Chapter VII

LARGE-SCALE ORGANIC SYNTHESIS USING CELL-FREE ENZYMES

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1 INTRODUCTION

The effectiveness with which cell-free enzymes can be applied as catalysts in practical organic synthetic procedures depends both on scale and reaction type. Cell-free enzyme preparations have been widely employed in a variety of types of small-scale syntheses, and used in several large-scale degradative transformations not involving cofactors. The partial hydrolysis of milk proteins by rennin and of soil proteins by protease-containing detergents, and the chillproofing of beer by papain, provide examples of the latter class. Enzymes are not commonly utilized in large-scale reactions that require cofactors or that carry out reactions more complex than hydrolysis or isomerization. The border between the regions of synthesis in which enzymic catalysis has been successfully used and those in which it has not has been defined primarily by economics [1]. In syntheses of small quantities of materials for research or laboratory use, feasibility and convenience are usually more important than enzyme cost: for example, the fact that it is possible to join deoxyribonucleotide chains enzymatically, but difficult

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or impossible to join them by conventional synthetic chemical techniques, renders the economics of the production and utilization of the appropriate ligase moot [2]. In the synthesis of large quantities of a substance, by contrast, economics becomes an important consideration, and the factors defining the cost of a reaction sequence requiring cell-free enzymic catalysis—production and isolation of the enzyme, enzyme operating lifetime and specific activity, cofactor replacement, reagents, supports, and equipment peculiar to enzymic reactors—may be such that alternative synthetic procedures utilizing chemical and/or microbiological transformations may appear more attractive than a cell-free enzymic route.

The purpose of this chapter is to illustrate briefly examples or representative reactions for the large-scale synthesis or processing of organic materials in which cell-free enzymic synthesis presently plays a role, to discuss considerations important to the successful design of new processes (particularly processes involving transformations more complex than those now employed), and to outline criteria useful in identifying situations in which cell-free enzymic catalysis might offer advantages over other synthetic strategies. Certain of the considerations important in using enzymic catalysis in large-scale applications -- particularly those involving the isolation and stabilization of enzymes-are central to their use in synthesis at any scale, although the requirements for successful large-scale utilization are almost always more stringent than those for small-scale reactions; others-cofactor regeneration, reactor design-are unique to large-scale processes. We are concerned here with the question of the application of enzymes to the synthesis of relatively small quantities of materials: this issue is discussed in detail elsewhere in this book (Part I, Chapters IV and VI). Rather, we will consider the distinct problems that must be solved in synthesizing large quantities (arbitrarily defined as ranging from one or several moles to commercial scale) of a substance using enzymic catalysis. Incorporation of the techniques required to solve these problems into the body of synthetic organic chemistry requires a blending of chemical procedures with those from enzymology and microbiology. These biological techniques are unfamiliar to most synthetic organic chemists. Nonetheless, despite the fact that synthesis requiring cell-free enzymic catalysis may be technically more complex than more familiar types of chemical or fermentation syntheses, the selectivity offered by enzymic catalysis. particularly for the many biologically, medicinally, and nutritionally important classes of materials containing carbohydrates, peptides, and nucleotides, cannot, in many cases, be equaled by conventional organic or fermentation synthesis.

2 SYNTHESES UTILIZING CELL-FREE ENZYMES: EXAMPLES

Existing processes that utilize enzymes for the production of discrete chemical compounds on a large scale have in common several features that simplify their operation: little or no enzyme purification is required; immobilization is

achieved simply using inexpensive supports; the reactions catalyzed are uncomplicated [3]. Reactions utilizing L-amino acid acylase, penicillin amidase, and aspartase illustrate types of enzymic catalysis that are actually used in largescale work and that provide examples of types of enzymic transformations that can be applied relatively easily in chemical synthesis.

L-Amino acid acylase is used in a process for resolving racemic amino acids [4]. A racemic mixture of D- and L-N-acylamino acid (obtained by chemical synthesis) is passed through a column containing enzyme immobilized by adsorption on DEAE cellulose. The L-enantiomer is hydrolyzed to free amino acid. Separation of the D-acylamino acid from the free L-amino acid is easily accomplished, and the D-acylamino acid is subsequently epimerized and returned to the column.



Penicillin amidase is used to remove the side chain of penicillin G in certain procedures for the production of 6-aminopenicillanic acid (6-APA) [5]. This



material is in turn used as a starting material for a variety of semisynthetic penicillins. Aspartase catalyzes the addition of ammonia to fumarate ion, yielding optically active L-aspartic acid [6, 7].



The most successful technique used to immobilize aspartase is one that, although not formally "cell-free," offers great promise for many types of enzymecatalyzed syntheses. The enzyme is not isolated. Instead, an intact microorganism containing the enzyme is incubated with acrylamide and appropriate crosslinking agent and radical initiator, and the entire suspension polymerized [6]. The resulting polyacrylamide gel containing immobilized, polymer-filled cells, is broken up, and transferred to a column. The resulting preparation shows good stability and activity. (A 10×100 -cm column, containing packed cells of *Escherichia coli* in polyacrylamide, can generate about 700 g of aspartic acid per hour) [7].

