Applications of cell-free enzymes in organic synthesis

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Abstract. Enzymes are a potentially useful addition to the group of catalysts used in practical organic synthesis for the preparation of biologically relevant substances. This paper outlines certain of the problems still limiting the use of enzymic catalysts, and illustrates successful applications to the synthesis of complex molecules: phosphoribosyl pyrophosphate, ribulose 1,5-bisphosphate and lactosamine. Certain problems peculiar to enzymic catalysis—product inhibition and specific activity—are discussed.

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This paper outlines current and potential applications of cell-free enzymes as catalysts in organic synthesis, and indicates the unsolved problems that limit these applications at present. Why should synthetic organic chemists now consider enzymes for their potential as catalysts in organic synthesis when they have not felt compelled to do so previously? The most compelling reason for re-examining the potential of enzymology in synthetic organic chemistry stems from certain new classes of problems which are now important in medicinal and biological chemistry. A substantial amount of biological chemistry related to human health has recently focused on the interaction of protein receptors and enzymes with their substrates. The biological activity of most drugs is due to their ability to interfere with these protein–substrate interactions, either by competition at the binding site or by some other type of reaction, and rational strategies for analysing and manipulating these interactions may offer new approaches to drug design. The focus on the molecular chemistry of protein receptors and enzyme active sites is leading medicinal chemistry into studies involving a range of substrates—polypeptides, proteins, sugars and sugar derivatives—which are difficult to manipulate with classical synthetic methodology. A major reason for considering the potential utility of biologically based synthetic techniques—genetic engineering, enzymology, tissue culture, advanced fermentation—is thus to try to develop practical preparative routes to these classes of compounds.

Why have biological methods not been more commonly used in organic synthesis in the past? First, because synthetic organic chemists are unfamiliar with many of the experimental techniques required by these methods. Second, because biological methodology has not been very useful for transformations of the predominantly hydrocarbon-based, water-insoluble substances which are the targets of much current activity in organic synthesis. Third, because biological methodology has not met a criterion of strategy considered important in university research. Much of current academic organic synthesis is concerned less with the development of practical routes to specific compounds than with the use of organic synthesis to explore new types of reactions and to improve understanding of molecular reactivity. Often, in practice, the complex multi-step organic synthetic schemes now developed in academic organic synthesis serve to demonstrate a relatively small subset of interesting new transformations. Biological methodology is much more a process-oriented set of techniques than it is a methodology convenient for flexible use in small-scale, exploratory laboratory synthesis. As such, it may provide direct, efficient routes from reagents to products, but it does not afford the same opportunity to explore new and chemically interesting transformations as do classical organic synthetic strategies.

The potential of enzymology to provide new catalysts for use in practical organic synthesis has, nonetheless, been recognized for many years, and this field has been the subject of research by a substantial number of groups (Whitesides & Wong 1983, Findeis & Whitesides 1984). The technical problems that have been believed to limit the development and application of enzyme methodology have shifted over time. The current status of some of these concerns is summarized in the following list:

Enzyme availability. The cost of production of enzymes is now commonly considered not to be limiting. If the enzymes cannot be obtained from naturally occurring sources, they can, according to current biological dogma, be obtained in large quantities from genetically engineered cells (Rastetter 1983). It is now practical to consider using for synthesis enzymes that were once thought to be too expensive and too scarce to be practical, provided that the importance of the problem justifies the development of an inexpensive method for their production.

Enzyme immobilization. Immobilization is a technique of established utility in applied enzymology for two reasons: first, it facilitates the recovery of the enzymes from reaction mixtures, and permits their economical reuse; second, by mechanisms which are still not fully understood, it often dramatically stabilizes the enzymes. A great deal of effort has been devoted to developing methods for immobilizing enzymes, and further methods are not needed at

this time for applications at laboratory or small pilot-plant scale (Chibata 1978, Pollak et al 1980). For large-scale processes, more durable supports and higher-yield immobilization procedures could still profitably be developed.

Applicability; substrate selectivity. Many reactants and products of interest in organic synthesis do not occur naturally. Can enzymes be used efficiently to effect transformations of *unnatural* starting materials? This question is one focus of current activity in applied enzymology, and its answer is still unclear. It is evident that many enzymes will accept a much broader range of substrates than those occurring naturally. The limitations to substrate structure must, however, be worked out for each individual enzyme. Current research in this area will outline empirical rules to permit some level of prediction of the acceptability of new substrates for many of the enzymes that are useful in organic synthesis.

In the future, it may be possible to modify substrate selectivity by site-specific mutagenesis, or other techniques of recombinant DNA methodology. These techniques are, however, now only beginning to be applied to the modification of enzymic activities.

