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THE USE OF IMMOBILIZED ENZYMES IN THE FOOD INDUSTRY: A REVIEW*

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INTRODUCTION

Enzymes are organic substances produced by living cells which catalyze physiologically significant reactions. Enzymes often are defined as biocatalysts, and they possess greater catalytic activity than chemical catalysts. All known enzymes are protein in nature and are generally colloidal, thermolabile, have relatively high molecular weights, exhibit high degrees of stereochemical and substrate specificities, and can usually be isolated from the living cell. There are a wide variety of enzymes which contribute, in part, to the biological diversity observed in nature.

The properties and reactions of enzyme catalysis were first recognized by G. S. C. Kirchoff in 1811; however, the word catalysis was not introduced into science until 1838 by Berzelius. Kühne in 1878 was the first person to propose the name "enzyme". During the 19th century, a number of enzymic studies were carried out, but the breakthrough came in the early 20th century when Michaelis and Menten⁹⁴ proposed a hypothesis for enzyme action in 1913. It was 13 years after this event before the first enzyme was isolated by Sumner in 1926. Since then, the proliferation of knowledge in the field of enzymology has been phenomenal. At the present time, it is estimated that nearly 1000 enzymes have been isolated and studied.

Enzymes and Their Uses in the Food Industry

Even though modern enzymology emerged in the 20th century, the application of enzymes in food processing predates biblical times. The art of cheesemaking is believed to originate from the practice of Nomads, who stored milk in containers made from the stomachs of animals. When stored in such containers, the milk coagulated into a tasty solid food, presumably due to the action of rennet in the stomach lining. Similarly, these people realized that meat wrapped in leaves from certain plants or treated with certain fruit juices became more tender and juicy. It is now known that plant proteases, such as papain, can tenderize meat effectively. Thus, even though the cause or mechanism of action was not understood, our ancestors employed enzymes in the

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processing of their food. With the advancement of science, enzymes were purified, and their modes of action were studied more thoroughly, leading to their enhanced use on food substrates. Accordingly, enzymes play a significant role in food science and technology,¹¹⁶ and currently, numerous enzymes are employed by the food industry, as in Table 1. Apart from the exogenous addition of enzymes, it must be realized that endogenous or inherent enzymes in foods as well as enzymes resulting from microbial growth play important roles in food processing. For example, fermented foods are a direct result of microbial enzymic activity.

Immobilized Enzymes

In the recent past, newer technologies have been developed which involve immobilizing enzymes. Immobilization of enzymes refers to the localization or confinement of enzymes during a process which allows the enzyme to be separated physically from substrate and product for reuse. An enzyme catalyzing a reaction in a beaker is confined, but not immobilized, because substrate and product cannot be added or removed. Classically, enzymes have been immobilized by associating them with a water-insoluble material; hence, insolubilization is sometimes used erroneously as a synonym.

Although it is thought that immobilized enzymes are relatively new, their origin preceded the isolation of urease by Sumner. In 1916, Nelson and Griffin¹⁰⁰ adsorbed invertase on charcoal and on alumina and demonstrated that this adsorbed enzyme was active even in the "immobilized" form. Unfortunately, this finding was not attributed any significance, and four decades elapsed before the concept of immobilization of enzymes reemerged. Between 1954 and 1961, McLaren and Estermann^{86,87} and Zittle¹⁶² worked on the adsorption of enzymes onto inorganic carriers, whereas Bar-Eli and Katchalski⁵ and Mitz and Summaria⁹⁵ approached the problem of covalent attachment of enzymes to organic copolymers and cellulose, respectively. In the post-1961 era, the field of immobilized enzymes gathered great momentum. The reason for the concentrated effort stems from the advantages offered by immobilized-enzyme systems which have been summarized by Messing⁹³ as follows: (1) multiple or repetitive use of a single batch of enzyme, (2) better process control — enzyme can be separated from reactants, (3) enhanced stability — contributes to stabilization of tertiary structure and antiturbulence factors, (4) products are enzyme free, (5) long half-lives and predictable decay rates, and (6) good model to study *in vivo* kinetics of enzymes.

Numerous articles have appeared in recent literature dealing with immobilization of enzymes. For a more thorough treatment, articles by Falb³³, Gabel and Porath³⁵, Mosbach⁹⁷, and books edited by Messing⁹³ and Zaborsky¹⁶⁰ are recommended.

Methods for Immobilizing Enzymes

There are numerous methods for achieving the immobilization of enzymes. For the sake of clarity in understanding, Weetall¹⁴³ classified the techniques into the following groups:

Adsorption — Enzymes can be absorbed on materials, such as charcoal, organic polymers, glass, mineral salts, metal oxides, or silica gel. Although this method was first employed by Nelson,^{100,101} the mechanisms involved in adsorption are not well understood. This method of immobilizing enzymes has the advantages of being inexpensive and chemically simple, since no reagents are required, and the process involves only a minimum of activation steps. Additionally, the enzyme is less likely to be denatured during the process of immobilization when compared with chemical methods of immobilizing enzymes. It has been speculated that the binding forces involved in adsorption are hydrogen bonds, coordinate bonds, and Van der Waal's forces. The weak nature of the binding does lead to some disadvantages. For example, desorption

TABLE 1

Use of Enzymes in the Food Industry

Industry	Enzymes used	Common sources	Ref.
Milling and baking	α -Amylase	Fungal	8
	β -Amylase	Malted grain, bacterial	
	Proteases	Fungal, bacterial, plant	
	Lipoxidase	Cereal grain, de-fatted soy flour	
	Lactase Pentosanase	Fungal Fungal	
Corn starch and syrups	α -Amylase	Bacterial	84
	β -Amylase	Fungal	
	Glucoamylase	Fungal	
	Glucose isomerase	Bacterial, fungal	
Dairy	Catalase	Animal, bacterial	110
	Rennin	Animal, fungal	
	Pepsin	Animal	
	Lipase, esterase	Animal, fungal, bacterial	
	Lactase	Fungal	
Fruits, fruit products, and wine	Pectinase	Fungal	102
	Pectin methylesterase	Fungal	
	Polygalacturonase	Fungal	
	Glucose oxidase	Fungal	
	Catalase	Bacterial	
	Naringinase	Fungal	
	Hesperidinase	Fungal	
	Pectin glycosidase	Fungal	
	Protopectinase	Fungal	
Distilled alcoholic beverages	α -Amylase	Malt, fungal	15
	Transglucosylase	Fungal	
Beer	α -Amylase	Fungal, bacterial	9
	Amyloglucosidase	Fungal	
	β -Glucanase	Bacterial	
	Proteases	Plant, animal	
Meat and other proteinaceous foods	Proteases	Bacterial, fungal, plant, animal	14
Miscellaneous			
Beer	Diacetyl reductase	Bacterial	115
Tea	Tannase	Fungal	
Beet sugar	α -Galactosidase	Fungal	
"Humanizing cow milk"	Lysozyme	Hen's egg white	
Bean	Stachyase	Fungal	
Shellfish	Cellulase	Fungal	
Sugarcane juice	Dextranase	Bacterial, fungal	

of the bound enzyme can occur due to changes in temperature, pH, ionic strength, or presence of substrate. Further, since the support does not selectively bind the enzyme, other extraneous matter can also be adsorbed onto the matrix which can cause a variety of problems including denaturation of the enzyme.

Entrapment — The enzymes can be trapped by processes such as the enveloping of an enzyme by polyacrylamide gel. This procedure has been delineated by Bernfeld and Wan.¹³ This mode of immobilizing an enzyme provides for a free diffusion of low molecular weight substrates, and end products and enzymes being relatively larger molecules cannot leak out of the beads. Other matrices used to entrap enzymes include polyurethane foams, silastic gels, and starch gels. Since there is no bond formation between the enzyme and the matrix, it would appear that the enzyme is subjected to a minimal of destructive forces. However, in many polymerization processes, free radicals are generated which, in turn, can adversely alter the properties of the enzymes. Although a wide variety of enzymes can be immobilized by entrapment, only low molecular weight substrates can be used in such processes. This may serve as a limitation for its use in food technology, since food systems often contain macromolecules. Theoretically, the pore size of the polymers should prevent leakage of the enzyme; in practice, however, a wide range of pore sizes are encountered which can lead to a loss of enzyme through diffusion.

Microencapsulation — This mode of immobilizing an enzyme described by Chang²⁰ resembles entrapment, but in essence is the formation of very small beads or capsules. The substrate in this instance, as in the previous mode, has to be low molecular weight substrate. It has been reported that the pore size can be varied at will, but more commonly, the envelopes are 200 Å thick, with a pore radius of 18 Å. Microencapsulation overcomes the problem of enzyme leakage because the pore sizes are uniform.

Ion-exchange — An ion-exchanger is an insoluble material containing chemically bound charged groups and mobile counter-ions. The counter-ions can be reversibly exchanged with other ions of the same charge without any changes in the insoluble matrix. Proteins and thus enzymes can carry a charge depending on the pH and type of protein. This property was utilized by Tosa et al.¹³⁵ to link enzymes with ion-exchangers via electrostatic interactions. Although changes in pH and ionic strength can cause leaching of the enzymes, a judicious set of operating conditions can overcome this problem. If the enzyme is denatured after repetitive use, the process of removing the old batch of enzyme and recharging the matrix with a fresh batch can be a relatively simple process.

Cross-linking — Avrameas and Guilbert² developed a process whereby molecules of the enzymes were intermolecularly linked with one another by the use of various bifunctional reagents, e.g., glutaraldehyde. Bifunctional reagents may contain two identical functional groups or two different functional groups or groups of different reactivities. The intermolecular linking of enzyme molecules is inefficient, as part of the enzyme will be functioning as a support, resulting in low activities. Since enzymes are often expensive biochemicals, the inefficient use of enzymes in this process leads to increased cost of the immobilized enzymes.

Adsorption and cross-linking — A combination of two methods reported by Haynes and Walsh⁵² involves the adsorption of the enzyme onto a material, such as silica gel, followed by cross-linking of the adsorbed enzyme. The resulting immobilized enzyme has been reported to be more stable than the enzyme immobilized by either method per se. Goldman et al.⁴⁰ adsorbed papain on collodion and then cross-linked the adsorbed enzyme using bisdiazobenzidine-2,2'-disulfonic acid.

Copolymerization — Polymerization is a process of changing the molecular arrangement so as to form new compounds having the same percentage elemental composition

as the original compound but of greater molecular weight and different properties. Enzymes have been copolymerized with a polymer of the matrix by Levin et al.⁷¹ The enzyme participated chemically in the formation of the copolymer and was thus immobilized. The difference between entrapment and copolymerization is that in the former process, the enzyme is physically confined, whereas, in the latter, the enzyme participates chemically in the formation of the polymer.

Covalent attachment — When two atoms share electrons, the resultant bond between the two atoms is termed a covalent bond. Enzymes have free amino groups, such as the ϵ -amine of lysine or the N-terminal group. They also have free carboxyl groups, such as the carboxyl of glutamic acid and the C-terminal group. By using a suitable matrix and appropriate chemicals, either the free carboxyl or the free amino groups can be reacted to form covalent linkages.^{121,143} The choice of the method is limited by the conditions of the reaction. The preferred reactions should cause minimal alterations to the active center of the enzyme and should be gentle enough to retain high enzyme activities after immobilization. The functional groups of enzymes available for covalent bonding include N-terminal amino groups, ϵ -amino groups of lysine and arginine, C-terminal group, β and γ carboxyl groups of aspartic and glutamic acids, phenol ring of tyrosine, the thiol group of cysteine, hydroxyl group of serine and threonine, imidazole of histidine, and the indole group of tryptophan. Covalent bonding of the enzyme to the matrix can be achieved by allowing them to react with acylating or alkylating agents, aldehydes, isocyanates, and diazonium salts. Some of these reactions will be discussed in greater detail elsewhere in this article. Since covalent bonds are relatively permanent bonds, enzymes immobilized in this manner generally do not leach out.

