Enzyme Kinetics and Engineering

This review article surveys representative literature on enzyme kinetics and engineering published from January 1969 to August 1971. Particular attention is given to methods of attaching enzymes to solid supports, and to factors, such as pH and temperature, which affect the reaction rate. Recent theories accounting for the high selectivity and high rate of enzyme reactions, calculations taking into consideration the effects of diffusion, methods for analyzing kinetic data, and proposals for enzyme reactors are evaluated. Also reviewed are recent methods for extraction of enzymes from cells, procedures for synthesizing enzymes artificially, and techniques for isolating and purifying enzymes.

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SCOPE

Enzymes are of great interest to scientists and engineers not only because of their essential role in the metabolic pathways of biological systems, but also because of the vast opportunities they open up for innovations in chemical technology. Their ability to enhance the rate of chemical reactions by many orders of magnitude at room temperature and their amazing specificity towards substrates make them ideal industrial catalysts. Enzymes provide a way of eliminating many of the operational difficulties of high pressures and temperatures often encountered in chemical catalytic processes while at the same time avoiding costly and time consuming product purifications.

One of the major obstacles to the industrial use of enzymes is the lack of fast, efficient methods of extracting enzymes from the cells of biological organisms. The enzyme purification methods currently available are suitable for the production of enzymes on a small scale for research applications. However, the isolation of large quantities of enzymes from microorganisms has not reached its full potential. Procedures for synthesizing enzymes artificially are being investigated, but these are still in a preliminary research stage.

Enzyme reactions usually follow the well-known Michaelis-Menten kinetics under steady state conditions. The constants in the rate equation are very dependent on the physical conditions of the media in which conversion is taking place. Most enzymes exhibit an optimum pH and temperature for reaction to occur and are very sensitive to small deviations from these optimum values. The ionic strength and the presence of metal ions and inhibitors in solution can affect the rate of reaction significantly. Unusual conditions of temperature, pressure, and the presence of some organic compounds can denature the protein and completely inactivate it. The enzyme environment must therefore be carefully controlled in order to obtain the largest possible rate of conversion.

In the few cases where enzymes are used industrially, the reaction is usually carried out in a homogeneous liquid phase. To isolate the products, the enzyme must be denatured by heating or by hydrolysis. It is very important to develop ways of insolubilizing enzymes to allow complete recovery of the catalyst. This would also facilitate the development of continuous heterogeneous enzymatic reaction processes. Many insolubilization procedures are being investigated, ranging from the covalent attachment of the protein to polysaccharides to physical adsorption to glass. In some cases, these have been very successful.

In the future, enzymes will continue to be of great interest to engineers because of their many applications to medicine, analytical chemistry, and the chemical industry. In this review article some aspects of enzyme catalysis are discussed, with particular emphasis on the kinetics of enzyme reactions.

CONCLUSIONS AND SIGNIFICANCE

A great deal of research effort has been spent in isolating enzymes, identifying the factors that influence their kinetics, and applying this knowledge to new processes and procedures. In this paper, an attempt is made to survey some of the important characteristics of enzyme catalysis by taking examples from the more recent literature in the field. The literature search for this review encompasses publications from January, 1969, to August, 1971. Older articles are included, when needed, to reference some aspect of the discussion not covered fully in

more recent papers. Because of space limitations many articles had to be excluded arbitrarily from this review. Those articles chosen are either representative of the approaches and methods currently employed, or they describe a significant innovation or advancement in enzyme technology. In most cases, possible engineering application was a criterion for the selection of papers to be discussed. Therefore, many topics with a stronger biological or medical rather than technological importance are either glossed over or omitted entirely.

An effort has been made to stress enzyme kinetics. However, it was felt that a proper perspective of the subject could not be realized without mentioning briefly some of the techniques of enzyme isolation and extraction. The first section on the production and purification of enzymes covers some of the more recent attempts at the extraction of enzymes from microbial and vegetable sources and the possibility of synthesizing enzymes artificially. The principles behind the most common enzyme purification techniques are described and some examples of their uses are given. Some promising new separation methods are also outlined.

The second section of the article deals in greater detail with many of the aspects of enzyme kinetics which have already been mentioned. After a preliminary discussion of Michaelis-Menten kinetics, attention is focused on the effect of *p*H, temperature, metal ions, inhibitors and activators on the reaction rate. Recent theories on the mode of action of enzymes, transient kinetic studies, analyses of kinetic data, and some problems in reactor design are also discussed.

Some of the recent publications in the development of water-insoluble enzyme derivatives are reviewed in the third section. Four basic methods of enzyme attachment to a solid matrix are considered: 1. covalent bonding to an insoluble carrier, 2. polymer entrapment, 3. covalent cross-linking, and 4. physical adsorption. Some of the differences between homogeneous and heterogeneous enzyme reactions are pointed out.

The fourth and last section is devoted to a short description of topics which require further investigation and to additional practical uses of enzymes. Some of the applications of enzymes to medicine, analytical chemistry, and the chemical industry are pointed out.

PRODUCTION AND PURIFICATION OF ENZYMES

Enzymes are found in the cells of biological organisms. Some enzymes are in solution in the cytoplasm, others are bound to membranes and organelles and yet others are excreted by the cell. The large-scale production of enzymes from microorganisms usually involves the purification of extracellular enzymes such as proteases and amylases. Microorganisms are difficult to cultivate and process in large quantities. However, their low cost has encouraged research in the growth of mutant strains with higher intracellular enzyme content and in the development of more efficient extraction techniques.

M.D. Lilly et al. (*1*) have described a pilot plant scale process for the production of aliphatic amidase from a mutant of the bacteria *Pseudomonas aeruginosa* containing 2% of its dry weight of the enzyme. By disruption of baker's yeast cells in an industrial homogenizer seven enzymes have been isolated (*2*) without any apparent loss of activity. Using sequential fractionation, chromatography and crystallization, Blair and co-workers isolated large quantities of relatively pure transaldolase from the yeast *Candida Utilis* (*3*). Zetelaki (*4*) has pointed out that proper aeration can cause a significant increase in the rate of production of glucose oxidase in mould cultures of *Aspergillus niger*. An improved technique for the isolation of 3-phosphoglycerate kinase from baker's yeast has been reported by Scopes (*5*). He was able to obtain 1 g. of the enzyme from 450 g. of granulated yeast with a fairly high specific activity. Other workers have isolated proteases (*6* to *8*) and α-D-mannosidase (*9*) from bacterial media.

Vegetable cells have also been investigated as possible sources of enzymes. Dunnill, Currie, and Lilly (*10*) developed a continuous milling and centrifugation process for the extraction of prolyl-tRNA synthetase from mung bean (*Phaseolus aureus*). Single plant cell cultures of red kidney bean (*Phaseolus vulgaris*) have been grown as a method of producing amylase and peroxidase, but the yield of enzyme is relatively low (*11*).

