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The use of enzymes as catalysts for synthesis in medicinal chemistry: chiral synthons and carbohydrates

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INTRODUCTION

Enzymes are slowly being accepted as catalysts useful in medicinal chemistry (1-3). Present applications range from relatively large-scale processes (conversion of penicillin G to 6-APA (4), synthesis of amino acids (5)) to analytical and research applications at a very small scale. Medicinal chemistry is increasingly facing problems in the synthesis and synthetic modification of classes of substances that present difficult targets for classical synthetic chemistry: nucleotides and related substances, proteins, polypeptides, and sugars. Techniques drawn from biology, including enzymology and fermentation using microorganisms or higher cells, are uniquely suited for these types of problems.

Where can enzymes best be used as catalysts in practical medicinal synthesis?

- Preparation of synthons, especially chiral synthons, for use in assembly of stereochemically complex drugs.
- Preparation of specific compounds that are intermediates in metabolism, and analogs of these intermediates.
- Preparation of small quantities of materials for use in research biochemistry and pharmacology.
- Modification of complex substances (especially proteins, polypeptides, polysaccharides and oligosaccharides) isolated from natural sources or prepared by fermentation.

Enzymes have, of course, several intrinsic liabilities as catalytic entities. They are often difficult to obtain in quantity, and may be expensive. They are intrinsically fragile, and only a small number withstand exposure to elevated temperatures (>70 °C) (6) or organic solvents (7,8). Most classes of enzymes function best in aqueous solution, pH ≈ 7 and room temperature: many substrates of interest in medicinal chemistry are insoluble or otherwise difficult to manipulate under these conditions. Enzymatic reactors have special practical problems associated with their operation, such as control of microbial contamination. Nonetheless, it is increasingly evident that enzymes can be used quite generally for certain types of organic syntheses. Their use, however, will be reserved for synthetic problems that cannot be addressed adequately using more classical synthetic methodology.

Given the identification of a problem that seems possible as an application of enzymology, there is an hierarchy of questions that should be considered in evaluating the practicality of this application.

1) Is an enzyme known that effects the transformation of interest? If such an enzyme is known, or if an enzyme is known that shows activity on a substrate similar to that of interest, this substance provides an obvious starting point for a program in catalyst development. Otherwise, it may be necessary to start from the beginning with a program designed to identify an appropriate enzyme by screening microbial sources. This type of program can, with luck, be relatively straightforward, but is generally outside the scope and interest of an organic synthetic laboratory.

2) Do the enzymes of interest have high specific activity? The specific activity of an enzyme is its catalytic activity, normally in units of micromoles of substrate transformed per minute per milligram of enzyme. The specific activity is a critically important parameter in considering enzymes as catalysts for large-scale synthesis, since it is the factor that most directly correlates with reactor productivity and reactor size. Certain classes of enzymes-for example, the cytochromes P_{450}-have very interesting catalytic activities (9). Many of these activities are associated, however, with enzymes having such low specific activities that it is difficult to imagine ever preparing a finite quantity of product using them. The specific activity of an enzyme being considered for use as a catalyst should be determined early in the program. If it is too low to be practical, there is, in general, little that can be done other than to search for new enzymes. The issue of reactor productivity is, of course, of less concern for laboratory-scale synthesis than for production.

3) Does the proposed synthesis require one enzyme or multiple cooperating enzymes (either in series or in parallel)? Current technology in enzyme-catalyzed synthesis can tolerate systems containing up to

perhaps 10 cooperating enzymes (10,11). In fact, one of the strengths of enzymology as a branch of catalysis is that, because most enzymes operate optimally under very similar reaction conditions, it is possible to assemble multi-catalyst systems. It is, nonetheless, the case that the complexity of a system increases markedly with the number of enzymes involved. A particular problem concerns systems in which the multiple cooperating enzymes have different relative stabilities. Under these conditions, maintaining stable operation of the system can be very difficult unless there are methods for monitoring the concentrations of critical intermediates during the synthesis. In general, the fewer the enzymes required to effect a given transformation, the simpler and more practical the system.

