Immobilized Enzymes and Cells as Practical Catalysts

Alexander M. Klibanov

Summary. Performance of enzymes and whole cells in commercial applications can often be dramatically improved by immobilization of the biocatalysts, for instance, by their covalent attachment to or adsorption on solid supports, entrapment in polymeric gels, encapsulation, and cross-linking. The effect of immobilization on enzymatic properties and stability of biocatalysts is considered. Applications of immobilized enzymes and cells in the chemical, pharmaceutical, and food industries, in clinical and chemical analyses, and in medicine, as well as probable future trends in enzyme technology are discussed.

There has been a great deal of excitement about various practical applications of biotechnology, including the production of chemicals, fuels, foods, and drugs; waste treatment; clinical and chemical analyses; toxicological assays; and uses in medicine. The question arises of which biological entities will serve as bioproducers and bioconverters in those areas. Analysis indicates that there are only two principal candidates for this role: whole cells (microbial, plant, or animal) and isolated enzymes.

Conceptually, it is easy to decide whether to employ growing cells or isolated enzymes as bioproducers and bioconverters in a particular process. Multistep transformations such as the synthesis of interferon or the production of ethanol from cellulose involve a number of different enzymes acting sequentially, and regeneration of cofactors is required; therefore, it is clearly advantageous to use whole cells. For one-step or two-step transformations, however, enzymes are probably superior because their use is free of drawbacks such as competing side reactions, sterility problems, and the cell lysis often associated with fermentations.

Microbe-catalyzed processes constitute industrial microbiology, which today is a diversified, multibillion-dollar industry (*1*). Several other articles in this issue are devoted to applications of living microbial, plant, and animal cells for the production of useful compounds. Therefore I will focus on practical uses for isolated enzymes and dead (nonviable) cells. The integrity and infrastructure of such dead cells are not required for the processes they catalyze; the main reason for using them is to save the cost and labor involved in isolating and purifying the needed enzymes.

The scope of enzyme technology is indicated by a list of the types of reactions catalyzed by enzymes. These include oxidation, reduction, inter- and intramolecular transfer of groups, hydrolysis, cleavage of covalent bonds by elimination, addition of groups to double bonds, and isomerization (*2*). Hence virtually all organic and many inorganic reactions can be catalyzed by an enzyme or several enzymes acting in sequence. Of course, most of these reactions can also be catalyzed by nonbiological, chemical catalysts (homogeneous or heterogeneous). A great number of such chemical catalysts have been developed, and their importance, power, and versatility are illustrated by the fact that more than 70 percent of all industrial chemical processes involve catalysis (*3*). One may therefore ask why there is a need for enzymes as industrial catalysts. The answer is that enzymes have several remarkable features lacking in most nonbiological catalysts: (i) extremely high catalytic activity (which may greatly exceed that of common chemical catalysts); (ii) unique specificity of action (substrate specificity, stereoselectivity, regiospecificity, geometric specificity, and so on); and (iii) ability to function under mild conditions (for instance, at ambient temperature, under normal pressure, and in aqueous solutions).

Despite such striking characteristics, enzymes have not been widely used instead of chemical catalysts. This is because enzymes also suffer from serious drawbacks. From a practical standpoint, the most important drawbacks are that (i) most enzymes are not sufficiently stable under operational conditions and (ii) enzymes are water-soluble molecules, hence difficult to separate from substrates and products and to use repeatedly.

Attempts to circumvent these problems led about 20 years ago to a major breakthrough in applied enzymology, that is, enzyme immobilization.

Immobilization of Enzymes and Cells

Immobilization of isolated enzymes. By definition, immobilization is the conversion of enzymes from a water-soluble, mobile state to a water-insoluble, immobile state. More than 100 immobilization techniques have been elaborated (*4*, *5*). They can be divided into the following five groups (Fig. 1).