Apart from extracting enzymes from biological materials, another interesting production possibility is to synthesize enzymes artificially. Because of the high molecular weight and large number of amino acid residues in protein molecules this is a very complicated procedure. Since the amino acid sequence or primary structure of the enzyme determines its three-dimensional configuration and this in turn controls its catalytic activity, the number of mistakes must be minimized in the synthesis. Groups led by Merrifield (*12*) and Denkewalter (*13*) have been able to synthesize independently the complete structure of ribonuclease with its 124 amino acids. The end products showed considerable enzymatic activity. Merrifield used the sequential solid phase synthesis method (*14*) in his procedure while the Denkewalter team synthesized the enzyme in solution from large polypeptide sections. At the present time other workers are attempting the synthesis of ribonuclease T1 and staphylococcus nuclease (*15*). Artificial enzyme sythesis is still far away from industrial scale applications, but it suggests the important goal of being able to design enzymes for specific purposes, perhaps using other enzymes in their preparation.

Once a homogenate of the biological system from which the enzyme is to be obtained has been formed, various methods have been devised to isolate the enzyme from the other proteins and smaller molecules in the mixture (*16*). The first step in the separation is usually a fractionation procedure that takes advantage of the solubility and acid-base properties of the enzyme. The isoelectric point of a protein is the *p*H at which the protein will have no net charge associated with it. If the *p*H of the suspension is adjusted to the isoelectric point of the enzyme desired, it may precipitate, leaving in solution all othed proteins with a different number of electrically charged groups. Isoelectric fractionation is a common first step in enzyme purification.

Since the solubility of proteins is strongly dependent on the dielectric constant and ionic strength of the solution,

a further precipitation can usually be obtained by adding to the mixture neutral organic solvents such as ethanol and monovalent or divalent salts. Sternberg (*17*) has shown that heteropolyacids such as tungstophosphoric acid can be very efficient in separating globular proteins, and Wilson et al. precipitated many proteins with the alkaloid vinblastine and calcium ions (*18*).

A knowledge of the effects of temperature on solubility is often useful in enzyme purification. If the enzyme being extracted is very stable to heat it is possible to coagulate the undesired proteins by raising the temperature of the solution (*19*). This cannot be done with large quantities of material since a delay in cooling the mixture can result in denaturation of the enzyme.

Usually, the enzyme extract obtained from fractionation procedures is not sufficiently pure for kinetic and equilibrium studies of reactions. Additional isolation techniques must then be used. Among the more common are ion exchange, adsorption, and molecular sieve chromatography on solids such as Sephadex or agarose. These methods take advantage of the variation in charge and molecular diameter of protein molecules. To obtain small quantities of very pure samples, density gradient ultracentrifugation and gel electrophoresis are normally utilized. Since different proteins have different mobilities under the influence of centrifugal and electric fields, these procedures, when used in conjunction with the fractionation and adsorption methods already mentioned, may lead to almost total purification of the enzyme.

At every step in the purification scheme the homogeneity and specific activity of the enzyme must be evaluated. The presence of a single band with catalytic activity in disk gel electrophoresis is usually a good indication that a reasonable degree of purification has been achieved. A crystalline product is no guarantee of purity since some protein mixtures are known to form regular crystals.

The use of many of these procedures is clearly illustrated by recent separations of amylase from *Bacillus subtilis* (*20*), catalase from beef erythrocites (*21*), β-glucuronidase from rat liver lysosomes (*22*), RNA polymerase from *Escherichia coli* (*23*), and fructokinase from rat liver (*24*).

In order to process large quantities of proteins, separation methods have been devised that do not subject the enzyme to unfavorable conditions for long periods of time. Also, the fewer the steps in the procedure, the higher may the yield of enzyme be. As a result, continuous purification processes can have an edge in efficiency over large scale batch systems. Ultrafiltration is one operation that seems to show great promise for the concentration of large quantities of biological macromolecules. It has already been shown to be useful for the purification of trypsin by Wang and co-workers (*25*). They found that the filtration flux increased with increasing pressure difference between the two sides of the membrane and with temperature but decreased with increasing salt concentration in the feed. A layer of protein is usually formed at the membrane surface with time and this decreases the flux of solvent through the membrane (*26*). To reduce this effect of concentration polarization it is advantageous to maintain a high shear stress at the membrane surface but this has been found to decrease the activity of the concentrated enzyme (*27, 28*). Kozinski and Lightfoot (*29*) were able to account for this phenomenon using a simple hydrodynamic model of stagnant flow. Charm and Lai (*28*) investigated the performance of several of the most common ultrafiltration systems currently in use. No one apparatus was found to be completely superior to any of the others for the concentration of enzymes.

Another rapid separation technique that has not reached industrial proportions but shows great promise is affinity chromatography (30). In this procedure, highly selective inhibitors of the enzyme to be separated are attached either directly, or by means of long side chains, to a solid support. The protein mixture is introduced into the matrix and the enzyme binds to the inhibitor while all other proteins remain in solution. The attached enzyme can then be eluted by *p*H gradient or by a stronger enzyme inhibitor. Berman and Young (*31*) used affinity chromatography very effectively in purifying electric eel and beef erythrocyte acetylcholinesterase with agarose as the solid support. Robinson *et al.* separated various trypsins by binding chicken ovomucoid to Sepharose (*32*).

Electrodecantation is essentially a method of carrying out electrolysis and isoelectric fractionation of proteins simultaneously. It appears to be amenable to industrial applications because of the simplicity of its design and its operational flexibility. Winchester, Caffrey, and Robinson used it successfully to separate various enzymes (33).

Edelman and Gall have described an automated separation scheme for proteins that is able to carry out multistep isolations in a continuous fashion (*34*). Such an approach would be very useful in industry since it would save a great deal of time in enzyme extractions. By taking advantage of the temperature dependence of the *p*H of a solution, Luner and Kolin have been able to produce very fast *p*H gradients and thus reduce the time required for isoelectric focusing of proteins from days to minutes (35). This technique seems to be very valuable and worthy of further study. Additional procedures used to measure physical parameters of enzymes such as molecular weight, diffusion coefficient, and sedimentation coefficient are often based on separation procedures (*36*). It is possible, for example, to calculate molecular weights from ultracentrifugation and molecular sieve chromatography (*37*). Sedimentation methods have received the most attention recently for the measurements of molecular weight (*38* to *41*) and diffusion coefficients (*42*).

Having a pure enzyme is desirable since it leads to uniformity and reproducibility in kinetic studies. If enzymes are to be used to carry out chemical reactions in industry it will be necessary to develop large-scale, safe, efficient and economic extraction and purification techniques.

THE KINETICS OF ENZYMES IN SOLUTION

The techniques available for kinetic studies of enzyme reactions can be conveniently grouped into two categories: steady state and transient. Steady state methods require less sophisticated equipment, but they are sufficiently sensitive to allow in most cases a complete determination of the overall reaction mechanism. However, direct accurate measurements of the rate constants of individual steps in the mechanism and of the formation of intermediates with time can only be obtained from transient techniques. This section deals mainly with the steady state behavior of enzyme reactions in solution.