4) Does the proposed synthesis require cofactor recycling? The development of schemes for recycling the nucleoside triphosphate (especially ATP) (12,13) and the nicotinamide cofactors (14-19) have progressed remarkably in the last several years. Flavin- and pyridoxal phosphate-based systems do not require recycling. Recycling of other important cofactors-CoASH (20), SAM (21), PAPS (22) - is presently more difficult or impractical. The requirement for, *e.g.*, ATP and NAD in a proposed synthesis is no longer a serious barrier to practicality, although it does, of course, complicate the catalytic system.

5) Are special circumstances associated with the enzymes suggesting low stability or other problems? Enzyme stability remains a problem (8). In general, the use of enzymes that are associated with membranes *in vivo*, or of enzymes involving strongly oxidizing intermediates, is still difficult or impossible.

We note that for large-scale synthesis the availability of an enzyme is not as serious a consideration as it once was. Recombinant DNA technology has progressed sufficiently so that it is possible (and often relatively speaking straightforward) to clone the gene coding for many enzymes. Thus, if a potential enzyme-catalyzed process is sufficiently important to warrant the effort, recombinant technology can be applied to the production of critical enzymes with attractive economics. For bench-scale work, the cost of rare enzymes remains a significant problem.

In this paper, we discuss a number of aspects of enzymatic catalysis as applied to two types of problems representative of those encountered in modern synthetic medicinal chemistry: the preparation of chiral synthons (23,24) and the synthesis of carbohydrates (25,26). These two applications of enzymes as catalysts illustrate many of the principles and techniques of enzyme-catalyzed synthesis. We, Jones, and others have recently prepared reviews of relevant literature (1,2). An OSTP report helps to place enzymatic synthesis in the broader perspective of

biotechnology (27). This paper emphasizes work with which we are most familiar: that is, our own.

CHIRAL SYNTHONS

Several factors have focused attention on the importance of chiral synthons in medicinal chemistry. The first concern is the increasing structural complexity of many targets in modern medicinal chemistry. Biological activity in a mixture of diastereometric compounds is usually concentrated in one or a few diastereomers. It is inefficient, uneconomical, and perhaps unsafe to prepare and use an active compound as one component of a mixture of compounds having different biological activities. The second concern is based on regulatory and economic issues. Increasingly, the diastereometric components in a proposed new drug must be considered as separate entities in proving safety and efficacy. The cost of testing for a mixture of diastereomers is greater than that of a single pure compound and strongly favors enantiospecific and diastereospecific syntheses. Moreover, of course, undesiderable biological activities can be associated with undesired enantiomers and diastereomers.

The enantioselective and diastereoselective synthesis of complex molecules is an area of high current activity throughout organic synthesis. One approach is centered on the development of efficient procedures for the preparation of chiral synthons to be incorporated into complex target molecules. The standards of the field are such that enantiomeric purities must be high (>95% enantiomeric excess, %ee) in order to be useful. Although it is difficult yet to make generalizations, it seems that the two major catalytic technologies showing promise for enantioselective synthesis are those based on enantioselective synthesis using transition metal complexes (for example, asymmetric hydrogenation (28) and asymmetric epoxidation (29), and biological methods. Methods based on disposable or recyclable chiral auxiliaries (30) are of course also being actively developed, but are not considered here. Biological procedures, when applicable, seem to be the more practical catalytic strategies, in part because they offer, in appropriate cases, high enantioselectivity. The generality of biological routes is however, often more limited than that of transition metal-based routes. A major current research activity in enzyme-catalyzed organic synthesis is the identification of enzymatic systems in which enantioselectivity is high and the exploration of the breadth of application of these systems. Good examples include lipases, (31), aldolases (32) (the non-cofactor requiring systems) and glycerol kinases (33), alcohol dehydrogenases (19), lactate dehydrogenases (34) (the cofactor-requiring systems); all of these enzymes are discussed below.

