3

Enzymes

3.1. INTRODUCTION

Enzymes are usually proteins of high molecular weight (15,000 < MW < several million daltons) that act as catalysts. Recently, it has been shown that some RNA molecules are also catalytic, but the vast majority of cellular reactions are mediated by protein catalysts. RNA molecules that have catalytic properties are called *ribozymes*. Enzymes are specific, versatile, and very effective biological catalysts, resulting in much higher reaction rates as compared to chemically catalyzed reactions under ambient conditions. More than 2000 enzymes are known. Enzymes are named by adding the suffix *-ase* to the end of the substrate, such as urease, or the reaction catalyzed, such as alcohol dehydrogenase. Some enzymes have a simple structure, such as a folded polypeptide chain (typical of most hydrolytic enzymes). Many enzymes have more than one subunit. Some protein enzymes require a nonprotein group for their activity. This group is either a cofactor, such as metal ions, Mg, Zn, Mn, Fe, or a coenzyme, such as a complex organic molecule, NAD, FAD, CoA, or some vitamins. An enzyme containing a nonprotein group is called a *holoenzyme*. The protein part of this enzyme is the *apoenzyme* (holoenzyme = apoenzyme + cofactor). Enzymes that occur in several different molecular forms, but catalyze the same reaction, are called *isozymes*. Some enzymes are grouped together to form enzyme complexes. Enzymes are substrate specific and are classified according to the reaction they catalyze. Major classes of enzymes and their functions are listed in Table 3.1.

TABLE 3.1 International Classification of Enzymes: Class Names, Code Numbers, and Types of Reactions Catalyzed

1. Oxidoreductases (oxidation-reduction reactions)
   - 1.1 Acting on $\diagdown$CH—OH$\diagup$
   - 1.2 Acting on $\diagdown$C=O$\diagup$
   - 1.3 Acting on $\diagdown$C=CH—$\diagup$
   - 1.4 Acting on $\diagdown$CH—$NH_2$$\diagup$
   - 1.5 Acting on $\diagdown$CH—NH—$\diagup$
   - 1.6 Acting on NADH; NADPH
2. Transferases (transfer of functional groups)
   - 2.1 One-carbon groups
   - 2.2 Aldehydic or ketonic groups
   - 2.3 Acyl groups
   - 2.4 Glycosyl groups
   - 2.7 Phosphate groups
   - 2.8 S-containing groups
3. Hydrolases (hydrolysis reactions)
   - 3.1 Esters
   - 3.2 Glycosidic bonds
   - 3.4 Peptide bonds
   - 3.5 Other C—N bonds
   - 3.6 Acid anhydrides
4. Lyases (addition to double bonds)
   - 4.1 $\diagdown$C=C$\diagup$
   - 4.2 $\diagdown$C=O$\diagup$
   - 4.3 $\diagdown$C=N—$\diagup$
5. Isomerases (isomerization reactions)
   - 5.1 Racemases
6. Ligases (formation of bonds with ATP cleavage)
   - 6.1 C—O
   - 6.2 C—S
   - 6.3 C—N
   - 6.4 C—C

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3.2. HOW ENZYMES WORK

Enzymes lower the activation energy of the reaction catalyzed by binding the substrate and forming an enzyme–substrate complex. Enzymes do not affect the free-energy change or the equilibrium constant. Figure 3.1 illustrates the action of an enzyme from the activation-energy point of view. For example, the activation energy for the decomposition of hydrogen peroxide varies depending on the type of catalysis. The activation energy of the uncatalyzed reaction at 20°C is 18 kilocalories per mole (kcal/mol), whereas the ΔE values for chemically catalyzed (by colloidal platinum) and enzymatically catalyzed (catalase) decomposition are 13 and 7 kcal/mol, respectively. That is, catalase accelerates the rate of reaction by a factor of about 10^8. The reader should note that this large change in rate for a relatively small change in activation energy is due to the exponential dependence of rate on activation energy. In this case, the ratio of the rates is $\exp(-7000/2 \cdot 293) \div \exp(-18{,}000/2 \cdot 293)$.

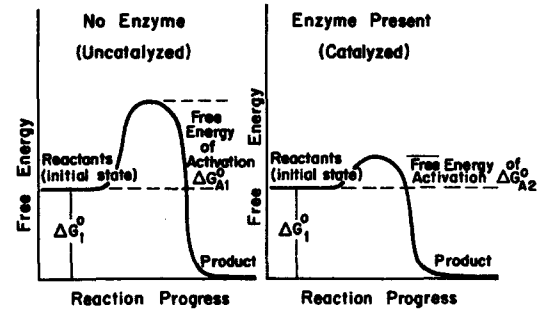

Figure 3.1. Activation energies of enzymatically catalyzed and uncatalyzed reactions. Note that $|\Delta G^\circ_{A_2}| < |\Delta G^\circ_{A_1}|$.

The molecular aspects of enzyme–substrate interaction are not yet fully understood. This interaction varies from one enzyme–substrate complex to another. Various studies using x-ray and Raman spectroscopy have revealed the presence of the enzyme–substrate (ES) complex. The interaction between the enzyme and its substrate is usually by weak forces. In most cases, van der Waals forces and hydrogen bonding are responsible for the formation of ES complexes. The substrate binds to a specific site on the enzyme known as the *active site*. The substrate is a relatively small molecule and fits into a certain region on the enzyme molecule, which is a much larger molecule. The simplest model describing this interaction is the lock-and-key model, in which the enzyme represents the lock and the substrate represents the key, as described in Fig. 3.2.

In multisubstrate enzyme-catalyzed reactions, enzymes can hold substrates such that reactive regions of substrates are close to each other and to the enzyme's active site, which is known as the *proximity effect*. Also, enzymes may hold the substrates at certain positions and angles to improve the reaction rate, which is known as the *orientation effect*. In some enzymes, the formation of an enzyme–substrate complex causes slight changes in the three-dimensional shape of the enzyme. This induced fit of the substrate to the enzyme molecule may contribute to the catalytic activity of the enzyme, too. The enzymes lysozyme and carboxypeptidase A have been observed to change their three-dimensional structure upon complexing with the substrate. Enzyme catalysis is affected not only by the primary structure of enzymes but also by the secondary, tertiary, and quaternary structures. The properties of the active site of enzymes and the folding characteristics have a profound effect on the catalytic activity of enzymes. Certain enzymes require coenzymes and cofactors for proper functioning. Table 3.2 lists some enzymes and their cofactors and coenzymes.

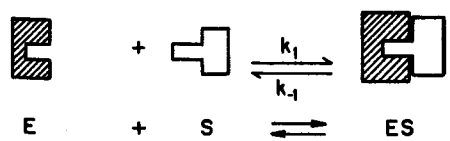

Figure 3.2. Schematic of the lock-and-key model of enzyme catalysis.

TABLE 3.2 Cofactors (Metal Ions) and Coenzymes of Some Enzymes

	Coenzyme	*Entity transferred*
Zn^{2+}	Nicotinamide adenine dinucleotide	Hydrogen atoms (electrons)
Alcohol dehydrogenase	Nicotinamide adenine dinucleotide	Hydrogen atoms (electrons)
Carbonic anhydrase	phosphate	Hydrogen atoms (electrons)
Carboxypeptidase	Flavin mononucleotide	Hydrogen atoms (electrons)
Mg^{2+}	Flavin adenine dinucleotide	Hydrogen atoms (electrons)
Phosphohydrolases	Coenzyme Q	Aldehydes
Phosphotransferases	Thiamin pyrophosphate	Acyl groups
Mn^{2+}	Coenzyme A	Acyl groups
Arginase	Lipoamide	Alkyl groups
Phosphotransferases	Cobamide coenzymes	Carbon dioxide
Fe^{2+} or Fe^{3+}	Biocytin	Amino groups
Cytochromes	Pyridoxal phosphate	Methyl, methylene, formyl,
Peroxidase	Tetrahydrofolate coenzymes	or formimino groups
Catalase		
Ferredoxin		
Cu^{2+} (Cu^{+})		
Tyrosinase		
Cytochrome oxidase		
K^{+}		
Pyruvate kinase (also requires Mg^{2+})		
Na^{+}		
Plasma membrane ATPase (also requires K^{+} and Mg^{2+})		

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3.3. ENZYME KINETICS

3.3.1. Introduction

A mathematical model of the kinetics of single-substrate-enzyme-catalyzed reactions was first developed by V. C. R. Henri in 1902 and by L. Michaelis and M. L. Menten in 1913. Kinetics of simple enzyme-catalyzed reactions are often referred to as Michaelis–Menten kinetics or *saturation* kinetics. The qualitative features of enzyme kinetics are similar to Langmuir–Hinshelwood kinetics (see Fig. 3.3). These models are based on data from batch reactors with constant liquid volume in which the initial substrate, $[S_0]$, and enzyme, $[E_0]$, concentrations are known. More complicated enzyme-substrate interactions such as multisubstrate–multienzyme reactions can take place in biological systems. An enzyme solution has a fixed number of active sites to which substrates can bind. At high substrate concentrations, all these sites may be occupied by substrates or the enzyme is *saturated*. Saturation kinetics can be obtained from a simple reaction scheme that involves a reversible step for enzyme–substrate complex formation and a dissociation step of the ES complex.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad (3.$$

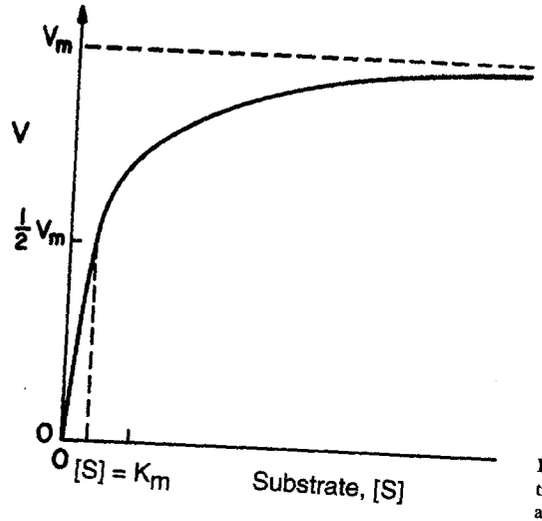


Figure 3.3. Effect of substrate concentration on the rate of an enzyme-catalyzed reaction.