1) Covalent attachment of enzymes to solid supports (Fig. 1a). A variety of supports have been used, including porous glass and ceramics, stainless steel, sand, charcoal, cellulose, synthetic polymers, and metallic oxides. Enzymes are usually immobilized through their amino or carboxyl groups. In most instances, the immobilization procedure consists of at least two steps: activation of the support, and enzyme attachment per se. For example, Corning Glass Works has used porous ceramics for immobilization of industrial enzymes such as glucose isomerase and lactase. The support is first treated with 3-aminopropyltriethoxysilane, $(C_2H_5O)_3Si(CH_2)_3NH_2$. This yields the activated support, ceramic-Si-$(C_2H_5O)_2(CH_2)_3NH_2$. The activation step is completed by reaction of the activated support with glutaraldehyde, OHC–$(CH_2)_3$–CHO, one of whose carbonyl groups forms a Schiff base with an NH_2 group of the support. Then the unreacted glutaraldehyde is washed off and an enzyme solution is added. The second, free carbonyl group of the support-bound dialdehyde reacts with amino groups of the enzyme.

2) Adsorption of enzymes on solid supports (Fig. 1b). Ion-exchangers readily adsorb most proteins, and therefore they have been widely employed for

Dr. Klibanov is assistant professor of applied biochemistry and Henry L. Doherty Professor in the Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge 02139.

enzyme immobilization. Such supports as the anion-exchangers diethylaminoethyl cellulose (DEAE-cellulose) or Sephadex and the cation-exchanger carboxymethyl cellulose (CM-cellulose) are used industrially for adsorption of enzymes. The appealing feature of adsorption immobilization is its simplicity: an enzyme solution is added to the support, the system is stirred for a few minutes, and then the enzyme remaining in solution is removed by washing.

3) Entrapment of enzymes in polymeric gels (Fig. 1c). In this approach, an enzyme is added to a solution of monomers before the gel is formed. Then gel formation is initiated by either changing the temperature or adding a gel-inducing chemical. As a result, the enzyme becomes trapped in the gel volume. The gels employed for immobilization of enzymes may be covalent (for instance, polyacrylamide cross-linked with *N*,*N*′-methylenebisacrylamide) or noncovalent (calcium alginate or kappa-carrageenan); these three gels have been used industrially.

4) Cross-linking of enzymes with bifunctional reagents (Fig. 1d). Among the most popular cross-linkers are glutaraldehyde, dimethyl adipimidate, dimethyl suberimidate, and aliphatic diamines. The first three directly cross-link enzymes through their amino groups. Diamines (for instance, hexamethylene diamine) cross-link enzymes through carboxyl groups following activation of these groups with carbodiimides. Cross-linking may be both intermolecular (forming water-insoluble aggregates) and intramolecular. In the former case, enzyme molecules can be cross-linked either with themselves or with other proteins present in solution. For example, immobilized glucose isomerase manufactured by Novo Industries in Denmark is produced by a glutaraldehyde treatment of pellets of homogenized *Bacillus coagulans* cells containing the glucose isomerase activity.

5) Encapsulation of enzymes (Fig. 1e). In this approach, pioneered by Chang (*6*), enzymes are enveloped within various forms of membranes that are impermeable for enzymes and other macromolecules but permeable for low molecular weight substrates and products. Typical examples include entrapment of enzymes in microcapsules (produced by interfacial polymerization, liquid drying, or phase separation), in liposomes, and in hollow fibers. The first two methods are intended for medical applications and the third for industrial ones. For instance, the Snamprogetti Company in Italy has used penicillin acylase, lactase, and aminoacylase entrapped in hollow fibers (*7*).

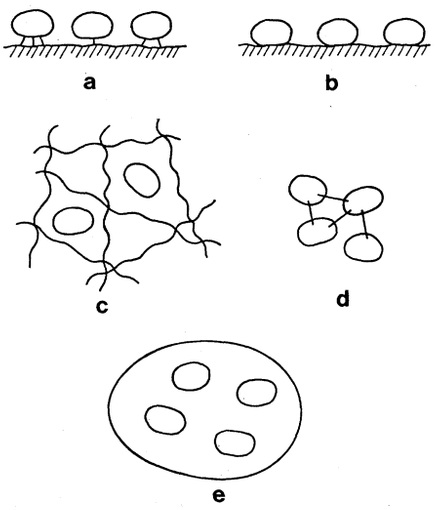

Fig. 1. Methods of enzyme immobilization: (a) covalent attachment to solid supports, (b) adsorption on solid supports, (c) entrapment in polymeric gels, (d) intermolecular cross-linking, and (e) encapsulation.