The aforementioned methods of immobilizing enzymes are illustrated in Figure 1. The question then arises "which method of immobilization should be followed?" Perhaps there is no universal answer to this question. The ultimate goal of any immobilizing process is to obtain maximum enzyme activity per unit of the matrix. In order to achieve this, the process of immobilization must not alter the active site or center of the enzyme, it should not denature the enzyme, and the process must be economical if industrial applications are being considered. Maximum enzyme activity is not synonymous with maximum amount of enzyme bound. In many instances, while greater enzyme loadings are achievable, the efficiency with which the enzyme acts compared to the efficiency of a similar amount of enzyme in solution decreases markedly above certain loadings. This loading limit has to be optimized for each system being considered. Another factor in the choice of the method of immobilizing enzymes deals with the end use for the products resulting from enzyme action. If the enzymatic process is designed for human or animal food uses, then toxic or carcinogenic substances should be avoided in the immobilization process. Also, if the immobilized enzymes are being used to modify foods, the immobilized enzyme should be easily separable and recoverable from the foods. Thus, there is no single method of immobilization that is universally applicable, and a number of factors have to be considered prior to choosing a method.

The commercially patented methods for immobilizing enzymes have been compiled by Gutcho,⁵¹ and a more detailed discussion of the various individual methods for immobilizing enzymes appears in a book edited by Messing.⁹³ The carriers (supports and matrix are also used as synonyms for carrier) have been broadly divided into two categories, namely, organic and inorganic. Some of the organic carriers used are cellulose,^{117,151} agarose,^{4,38} collodion,^{40,41} starch,¹⁰ polyacrylamides,¹⁷ dextran,³ nylon,⁵⁴ collagen,⁶⁰ copolymers, such as maleic anhydride and ethylene,⁶² and DEAE-cellulose.¹³⁴ A sampling of the inorganic supports used to immobilize enzymes revealed

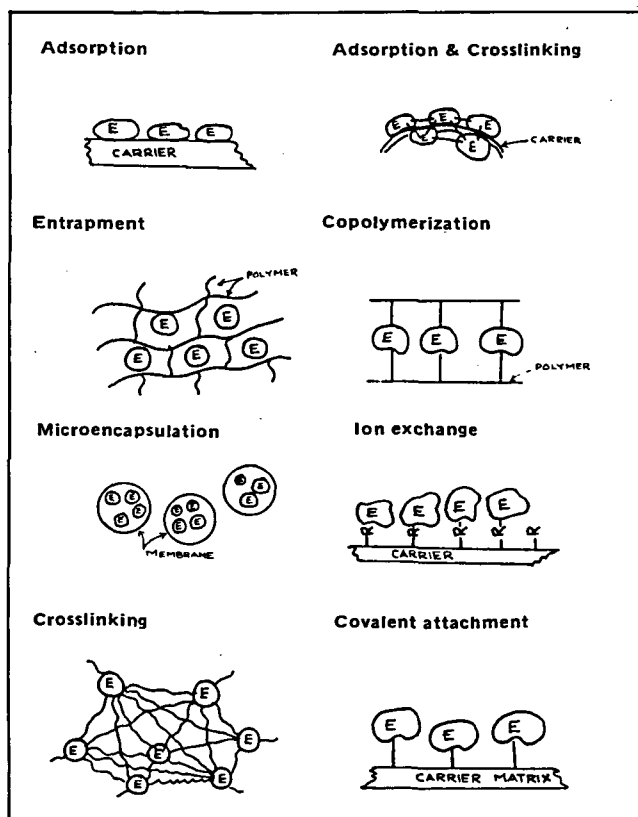


FIGURE 1. Different methods of immobilizing enzymes.

materials, such as kaolinite,^{86,87} colloidal silica,⁵¹ glass particles,⁵⁶ controlled-pore glass,⁸⁹ alumina,¹³² controlled-pore alumina,⁹⁰ controlled-pore titania,¹⁰¹ nickel oxide,¹⁴³ controlled-pore zirconia,⁹⁰ charcoal,¹⁰¹ hydroxyapatite,¹³⁷ and iron oxide.³⁷ Brown and Hasselberger¹⁶ have reviewed the immobilization of specific types of enzymes.

The choice of a carrier is dependent upon certain factors which play a vital role in industrial processing applications. According to Messing,⁹³ these factors are

Mechanical strength — The carrier should be able to withstand compression and shear or stress forces. These forces, if not controlled, can lead to the denaturation of the enzyme. The carrier should thus be able to absorb the pressure forces which normally may be exerted on the enzyme itself.

Microbial resistance — Continuous reactors are operated for extended periods of time at temperatures optimal for enzyme activity. Often this temperature coincides with the temperature optimum for the growth of microorganisms. Substrates, especially if food systems are considered, are rich sources of nutrients for the growth of organisms. The repercussions of microbial growth may thus be manifested in a number of forms, such as plugging of columns, degradation of the enzyme through microbial metabolism, degradation of the carrier, and microbial contamination of outgoing products. The latter point is of particular significance to the food industry because the deleterious effects of contamination may lead to a loss of shelf life and unsafe foods if the organisms are pathogens.

Thermal stability — Thermal stability has a direct bearing on the dimensional sta-

bility of enzymes. One aspect deals with the coefficient of expansion of the carrier; the larger the coefficient of expansion of the carrier, the greater the distortion of active sites and centers of the enzyme. A second aspect involves denaturation of the protein. Generally, thermal denaturation of the immobilized enzyme is less likely to occur because the operational temperature range of immobilized enzyme reactors is confined to relatively narrow temperature range below the temperature for denaturation.

Chemical durability — The carriers should be able to withstand a wide variety of solvents. This point becomes crucial when the investigator subjects the matrix to many solvent systems for the purposes of derivatizing the support or in reactions which prepare the carrier to bind the enzymes. Of particular importance in this respect is the process of covalent attachment of enzymes to matrix which often necessitates the use of organic solvents.

Additionally, Royer and Andrews^{112,113} considered some other aspects for choosing carriers, as follows:

Chemical functionality — The chemical reactivity of the matrix should be sufficiently general to permit reactions with a wide variety of groups in proteins.

Low cost — Obviously, in some cases, the large-scale applicability of immobilized enzymes could be limited by the cost of the carrier itself. The ligand or pure enzyme in most instances is relatively expensive, and an expensive matrix would further increase the cost of the immobilized enzyme.

Hydrophilicity — Direct attachment of the enzyme to the matrix may limit access of the substrate to the active center of the enzyme through stereochemical hindrance. For this reason, the support is derivatized to provide a hydrocarbon arm. The enzyme is then attached to the arm. The length of the hydrocarbon arm is determined by the size of the macromolecule being attached. It should be cautioned that hydrophilicity of the arm plays an important role in maintaining biological activity. Some proteins denature at the hydrocarbon-water interface.⁹⁸

Regeneration — This is another important factor which aids the economic feasibility of large-scale immobilized-enzyme reactors. If for some reason the enzyme should denature after immobilization or after limited use, the capability of the carrier to be regenerated to bind a new charge of enzyme will aid in reducing operational costs of the process.

High capacity for enzyme — The matrix should have a large number of sites available for the attachment of the enzyme. The greater the amount of enzyme attached per unit weight of the matrix, the greater will be the capacity of a given quantity of the immobilized enzyme to convert substrates.

While all of these factors do not occur in any known single matrix, the selection of a carrier should be based on an optimization of these considerations. Two commonly used supports are agarose and glass beads, and neither meets all of the above requirements. Agarose, for example, is compressible, expensive, and susceptible to microbial attack. Fluidized-bed reactors may overcome the problem of compressibility, and the intermittent flushing of reactors with antibacterial agents may limit the hazards of microbial growth. Porous glass, on the other hand, may have many good qualities listed, but its cost for high-volume use can be prohibitive. Derivatives of phenol-formaldehyde resins and conjugates of various polymers with silica beads have been discussed as alternates to "ideal" supports by Royer.¹¹²

After the selection of a carrier or matrix, the method of immobilizing the enzyme must be considered. Covalent bonding or linking of enzymes to supports has been most widely investigated due to the stability offered by covalent bonding. Some of the common methods for immobilizing enzymes through covalent linkage to organic carriers are as follows:

Carboxymethylcellulose-azide reaction — This usually involves the ϵ -amino group of lysine and possibly amines of tyrosine, cysteine, and serine.^{30,73,95} The reaction is illustrated in Figure 2A. Carboxymethylcellulose is used as an acylating agent by the reaction of the acid group with hydrazine to obtain an acyl hydrazide which is then treated with nitrous acid, resulting in a reactive acid azide derivative. The coupling of the protein occurs at alkaline pH values of 8 to 9 — a pH which approximates the pK_a of the amino groups mentioned above.

Carbodiimides — The basicity of amines determines the formation of amide linkage with carboxyl groups which have been activated by carbodiimides.^{74,151} The reactions are carried out under relatively mild conditions, and both water-soluble and water-insoluble carbodiimides can be used (Figure 2B). In most cases, the matrix is in the carboxylated form to react with the amino group of enzymes to be immobilized.

Diazotization — Various supports have been converted to polydiazonium salts which are then used for coupling phenolic residues of amino acids (Figure 2C). Diazonium chloride derivatives of the matrix can be prepared with sodium nitrite and hydrochloric acid. Reduction of the azo-linkage gives rise to hydrazo compounds or upon complete hydrolysis will yield two primary amines.^{19,28,80,141,142,150}

Glutaraldehyde — Glutaraldehyde is a bifunctional reagent, and the reaction involves the formation of a Schiff's base.¹⁴⁹ The complete mechanism of the reaction is not well understood, but the reaction is gentle (pH 7.0), and the introduction of a washing step minimizes the chance of cross-linking. Thus, glutaraldehyde facilitates the reaction between the aminated carrier and the amine group of the enzyme (Figure 2D).

Cyanogen bromide activation — This method is most commonly used for the activation of cross-linked dextrans (such as Sephadex®) or other polyhydric compounds, such as agarose and cellulose. It offers the versatility of directly attaching the enzyme to the matrix (Figure 2E) or attaching the enzyme indirectly through an intermediate molecule at a pre-chosen distance from the matrix.^{3,4,98} The coupling usually occurs through the ϵ -amino group of lysine. When a hydrocarbon arm is interposed between the matrix and the ligand, the schematic is changed as in Figure 2F. The proposed mode of action of cyanogen bromide involves the formation of a hydrocyanate ester which is then converted to a cyclic imidocarbonate with an adjacent hydroxyl group which can further react with ligands.

Often a combination of these reactions are employed. For example, agarose can be activated with cyanogen bromide, and the ligand can be attached through either the carbodiimide procedure or through diazotization.²⁵

Using glass as an example of an inorganic carrier, the following are some of the common routes of achieving covalent attachment of enzymes:

Silanization of the carrier — This is the first step in the preparation of glass for immobilizing ligands and consists of a treatment with an organo-functional silane.^{141,142} For instance, if γ -aminopropyltriethoxy silane is used in this reaction, the silane reacts with the available silanol or oxide groups on the carrier thereby leaving the functional group available for coupling (Figure 3A). The amino derivative thus generated can be covalently coupled to proteins by a wide variety of methods, including carbodiimide, glutaraldehyde, and isothiocyanate reactions.

Arylamine derivatization — The alkylamine can be modified to an arylamine derivative which can then be used to couple proteins via the azo linkage^{141,142} as in Figure 3B.

Carboxyl derivative — If alkylamine glass is reacted with succinic anhydride, a carboxyl derivative can be formed.⁸³ The protein can then be coupled by carbodiimide, acid chlorazide, or the azide methods (Figure 3C).

Cyanogen bromide on glass carriers — The method for glass carriers is analogous to the activation of organic matrices, and the process is schematically illustrated in Figure 3D.

It should be emphasized that not all the known reactions for covalent attachments have been considered in this section. The processes cited are to serve as examples of some of the various means available for achieving the covalent immobilization of enzymes. The reaction chemistry for many other cases has been delineated. In any process, however, the mode of immobilization and the chemistry used to achieve this immobilization directly alter the properties of the immobilized enzyme. Therefore, it would be ideal to select a process which introduces minimal changes in the structure, functions and properties of the enzyme.