The steady state assumption (*43, 44*) is frequently used in the kinetic theory of enzyme reactions and leads to the Michaelis-Menten kinetic expression

$$v = dP/dt = k_p E_0 S/(K_m + S) \qquad (1)$$

where v is the velocity of the enzyme reaction, K_m is the Michaelis constant, k_p is a rate constant, E_0 is the total

enzyme concentration, and P and S are the concentrations of product and substrate, respectively (*44*). When the concentration of substrate is much larger than K_m, then $v = V = k_pE_0$ and the rate of reaction is at its maximum. At low substrate concentrations where $S << K_m$, the rate $v = VS/K_m$ and the rate is linear in S. The parameter K_m is the substrate concentration at which $v = V/2$. The parameter k_p is occasionally called the turnover number. It is approximately the number of substrate molecules converted into product per enzyme molecule per unit time when $S >> K_m$. For very active enzymes such as catalase its value can be as high as 10^7 sec.$^{-1}$. The parameters k_p and K_m may be highly dependent on the pH, temperature, ionic strength, and concentration of inhibitors and activators in solution.

The measurement of k_p and K_m is usually carried out by plotting the initial velocity data in a double reciprocal plot of $1/v$ versus $1/S$ called a Lineweaver-Burke plot. A straight line results that intersects the abscissa at $-1/K_m$ and the ordinate at $1/V$. E. J. del Rosario and G. G. Hammes (*45*) studied the hydrolysis of uridine 2′,3′-cyclic phosphate by ribonuclease. The initial rate data was fitted to a Lineweaver-Burke plot by a weighted least-squares analysis (*46*). They obtained values of $k_p = 4.20$ sec.$^{-1}$, and $K_m = 3.87 \times 10^{-8}$M at pH 6.5 and 25°C. in a 0.1M Tris-acetate, 0.1M NaCl solution. Similarly, Hillman and Mautner (*47*) obtained values of K_m and V for the hydrolysis of various substrates of electric eel acetylcholinesterase. This is a more complicated reaction because an acyl-enzyme intermediate is formed. As a result the constants k_p and K_m have a more complex dependence on the rate constants of individual steps in the mechanism. However, the functional form of Equation (1) is still valid. Even when the reaction mechanism consists of several successive steps, it can be shown that an equation of the form of the Michaelis-Menten rate expression will be obtained (*48*).

Many enzymatic reactions involve a reaction between two substrates and the formation of two products. Reactions in which the rate of adsorption and desorption of both substrates is much faster than the rate of decomposition of the enzyme-substrate complex into products, may have a steady state rate equation of the form (*49*)

$$v = VS_1S_2/(K_1S_2 + K_2S_1 + S_1S_2 + K_2K_3) \qquad (2)$$

If the concentration of one of the substrates S_2 is held constant, while the concentration of the other S_1 is varied, a plot of $1/v$ versus $1/S_1$ for various values of S_2 will give a series of lines which will intersect at the left of the ordinate. A plot of the slopes or intercepts of these lines against $1/S_2$ allows the evaluation of all the constants in the rate expression (*50*). Zeijlemaker et al. (*51*) studied the rate of oxidation of succinate catalyzed by succinate dehydrogenase with phenazine methosulfate (PMS) as the acceptor. Plots of $1/v$ versus the reciprocal of the concentration of succinate were linear for various concentrations of PMS. The plots of $1/v$ against the reciprocal of PMS concentration for several concentrations of succinate were also linear. All the lines intersected in the third quadrant. From secondary plots of the slopes and intercepts the various kinetic parameters were obtained. Pig liver glycolic acid oxidase can catalyze the oxidation of glycolate with either oxygen or 2,6-dichlorophenolindophenol (DCIP) as the electron acceptors. The reaction with DCIP was studied by Schuman and Massey (*52*). Double reciprocal plots of $1/v$ versus the inverse of glycolate concentration with the DCIP concentration as a parameter were all linear and intersected in the third quadrant. By replotting the intercepts as a function of the inverse of DCIP concentration, they were able to calculate the value of V and the values of the K's in the rate expression. Garces and Cleland (*53*) examined the kinetics of the reaction MgATP + MgUDP ⇄ MgADP + MgUTP catalyzed by yeast nucleoside diphosphate kinase. Reciprocal plots of $1/v$ as a function of the inverse of the substrate concentrations were found to be not only linear but parallel. A stable phospho-enzyme intermediate was isolated after reaction with MgATP. Inhibition studies were consistent with their reaction model. The reaction of β-D-glucose with NAD on rat liver microsomal glucose dehydrogenase (*54*) is an example of a reaction where the double reciprocal plots intersect on the abscissa. As has been pointed out by Cleland (*49*), such results may indicate that the Michaelis constant for the substrate is almost identical with its dissociation constant and no additional mechanistic inferences can be drawn from this data alone.

Equation (1) states that as the concentration of substrate is increased, the rate of reaction also is increased. However, it is noticed in many enzyme systems that further increase of substrate concentration beyond a certain value causes a decrease in the steady state rate of reaction. This effect has been termed substrate inhibition, and is usually associated with nonproductive binding of substrate molecules to the enzyme (*55*). Some of the details of this behavior have been thoroughly discussed by Cleland (*56*). It has been found (*53*) that for very high MgUDP concentrations (1 mM − 4 mM), the reciprocal plots of $1/v$ versus the inverse of MgATP concentration for yeast nucleoside diphosphate kinase, as discussed above, cease to be parallel. The set of curves for high MgUDP have the same ordinate intercept but their slopes increase with increasing MgUDP concentrations. This has been interpreted as being due to a competition for the active site between MgATP and MgUDP at high concentrations. Schuman and Massey (*52*) also reported that at high glycolate concentrations this substrate became inhibitory of its reaction with DCIP on pig liver glycolic oxidase.

A substrate can also activate an enzyme, so that as the substrate concentration increases, the rate of reaction is accelerated to a higher extent than can be explained by the Michaelis-Menten equation. Presumably, the substrate can bind to a different part of the enzyme to make it more reactive, or it can cause the release from the active site of a non-reactive intermediate. Duke et al. (*57*) used integrated rate expressions to analyze the kinetics of the reaction between β-D-glucose and O_2 in glucose oxidase and found that their data could be explained by a model invoking activation by substrate. According to the mechanism they proposed, the substrate β-D-glucose activates the release of the product δ-gluconolactone from the active site, thus increasing the amount of free enzyme available for reaction. Substrate inhibition is more common than substrate activation, and its possibility must be kept in mind when high substrate concentrations are being used.

So far we have considered the effect of the substrate concentration on the rate of enzymatic reactions. The steady state reaction velocity can also be influenced to a great extent by the pH, temperature, and ionic strength of the solution in which the reaction is taking place, and by the presence of inhibitors or activators in the reaction media.

Many enzymes exhibit an optimum pH for reaction so that a plot of v versus pH has a positive slope at very low pH, a negative slope at very high pH, and a maximum in the intermediate range of pH (*58*). Some enzymes are more sensitive to this effect than others so that the pH-activity curve can take a variety of shapes. Changes in hydrogen ion concentration can affect the parameter V

only, or K_m only, or both simultaneously. Activity changes in pH can be associated with denaturation of the enzyme or with a decrease in the affinity of the substrate for the active site. When very high substrate concentrations are used, and when the kinetic measurements are made right after the pH has been altered, these affinity and denaturation contributions may be neglected, so that the effect of pH on V and K_m may be singled out. Enzyme kinetic data is usually interpreted in terms of a model where only one ionization state of the enzyme-substrate complex is reactive. Ionization of this complex is responsible for the drop in activity. According to this model, the effect of pH on V can be described by the equation

$$V = V_0/(1 + H/K_a + K_b/H) \qquad (3)$$

where H is the hydrogen ion concentration, V_0 is the maximum velocity of the reaction and K_a and K_b are the ionization constants of the complex in the acid and basic side, respectively. The denominator of Equation (3) is called the Michaelis pH function. By plotting $\log_{10}V$ versus pH, one can find values for pK_a and pK_b. At very large hydrogen ion concentration, the slope of the curve of $\log_{10}V$ is $+1$, while for very low H concentrations the slope is -1. In the intermediate region of pH, $\log_{10}V \approx \log_{10}V_0$ and the plot is horizontal. The intersection of this horizontal line with the lines of slope $+1$ and -1 can be shown to correspond to the values of pK_a and pK_b, respectively. Similar effects can be found for pH dependence of K_m, but they are much more difficult to interpret (*59*).