Non-cofactor requiring systems (lipases and aldolases)

Lipases are a broad group of enzymes that hydrolyze ester linkages. Most are active only at the interface between water and an organic liquid, and are thus uniquely attractive for reactions involving water-insoluble substrates. An example of a useful enzyme-catalyzed kinetic resolution is that of glycidyl butyrate (35). This procedure for the preparation of a very useful C_3 synthon has been explored extensively and is now in commercial production. The enantioselectivity of this hydrolysis is high.

Lipases have the attractive feature that they exhibit very broad substrate specificity. Are hydrolyses of substrates other than glycidyl butyrate also highly enantioselective? Briefly, the answer to this question is that the enantioselectivity of lipase-catalyzed hydrolyses is highly dependent upon the structure of the substrate. A number of substrates can be hydrolyzed with enantiomeric excesses in the range of 50-90%, and a smaller number with enantioselectivities from 90 to >95% (36). There is presently no model for the active site of lipase that is useful in predicting the enantioselectivity of hydrolysis of a new substrate. Enantiomeric excesses below 90% are, as indicated previously, not very useful synthetically. We are trying to determine if the enantioselectivity of a lipase-catalyzed hydrolysis can be improved by manipulations of reaction conditions (pH, temperature, added surfactants, added cosolvents). We have relied heavily on statistically-designed experiments (37) to guide this program: Statistical design is particularly useful in optimizing properties in which the variables interact with one another. Figure 1 summarizes a typical 3 x 3 block focusing on temperature, pH and added organic cosolvent (used primarily to prevent freezing of the aqueous phase at low temperature) (38). The parameter E is a conversion-independent measure of enantioselectivity. Briefly, a value of $E \geq 12$ is required for the %ee to be greater than 90% in the remaining product at a 60% conversion of racemic substrate (38).

The conclusion from this work is an encouraging one. It *is* possible to significantly improve the enantiomeric excess of a compound generated by kinetic hydrolysis by carrying out that hydrolysis under «unnatural» conditions: that means, under conditions clearly distinct from the biological norm of pH 7, room temperature. In the particular instance summarized in Figure 1, the change is not major, but it is sufficiently large so that it can make the difference between a practical and an impractical preparation of an enantiomerically enriched synthon. The generality of this conclusion is unclear, but our current expectation is that enzyme-catalyzed processes will be amenable to some degree of manipulation by altering conditions from those encountered *in vivo*.

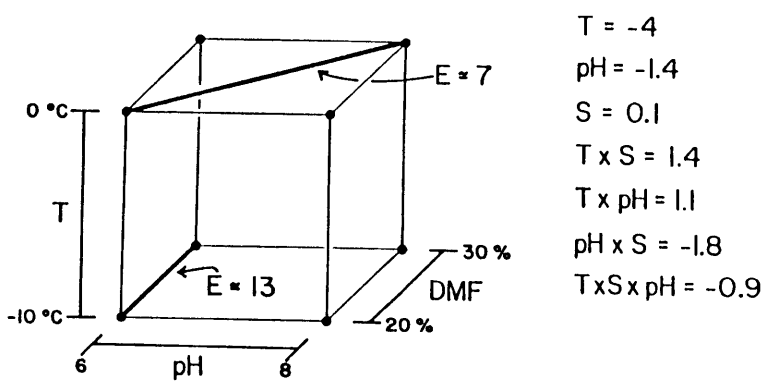

Figure 1. Optimization of enantiomeric excess: 2^3 factorial design.

The manipulation of enantioselectivity through changes in reaction conditions should be applicable to a number of other enzymes useful in kinetic resolution, especially proteases. The utility of lipases and related species (for example, the very useful cholesterol esterase) would be improved if the manipulation of reaction conditions were based on a firm understanding of reaction mechanism. Unfortunately, there is presently very little known about the details of the mechanism of action of lipases, or concerning the shape of their active site. The obvious importance of this class of enzymes to organic synthesis warrants more detailed mechanistic understanding of their catalytic activity.

