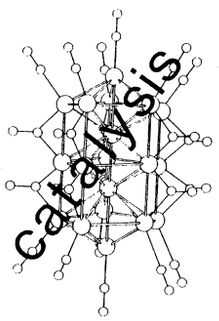
A Renewed Interest in Immobilized Enzymes

A host of potential new applications for immobilized enzymes and cells presages what some call "a new industrial revolution"



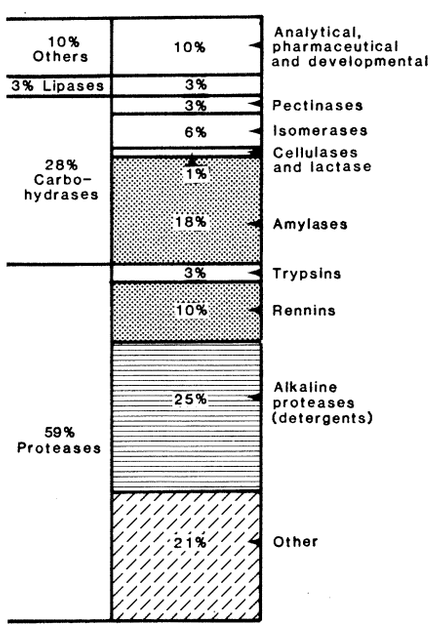
"Ten years ago, immobilized enzymes were looked on as a panacea for industrial development," says Lemuel Wingard of the University of Pittsburgh. "There was a lot of enthusiasm, but not much realism." Realism sank in slowly as investigators learned that enzymes, bound or otherwise, simply could not compete economically with large-scale chemical processes. In specialty areas where enzymes might have been able to compete, as in the production of pharmaceuticals and more expensive chemicals, the desirable enzymes were generally too expensive or, more important, too scarce to be of commercial importance.

That situation is changing rapidly. "In the past couple of years," says Wingard, "there has been a strong resurgence of interest in immobilized enzymes." This renewed interest in biological catalysts results primarily from the development of genetic engineering techniques that promise to provide substantial quantities of virtually any desired enzyme at reasonable prices.*

The technology for immobilizing enzymes is relatively mature (see box). There are, says Wayne Pitcher of Genencor Corporation, "literally hundreds of ways to immoblize enzymes, most of which have been available for some time." Adds Howard Weetall of Corning Glass Works: "There haven't been any real breakthroughs recently, and there is nothing exciting coming along. The real interest now is in applications of immobilized enzymes." The key thing now, says Oscar Zaborsky, a consultant who recently retired from the National Science Foundation, "is to come up with new uses and new enzymes that can fill specific needs. If you can find an enzyme that will help you, the chances are that it can be immobilized with some reasonable success."

Among the number of reasons to immobilize enzymes, the most common is simply to facilitate recovery of the enzyme or separation of the product from the catalyst. Immobilization frequently also stabilizes the enzymes. Proteases, for example, catalyze their own destruction; if they are attached to a support, that destruction becomes more difficult. Cross-linking reactions may also stabilize the three-dimensional structure of the protein, rendering it less susceptible to thermal degradation. Immobilization may also make the enzymes less susceptible to oxidation. Ephraim Katchalski of the Weizmann Institute in Israel has shown that the ionic strength of the microenvironment surrounding an enzyme on a highly charged support excludes dissolved oxygen.

The use of enzymes as catalysts seems poised for a remarkable growth. In 1983, enzyme sales worldwide were reported to be about $390 million, up nearly 25 percent from 1979. A 1982 report from the Office of Technology Assessment predicted that, within 20 years, enzymes would be used in the production of $15 billion worth of chemicals and pharmaceuticals. "We are on the verge of a second industrial revolution," says William Amon, Jr., of Cetus Corporation.



Enzyme uses

The major use of proteases is in detergents; rennins are used in making cheese, and other proteases are used to tenderize meat and for production of pharmaceuticals. Amylases are used primarily to hydrolyze starch; other carbohydrases are used to produce invert sugar, to convert glucose to fructose, to hydrolyze pectic substances, and to oxidize glucose to gluconic acid. Lipases are used to hydrolyze fats and fatty acid esters. Catalases are used to decompose hydrogen peroxide. [Adapted from Industrial Enzymology *(Nature Press, New York, 1983)]*

On a dollar basis, about 80 percent of enzyme sales are to the food industry. On a volume basis, about 40 percent of sales are accounted for by starch conversion enzymes and another 30 percent by proteolytic enzymes used in laundry detergents. Yet with all these sales, there are only four significant industrial applications of immobilized enzymes.

