ* regeneration of ATP from ADP. Both of these schemes are well known, and have been used on small scales in analytical and mechanistic enzymology for many years. The trick in developing procedures valuable in large-scale synthesis was to find convenient routes to the phosphate donors, acetyl phosphate and phosphoenol pyruvate. Acetyl phosphate can now be made by a very simple procedure involving acylation of phosphoric acid with acetic anhydride, removal of the excess acetic acid by extraction, and neutralization (Scheme VIII).[41] Phosphoenol pyruvate (PEP) requires a slightly more complex synthesis,[29] but can also be made easily on a mole scale. Both of these regeneration procedures have their specific applications. The preparation of acetyl phosphate is experimentally the simpler of the two, and the corresponding regeneration procedure is the more commonly used. PEP is, however, much more stable in solution, and a stronger phosphorylating agent than AcP. It is used when either of these characteristics is a convenience or a requirement.

A number of reactions which consume ATP generate AMP as a product. A simple modification of the scheme illustrated above makes possible the regeneration of ATP from AMP (Scheme IX).[42,43] The same enzymes and cofactors are required, and only one other component is added — the enzyme adenylate kinase, which catalyzes the phosphorylation of AMP to ADP by ATP.

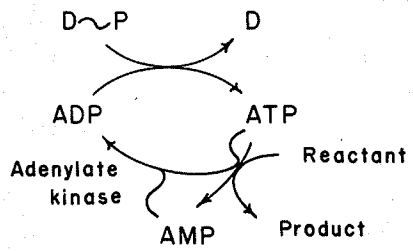
Acetate kinase and pyruvate kinase will accept all of the nucleoside diphosphates as substrates, and catalyze their conversion to nucleoside triphosphates.[2,44] This fact provides the basis for regeneration of all of the nucleoside triphosphates from nucleoside diphosphates. Relatively few reactions generate XMP (X = U, G, C); for these few, at present, no truly practical regeneration scheme exists since adenylate kinase is specific for AMP.

Scheme X provides examples of applications of the ATP regeneration systems. The selective phosphorylation of glucose at C_6 illustrates the selective derivatization of an unprotected carbohydrate;[27] the conversion of glycerol to *sn*-glycerol-3-phosphate is enantiospecific and is the best route presently available to the chiral synthon required for enantiomerically pure phospholipids.[43] Phosphoribosyl pyrophosphate (PRPP) is an important intermediate in nucleoside and nucleotide biosynthesis, and should be valuable in a number of synthetic applications.[45]