A second class of enzymes that shows promise for utility in diastereoselective synthesis is the aldolases. The readily available rabbit muscle enzyme catalyzes the aldol condensation of dihydroxyacetone phosphate with aldehydes (32). The reaction is specific for dihydroxyacetone and a small number of very close structural analogs; a variety of aldehyde structures are accepted (39). The reaction catalyzed by rabbit muscle enzyme seems to be diastereospecific. Thus, one can form two chiral centers diastereospecifically in a single step, using unprotected starting materials. We believe this reaction has very broad potential for utility in the synthesis of carbohydrates and related structures. Figure 2 illustrates the application of rabbit muscle enzyme to a synthesis of two 6-deoxyhexoses, used as intermediates in the synthesis of the flavoring agent Furaneol (40). Aldolase is now attracting wide attention, and has been applied to syntheses of a wide variety of substances, including rare, unnatural, and isotopically labeled sugars (41-42).

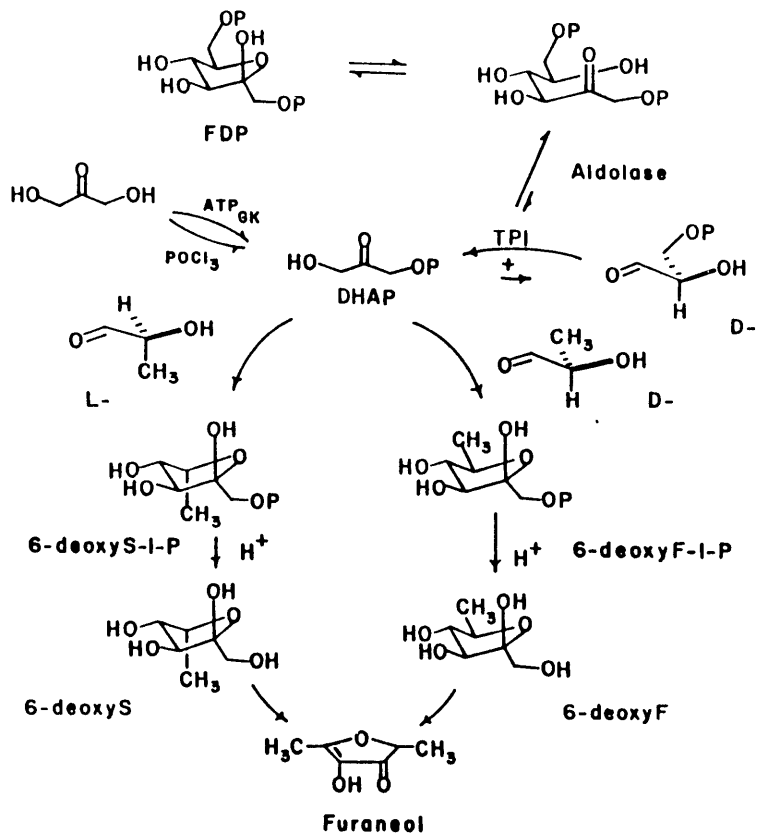

Figure 2. Enzyme-catalyzed synthesis of 6-deoxyhexoses and furaneol: F = fructose.

Cofactor-requiring systems (glycerol kinases, lactate dehydrogenases and alcohol dehydrogenases)

The most important cofactors from the vantage of enzyme-catalyzed synthesis are those that associate with and dissociate from the enzyme active site as an obligatory part of the mechanism. The nucleoside triphosphates (especially ATP), the nicotinamide cofactors (NAD(P)H) and CoASH are important examples. Other cofactors-especially the flavins and pyridoxal phosphate-remain associated with the enzyme active site and are regenerated *in situ*; these cofactors pose relatively little problem in applied enzymology.