An obvious advantage of this technique is its simplicity: no enzyme isolation is required. Less obvious advantages also apparently attend it: the enzyme immobilized by polymerization of the intact organism is very stable. It is possible, although not established, that this stability reflects permeation of the cell interior by acrylamide, with subsequent polymerization to a polyacrylamide gel throughout the cell matrix. This gel must have pores sufficiently small that any proteases present at the time of polymerization, or released by the polymerization, are unable to attack the aspartase. In addition, the environment provided for the enzyme—its natural matrix with a low concentration of polyacrylamide may discourage conformational changes that lower its activity.

This procedure, in its present form, is not applicable to a number of classes of enzyme-catalyzed reactions. Any process that requires diffusion of enzymes or other macromolecules (e.g., ribosomal protein synthesis) will be hindered or prohibited by the polymer matrix. The cell wall of the microorganism might in principle also restrict diffusion of certain substrates or products to or from the interior of the cell. The condition of the cell wall in these preparations is not known. The catalytic activity of the aspartase preparation, is however, increased by an activation procedure involving, inter alia, heating: this activation may serve to disrupt cell membranes sufficiently to permit free passage of substrates and products. Acrylamide reacts rapidly with several functional groups of enzymes. particularly the -SH moiety of cysteine and other mercaptan-containing molecules, and is itself capable of modifying or destroying the activity of certain enzymes on very short contact [8]. Polyacrylamide gels have relatively poor flow and mechanical characteristics, and are not ideal for processes requiring the processing of very large volumes of solution. Finally, the acceptability to many of the world's food and drug administration bodies of materials, intended for human use, prepared by a step involving immobilized whole cells is not entirely

clear. Nonetheless, for processes that involve relatively simple catalytic transformation of low-molecular-weight substrates, the immobilization of intact organisms offers a very practical and attractive method of utilizing enzymic catalysis.

These three examples are processes sufficiently developed to be used in commercial-scale synthesis. Many examples of smaller-scale syntheses also exist [9], and representative examples may be found in *Methods in Enzymology*, *Biochemical Syntheses*, and similar sources, as well as in other parts of this volume.

3 CONSIDERATIONS IN DEVELOPING NEW PROCESSES UTILIZING CELL-FREE ENZYMIC CATALYSIS

Five principal factors must be considered in designing and developing a practical synthesis based on enzymic catalysis: (1) the availability, ease of isolation, and purification of the enzyme(s) required; (2) the lifetime of the enzyme(s) under operating conditions and the efficacity of stabilization protocols; (3) the intrinsic activity of the enzyme(s), and the efficiency with which enzymic activities can be coupled in reactions requiring multiple enzyme-catalyzed steps; (4) the cofactors required for enzymic activity; and (5), the design of reactors compatible with the transformation to be achieved. We consider each of these subjects in turn.

Enzyme Isolation

Enzymes are, in principle, available from all living organisms. For synthetic work requiring large quantities of enzymes, certain types of sources are better than others. The most convenient sources are microorganisms. Yeasts, bacteria, and fungi can be grown in quantity using well-established fermentation techniques; alternatively, certain commonly used lines-particularly Baker's yeast and E. coli-are commercially available. Starter quantities of large numbers of standard cultures are available from the American Type Culture Collection. (See also Appendix 1, Part I for a list of other culture collections.) In addition. microorganisms offer an advantage over plant or animal sources that is particularly valuable when large quantities of enzymes are required; namely that selective mutation and genetic manipulation can often be used to develop strains that produce relatively large quantities of the enzymes of interest, and enzyme induction is easier to accomplish with microorganisms than with higher organisms. A final, significant advantage of microorganisms as enzyme sources is that the convenience with which they can be handled has, in the past, made them favorite subjects for mechanistic enzymology and biology. Thus, large quantities of data are already available on the preparation and properties of many interesting enzymes. Unfortunately, some of the microorganisms most commonly exploited as enzyme sources for mechanistic work, particularly Salmonella typhimurium, are sufficiently pathogenic to make their growth in quantity inconvenient or hazardous; alternative sources for these enzymes can, however, usually be found.

Plant and animal sources of enzymes are less convenient than microbial sources; they may, however, offer the only source of enzymes of interest. Plant sources, in particular, may become increasingly attractive as the techniques for plant tissue culture develop [10].