It is assumed that the ES complex is established rather rapidly and the rate of the reverse reaction of the second step is negligible. The assumption of an irreversible second reaction often holds only when product accumulation is negligible at the beginning of the reaction. Two major approaches used in developing a rate expression for the enzyme-catalyzed reactions are (1) rapid-equilibrium approach and (2) quasi-steady-state approach.

3.3.2. Mechanistic Models for Simple Enzyme Kinetics

Both the quasi-steady-state approximation and the assumption of rapid equilibrium share the same few initial steps in deriving a rate expression for the mechanism in eq. 3.1, where the rate of product formation is

$$v = \frac{d[P]}{dt} = k_2[ES] \qquad (3.2)$$

where v is the rate of product formation or substrate consumption in moles/l-s.

The rate constant k_2 is often denoted as k_{cat} in the biological literature. The rate of variation of the ES complex is

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_2[ES] \qquad (3.3)$$

Since the enzyme is not consumed, the conservation equation on the enzyme yields

$$[E] = [E_0] - [ES] \qquad (3.4)$$

At this point, an assumption is required to achieve an analytical solution.

3.3.2.1. The rapid equilibrium assumption. Henri and Michaelis and Menten used essentially this approach. Assuming a rapid equilibrium between the enzyme and

substrate to form an [ES] complex, we can use the equilibrium coefficient to express [ES] in terms of [S].

The equilibrium constant is

$$K'_m = \frac{k_{-1}}{k_1} = \frac{[E][S]}{[ES]} \tag{3.5}$$

Since $[E] = [E_0] - [ES]$ if enzyme is conserved, then

$$[ES] = \frac{[E_0][S]}{(k_{-1}/k_1) + [S]} \tag{3.6}$$

$$[ES] = \frac{[E_0][S]}{K'_m + [S]} \tag{3.7}$$

where $K'_m = k_{-1}/k_1$, which is the dissociation constant of the ES complex. Substituting eq. 3.7 into eq. 3.2 yields

$$v = \frac{d[P]}{dt} = k_2 \frac{[E_0][S]}{K'_m + [S]} = \frac{V_m[S]}{K'_m + [S]} \tag{3.8}$$

where $V_m = k_2[E_0]$.

In this case, the maximum forward velocity of the reaction is V_m. V_m changes if more enzyme is added, but the addition of more substrate has no influence on V_m. K'_m is often called the Michaelis–Menten constant, and the prime reminds us that it was derived by assuming rapid equilibrium in the first step. A low value of K'_m suggests that the enzyme has a high affinity for the substrate. Also, K'_m corresponds to the substrate concentration, giving the half-maximal reaction velocity.

An equation of exactly the same form as eq. 3.8 can be derived with a different, more general assumption applied to the reaction scheme in eq. 3.1.

3.3.2.2. The quasi-steady-state assumption. In many cases the assumption of rapid equilibrium following mass-action kinetics is not valid, although the enzyme–substrate reaction still shows saturation-type kinetics.

G. E. Briggs and J. B. S. Haldane first proposed using the quasi-steady-state assumption. In most experimental systems a closed system (batch reactor) is used in which the initial substrate concentration greatly exceeds the initial enzyme concentration. They suggest that since $[E_0]$ was small, $d[ES]/dt \approx 0$. (This logic is flawed. Do you see why?) Computer simulations of the actual time course represented by eqs. 3.2, 3.3, and 3.4 have shown that *in a closed system the quasi-steady-state hypothesis holds* after a brief transient *if* $[S_0] \gg [E_0]$ (for example, 100×). Figure 3.4 displays one such time course.

By applying the quasi-steady-state assumption to eq. 3.3, we find

$$[ES] = \frac{k_1[E][S]}{k_{-1} + k_2} \tag{3.9}$$

Substituting the enzyme conservation eq. 3.4 in eq. 3.9 yields

$$[ES] = \frac{k_1([E_0] - [ES])[S]}{k_{-1} + k_2} \tag{3.10}$$

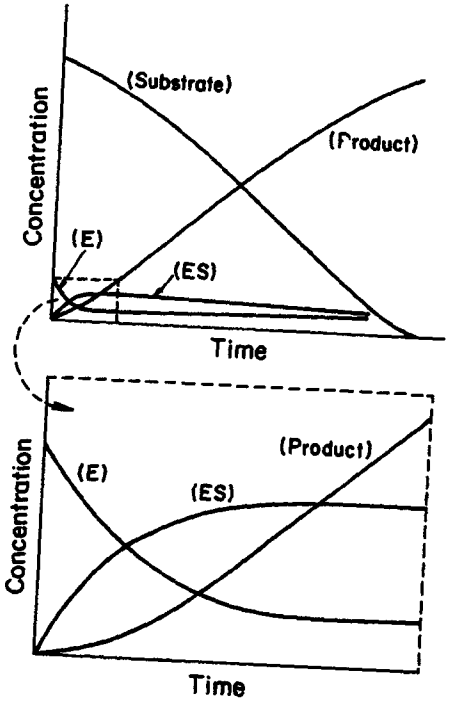


Figure 3.4. Time course of the formation of an enzyme/substrate complex and initiation of the steady state, as derived from computer solutions of data obtained in an actual experiment on a typical enzyme. The portion in the dashed box in the top graph is shown in magnified form on the lower graph. (With permission, adapted from A. Lehninger, *Biochemistry*, 2d ed., Worth Publishers, New York, 1975, p. 191.)

Solving eq. 3.10 for [ES],

$$[ES] = \frac{[E_0][S]}{\frac{k_{-1} + k_2}{k_1} + [S]} \tag{3.11}$$

Substituting eq. 3.11 into eq. 3.2 yields

$$v = \frac{d[P]}{dt} = \frac{k_2[E_0][S]}{\frac{k_{-1} + k_2}{k_1} + [S]} \tag{3.12a}$$

$$v = \frac{V_m[S]}{K_m + [S]} \tag{3.12b}$$

where K_m is $(k_{-1} + k_2)/k_1$ and V_m is $k_2[E_0]$. Under most circumstances (simple experiments), it is impossible to determine whether K_m or K'_m is more suitable. Since K_m results from the more general derivation, we will use it in the rest of our discussions.

3.3.3. Experimentally Determining Rate Parameters for Michaelis–Menten Type Kinetics

The determination of values for K_m and V_m with high precision can be difficult. Typically, experimental data are obtained from *initial-rate experiments*. A batch reactor is charged with a known amount of substrate $[S_0]$ and enzyme $[E_0]$. The product (or substrate concentration) is plotted against time. The initial slope of this curve is estimated (i.e., $v = d[P]/dt|_{t=0} = -d[S]/dt|_{t=0}$). This value of v then depends on the values of $[E_0]$ and $[S_0]$ in the charge to the reactor. Many such experiments can be used to generate many pairs of v and $[S]$ data. These could be plotted as in Fig. 3.3, but the accurate determination of K_m from such a plot is very difficult. Consequently, other methods of analyzing such data have been suggested.

3.3.3.1. Double-reciprocal plot (Lineweaver–Burk plot). Equation 3.12b can be linearized in double-reciprocal form:

$$\frac{1}{v} = \frac{1}{V_m} + \frac{K_m}{V_m}\frac{1}{[S]} \tag{3.13}$$

A plot of $1/v$ versus $1/[S]$ yields a linear line with a slope of K_m/V_m and y-axis intercept of $1/V_m$, as depicted in Fig. 3.5. A double-reciprocal plot gives good estimates on V_m, but not necessarily on K_m. Because the error about the reciprocal of a data point is not symmetric, the reader should be cautious in applying regression analysis (least squares) to such plots. Data points at low substrate concentrations influence the slope and intercept more than those at high substrate concentrations.

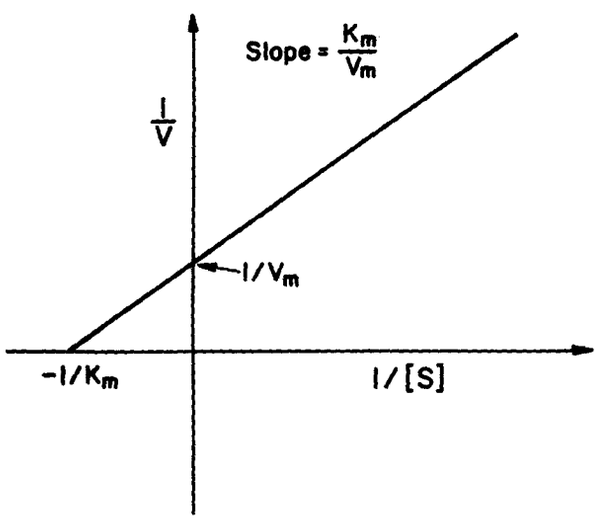


Figure 3.5. Double-reciprocal (Lineweaver–Burk) plot.

3.3.3.2. Eadie–Hofstee plot. Equation 3.12b can be rearranged as

$$v = V_m - K_m\frac{v}{[S]} \tag{3.14}$$

A plot of v versus $v/[S]$ results in a line of slope $-K_m$ and y-axis intercept of V_m, as depicted in Fig. 3.6. Eadie–Hofstee plots can be subject to large errors since both coordinates contain v, but there is less bias on points at low $[S]$.

3.3.3.3. Hanes–Woolf plot. Rearrangement of eq. 3.12b yields

$$\frac{[S]}{v} = \frac{K_m}{V_m} + \frac{1}{V_m}[S] \tag{3.15}$$

A plot of $[S]/v$ versus $[S]$ results in a line of slope $1/V_m$ and y-axis intercept of K_m/V_m, as depicted in Fig. 3.7. This plot is used to determine V_m more accurately.

3.3.3.4. Batch kinetics. The time course of variation of $[S]$ in a batch enzymatic reaction can be determined from

$$v = -\frac{d[S]}{dt} = \frac{V_m[S]}{K_m + [S]} \tag{3.12b}$$

by integration to yield

$$V_m t = [S_0] - [S] + K_m \ln\frac{[S_0]}{[S]} \tag{3.16}$$

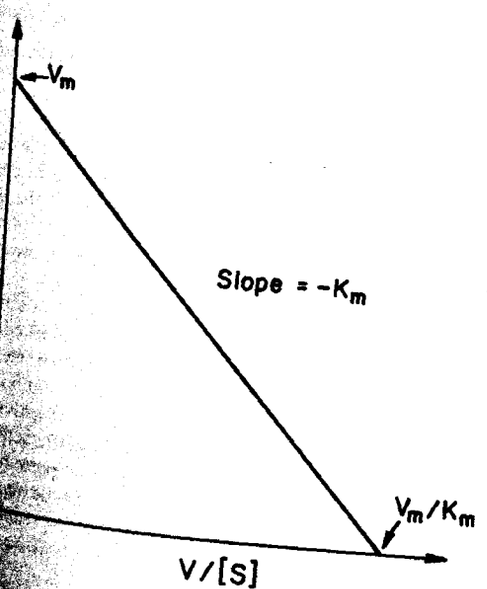


Figure 3.6. Eadie–Hofstee plot.