The major concerns in cofactor-requiring enzymology are economic. The cofactors are too expensive to be used stoichiometrically in most reactions; they must, therefore, be regenerated *in situ*. The regenera-

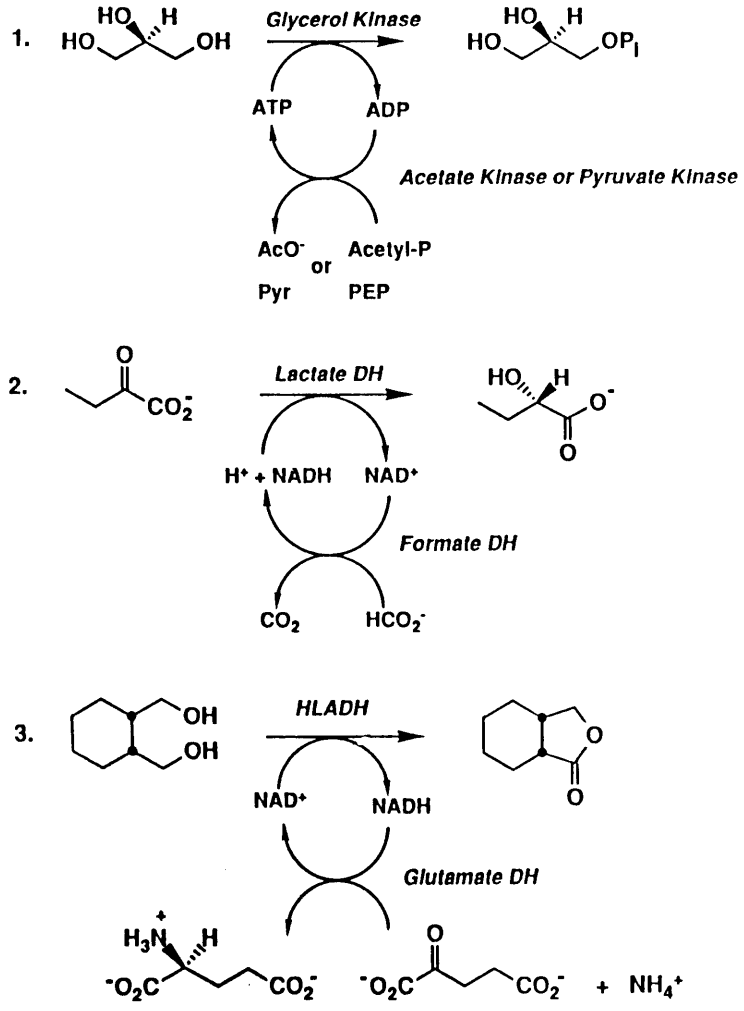

Figure 3. Enzyme-catalyzed syntheses requiring the regeneration of cofactors.

tion processes must be based on readily available reagents and enzymes and must reduce the cost of the cofactor to an acceptable level. Good regeneration schemes are available for the nucleoside triphosphates (12) and the nicotinamide cofactors (14,17); syntheses generating kilograms of products based on these cofactors (13,16) are now practical. The most useful systems for regeneration of ATP, NAD, and NADH are illustrated in Figure 3.

The enantiospecific conversion of glycerol to *sn*-glycerol-3-phosphate

has been described (43). This synthesis can be carried out using either phosphoenol pyruvate or acetyl phosphate as the ultimate phosphorylating agent; a practical synthesis for each is available (44). The asymmetric reduction of 2-ketobutyrate by lactate dehydrogenase illustrates a typical enantioselective reduction (45). The most attractive cofactor regeneration system for NADH is one based on formate and formate dehydrogenase (15). It is important to recognize that several formate dehydrogenases are commercially available and vary widely in their stability (46). The kinetic resolution by oxidation of a *meso-cis*-cyclopentane-1,2-diol (17) illustrates both the most practical of the regeneration systems for use with NAD and an important generic problem-product inhibition-encountered in this type of enzymology. A number of regeneration systems have been proposed and demonstrated for the regeneration of NAD from NADH, but only the systems based on FMN/0_2 (19) and various dyes (47) have been used with any regularity. The dye-based systems are impractical for large-scale applications, primarily because their kinetics are slow and very large quantities of dye can be required. The regeneration system based on 2-ketoglutarate/glutamate dehydrogenase is a very practical one and suffers only from occasional difficulties: separation of the components of the regeneration system from the products of the reaction; incomplete equilibrium conversions, reflecting the fact that 2-ketoglutarate is not a very strong oxidizing agent; reagent instability, cost and complexity. A more important constraint than the deficiencies of the cofactor regeneration system in asymmetric oxidations is, however, that imposed by product inhibition (7). Many alcohol dehydrogenases are inhibited by their products in the oxidizing direction. Those enzymes that operate by an ordered bi-bi mechanism often exhibit non-competitive or incompetitive inhibition. This type of inhibition cannot be overcome simply by increasing the concentration of the starting material; in fact, since the enzyme ceases to work at a characteristic concentration of product, high concentrations of reagents typically cause difficulties in product isolation and are not desirable. No satisfactory protocol for overcoming this type of inhibition has been developed, other than procedures relying on the removal of the product during reaction, either by extraction or by subsequent reaction.