Enzyme stabilization. The stability of enzymes under the reaction conditions encountered during organic synthesis is sometimes unacceptably low. A number of empirical procedures have been developed for stabilizing enzymes, but these are not always satisfactory. The basis for deactivation of enzymes is only partially understood, and undoubtedly varies from instance to instance (Klibanov 1983). Stabilization of enzymes is an area in which research is being conducted (although probably not as much nor at as basic a scientific level as would be desirable). There is hope that it may be possible to obtain substantial improvements in stability through site-specific mutagenesis, or by developing thermophilic and halophilic organisms as sources of enzymes.

Cofactor regeneration. The major cofactors of interest in organic synthesis are ATP and the nicotinamide cofactors, NAD(P)(H). The problem of regenerating ATP *in situ* is essentially solved, as discussed below. Regeneration of the nicotinamide cofactors is also practical on a scale adequate for most laboratory uses. Large-scale methodology for NAD(P)(H) regeneration is still being developed.

Product inhibition. Product inhibition is a problem which has been largely ignored in most work on small-scale applications of enzymology in organic synthesis. It becomes, in fact, important only in large-scale reactions. The basis of the phenomenon is a reduction in the activity of the enzyme at some concentration of product, due either to competitive occupancy of the enzyme

active site by the product, or to a conformational change induced in the enzyme by association with the product. If enzyme activity is strongly reduced at low concentrations of product, it may then be impractical to obtain solutions containing high concentrations of product. When product inhibition is severe, it is necessary to work with dilute solutions of reagents and products, to suffer with large reaction volumes, and to isolate products from the resulting dilute solutions. There is presently no general strategy for circumventing product inhibition, other than to remove product continuously as it is formed (often impractical).

Process design. As techniques for enzymic synthesis on a laboratory scale become more successful, the problems of scaling up these reactions to produce large quantities of product become more significant. Scale-up involves a substantial number of important problems in biochemical engineering, centering on separation and purification of products, reactor design and control, and related issues.

Examples of enzyme-catalysed organic synthesis

This paper concentrates on uses of enzymes as catalysts in reactions relevant to the synthesis of relatively complex molecules—those containing chiral centres, requiring cofactors or multi-enzyme systems for synthesis, or having high degrees of structural complexity. Applications of enzymes to semi-commodity chemicals (amino acids, starch and glucose derivatives, fatty acids, food acidulants, penicillin derivatives) are not discussed.

Enzymes not requiring cofactors: lipase and aldolase

Enzymes that do not require cofactor regeneration were the first class to be important in enzyme-catalysed organic synthesis. They will probably continue to occupy this position for some time. Many of these enzymes are commercially available, stable and inexpensive. They are, in general, more convenient to use than cofactor-requiring enzymes.

Lipase is a useful example from this class of enzymes. It and other esterases have been exploited previously in chiral synthesis based on enantioselective hydrolysis. We have begun a programme to use lipase (from hog pancreas) to prepare optically active epoxy alcohols (equation 1) by kinetic resolution of enantiomeric mixtures (Ladner & Whitesides 1984).

(1)

This reaction is interesting for two reasons. First, it provides a practical synthetic route to a group of compounds which are valuable synthetic intermediates. Second, it permits a comparison of the enzymic technology for preparing chiral synthons with the useful transition metal-based procedure for asymmetric epoxidation. Our studies are not yet complete, and all points of the comparison between enzymic and metal-catalysed processes have not been explored. It is, however, clear that enzymology has an important place in this type of transformation. When applicable, it is substantially easier to carry out than the transition metal-catalysed chemistry. For example, the transformation summarized in equation (1) represents the most practical procedure presently available for the preparation of glycidol. Enzymic methodology can be easily used to prepare enantiomerically pure (>95% enantiomeric excess) glycidol in hundred-gram quantities. The transition metal-catalysed route to enantiomerically enriched glycidol is considerably more cumbersome, for chemically uninteresting but practically important reasons (for example, the water solubility of the product, and the requirement for separating water-soluble product from the large quantities of tartaric acid used as the chiral influence).

The areas in which lipase-catalysed transformations will ultimately prove superior to other methods for preparing chiral substrates are not yet defined, and one important uncertainty concerning the utility of this and other esterases involves substrate acceptability. Current evidence suggests that a broader range of compounds can be prepared by transition metal-catalysed reactions than by enzyme-catalysed hydrolyses. There are, however, many different lipases from many different sources, and very few of these have been explored for utility in organic synthesis. Further, many other esterases are available. The range of chiral compounds that could be prepared if all the available enantioselective esterases were used remains to be established, but is probably large. A second annoying problem with esterases concerns the reproducibility of enzyme-catalysed hydrolyses. Certain enzymes consist of mixtures of isozymes. Ageing, autoxidation or species of origin may influence the structure and activity of other enzymes. Defining the composition of synthetically useful enzymes in sufficient detail that they can be used reproducibly by synthetic organic chemists remains a problem.