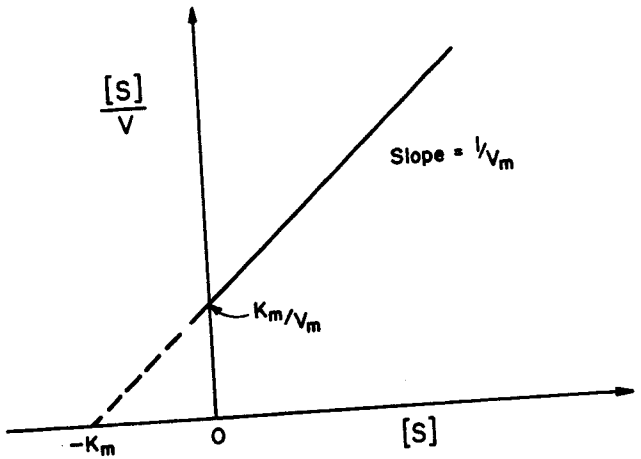


Figure 3.7. Hanes–Woolf plot.

or

$$V_m - \frac{[S_0]-[S]}{t} = \frac{K_m}{t}\ln\frac{[S_0]}{[S]} \tag{3.17}$$

A plot of $1/t \ln[S_0]/[S]$ versus $\{[S_0] - [S]\}/t$ results in a line of slope $-1/K_m$ and intercept of V_m/K_m.

3.3.3.5. Interpretation of K_m and V_m. While K_m (or K_m') is an intrinsic parameter, V_m is not. K_m is solely a function of rate parameters and is expected to change with temperature or pH. However, V_m is a function of the rate parameter k_2 and the initial enzyme level, $[E_0]$. As $[E_0]$ changes, so does V_m. Of course, k_2 can be readily calculated if $[E_0]$ is known. For highly purified enzyme preparations it may be possible to express $[E_0]$ in terms of mol/l or g/l.

When the enzyme is part of a crude preparation, its concentration is in terms of "units." A "unit" is the amount of enzyme that gives a predetermined amount of catalytic activity under specific conditions. For example, one unit would be formation of one μmol product per minute at a specified pH and temperature with a substrate concentration much greater than the value of K_m. The *specific activity* is the number of units of activity per amount of total protein. For example, a crude cell lysate might have a specific activity of 0.2 units/mg protein which upon purification may increase to 10 units/mg protein. One enzyme that remains catalytically active will be measured. The enzyme may be *denatured* if it unfolds or has its three-dimensional shape altered by pH extremes or temperature during purification. The denatured enzyme will have no activity.

Example 3.1.

To measure the amount of glucoamylase in a crude enzyme preparation, 1 ml of the crude enzyme preparation containing 8 mg protein is added to 9 ml of a 4.44% starch solution. One unit of activity of glucoamylase is defined as the amount of enzyme which produces 1 μmol of glucose per min in a 4% solution of Lintner starch at pH 4.5 and at 60°C. Initial rate experiments show that the reaction produces 0.6 μmol of glucose/ml-min. What is the specific activity of the crude enzyme preparation?

Solution The total amount of glucose made is 10 ml × 0.6 μmol glucose/ml-min or 6 μmol glucose per min. The specific activity is then:

$$\text{specific activity} = \frac{6 \text{ units}}{1 \text{ ml protein solution} \cdot 8 \text{ mg/ml}}$$
$$= 6 \text{ units/8 mg protein}$$
$$= 0.75 \text{ units/mg protein}$$

V_m must have units such as μmol product/ml-min. Since $V_m = k_2E_0$, the dimensions of k_2 must reflect the definition of units in E_0. In the above example we had a concentration of enzyme of 8 mg protein/10 ml solution · 0.75 units/mg protein or 0.6 units/ml. If, for example, V_m = 1 μmol/ml-min, then k_2 = 1 μmol/ml-min ÷ 0.6 units/ml or k_2 = 1.67 μmol/unit-min.

3.3.4. Models for More Complex Enzyme Kinetics

3.3.4.1. Allosteric enzymes. Some enzymes have more than one substrate binding site. The binding of one substrate to the enzyme facilitates binding of other substrate molecules. This behavior is known as *allostery* or *cooperative binding,* and regulatory enzymes show this behavior. The rate expression in this case is

$$v = -\frac{d[S]}{dt} = \frac{V_m[S]^n}{K_m'' + [S]^n} \tag{3.18}$$

where n = cooperativity coefficient and $n > 1$ indicates positive cooperativity. Figure 3.8 compares Michaelis–Menten kinetics with allosteric enzyme kinetics, indicating a sigmoidal shape of υ –[S] plot for allosteric enzymes.

The cooperativity coefficient can be determined by rearranging eq. 3.18 as

$$\ln\frac{v}{V_m - v} = n\ln[S] - \ln K_m'' \tag{3.19}$$

and by plotting $\ln v/(V_m - v)$ versus ln[S] as depicted in Fig. 3.9.

3.3.4.2. Inhibited enzyme kinetics. Certain compounds may bind to enzymes and reduce their activity. These compounds are known to be enzyme inhibitors. Enzyme inhibitions may be irreversible or reversible. Irreversible inhibitors such as heavy metals (lead, cadium, mercury, and others) form a stable complex with enzyme and reduce enzyme activity. Such enzyme inhibition may be reversed only by using chelating agents such as EDTA (ethylenediaminetetraacetic acid) and citrate. Reversible inhibitors may dissociate more easily from the enzyme after binding. The three major classes of re-

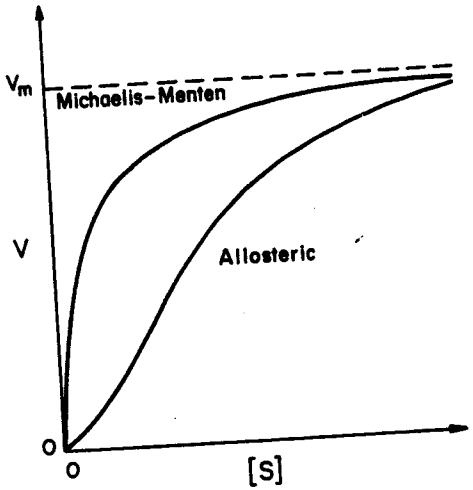


Figure 3.8. Comparison of Michaelis–Menten and allosteric enzyme kinetics.

versible enzyme inhibitions are competitive, noncompetitive, and uncompetitive inhibitions. The substrate may act as an inhibitor in some cases.

Competitive inhibitors are usually substrate analogs and compete with substrate for the active site of the enzyme. The competitive enzyme inhibition scheme can be described as

$$\begin{array}{ccccc} E+S & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & ES & \xrightarrow{k_2} & E+P \\ + & & & & \\ I & & & & \\ \Updownarrow K_I & & & & \\ EI & & & & \end{array} \qquad (3.20)$$

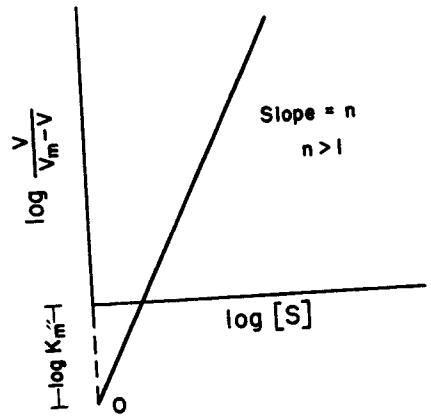


Figure 3.9. Determination of cooperativity coefficient.

Assuming rapid equilibrium and with the definition of

$$K_m' = \frac{[E][S]}{[ES]}, \qquad K_I = \frac{[E][I]}{[EI]} \qquad (3.21)$$

$$[E_0] = [E] + [ES] + [EI] \quad \text{and} \quad v = k_2[ES]$$

we can develop the following equation for the rate of enzymatic conversion:

$$v = \frac{V_m[S]}{K_m'\left[1 + \dfrac{[I]}{K_I}\right] + [S]} \qquad (3.22)$$

or

$$v = \frac{V_m[S]}{K_{m,\,app}' + [S]} \qquad (3.23)$$

where $K_{m,app}' = K_m'\left(1 + \dfrac{[I]}{K_I}\right)$.

The net effect of competitive inhibition is an increased value of $K_{m,\,app}'$ and, therefore, reduced reaction rate. Competitive inhibition can be overcome by high concentrations of substrate. Figure 3.10 describes competitive enzyme inhibition in the form of a double-reciprocal plot.