The pH-dependent trypsin and α-chymotrypsin catalyzed hydrolysis of esters has been carefully studied by del Castillo et al. (*60*). They found that the maximum value of k_p occurred at a pH of 6.5 for the trypsin hydrolysis of L-lysine methyl ester and the α-chymotrypsin hydrolysis of L-phenylalanine ethyl ester. Plots of K_m versus pH decreased very fast in the pH range of 4 to 6 and remained flat from pH 6 to pH 9 in both cases. Auld and Vallee (*61*) studied the pH dependence of hydrolysis of tripeptide substrates by carboxypeptidase A. Symmetrical v/Eo versus pH profiles were found for these substrates with a maximum at pH 8. The plots of $\log_{10}k_p$ as a function of pH increased in the region of pH 5 to pH 8 and remained level from pH 7 to pH 11. The $\log_{10}K_m$ remained level from pH 5 to pH 8 and dropped off in the region of pH 8 to pH 11. A simple reaction and ionization model was able to account for their results. Another good example of the use of the Michaelis pH functions is given in the work of Spomer and Wooton (*62*) on the hydrolysis of α-N-benzoyl-L-argininamide catalyzed by trypsin and acetyltrypsin. By postulating the ionization of groups in the free enzyme and the enzyme-substrate complex they were able to account for the k_p/K_m versus pH profiles obtained experimentally.

The effect of temperature on enzymatic reactions is much more complicated than that of the more common homogeneous and heterogeneous reactions. When Arrhenius plots of ln V versus $1/T$ are constructed, it may be found that sharp transitions occur at given temperatures (*63*), so that two or even three energies of activation can be calculated for the system over a relatively small temperature range (in the order of 30 C. degrees). Similar transitions are found for Arrhenius plots of K_m. Of course, nonlinear Arrhenius plots are not unfamiliar to chemical engineers since they arise quite naturally in the theory of heterogeneous reactions in catalyst pellets (*64*). However, the situation with enzymes is somewhat more complicated. Both V and K_m are complex functions of several rate constants for individual steps in the reaction mechanism, and this prevents a straightforward interpretation of the temperature effects. In addition, enzymes tend to denature with heat and this is accompanied by a complementary loss in activity. Sugimoto and Nosoh (*65*) found that the activity of fructose-1,6-diphosphate aldolase increased continually up to a maximum at a temperature of 70°C., after which the activity dropped dramatically. Denaturation is a kinetic process, and in identifying the activity of an enzyme at a given temperature it is usually desirable to consider how long the enzyme has been in that particular state. Again in the case of fructose-1,6-diphosphate aldolase, it took only 30 min. to completely deactivate the enzyme at 75°C. while at 50°C. after 1.5 hr., only 5% of the activity had been lost (*65*). Kapuler (*66*) found that RNA polymerase has a temperature optimum at 50°C., and an Arrhenius plot of the rate with two transitions, one at about 39°C., the other at 49°C. This was found to be the case for a series of substrates. All showed the same apparent energy of activation in each of the three regions and the same transition temperatures. By studying the effect of temperature on the pH-activity profiles for tripeptide hydrolysis catalyzed by carboxypeptidase A, Auld and Vallee (*67*) obtained the values of the pK's of ionizable groups in the enzyme as a function of temperature and calculated their heats of ionization from experimental data. They reported that linear Arrhenius plots of the activity were obtained and they calculated the free energy, enthalpy and entropy of activation for the reaction of the enzyme with several substrates. The plot of ln V versus $1/T$ for the reaction of succinate dehydrogenase showed two transitions, one at 18°C., the other at 27°C. when the enzyme was not activated (*68*). When the protein was activated by incubation with succinate, only one transition was obtained at a temperature of 18°C. The results were tentatively explained in terms of temperature dependent conformational transitions in the enzyme.

The physical properties of the solvent in which reaction is taking place can also influence the rate of reaction. One excellent example of this is the study of Bolen and Fisher (*69*) on the reaction of adenosine deaminase in mixed aqueous solvents. From optical rotatory dispersion and solvent perturbation difference measurements they established that the presence of ethylene glycol in concentrations up to 80 volume percent did not alter the conformation of the enzyme. However, Lineweaver-Burke plots of the reaction velocity at different volume percents of ethylene glycol showed that the maximum velocity of the reaction decreased with increasing glycol concentration. The values of the kinetic parameters in the rate expression were highly dependent on the concentration of ethylene glycol in the solvent. It was demonstrated that the logarithm of the reaction rate was linearly dependent on the inverse of the dielectric constant of the solution when five different solvents were tested. Davies et al. (*70*) have shown that the activity of lysozyme towards cell suspensions of *Micrococcus lysodeikticus* decreases significantly with increasing ionic strength of the solution. The degree of inhibition of the reaction by some inhibitors also decreased with increasing ionic strength. Related studies were made by Saint-Blancard and co-workers (*71*) with six human and four avian lysozymes again acting on *Micrococcus lysodeikticus*.

Many enzymes require the presence of metal ions for high activity (*72*). Some ions enhance the rate of many enzyme reactions while they inhibit others. If the rate of reaction v is plotted as a function of salt concentration, a Michaelis-Menten type curve is often obtained. At very high salt concentrations the ions can be inhibitory to the reaction, an effect very similar to substrate inhibition. The exact reason for ion activation has not be elucidated

in most cases, but there is no lack of kinetic models (73) that can account favorably for the observed experimental results. The ion very often forms an essential part of the enzyme's active site. In other systems it may bind to the substrate or be adsorbed by the active site in order to increase substrate binding or to enhance chemically the rate of reaction. Anions are capable of affecting the kinetics of the reaction, but metal cations are by far the most important class of ions influencing enzyme catalysis. Kelemen and Muhlrad (74) studied the activation of myosin ATPase by monovalent cations. They found that values of K_m and V increased with increasing concentrations of K^+ and NH_4^+ but decreased with increasing concentrations of Mg^{++}. Bothwell and Datta (75) have found that the rate constant for inactivation at 25°C. of homoserine dehydrogenase from *Pseudomonas fluorescens* is 25 times lower when the concentration of K^+ ions is 20mM than when it is 1mM. Potassium ions also help to prevent the inhibition of the reaction by L-threonine. The activity of β-galactosidase is also influenced by K^+ and Na^+ ions (76). Satchell and White (77) studied the kinetics of acetate kinase and concluded from the available data that Mg^{++} binds itself to the substrate ADP prior to binding to the active site of the enzyme. Horne and Nordlie found that many anions activate rat liver microsomal glucose dehydrogenase (54) while Bishop and Gill (78) found that *E. coli* DNA polymerase was inhibited by Li^+, Na^+, K^+, Rb^+ and Cs^+ ions and the degree of inhibition increased with increasing salt concentration.