Effects of Immobilization on the Operating Parameters of Enzymes

Once an enzyme has been immobilized, a meticulous investigation should be undertaken to determine the effects of immobilization on the operational parameters of the enzymes. The operational parameters most frequently investigated are pH and temperature of assay, thermal stability, kinetic parameters, and operational half-life. The process of immobilization can affect the above parameters while altering substrate, activator, and ionic strength requirements also. The main difference between a soluble enzyme and its immobilized state is that the enzyme, once immobilized, is no longer completely surrounded by an aqueous environment. In other words, there is no guarantee that the conditions in the immediate vicinity of the enzyme are the same as those in external solution.^{59,93} This situation parallels the existence of enzymes *in vivo*, where very few enzymes exist as free molecules in an aqueous environment. More often, enzymes *in vivo* are membrane bound or associated with organelles, such as the mitochondria. These conditions give rise to phenomena referred to as microenvironmental effects. Srere and Mosbach¹²¹ attributed these microenvironmental effects to two principal factors: (1) the chemical nature of the carrier and (2) the local concentrations of substrates and products which directly affect the activity of the enzyme through mechanisms, such as substrate or end-product inhibitions.^{119,128}

Microenvironmental matrix effects arise from the chemical and physical nature of the matrix *per se*. In a biological system, membrane-bound enzymes are in an environment where the surface is highly charged. The charges on membranes result from phospholipids, mucoproteins and mucopolysaccharides, glycopeptides, and lipopolysaccharides.⁶¹ In immobilized enzyme systems, charged matrices have been used to adsorb enzymes. McLaren and Estermann^{86,87} studied the action of chymotrypsin, using lysozyme as the substrate. Both the enzyme and the substrate were adsorbed on Kaolinite particles which carry a high negative charge. For comparative evaluations, the action of chymotrypsin on lysozyme, both in solution, was investigated. The pH value required for half maximum activity of the immobilized system was shifted by two units towards the alkaline side when compared with the soluble system. It was felt that the hydrogen-ion concentration on the surface of the ionizable Kaolinite particles was greater than that in the surrounding medium. This localized increase in hydrogen-ion concentration may have resulted in the observed pH shift.⁸⁷ Several proteolytic enzymes were immobilized on both polyanionic and polycationic carriers, and the pH activity profiles of these immobilized proteases were determined using low molecular weight substrates.^{43,45} The polyanionic-bound proteases showed a pH shift of 1 to 2.5 units towards the alkaline side when the ionic strength of the assay system was 0.01. The polycationic-bound proteases exhibited a pH shift of similar magnitude, but towards the acidic side. If, however, the ionic strength of the assay system was increased to 1.0, these pH shifts could no longer be observed. Similar polyelectrolyte effects of

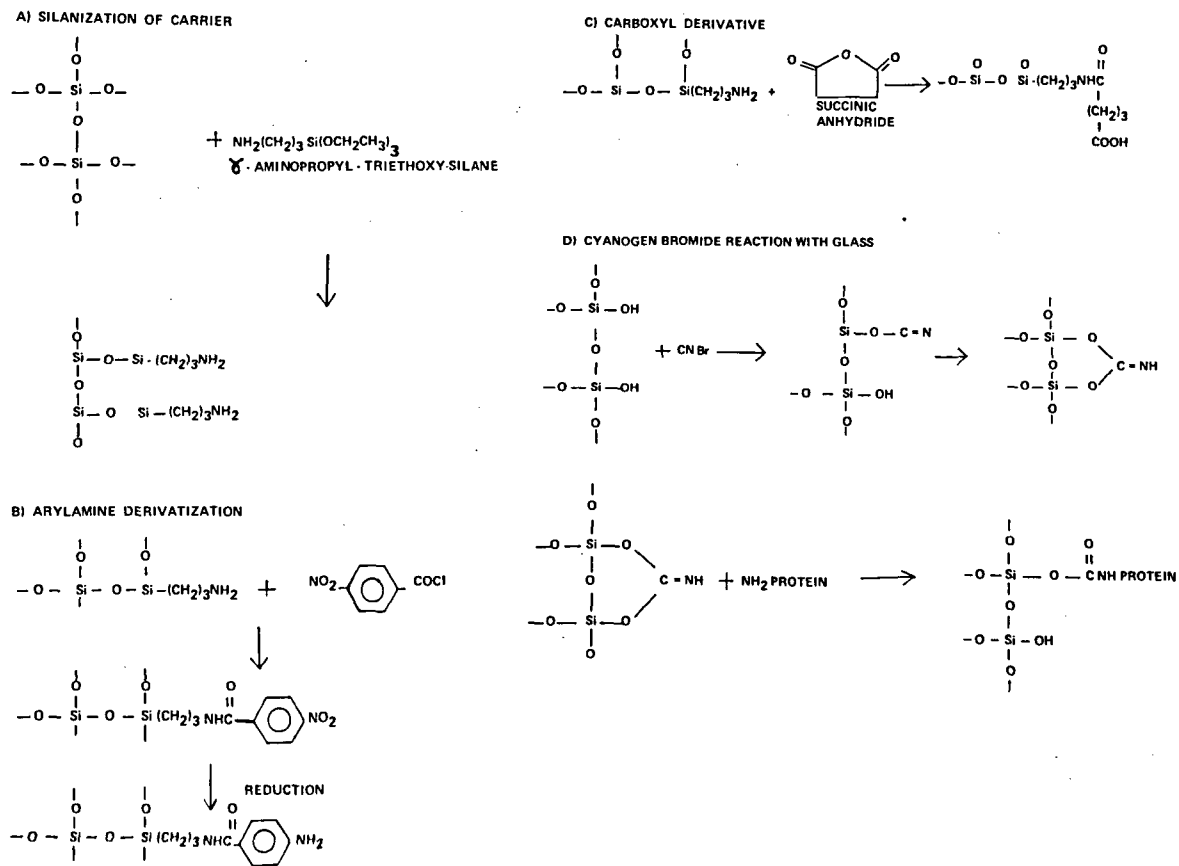


FIGURE 3. Schematic representation of covalent bonding of enzymes to inorganic matrices (using glass as an example).

the carrier on pH-activity profiles have been demonstrated by Suzuki et al.¹²⁵ and Chung et al.²³ Suzuki et al.¹²⁵ adsorbed invertase on DEAE-cellulose and observed that the bound enzyme had a pH optimum of 3.4 as compared to the pH optimum for soluble enzyme which was pH 5.4. When ATP deaminase was adsorbed on DEAE-cellulose,²³ the pH optimum for the immobilized enzyme (pH 3) was two units lower than that of the soluble enzyme (pH 5). These changes in pH optima are more pronounced if the matrix is highly charged, and the phenomena have been explained on the basis of unequal distribution of hydrogen and hydroxyl ions and of charged substrates between the surface of the immobilized enzyme and the outer solution.⁴⁵ It is interesting to note that pH optimum shifts can also occur in cases where enzymes have been immobilized by covalent attachment to neutral carriers.^{4,45} Axén et al.⁴ immobilized α -chymotrypsin onto Sephadex®. Sephadex® by itself is electrically neutral, but upon activation with cyanogen bromide assumes a slight net negative charge. The pH optimum of α -chymotrypsin so immobilized was shifted to alkaline side when compared to the pH optimum of soluble chymotrypsin. Using diazotized dialdehyde starch-methylenedianiline resins (S-MDA), Goldstein et al.⁴⁵ covalently bound polytyrosyl trypsin, papain, and subtilopeptidase. S-MDA is an uncharged carrier, but the immobilized enzymes had displaced pH optima toward the alkaline side when compared to their respective soluble forms. These observations were explained on the basis of the net residual charge on the enzymes postimmobilization. Both derivatized matrices, imidocarbonate form of Sephadex® and the diazotized S-MDA, carry slightly net negative charges which may tend to be neutralized by interaction with the positively charged groups on the enzyme. This, in turn, results in an enzyme molecule with less positively charged groups, causing a shift in the pH-activity profile towards the alkaline side. The significant difference between the pH shifts observed with enzymes immobilized on charged matrices and enzymes immobilized on uncharged matrices was that in the former case, high ionic strengths eliminated these shifts; whereas, in the latter instances, ionic strength did not influence the magnitude of the shifts. It is generally believed that the pH shifts observed with enzymes immobilized on uncharged carriers are generated by localized electrostatic interactions induced perhaps by the chemistry of the immobilization process.^{4,45}

The matrix used for immobilizing the enzyme may also generate kinetic changes. One important kinetic parameter in enzymology is designated as the Michaelis Constant (K_m) and is defined as the substrate concentration required to achieve one half of the maximal velocity (V_m). The kinetics of many enzymatic reactions can be described by the Michaelis-Menten equation

$$\frac{-ds}{dt} = \frac{K.E \times S}{K_m + S} = \frac{V_m \times S}{K_m + S}$$

Where E and S are concentrations of enzyme and substrate, respectively, and V_m or K.E. represents the reaction rate when the substrate concentration is much higher than K_m , a constant characteristic of the reaction. The values of both V_m and K_m are pH dependent. In the case of immobilized enzymes, V_m and K_m depend not only on the nature of the enzyme and substrate and external pH, but also on the nature of the carrier and its particle size, enzyme concentration, and also the concentration, charge, and molecular dimensions of all species involved directly or indirectly in the reaction. Since many of the supports used for immobilizing enzymes are porous, the process of diffusion plays an important role in the determination of kinetic parameters. Immobilized-enzyme particles are enveloped by a quiescent layer of solvent referred to as the Nernst layer which leads to a substrate concentration gradient across this layer. This

concentration gradient, in turn, is responsible for the higher substrate concentration required to saturate the immobilized enzyme when compared to the soluble form of the enzyme. These higher substrate concentrations are reflected in higher K_m values. Since this requirement for higher substrate concentrations results from the Nernst layer rather than a chemical modification of the active center, the K_m values must be referred to as K_m apparent or $K_m(\text{app.})$. If particle size of the immobilized enzyme can be reduced or if the substrate and enzyme were brought in thorough contact by agitation, then the $K_m(\text{app.})$ value can be lowered to approximate the true K_m of the enzyme. Thus, in kinetic investigations of immobilized enzymes, care should be exercised in interpreting the results obtained. Genuine changes in kinetic parameters must be differentiated from those arising from diffusional or other limitations.

Goldstein et al.,^{42,43} using polyanionic derivatives of trypsin under low ionic-strength conditions and positively charged low molecular weight substrates (benzoyl-L-arginine amide), observed that the $K_m(\text{app.})$ of immobilized trypsin decreased by more than one order of magnitude. The $K_m(\text{app.})$, with uncharged substrates or under high ionic strength conditions, remained unaltered. Thus, low ionic strength and substrates with a charge opposite to that of the matrix were found to be critical factors in altering the $K_m(\text{app.})$. From the shifts in pH-activity profiles observed and assuming a Maxwell-Boltzmann distribution of charge, they calculated the electrostatic potential existing in the polyelectrolyte-enzyme phase. The electrostatic potential was also calculated by insertion of the Maxwell-Boltzmann distribution into the Michaelis-Menten equation using the changes in $K_m(\text{app.})$. A close agreement was obtained in the electrostatic potentials calculated by either of the two methods, lending support to the observation that when the substrate is positively charged and the carrier is negatively charged or vice versa, then $K_m(\text{app.}) < K_m$. If, however, the substrate and the polyelectrolyte-enzyme conjugate carry the same charge, then the $K_m(\text{app.})$ will be greater than the true K_m of the enzyme.⁴¹

In the case of uncharged matrices, Axén et al.⁴ covalently bound α -chymotrypsin to Sephadex® activated a priori with cyanogen bromide. Sephadex® had been activated at two different pH values of 10.3 and 9.8. With N-acetyl tyrosine ethyl ester (ATEE) as the substrate, chymotrypsin bound to Sephadex® activated at pH 10.3 showed a $K_m(\text{app.})$ of 25 to 30 mM. The $K_m(\text{app.})$ value for α -chymotrypsin bound to Sephadex® activated at pH 9.8 was in the 30- to 40-mM range, while the K_m for the soluble enzyme was 3.3 mM. Sephadex® was then solubilized by the action of the enzyme dextranase, and the $K_m(\text{app.})$ values for the immobilized chymotrypsin dropped markedly to approximate the true K_m of the enzyme.