Comparison of the methods of enzyme immobilization listed above leads to some important conclusions. The advantage of covalent methods 1 and 4 is that they result in strong chemical bonds between the enzyme and the support. The disadvantages are that covalent binding is relatively laborious and expensive and often leads to significant inactivation of enzymes due to attachment through their active centers. The latter problem, however, can be alleviated in many cases if immobilization is carried out in the presence of substrates or other ligands (inhibitors, cofactors, and so on) that selectively protect the active center from the attachment. Methods of immobilization such as adsorption and gel entrapment are very simple and efficient, but since such methods create no strong bonds between the enzyme and the matrix, enzymes often leak from the supports. This problem can be overcome by treatment of adsorbed or entrapped enzymes with a cross-linking reagent such as glutaraldehyde.

Immobilization of whole cells. All five of the methods listed above have been employed for immobilization of both dead and living whole cells (*8*). By far the most fruitful technique has been entrapment of cells in gels; used with whole cells, gel immobilization is free of its major shortcoming when used with enzymes, that is, leakage from the matrix.

In the case of dead cells, the major reasons for immobilization are the same as for enzymes: to facilitate separation of the biocatalyst from products and to make the biocatalyst more stable. These objectives have often been achieved, and consequently several processes involving immobilized microbial cells have found industrial applications. For instance, polyacrylamide gel–immobilized *Escherichia coli* (possessing aspartase activity) is used for the synthesis of L-aspartic acid and *Brevibacterium ammoniagenes* (possessing fumarase activity) for the production of L-malic acid (*8*).

From the biotechnological standpoint, a dead cell can be considered a bag filled with enzymes; hence the only objective is to maintain the desired enzymatic activity. In contrast, living cells must have their metabolic machinery substantially intact and also be capable of reproduction. Therefore immobilized living cells represent an alternative to traditional fermentations and have been considered to have certain advantages such as increased cell densities, superior performance in continuous processes, and easier reactor control. Immobilized (gel-entrapped or adsorbed) living cells—microbial (*9*), plant (*10*), and animal (*11*)—have been successfully used for various biotransformations.

Immobilization techniques have been refined to the point where virtually any enzyme or whole cell can now be immobilized with sufficient retention of enzymatic activity. Immobilization almost always dramatically improves the technological properties of biocatalysts, converting them from water-soluble to water-insoluble molecules and thereby permitting their use in conventional chemical reactors (*12*). However, with respect to the second major incentive for immobilization of enzymes—their stabilization—the situation is not as straightforward or favorable.

Stabilization of Enzymes by Immobilization

One of the most important biotechnological characteristics of an enzyme is its longevity or stability. Since the environment in chemical reactors is usually much harsher than that in vivo (higher temperature, the absence of a protective milieu, inactivating impurities, aggressive surfaces, and so on), most enzymes are not sufficiently stable under operational conditions. Relatively little is known about mechanisms of enzyme inactivation; therefore stabilization of enzymes is a difficult task.

In a few cases, immobilization assuredly stabilizes enzymes. For in-

stance, the major cause of inactivation of proteases is proteolytic self-degradation (autolysis). Binding proteases to a solid support makes them lose their capacity for intermolecular self-digestion and therefore stabilizes them against autolysis. The same mechanism—mutual spatial fixation of enzyme molecules—affords stabilization against another intermolecular process, aggregation. Another case in which enzyme stabilization has been achieved involves immobilization of whole cells. Many enzymes are much more stable in their natural cellular environment than in the isolated state—for example, by virtue of stabilization by biological membranes (*13*). Stirring free cells in an aqueous solution eventually results in their rupture, which leads to solubilization and, consequently, destabilization of the enzymes. Entrapment of such cells in a gel makes them mechanically more resistant and hence stabilizes the enzymes by helping to maintain their favorable environment, as shown for *E. coli* aspartase (*14*).