There are many substances that prevent the normal reaction of the enzyme with the substrate (79). These inhibitors have been very useful in determining the reaction mechanism of enzymes. They are also of practical significance since their presence in a reaction media may greatly affect the kinetics of the reaction. Most inhibitors are structurally or chemically similar to the substrate. As a result, they are able to interact with the active site and interfere with substrate binding. Inhibitors can be classified as reversible or irreversible. The concentration of reversible inhibitor in solution is in equilibrium with the concentration of enzyme-inhibitor complex, while an irreversible inhibitor is chemically attached to the enzyme. There are three basic types of reversible inhibitors that may be distinguished by the types of Lineweaver-Burke plots obtained at various inhibitor concentrations. If the lines obtained by plotting $1\text{-}v$ as a function of $1/S$ at different concentrations I of inhibitor have different slopes but the same intercept, the inhibitor is called competitive. The value of the inhibition constant K_i can be evaluated from the intersection of the line with the $1/S$ axis. If the slopes of the lines are the same but the ordinate intercepts are different, parallel lines result and the system is called uncompetitive (80). Finally, if both the slopes and the intercepts vary with inhibitor concentration the set of lines will intersect to the left of the ordinate either above, below, or on the base line. If the lines intersect on the base line the inhibitor is called noncompetitive. If the intersection is either above or below the base line, the inhibition is called mixed. When the plots of the slope or the intercepts of the reciprocal lines as a function of inhibitor concentration are linear, the inhibition is called linear competitive, etc. Parabolic and hyperbolic inhibition are also possible (80). Competitive and noncompetitive inhibition are common forms and the kinetics of the reaction in the presence of such inhibitors is given by Equations (4) and (5), respectively.

$$v = k_p E_0 S/[S + K_m(1 + I/K_i)] \qquad (4)$$

$$v = k_p E_0 S/[(S + K_m)(1 + I/K_i)] \qquad (5)$$

The product formed in a reaction may be an inhibitor to its own formation. This is called product inhibition and is a special case of the mechanism of feedback inhibition that is common in complex enzyme systems. It is desirable to carry out enzyme reactions by continually removing the product from solution in order to prevent this. If product inhibition is competitive, the use of very high substrate concentrations will shift the equilibrium towards enzyme-substrate complexes and prevent product binding to the active site.

Alridge and Reiner (81) studied the irreversible inhibition of acetylcholinesterase by haloxon, coroxon, and other compounds. They found that an irreversible, inactive phosphorylated enzyme intermediate was formed during inhibition by haloxon, which blocked subsequent binding and reaction of acetylcholine. Similar intermediates were obtained from inhibition by neostigmine and other carbamates (82). Goldberg and Fruton (83) found that inhibition of the hydrolysis of methyl β-phenyl propionate and methyl β-phenyl-D-lactate by isoleucine methyl ester on beef liver esterase is noncompetitive since Lineweaver-Burke plots at different inhibitor concentrations intersected on the base line. Replots of the slopes and intercepts as a function of inhibitor concentration subsequently showed that the inhibition was linear. Hall and co-workers (84) have found that product inhibition of the hydrolysis of O-(trans-cinnamoyl)-L-β-phenyllactate by carboxypeptidase A is competitive in nature and described by Equation (4). The yeast nucleoside diphosphate kinase system studied by Garces and Cleland (53) showed linear mixed and competitive inhibition by the two products as well as the substrate inhibition already discussed.

The short outline of enzyme kinetics given here stresses the importance and validity of the Michaelis-Menten equation. This is the expression that will probably be applicable for most engineering design applications as well as for reaction rate studies. Cha (85) has pointed out that errors may be generated by Equation (1) when the concentration of enzyme is large. He proposed other alternatives for the rate expression in this case.

A common method of obtaining kinetic data is by initial velocity measurements. These have the disadvantage of requiring many runs, and as a result, can be costly and time consuming if the reacting materials are expensive or hard to obtain. Recently, attention has been given to methods of obtaining kinetic constants from progress curves by parameter fitting (86) and integrated rate expressions (87, 88). However, initial velocity data allows the investigator to distinguish different reaction mechanisms with more accuracy if regression analysis programs are used such as the one developed by Arihood and Trowbridge (89). Steady state theories of enzyme reactions have been developed recently for reactions between a substrate and a metal (90), between three substrates (91), and for inhibition with tight-binding inhibitors (92). These types of analyses can be very useful in interpreting enzyme kinetic data.

Unsteady state methods of studying enzyme reactions such as stopped flow, continuous flow, and relaxation techniques (93) are very powerful and generate considerable insight into an enzymatic reaction. Finlayson and Taylor (94) studied the hydrolysis of nucleoside triphosphates by myosin using stopped-flow methods. Hammes and co-workers have studied the interaction of various substances with tryptophan synthetase (95, 96) and aspartate transcarbamylase (97, 98) using temperature-jump and stopped-flow techniques. An unsteady state

theory has been developed by Rubinow and Lebowitz (99) for the kinetics of an enzyme-substrate-inhibitor system. Walter (*100*) has discussed the possibility of using a power series approximation in obtaining presteady state information from rate data.

One of the most baffling and important problems is the determination of the molecular mechanism underlying enzyme catalysis. The relationship between the structure of an enzyme, the details of the binding, reaction, and desorption steps and the increased rate of enzymatic reactions is still unknown. This problem has been approached from many different directions. X-ray crystallographic studies of purified enzymes (*101, 102*) have shed considerable light upon the subject. Hess and Rupley reviewed some of the current theories regarding the mode of action of enzymes whose structure is known (*103*). Recently, Wyckoff et al. (*104*) published the results of a 2 Å. resolution electron density map of ribonuclease S. Surveys of the chemical approaches to the study of the active sites of enzymes were carried out by Vallee and Riordan (*105*) and Cohen (*106*). Isotope effects in enzymatic reactions have been one of the more flexible and useful tools in determining enzyme mechanisms (*107*). O'Leary (*108*) used carbon-13 isotopes to study the rate determining step in the oxidative decarboxylation of isocitric acid by isocitrate dehydrogenase. Other isotope effect studies have been presented by Page and Vanetten (*109*) for L-amino acid oxidase. Nuclear spin relaxation (*110*) and electron spin resonance (*111*) have also proven useful in mechanism studies.

Detailed investigations of the specificity of enzymes towards substrates (*112*) and of the binding of inhibitors and cofactors can give significant insights into the nature of the active site of the enzyme. Brockerhoff (*113*) studied the specificity of pancreatic lipase to many different esters. Other specificity studies were carried out by Brot and Bender (*114*) for the α-chymotrypsin catalyzed hydrolysis of several substrates, and the results were compared in terms of the specificity constant k_p/K_m. Price and Radda (*115*) were able to investigate the interaction of NAD with rabbit muscle glyceraldehyde-3-phosphate dehydrogenase by protein fluorescence quenching. The binding of the same cofactor to the same enzyme was also studied by de Vijlder and Harmsen (*116*) using circular dichroism and optical rotatory dispersion and by Velick and coworkers (*117*) using calorimetric and fluorometric titration. The thermodynamics of binding of inhibitors to trypsin (*118*), lysozyme (*119*), α-chymotrypsin (*120, 121*) and rabbit muscle aldolase (*122*) have also been investigated.