Isolation of enzymes for large-scale synthetic work presents economic problems not encountered in small-scale preparation. In general, enzyme isolation and purification is an expensive process, and the less manipulation required in reaching a useable preparation, the better. The least complex technique conceptually is the use of whole cells [6, 11-13]. Although this technique will certainly be widely exploited when applicable, it is not yet clear whether it can be employed for other than very simple transformations. Next least complex is to use the crude protein mixture obtained on breaking cells with a minimum of purification. Typically, autolysis, lysis with an added enzyme, or mechanical or osmotic disruption of cells yields a mixture of cell components containing, in addition to the enzyme(s) of interest, various proteases, nucleic acids, lipids, carbohydrates, and proteins that may bind or degrade the products or starting materials of the reaction of interest, the enzymes required to effect the desired transformations, or essential cofactors (Scheme I). Often, however, either no or surprisingly little further treatment is required to obtain useable preparations. Thus, if the enzyme of interest is thermally stable, brief heating may denature many of the other proteins present. Enzyme immobilization by adsorption, entrapment, or covalent attachment of crude preparations to supports may decrease the mobility of the proteases sufficiently that they become unimportant in determining enzymic lifetimes; alternatively, addition of protease inhibitors may decrease their activity (see below). Nucleic acids can be removed by precipitation (protamine sulfate, streptomycin) or enzymically degraded. Ammonium sulfate or organic solvent fractionation serves to remove some of the unwanted proteins from the crude preparation. These and related elementary purification techniques are described in a variety of sources [14, 15]. Alone, they may prove insufficient or unsatisfactory in many cases; the important point to realize, however, is that it is not necessarily required (or even desirable) to purify an enzyme to homogeneity for it to be useful in a synthetic procedure. and it is usually well worth the effort to examine the activity and stability of crude preparations.

Enzyme Stabilization

The stabilization of enzyme preparations is a topic of central importance in enzymic synthesis: even relatively inaccessible and expensive enzymes can, in principle, be used in a practical synthesis provided the lifetime of the enzyme under operating conditions is long. Unfortunately, in most instances, less is



Scheme 1. Representative Enzyme Isolation Procedure

known about mechanisms of the processes leading to losses in enzymic activity than about the mechanism of the enzymic activity itself. Consequently, enzyme stabilization is presently as much art as science. It is possible to point to a number of experimental protocols that are known to increase the stability of enzymes, and in some cases to rationalize these procedures on plausible mechanistic grounds. In any new case, however, the process of devising a set of experimental conditions resulting in good operating lifespans for an enzyme remains largely empirical [16].

Proteases

A major contributor to the loss of enzymic activity in crude enzyme-containing solutions (and in many "pure" preparations) is destruction of the enzyme by proteases. Proteases occur in a wide range of molecular weights, and operate over a range of values of pH [17-19]. The ability of proteases to persist through

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even careful chromatographic purification procedures [20-22] has led to an only partially joking feeling among enzymologists that for every protein there is an almost identically constituted protease. There are three effective strategies for dealing with protease contamination of an enzyme. First, the protease can be inhibited by addition of substances that irreversibly block or completely inhibit the protease active sites. Two broadly effective compounds for this purpose are PhCH₂SO₂F [23] (called phenyl methyl sulfonyl fluoride in biochemical circles) and diisopropylphosphofluoridate (DIFP). Both inhibit serine proteases (e.g., trypsin, chymotrypsin); the former is less toxic than the latter, which is also a very powerful acetyl cholinesterase inhibitor. Benzamidine and related materials are also useful in inhibiting proteases related to thrombin [24]. Protein protease inhibitors [25] are too specific to be useful for broad-spectrum protease inhibition. Second, immobilization of the enzyme preparation usually renders protease activity unimportant, since the solution protein-protein encounters that permit attack by the proteases are unimportant with immobilized enzymes [26]. Third, careful chromatography-particularly affinity chromatography-may reduce protease activity to insignificant levels.

Autoxidation: Oxidation-Reduction Buffering

Many enzymes require -SH or -S-S- moieties as essential structural or catalytic elements [8]. Both can be oxidized rapidly under conditions commonly encountered in enzymic reactors. Although the detailed mechanisms of these oxidations are not known, several of their qualitative characteristics have been identified. Thus, the initial oxidation of cysteine to cystine occurs more rapidly

$$\begin{array}{cccc} R-SH \\ R-SH \\ R-SH \end{array} \xrightarrow[R-S]{} R-S \\ R-$$

at high than at low pH, is strongly catalyzed by traces of transition metals [particularly Fe(III) and Cu(II)], and can be reversed with suitable reducing agents. The subsequent oxidation of cystine disulfide linkages (ultimately to cysteine sulfonic acid) is usually slower, but is not easily reversed. Autoxidation may also destroy proteins by routes not involving SH groups [27].

The significance of these observations for enzymes that are active in the fully reduced form (i.e., with functional cysteines but without important cystine groups) is clear: the enzyme should be stored and used under anaerobic conditions (a nitrogen or argon atmosphere); the reagents used should be free of transition-metal contaminants; a suitable reducing agent should be present in the solution to reverse any adventitious oxidation. The first condition is difficult to satisfy adequately; 1 μ 1 of air may contain enough oxygen to oxidize the cysteine moieties of 1 mg of protein. The second is also difficult to meet. Although buffers and reagents may be reasonably stripped of transition-metal ions by passing them through a resin containing SH groups before use, the enzyme itself usually cannot be. Since many proteins bind transition-metal ions strongly, it is often impractical to try to keep solutions containing enzymes free of transition metals. Thus, the major burden of protecting enzymes against deactivation by autoxidation usually falls on the reducing agent added to the solution.