Aldolase represents a second example of an enzyme that is useful in organic synthesis and that does not require cofactors. The aldolases are a broadly distributed class of enzymes which effect a substantial number of important transformations in intermediary metabolism. Only a few of these enzymes have been examined for substrate specificity and possible utility as catalysts in organic synthesis. We have concentrated our own work on the commercially available aldolase from rabbit muscle. This enzyme catalyses the condensation of dihydroxyacetone phosphate with a number of aldehydes. The enzyme

shows narrow specificity for dihydroxyacetone phosphate; it will, however, accept a wide range of aldehydes and will form a carbon–carbon bond between the two reactants with well-defined stereochemistry by directed aldol condensation. An example illustrating the use of aldolase in organic synthesis is the conversion of dihydroxyacetone phosphate (easily prepared by several routes, including phosphorylation of dihydroxyacetone by ATP and hexokinase with *in situ* ATP regeneration) to glucose (Fig. 1). We have found this

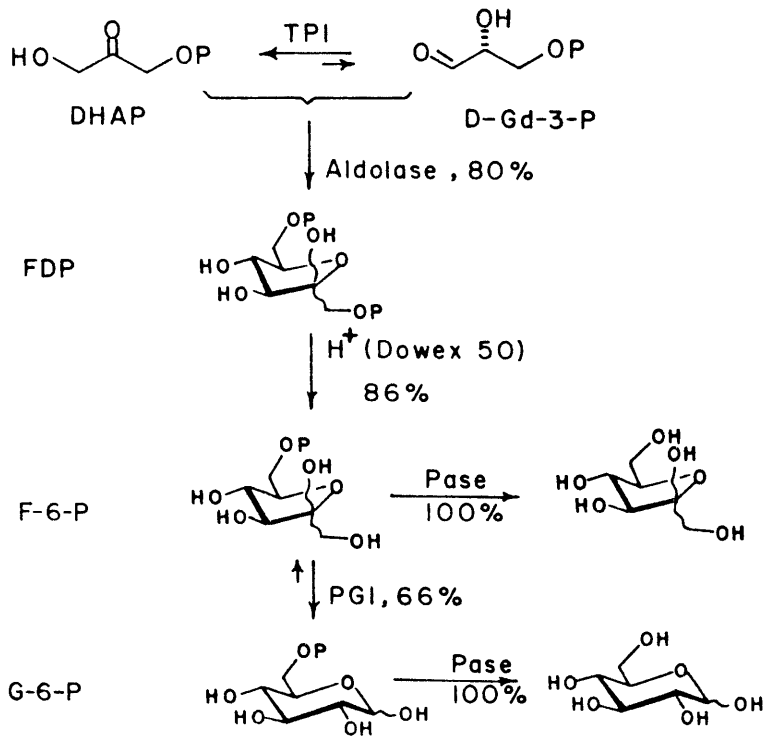

FIG. 1. Conversion of dihydroxyacetone phosphate to glucose based on aldolase. Abbreviations: DHAP, dihydroxyacetone phosphate; D-Gd-3-P, D-glyceraldehyde 3-phosphate; FDP, fructose 1,6-bisphosphate; F-6-P, fructose 6-phosphate; G-6-P, glucose 6-phosphate; TPI, triosephosphate isomerase; Pase, acid phosphatase; PGI, glucosephosphate isomerase.

transformation useful for the synthesis of labelled fructose and glucose derivatives, and for the preparation of unnatural sugars and sugar analogues (Wong & Whitesides 1983a,b).

Enzymes requiring in situ cofactor regeneration

The enzymes that require separate systems for cofactor regeneration and that are presently useful in organic synthesis work with ATP or NAD(P)(H). Other potentially useful cofactor-requiring enzymes use flavins, pyridoxal phosphate, porphyrins and other cofactors, but most of these cofactors are self-regenerating and require no separate regeneration system. Both ATP and the nicotinamide cofactors are sufficiently expensive that they cannot be used stoichiometrically, but must be regenerated *in situ* if they are to constitute an economically acceptable component of a practical synthetic scheme. The problem of ATP regeneration has now been solved (Fig. 2).