Koshland and Neet (*123*) and Bruice (*124*) have reviewed some of the current theories regarding the mode of action of enzymes. The reduced orientational freedom of the substrate when bound to the active site (*125, 126*), the proximity of reacting groups (*127*) and electronic effects (*124*) are all believed to contribute to the high rates of enzymatic reactions. Storm and Koshland proposed that orbital steering could account for the rate enhancement (*128*) but Bruice and co-workers have presented several arguments (*129*) which they claim would make this mechanism implausible.

Kinetic rate expressions have important implications for the design and analysis of large scale enzyme processes. Their complex functional form and strong dependence on variables such as *p*H and temperature can cause some interesting problems in optimal control and stability analysis. Ho and Humphrey (*130*) studied the optimization of the production of 6-aminopenicillic acid from penicillin G by penicillin amidase in a batch process. After a suitable formulation of the kinetics, including the effects of temperature, *p*H and time dependent enzyme denaturation, optimization policies were obtained by maximizing the yield of product within a fixed operating time while minimizing the degradation of the enzyme. O'Neill, Lilly, and Rowe (*131*) showed that the conversion of an enzymatic reaction taking place in a continuous feed stirred tank reactor subject to substrate inhibition is capable of exhibiting multiple steady states. O'Neill (*132*) expanded this work to include a more complete stability analysis and a study of the approach to steady state of this enzyme reaction system.

The use of steady state kinetic expressions to model biological processes also offers some interesting possibilities. Denbigh (*133*) has noted the possibility of using enzyme kinetics in a continuous stirred tank reactor as a model for the living cell. Tanner has suggested the use of enzyme kinetic expressions to model fermentation processes (*134*). His approach includes the rate of formation of ribosomes and the rate of production of enzymes within the cell as well as the rate of the chemical reactions catalyzed by these enzymes. In the limiting case where ribosome and enzyme production are linear in cell concentration, his equations reduce to Monod's cell growth equation. Some contact was made with experimental data from the gluconic acid fermentation process.

When diffusion steps are involved in the reaction, the microscopic conservation equations must be solved with the rate expressions already discussed. This is of special importance in heterogeneous reaction systems where the enzyme is bound to a solid support. In the next section we discuss some aspects of enzyme immobilization and some of the work that has been done in the analysis of heterogeneous enzyme reactions.

ENZYME REACTIONS ON SOLID SURFACES

Active insolubilized derivatives of enzymes are of great technological and scientific importance. *In vivo*, most enzyme reactions are heterogeneous. Enzymes are usually bound to membranes made up of lipids and proteins or embedded in some other solid surroundings. If an enzyme can be attached either physically or chemically to a well defined surface, and the kinetics of the insolubilized derivative compared to the reaction in solution, a great deal may be learned about the behavior of the enzyme in its natural enviroment. Furthermore, the development of solid matrices with enzymatic activity are beneficial for the increased use of enzymes in the chemical industry. Most industrial enzyme reactions are now carried out in a homogeneous liquid phase. In order to stop the reaction and separate the products, the enzyme must often be destroyed either by heating or by hydrolysis. Attaching enzymes to a solid support would allow almost complete recovery of the catalyst and would facilitate the design of continuous enzyme processes.

There are many methods of insolubilizing enzymes and these have been very well reviewed by McLaren and Packer (*135*), Silman and Katchalski (*136*), and Kay (*137*). All of the insolubilization procedures usually bring about marked changes in the kinetic behavior of enzymes when compared to the enzymes in solution. The most obvious effect is the decrease in the specific activity of the insoluble enzyme derivative. Many things could account for the lower observed rates of reaction in the solid phase. It is not unreasonable to suspect that the enzyme undergoes a change in its tertiary structure upon binding. This in turn can affect both the specificity of the enzyme and

its maximum velocity.

It has been observed that the *p*H-activity curves for solid phase enzymatic reactions are usually shifted one or two *pH* units to the alkaline or acidic side with respect to the activity curve for the enzyme in solution. The insolubilized enzyme is generally more stable and can withstand attack by denaturating agents and high temperatures much better than the enzyme in solution. The rigidity of the support apparently allows the protein molecule to preserve its active conformation under more adverse conditions.

Four principal insolubilization procedures have been investigated: 1. covalent attachment of the enzyme to an inert organic or inorganic support such as cellulose or glass, 2. mechanical entrapment of the enzyme in a polymer matrix, 3. covalent cross linking of the protein molecules using bifunctional reagents, and 4. physical adsorption of the enzyme to an insoluble support. In this section we consider each one of these in turn.

In order to bind enzymes covalently to solid carriers it is important that the reaction does not impair the behavior of functional groups that may be essential for catalytic activity. It is also important that the reaction conditions do not denature the protein to any significant extent. Wilson and Lilly (*138*) bound amyloglucosidase to diethyl-aminoethyl cellulose (DEAE-cellulose) which had been activated with 2-amino-4,6-dichloro-s-triazine. O'Neill, Dunnill, and Lilly (*139*) later used this insolubilized enzyme in a packed bed reactor and in a continuous stirred tank reactor for the conversion of maltose to glucose. At *p*H 8, they were able to attach 120 mg. protein/g. of support. The *p*H optimum for reaction was shifted to a value of 4 for the immobilized enzyme from a value of 5 for the enzyme in solution. The water-insoluble enzyme was approximately ¼ as active as in solution but it was able to retain its activity for a period of 21 days of continuous operation in the packed column. A mathematical model using the plug flow assumption was not adequate in explaining the results obtained from the packed bed reactor; however, the perfect mixing assumption in the analysis of the data from the CSTR was able to account well for the conversion obtained. Again using 2-amino-4, 6-dichloro-s-triazine, Kay and Lilly (*140*) attached chymotrysin to cellulose, DEAE-cellulose, carboxymethyl (CM) cellulose, Sephadex and Sepharose. They found that under certain conditions the rate of reaction was enhanced by a reduction of the size of the solid particles. This indicates that intraparticle diffusion may be rate controlling for the larger particles. Penicillin amidase from *E. coli* was bound to DE52-cellulose and cellulose filter paper by Self and co-workers (*141*) using chloro-s-triazine. A packed bed reactor for the conversion of benzylpenicillin to 6-amino-penicillic acid with 25% of the original enzymatic activity was operated for 11 weeks with no appreciable loss of enzyme. The optimum *p*H for the reaction was shifted from a *p*H of 8 to a *p*H of 7.5 by insolubilization. Wheeler, Edwards, and Whittam (*142*) were able to prepare active cellulose derivatives of apyrase (potato ATPase) but were not successful in binding rabbit kidney ATPase. The bound apyrase had 18-33% of the specific activity of the soluble enzyme and, just as the free apyrase, it needed 2-3 mM $CaCl_2$ for activation. The kinetics of β-galactosidase from *E. coli* covalently attached to cellulose sheets was studied by Sharp, Kay, and Lilly (*143*). Using a single active sheet as the reactor they studied the effect of flow rate, substrate concentration, and temperature on the hydrolysis of lactose and O-nitro-phenyl galactoside. They found that Michaelis-Menten kinetics were applicable to this system, but there was evidence of some diffusional limitations since the values obtained for the kinetic parameters depended on the flow rate through the reactor.