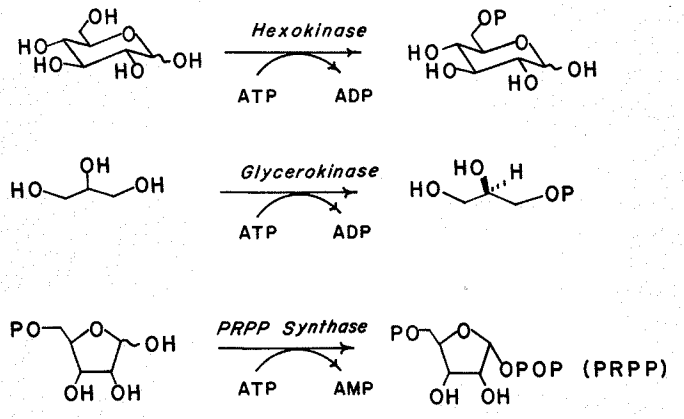
Scheme VIII



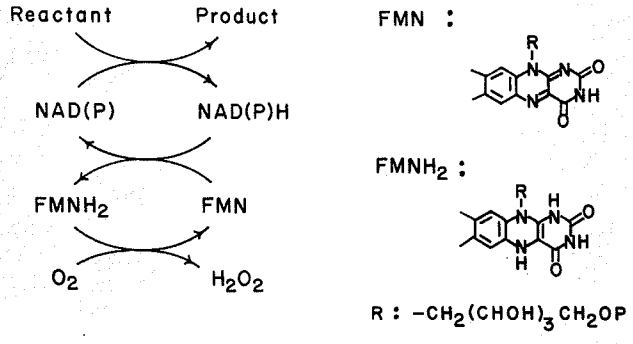
Scheme IX



Scheme X



Scheme XI



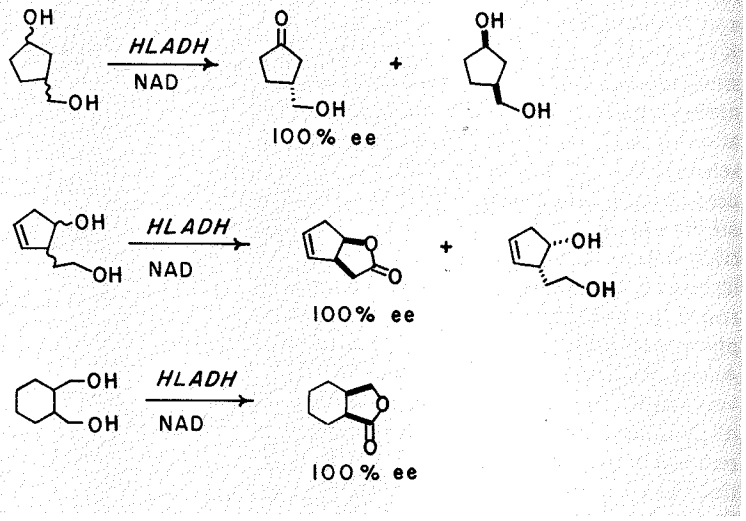
The ATP used in these syntheses is typically cycled *in situ* approximately 100 times; this value is limited only by achievement of a convenient rate in the reaction — the

nucleoside triphosphate cofactors are themselves intrinsically stable in solution. The total turnover numbers obtained for the enzymes (total turnover number ≙ TTN = mol of product/mol of enzyme) is usually in the range of 10^6 to 10^8. The enzymes can usually be recovered in good yield and reused if immobilized.

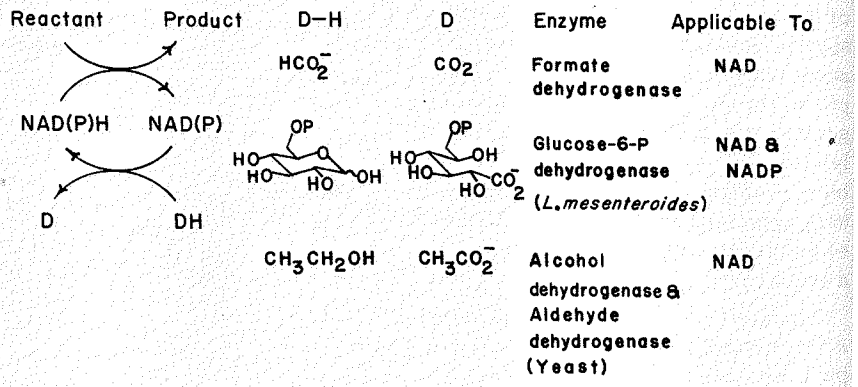
Regeneration of the nicotinamide cofactors presents an intrinsically more complex problem than that for the nucleoside triphosphates, both because many of these materials are more expensive than the nucleoside triphosphates and because their stability in solution is only modest. Nonetheless, satisfactory routes for regeneration of all of these species are now available. For oxidative regeneration from reduced cofactors, the best procedure is that developed by Jones and co-workers, utilizing an intermediate flavin with dioxygen as the ultimate oxidizing agent (Scheme XI).[46] When oxygen cannot be used in the system due to the sensitivity of one of the constituent enzymes, an alternative system based on conversion of α-ketoglutarate to glutamic acid can be used.[28] Scheme XII gives examples of synthetic applications which require regeneration of oxidized nicotinamide cofactors. The most widely explored system is that based on the ability of horse liver alcohol dehydrogenase (HLAD) to oxidize alcohols selectively to ketones or aldehydes.[47–49]

The regeneration of reduced from oxidized nicotinamide cofactors is a more difficult problem, for several technical reasons. A number of routes have been explored to accomplish this regeneration. The most practical for use in organic synthesis involve glucose-6-phosphate,[31] formate,[32] or ethanol[39] as the ultimate reducing agents (Scheme XIII). The advantages of the route based on formate dehydrogenase are that no byproduct is formed in the reaction and workup is very simple. Its disadvantages are that it is applicable only to the reduction of NAD and the enzyme is relatively expensive. The scheme utilizing glucose-6-phosphate dehydrogenase from *L. mesenteroides* is applicable to both NAD and NADP, and the enzyme is readily available, sturdy, and inexpensive. The substrate for the reaction (glucose-6-phosphate) is, however, less readily available than formate or ethanol and the product — 6-phosphogluconate — may complicate the workup in some circumstances. The procedure using ethanol as a starting material has the advantages of a readily available reducing agent and an innocuous product. However, two relatively expensive enzymes are required, and it is applicable only to NAD.