Noncompetitive inhibitors are not substrate analogs. Inhibitors bind on sites other than the active site and reduce enzyme affinity to the substrate. Noncompetitive enzyme inhibition can be described as follows:

$$\begin{array}{ccccc} E+S & \underset{K_m'}{\rightleftharpoons} & ES & \xrightarrow{k_2} & E+P \\ + & & + & & \\ I & & I & & \\ \Updownarrow K_I & & \Updownarrow & & \\ EI+S & \underset{K_m'}{\rightleftharpoons} & ESI & & \end{array} \qquad (3.24)$$

With the definition of

$$K_m' = \frac{[E][S]}{[ES]} = \frac{[EI][S]}{[ESI]}, \qquad K_I = \frac{[E][I]}{[EI]} = \frac{[ES][I]}{[ESI]} \qquad (3.25)$$

$$[E_0] = [E] + [ES] + [EI] + [ESI] \text{ and } v = k_2[ES]$$

we can develop the following rate equation:

$$v = \frac{V_m}{\left(1 + \dfrac{[I]}{K_I}\right)\left(1 + \dfrac{K_m'}{[S]}\right)} \qquad (3.26)$$

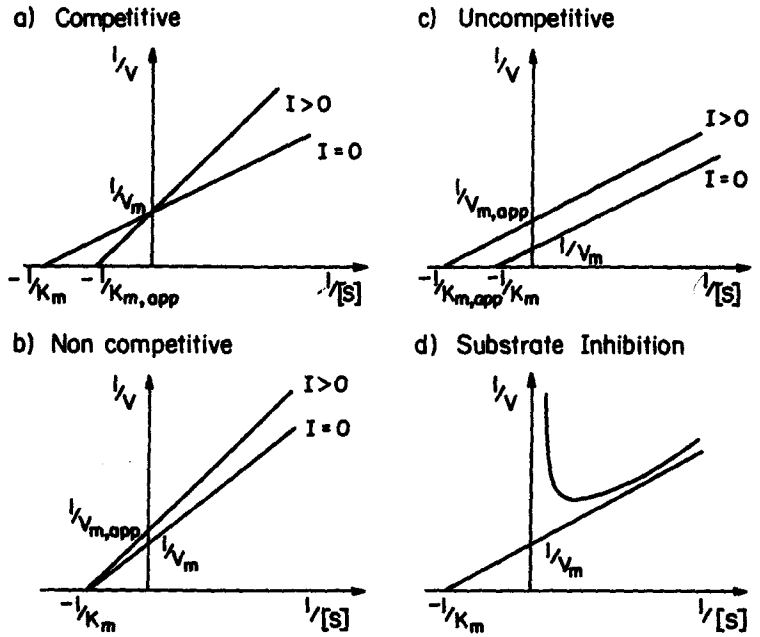


Figure 3.10. Different forms of inhibited enzyme kinetics.

or

$$v = \frac{V_{m,app}}{\left(1 + \frac{K'_m}{[S]}\right)} \tag{3.27}$$

where $V_{m,app} = \dfrac{V_m}{\left(1 + \dfrac{[I]}{K_1}\right)}$

The net effect of noncompetitive inhibition is a reduction in V_m. High substrate concentrations would not overcome noncompetitive inhibition. Other reagents need to be added to block binding of the inhibitor to the enzyme. In some forms of noncompetitive inhibition V_m is reduced and K'_m is increased. This occurs if the complex ESI can form product.

Uncompetitive inhibitors bind to the ES complex only and have no affinity for the enzyme itself. The scheme for uncompetitive inhibition is

$$\begin{array}{ccccc} E + S & \underset{}{\overset{K'_m}{\rightleftharpoons}} & ES & \xrightarrow{k_2} & E + P \\ & & + & & \\ & & I & & \\ & & \Big\| K_1 & & \\ & & ESI & & \end{array} \tag{3.28}$$

With the definition of

$$K'_m = \frac{[E][S]}{[ES]}, \quad K_1 = \frac{[ES][I]}{[ESI]}$$
$$[E_0] = [E] + [ES] + [ESI] \text{ and } v = k_2[ES] \tag{3.29}$$

we can develop the following equation for the rate of reaction:

$$v = \frac{\dfrac{V_m}{\left(1 + \dfrac{[I]}{K_1}\right)}[S]}{\dfrac{K'_m}{\left(1 + \dfrac{[I]}{K_1}\right)} + [S]} \tag{3.30}$$

or

$$v = \frac{V_{m,app}[S]}{K'_{m,app} + [S]} \tag{3.31}$$

The net effect of uncompetitive inhibition is a reduction in both V_m and K'_m values. Reduction in V_m has a more pronounced effect than the reduction in K'_m, and the net result is a reduction in reaction rate. Uncompetitive inhibition is described in Fig. 3.10 in the form of a double-reciprocal plot.

High substrate concentrations may cause inhibition in some enzymatic reactions, known as *substrate inhibition*. Substrate inhibition is graphically described in Fig. 3.11.

The reaction scheme for uncompetitive substrate inhibition is

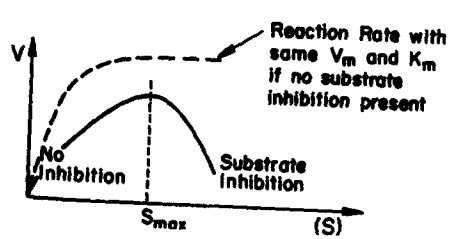
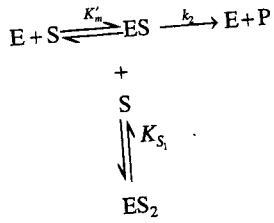


Figure 3.11. Comparison of substrate-inhibited and uninhibited enzymatic reactions.



(3.32)

With the definitions of

$$K_{S_1} = \frac{[S][ES]}{[ES_2]}, \quad K'_m = \frac{[S][E]}{[ES]} \tag{3.33}$$

the assumption of rapid equilibrium yields

$$v = \frac{V_m[S]}{K'_m + [S] + \dfrac{[S]^2}{K_{S_1}}} \tag{3.34}$$

A double-reciprocal plot describing substrate inhibition is given in Fig. 3.10.

At low substrate concentrations, $[S]^2/K_{S_1} << 1$, and inhibition effect is not observed. The rate is

$$v = \frac{V_m}{\left[1 + \dfrac{K'_m}{[S]}\right]} \tag{3.35}$$

or

$$\frac{1}{v} = \frac{1}{V_m} + \frac{K'_m}{V_m}\frac{1}{[S]} \tag{3.36}$$

A plot of $1/v$ versus $1/[S]$ results in a line of slope K'_m/V_m and intercept of $1/V_m$.

At high substrate concentrations, $K'_m/[S] << 1$, and inhibition is dominant. The rate in this case is

$$v = \frac{V_m}{\left(1 + \dfrac{[S]}{K_{S_1}}\right)} \tag{3.37}$$

or

$$\frac{1}{v} = \frac{1}{V_m} + \frac{[S]}{K_{S_1}V_m} \tag{3.38}$$

A plot of $1/v$ versus $[S]$ results in a line of slope $1/K_{S_1} \cdot V_m$ and intercept of $1/V_m$.

The substrate concentration resulting in the maximum reaction rate can be determined by setting $dv/d[S] = 0$. The $[S]_{max}$ is given by

$$[S]_{max} = \sqrt{K'_m K_{S_1}} \tag{3.39}$$

Example 3.2

The following data have been obtained for two different initial enzyme concentrations for an enzyme-catalyzed reaction.

$v([E_0] = 0.015$ g/l) (g/l-min)	[S] (g/l)	$v([E_0] = 0.00875$ g/l) (g/l-min)
1.14	20.0	0.67
0.87	10.0	0.51
0.70	6.7	0.41
0.59	5.0	0.34
0.50	4.0	0.29
0.44	3.3	
0.39	2.9	
0.35	2.5	

a. Find K_m.
b. Find V_m for $[E_0] = 0.015$ g/l.
c. Find V_m for $[E_0] = 0.00875$ g/l.
d. Find k_2.

Solution A Hanes–Woolf plot (Fig. 3.12) can be used to determine V_m and K_m.

$$\frac{[S]}{v} = \frac{K_m}{V_m} + \frac{1}{V_m}[S]$$

$[S]/v$ ($E_0 = 0.015$) (min)	$[S]/v$ ($E_0 = 0.00875$) (min)	[S] (g/l)
17.5	30	20.0
11.5	20	10.0
9.6	16	6.7
8.5	15	5.0
8.0	14	4.0
7.6		3.3
7.3		2.9
7.1		2.5

From a plot of $[S]/v$ versus $[S]$ for $E_0 = 0.015$ g/l, the slope is found to be 0.6 min/g/l and $V_m = 1/0.6 = 1.7$ g/l min. The y-axis intercept is $K_m/V_m = 5.5$ min and $K_m = 9.2$ g [S]/l.

Also, $V_m = k_2E_0$ and $k_2 = 1.7/0.015 = 110$ g/g enzyme-min. The Hanes–Woolf plot for $E_0 = 0.00875$ g/l gives a slope of 1.0 min/g/l and $V_m = 1.0$ g/l-min; $k_2 = V_m/E_0 = 1.0/0.00875 = 114$ g/g enzyme-min.

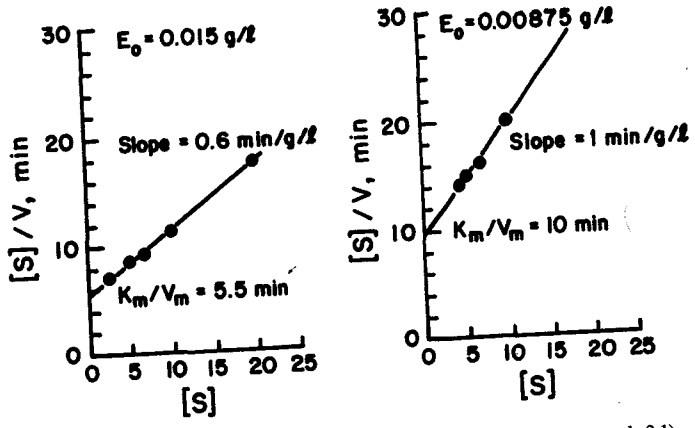


Figure 3.12. Hanes–Woolf plots for $E_0 = 0.015$ g/l and $E_0 = 0.00875$ g/l (Example 3.1).

Example 3.3

The hydrolysis of urea by urease is an only partially understood reaction and shows inhibition. Data for the hydrolysis of the reaction are given next.

Substrate concentration:	0.2 *M*		0.02 *M*	
	1/*v*	I	1/*v*	I
	0.22	0	0.68	0
	0.33	0.0012	1.02	0.0012
	0.51	0.0027	1.50	0.0022
	0.76	0.0044	1.83	0.0032
	0.88	0.0061	2.04	0.0037
	1.10	0.0080	2.72	0.0044
	1.15	0.0093	3.46	0.0059

where v = moles/l-min and I is inhibitor molar concentration.

a. Determine the Michaelis–Menten constant (K'_m) for this reaction.

b. What type of inhibition reaction is this? Substantiate the answer.

c. Based on the answer to part b, what is the value of K_i?