Hydrophobicity of the carrier can also generate differences in $K_m(\text{app.})$ values. When alcohol dehydrogenase was immobilized by copolymerization with acrylamide and methacrylate, the degree of hydrophobicity of the enzyme-copolymer could be varied.¹²¹ Using *n*-butanol as the substrate, it was observed that as the hydrophobicity of the copolymer increased, the $K_m(\text{app.})$ values decreased.¹²¹ Apart from these matrix-generated changes in the properties of the enzyme, the matrix may also be responsible for inducing changes in the medium surrounding the matrix. Such changes may be manifested by altered solvation of reactants and intermediates as would occur in a medium containing both polar and nonpolar solutes. Since experimental proof of this phenomenon is lacking, it has been hypothesized that a hydrophobic matrix would attract a more lipophilic medium because of its low dielectric constant.¹²¹

Next, the microenvironmental effects due to enzymic activity should be considered briefly. It has been explained by Srere and Mosbach¹²¹ that the activity of an enzyme or its neighboring enzymes could alter the microenvironment by creating different local concentrations of substrate, product, proton, effectors, etc. Enzyme action can gen-

erate pH gradients within the immediate vicinity, and the pH-activity curves can thus be displaced. The degree of shift in the pH activity is dependent on the activity of the enzyme and the rate of diffusion of the substrate and end product from the site of the reaction.¹⁵⁸ Although more experiments demonstrating this pH shift have involved a membrane-bound enzyme system, evidence for such pH-activity displacements have been delineated in other systems as well. Thomas et al.¹²⁸ cross-linked urease into plasma albumin and evaluated the properties of the immobilized enzyme. Urea, a weak monoacid, was used as the substrate and the formation of ammonium carbonate (an ionized product) was determined by a pH-stat method. A substantial increase in pH in the interior of the membranes was observed. Katchalaski and associates^{41,61,120} used papain immobilized on collodion membrane with a proton-generating substrate and observed a substantial decrease in pH in the interior of the membrane. The substrate used was benzoylarginine ethyl ester and the membrane-bound papain could increasingly hydrolyze the substrate up to pH 9.6, whereas the soluble papain had a characteristic bell-shaped pH-activity profile with optimal activity occurring at pH 6.5. The difference in apparent activities was attributable to the accumulation of protons within the membrane, thereby creating a difference of several pH units between the interior and the exterior of the membranes.¹²⁰ Axén et al.⁴ immobilized several proteases, including trypsin and chymotrypsin, on cyanogen halide activated agarose. The pH-activity profiles for these immobilized proteases were narrower and more alkaline than those obtained with soluble enzymes. For example, when ATEE was used as the substrate for chymotrypsin, the pH optimum for immobilized chymotrypsin was pH 9.7, whereas that for the soluble form of the enzyme was pH 8.0. When a macromolecular substrate, such as casein, was used, the pH optima of the immobilized proteases were not altered significantly. It was felt that with synthetic substrates, such as ATEE, the generation of protons was very rapid and thus led to the establishment of a pH gradient. If the generation of protons could be minimized by using suitable substrates, such effects would not be manifested.

These investigations measured the effect on an enzyme by changing proton concentrations resulting from activity of an enzyme. Since many enzymes produce or utilize protons, it is conceivable that such action could modify or regulate activities of neighboring enzymes in multienzymic systems. Other factors have also been shown to contribute to the microenvironment of an immobilized enzyme. However, these deal with diffusional influences or mass transfer characteristics and have been accorded extensive theoretical treatment.^{69,72,104,119}

Weetall¹⁴⁶ compiled the literature values for $K_m(\text{app.})$ of immobilized enzymes which indicated them to be higher than the K_m of their respective soluble forms (Table 2). There were also instances where $K_m(\text{app.})$ values remained unchanged or decreased. Presently, there is no method to predict the effect of immobilization on the properties of the enzymes. This must be determined experimentally for each given set of conditions. Thus far, the discussion has dealt with effects of immobilization on pH-activity profiles and the kinetic parameters. Another important parameter for immobilized enzymes is the operational half-life.

The half-life of an immobilized enzyme may be defined as the time required to lose one half of the initial activity. It is an important parameter for determining commercial utility of an immobilized enzyme system. Half-life for soluble enzymes used in food processing is of no value because after achieving the desired conversion, they are destroyed or denatured. Since it is the intent to reuse immobilized enzymes, half-life is a very useful parameter. To calculate this, a given amount of immobilized enzyme is used in a continuous reactor under constant-flow-rate conditions. The amount of substrate being converted or the amount of product being formed can be measured as a function of time to determine the enzyme activity in the column. Then a semilogar-

TABLE 2

 K_m Values of Some Soluble and Immobilized Enzymes^{63,64,145}

Enzyme	Substrate	K_m (mM)	
		Soluble	Immobilized
Alkaline phosphatase	<i>p</i> -Nitrophenyl phosphate	0.10	2.90
L-Amino acid oxidase	L-Leucine	1.00	4.00
Arylsulfatase	<i>p</i> -Nitrophenyl sulfate	1.85	1.57
Glucoamylase	Starch	1.22	0.30
Glucose oxidase	Glucose	7.70	6.80
Invertase	Sucrose	0.448	0.448
Lactase	ONPG*	3.62	3.62
Lactase	Lactose	39.80	25.70
Papain	BAEE*	0.0519	0.0687
Urease	Urease	10.00	7.60

* ONPG = O-nitrophenyl- β -D-galactopyranoside* BAEE = α = N-benzyl-L-arginine ethyl ester hydrochloride

ithmic plot of activity (on the log scale) vs. time (on linear scale) can be obtained. Assuming that reaction is of the first order, then the slope of this line will be $-k/2.303$, where k is the rate constant. The half-life, in turn, can be calculated by the relationship

$$k = \frac{0.693}{t_{1/2}}$$

where k = rate constant determined from the slope of the plot, and $t_{1/2}$ is the half-life. The half-life of an immobilized reaction is a function of temperature. Using zirconium oxide (ZrO_2) coated glass beads of 40- to 80-mesh size and a mean pore diameter of 550 Å as the support, Weetall¹⁴⁶ immobilized five enzymes. These immobilized enzymes were then used to determine half-lives with the appropriate substrates and temperatures (Table 3). The immobilized glucoamylase at 45°C, using starch as the substrate, had an extremely long half-life of 645 days. This is of great practical significance, but is not a common feature of immobilized enzymes. To illustrate the temperature dependence of half-life, Weetall et al.¹⁴⁶ determined that at 60°C the operational half-life for glucoamylase was 15 days; however, as the operational temperatures were lowered, the half-life increased so much that at 40°C the half-life was 900 days. As was expected, the reaction rate dropped with the reduction in temperature. Taking the activity of immobilized glucoamylase at 60°C to be 100%, the activity at 40°C was 25% of the original. It was felt that the substantial increase in half-life offset the disadvantage of a lowered reaction rate.

Since half-life of an immobilized enzyme is temperature dependent, the thermal stability of the immobilized enzyme should be determined. Thermal stability can be determined by keeping the temperature constant and varying the time or by varying the temperature and keeping the time constant. The relative merits and demerits of these methods are beyond the scope of this investigation. For illustrative purposes, the temperature dependence of reaction-rate constants will be briefly described. Aliquots of immobilized enzyme are tempered to different temperatures, and the activity of the enzyme is determined. The time of reaction of enzyme with substrate is kept constant.

TABLE 3
Half-lives of Some Enzymes Immobilized on Porous Glass.^{145,146}

Enzyme	Temperature (°C)	Substrate	Half-life (days)
Alkaline phosphatase	23	<i>p</i> -Nitrophenyl phosphate	55
Lactase (yeast)	50	Lactose	20
Glucoamylase	50	Starch	100
Papain	45	Casein	35
Pepsin	—	Milk	>30

Then a graph can be plotted between the log of the activity vs. the reciprocal of the absolute temperature. The dependence of the specific reaction rate on temperature is given by the Arrhenius equation where

$$k = Ae^{-E_a/RT}$$

$$\text{of } \log k = \log A - \frac{E_a}{2.303 RT}$$

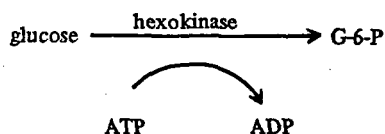
or integrating the equation between limits of k_1 and k_2 at T_1 and T_2 to obtain

$$\log \frac{k_2}{k_1} = \frac{E_a}{2.303 R} \frac{T_2 - T_1}{T_2 \cdot T_1}$$

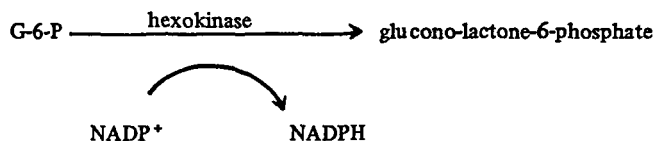
where k_1 is the reaction rate constant at absolute temperature T_1 and k_2 is the reaction rate constant at absolute temperature T_2 . E_a is usually referred to as the activation energy, and any parameter proportional to k (the reaction rate constant) may be substituted in the equation above. Since activity of the enzyme would be proportional to k , the plot of log activity vs. reciprocal temperature in absolute degrees would give a line the slope of which will be $E_a/2.303$. Thus, the activation energy for the immobilized enzyme can be calculated and compared with the values for soluble enzymes. In some instances where temperature is increased beyond certain limits, there are deviations in the straight line plot. These deviations do not necessarily manifest enzyme denaturation, but could occur due to a limitation of substrate or end product inhibition. At higher temperatures, the enzyme turnover rate exceeds the diffusion rate of substrates and end products.¹⁴⁶ It has been suggested that plotting half-life as a function of reciprocal temperature in an Arrhenius-type plot can permit the extrapolation of half-lives to any desired temperature. Generally, as a result of immobilization, the thermal stability of enzymes is enhanced. Increased temperatures may result in the expansion of the matrix which can contribute to the denaturation of the enzyme. Other important parameters that must be determined for an immobilized enzyme system are pH and temperature stabilities which will be useful in understanding the storage stability of the bound enzymes. It should be remembered that every enzyme parameter may be affected by the immobilization process. Only a very careful study of a particular system and a thorough understanding of the physical and chemical properties of the system in question will permit the determination of the precise alterations to the enzymes due to immobilization. Evaluation of the microenvironmental effects on an immobilized multienzyme is a more complex task.

Immobilized Multienzymes

Immobilized multienzymes refers to the immobilization of more than one enzyme on the same support. Even though the concept is not new, experimental work in this area is relatively recent. In 1968, Brown et al.¹⁷ studied several entrapped glycolytic enzymes, with each enzyme being arranged in a separate section within a column. Also in 1968, Wilson et al.¹⁵⁴ reported kinetic investigations on a two-enzyme system consisting of pyruvate kinase and lactate dehydrogenase, with each enzyme being bound to separate filter paper discs. Until 1970, no reports had appeared on the binding of more than one enzyme on the same matrix surface. In nature, however, the existence of multienzyme systems includes (1) enhanced substrate transfer efficiencies; (2) production, enhancement, or inhibition of reactions by aggregation; (3) stabilization of intermediate; (4) sharing of cofactors; (5) unidirectional reactions or controls; (6) establishment of a hydrogen ion gradient or a redox potential; and (7) provision of hydrophobic areas of reactions.⁵⁵ The enzyme aggregates in nature carry out sequential reactions; i.e., the product of one enzyme reaction serves as the substrate for the next reaction and so on. In all such processes, the influences due to diffusion of substrate and/or products can be minimized greatly. In an attempt to simulate *in vivo* conditions, Mosbach and Mattiasson⁹⁸ initiated studies on immobilized multienzymes. In this study, hexokinase and glucose-6-phosphate (G-6-P) dehydrogenase were simultaneously immobilized on agarose or cross-linked copolymer of acrylamide and acrylic acid. Hexokinase catalyzes the reaction:



using ATP to derive phosphorylation. G-6-P dehydrogenase catalyzes the reduction of G-6-P to gluconolactone-6-phosphate.



Thus, the products of the first reaction serve as the substrate for the second reaction. These two enzymes were also individually immobilized on the two supports. To determine the activity of hexokinase a coupled-enzyme assay was used. Glucose and ATP were provided as the substrate and an excess of soluble G-6-P dehydrogenase and NADP⁺ were added to the reaction mixture. The formation of NADPH was monitored spectrophotometrically by recording the increase in absorbance at 340 nm. For determining the activity of the immobilized two-enzyme system, the substrate solution contained glucose, ATP, and NADP⁺, and a coupled assay was utilized following which individual activities of each enzyme were determined. Soluble enzymes were used for comparative purposes. Taking the number of moles of NADPH formed per minute per milliliter in the soluble system as 100%, an increase of 40 to 100% in the formation of NADPH/min/ml with the immobilized two-enzyme system was observed with agarose as the matrix. This indicated that the G-6-P formed was being immediately transformed to the lactone before it could equilibrate with the surrounding medium. Alternately, it could be inferred that the localized concentration of G-6-P in the vicinity of

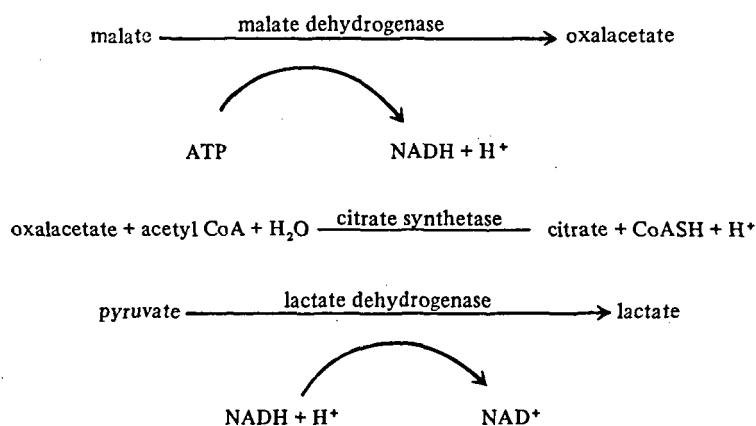
G-6-P dehydrogenase molecules was higher in the immobilized two-enzyme system than in the corresponding soluble system. When the formation NADPH/ml was plotted as a function of time, it was observed that all immobilized-enzyme systems reached their maximal velocity, while the corresponding soluble systems were still in their lag phase.