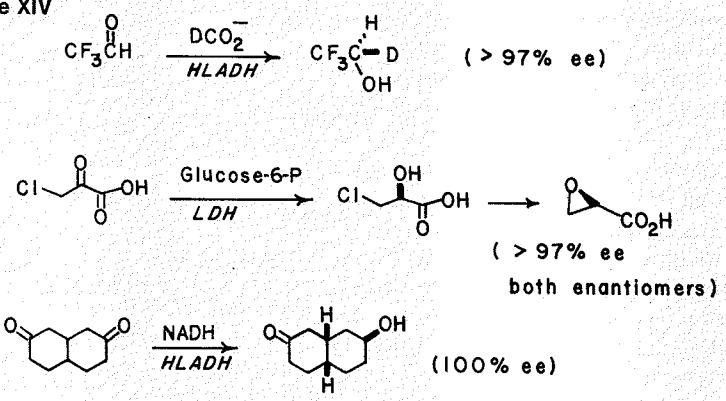
Scheme XII



Scheme XIII



Scheme XIV



In our laboratory, all of these three systems are commonly used. Scheme XIV gives examples of applications of the reducing regeneration systems to typical problems in organic synthesis.[48,50,51]

Multistep syntheses involving cofactors. The examples given so far have been primarily those involving one-step transformations of simple molecules. An important use of synthetic enzymology is the construction of schemes involving multiple, coupled enzymatic steps and which carry out complex syntheses. Scheme XV outlines the conversion of glucose to ribulose-1,5-diphosphate;[28] Scheme XVI illustrates the synthesis of lactosamine.[52] These represent the most complex syntheses which are practical at this time. The synthesis of ribulose-1,5-diphosphate is a particularly interesting example. This substance is important as a substrate in studies of the enzymology of

ribulose diphosphate carboxylase, the critical enzyme in carbon dioxide fixation in plants. The characteristics of the molecule are such that development of a practical synthesis based on conventional chemical transformations seems improbable. The enzymatic route, despite its apparent complexity, has been carried out on a scale yielding several-hundred-gram quantities of ribulose diphosphate. The synthesis of lactosamine, however, represents the first successful step in a program utilizing the Leloir pathway enzymes for carbohydrate synthesis.

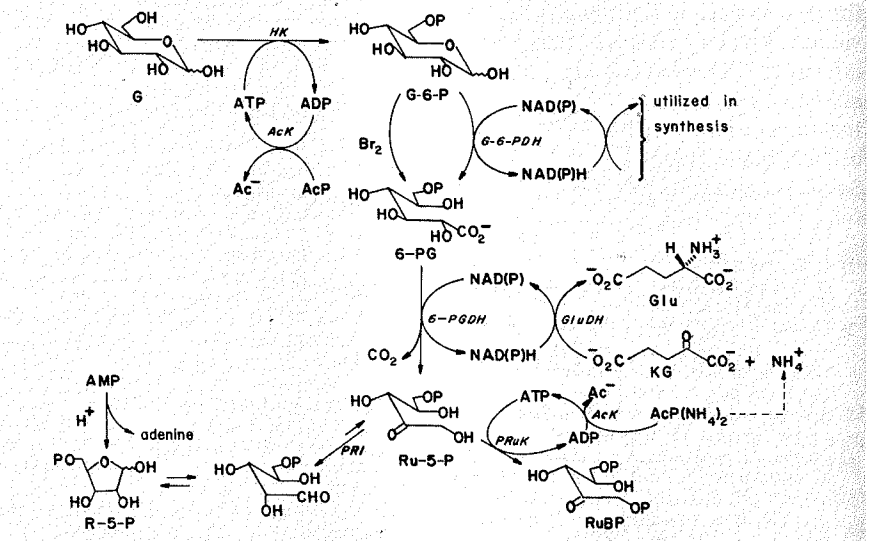
Summary. For what types of reactions should organic chemists consider enzymes as catalysts? The range of applications in the preceding treatise suggests that syntheses involving (or producing) sugars, chiral substances, polysaccharides, amino acids,[53] proteins, nucleic acids, and intermediates in major metabolic pathways are all plausible candidates for enzymatic reactions. Enantioselective or regioselective hydrolyses of esters and amides,[54] and selective oxidation of alcohols to ketones or reduction of ketones to alcohols are also plausible candidates for current enzymology. Reactions for which enzymology is not applicable are those which produce water-insoluble hydrocarbons and related species and those in which the principal technical problem centers on the construction of carbon-carbon bonds.