The cofactor regeneration systems illustrated in Figure 3 represent practical but not universally applicable solutions to the problem of cofactor cost. Their economics varies with the system. It is, nonetheless, necessary to consider the kinetic characteristics of the enzymes to which the cofactor systems are coupled in order to evaluate the practicality of a proposed synthesis.

APPLICATIONS OF ENZYMATIC CATALYSIS TO THE SYNTHESIS OF CARBOHYDRATES

Synthesis and synthetic modification of carbohydrates is one of the more difficult problems in classical synthetic chemistry (25). The available methods of synthesis produce impressive results, but the extreme difficulties in protection, deprotection, separation, and characterization make this technology applicable only with difficulty to the synthesis of large quantities of materials. We are examining a number of enzyme-based routes to carbohydrates. The generality of these methods is still much smaller than that of the chemical methods. As routes to specific compounds they may, however, have substantial advantages in terms of practicality and ease of operation relative to classical synthetic procedures.

We have previously mentioned the use of aldolases as catalysts for the synthesis of monosaccharides. A number of different types of enzymes having aldolase activity are available (2) and only a few have been explored for synthetic applicability. We believe, however, that this class of enzymes will, as a group, permit the diastereoselective synthesis of a very broad range of carbohydrates and carbohydrate-like substances.

A separate problem in sugar chemistry is the formation of glycosidic linkages. *In vivo*, oligosaccharides (and, often, polysaccharides) are biosynthesized by the Leloir pathway: that is, through processes involving nucleoside diphosphate sugars and nucleoside monophosphate sugars as the reactive intermediates (48). Is it possible to use these Leloir pathway biosyntheses for *in vitro* preparations of oligosaccharides?

The answer to this question is «yes,» but the applicability is still limited. Figure 4 outlines a synthesis of the disaccharide lactosamine (49). A substantial number of cooperating enzymes are required to conduct this synthesis. Nonetheless, all of the components can be assembled and operate smoothly together. Although this synthesis demonstrates the preparation of only one nucleoside diphosphate sugar, this type of process has been extended successfully to tri- and tetrasaccharides (50). With sufficient effort, there is no doubt that one could provide routes to the 10-15 nucleoside di- and monophosphate sugars required to synthesize the majority of the oligo- and polysaccarides encountered in nature. The principal issue hindering the development of this type of synthesis is the limited availability and stability of the glycosyl transferase enzymes responsible for formation of glycosidic bonds and, to a lesser extent, of the glycosyl transferases responsible for certain trimming and modification processes sometimes encountered (particularly in the biosynthesis of the oligosaccharide moities of glycoproteins (51)). The glycosyl transferases in mammalian systems are often membrane-bound and