The most popular and effective material for this purpose is dithiothreitol (Clelland's reagent, DTT), ordinarily added in concentrations approximately 1000 times greater than that of the enzyme [28]. DTT is unfortunately an expensive material, and the less expensive but less effective 2-mercaptoethanol is often used in its place.



The stabilization of enzymes that require an intact cystine disulfide linkage against oxidation is a problem that has not been satisfactorily solved. The presence in solution of a large excess of an added material containing a disulfide linkage should stabilize an essential disulfide in a protein, but the irreversibility of oxidation proceeding past the disulfide oxidation level presents obvious problems. The coupling of two or more enzyme systems operating at different oxidation levels has not been attempted.

Environment

A variety of solution variables are capable of influencing the stability of enzymes: pH, temperature; ionic strength, buffer composition, solvent character (and immobilization support, for immobilized enzymes), the nature of interfaces present in solution, and the nature and concentration of other solution components. Most of these variables are presumed to act by influencing the tertiary structure of the enzyme. No generalizations concerning most of these inter-

actions are presently possible: indeed, no generalizations may exist, since the relative importance of various factors will differ for different proteins. It is, however, worthwhile to question one assumption that is commonly tacit in much experimental work, namely, that most enzymes exist in the cell in a predominantly aqueous environment, and that, once removed from the cell, enzymes are likely to be most stable in water. There is little question that for enzymes that are clearly associated with membranes, the presence of lipids in solution may be essential for stability and activity [29]. At another extreme, extracellular enzymes probably are most stable in water. For the intermediate class of enzymes that exist in the interior of the cell, it is by no means clear what solution composition would be expected most closely to duplicate their native environment. The fluid portions of cells may contain high concentrations of many components other than water, and duplication in vitro of the cellular microenvironment of an enzyme, even in the uncommon event in which the physical location of the enzyme within the cell is known, cannot presently be carried out by design. Thus, many of the standard empirical tricks used to stabilize enzymes in solution may be effective by virtue of providing the type of mixed aqueous-nonaqueous environment found in the cell. In fact, it seems possible that investigation of the balance of hydrophilic and lipophilic character that is most effective in stabilizing an enzyme might provide an indirect approach to inferring the character of the cell interior.

Brief listing and comment on several of these tricks is worthwhile. Enzymes may denature at interfaces [30]. Interfacial denaturation may often be controlled by allowing an inexpensive, inert protein (commonly bovine serum albumin) to adsorb on the surface of glassware to be used with enzymes or by using polyethylene or Teflon rather than glass apparatus. Since interfacial denaturation of susceptible enzymes may also occur at the solution-air (or inert gas) interface, gases should not be bubbled through an enzyme solution, nor should stirring be so vigorous as to cause strong vortex formation. In controlling the pH and ionic strength of enzyme solutions, it is important to remember that buffer components [31] and salts used to adjust ionic strength influence many solution properties other than those of immediate concern. Thus, solutions containing, for example, phosphate buffer or tris buffer of the same pH do not have the same values of surface tensions, internal pressures, or many other parameters that might influence the conformations of enzymes. The subject of hydrophobic interactions involving proteins is discussed in greater detail in Chapter VIII of this volume and elsewhere [32-36]. Addition of glycerol, sorbitol, polyvinyl alcohol, or other neutral, hydrophilic polymers (often in high concentrations), stabilizes many enzymes [37-39]. In at least one instance, this stabilization has been correlated with a change in tertiary structure [39]. Polyelectrolytes may also strongly influence enzyme stability and activity [40]. The influence of detergents on enzyme stability has not been extensively investigated, and judicious choice of surface active agents will probably provide significant stabilization for enzymes not classified as "membrane bound" but still associated weakly with membranes, lipids, or hydrophobic portions of other proteins in vivo. Studies of the sensitivity of the activity of D-alanine carboxy-peptidase to detergent structure suggests that considerable care may be required to achieve the best balance of hydrophilic and lipophilic interactions and "structure-making" and "structure-breaking" properties for such stabilization [41, 42].