One of the reasons for this lack of use is that many of the applications involve the breakdown of insoluble biopolymers; only a soluble enzyme can efficiently attack such materials. Second, many of the enzymes now used are so inexpensive that it is cheaper to throw them away than to immobilize them. And finally, many potential enzymatic processes are simply too expensive to compete with more conventional homogeneous or heterogeneous catalysis. That situation seems likely to change, however, as more complicated structures with greater stereochemical requirements achieve greater use.

The "granddaddy" of immobilized enzyme processes is the use of glucose isomerase to convert corn-derived glucose into high-fructose corn syrup, which is used in soft drinks and many other packaged products. Glucose isomerase use started under a unique set of conditions: in 1974, the price of sugar skyrocketed, making the cost of this conversion process competitive. By the time the cost of sugar came back down, the enzyme makers were able to reduce costs enough to remain competitive, and today about $40 million worth of immobilized glucose isomerase is used yearly to produce 6 billion pounds of high-fructose corn syrup.

Tanabe Seiyaku of Osaka, Japan, has for many years used an immobilized aminoacylase to resolve racemic mixtures of amino acids. More recently, the company has also begun using immobilized cells to produce L-amino acids, such as alanine.

*Modifications of enzymes are discussed in articles appearing in the issues of 13 January, p. 154, and 20 January, p. 269. Preceding articles in this series are cited in those issues. This is the last article of the series.

A third, much smaller market, is the use of immobilized penicillin acylase to convert penicillin to 6-aminopenicillanic acid, a precursor of many partially synthetic antibiotics. This technique is used primarily in Europe, especially in Germany, although the technology was developed in the United States by Miles Laboratories. It also is a case where gradual improvements in the enzymatic process eventually brought it to the stage where it could compete economically with conventional chemistry.

The fourth and newest process is one used by Nutrisearch Company to recover usable products from cottage cheese whey produced in Kroger dairies. Nutrisearch, a joint venture of Kroger Company and Corning Glass Works, opened a plant in Winchester, Kentucky, in October 1983, where the firm uses an immobilized lactase to convert lactose from the whey to glucose and galactose. These sugars are then used to grow bakers' yeast, which is subsequently sold at the Kroger stores. The company uses a commercially available lactase that is chemically linked to silicate particles produced by Corning. This process could help solve a pollution problem also, since half the whey produced in this country is now dumped into sewers.

Other potential applications of immobilized enzymes are aimed at much smaller markets and unusual niches that can be filled only by enzymes. Investigators at the Midwest Research Institute (MRI), for example, use an immobilized cholinesterase as a detector for pesticides in air, water, and soil. The pesticides inhibit enzyme activity and that inhibition can be monitored electrochemically or colorimetrically. MRI has recently established a marketing arm to sell individual air and water monitors,

How Do You Immobilize an Enzyme?

Enzymes and cells have been immobilized on substances ranging from paper, wood chips, and crushed red brick through conventional ion exchange resins to sophisticated and costly ceramic and glass beads. But in general, says Alexander Klibanov of the Massachusetts Institute of Technology, immobilization techniques can be divided into five major classes.

▶ Adsorption on solid supports. The most popular supports are ion-exchange resins, but many other materials can be used. This is the simplest and cheapest approach: an enzyme solution is added to the support, the system is stirred, and the unattached enzyme is washed away. The adsorbed product is not very stable, however, and cells are more easily adsorbed than enzymes.

▶ Covalent attachment to supports. Many different supports can be used, but the most popular are porous ceramics such as those developed by Corning Glass Works. A major problem is the potential for inactivation of the active site by chemical reagents, but this can often be avoided by binding a substrate or ligand to the active site during the immobilization process. This technique is generally more useful for enzymes than for cells.

▶ Entrapment in polymeric gels. The enzyme is added to a solution of monomer before polymerization is initiated, so that the enzyme is trapped in the gel volume. Entrapment is inexpensive, can be performed under mild conditions, and is especially good for cells, but the reactants may not always reach the catalyst.