Solution A double-reciprocal plot of 1/v versus 1/[S] for inhibitor concentrations I = 0, 0.0012, 0.0044, and 0.006 indicates that the inhibition is noncompetitive (Fig. 3.13). From the x-axis intercept of the plot, $-1/K'_m = -13$ and $K'_m \approx 7.77 \times 10^{-2}$ *M*. For [S] = 0.2 *M* and I = 0 from the intercept of 1/V versus 1/S, $1/v_m = 0.2$ and $V_m \cong 5$ moles/l-min. For I = 0.0012 *M* and [S] = 0.2 *M*, $v = 3$ moles/l-min. Substituting these values in

$$v = \frac{V_m}{\left(1+\frac{[I]}{K_I}\right)\left(1+\frac{K'_m}{[S]}\right)}$$

gives $K_I = 6 \times 10^{-3}$ *M*.

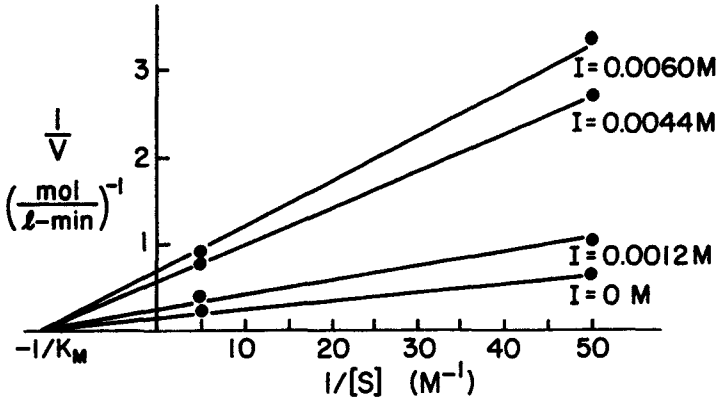


Figure 3.13. Double-reciprocal plot for different inhibitor concentrations (Example 3.2).

3.3.5. Effects of pH and Temperature

3.3.5.1. pH effects. Certain enzymes have ionic groups on their active sites, and these ionic groups must be in a suitable form (acid or base) to function. Variations in the pH of the medium result in changes in the ionic form of the active site and changes in the activity of the enzyme and hence the reaction rate. Changes in pH may also alter the three-dimensional shape of the enzyme. For these reasons, enzymes are only active over a certain pH range. The pH of the medium may affect the maximum reaction rate, K_m, and the stability of the enzyme. In some cases, the substrate may contain ionic groups, and the pH of the medium affects the affinity of the substrate to the enzyme.

The following scheme may be used to describe pH dependence of the enzymatic reaction rate for ionizing enzymes.

$$\begin{array}{ccccc} & & E^- + H^+ & & \\ & & K_2 \uparrow\downarrow & & \\ EH + S & \underset{}{\overset{K'_m}{\rightleftharpoons}} & EHS & \xrightarrow{k_2} & EH + P \\ + & & & & \\ & & H^+ & & \\ & & K_1 \uparrow\downarrow & & \\ & & EH_2^+ & & \end{array} \qquad (3.40)$$

With the definition of

$$K_m' = \frac{[EH][S]}{[EHS]}$$

$$K_1 = \frac{[EH][H^+]}{[EH_2^+]} \tag{3.41}$$

$$K_2 = \frac{[E^-][H^+]}{[EH]}$$

$$[E_0] = [E^-] + [EH] + [EH_2^+] + [EHS], \quad v = k_2[EHS]$$

We can derive the following rate expression:

$$v = \frac{V_m[S]}{K_m'\left[1 + \frac{K_2}{[H^+]} + \frac{[H^+]}{K_1}\right] + [S]} \tag{3.42}$$

or

$$v = \frac{V_m[S]}{K_{m,app}' + [S]} \tag{3.43}$$

where $K_{m,app}' = K_m'\left[1 + \frac{K_2}{[H^+]} + \frac{[H^+]}{K_1}\right]$

As a result of this behavior, the pH optimum of the enzyme is between pK_1 and pK_2.

For the case of ionizing substrate, the following scheme and rate expression can be developed:

$$SH^+ + E \underset{}{\overset{K_m'}{\rightleftharpoons}} ESH^+ \xrightarrow{k_2} E + HP^+ \tag{3.44}$$

$$\downarrow K_1$$

$$S + H^+$$

$$v = \frac{V_m[S]}{K_m'\left(1 + \frac{K_1}{[H^+]}\right) + [S]} \tag{3.45}$$

Theoretical prediction of the pH optimum of enzymes requires a knowledge of the active site characteristics of enzymes, which are very difficult to obtain. The pH optimum for an enzyme is usually determined experimentally. Figure 3.14 depicts variation of enzymatic activity with pH for two different enzymes.

3.3.5.2. Temperature effects. The rate of enzyme-catalyzed reactions increases with temperature up to a certain limit. Above a certain temperature, enzyme activity decreases with temperature because of enzyme denaturation. Figure 3.15 depicts the variation of reaction rate with temperature and the presence of an optimal temperature. The ascending part of Fig. 3.15 is known as *temperature activation*. The rate varies according to the Arrhenius equation in this region.

$$v = k_2[E] \tag{3.46a}$$

$$k_2 = Ae^{-E_a/RT} \tag{3.46b}$$

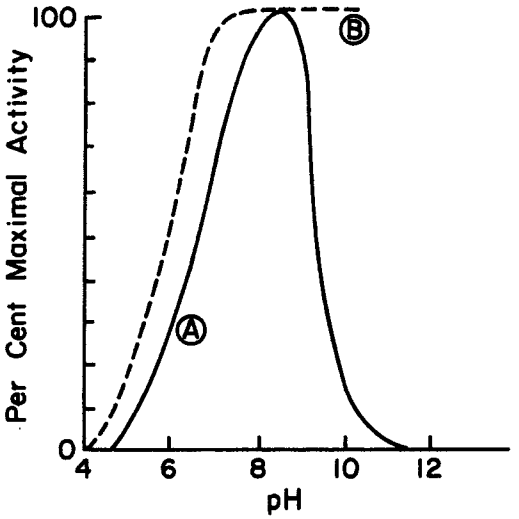


Figure 3.14. The pH-activity profiles of two enzymes. (A) approximate activity for trypsin; (B) approximate activity for cholinesterase.

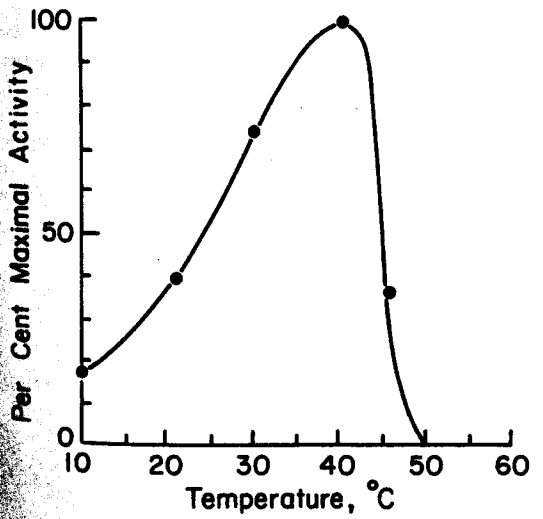


Figure 3.15. Effect of temperature on the activity of an enzyme. Here we have assumed a value of E_a = 11 kcal/g-mol and E_d = 70 kcal/g-mol. The descending portion of the curve is due to thermal denaturation and is calculated assuming a 10-min exposure to the temperature. Note that the nature of the plot will depend on the length of time the reaction mixture is exposed to the test temperature.

where E_a is the activation energy (kcal/mol) and [E] is the active enzyme concentration. A plot of ln υ versus $1/T$ results in a line of slope $-E_a/R$.

The descending part of Fig. 3.15 is known as *temperature inactivation* or *thermal denaturation*. The kinetics of thermal denaturation can be expressed as

$$-\frac{d[E]}{dt} = k_d[E] \tag{3.47}$$

or

$$[E] = [E_0]e^{-k_d t} \tag{3.48}$$

where $[E_0]$ is the initial enzyme concentration and k_d is the denaturation constant. k_d also varies with temperature according to the Arrhenius equation.

$$k_d = A_d e^{-E_a/RT} \tag{3.49}$$

where E_d is the deactivation energy (kcal/mol). Consequently,

$$v = Ae^{-E_a/RT}E_0 e^{-k_d t} \tag{3.50}$$

The activation energies of enzyme-catalyzed reactions are within the 4 to 20 kcal/g mol range (mostly about 11 kcal/g mol). Deactivation energies E_d vary between 40 and 130 kcal/g mol (mostly about 70 kcal/g mol). That is, enzyme denaturation by temperature is much faster than enzyme activation. A rise in temperature from 30° to 40°C results in a 1.8-fold increase in enzyme activity, but a 41-fold increase in enzyme denaturation. Variations in temperature may affect both V_m and K_m values of enzymes.

3.3.6. Insoluble Substrates

Enzymes are often used to attack large, insoluble substrates such as wood chips (in biopulping for paper manufacture) or cellulosic residues from agriculture (e.g., cornstalks). In these cases access to the reaction site on these biopolymers by enzymes is often limited by enzyme diffusion. The number of potential reactive sites exceeds the number of enzyme molecules. This situation is opposite that of the typical situation with soluble substrates, where access to the enzyme's active site limits reaction. If we consider initial reaction rates and if the reaction is first order with respect to the concentration of enzyme bound to substrate (i.e., [ES]), then we can derive a rate expression:

$$v = \frac{V_{\max,S}[E]}{K_{eq} + [E]} \tag{3.51a}$$

where

$$V_{\max,S} = k_2[S_0] \tag{3.51b}$$

and

$$K'_{eq} = k_{des}/k_{ads} \tag{3.51c}$$

The previous equation assumes slow binding of enzyme (i.e., $[E] \approx [E_0]$), S_0 is the number of substrate bonds available initially for breakage, and k_{des} and k_{ads} refer to rates of enzyme desorption and adsorption onto the insoluble matrix, respectively.

3.4 IMMOBILIZED ENZYME SYSTEMS

The restriction of enzyme mobility in a fixed space is known as *enzyme immobilization*. Immobilization of enzymes provides important advantages, such as enzyme reutilization and elimination of enzyme recovery and purification processes, and may provide a better environment for enzyme activity. Since enzymes are expensive, catalyst reuse is critical for many processes. Since some of the intracellular enzymes are membrane bound, immobilized enzymes provide a model system to mimic and understand the action of some membrane-bound intracellular enzymes. Product purity is usually improved, and effluent handling problems are minimized by immobilization.