The storage stability of solutions of many enzymes is increased by the presence of substrates, cofactors, or products, and both stability and activity of enzymes may require metal ions [43]. The former characteristic may reflect either stabilization of tertiary structure or protection of the active site region from attack by oxygen, metal ions, or other solution components. The frequent requirement for significant concentrations of free Mg(II) or other main-group metals for activity places some limitation on the extent to which the activity of transition metal ions can be suppressed by adding chelating agents such as EDTA. The association constants between EDTA and Cu(II) and Mg(II) are about 10¹⁹ and 10⁹ mol/liter, respectively [44]. Thus, if the original concentration of Cu(II) in an enzyme solution is 10^{-4} M, addition of enough EDTA to reduce the concentration of uncomplexed Cu(II) to $10^{-12} M$ will have very little influence on the concentration of free Mg(II) $[(Mg(II))/(Mg(II) \cdot EDTA) = 10^2]$. Addition of enough more EDTA to reduce the Cu(II) concentration to 10^{-14} M will, however, significantly perturb the concentration of free Mg(II) [(Mg(II))/- $(Mg(II) \cdot EDTA) = 1$. Thus, there is a lower limit below which it is not practical to reduce the concentration of transition-metal ions in solution by chelation. Although this limit is low, catalysis of autoxidation of enzyme sulfhydryl groups by these metals might, in principle, still be significant. Further, the metal-EDTA complexes may be autoxidation catalysts in their own right. Thus, although addition of chelating agents should (and does) greatly decrease the rate of metal-catalyzed autoxidation, it may not completely eliminate it. In fact, the use of EDTA in solutions containing DTT may be superfluous, since the latter is probably a very strong chelating agent in its own right.

Enzymic Activity

The activity of an enzyme to be used in a synthetic reaction determines the amount of enzyme required to achieve synthesis of a given amount of product in a set time. For calibration, 1 mg of an enzyme having specific activity of about 700 international units (I. U.) will catalyze the formation of 1 mole of product per day, when operating at maximum velocity (a specific activity of 1 I. U. = 1 μ mol substrate transformed/(min) (mg) of protein at V_{max}) [45]. The specific activity of enzymes varies widely. Table 7.1 lists specific activities of enzymes catalyzing reactions representative of those that might be useful in organic

	Ref.	46	47	47	47	47
Transformations.	Specific Activity	7,800,000	1,100	700	416	193
Representative Synthetic	Enzyme	∆ ⁵ -3-Ketosteroid isomerase	Lactate dehydrogenase	Glucose-6-phosphate dehydrogenase	Yeast alcohol dehydrogenase	Lipoxidase
Table 7.1. Approximate Specific Activities of Enzymes Involved in	Transformation		$H_{3}C \xrightarrow{OPO_{3}^{2}} OPO_{3}^{2} \xrightarrow{OPO_{3}^{2}} OPO_{3}^{2}^{2}$	HO H	CH ₃ CH ₂ OH <u>NAD</u> → CH ₃ CH OOH	

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	Ref.	47	47	4 8	
etic Transformations.	Specific Activity	0	S	1.6	
ved in Representative Synthe	Enzyme	Try ptophane synthetase	Aldehyde Oxidase	Phosphopantothenoyl- cysteine synthetase	
Table 7.1. cont. Approximate Specific Activities of Enzymes Invol	Transformation	$ \begin{array}{c} $	$CH_3CH \xrightarrow{O_1}{H_2O} \rightarrow CH_3CO_2H$	$(HO)_2 PO O O O O O O O O O O O O O O O O O O$	(HO) ₂ PO H ₃ C CII ₃ CII ₃

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synthesis. Many of these activities are for impure enzymes, and activities for pure enzymes may vary widely depending on the source. Moreover, several of these transformations may involve more than one step. These data should thus be considered only as lower limits. Nonetheless, the total range of specific activities shown covers almost 10⁹. Enzymes are known that are appreciably slower than the slowest listed. It is difficult to draw generalizations concerning which types of transformations are likely to be fast and which slow. Proton transfer, hydration and dehydration, and phosphate transfer are often fast; individual steps in the biosynthesis of complex molecules may be very slow. Clearly, the practicality of any scheme for synthesizing appreciable quantities of a substance using enzymatic catalysis must take into consideration the activity of the enzyme: enzyme-catalyzed reactions are, of course, faster than uncatalyzed reactions, but they may still be very slow.

Cofactor Requirements

The requirement of many biosynthetic reactions for cofactors places important economic restrictions on the use of these reactions for large-scale syntheses (Chapter I of Part I of this book outlines the functions of most of the common cofactors). Although certain cofactors (CoA, thiamine pyrophosphate, biotin, tetrahydrofolic acid, B_{12}) act as true catalysts—that is, they are regenerated unchanged at the conclusion of the reactions in which they are involved—others (NAD⁺, NADP⁺, nucleoside triphosphates, flavins, pyridoxal phosphate) are involved as stoichiometric reagents. All of these cofactors are expensive. For small-scale syntheses in which the costs of reagents is not a major concern, cofactors can be added in quantities stoichiometrically equivalent to those of the starting materials, and considered to be consumable reagents. For large-scale synthesis, it will be necessary to recycle the cofactors to achieve an economically acceptable cost.