Axen and Ernback (*144*) attached enzymes covalently to polysaccharides by using cyanogen halides as activators. They attached chymotrypsin, trypsin, and papain to Sephadex, beads of agarose and cellulose powder using CNBr and CNI. Because of the relatively mild reaction conditions, up to 82% of the activity of soluble papain towards N-benzoyl-L-arginine ethyl ester was maintained when bound to agarose at 66 mg. of enzyme per gram of solid. The *p*H maximum for reaction was shifted about 2 *p*H units towards the alkaline side for trypsin, chymotrypsin, and papain bound to agarose. Relatively good stability in storage was observed when in the presence of humectants. Gabel and Hofsten (*145*) bound an extracellular bacterial proteinase to agarose beads using CNBr, and were able to obtain 20 mg. of enzyme per gram of conjugate. With casein as substrate this insolubilized derivative showed 65% of the proteolytic activity it had in solution. The *p*H optimum was shifted 2 *p*H units towards the alkaline side, and the stability of the enzyme at 55°C. was tremendously increased. Mosbach and Mattiasson attached hexokinase and glucose-6-phosphate dehydrogenase to polymers using CNBr and carbodiimide (*146, 147*). These enzymes catalize two sequential reactions. The insolubilized derivative was more efficient in converting substrate than the enzymes in solution. A three enzyme system (*148*) was also covalently attached to a carrier, and high activity resulted. Cyanogen halides have also been used to covalently bind ribonuclease T_1 (*149*), rennin, and chymotrypsin (*150*) to polysaccharides.

One very successful covalent attachment of an enzyme was reported by Glassmeyer and Ogle (*151*) who bound trypsin to aminoethyl cellulose using glutaraldehyde. The proteolytic activity of the bound trypsin towards benzoyl-DL-arginine-p-nitrophenyl-anilide (BAPA) was about 95% of its activity in solution. Storage at 4°C. for several months resulted in only a 15% loss in activity. Experiments indicated that many of the enzymes within the matrix were relatively inaccessible to large substrate and inhibitor molecules. Filippusson and Hornby (*152*) attached β-fructo-furanosidase to polystyrene tubes and beads by nitration and diazotization of the polymer. The *p*H-activity profiles for the bound derivatives had the same *p*H optimum as the enzyme in solution. Inman and Dintzis (*153*) have described several procedures for the activation of polyacrylamide beads for use in insolubilizing proteins. Goldstein et al. (*154*) reported a method for synthesizing carriers made of diazotizable resins. They were able to attach approximately 100 mg. of protein/g. of resin. Papain, trypsin, and subtilopeptidase A were attached, and maintained a considerable amount of activity. The insolubilized derivatives showed significant stability, and the *p*H-activity curves were displaced by one or two *p*H units to the alkaline side depending on the substrate. The kinetics of apyrase, deoxyribonuclease, and cholinesterase bound to several polymers (*155*) and of peroxidase attached to CM-cellulose (*156*) have also been studied.

The attachment of enzymes to inorganic carriers presents a suitable alternative to the covalent binding of the protein to organic supports. Weetall and Hersh (*157*) coupled urease to silica glass particles by preparing the glass in a toluene solution of γ-aminopropyltriethoxysilane followed by reaction with p-nitrobenzoic acid, reduction, and diazotization. The enzyme was found to be active, but its kinetics were not studied in detail. Alkaline phosphatase was also insolubilized using a similar procedure (*158*). Trypsin and papain were bound to porous glass in an amount of 0.12 to 2.60 mg./g. of glass (*159*), generally a lower yield than enzymes covalently bound to organic

carriers. Most of the activity of bound trypsin had been lost after 347 hr. of continuous hydrolysis of benzyol arginine ethyl ester (BAEE) at 23°C. L-amino acid oxidase bound to silica glass and packed in a column was able to maintain its activity during 35 days of continuous reaction (*160*). The *p*H-activity curve was shifted 0.3 units to the alkaline side by the binding of the protein. The enzyme lost all its activity towards some of its substrates when attached to the glass. Crystalline and crude native pepsin were also covalently bound to glass (*161*).

Another way of insolubilizing enzymes is to entrap them mechanically into a solid matrix with no covalent bonds formed between the enzyme and the support. Bernfeld and Bieber (*162*) embedded rabbit muscle enolase into a synthetic polymer matrix. The soluble and insoluble enzymes showed essentially the same *p*H-activity curve and the same dependence of the rate on the substrate and enzyme concentration, but showed different dependences on magnesium and temperature. Phosphoglycerate mutase from rabbit muscle was also imbedded in a carrier of crosslinked polyacrylamide (*163*). The insolubilized enzyme had a lower specific activity than the soluble enzyme but it showed the same variations of the rate with respect to temperature and substrate concentration. The optimum *p*H for reaction was shifted from *p*H 7 to *p*H 6.3 upon insolubilization. Mosbach and Larsson (*164*) entrapped a steroid dehydrogenase from *Corynebacterium simplex* in a crosslinked polyacrylamide gel. The embedded enzyme was able to retain only 7% of its activity towards Δ' dehydrogenation of cortisol.

Cross-linking the protein using bifunctional reagents is a fairly efficient way of making the enzyme insoluble. Schejter and Bar-Eli (*165*) reacted catalase with glutardialdehyde and suspended the precipitate in a column with Geon, a polyvinyl resin. There was considerable loss of activity upon cross-linking, but the insolubilized derivative retained 80% of its activity after five months when stored at 4°C. Haynes and Walsh (*166*) adsorbed bovine trypsin on silica particles in suspension and cross-linked the enzyme molecules with glutaraldehyde. They claimed that this procedure formed a monolayer of adsorbed enzyme on the surface of the particle and as a result, intraparticle diffusional effects were reduced. The enzyme derivative retained 80% of its activity towards benzoyl-L-arginine ethyl ester (BAEE) but only 17% of its protease activity with casein as the substrate. Goldman et al. (*167*) bound papain to a collodion membrane. Adsorption of the enzyme on the matrix was followed by cross-linking of the molecules using bisdiazobenzidine 3,3′-disulfonic acid. The resulting enzyme membrane consisted of three layers, two outer protein layers about 70 microns in thickness and the inner collodion layer, which was about 400 microns thick. 5% of the original activity towards BAEE and 40% towards benzoyl-L-arginine amide (BAA) was maintained for this insoluble papain derivative. The membrane showed typical bell-shaped *p*H-activity curves for all the substrates studied except BAEE whose rate of hydrolysis increased continually up to *p*H 9.6. The thickness of papain layers cross-linked to a collodion membrane with bisdiazobenzidine 2,2′-disulfonic acid was controlled by the amount of enzyme in solution (*168*). It was noted that the *p*H of the papain membrane became more acidic as a result of hydrolysis of the substrate BAEE. Broun, Selegny, and co-workers have bound β-D-glucose oxidase to cellophane sheets using glutaraldehyde as the bifunctional reagent (*169*), and obtained essentially the same activity as in solution.