3.4.1. Methods of Immobilization

Major methods of immobilization are summarized in Fig. 3.16. The two major categories are entrapment and surface immobilization.

3.4.1.1. Entrapment. Entrapment is the physical enclosure of enzymes in a small space. Matrix entrapment and membrane entrapment, including microencapsulation, are the two major methods of entrapment.

Matrices used for enzyme immobilization are usually polymeric materials such as Ca-alginate, agar, κ-carrageenin, polyacrylamide, and collagen. However, some solid matrices such as activated carbon, porous ceramic, and diatomaceous earth can also be used for this purpose. The matrix can be a particle, a membrane, or a fiber. When immobilizing

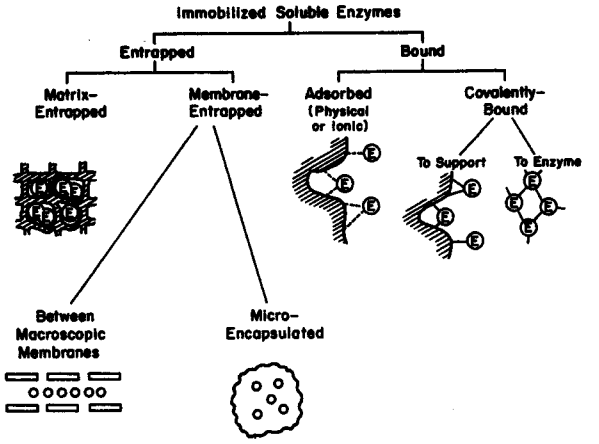


Figure 3.16. Major immobilization methods.

in a polymer matrix, enzyme solution is mixed with polymer solution before polymerization takes place. Polymerized gel-containing enzyme is either extruded or a template is used to shape the particles from a liquid polymer-enzyme mixture. Entrapment and surface attachment may be used in combination in some cases.

Membrane entrapment of enzymes is possible; for example, hollow fiber units have been used to entrap an enzyme solution between thin, semipermeable membranes. Membranes of nylon, cellulose, polysulfone, and polyacrylate are commonly used. Configurations, other than hollow fibers, are possible, but in all cases a semipermeable membrane is used to retain high-molecular-weight compounds (enzyme), while allowing small-molecular-weight compounds (substrate or products) access to the enzyme.

A special form of membrane entrapment is *microencapsulation.* In this technique, microscopic hollow spheres are formed. The spheres contain the enzyme solution, while the sphere is enclosed within a porous membrane. The membrane can be polymeric or an enriched interfacial phase formed around a microdrop.

Despite the aforementioned advantages, enzyme entrapment may have its inherent problems, such as enzyme leakage into solution, significant diffusional limitations, reduced enzyme activity and stability, and lack of control of microenvironmental conditions. Enzyme leakage can be overcome by reducing the MW cutoff of membranes or the pore size of solid matrices. Diffusion limitations can be eliminated by reducing the particle size of matrices and/or capsules. Reduced enzyme activity and stability are due to unfavorable microenvironmental conditions, which are difficult to control. However, by using different matrices and chemical ingredients, by changing processing conditions, and by reducing particle or capsule size, more favorable microenvironmental conditions can be obtained. Diffusion barrier is usually less significant in microcapsules as compared to gel beads.

3.4.1.2. Surface immobilization. The two major types of immobilization of enzymes on the surfaces of support materials are adsorption and covalent binding.

Adsorption is the attachment of enzymes on the surfaces of support particles by weak physical forces, such as van der Waals or dispersion forces. The active site of the adsorbed enzyme is usually unaffected, and nearly full activity is retained upon adsorption. However, desorption of enzymes is a common problem, especially in the presence of strong hydrodynamic forces, since binding forces are weak. Adsorption of enzymes may be stabilized by cross-linking with glutaraldehyde. Glutaraldehyde treatment can denature some proteins. Support materials used for enzyme adsorption can be inorganic materials, such as alumina, silica, porous glass, ceramics, diatomaceous earth, clay, and bentonite, or organic materials, such as cellulose (CMC, DEAE-cellulose), starch, activated carbon, and ion-exchange resins, such as Amberlite, Sephadex, and Dowex. The surfaces of the support materials may need to be pretreated (chemically or physically) for effective immobilization.

Covalent binding is the retention of enzymes on support surfaces by covalent bond formation. Enzyme molecules bind to support material via certain functional groups, such as amino, carboxyl, hydroxyl, and sulfhydryl groups. These functional groups must not be in the active site. One common trick is to block the active site by flooding the enzyme solution with a competitive inhibitor prior to covalent binding. Functional groups on support material are usually activated by using chemical reagents, such as cyanogen bromide, carbodiimide, and glutaraldehyde. Support materials with various functional groups and the chemical reagents used for the covalent binding of proteins are listed in Table

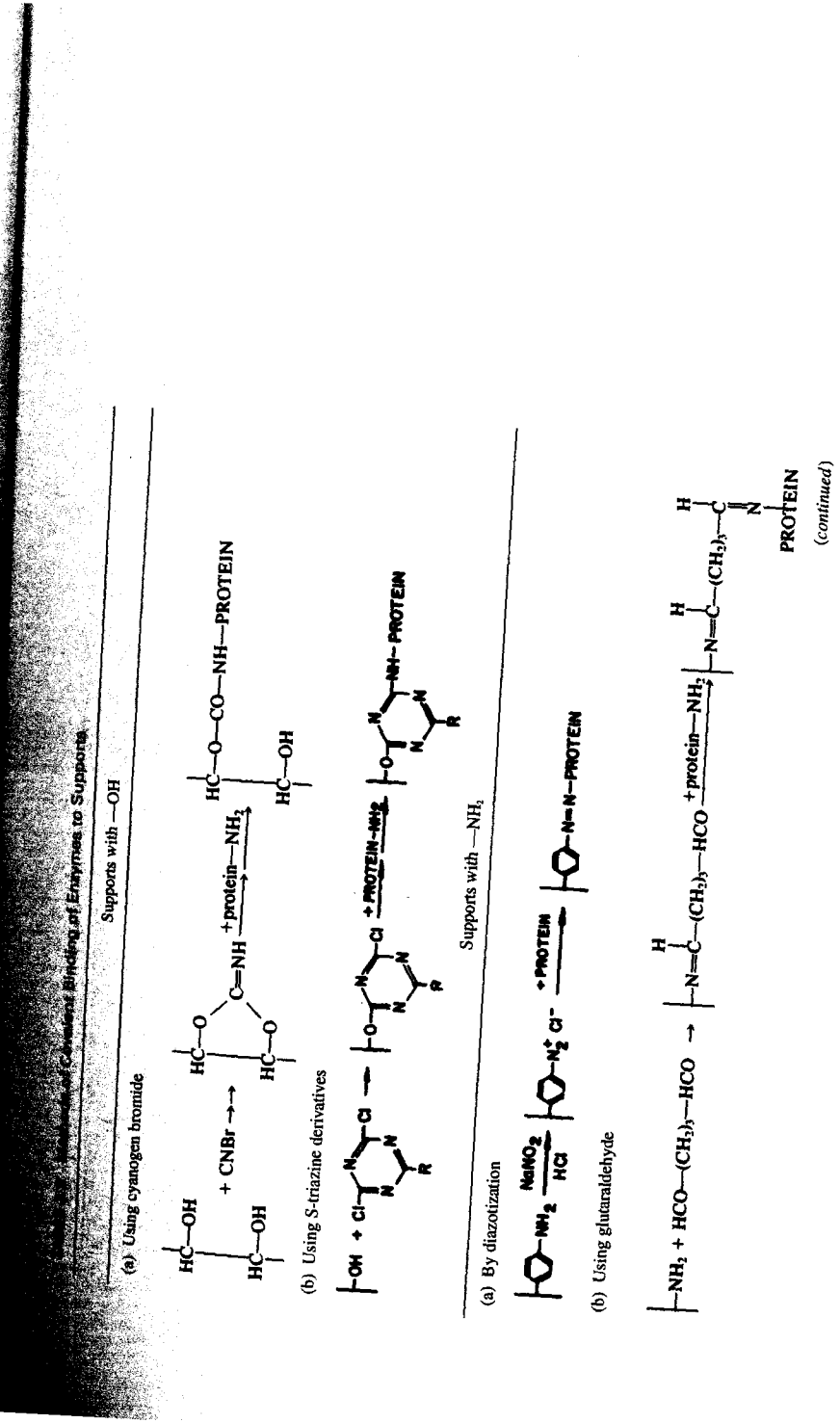
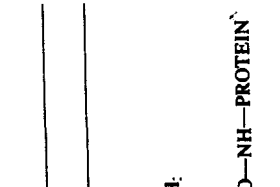
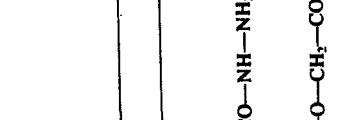


TABLE 3.3 Methods of Covalent Binding of Enzymes to Supports (*Continued*)

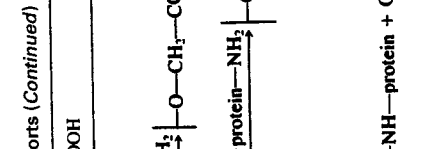
Supports with —COOH

(a) Via azide derivative

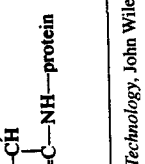




(b) Using a carbodiimide



Supports containing anhydrides



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Binding groups on the protein molecule are usually side groups (R) or the amino or carboxyl groups of the polypeptide chain.

The cross-linking of enzyme molecules with each other using agents such as glutaraldehyde, *bis*-diazobenzidine, and 2,2-disulfonic acid is another method of enzyme immobilization. Cross-linking can be achieved in several different ways: enzymes can be cross-linked with glutaraldehyde to form an insoluble aggregate, adsorbed enzymes may be cross-linked, or cross-linking may take place following the impregnation of porous support material with enzyme solution. Cross-linking may cause significant changes in the active site of enzymes, and also severe diffusion limitations may result.