Processes for catalytic regeneration of two classes of cofactors, nucleoside triphosphates and NAD(P)H derivatives, have been developed to the stage of successful laboratory demonstrations. The most important of the first of these classes is ATP, which is in some sense the biochemical equivalent of DCC or tosyl chloride; that is, phosphate or adenylate transfer from ATP to oxygen centers serves to activate oxygen as a leaving group [51]. For example, carboxyl group activation can proceed by either path [51, 52], and a very wide range of important substances—proteins, carbohydrates, nucleotides, nucleic acids, terpenes, and others—require ATP at one or several stages in their biosynthesis. NAD⁺/NADH and NADP⁺/NADPH are similarly widely used as hydride donors and hydride acceptors.

A useful scheme for ATP regeneration must combine two features: it must be able to accept either ADP or AMP as the starting material, since either or both may be generated in a particular biosynthesis, and it must utilize an inexpensive



substance as phosphorylating agent. The most highly developed schemes for ATP regeneration are based on the coupled activity of adenylate kinase [53] and another kinase utilizing the ultimate phosphate donor as substrate. In one regeneration scheme, X-P is acetyl phosphate, generated by reaction of ketene with

$$AMP + ATP \xrightarrow{adenylate} 2ADP$$
$$2ADP + 2X-P \xrightarrow{X \text{ kinase}} 2ATP + 2X$$

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phosphoric acid [54], and X kinase is acetate kinase [55]; in the second X-P is carbamyl phosphate, produced by reaction between potassium cyanate and phosphate; here, carbamyl phosphokinase is used as catalyst [56]. The estimated cost of regenerating ATP (in very large quantities) by a route based on acetyl phosphate is less than \$1.00 per pound [57]; detailed economic estimates based

$$\begin{array}{c} O \\ H_{3}C \\ \hline CH_{3} \\ \hline -CH_{4} \\ \hline CH_{2}=C=O \\ \hline (1) H_{3}PO_{4} \\ \hline (2) NH_{3} \\ \hline CH_{3}COPO_{3}^{2} \\ \hline CH_{3}COPO_{3}^{2} \\ \hline \\ \hline N=C=O \\ + \\ HPO_{4}^{2^{-}} \\ \hline H_{2}N \\ \hline O \\ PO_{3}^{2^{-}} \\ \hline \end{array}$$

on carbamyl phosphate have not been made. Similar processes could be used to regenerate nucleotide triphosphates other than ATP, or ATP could be used. together with appropriate phosphotransferases, to effect these phosphorylations.

$$XDP + ATP \rightleftharpoons XTP + ADP$$

Schemes for regeneration of cofactors in the NADPH (NADH) series rely on an alcohol dehydrogenase to catalyze hydrogen transfer [58].



Many problems, not the least of which is that of coupling the cofactor regeneration step to the enzymic synthesis step(s), remain to be solved before processes for regenerating ATP and NAD(P)H derivatives can be used directly for largescale synthesis. Nonetheless, many of the fundamental uncertainties in these schemes have been resolved, and it seems clear that these cofactors can be economically regenerated in quantity, if required for a large-scale synthesis. No attention has been devoted to the problems of regenerating other cofactors, and the practicality of these regenerations remains to be demonstrated.

Reactor Design [59]

Enzymes to be used as catalysts in biosynthetic reactors will be expensive. A biosynthetic reactor using cell-free enzymes as catalysts should be designed to conserve enzymic activity: specifically, a continuous process should retain enzymic activity in the synthetic reactor with high efficiency, and a batch process should permit easy separation of products from catalysts. In either type, the reactor should be designed to permit maximum stabilization and operating lifetime of the enzymes. Two strategies are available for design of biosynthetic reactors: either the enzymes may be immobilized on an insoluble support, or they may be used in solution, and reisolated from products (by ultrafiltration, microencapsulation, or possibly by precipitation). Of the two, immobilization seems the more effective approach when applicable: not only are problems of isolation minimized by immobilization, but immobilized enzymes automatically enjoy a high degree of protection against hydrolysis by proteases and are subjected neither to the potential for shear and surface degradation present during ultrafiltration, nor to the rigors of precipitation or microcapsule formation. The subject of enzyme immobilization is discussed in Chapter IX of this volume; here it is worthwhile to make only a few general points about the characteristics

required of a support for immobilizations for large-scale synthetic reactors. The support should present a balance of hydrophilic and lipophilic character that permits maximum stabilization of the active conformation of the enzyme; its functionalization should be straightforward, and the coupling of the enzyme to the support should ideally preserve the charge type of the amino acid involved (usually the γ -amino group of lysine) and produce a permanent link between enzyme and support; the support should have good mechanical strength and flow characteristics for use in large columns and stirred or fluidized reactors; it should permit rapid access of starting materials to the enzyme, and rapid diffusion of the product from the enzyme; it should tolerate sterilization; it should be inexpensive.