It should be stressed, though, that in general immobilization is not a method of enzyme stabilization, and the latter, when observed, can often be attributed to artifacts (*15*). Statistical analysis indicates (*16*) that immobilization is as likely as any other random treatment to increase, decrease, or have no effect on enzyme stability. However, as illustrated below, if one uses immobilization to realize a rational stabilization strategy, then it should indeed produce more stable enzyme preparations (*15*).

By far the most important cause of enzyme inactivation in industrial reactors is heat, because it is often imperative to operate reactors at elevated temperatures to increase productivity and prevent microbial contamination. Although details of the mechanisms of thermal inactivation of enzymes remain obscure, some aspects are clear. For example, there is no doubt that thermal inactivation involves considerable conformational changes in the protein molecules, that is, partial unfolding (Fig. 2, transition a → b). Imagine now that an enzyme molecule is linked to a solid support by several chemical bonds (Fig. 2c). The structure of such a molecule is much more rigid than that of its free predecessor; therefore the attached molecule unfolds and is inactivated much less readily. The simplified model shown in Fig. 2 turns out to be realistic. It has been found that multipoint attachment—both covalent and noncovalent—of enzymes to polyacrylic matrices dramatically enhances their resistance to thermal inactivation (*17*). Since unfolding is a common

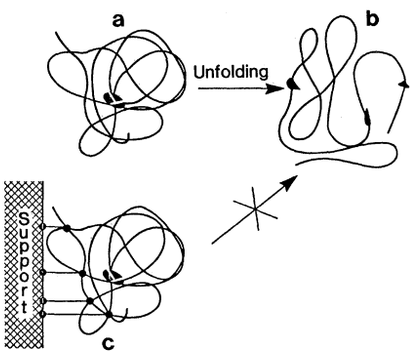

Fig. 2. Schematic representation of thermal unfolding (and consequently inactivation) of an enzyme (a → b). For the enzyme attached by many links to a solid support (c), the unfolding becomes greatly hindered. Filled region depicts the enzyme active center.

feature of several different modes of enzyme inactivation (for instance, by *p*H, denaturing agents, and organic solvents), making the molecules more rigid through multipoint binding to solid supports appears to be a general approach to enzyme stabilization (*15*).

Another example of stabilization through immobilization concerns oxygen-labile enzymes. While most enzymes are stable in the presence of oxygen, some potentially important ones such as nitrogenase, hydrogenase, and formate dehydrogenase are not. Unless oxygen-labile enzymes are made stable it is not practical to use them in air. An approach to stabilization of oxygen-sensitive enzymes by immobilization has been suggested (*18*) which takes advantage of the fact that oxygen is much less soluble in concentrated salt solutions than in pure water. If an enzyme is placed on the surface of a highly charged support, the effective ionic strength in the microenvironment of the enzyme will be very high and hence the concentration of dissolved oxygen will be much lower than in the bulk solution. This should result in enhanced oxygen stability of the immobilized enzyme. This approach has been used successfully with clostridial hydrogenase; the half-life of the enzyme under air was greatly increased by adsorption on ion-exchangers, being 2 weeks for the hydrogenase immobilized on polyethyleneimine-cellulose compared to 5 minutes for the free enzyme.

It appears that in many other cases, too, rational strategies for enzyme stabilization can be developed (*15*). Stabilization of enzymes remains one of the most challenging areas of biotechnology, and its importance will increase as more and more enzymes are used commercially.

Properties of Immobilized Enzymes and Cells

Immobilization often significantly alters the behavior of biocatalysts. The most important examples and reasons for such changes are briefly considered below.

Partitioning. The properties of immobilized enzymes and their free predecessors differ primarily because the former no longer constitute a homogeneous and isotropic enzymatic system. Instead, they represent an individual phase separate from the outer solution. The physicochemical properties of that new phase (for instance, an enzyme in a polymeric gel or on an ion-exchanger) are generally different from those of the bulk solution. Therefore, all components of the enzymatic process—hydrogen ions, substrates, products, inhibitors, activators, cofactors, and so on—are partitioned between the immobilized enzyme phase and the aqueous phase. This should significantly affect characteristics of an enzymatic reaction even if the enzyme molecule itself is not altered by immobilization.