In another study by Mattiasson and Mosbach,⁵⁵ three enzymes were simultaneously immobilized on Sephadex® G-50. The enzymes were β -galactosidase, hexokinase, and G-6-P dehydrogenase. β -Galactosidase is responsible for the hydrolysis of lactose to its constituent monosaccharides, glucose, and galactose. Glucose thus generated could be phosphorylated by hexokinase, and the resulting G-6-P could be reduced to the lactone formed by G-6-P dehydrogenase. The results from this investigation revealed that prior to reaching steady state, the rate of the coupled reaction carried out by the immobilized three-enzyme system was markedly higher than that catalyzed by the corresponding soluble system. This increase in the efficiency of substrate conversion was deduced to be a cumulative one rather than the increase in efficiency of any single given component.

Gestrelus et al.³⁸ entrapped glucose oxidase, hexokinase, trypsin, and urease either separately or together in polyacrylamide beads. The pH optima of the immobilized enzymes were displaced compared to those of the enzymes in solution. Using benzoyl-L-arginine ethyl ester (BAEE) as the substrate for trypsin, the pH optimum for immobilized trypsin was observed to be pH 9.6 as compared to the value of pH 8.3 for soluble trypsin. Similarly, the pH optima of glucose oxidase, using glucose as the substrate, were pH 6.9 and 6.6 for the immobilized and soluble forms of the enzyme, respectively. While these two enzymes showed pH shifts to the alkaline side, the pH optimum of immobilized urease was displaced 0.4 unit to the acidic side, to pH 5.8, in relation to the pH optimum of soluble urease. They also studied the effects on coentrapping trypsin, urease, and glucose oxidase on the pH-activity profiles of the enzymes. The reaction of trypsin with BAEE leads to the generation of protons, and the glucose oxidase converts glucose to gluconic acid, producing hydrogen peroxide. The reaction of urease with urea produces CO₂ and ammonia, leading to a consumption of protons in the reaction mixture for neutralizing ammonia. When BAEE was added along with glucose, the glucose oxidase activity in the alkaline pH ranges (pH >7.0) was stimulated. If the activity of the coentrapped glucose oxidase at pH 6.9 was taken to be 100%, then during simultaneous trypsin and glucose oxidase reactions at pH 8.6, the glucose oxidase retained 75% of its original activity. In a system where glucose oxidase and urease were active, then at pH 6.0, glucose oxidase retained 80% of its activity. Soluble glucose oxidase at pH 8.6 retained only 25% of the activity, and at pH 6.0 only 20% of the activity of that observed at pH 6.9. When the effects of pH on simultaneous action of all three enzymes, glucose oxidase, urease, and trypsin, were tested, then a "two-peak" effect was observed. All these observations were valid only when low ionic strength 5-mM buffers were used in assays. Increasing buffer strength to 100 mM, for example, eliminated these effects, suggesting that this category of reactions were similar to the ones discussed earlier in the preceding section based on Katchalski's work.⁶¹ In the same study,³⁸ a model system containing coentrapped glucose oxidase, hexokinase, and trypsin was studied. Both hexokinase and glucose oxidase require the same substrate, glucose. At an assay pH of 8.6 (optimum for hexokinase), 15% of the added glucose converted was phosphorylated and the remainder was oxidized by glucose oxidase (pH optimum 6.6). A trypsin substrate (BAEE) was then added which upon hydrolysis produced protons in the microenvironment of the two glucose utilizing enzymes. The external pH of the reaction mixture was kept constant at pH 8.6 using a pH-stat. The acidification of the microenvironment through

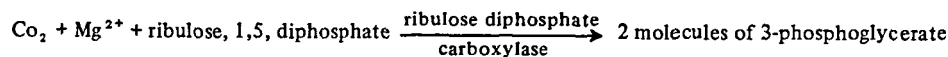
trypsin activity caused a simultaneous increase in glucose oxidase activity so that all of the available glucose was then oxidized. The salient features of this study were to demonstrate the action of protons as regulators of enzymic activities and to further reinforce the observations made earlier by Katchalski's group.⁶¹

In recent reports by Mosbach et al.⁹⁹ and Srere et al.,¹²² an immobilized three-enzyme system consisting of malate dehydrogenase, citrate synthetase, and lactate dehydrogenase was studied as a model for the rate of oxaloacetate production and utilization in the mitochondria. The reactions catalyzed by these enzymes occur in the citric acid or tricarboxylic acid cycle and can be written as follows:



Since the malate dehydrogenase reaction utilizes NAD, the lactate dehydrogenase was included to regenerate NAD, thereby simulating reoxidation of NADH which occurs in the mitochondria. Four different immobilized malate dehydrogenase-citrate synthetase-lactate dehydrogenase systems were tested, and the rates of citrate formation in all cases were faster than that observed in a corresponding soluble system. Addition of pyruvate further enhanced the rate of citrate formation in the immobilized enzyme systems, whereas the rate of citrate formation was unaffected in the soluble-enzyme system. These experiments were conducted using 100-mM buffers sufficient to negate microenvironmental pH effects, and the enhancement in rate of citrate production was dependent on the ratios of the three enzymes used.

In a similar study, ribulose diphosphate carboxylase and urease were found in the vicinity of one another.⁵⁹ The enzyme ribulose diphosphate carboxylase participates in CO₂ fixation in the "dark reaction" of photosynthesis. The reaction can be written as follows:



The enzyme obtained from spinach had a molecular weight of 550,000, and the K_m for HCO₃⁻ was 22 mM. The true substrate is actually CO₂ for which the K_m is uncertain, but has been estimated at about 0.15 mM. The high physiologically unattainable K_m and low-turnover number (1300/min at V_m) may be compensated for by the abundance of the enzyme (>15% of total spinach protein) and by local high Mg²⁺ concentrations which may tend to lower the K_m value. However, an alternate explanation may be discerned from this experiment where urease was immobilized in the vicinity of ribulose diphosphate carboxylase. The K_m of this enzyme for CO₂ was lowered. While

the K_m of the isolated enzymes for HCO_3^- was 22 mM, the K_m of the enzyme in intact chloroplasts for HCO_3^- was only 0.6 mM. It was hypothesized that the carboxylase in the chloroplast was associated with a CO_2 -generating enzyme, such as maleic dehydrogenase, with the two enzymes sharing the same microenvironment. Thus, no special transcarboxylase was required to provide the ribulose diphosphate carboxylase with high concentrations of CO_2 .¹²¹

Gestrelus et al.³⁸ cautioned experimenters on applying overall pH optima obtained for soluble sequential enzymic reactions to the natural membrane-bound situation. In this study, amylo- α -1,6-glucosidase (pH optimum 4.8) and glucose oxidase (pH optima 6.4) were covalently attached to Sepharose® 4-B. The pH optimum of the immobilized coupled reaction differed by 0.3 unit from that of the corresponding soluble system. When the ratios of the enzymic activities bound were varied, this displacement in pH optimum could be enhanced to 0.75 unit. The local enrichment of intermediate substrate concentration occurred, thereby changing the pH optimum for one of the enzymes. As the next enzyme in sequence obtained more favorable substrate concentrations, its pH optimum changed, and this resulted in a change in the pH optimum for the entire system. Since the ratios of enzymes immobilized were varied, the ratios of substrate utilized or products evolved also differed which, in turn, altered the degree of difference in the microenvironment. Most of the studies discussed up to this point were conducted to gain a better understanding of the life processes. They entailed simulating *in vivo* conditions or creating model systems which would be applicable to the delineation of biological processes *in vivo*. These studies will definitely be advantageous in developing heterogeneous catalysts capable of catalyzing a sequence of reactions on the same support. Knowledge of the effect of internal mass transfer resistance on the effectiveness of the catalyst particle would be of practical importance in developing the catalyst and the reaction environment to maximize productivity of the system. Theoretical considerations for diffusion and reactions in dual-enzyme catalyzed reactions have also been developed.

From an industrial or commercial point of view, very few studies have concentrated on developing immobilized multiple-enzyme systems. Messing⁹² reported immobilizing glucose oxidase and catalase on controlled-pore titania by adsorption. The system so developed was very stable, with catalase acting both as a stabilizer and an activator for glucose oxidase. Hultin⁵⁵ reported a similar system in which glucose oxidase and catalase were immobilized on 5% nickel-impregnated silica alumina support. The rate of utilization of hydrogen peroxide was used as an index to calculate efficiency of the system. The rate of utilization of hydrogen peroxide in the presence of excess of catalase in the dual enzyme system was taken to be 100%. By a batch method of assay, it was determined that the efficiency of the system in which glucose oxidase and catalase were coimmobilized on the same support was greater than the one observed with a corresponding soluble system. In their study, the ratio of glucose oxidase to catalase immobilized was less important than the amounts of activities immobilized. This was explained on the basis of proximity effects. When there was a greater activity of each enzyme immobilized, the H_2O_2 generated by glucose oxidase would have a lesser tendency to escape into the surrounding medium than if the enzyme molecules were separated by considerable distances; whereas when the molecules of glucose oxidase and catalase are packed near one another, the probability of the hydrogen peroxide being utilized immediately by catalase would be greater. Catalase is a very rapidly acting enzyme and has a very high turnover rate.

As evidenced by the lack of literature in this area, a real need exists for development and critical evaluation of immobilized multiple-enzyme systems which can be applied to industrial food processing.⁶⁵ It would be interesting to study an immobilized multiple-enzyme system containing metabolically unrelated enzymes.

Use of Immobilized Enzymes in the Food Industry

The techniques and methodologies for immobilizing enzymes originated due to interest in basic life processes. Scientists were trying to chemically synthesize peptides and were also attempting to study *in vivo* kinetics of enzymes by using *in vitro* techniques. This work was then extended into the areas now called affinity chromatography and immobilized enzymes. The immobilized enzyme technology shows a great deal of promise for commercial applicability. When considering the use of immobilized enzymes for the treatment of foods and feeds, six factors must be surveyed. According to Olson and Richardson,¹⁰⁵ these are as follows: (1) economics of immobilization, (2) suitability of components, (3) activity of the immobilized enzyme, (4) characteristics of the substrate and medium being treated, (5) stability of the immobilized enzyme, and (6) potential for microbial growth during continuous operation.

For successful commercialization of an immobilized-enzyme system, the economics of the overall process would be a key factor. Some of the items that contribute to the cost of the immobilization, such as price of support, cost of enzyme, cost of chemicals involved in the immobilization of the enzymes, and the specialized equipment that may be required for immobilizing the enzymes, are ones that can be listed directly. An equally important economic consideration would be the stability and half-life of the immobilized enzyme and other operating parameters. In the case of immobilized enzymes for the food industry, perhaps crude enzyme extracts can be utilized with good functionality. Methods to regenerate the initial charge of enzyme, the mechanical properties of the support have been discussed in the preceding sections of this article. The net outcome of an immobilized-enzyme process should be to produce a product which is less expensive than a comparable product produced by a soluble-enzyme process. The additional factors, such as the cost of the raw material and the market for an enzyme-treated product, are also critical. In some instances, even though the technology for using an immobilized enzyme is available, the market for an enzyme-treated product has been the limiting factor in commercializing the process. For example, in the corn syrup industry, the production of high fructose corn syrups (HFCS) was limited because of the cost of the product. However, when sucrose prices were elevated, the HFCS production became a reality.⁸⁹ Due to many such economic considerations, enzyme technology has been called a solution in search of a problem.