▶ Cross-linking with bifunctional reagents. This approach may be used to form agglomerations of enzymes (or of enzymes and other proteins) large enough to precipitate from solution, or to incorporate the enzymes chemically into a polymer. This technique produces very stable systems, but it is very difficult to do, attachment can destroy catalytic activity, and reactants may have problems reaching the catalyst. The approach does not work well with cells.

▶ Encapsulation in membranes that trap the enzymes but permit diffusion of reactants and products. Encapsulation may be with microcapsules, liposomes, hollow fibers, or dialysis membranes. This technique works very well, but it is quite expensive and is probably best suited to medical applications.

All the immobilization techniques typically reduce activity by 10 to 50 percent, but this is not necessarily a problem. "Even if you lose half the activity," says Howard Weetall of Corning, "if you use the enzyme twice as long, you get it all back, and if you use it 100 times as long, the loss is meaningless."

Unfortunately, though, "There is no ideal technique for immobilization," says William Amon, Jr., of Cetus Corporation, and "it is impossible to make generalizations about what types of enzymes are best suited for which techniques." Typically, "one would initially look at techniques which are the most gentle to the enzymes," says Weetall. "Only if these do not work would one go on to harsher techniques."

That is not to say that there are no problems associated with immobilized enzymes. One major concern is contamination. Because most enzymatic reactions must be carried out under near physiological conditions, the supports become an ideal breeding ground for microbes. Great care must be taken to minimize such growth. Leakage of unreacted monomer or other contaminants from polymer-bound enzymes can be a problem, particularly in the food industry. And finally, designing the reactor is a critical problem, says Walter Goldstein of Miles Laboratories: "There must be room for the fluid to pass through; the immobilizing agent needs physical durability, but it can't be so stiff that it blocks the flow." Adds another investigator: "The engineering of immobilized enzyme systems is still more art than science."

Perhaps the most serious problems, says Thomas Chang of McGill University, involve membrane-bound enzymes, multienzyme systems, and enzymes that require cofactors. Such systems have been particularly difficult to work with and have, in most cases, required the use of immobilized cells. The cells, whether living or dead, can keep the enzymes in the proper spatial arrangement with respect to each other and can regenerate cofactors. This approach entails getting reactants into the cell and products out. In many cases, though, the desired product may be an intermediate in cellular metabolism and further metabolic activity must be blocked so that the desired product is not used up; this, notes Lemuel Wingard of the University of Pittsburgh, has been easier in concept than in practice.

—T.H.M.

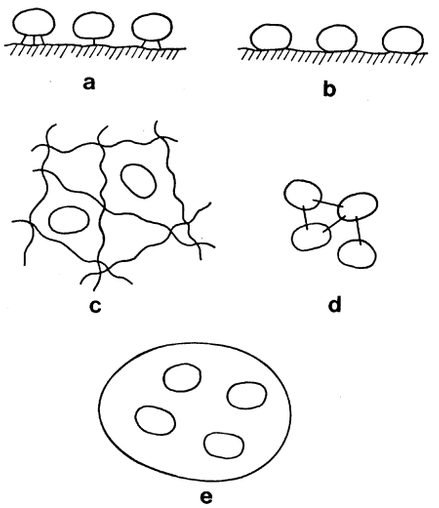
custom-built portable monitors, and "enzyme tickets" that can be dipped in water like *p*H paper to check for pesticides.

MRI is now working under a contract from the U.S. Environmental Protection Agency (EPA) to screen other commercially available enzymes against each of the chemicals on EPA's list of 129 toxic substances to develop new detection techniques. They have found, for example, that carbonic anhydrase is sensitive to chlorinated hydrocarbons in low concentrations and hexokinase to chlordane, lindane, and toxaphene.

Another approach to toxic chemicals has been developed by Francis Hoskin of the Illinois Institute of Technology. Hoskin has isolated an enzyme, diisopropylphosphofluoridase, from squid nerve cells; this enzyme detoxifies the organophosphate nerve gases Soman (produced by the United States) and Sarin (produced by the Soviet Union). Preliminary studies suggest that an immobilized form of Hoskin's enzyme could be used to destroy U.S. stockpiles of obsolete Soman as well as to protect soldiers from the effects of Sarin. Hoskin is now using genetic engineering techniques to try to obtain enough of the enzyme for further studies.