Various enzymes have been insolubilized by physical adsorption to solid carriers. Bachler et al. (*170*) found that glucoamylase remained active when adsorbed to DEAE-cellulose. The most active preparation obtained had 55% of the activity of the enzyme in solution. It was found that the optimum temperature for reaction was displaced from 60°C. to 50°C. by binding the enzyme. Smiley (*171*) utilized a CSTR for the continuous production of glucose using charge-bound glucoamylase. Tosa et al. (*172*) have been able to bind aminoacylase to DEAE-Sephadex by adsorption. The *p*H optimum for various substrates was shifted 0.5 to 1.5 *p*H units towards the acidic side by immobilizing the enzyme. No difference was obtained in other kinetic parameters but the heat stability of the water insoluble derivative was larger. Urea, normally a protein denaturant, activated the bound enzyme. This has also been observed with many other denaturing agents (*173*). By binding aminoacylase to DEAE-Sephadex, Tosa and coworkers were able to carry out the continuous optical resolution of acyl-DL-methionine (*174*). Adsorption of leucine aminopeptidase into calcium phosphate gel is simple to carry out (*175*) and the enzyme remains very active. However, in this case, the calcium phosphate gel-enzyme complex is more susceptible to denaturation at high temperatures and organic solvents than the free enzyme. Messing (*176*) adsorbed many macromolecules to porous glass surfaces. Only 7.5 micro grams of RNAase per gram of glass remained permanently bound to porous glass (*177*) but it maintained its activity for more than 100 days. It has been found that the amount of papain and glucose oxidase that can be bound to glass is dependent on the surface area per gram of glass and the pore size diameter (*178*).

Other very appealing approaches to enzyme insolubilization have been put forward. Chang (*179, 180*) used semi-permeable microcapsules which allowed diffusion of the smaller substrates and products but prevented the large enzyme molecules from diffusing out. Rony (*180*) has recently proposed the use of hollow fibers to enclose enzyme solutions. He presented no experimental results, but solved the appropriate diffusion equations in rectangular, cylindrical, and spherical coordinates. Expressions were obtained for the effectiveness factor and the conversion in a packed bed and continuous stirred tank reactor full of hollow fiber catalysts.

Combined diffusion and reaction steps in heterogeneous enzyme reactions have just begun to be studied. Selegny et al. (*181*) solved the steady state one-dimensional diffusion equation for the case of an enzyme membrane separating two solvent compartments with different substrate concentrations. They obtained expressions for the flux of substrate into the two sides of the membrane and their calculations agreed very well with experimental data on an enzyme membrane of glucose oxidase cross-linked to cellophane. Selegny et al. (*182*) also derived a method for calculating the Michaelis constant from experimental measurements on enzyme membranes by solving for the flux of substrate from the steady state diffusion equation. Goldman, Katchalski et al. studied the kinetics of enzyme membranes with one and two layers of bound enzyme (*168, 183, 184*). Neglecting boundary layer diffusion on the solution-membrane interface (*183*), they were able to account quite well for the change in *p*H within the membrane as the reaction proceeds (*167, 168*). These results were later modified by including in the analysis the effect of a Nernst diffusion layer adjacent to the membrane (*184*). Theoretical kinetic analyses have also been carried out for two consecutive or sequential enzyme reactions in a membrane (*185*). Sluyterman and De Graaf (*186*) considered the case where reaction takes place within enzyme crystals suspended in a substrate solution. A critical size for

intraparticle diffusion control was derived along the same lines of an effectiveness factor calculation. Several of their assumptions such as that the concentration of free enzyme is independent of position remain to be investigated.

ADDITIONAL TRENDS

Through the painstaking work of biochemists and biologists, a great deal has been learned about the reactions of enzymes, their production in living cells (*187*), and the complex control processes that regulate their reactions in biological organisms (*188* to *190*). Enzymes have been found to be useful in medicine for the treatment of metabolic diseases (*191*). Industrial applications of enzymes have just begun to be explored. Wang and Humphrey (*192*) present an excellent introduction to basic concepts and current and potential uses for enzymes.

Increasing the production of enzymes for research as well as for industrial purposes will probably be one of the major goals of investigators in the near future. Ultrafiltration, affinity chromatography, and fractionation with heteropolyacids have already been mentioned as new purification procedures that hold out a great promise for the isolation of enzymes in large quantities.

For engineering purposes, better knowledge of enzyme kinetics and the factors that influence the rate of reactions is needed. This is specially true if large scale production processes are to be designed with enzymes as the catalysts. Automatic apparatus for recording enzyme kinetic data (*193*) will be a great aid in this task. Besides efforts aimed at increasing our understanding of the intrinsic kinetics of enzymes, a great deal of work remains to be done on heterogeneous enzyme reactions where coupled diffusion and reaction steps are important. Optimal control and stability analyses of enzyme reactors will become increasingly important as new reaction systems are designed. More efficient computer methods of analyzing reaction data (*194*) will increase the capability of the researcher in elucidating reaction mechanisms.

If enzymes are to be introduced in fluid flow fields in chemical processes, the effects of shear stress on the activity of the enzymes need to be studied (*27, 28*). Possible changes in conformation might be studied by using methods for the analysis of the structure of proteins in solution (*195*).

The advantages of having insolubilized enzymes have been mentioned. The binding of enzymes that catalyze sequential or parallel reactions will greatly increase the flexibility of continuous packed bed or stirred tank reactors. Combined utrafiltration and reaction systems have achieved some success in separating the products and unreacted substrates from the enzyme without insolubilizing or denaturing the protein (*196, 197*). This type of design offers a suitable alternative for catalyst recovery.

It is well recognized that the potential uses for enzymes are numerous (*198*). By lysing the cell walls of microorganisms and releasing proteins suitable for human consumption, enzymes may be used to increase the world food supply. One attempt at a process of this type has been published by Carenberg and Heden (*199*). Enzymes have application in chemical analysis and are currently being used as electrodes for detection of substances such as urea (*200*). Industrially, enzymes may be used to catalyze difficult reactions for food processing. The increased use of enzymes in consumer products such as detergents is dependent on the results of further investigations on the effects of enzymes on health and the environment. In short, there is no lack of work to be done in the area of enzyme technology. During the coming years our knowledge of enzymes will surely expand, and hopefully, a new dimension will have been added to the already vast activities encompassed by chemical engineering.

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NOTATION

E_0 = total enzyme concentration
H = hydronium ion concentration
I = inhibitor concentration
K_m = Michaelis constant in the steady state rate equation
K_1, K_2, K_3 = constants in the two-substrate steady state rate equation
K_a, K_b = equilibrium constants for protonation and deprotonation of the enzyme-substrate complex
K_i = dissociation constant for enzyme-inhibitor complex
k_p = rate constant in Michaelis-Menten equation
M = concentration in molar units
P = product concentration
S = substrate concentration
T = temperature
t = time
V = maximum velocity, k_pE_0
V_0 = maximum velocity at optimum pH
v = rate of formation of products at steady state

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