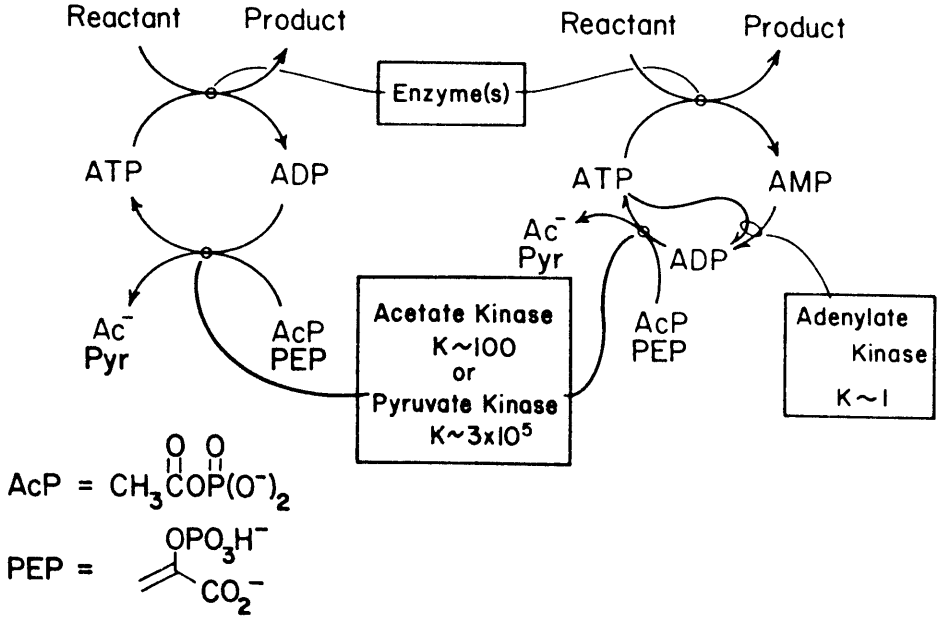

FIG. 2. Scheme for organic synthesis based on ATP-requiring enzymes coupled with *in situ* ATP regeneration. K, equilibrium constant.

Two systems have proved useful: one is based on acetyl phosphate as the ultimate phosphorylating agent, the second on phosphoenolpyruvate (PEP). Both these materials are readily prepared. In practice, schemes based on acetyl phosphate have the advantage that this material is very readily synthesized in multi-mole quantities (Crans & Whitesides 1983). The disadvantage of acetyl phosphate is that it is slightly unstable in aqueous solution, and that it is only a modestly strong phosphate donor. PEP is a very

strong phosphate donor, and very stable in aqueous solution; it is, however, somewhat complicated to prepare. We routinely use both procedures to prepare multi-mole quantities of products.

Both the procedures summarized in Fig. 2 represent development and optimization of general approaches known in enzymology for many years. Even within this well-explored area of ATP regeneration, there is, however, opportunity for the development of new techniques. For example, we have circumvented the problem of the low phosphate donor potential of acetyl phosphate by the preparation of an analogue, methoxycarbonyl phosphate ($CH_3OCOOPO_3H^-$). This substance is as strong a phosphate donor as PEP, but is as easily prepared as acetyl phosphate. It has the additional attractive characteristic that the substance formed by phosphate transfer to ATP—methyl carbonate—decomposes spontaneously to methanol and carbon dioxide. Neither of these compounds causes problems in the work-up of the enzyme-catalysed reaction mixture, nor is either an inhibitor of the kinases used to catalyse phosphate transfer to ADP. The modest acetate inhibition observed for acetate kinase is thus circumvented. Methoxycarbonyl phosphate is not an ideal reagent; its particular weakness is a relatively limited stability in aqueous solution (half-life = 0.3 h at 25°C, pH 7). Nonetheless, it represents an example of the successful design of an unnatural substrate for an enzyme to solve specific problems present with the natural substrate.

Regeneration of the nicotinamide cofactors continues to cause problems. For reductive regeneration [$NAD(P)^+$ to NAD(P)H] the best presently available procedure uses formate and formate dehydrogenase (Shaked & Whitesides 1980, Kula et al 1982). This procedure works well on the multi-mole scale, and is being actively developed by several groups. In the oxidizing direction [NAD(P)H to $NAD(P)^+$], the best strategies are still unclear. All the procedures now available have certain disadvantages. The most commonly used in our laboratory is that based on conversion of 2-oxoglutarate to glutamic acid (see below). Enzyme-catalysed syntheses based on *in situ* regeneration of oxidized nicotinamide cofactors will require further research and development before they become practical for very large-scale synthesis (Jones & Taylor 1976, Takemura & Jones 1983, Hartley et al 1983).

Multi-step syntheses

I illustrate the use of these enzymic techniques with three examples. Fig. 3 shows a synthesis of phosphoribosyl pyrophosphate (PRPP) from ribose 5-phosphate (Gross et al 1983). PRPP is an important intermediate in nucleotide biosynthesis. It is unstable in solution, and impractical to make by