A theory of the effect of the microenvironment on the catalytic properties of immobilized enzymes has been developed by Katchalski and co-workers (*19*). Suppose that an enzyme is immobilized in a negatively charged support. Due to electrostatic attraction, the concentration of hydrogen ions in the enzyme's microenvironment will always be higher than in the bulk solution and hence the *p*H will be lower. This will result in a shift of the profile of enzymatic activity versus *p*H to a more alkaline *p*H. The opposite will happen when an enzyme is immobilized in a positively charged support, where there is electrostatic repulsion between protons and the matrix. Quantitatively, the difference in *p*H between a charged support and the surrounding aqueous solution is determined by the equation $\Delta p\mathrm{H} = 0.43\ e\Psi/RT$, where e is the positive electron charge, Ψ is the electrostatic potential of the immobilized enzyme phase, R is the Boltzmann constant, and T is the absolute temperature. Experimentally observed shifts in activity-*p*H profiles (or in the *p*H optima of enzyme action) have been as high as three *p*H units. In agreement with the electrostatic theory, such changes resulting from immobilization in charged supports greatly decrease when salts are added.

The same approach can be used to describe qualitatively and quantitatively the interaction of enzymes immobilized in charged matrices with charged sub-

strates, inhibitors, and other ligands. For example, the observed binding constants for substrates and inhibitors having a charge opposite to that of the matrix will be lower (that is, the observed affinity will be higher) than those for the free enzyme.

The concept of nonequal distribution of components of enzymatic reactions between the immobilized enzyme and outer solution phases is applicable not only to electrostatic but to other types of interactions as well. For instance, hydrophobic ligands will concentrate around enzymes immobilized in hydrophobic supports, and the opposite will occur in the case of hydrophilic ligands.

Diffusional limitations. Immobilization converts enzymes from homogeneous to heterogeneous catalysts, which results in the appearance of some novel features. In particular, the transport of substrates to catalysts becomes subject to diffusional resistances. The classical consideration of mass transfer in catalytic chemical processes has been successfully applied to immobilized biocatalysts (*20, 21*). Diffusional resistances can be divided into two categories: (i) external diffusional limitations arising from the fact that substrates must be transported from the bulk solution to the immobilized biocatalyst's surface across a boundary layer of water, and (ii) internal diffusional limitations stemming from the fact that substrates must diffuse inside the immobilized enzyme particle (including the gel, porous support bead, microcapsule, or hollow fiber).

The existence of diffusional limitations reduces the catalytic efficiency of immobilized biocatalysts and therefore should be minimized. This can be achieved by decreasing the size and optimizing the geometry of immobilized biocatalyst particles, increasing the substrate concentration, enhancing the stirring or flow rate, increasing the porosity and optimizing the biocatalyst distribution in the beads, and so on (*20*). However, diffusional resistances may sometimes have a beneficial effect. For example, in the case of coimmobilized multienzyme systems catalyzing consecutive reactions, such as $A \rightarrow B \rightarrow C \rightarrow \cdots \rightarrow Z$, reduced diffusion of B, C, and other intermediates from the matrix will result in their buildup in the bead and thus increase the overall rate of production of Z.

Steric hindrances. I mentioned earlier that virtually any enzyme can be immobilized with satisfactory retention of its catalytic activity. Although this assertion is valid in the case of low molecular weight substrates, it does not always hold for enzymatic reactions involving high molecular weight substrates. For example, many hydrolases covalently attached to solid supports exhibit markedly lower enzymatic activity toward polymeric substrates (proteins, polysaccharides, and nucleic acids) than expected on the basis of their reactivity to small substrates (*21*). This effect is due to steric hindrances involving the support's surface, and it can be greatly reduced by attaching enzymes through longer "arms." Similarly, interactions of biocatalysts entrapped in polymeric gels with macromolecules are often severely diminished because of the slow diffusion of the latter in the gel matrices. The ultimate decrease in enzymatic activity upon immobilization occurs when substrates are insoluble in water (for instance, cellulose, starch, and keratin). It appears that such cases call for the use of free enzymes (either native or chemically modified and stabilized) as opposed to their immobilized counterparts.