The most suitable support material and immobilization method vary depending on the enzyme and particular application. Two major criteria used in the selection of support material are (1) the binding capacity of the support material, which is a function of charge density, functional groups, porosity, and hydrophobicity of the support surface, and (2) stability and retention of enzymatic activity, which is a function of functional groups on support material and microenvironmental conditions. If immobilization causes some conformational changes on the enzyme, or if reactive groups on the active site of the enzyme are involved in binding, a loss in enzyme activity can take place upon immobilization. Usually, immobilization results in a loss in enzyme activity and stability. However, in some cases, immobilization may cause an increase in enzyme activity and stability due to more favorable microenvironmental conditions. Because enzymes often have more than one functional site that can bind the surface, an immobilized enzyme preparation may be very heterogeneous. Even when binding does not alter enzyme structure, some enzyme can be bound with the active site oriented away from the substrate solution and toward the support surface, decreasing the access of the substrate to the enzyme. Retention of activity varies with the method used. Table 3.4 summarizes the retention of activity of aminoacylase immobilized by different methods.

TABLE 3.4 Effect of Immobilization Methods on the Retention of Enzymatic Activity of Aminoacylase

Support	Method	Observed activity (units)	Enzyme activity immobilized (%)
Polyacrylamide	Entrapment	526	52.6
Nylon	Encapsulation	360	36.0
DEAE-cellulose	Ionic binding	668	55.2
DEAE-Sephadex A-5O	Ionic binding	680	56.2
CM-Sephadex C-5O	Ionic binding	0	0
Iodoacetyl cellulose	Covalent binding	472	39.0
CNBr-activated Sephadex	Covalent binding	12	1.0
AE-cellulose	Cross-linked with glutaraldehyde	8	0.6

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3.4.2. Diffusional Limitations in Immobilized Enzyme Systems

Diffusional resistances may be observed at different levels in immobilized enzymes. These resistances vary depending on the nature of the support material (porous, nonporous), hydrodynamical conditions surrounding the support material, and distribution of the enzyme inside or on the surface of the support material. Whether diffusion resistance has a significant effect on the rate of enzymatic reaction rate depends on the relative rate of the reaction rate and diffusion rate, which is characterized by the Damköhler number (Da).

$$\mathrm{Da} = \frac{\text{maximum rate of reaction}}{\text{maximum rate of diffusion}} = \frac{V_m'}{k_L[S_b]} \tag{3.52}$$

where $[S_b]$ is substrate concentration in bulk liquid (g/cm^3) and k_L is the mass-transfer coefficient (cm/s).

The rate of enzymatic conversion may be limited by diffusion of the substrate or reaction, depending on the value of the Damköhler number. If Da >> 1, the diffusion rate is limiting. For Da << 1, the reaction rate is limiting, and for Da ≈ 1, the diffusion and reaction resistances are comparable. Diffusion and enzymatic reactions may be simultaneous, with enzymes entrapped in a solid matrix, or may be two consecutive phenomena for adsorbed enzymes.

3.4.2.1. Diffusion effects in surface-bound enzymes on nonporous support materials. Assume a situation where enzymes are bound and evenly distributed on the surface of a nonporous support material, all enzyme molecules are equally active, and substrate diffuses through a thin liquid film surrounding the support surface to reach the reactive surfaces, as depicted in Fig. 3.17. Assume further that the process of immobilization has not altered the protein structure, and the intrinsic kinetic parameters (V_m, K_m) are unaltered.

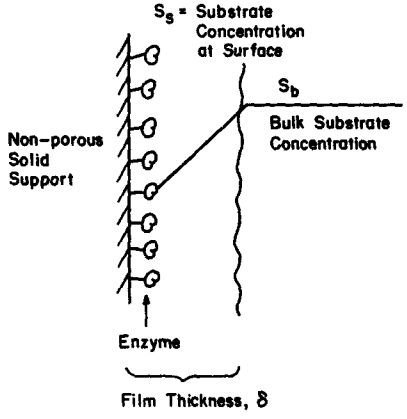

Figure 3.17. Substrate concentration profile in a liquid film around adsorbed enzymes.

At steady state, the reaction rate is equal to the mass-transfer rate:

$$J_s = k_L([S_b]-[S_s]) = \frac{V_m'[S_s]}{K_m + [S_s]} \tag{3.53}$$

where V_m' is the maximum reaction rate per unit of external surface area and k_L is the liquid mass-transfer coefficient. This equation is quadratic in $[S_s]$, the substrate concentration at the surface. It can be solved analytically, but the solution is cumbersome. Furthermore, the value of $[S_s]$ is not amenable to direct experimental observation.

Equation 3.53 can be solved graphically as depicted in Fig. 3.18. Such a plot also makes it easy to visualize the effects of parameter changes such as stirring rate, changes in bulk substrate concentration, or enzyme loading.

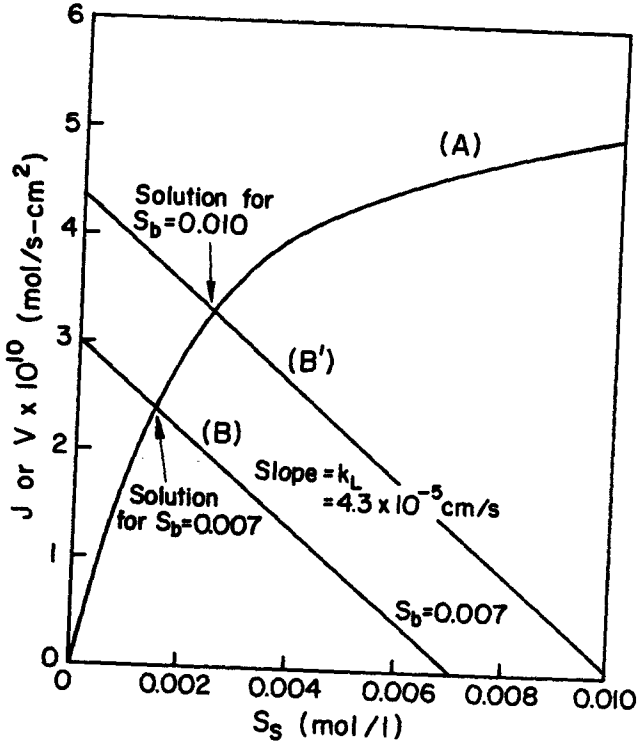

Figure 3.18. Graphical solution for amount of reaction per unit surface area for enzyme immobilized on a nonporous catalyst. Curve A results from a knowledge of the intrinsic solution-based kinetic parameters and the surface loading of enzyme (right side of eq. 3.53). Line B is the mass transfer equation (left side of eq. 3.53). The intersection of the two lines is the reaction rate, υ, that can be sustained in the system. The responses for two different bulk substrate concentrations are shown.

When the system is strongly mass-transfer limited, $[S_s] \approx 0$, since the reaction is rapid compared to mass transfer, and

$$v \approx k_L[S_b], \quad (\text{for Da} >> 1) \tag{3.54}$$

and the system behaves as pseudo first order.

When the system is reaction limited (Da << 1), the reaction rate is often expressed as

$$v = \frac{V'_m [S_b]}{K_{m,app} + [S_b]} \tag{3.55a}$$

where, with appropriate assumptions,

$$K_{m,app} = K_m \left\{ 1 + \frac{V'_m}{k_L([S_b] + K_m)} \right\} \tag{3.55b}$$

Under these circumstances, the apparent Michaelis–Menten "constant" is a function of stirring speed. Usually, $K_{m,app}$ is estimated experimentally as the value of $[S_b]$, giving one-half of the maximal reaction rate.

Example 3.4

Consider a system where a flat sheet of polymer coated with enzyme is placed in a stirred beaker. The intrinsic maximum reaction rate (V_m) of the enzyme is 6×10^{-6} mol/s-mg enzyme. The amount of enzyme bound to the surface has been determined to be 1×10^{-4} mg enzyme/cm^2 of support. In solution, the value of K_m has been determined to be 2×10^{-3} mol/l. The mass-transfer coefficient can be estimated from standard correlations for stirred vessels. We assume in this case a very poorly mixed system where $k_L = 4.3 \times 10^{-5}$ cm/s. What is the reaction rate when (a) the bulk concentration of the substrate is 7×10^{-3} mol/l? (b) $S_b = 1 \times 10^{-2}$ mol/l?

Solution The solution is given in Fig. 3.18. The key is to note that the mass-transfer rate equals the reaction rate at steady state, and as a consequence the right side of eq. 3.53 must equal the left side. In case (a), this occurs at a substrate surface concentration of about 0.0015 mol/l with a reaction rate of 2.3×10^{-10} mol/s-cm^2. By increasing the bulk substrate concentration to 0.01 mol/l, the value of $[S_s]$ increases to 0.0024 mol/l with a reaction rate about 3.3×10^{-10} mol/s-cm^2.

3.4.2.2. Diffusion effects in enzymes immobilized in a porous matrix. When enzymes are immobilized on internal pore surfaces of a porous matrix, substrate diffuses through the tortuous pathway among pores and reacts with enzyme immobilized on pore surfaces. Diffusion and reaction are simultaneous in this case, as depicted in Fig. 3.19.

Assume that enzyme is uniformly distributed in a spherical support particle; the reaction kinetics are expressed by Michaelis–Menten kinetics, and there is no partitioning of the substrate between the exterior and interior of the support. Then we write the following equation, stating that diffusion rate is equal to reaction rate at steady state:

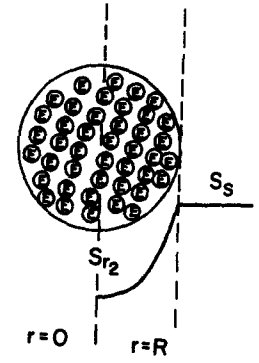

Figure 3.19. Substrate concentration profile in a porous support particle containing immobilized enzymes. Here it is assumed that no external substrate limitation exists so that the bulk and surface concentrations are the same.

$$D_e\left(\frac{d^2[S]}{dr^2} + \frac{2}{r}\frac{d[S]}{dr}\right) = \frac{V''_m [S]}{K_m + [S]} \tag{3.56}$$

with boundary conditions $[S] = [S_s]$ at $r = R$ and $d[S]/dr = 0$ at $r = 0$, where V''_m is the maximum reaction rate per unit volume of support, and D_e is the effective diffusivity of substrate within the porous matrix.