One of the most intriguing potential uses of immobilization technology is the production of an "artificial gill." Joseph Bonaventura, Celia Bonaventura, and their colleagues at Duke University have developed a technique for entrapping hemoglobin (a protein, but not an enzyme) in a polyurethane matrix to produce what they call a "hemosponge." The hemosponge can extract oxygen directly from seawater; the oxygen can then be released from the polymer by exposing the hemosponge to a vacuum or by passing a weak electrical charge through it. Bonaventura says that the immobilized hemoglobin operates with an efficiency of about 80 percent, and that the immobilization greatly stabilizes the protein.

A small hemosponge unit carried on the back, says Bonaventura, could allow a diver to work under water indefinitely. A container 3 feet in diameter and 10 feet long could provide oxygen to 150 men on the ocean floor. But even more important, he says, is the possibility of supplying oxygen for underwater combustion. "Now, there is nothing between nuclear reactors and batteries for underwater propulsion. But gasoline or kerosene provides 300 times the energy per weight of a battery if you could get oxygen to burn it with. A reasonably sized hemosponge could do this." In November, the



Enzyme immobilization

Methods of immobilization: (a) covalent attachment to solid supports, (b) adsorption on solid supports, (c) entrapment in polymeric gels, (d) intermolecular cross-linking, and (e) encapsulation. [Source: Alexander Klibanov, Massachusetts Institute of Technology]

Bonaventuras and Duke signed a contract giving Aquanautics Corporation of San Francisco an exclusive license on the technique. The company introduced an engineering prototype of a respirator for divers in January.

Robert Langer of the Massachusetts Institute of Technology has developed a system in which the enzyme heparinase has been immobilized on Sepharose beads. Heparin is an anticoagulant that is used to keep clots from forming when blood is circulated extracorporeally, as through a heart-lung machine. The heparin stays in the blood after it is returned to the body, however, and can cause internal bleeding. The immobilized heparinase could be used, Langer says, to destroy heparin before blood is returned to the patient. Tests in dogs, for example, show that the immobilized enzyme can destroy 99 percent of heparin in the blood within 2 minutes.

To date, Langer says, the system looks very good, and MIT is negotiating with several companies that would like to produce it. Langer speculates that the approach could have many other applications. He has already developed a prototype system in which immobilized bilirubin oxidase is used to remove bilirubin from blood; this could be used to treat jaundice in infants.

Investigators at Corning have developed a process in which cells of three different microorganisms are immobilized in the pores of ceramics and used to treat municipal sewage. Laboratory scale and small pilot studies show that the system can convert 90 percent of the sewage to fuel-quality methane in 2 to 6 hours. Both the identity of the microorganisms and the methods of immobilization are proprietary. Corning recently signed an agreement with NGK Insulators of Japan for joint development of the process. After further studies, the process will presumably be used to supply methane to fuel-short industries in Japan.

Thomas Chang of McGill University has developed a complex system in which three different enzymes enclosed in a microcapsule could be used to remove urea from the blood of kidney failure patients or ammonia from the blood of liver failure patients. The capsules contain urease, glutamate dehydrogenase, and glucose dehydrogenase. The urease converts urea to ammonia. Glutamate dehydrogenase then adds the ammonia to alpha ketoglutarate to form glutamic acid. In this process, reduced nicotinamide adenine dinucleotide (NADH) is oxidized to NAD. Glucose dehydrogenase is therefore included in the capsule to reduce it back to NADH, with glucose being used as an energy source. Alternatively, Chang says, alcohol dehydrogenase can be used for the same purpose. Chang uses a cellulose nitrate membrane coated with a monolayer of lipids to encapsulate the enzymes. He has not yet tested the microcapsules in humans.

Less is known about several other potential processes. W. R. Grace & Company is working on a system in which dead cells are immobilized in a hydrophilic polyurethane to produce amino acids. The company hopes to begin selling amino acids produced in this fashion within a year. Kraft Company is working on processes that use immobilized rennin and pepsin to clot milk for cheese production and immobilized lipase and esterase to hydrolyze butterfat. A consortium of Japanese companies is developing a technique in which immobilized yeast cells are used to shorten the time required to ferment biomass into alcohol. Many other processes are also thought to be under development in even greater secrecy.

"There is a great deal of enthusiasm that now surrounds immobilized enzymes," says Robert Jackson of Nutrisearch, "and everyone has a tendency to make extreme projections about how successful the technique will be. In theory, the technique has a great deal of potential to reduce the costs of many chemical processes. In practice, it may take a lot more hard work before those cost savings can be achieved."

—THOMAS H. MAUGH II