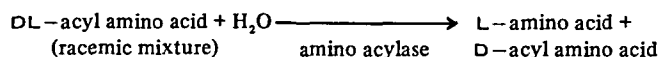
The majority of the enzymes used in the food industry are hydrolases; some isomerases and oxidoreductases are also employed. The immobilized-enzyme systems can thus be used to replace existing soluble enzyme processes. There are some special considerations which have to be accorded to a food system. From a safety angle, the process should not contaminate the food chemically or microbiologically. This can occur if hazardous chemicals are used in the immobilization process, and through attrition or other phenomena, these chemicals find their way into the substrate being processed. The microbiological contamination can occur during continuous processing of the food, and if this contamination consists of pathogens, it can pose serious public health problems. Apart from such safety considerations, the current limitation of using immobilized enzymes in foods is the nature of the substrate itself. It is only possible to utilize fluid substrates, liquids in particular. Solid substrates are not amenable for processing because of technological problems in separating the enzyme from the substrate. Even in certain liquid systems, such as the treatment of milk with protease or lactase (β -galactosidase), protein-protein interactions may adversely affect the activity of enzyme or clog columns. With advances in technology, it is hoped that many of these obstacles can be overcome.

Currently, there are three immobilized systems in commercial use. These are the glucose isomerase system for producing HFCS, the amino acylase system used for the

resolution of D- and L-amino acids, and the penicillin acylase system used in the pharmaceutical industry to obtain 6-amino penicillanic acid.

Amino Acylase System

Developmental work on the first commercially successful process using an immobilized enzyme was initiated by Tosa et al.¹³⁰⁻¹³⁶ in 1966 and consisted of an L-amino acid acylase. The reaction catalyzed by the enzyme L-amino acid acylase can be represented as follows:



In the production of amino acids by microbial fermentation, a racemic mixture of acyl amino acids is produced. The desired product is the L-amino acid, which is used to supplement proteins in foods and in pharmaceutical preparations. The resolution of racemic mixtures was performed by a soluble enzyme in a batch operation. However, since 1969 an immobilized-enzyme process has been utilized. The most satisfactory method of immobilizing the amino acid acylase was by linking the enzyme to DEAE-Sephadex® A-25 at pH 7.0. Approximately 333 units of acylase could be bound per milliliter of the carrier, and yield of enzymic activity was reported to be 47%. Immobilization of the enzyme enhanced the thermal stability of the catalyst, and the Sephadex®-aminoacylase column could be operated continuously at 50°C for 32 days with less than 40% loss of initial activity. The lost activity was compensated by the addition of an amount of fresh enzyme, corresponding to the deteriorated activity. The operating parameters for this system as reported by Weetall³⁸ are in Table 4. A schematic for the continuous resolution of racemic mixtures of amino acids is in Figure 4. The acylated DL-amino acids are tempered to 50°C prior to passage through immobilized-enzyme reactor. The reaction mixture after enzymic treatment is concentrated, and the L-amino acids are crystallized and separated. The acylated D-amino acids are then allowed to undergo chemical racemization.

Also, Tosa et al.¹³⁵ observed conversions with racemic methionine mixtures to be 88% of the calculated theoretical yields. The use of an ion exchange rather than covalent linking provided an advantage in regenerating the system. Circulation of a fresh enzyme charge through the reactor was sufficient to regenerate the system. The substrate used was easier to work with as it was devoid of macromolecules, such as proteins and nucleic acids. The economies affected by the use of immobilized amino acylase show the cost of producing a given quantity of the product by the immobilized enzyme system was only 60% of that of a conventional batch process using soluble enzyme. In the batch process, purification steps have to be included in order to separate the product in a pure form from contaminating proteins and pigments, and this led to a decrease in the yield of the product. Automation of the process using immobilized acylase and the elimination of the purification step helped lower labor costs and increased yields of the product. Later in 1971, Sato et al.¹¹⁴ covalently coupled amino acylase to halogenacetyl groups on cellulose and reported this system to be as effective as the DEAE Sephadex®-acylase system. The use of D-amino acid oxidase to resolve L-amino acids from racemic mixtures has also been investigated.¹³⁸

Glucose Isomerase System

Another immobilized enzyme system that has been applied successfully on a commercial scale utilizes glucose isomerase. This enzyme isomerizes glucose to fructose and is used in the corn syrup industry for producing HFCS. Glucoamylases introduced

TABLE 4

Operating Parameters for L-Amino Acid Acylase System

Enzyme	Microbial L-amino acylase
Carrier	DEAE-Sephadex® A-25
Buffer	pH 7.0 phosphate with 0.1 M amino acid
Activity added	333 units/ml carrier
Activity coupled	157 units/ml carrier
Yield	47%
Operating temperature	50°C
Operation time before re-charge	32 days
Stability of carrier	>2 years
Reactor size	1000 l +

From Weetall, H. H., *Immobilized Enzymes for Industrial Reactors*, Messing, R. A., Ed., Academic Press, New York, 1975, 201. With permission.

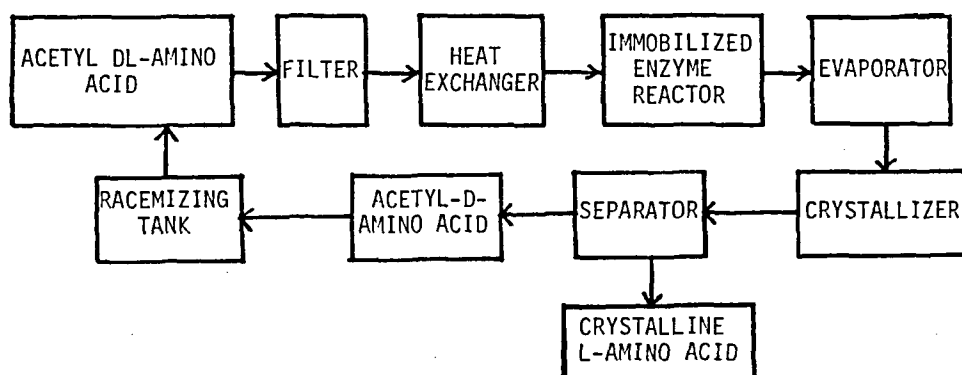


FIGURE 4. Schematic of process using immobilized aminoacylase.

in the 1950s were capable of hydrolyzing corn starch to yield corn syrups with 95 to 97 dextrose-equivalent (DE) sweeteners, but these high DE syrups were still not as sweet as sucrose, and the product tended to crystallize at room temperature. The isomerization of glucose to fructose not only eliminates crystallization problems encountered during shipping and storage, but also enhances the sweetening power of the syrup. Takasaki et al.¹²⁶ in the 1960s reported that organisms of the *Streptomyces* species isolated from soils could produce high levels of glucose isomerase when grown in culture media containing sources of xylan, such as wheat bran, corn cob, or corn hull. This led to the economical production of the enzyme which could be used on an industrial scale to produce HFCS. The specific organism isolated was identified as *Streptomyces albus* which was generally cultivated in a medium containing 3% wheat bran, 2% corn steep liquor, and 0.024% $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ at pH 7.0 and 30°C. The production of glucose isomerase could be induced by xylose, but xylan was preferable to xylose, whereas glucose, fructose, or mannose did not serve as an inducer for this enzyme. Maximal amounts of enzymes were produced after 25 to 30 hr of cultivation when cell yields were 10 g/l. At this stage, the cells were harvested and could be used as a source of the enzyme. The enzyme could also be isolated and purified. The molecular weight of the purified enzyme was calculated to be 157,000 daltons and had a pH

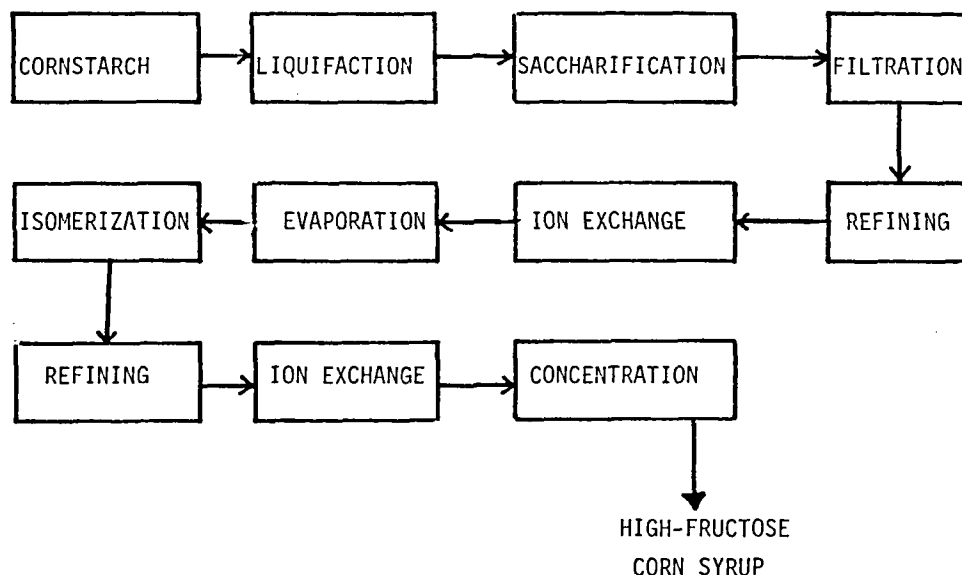


FIGURE 5. Schematic of process to produce high-fructose corn syrups.

optimum of 8.0 to 8.5 in phosphate buffer, with an observed temperature optimum of 80°C. There were no apparent differences in the ability to isomerize glucose to fructose between the intracellular enzyme and the cell-free enzyme extracts. Glucose isomerase could be fixed in the cells by heating the cells to 65°C for 15 min. These findings were utilized by Takasaki et al.¹²⁶ and Lloyd and Logen⁷⁶ to entrap heat-fixed *Streptomyces* cells in a filter bed. Other methods of fixing glucose isomerase in cells include using glutaldehyde¹⁶¹, entrapping cells in polyacrylamide gels,¹²³ or covalently coupling the enzyme to porous glass beads.¹²⁴ Glucose isomerase preparations used in a continuous manner enhanced stability of the immobilized enzyme, whereas batch processing was detrimental to enzyme stability. Commercial processes have been patented by various workers.^{11,24,28,76,77,127,129,161} Based on the success and economics of such processing, the Institute of Food Technologists awarded their 1975 Industrial Achievement Award to Clinton Corn Processing Co., a division of Standard Brands Inc.⁸⁸ Clinton first produced a HFCS containing 15% fructose by a batch method using soluble glucose isomerase. This process was later improved to produce a corn syrup containing 42% fructose on a continuous process using immobilized glucose isomerase. Cornstarch was liquified and hydrolyzed to dextrose which was refined, isomerized, refined again, and concentrated to obtain the syrup. The process is shown schematically in Figure 5. The entire process is a combination of batch and continuous operations in which hydrolysis of starch and subsequent preparation for enzymic isomerization are batch processes, whereas the isomerization, refining, and evaporation are continuous operations, and the remainder of the process is semicontinuous. The entire operation is automated and controlled by extensive on-line process-control systems. The price of the HFCS has been lower than sucrose and has thus made these syrups an attractive economical substitute for sucrose in various food formulations.