Applications of Immobilized Biocatalysts

The most significant practical applications of immobilized biocatalysts are discussed briefly in this section in terms of some representative examples that clearly show the scope, possibilities, and limitations of immobilized enzyme technology.

Chemical and pharmaceutical industries. Biotechnology in general and immobilized biocatalysts in particular are unlikely to have an appreciable impact on the production of bulk (commodity) chemicals as long as such conventional feedstocks as oil, natural gas, and coal are used. This conclusion is based on the following facts. (i) Up to 60 to 80 percent of the cost of bulk chemicals is due to the cost of the raw material, so a change in the process technology would have a rather small effect on the economics. (ii) There are excellent chemical catalysts for the synthesis of bulk chemicals and—considering the progress made over the past two decades—the opportunities here appear to be unlimited. (iii) Enormous capital investments have been made in existing chemical plants, and since most of them currently function well below capacity, chances of sizable investments in novel, competing technologies are slim. Only when alternative feedstocks such as biomass and wastes become a major factor can one expect biotechnology to play an important role. And even when that happens, which will probably not be for 30 to 50 years, the contribution of immobilized biocatalysts is questionable in comparison with conventional fermentations.

Therefore, the chief target for immobilized biocatalysts could only be the production of fine or specialty chemicals, that is, relatively low-volume, high-cost compounds. In such cases enzymes seem to be attractive catalysts because of their specificity of action and ability to function under mild conditions (*22*). Several processes employing immobilized biocatalysts have been or are being commercialized, all of them to produce specialty chemicals.

Immobilized biocatalysts have proved particularly useful for the production of L-amino acids, which are widely used as food additives, as animal feed, and in medicine. The L- (but not D-) amino acids have nutritive value and have traditionally been manufactured by fermentation. Chemical syntheses are simpler, faster, and cheaper than fermentations but almost always result in racemic mixtures of amino acids (*23*). Chibata and co-workers (*5*) developed an enzymatic method for the conversion of such racemic mixtures to pure L-amino acids, and that method is now used industrially in Japan. Chemically synthesized amino acids are first acylated and then passed through a column packed with immobilized aminoacylase, which selectively hydrolyzes (deacylates) the L-isomer. The L-amino acid is then readily separated from the acyl-D-amino acid on the basis of solubility differences. This is followed by racemization of the acyl-D-amino acid by heating and its reuse in the resolution procedure. As in many other immobilized enzyme processes, the major cost savings over the free-enzyme-based batch reactor process come from reductions in labor and enzyme costs.

Other transformations aimed at the production of L-amino acids and catalyzed by immobilized enzymes and cells include (*24*) the conversion of ammonium fumarate to L-aspartic acid; synthesis of L-tryptophan from indole and either acetic acid and ammonia or D,L-serine; synthesis of L-tyrosine from phenol, acetic acid, and ammonia; conversion of L-arginine to L-citrulline or L-ornithine; and synthesis of L-glutamic acid from glucose.

Another successful use of immobilized biocatalysts is in the production of 6-aminopenicillanic acid (6-APA)—the starting material for industrial manufacture of the semisynthetic penicillins—from penicillin G, which is readily obtained by fermentation (*24, 25*). A unique feature of the enzyme penicillin acylase is that (in contrast to OH^- or H^+) it hydrolyzes the more stable of the two

amide bonds in penicillin G, which happens to be the desirable reaction. Currently, immobilized penicillin acylase is employed on an industrial scale not only to produce 6-APA but also to acylate 6-APA to form novel penicillins and to deacylate and acylate cephalosporins, which are more potent structural analogs of penicillins (*24, 25*).

Food industry. Immobilized biocatalysts hold particular promise for use in food processes. Technologically, the food industry is grossly underdeveloped compared with the chemical and pharmaceutical industries. The competitors of enzymes, chemical catalysts, have not played and are not expected to play a significant role in food processing because of their "incompatibility" with food materials and for safety reasons. Enzymes, on the other hand, have been widely used in food technology for many years (*26*). The introduction of immobilization technology has created several novel applications for biocatalysts (*27*).