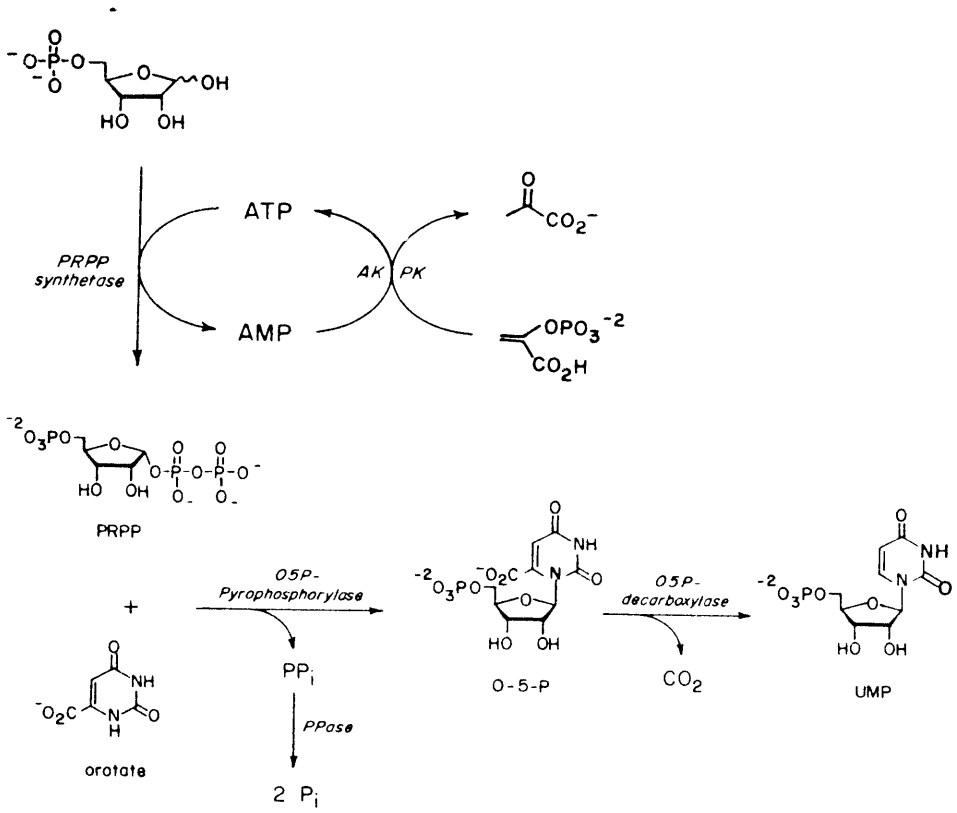

FIG. 3. Synthesis of phosphoribosyl pyrophosphate (PRPP). Abbreviations: AK, acetate kinase; PK, pyruvate kinase; O-5-P, orotidine 5'-phosphate; PPase, inorganic pyrophosphatase.

conventional chemical or fermentation methods. Its synthesis with cell-free enzymes is straightforward. In this synthesis, the only step presenting any difficulty was that of isolating PRPP synthetase. Manipulation of the enzyme, once isolated, and the operations involved in the enzyme-catalysed synthesis were straightforward.

Figure 4 outlines two syntheses of ribulose diphosphate (RuDP; ribulose 1,5-bisphosphate) (Wong et al 1982a). This substance is the substrate for ribulose bisphosphate carboxylase, the enzyme which fixes CO_2 in plants. The poor availability of RuDP has hindered research on this important enzymic system for some years. The syntheses outlined are straightforward, and RuDP has been prepared in 100g quantities by these procedures. An interesting technical detail in this synthesis concerns the regeneration of NAD^+ from NADH during the oxidation and decarboxylation of 6-phospho-

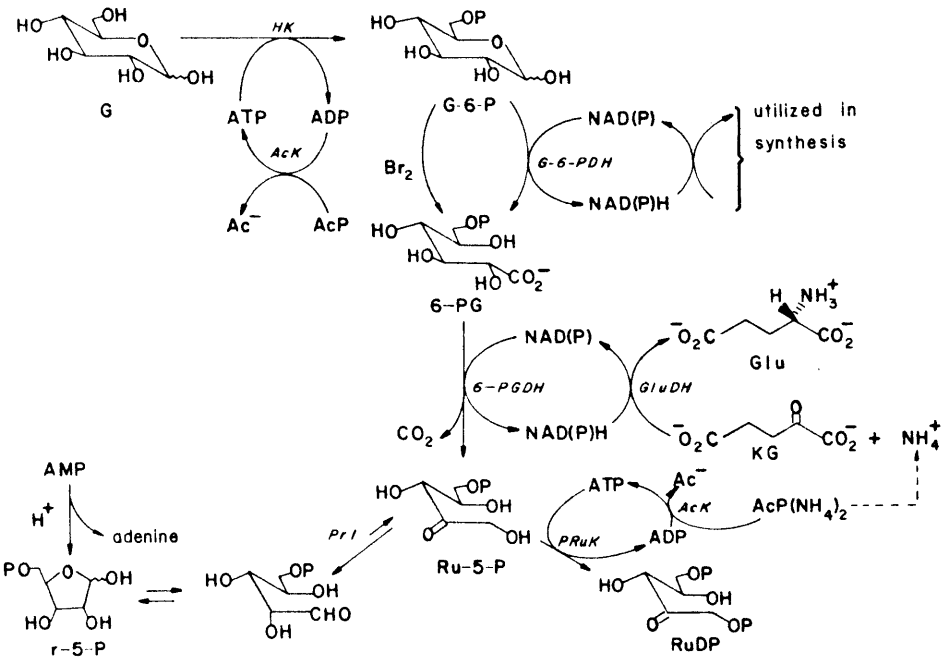

FIG. 4. Syntheses of ribulose 1,5-bisphosphate (RuDP). Abbreviations: G, glucose; HK, hexokinase; AcK, acetate kinase; AcP, acetyl phosphate; G-6-P, glucose 6-phosphate; G-6-PDH, glucose-6-phosphate dehydrogenase; 6-PG, 6-phosphogluconate; 6-PGDH, 6-phosphogluconate dehydrogenase; GluDH, glutamate dehydrogenase; Glu, glutamate; KG, ketoglutarate (2-oxoglutarate); Ru-5-P, ribulose 5-phosphate; PRuK, phosphoribulokinase; r-5-P, ribose 5-phosphate; Prl, ribosephosphate isomerase.