THE FUTURE: MORE COMPLEX SYNTHESES

A very large number of enzymes are known. The majority of the enzymes which so far have been exploited in synthesis are those which are commercially available and inexpensive. This group of enzymes is now accessible to all synthetic organic chemists at the modest cost of learning the experimental techniques required to assay, manipulate, and immobilize them. An enormously larger number of catalytic activities are available to those willing to carry out simple isolations (often from commercially available cell sources such as yeast, animal and plant tissue, or *E. coli*) and small-scale fermentations. Quantities of enzymes are often now limiting, but it is important to remember that, in principle, virtually any enzyme can be made in quantity using recombinant DNA techniques if the demand for that enzyme justifies the effort.

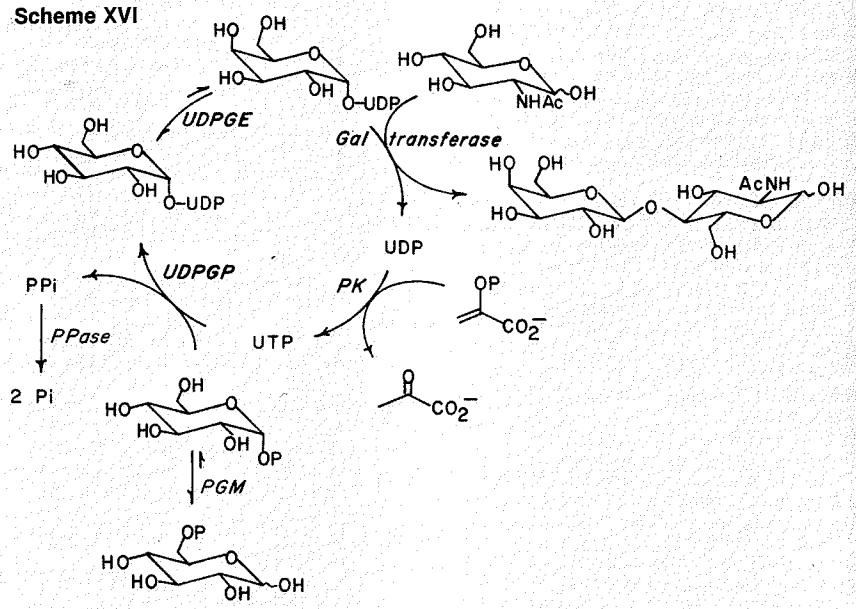
Many of the most exciting areas of biology and pharmacology — immunology, neurobiology, endocrinology, molecular genetics, membrane biology — are becoming more molecular. Plant and insect biology are also being explored actively at the molecular level. An increasing number of applications which will depend upon the availability of biological substrates will arise in these areas. Synthetic enzymology will play an important role in the synthesis of these substances. The compounds to which it is best applied — water-soluble biological molecules or molecules analogous to biological molecules, especially carbohydrates, nucleic acids, lipids, and proteins — are those for which conventional "abiological" chemistry has not (yet) developed satisfactory synthetic methods. Applied enzymology will thus complement conventional chemistry on the one hand, and more biological synthetic techniques (fermentation, recombinant DNA technology, and tissue culture) on the other. This entire group of biologically derived synthetic techniques will represent an important part of organic synthesis in the future, and an essential set of techniques for those who wish to work at the boundary between molecular biology and biochemistry or medicinal chemistry.

Scheme XV



Abbreviations: HK, hexokinase; AcK, acetate kinase; G-6-PDH, glucose-6-phosphate dehydrogenase; 6-PGDH, 6-phosphogluconate dehydrogenase; GluDH, glutamic dehydrogenase; PRuK, phosphoribulokinase; PRI, phosphoriboisomerase.

Scheme XVI



Abbreviations: Gal transferase, galactosyltransferase; PK, pyruvate kinase; PGM, phosphoglucomutase; UDPGP, UDP-glucose pyrophosphorylase; UDPGE, UDP-glucose epimerase; PPase, inorganic pyrophosphatase.

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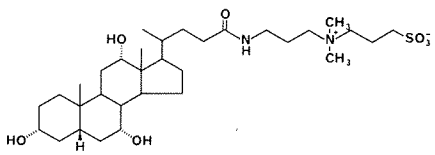
His research interests include biocatalysts in organic synthesis, protein chemistry and biochemistry, carbohydrate and peptide syntheses, and immunology.

Membrane Protein Solubilizing Agent



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