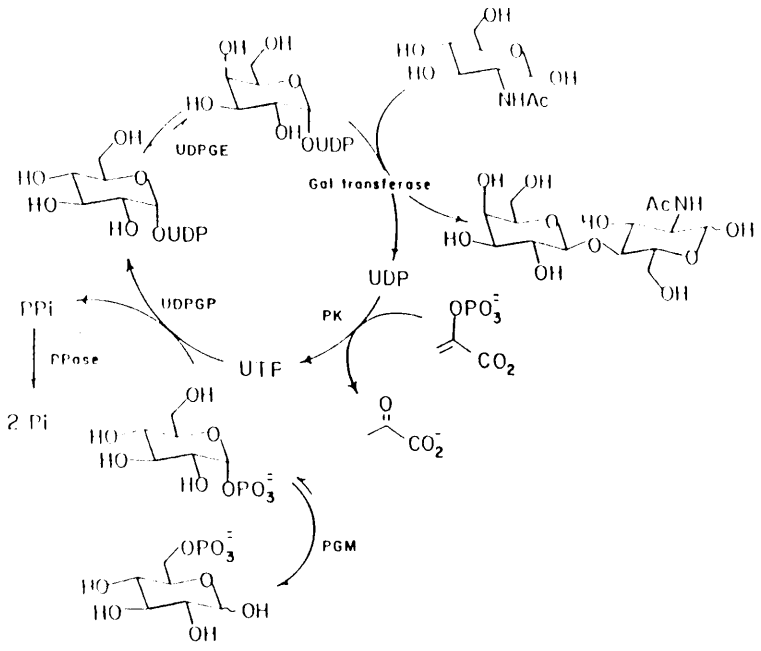

Figure 4. Synthesis of N-acetylactosamine.

associated with the Golgi apparatus. Few of these enzymes have been isolated in any finite quantity, and indications are that they are not, as a class, particularly stable. If these enzymes were more readily available (and it may, in fact, ultimately be possible to make important members of this class using recombinant techniques), Leloir pathway biosynthesis of oligosaccharides appears to be a practical proposition. In the absence of such enzymes, Leloir pathway enzymatic synthesis remains a laboratory exercise for oligosaccharides. For particular polysaccharides it may, nonetheless, prove more attractive.

CONCLUSIONS

This paper emphasizes the application of enzymes as catalysts for enantioselective or enantiospecific synthesis of chiral synthons and as catalysts useful in the synthesis of sugars and, perhaps, of oligo- and polysaccharides. We note here, only in passing, the applicability and importance of enzymes to other classes of problems-for example, those in nucleotides and proteins-relevant to medicinal chemistry. For example, synthesis and modification of nucleic acids depends very heavily on enzymes at critical steps (52); the Novo process for semisynthetic human

insulin illustrates an important example of the application of enzymes in modification of proteins (53). This latter type of application of enzymes-to protein modification-may become an important one, but its development is heavily dependent on the course of current activities in recombinant DNA technology. Present experience is that production of proteins present in mammalian cells using microorganisms as production vehicles is not as straightforward as might be hoped. Levels of expression are often low; the proteins sometimes show toxicity toward the production organism; they appear to be susceptible to proteolysis by endogenous proteases; posttranslational modification is often incomplete or inappropriate. Of the many solutions being considered for these problems, two represent relevant extremes. In one extreme, the technology would emphasize production in mammalian cells. This mammalian cell culture technology is intrinsically more expensive and complex than that based on microorganisms, but given the expense and difficulty in isolation and purification of proteins (especially for human use) it is not obvious that the cost of the production system itself should be considered a determining factor in choosing between mammalian tissue culture and microbial fermentation for protein production. The mammalian tissue culture systems would, presumably, carry out the posttranslational modification of the proteins in a more satisfactory way than the microorganisms. It might, however, be preferable to stay with microbial production systems and rely on enzymes *ex vivo* to accomplish the required posttranslational modifications. In one case, applied enzymology may become a critical part of protein production by recombinant methods; in the second, it would play a relatively minor role. It is presently impossible to predict the resolution of this issue.

In summary, it is clear that classical methods for organic synthesis are limited in their usefulness and practicality when applied to classes of compounds such as sugars, proteins, nucleic acids and chiral synthons of central interest in modern medicinal and pharmacological chemistry. Enzymatic systems, either *in vivo* or *in vitro*, may offer the best solutions to certain synthetic problems posed by these classes of compounds. Certainly, the medicinal chemist familiar with the techniques of enzymology and fermentation will enjoy an advantage over one familiar only with classical synthetic techniques in his or her ability to tackle important problems in medicinal chemistry without regard to constraints imposed by compound type.

ACKNOWLEDGEMENTS

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