gluconate (6-PG) to ribulose 5-phosphate (Ru-5-P). This reaction was coupled with the phosphorylation of ribulose 5-phosphate to RuDP. The enzyme responsible for this phosphorylation (phosphoribulokinase) is sensitive to autoxidation, and this sensitivity precluded the use of oxygen-based regeneration systems for the nicotinamide cofactors. We have developed a nicotinamide-cofactor regeneration system based on the conversion of 2-oxoglutarate to glutamic acid by glutamate dehydrogenase (GluDH) to solve this specific problem of the oxygen sensitivity of phosphoribulokinase. We find, moreover, that this regeneration system is conveniently and broadly applicable to the problem of NAD^+ regeneration during enzyme-catalysed synthesis. In fact, its most serious deficiency is that it requires a separation of unused 2-oxoglutarate, glutamic acid and product at the conclusion of the reaction; in some instances this separation is straightforward but in others it is complicated.

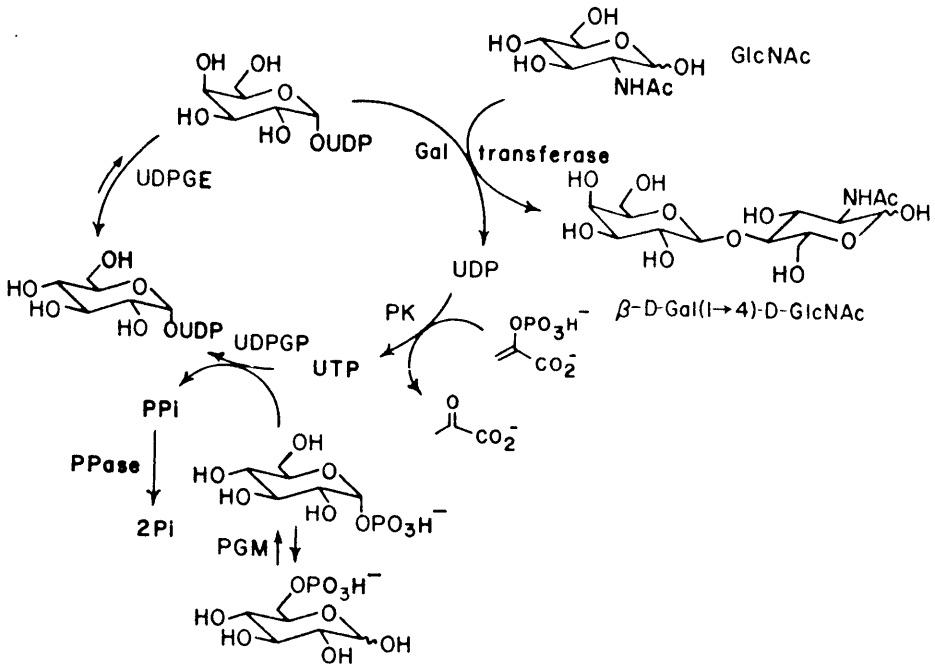

FIG. 5. Synthesis of lactosamine from glucose 6-phosphate and *N*-acetylglucosamine. Abbreviations: PGM, phosphoglucomutase; PPase, inorganic pyrophosphatase; UDPGP, UDPglucose pyrophosphorylase; UDPGE, UDPglucose 4-epimerase; Gal, galactose; PK, pyruvate kinase.

Figure 5 outlines a synthesis of the disaccharide lactosamine [β-D-gal(1→4)D-GlcNAc] (Wong et al 1982b). This substance is interesting as a core disaccharide in glycoproteins, and its preparation provides an example of the use of the Leloir pathway enzymes in organic synthesis. This synthesis requires the regeneration of UTP from UDP *in situ*, and the conversion of UTP to UDPglucose. Despite its complexity, this synthesis is relatively uncomplicated experimentally. In fact, one of its major interests is its demonstration of one of the important strategic differences between enzyme-catalysed synthesis and synthesis based on more traditional types of catalysts. The synthesis of lactosamine involves 11 enzymes operating cooperatively in the same reaction mixture. This type of multi-catalyst, cooperative system is entirely practical to develop with enzymes, because the operating conditions optimal for most enzymic reactions are, by nature, similar. It is, in practice, difficult to find a group of non-enzymic catalysts all of which can be made to operate usefully and efficiently under similar reaction conditions.