Glucoamylase System

Another carbohydrase used in the wet corn milling industry is glucoamylase. This enzyme hydrolyzes α -1,4 glucan links in nonreducing ends of the starch chains. Weetall

TABLE 5

Parameters for Dextrose Production Pilot Plant With a Capacity of Producing 10 Million lb. of Dextrose Per Year

Enzyme	Partially purified glucoamylase
Substrate	30% (dry weight) enzyme thinned corn starch
Carrier	Silanized inorganic support
Operating pH	4.5
Operating temperature	40—50°C
Reactor size	4.0—7.5 ft ³
Specific activity	3000 units/g derivative (1 unit produces 13.8 mg dextrose/hr at 60°C)
K _m (app.)	3 × 10 ⁻⁴ M
Reactor type	Plug flow

From Weetall, H. H., *Immobilized Enzymes for Industrial Reactors*, Messing, R. A., Ed., Academic Press, New York, 1975, 201. With permission.

and Havewala¹⁴⁷ conducted preliminary studies with glucoamylase immobilized on porous silanized support. The crude enzyme was obtained from a commercial supplier and was used either as is or was further purified prior to immobilization. The enzyme was immobilized by two methods: (1) diazo linkage or (2) cross-linking enzyme to glass using 2.5% glutaraldehyde. The enzyme activity was quantitated by using either 4% starch or 25% DE cornstarch containing 30% solids. When crude enzyme preparations were immobilized by diazo linkage to porous glass, 244 units of glucoamylase could be coupled to 1 g of support. When purified enzyme was used, 2950 units of glucoamylase could be coupled per gram of porous glass. Studies conducted with kinetic parameters, operational half-lives, and other parameters led to the planning of a pilot plant capable of producing 10 million lb dextrose per year. The parameters of the immobilized-enzyme system to be used in such a pilot plant were given in Table 5.¹⁴⁷ Pitcher and Weetall¹⁰⁸ reported that under the conditions of (1) using seven columns in sequence, (2) operational temperature of 50°C, (3) a 100-day half-life, (4) immobilized-enzyme cost of \$5/lb, and (5) a plant capacity of 10 million lb dextrose per year, the cost of 100 lb dextrose would be between 10 and 15c. Lee et al.⁷⁰ have also covalently immobilized glucoamylase using porous glass as the support. In a 1 ft³ packed column that could yield 1000 lb glucose/day operating at 40°C, it was observed that 87 to 93% of the substrate could be converted to glucose. The variability observed was attributed to the dextrose equivalent and the extent of retrogradation that had occurred in the substrate. This column could be operated continuously for 80 days without significant losses in the activity of the immobilized enzyme. The enzyme column was sterilized with chloroform prior to start-up, and the substrate was also sterilized by heating to 120°C for 3 to 4 min. These sterile conditions resulted in low bacterial counts. These encouraging pilot-plant studies suggest that glucoamylase could be the third enzyme system to be used on a commercial scale.

Although only two or three immobilized enzyme processes are being used commercially by the food industry, research is in progress to develop newer systems. Monsan and Durand⁹⁶ immobilized invertase by a combination of adsorption and cross-linking using bentonite as the support. Invertase or β -fructofuranosidase, now called β -D-fructofuranoside fructohydrolase, hydrolyzes sucrose to glucose and fructose. Suzuki et al.¹²⁵ have adsorbed invertase on DEAE cellulose, whereas others have covalently bound this enzyme to polystyrene tubes³⁴ and porous glass.⁶⁴ Mason and Weetall's studies⁶⁴ revealed that when invertase was bound to porous glass, no activity was lost even after 28 days of operation at 23°C; however, invertase covalently coupled to

cellulose showed a steady loss of activity. Invert sugar does not crystallize out and is thus used in jam making, and invertase is also used in soft-centered candies. However, the use of immobilized invertase is still in experimental stages, and the demand for an industrial process to invert sugar by immobilized invertase is not that great.

Amylases are important to the starch industry, and attempts have been made to adsorb α -amylase on cellophane, prior to cross linking with glutaraldehyde, onto diazotized polyamino styrene⁴⁷ and on a variety of other supports,¹⁵⁹ including polyacrylamide entrapment.¹³ Two problems that have been encountered are loss of activity of the enzyme postimmobilization and the leaching of enzyme from the support. α -Amylase attacks the α -1,4 glucan linkages in polysaccharides, e.g., starch, to produce oligosaccharides, glucose, maltose, maltotriose, etc. Immobilization of this enzyme led to a change in specificity of the enzyme as evidenced by the enhanced rate of glucose production from starch.⁶⁶ It was theorized that the particulate nature of the *p*-aminobenzylcellulose and polystyrene supports cause steric effects which result in the enzyme acting on many more α -1,4 glucan links. Immobilized α -amylase produced by covalent linking of enzyme to polyacrylamide derivatives possessed enhanced heat and storage stabilities.^{31,74} The use of α -amylase in ultrafiltration reactors has also been investigated extensively.^{18,81,159} and it has been suggested that coupling of α -amylase to form soluble-polymer derivatives is more desirable.¹⁵⁹ Another amylase, called β -amylase, degrades α -1,4 glucan linkages, starting from the nonreducing ends of starch, to produce maltose. This enzyme has been immobilized on polyacrylamide derivatives.⁷ In one such study, only 6.6% of the original activity was retained.¹³ Marshall and Whelan⁸¹ have suggested that the degradation of potato starch by β -amylase in the presence of pullulanase to yield maltose was a feasible proposition. Pullulanase cleaves β -1,6 glycosidic linkages and has been recently purified and immobilized by cross linking to a copolymer of acrylamide and acrylic acid.⁸² Further development of such a process would lead to the utilization of amylopectins to produce amylose. The overall developments in starch degrading enzymes have resulted in many potential uses. The obvious benefits of utilizing these enzymes in the corn milling, brewing, grain milling, and baking industries and in the production of distilled alcoholic beverages can be readily visualized. However, some other potential uses for these enzymes lie in the area of waste-treatment of starchy effluents from these industries and others such as effluents from paper mills.

Lactase Systems

Another carbohydrase of particular interest to the dairy industry is lactase or β -galactosidase which hydrolyzes the glycosidic bond between glucose and galactose in the milk sugar-lactose. This interest stems from reports that indicate individuals of certain ethnic groups suffer from a malady called lactose intolerance. This condition is believed to arise from a lack of β -galactosidase in the intestinal microvilli.⁶⁷ Also, lactose poses technological problems in the processing of dairy products, such as ice cream and concentrated milk. More recently, intensive efforts have been made to utilize lactase in treating cheese whey. Cheese whey is a by-product of the cheesemaking industry and contains 63 to 69% of lactose. It has been estimated that in the U.S. alone, nearly 30 billion lb of whey are produced. Of this, nearly 40% is not utilized and is thus wasted as an effluent. This wasted whey imposes a high biochemical oxygen demand on receiving waters. Apart from the pollution potential of whey, it also constitutes a loss of valuable nutrients. In an effort to reclaim these lost nutrients, immobilized lactases have been prepared and evaluated. Kay et al.⁶² immobilized lactase on cellulose sheets using a chloro-5-triazinyl linking agent. Although this derivative was stable for several months, up to 81% of the lactase activity was lost within 3 years at

TABLE 6

Kinetic Parameters for Lactase Systems¹⁴⁵

Enzyme preparation	$K_m(\text{app})$ M	$K_i(\text{app.})$ mM	E_a (kcal/ mol)	pH optimum
Soluble lactase Y	0.112	0.666	10.5	6.25
Immobilized lactase Y	0.0714	3.27	11.3	3.0
Soluble lactase M	0.04	1.25	—	4.0
Immobilized lactase M	0.05	3.22	6.54	3.5

2 to 5°C.⁷³ Further, when the porous sheets were freeze dried, the enzyme activity was severely decreased.¹¹⁷ Lactase has also been immobilized on porous glass beads,¹⁵⁸ with a 75% retention of original activity. Further, no loss in enzyme activity was observed when the preparation was stored at 4°C for 2 months. Galactose, one of the monosaccharides resulting from lactose hydrolysis, acted as a competitive inhibitor for the immobilized enzyme, but the sensitivity of the enzyme to competitive inhibition is a function of the source of the enzyme. Lactase can be produced by bacteria (e.g., *Escherichia coli*), yeast (e.g., *Saccharomyces lactis*), or by mold (e.g., *Aspergillus niger*). Due to differences in origin of these lactases, they exhibit differing physicochemical and catalytic properties. For example, the maximum activity of *E. coli* lactase can be observed at pH 7.0 and 50°C, whereas *S. lactis* lactase exhibits maximal activity at pH 6.5 and 32°C, and *A. niger* lactase at pH 4.0 and 55°C. These different properties are extended into kinetic aspects of the lactases as well. The most commonly used lactases for immobilization are those derived from *E. coli* and *A. niger*. Kay et al.⁶² and Hustad et al.^{57,58} used *E. coli* lactase. When this enzyme was immobilized on teflon stirring bars coated with a polyisocyanate polymer, it was stable up to pH 8.75 and could be used continuously for 137.6 hr without appreciable losses in activity. The pH optimum of the soluble enzyme was 6.5, while that of the immobilized enzyme was 5.8. The apparent Michaelis constant (K_m) for the soluble and immobilized enzymes was 13.1 mM and 22.1 mM, respectively, and the pH optimum of the immobilized enzyme shifted upward by 0.8 pH unit compared to the soluble enzyme.⁵⁸ The current catalog price of partially purified *E. coli* lactase is \$80/g and is therefore prohibitively expensive for industrial use. Weetall et al.¹⁴⁵ immobilized lactase obtained from two different sources: one was from a fungus (called lactase M) and the other from a yeast (lactase Y). The kinetic parameters for these lactases are in Table 6. In these systems when galactose was added at a concentration of 20 mM, the apparent velocity decreased by as much as 50%. It is also interesting to note that in both cases, upon immobilization there was a change in pH optimum of the enzyme. The lactase Y which had a near neutral pH optimum in the soluble form showed a dramatic shift of more than 3 pH units toward the acidic side (from the pH optimum of 6.15 to 3.0) upon immobilization. Inhibition of immobilized lactase by galactose has also been reported by Okos and Harper.¹⁰³ Lactase has also been immobilized on collagen membranes³⁹ in which case an additional factor contributing to the inhibition of bound lactase has been suggested to be the presence of divalent cations. Wierzbicki et al.¹⁵² immobilized lactase from various microbial sources onto porous glass and concluded that many lactases, including one from *A. niger*, possessed enough activity and stability to be of economic use in the hydrolysis of lactose. They did not, however, present an economic analysis of their process.

Using immobilized *A. niger* lactase, Weetall et al.¹⁴⁶ and Pitcher¹⁰⁷ have scaled up operations from a bench scale to pilot-plant scale. Sufficient data were gathered to suggest the technoeconomic feasibility of their process. Under operating conditions

described by Pitcher,¹⁰⁷ a hydrolyzed lactose product could be produced at the cost of 8 to 10¢/lb (dry basis). However, this process was applicable only to acid whey. Neutral pH substrate, such as sweet whey and milk, could not be used in this process. Research at Lehigh University, Bethlehem, Pa., deals with *A. niger* lactase immobilized on various supports, including alumina and stainless steel.²¹ All the preparations tested had acidic pH optima, and in no instance was the support reused. From a technoeconomic point of view, there is a need for a stable, economic, immobilized-lactase system, one that is able to hydrolyze lactase in a wide range of substrates. The lactase from *A. niger* is also a very expensive enzyme \$800 to 1000/kg. The cost of the native or soluble enzyme should be low, and the process must be adaptable to large scale use. The lactase obtained from *S. lactis* is available at a cost of 27¢/g or \$270/kg. Besides being relatively inexpensive, this lactase has a near neutral pH optimum and has approximately 2.5 times more activity per unit weight than *A. niger* lactase.

Dahlqvist et al.²⁶ entrapped this neutral pH optimum lactase in polyacrylamide gels, and Woychik et al.¹⁵⁷ immobilized this enzyme by covalent attachment to porous glass as well as collagen. The operational stability of these immobilized lactase preparations were reported to be not conducive to industrial use. Furthermore, these processes for immobilizing the enzyme caused the pH optimum of the neutral lactase to shift to the acidic range of pH 6.0. Agarose has also been used as a matrix to immobilize the neutral pH 6.0. Agarose has also been used as a matrix to immobilize the neutral pH optimum lactase.⁶³ Agarose was derivatized to the succinylaminoethyl form, and the enzyme was linked to the spacer arm by using *N,N'*-dicyclohexylcarbodiimide. These techniques resulted in an insoluble derivative of lactase in which there were no significant changes in either pH and temperature optima or the kinetic parameters. This enzyme preparation was stable when stored at pH 7.5 and 4°C for periods of up to 1 year, with no apparent loss in activity. Further, this immobilized lactase was capable of hydrolyzing lactose in whey and milk.

The safety aspects of many immobilized-enzyme systems, including immobilized lactase, and the Food and Drug Administration (FDA) approval of such preparations are not very clear. In many processes for immobilization, however, bound toxic factors are converted to nontoxic forms during chemical reactions, and products which are not converted to nontoxic compounds are subsequently washed out. It is possible that at some future date, if the processes are economical, the FDA may grant approval for the use of such systems.