These characteristics are. of course, also desirable for supports to be used for immobilizations on a small scale. It seems most unlikely that a single support will ever serve every need adequately, and it is difficult to judge a priori the best support for a new application. Nonetheless, certain differences between largeand small-scale processes are common. For one, the nature of large-scale processes places a greater premium on the mechanical and flow characteristics of the support. These considerations alone are probably sufficient to exclude the crosslinked agarose and dextran gels commonly used in small-scale work from wide application in large-scale processes. Second, since the enzyme preparations used in large-scale biosynthesis will probably be less pure than those in smaller syntheses, the capacity of the support is of greater importance in the former than the latter. Since the activity per unit of reactor volume that can be achieved with any enzyme preparation will depend upon the support capacity, and since both the expense of the reactor and its operating characteristics (particularly pressure drop across large columns) depends upon the size required, there is a strong economic incentive to work with high-capacity supports. Finally, since the lifetime of the enzyme under operating conditions is central to large-scale processes, selection of a support and coupling procedure that maximizes this parameter is important.

There is presently no common agreement concerning the best supports for large-scale immobilizations. In the relatively rare applications in which physical adsorption on an inexpensive, high-surface area inorganic (diatomaceous earth) or organic (DEAE cellulose) support is successful, this procedure will probably be difficult to beat. In the more frequent applications in which the enzyme must be manipulated more carefully, organic polymer supports derived from vinyl monomers seem likely to prove most useful. Supports derived from vinyl polymerization have the combined advantages of relatively low cost and great flexibility in properties. Thus, polyacrylamide crosslinked with N,N'-methylene-bisacrylamide provides a hydrophilic polymer with fair but not outstanding mechanical properties. Inclusion of more hydrophobic monomers (2-hydroxy-

ethyl methacrylate, glycidyl methacrylate, methyl methacrylate), or cross



linking with more hydrophobic materials (hexamethylene N,N'-bisacrylamide) may provide improved structural and mechanical properties, although possibly with some decrease in suitability of the resulting polymer matrix as a support. Coupling of the enzyme to the gel can be accomplished either by including appropriate monomers (glycidyl methacrylate, N-acryloxysuccinimide) in the initial polymerization or by functionalization after polymerization. Since acrylic acid, methacrylic acid, and acrylamide are all readily available and subject to facile derivatization of a wide range of types, it should be possible to tailor the resulting polymers to have those properties most appropriate for a particular enzyme.

Of the other materials commonly considered as potential supports for largescale reactors, only the controlled-pore ceramics recently introduced by Corning seem likely to be useful [60]. Controlled-pore glasses from the same source seem too expensive to be useful [60, 61].

4 ALTERNATIVES AND PROSPECTIVES

Experimental procedures for enzyme isolation, stabilization, and immobilization are sufficiently advanced that it is practical to consider catalysis by cell-free enzymes as an integral, if relatively unexploited, technique in intermediate- and large-scale organic synthesis. Given the availability of the technique, it is interesting to ask in what types of reactions enzymic catalysis might be useful. This area requires a sophisticated blending of techniques from organic chemistry, microbiology, enzymology, and polymer chemistry. Its technical complexity is such that it will only be useful when competitive "standard" synthetic tech-

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niques, conventional organic synthesis and fermentation, are unsatisfactory. Organic synthetic procedure has the virtue of great flexibility. It is, however, notably unsuccessful when applied to problems requiring the selective manipulation of similar functionalities, and particularly for materials that are soluble in water and related solvents. Thus, the modification and synthesis of carbohydrates, nucleotides, and certain types of polypeptides is seldom easily accomplished by organic synthetic techniques. Further, the procedures developed for low-molecular-weight substances are not adequate for easily synthesizing and characterizing proteins, nucleic acids, and other biological macromolecules. Fermentation often offers a very successful approach to these types of problems. It is difficult to improve on a successful fermentation for simplicity. Fermentation suffers, however, from disadvantages of its own. It is intrinsically a slow and inflexible technique: the starting materials that are acceptable for a fermentation, and the products of the fermentation, are relatively fixed by the metabolism of the microorganism. It is sometimes difficult to isolate a particular transformation of interest from the myriad other reactions usually taking place in the cell, and, in consequence, improving a fermentation yield may be a very difficult task. Fermentation is also intrinsically inefficient, in the sense that the particular material of interest in a fermentation is usually only a small fraction of the cell's synthetic output. Particularly when fermentation yields are low. the disposal of fermentation residues may present a significant problem, and other aspects of fermentation processes present environmental problems.

Enzymic synthesis offers a compromise between fermentation and chemical synthesis having distinct potential advantages. It affords higher selectivity than chemical synthesis, but is more amenable to control and modification than fermentation. It permits single reactions or reaction sequences in complex metabolic pathways to be isolated and utilized, and promises higher yields, higher purity, and greater ease of isolation than either fermentation or chemical synthesis. Enzymic reactions are run at room temperature in water: enzymic catalysis thus combines the attractive process characteristics of fermentation with the efficiency of chemical synthesis, although at the cost of high technical complexity.