Conclusions

Are enzymes practical as catalysts for organic synthesis? This question is one of considerable practical importance for synthetic chemistry. It is evident that the future of organic synthesis will require techniques for the preparation and modification of sugars, nucleic acids, polypeptides, and a variety of other water-soluble, biologically active materials. If enzymology (and, of course, other biologically based methods) provide the best or, at least, competitive techniques for preparing certain of these classes of compounds, then enzymology, fermentation, microbiology and tissue culture should probably be included in the formal education of aspiring synthetic organic chemists. If enzymic routes are less practical than those derived from classical synthetic methodology, then this dislocation of the educational and research system need not be accomplished. My conclusion is that enzymes represent a valuable class of catalysts for use in organic synthesis, and are, in fact, a class whose use may be *required* for the synthesis of many biologically active materials. I emphasize again that applied enzymology is fundamentally a process technology. It is frequently less convenient to develop an enzymic route to a substance required in milligram quantities than to carry through a classical synthesis, no matter how cumbersome. When large quantities of that material are required, however, it will be worth the effort to try to find appropriate enzymes and to develop conditions for their isolation and use.

Where will enzymes be used? Certain areas of application are already clear. Several amino acids are already prepared on a large scale by enzymic methods. The conversion of cornstarch to high-fructose corn syrup is an enzymic process, and demonstrates that enzymology may provide the best route to a commodity chemical. The uses of enzymes in the coupling and modification of sugars, oligosaccharides, and polysaccharides will, I am confident, be important. A variety of transformations effected in food chemistry may be best carried out enzymically. Certain problems in the synthesis of fine chemicals and medicinals will certainly be best handled by enzymology. In particular, many of the most practical routes to optically active compounds now seem to be those based on enzymes. In fact, somewhat to the surprise of many organic chemists, enzymes presently seem more generally useful than chiral metal-based catalysts in the preparation of chiral synthons. Regioselective transformations of complex molecules can certainly benefit from enzymic catalysis. Many problems in protein chemistry (especially the modification of protein products obtained from recombinant DNA technology) can probably be solved only by enzymic methods. Nucleic-acid chemistry already relies heavily on enzymic procedures.

Once a decision has been taken to consider using enzymes to effect a given transformation, how should the synthetic chemist analyse the problems of

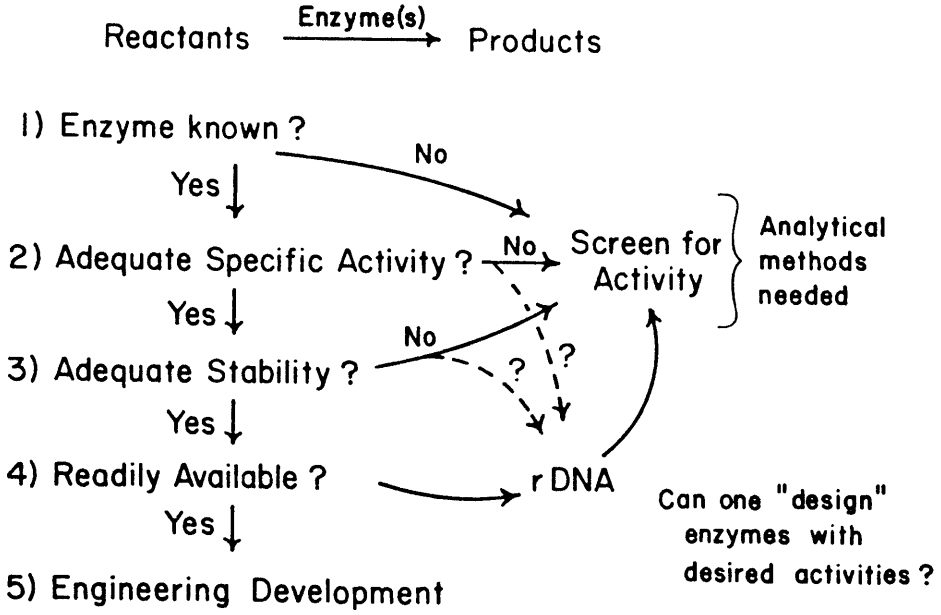

FIG. 6. Decisions important in designing an enzyme-based synthesis; rDNA, recombinant DNA.

selecting an appropriate enzyme and deciding if its properties are such that it represents a realistic candidate for the transformations being considered? Fig. 6 outlines the process. The first step—finding if one or more appropriate enzymes are known—is often difficult. There is, unfortunately, no reliable catalogue of enzymes classified by synthetic use. Further, the nomenclature used to name enzymes is extremely unhelpful for guessing potential new uses of a particular enzyme in organic synthesis.