The greatest commercial success of immobilized biocatalyst technology has been in the production of so-called high-fructose corn syrup (HFCS) catalyzed by immobilized glucose isomerase (*28*). This process converts glucose (produced by enzymatic hydrolysis of starch) to an approximately equimolar mixture of fructose and glucose, HFCS. Because fructose is sweeter than glucose, HFCS is about as sweet as a sucrose syrup of the same solid content, and it has found a wide and growing use in soft drinks (for instance, those manufactured by Coca-Cola and Pepsi-Cola). In 1980 more than 1 million metric tons of HFCS (based on dry weight) was produced in the United States, and by 1985 this figure is expected to double.

The history of the development of HFCS production catalyzed by immobilized glucose isomerase (*29*) is instructive and indicative of the role of the economic environment in biotechnology. Although the technology was ready by 1970, the price of raw sugar then was less than 10 cents a pound. Since HFCS could not be produced cheaper than that, the process was not commercialized. In November 1974 the price of raw sugar jumped to 50 cents a pound, and the glucose isomerase process suddenly became highly profitable. When the price of sugar plummeted below 10 cents a pound at the end of 1976, industrial HFCS production survived and even expanded, reaching the level stated above and capturing much of the liquid sweetener markets previously held by sucrose.

Another recently commercialized process is the hydrolysis of lactose in whey (a by-product of cheese manufacturing) to a mixture of glucose and galactose, catalyzed by lactase covalently attached to microporous silica beads. The whey glucose-galactose syrup produced is used as a protein-rich sweetener for baked goods, ice creams, candies, and jams. Like glucose isomerase, immobilized lactase converts a nonsweet sugar to sweet ones in a reaction that cannot be readily achieved by conventional chemical means.

Analytical applications of immobilized biocatalysts. Many enzymes exhibit a unique substrate specificity, reacting with only one substrate out of many. This feature (absent in almost all chemical catalysts) is especially valuable in analytical work, where it is commonly wished to measure the concentration of one compound in the presence of many others. The potential of immobilized biocatalysts in chemical and clinical analyses can be best illustrated by "enzyme electrode" technology (*30*).

Determination of particular compounds in complex, multicomponent fluids (such as blood or industrial waste streams) is a difficult task, usually requiring many laborious and time-consuming operations. This is quite different from the ideal determination, which would involve simply placing an electrode in a sample and immediately reading the concentration of the compound of interest. Unfortunately, the electrodes now available are limited to a very narrow range of species—H^+, O_2, NH_4^+, CO_2, and a few others—and cannot be used to directly determine more complex molecules such as amino acids. A revolutionary idea was to couple such an electrode with an immobilized enzyme: an electrode is wrapped with a polymeric film containing an enzyme, which converts the determined compound to a species that can be directly measured by the electrode. For example, the enzyme L-amino acid oxidase produces one ammonium ion per molecule of L-amino acid oxidized. Hence, coupling this enzyme with an NH_4^+-sensitive electrode creates an "enzyme electrode" that can directly assay L-amino acids. This principle has a general applicability, and it has already been used to assay a wide variety of diagnostically, environmentally, or otherwise important compounds including individual amino acids, glucose and other sugars, phenols, organophosphates, urea, cholesterol, penicillin, and hydrogen peroxide (*30*). In principle, virtually any compound can be assayed by an enzyme electrode composed of a suitable electrochemical probe and an immobilized enzyme or a combination of enzymes acting in sequence (or immobilized whole cells) (*31*).

Medical applications of immobilized enzymes. Most studies of medical uses of immobilized enzymes have focused on removing undesirable compounds from the blood. This can be done either by administering immobilized enzymes into the body or by using various extracorporeal devices (*4, 5*). For instance, microencapsulated (to prevent proteolytic degradation and immunological reactions) asparaginase has been injected intraperitoneally to treat leukemia. Such a treatment is based on the ability of the enzyme to decompose L-asparagine, an amino acid that is required by tumor cells much more than by normal cells.