Protease System

The broad class of hydrolases includes proteases of various types which are of economic consequence to segments of the food industry. Reports on the methods to immobilize proteases and the effects of immobilization on their operational parameters have been extensively reviewed by Zaborsky.¹⁶⁰ In general, proteases are very expensive enzymes, and many of them are not listed as generally recognized as safe (GRAS).¹⁴⁴ The practical applicability of immobilized proteases in food processing is restricted to fluid processing, such as the chillproofing of beer, continuous coagulation of milk, and the oxidative rancidity in milk.^{53,105,106} Proteases immobilized to an ethylenemaleic anhydride copolymer have been used to chillproof beer on a pilot-plant scale.¹⁵³ In the manufacture of beer, soluble proteins from the malt produce a clouding of the beer when the product is chilled. This defect is called chill hazing and can be overcome by hydrolyzing the proteins. The immobilized-enzyme treatment was carried out during the fermentation step. Glutaraldehyde cross-linked papain has also been tried by Witt et al.¹⁵⁵ to chillproof beer.

Olson and Richardson¹⁰⁶ used immobilized pepsin to study the feasibility of this

system for the continuous coagulation of milk.³² The primary phase is an enzymatic step which has a temperature coefficient (O_{10}) of 1.5 to 2.0,³² and the secondary step is a nonenzymatic step which requires the presence of divalent cations and has a Q_{10} of 15.²² The temperature coefficient Q_{10} may be described as the enhancement in the rate of a reaction when the temperature is raised by 10°C. By taking advantage of this property of milk coagulation, the primary or enzymatic step could be carried out at low temperatures so as to prevent the secondary step from occurring, thereby avoiding the coagulation of milk in the reactor. Olson and Richardson¹¹¹ used pepsin immobilized on porous glass to develop the process of continuous coagulation of milk. Milk was acidified to pH 5.6 or 5.9 before enzymic reaction. These pH values also enhanced the activity of pepsin at temperatures ranging from 5 to 20°C. The peptic activity declined dramatically above pH 6.2. After low-temperature enzyme treatment, the milk could be warmed to obtain a coagulation by activating the secondary phase of coagulation. Others have immobilized rennin and chymotrypsin⁴⁶ and pepsin⁷⁵ for the coagulation of milk, but these studies have had limited success due to losses in enzymic activity either during the immobilization process or due to leaching of the immobilized enzyme. Green and Critchfield⁴⁶ prepared enzymically active immobilized chymotrypsin and rennin, using cyanogen bromide activated agarose as the matrix. They also immobilized rennin on aminoethylcellulose. The agarose-chymotrypsin conjugates were found to be stable over the pH range of 2 to 9, but the agarose-rennin conjugates released the bound enzyme above pH 2; the aminoethylcellulose-rennin derivatives were also unstable at similar pH values. They concluded that the characteristics of the supports used render them unsuitable for use in the coagulation of milk. Shipe et al.¹¹⁸ immobilized trypsin by covalent binding to porous glass. Such a system was then used to treat milk for the prevention of oxidized flavors. The enzyme-treated milk developed flavor defects more slowly than untreated milk, but overtreatment of milk resulted in a bitter flavor.

Another area where proteases can be effectively utilized is in the production of protein hydrolysates. Aminopeptidases hydrolyze peptides splitting off N-terminal residues, whereas carboxypeptidases hydrolyze peptide, splitting off C-terminal residues. The aminopeptidases and carboxypeptidases have been immobilized^{113, 109, 160} and have been used in conjunction with general proteases to produce protein hydrolysates by Bennett et al.¹² When prolidase, aminopeptidase-M, trypsin, and chymotrypsin were bound individually to agarose and utilized for digestion of β ,1-24 adrenocorticotrophin at 25°C for 24 hr, the molar proportions of amino acids released varied by less than 5% per residue from that of the theoretical standard.¹² Although this work has a direct bearing on methods of preparing samples for amino acid analyses, similar techniques could conceivably lead to the production of protein hydrolysates. All these experiments are still in the preliminary stages of investigation, and the transition from laboratory bench scale to pilot-plant scale is expected to take a considerable amount of time. Recent experiments performed with papain immobilized covalently with succinylaminoethyl agarose revealed that the immobilization of papain did not significantly alter any of the kinetic parameters of the enzyme.⁶⁴ It was very interesting to note that when immobilized papain was treated with 6 M urea for periods of 270 min and then the urea was removed, the enzyme regained 100% of its initial activity. A similar treatment of the soluble enzyme resulted in the retention of only 17% of the initial activity. This study suggested that the immobilization of papain conferred exceptional confirmational stability on the enzyme and that the immobilized enzyme was only reversibly denatured, whereas the soluble enzyme was irreversibly denatured. Thus, there exists a great potential for the use of proteases in processing liquid substrates; just when this potential will be realized is not entirely certain.

Flavor Modification Systems

Apart from proteases and carbohydrases, lipases are also important to the food industry. Lipases are used extensively for the enzymic modification of flavor of foods.^{1,117} A search of the literature revealed very little work pertaining to the immobilization of lipase. Kosugi and Suzuki⁶⁶ have been successful in fixing cell-bound lipase of *Pseudomonas mephitica* var. *lipolytica*. The fixed lipase retained 83% of the soluble lipase activity, and the optimum temperature and heat stability of the fixed lipase were the same as those of the soluble lipase extracted from the microbial cells. The fixed lipase could be used continuously for the hydrolysis of tributyrin or triacetin. Datta and Ollis²⁷ immobilized porcine pancreatic lipase by entrapment in polyacrylamide gel. When the number of coupling groups on the surface of the beads were increased, a concomitant decrease in the specific activity of the bound enzyme was recorded. Additionally, the diazotization of lipase prior to coupling was also detrimental to enzyme activity. In another study, Kilara et al.⁶⁴ immobilized a fungal lipase on succinylaminoethyl agarose. The immobilization of this lipase did alter significantly any kinetic properties of the enzyme. However, this particular enzyme preferentially hydrolyzed olive oil rather than butter oil. These experiments, preliminary in nature, may lead to newer methods of flavor modification.

Among the other uses for enzymes in the modification of flavor are the use of naringinase and adenosine deaminase. Naringin is a glycoside of the flavonoid family which imparts bitterness to fruits, such as grapefruits and sour oranges. Naringinase is the enzyme which hydrolyzes naringin to yield naringin-7, β -glucoside (also called prunin) which is not bitter. Goldstein et al.⁴⁴ immobilized naringinase which could be employed in debittering clarified grapefruit juice. The other flavor-modifying enzyme studied is adenosine deaminase. Adenosine deaminase converts adenosine to inosine and has been immobilized using polyacrylamide gels.⁷⁸ Inosine so produced could be chemically phosphorylated to form inosine 5' monophosphate (IMP) which is a strong flavor potentiator. Alternately, an immobilized ATP deaminase has been employed to convert adenosine triphosphate to inosine monophosphate.²³

Other Enzyme Applications

Most of the enzymes used in the food industry are hydrolytic or isomeric in nature; however, certain other processes utilize oxidoreductases. Notable among these oxidoreductases are catalase and glucose oxidase. The use of an immobilized two-enzyme glucose oxidase-catalase system was mentioned in the section entitled Immobilized Multienzymes. The use of catalase in the food industry, particularly the dairy industry, has been extended to the removal of added hydrogen peroxide. Hydrogen peroxide has been used as a bactericidal agent in milk and in the processing of egg products.¹¹⁵ Olson and Richardson¹⁰⁶ have investigated various peroxidases for their efficacy to reduce bacterial populations. The effectiveness of such treatments was found to be dependent on the type of peroxidase, the mode of immobilization, and the type of reactor involved. For example, a continuous-stirred tank reactor was found to be better than a plug-flow reactor. Apart from the use of immobilized enzymes in food processing, they are also used in the pharmaceutical industry and in analytical techniques. For a more detailed discussion on these aspects, several review articles and books are available.^{48-50,139,140,160}

CONCLUSIONS

The use of soluble enzymes in food processing has been well established. Recent developments in solid-phase peptide synthesis have been translated into methods for

TABLE 7

Market Sales of Enzymes (in Millions of Dollars)

Enzymes	1975	1977	1980*	1985*
Proteases	38.7	43.6	52.5	71.5
Amylases	14.0	24.8	29.1	38.0
Others	19.8	45.4	56.6	73.6
Total	72.5	113.8	138.2	183.1

* Estimate.

From Wolnak, B., *Present Status of Enzyme Industry in the United States*, Proceedings Conf. Enzyme Economics, Barnard Wolnak and Associates, Chicago, Ill., 1978. With permission.

immobilizing enzymes. Thus, the chemistry for immobilizing enzymes has almost been standardized into "cookbook" recipes. Additionally, numerous enzymes have been immobilized and tested for various applications in the food processing industry. Thus, two processes, one utilizing amino acid acylase and another glucose isomerase, have become practical and economical. Various other enzymes and their applications are at different stages of development. Besides the applications discussed above, it has been suggested that enzymes may find extensive applications in the production of flavorants in bread, beer, wine, and other fermented foods as well as production of synthetic foods. Of course, numerous novel concepts have been attempted or are being pursued, such as (1) descaling of fish, (2) modification of wort, (3) beverage clarification, (4) the production of hydrolysate-based beverages for infants, geriatrics, and invalids, (5) enzymic determination as an index of food quality, (6) food analyses, and (7) removal of antinutritive factors from foods. Certainly, the use of enzymes from thermophilic organisms and low-temperature enzymes provide still further potentials. The future of such processes and applications will depend heavily on economics and regulatory decisions.

The total market for enzymes used in food and pharmaceutical processes has been summarized in Table 7. As can be seen from this illustration, proteases make up the largest share of the market, followed by amylases. These markets can further be fractionated as shown in Tables 8, 9, and 10. There are five major companies that produce enzymes in the U.S. These are: Wallerstein Division of GB Fermentation Industries, Miles Laboratories, Rohm and Haas, Dairyland Foods, and Chas. Pfizer and Company, whereas Novo and Enzyme Development Corporation both market enzymes produced in Japan and Europe.

In the U.S., the enzyme sales increased from \$25 million in 1960 to \$113 million in 1977 and are expected to climb to \$138 million in 1980 and \$183 million in 1985. Figuratively, it may mean a considerable increase. However, because of inflation, the actual increase does not seem to be as significant. Wolnak¹⁵⁶ states that this less-than-reasonable increase in the use of enzymes may be due to the lack of research work done for commercialization of enzymes. It has been estimated that in the U.S., funds matching only 0.5 to 1.0% of the total sales of enzymes are expended on their research and developmental activities. This amount is considered inadequate, especially in view of the government subsidized applied research conducted in several countries. How-

TABLE 8

Markets for Amylase (in Millions of Dollars)				
Enzymes	1975	1977	1980*	1985*
Amyloglucosidase	6.0	12.0	14.3	19.1
Alpha amylases	5.5	10.0	11.6	14.8
Beta amylases	2.5	2.8	3.2	4.1
Total	14.0	24.8	29.1	38.0

* Estimate.

From Wolnak, B., *Present Status of Enzyme Industry in the United States*, Proc. Conf. Enzyme Economics, Bernard Wolnak and Associates, Chicago, Ill., 1978. With permission.

TABLE 9

Markets for Proteases (in Millions of Dollars)				
Origin	1975	1977	1980*	1985*
Fungal	0.9	1.0	1.1	1.4
Bacterial	4.9	5.2	6.2	8.2
Pancreatin	4.6	5.1	5.9	7.5
Rennins	14.9	16.7	19.9	26.7
Pepsin	3.5	3.8	4.5	5.8
Papain	10.1	11.8	14.9	21.9
Total	38.7	43.6	52.5	71.5

* Estimate.

From Wolnak, B., *Present Status of Enzyme Industry in the United States*, Proc. Conf. Enzyme Economics, Bernard Wolnak and Associates, Chicago, Ill., 1978. With permission.

TABLE 10

Markets for Other Enzymes (in Millions of Dollars)

Enzymes	1975	1977	1980*	1985*
Glucose oxidase	0.7	0.8	1.1	1.3
Cellulase	0.3	0.3	0.4	0.5
Invertase	0.3	0.3	0.3	0.3
Glucose isomerase	15.0	40.0	50.0	65.0
Pectinase	2.0	2.3	2.7	3.6
Total	18.3	43.7	54.5	70.7

* Estimate.

From Wolnak, B., *Present Status of Enzyme Industry in the United States*, Proc. Conf. Enzyme Economics, Bernard Wolnak and Associates, Chicago, Ill., 1978. With permission.

ever, due to expanding human population and a concomitant reduction in natural resources, the future use of enzymes, immobilized or otherwise, may increase significantly. As stated previously, enzyme technology is a solution in search of a problem.

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