Because there is still so little literature relevant to the use of enzymes in organic synthesis, my best advice to synthetic chemists interested in using enzymes in synthetic applications, but also in avoiding becoming enzymologists, is to strike up an acquaintance with one or more biochemists, to examine catalogues for commercially available enzymes, and to read a standard enzymology textbook. With that background, one can be prepared to guess whether there is available an enzyme capable of carrying out a given transformation. An astonishing number of enzymes are already commercially available, but many more are known. In the event that it is necessary to carry out the isolation of an enzyme, one should remember that pure enzymes are not necessarily required for synthetic applications, and that the purifications may be relatively simple. Further, biochemical isolations are considerably less difficult (although more tedious) than one might expect.

Given the availability of an enzyme which catalyses a transformation of interest, the next step—evaluating the specific activity of this enzyme—is a crucial but often neglected one. The specific activity of an enzyme is a measure of its catalytic activity: that is, the quantity of product formed per milligram of enzyme per unit of time. If this quantity is too low (and certain enzymes have intrinsically very low catalytic activities) it may be that the quantity of enzyme required to prepare the required quantity of the product of interest is unrealistically large (even given the best efforts of genetic engineering). In that event, it is necessary either to find a more active enzyme, or to abandon the project to classical chemical methodology. If the specific activity of the enzyme *is* high enough to be practical, the remaining steps are (relatively) straightforward. One can usually find procedures which will stabilize an enzyme adequately for bench-scale synthesis. Immobilization is usually straightforward, and when it is unsuccessful it may be perfectly practical to use the enzyme in homogeneous solution. Most of the cofactors required for use in organic enzymology can now be obtained or regenerated, if required. On the basis of this information, it is usually possible to construct a laboratory-scale synthesis. Whether this synthesis can be easily scaled up to a full, multi-kilogram process is, of course, another matter.

Acknowledgements

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DISCUSSION

Jones: There are so many methods now for coenzyme recycling that it is difficult for the uninitiated to decide which are the good ones. You have identified the formate dehydrogenase method as the best for converting NAD^+ back to NADH, and Kula has obviously come to the same conclusion (Kula et al 1982). How many moles of product per hour will that system give, and for how many hours? How many coenzyme recycles can you expect before it dies?

Whitesides: We repeatedly get 1000–10000 real turnovers of cofactor, depending on the circumstances. The problem is that formate dehydrogenase is an enzyme of low specific activity, less than 20 units/mg protein (1 unit = 1 μmol product formed/min). It would be nice to have a formate dehydrogenase of higher specific activity and one that was commercially available at lower cost.

Cornforth: Have you calculated the cost of attrition of the cofactor compared with the cost of attrition of the enzyme in a reaction which requires regeneration of the nicotinamide nucleotide?

Whitesides: Economic analysis is critically important. In some cases the cofactor is the expensive component and in others the enzyme is. There may be uncertainty about the economics, but there are now enough cofactor regeneration systems available to solve the problems being faced in laboratory-scale synthesis–one or other system will work. The real question is, what important compounds does one want to make? For example, some of the elegant chiral reductions that have been done with alcohol dehydrogenase and other redox enzymes will probably ultimately be done instead by non-enzymic reduction and then hydrolysis, perhaps with a lipase. Where is the dividing line? Where will the cofactor systems with their particular characteristics be preferable to hydrolytic systems or something simpler? The ultimate drive in this, as in much process chemistry, will be towards the simplest possible system. The hydrolytic systems are certainly that.

Battersby: Yes, but the reductive approach is extremely valuable for labelling methylene groups with H-isotopes, so converting what would otherwise be a prochiral centre into a chiral one. The reductive approach can't be used on a huge scale but for this rather specific application it is very useful.

Mosbach: For some reactions, it may be better to use isolated enzymes than whole cells, for example for steroid hydroxylation which requires NADPH, or for steroid dehydrogenation. Provided a stable enzyme system and a good recycling method are found, the capacity per unit volume is superior to that of a cell system, unless the cells are genetically engineered. However, for genetically engineered cells you would need to engineer into the system enzymes that could continuously produce coenzymes and/or regenerate them.

Whitesides: I would wager that in our lifetime steroid hydroxylations will be done entirely by fermentation. The benzene diol synthesis that we heard about earlier (see p 71) is a genuinely new result. It should be possible to develop more systems like that, and perhaps improve some of the current fermentations if necessary. I can't think of any reason for taking such systems out of the cells and trying to put together all the machinery to make them work *in vitro*. If I were directing research, I would certainly choose fermentation for oxidative transformations of this type.

Mosbach: With penicillin amidase, there is a choice between isolating the enzyme or using whole cells. Some companies prefer isolated enzymes because they get better control and a higher capacity per unit volume. If it is possible to get a stable hydroxylase, for example for 11β- or 11α-hydroxylation, I think the isolated enzyme method will be preferable to the conventional fermentation process.

Jones: The specific activity of the hydroxylases is dismal. Hydrolytic enzymes are 10^4 to 10^6 times as active as the hydroxylases.

Whitesides: A typical specific activity for a fungal hydroxylase is about 0.001 units/mg, which is not impressive. Further, the coupling efficiency is low: 99%