Large-scale enzymic synthesis holds promise in a number of areas of fine chemicals synthesis, particularly in areas relevant to biologically active compounds. Specific areas in which research could produce useful synthetic methods include these:

1. Synthesis of nucleotides and nucleosides from sugars and purines, pyrimidines, or analogs might be carried out efficiently using enzymes. The availability of enzymes from the so-called "salvage pathways" that accept the preformed nitrogen heterocycles bypasses many of the steps in the main biosynthetic pathways [62], and would capitalize on the ability of cell-free enzymic synthesis to select one, minor, enzymic pathway from among the large number of paths

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competing in vivo for the same substrate. Unlike chemical syntheses, no protective groups would be involved in the enzymic syntheses.

2. Steroid modification, particularly oxidative functionalization, will probably present unusual and difficult problems in isolation and stabilization of the enzymes involved [63]. The area is nonetheless one of great practical importance, and fermentation methods, although extensively developed [64]. do not always produce the desired products in high yield or purity.

3. Asymmetric and stereoselective syntheses exploit the ability of enzymes to catalyze the formation of optically active products. This area is fully described in Chapter IV of Part I of this book.

4. The synthesis of nonribosomal peptides appears possible [65]. An example of an important member of this class of compounds that is within reach of present techniques is the animal food antibiotic bacitracin [66]. The biosyntheses of penicillin and cephalosporin are not presently understood, and the large-scale cell-free enzymic synthesis of these types of materials lies in the future. Studies directed toward the simpler but related antibiotic Gramicidin S have been described [65].

5. Intermediates useful in the synthesis of semisynthetic antibiotics are presently obtained by chemical or enzymic degradation of fermentation products. In principle, it should also be possible to produce these or complementary materials by cell-free enzymic synthesis. Such reactions would require a firm knowledge of the biosynthesis of the target antibiotic, and would interrupt this biosynthesis by using only those enzymes required to synthesize intermediates along the path to the product obtained by fermentation [67]. The availability of intermediates to penicillins, cephalosporins, or the aminoglycoside antibiotics would be particularly useful, and the enzymic synthesis or subsequent modification of these intermediates might present an easier problem than the total synthesis of the complete antibiotic.

6. Carbohydrates are particularly difficult to modify chemically. A large number of enzymic transformations have recently been catalogued [68], but most have not been exploited for synthesis.

7. A less specific application of enzymic synthesis lies in the area of drug metabolism. The usual approach to the isolation of drug metabolites is to feed the drug to an appropriate animal, and to isolate metabolites from blood or urine. These isolations often present severe experimental difficulties. A simpler (although not entirely equivalent) preliminary approach is to examine transformation of the drug of interest by enzymes or enzyme mixtures believed to be involved in metabolism (e.g., crude oxidase preparations from liver). The materials generated by such a procedure would probably, at least, be related to those formed in vivo, and their isolation would be less difficult.

8. It may prove possible to use enzymes ordinarily involved in hydrolysis to form peptide, phosphodiester, or related bonds by changing solvent or other

environmental parameters. These hydrolyses are reversible, and the practical problems to be solved in using enzymes to catalyze the reverse reactions are those of stabilizing the enzyme under the conditions necessary to run the reaction, dealing with the substrate specificity of the enzymes, and preventing multiple additions through suitable blocking groups. Several examples of reactions of these types have been reported [9, 69].

9. Ribosomal peptide synthesis of proteins represents a possibility for the distant future. Elegant studies by Schechter have pointed to a general procedure for isolating specific *m*-RNA's from eukaryotic cells, by precipitating the *m*-RNA, together with associated ribosomes and growing polypeptide chains, using antibodies against these polypeptides [70]. These *m*-RNA's can then, in principle, be used in combination with mixtures of ribosomes, *t*-RNA's, amino acids, and nucleoside triphosphates to generate proteins. This system would clearly be a difficult one to develop for practical synthesis, but for sufficiently valuable proteins (e.g., human growth hormone), it might prove very useful.

· These and similar subjects all are described in terms of cell-free enzymes. Progress in large-scale enzymic synthesis will also depend on two related and complementary subjects. The first is the development of more general techniques for synthesis using immobilized whole cells. As presently practiced, this technique will probably be limited in its application to relatively simple reactions. Combined with the procedures developed to protect, immobilize, and stabilize cell-free enzymes, it might have very great usefulness by providing a method of circumventing the labor required to isolate and (possibly) stabilize cell-free enzymes. Alternatively, a compromise between the use of immobilized enzymes and immobilized whole cells-synthesis using, say, immobilized intact mitochondria or membrane fragments-might offer important advantages. The second is the refinement of microbiological techniques for isolating and/or mutating new strains of organisms capable of carrying out new types of transformations. One important advantage of synthesis using immobilized enzymes relative to synthesis by fermentation is the ability of the former to take advantage of relatively unimportant metabolic pathways. Thus, if microorganisms can be developed that show detectable activity of a particular type, isolation and stabilization of the enzymes responsible offers a possible synthetic route even if the enzymes are present at such low concentrations that fermentation would not be practical.

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