A promising example of use of immobilized enzymes in extracorporeal therapy comes from a recent study by Langer *et al.* (*32*). Patients' blood that is to be perfused in an artificial kidney or a pump-oxygenator is usually heparinized to keep it from clotting in the device. Before the blood reenters the body, the heparin must be removed to avoid hemorrhagic complications. This problem was solved by using a blood filter containing the immobilized enzyme heparinase, which degrades 99 percent of heparin's anticoagulant activity within minutes (*32*).

Future Developments: Opportunities and Challenges

As Niels Bohr asserted, "Prediction is very difficult, especially about the future." Therefore, rather than trying to forecast specific events in the area of enzyme technology, I will attempt to identify and rationalize some key trends that may prove important.

Immobilized biocatalysts can be used either to improve existing processes or in novel processes. There are serious practical restrictions in both of these directions: in the former, the immobilized biocatalyst technology must be far superior to the conventional one to be competitive; in the latter, there is uncertainty concerning the market for the new product.

One way to improve a process is to replace a vital component that is expensive or in short supply. For example, Klibanov and Huber (*33*) proposed the use of immobilized microbial hydrogenases in place of platinum compounds as catalysts in the detritiation of aqueous effluents from nuclear power plants and in heavy-water production. Use of a biocatalyst can also make a process more efficient—for example, by making it less

labor- and energy-intensive. This approach is illustrated by the use (*34*) of nitrile hydratase to convert acrylonitrile to acrylamide, a monomer used in manufacturing synthetic fibers. The enzyme-based process for acrylamide production has advantages over conventional methods such as the hydration of acrylonitrile catalyzed by copper salts or sulfuric acid, as the latter methods yield several by-products or require high temperatures.

The production of HFCS catalyzed by immobilized glucose isomerase is perhaps the best example of a novel process whose product has found its market. Most developments in the medical and analytical areas will likewise be novel ideas rather than improvements.

An important trend in enzyme technology is the use of nontraditional catalytic properties of enzymes—that is, the ability of many enzymes to catalyze reactions quite different from those they catalyze in vivo (*35*). The enzymatic production of HFCS is a good illustration: in nature the enzyme catalyzing this process isomerizes xylose, not glucose. To identify such unnatural reactions, one must screen known enzymes for unknown catalytic activities; the results of such a search will add to the arsenal of approaches at our disposal.

For several reasons, genetic engineering is expected to contribute significantly to the further development of enzyme technology. Some of the enzymes currently used commercially are from plant and animal sources. To make the supply of such enzymes more stable and abundant, it would be beneficial to clone the genes for them into efficient enzyme-producing microorganisms. The same strategy applies to human enzymes for therapeutic applications and to enzymes needed in a purified state (such as those that are to be covalently attached to solid supports). Finally, it is often desirable to alter the catalytic characteristics of commercial enzymes—for instance, to enhance or reduce thermostability, to shift the *p*H optimum of the enzymatic activity, or to modify the substrate specificity. Once the genes for such enzymes have been cloned and we know what structural changes in the enzyme molecule will yield the desired result, the genes can be chemically changed correspondingly.

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23. A spectacular exception to this rule is the Monsanto process for the production of some L-amino acids with chiral Wilkinson-type catalysts [reviewed in R. E. Merrill, *CHEMTECH* **11**, 118 (1981)]. For example, L-phenylalanine (an essential amino acid used in the synthesis of the sweetener aspartame) can be prepared by the condensation of benzaldehyde with *N*-acetylglycine, followed by asymmetric hydrogenation catalyzed by rhodium-phosphine complexes and subsequent hydrolysis of the resulting *N*-acetyl-L-phenylalanine. The major drawback of this process is the high cost and instability of the catalyst.
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31. The biggest problem associated with the use of enzyme electrodes is due to interfering substances, which are often present in "real" samples. Such substances (proteins in the case of biological fluids) interact not with the enzyme (which is indeed specific) but with the surface of the electrode. In principle, this obstacle can be overcome by using semipermeable polymeric membranes to shield the electrode from interfering substances, as was done in the commercial Yellow Springs Instrument Co. glucose analyzer (based on immobilized glucose oxidase coupled with an oxygen-sensitive electrode).
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