Equation 3.56 can be written in dimensionless form by defining the following dimensionless variables:

$$\bar{S} = \frac{[S]}{[S_s]}, \quad \bar{r} = \frac{r}{R}, \quad \beta = \frac{K_m}{[S_s]}$$

$$\frac{d^2\bar{S}}{d\bar{r}^2} + \frac{2}{\bar{r}}\frac{d\bar{S}}{d\bar{r}} = \frac{R^2 V''_m}{S_s D_e}\left(\frac{\bar{S}}{\bar{S} + \beta}\right) \tag{3.57a}$$

or

$$\frac{d^2\bar{S}}{d\bar{r}^2} + \frac{2}{\bar{r}}\frac{d\bar{S}}{d\bar{r}} = \phi^2 \frac{\bar{S}}{1 + \bar{S}/\beta} \tag{3.57b}$$

where

$$\phi = R\sqrt{\frac{V''_m / K_m}{D_e}} = \text{Thiele modulus} \tag{3.57c}$$

With boundary conditions of $\bar{S} = 1$ at $\bar{r} = 1$ and $d\bar{S}/d\bar{r} = 0$ at $\bar{r} = 0$, eq. 3.57 can be numerically solved to determine the substrate profile inside the matrix. The rate of substrate consumption is equal to the rate of substrate transfer through the external surface of the support particle at steady state into the sphere.

$$r_s = N_s = 4\pi R^2 D_e \left.\frac{d[S]}{dr}\right|_{r=R} \tag{3.58}$$

Under diffusion limitations, the rate per unit volume is usually expressed in terms of the effectiveness factor as follows:

$$r_s = \eta \frac{V_m'' [S_s]}{K_m + [S_s]} \tag{3.59}$$

The *effectiveness factor* is defined as the ratio of the reaction rate with diffusion limitation (or diffusion rate) to the reaction rate with no diffusion limitation. The value of the effectiveness factor is a measure of the extent of diffusion limitation. For $\eta < 1$, the conversion is diffusion limited, whereas for $\eta \approx 1$ values, conversion is limited by the reaction rate and diffusion limitations are negligible. The factor is a function of ϕ and β as depicted in Figure 3.20.

For a zero-order reaction rate ($\beta \rightarrow 0$), $\eta \approx 1$ for a large range of Thiele modulus values such as $1 < \phi < 100$. For a first-order reaction rate ($\beta \rightarrow \infty$), $\eta = (\phi,\beta)$ and η is approximated to the following equation for high values of ϕ.

$$\eta = \frac{3}{\phi}\left[\frac{1}{\tanh\phi} - \frac{1}{\phi}\right] \tag{3.60}$$

When internal diffusion limits the enzymatic reaction rate, the rate-constant $V_{m,app}$ and $K_{m,app}$ values are not true intrinsic rate constants, but apparent values. To obtain true intrinsic rate constants in immobilized enzymes, diffusion resistances should be eliminated by using small particle sizes, a high degree of turbulence around the particles, and high substrate concentrations.

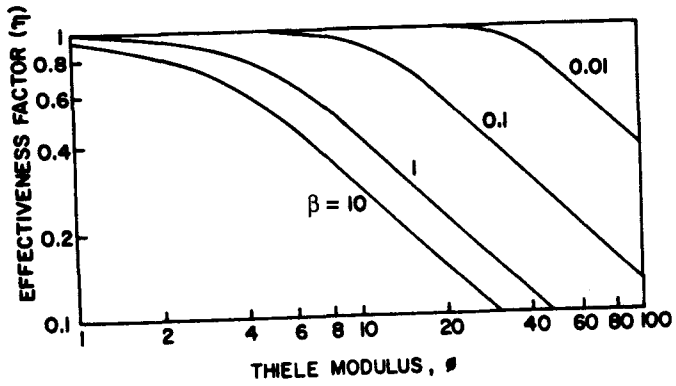

Figure 3.20. Theoretical relationship between the effectiveness factor η and first-order Thiele modulus, ϕ, for a spherical porous immobilized particle for various values of β, where β is the dimensionless Michaelis constant. (With permission, from D. I. C. Wang et al., *Fermentation and Enzyme Technology*, John Wiley & Sons, Inc., New York, 1979, p. 329.)

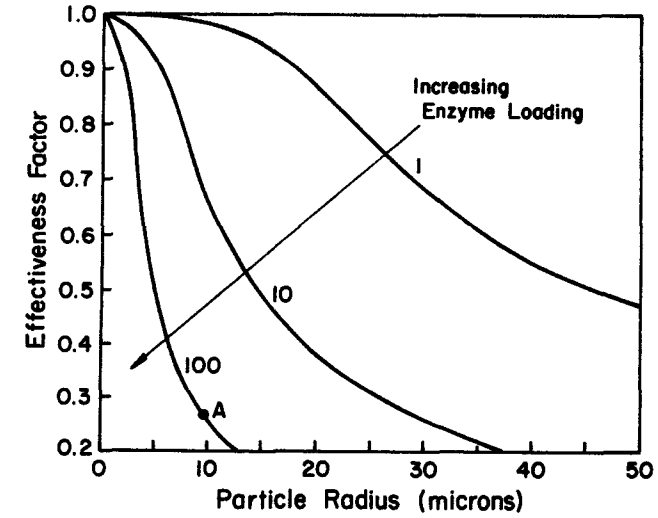

Figure 3.21. The effectiveness factor decreases with increases in enzyme loading or with increases in particle diameter. Point A represents the value of the effectiveness factor for a particle radius of 10 μm with an enzyme loading of 100 mg/cm^3, an enzyme activity of 100 μmol/min per mg enzyme, a substrate diffusivity of 5×10^{-6} cm^2/s, and a bulk substrate concentration tenfold higher than K_m.

When designing immobilized enzyme systems using a particular support, the main variables are V_m and R, since the substrate concentration, K_m, and D_e are fixed. The particle size (R) should be as small as possible within the constraints of particle integrity, resistance to compression, and the nature of the particle recovery systems. The maximum reaction rate is determined by enzyme activity and concentration in the support. High enzyme content will result in high enzyme activity per unit of reaction volume but low effectiveness factor. On the other hand, low enzyme content will result in lower enzyme activity per unit volume but a high effectiveness factor. For maximum conversion rates, particle size should be small ($D_p \leq 10$ μm) and enzyme loading should be optimized. As depicted in the example in Fig. 3.21, $D_p \leq 10$ μm and enzyme loadings of less than 10 mg/cm^3 are required for high values of the effectiveness factor ($\eta \geq 0.8$).

Example 3.5

D. Thornton and co-workers studied the hydrolysis of sucrose at pH = 4.5 and 25°C using crude invertase obtained from baker's yeast in free and immobilized form. The following initial velocity data were obtained with 408 units of crude enzyme (1 unit = quantity of enzyme hydrolyzing 1 μmol of sucrose/min when incubated with 0.29 *M* sucrose in a buffer at pH 4.5 and 25°C).

V_0 (mmol hydrolyzed/l-min)		S_0 (mol/l)
Free enzyme	Immobilized enzyme	
0.083	0.056	0.010
0.143	0.098	0.020
0.188	0.127	0.030
0.222	0.149	0.040
0.250	0.168	0.050
0.330	0.227	0.100
0.408	0.290	0.290

a. Determine the K_m and V_m for this reaction using both free and immobilized enzyme.

b. Do the data indicate any diffusion limitations in the immobilized enzyme preparation?

Solution From a double-reciprocal plot of $1/v$ versus $1/S$ for free enzyme (Fig. 3.22), $-1/K_m = -20$ and $K_m = 0.05$ M. $1/V_m = 2$ and $V_m = 0.5$ mmol/l min. From a double-reciprocal plot of $1/v$ versus $1/S$ for the immobilized enzyme, $-1/K_m = -20$ and $K_m = 0.05$ M. $1/V_m = 3$ and $V_m = 0.33$ mmol/l-min. Since the K_m values for free and immobilized enzymes are the same, there is no diffusion limitation.

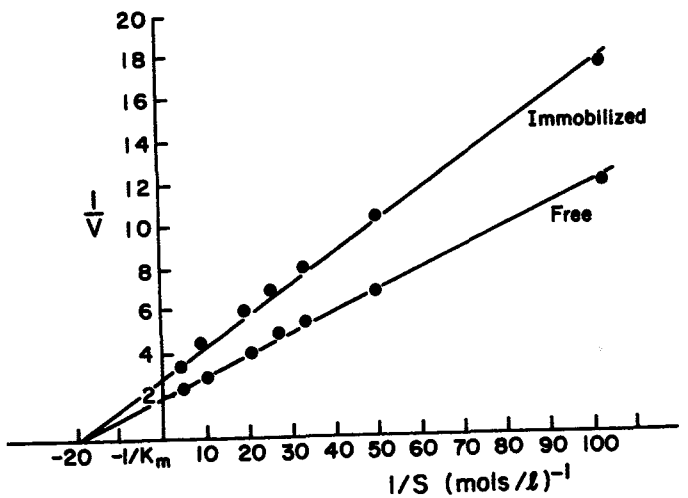

Figure 3.22. Double-reciprocal plots for free and immobilized enzymes (Example 3.4).

3.4.3. Electrostatic and Steric Effects in Immobilized Enzyme Systems

When enzymes are immobilized in a charged matrix as a result of a change in the microenvironment of the enzyme, the apparent bulk pH optimum of the immobilized enzyme will shift from that of soluble enzyme. The charged matrix will repel or attract substrates, product, cofactors, and H^+ depending on the type and quantity of surface charge. For an enzyme immobilized onto a charged support, the shift in the pH-activity profile is given by

$$\Delta pH = pH_i - pH_e = 0.43\frac{zF\psi}{RT} \tag{3.61}$$

where pH_i and pH_e are internal and external pH values, respectively; z is the charge (valence) on the substrate; F is the Faraday constant (96,500 coulomb/eq. g); ψ is the electrostatic potential; and R is the gas constant. Expressions similar to eq. 3.61 apply to other nonreactive charged medium components. The intrinsic activity of the enzyme is altered by the local changes in pH and ionic constituents. Further alterations in the apparent kinetics are due to the repulsion or attraction of substrates or inhibitors.

The activity of an enzyme toward a high-molecular-weight substrate is usually reduced upon immobilization to a much greater extent than for a low-molecular-weight substrate. This is mainly because of steric hindrance by the support. Certain substrates, such as starch, have molecular weights comparable to those of enzymes and may therefore not be able to penetrate to the active sites of immobilized enzymes.

Immobilization also affects the thermal stability of enzymes. Thermal stability often increases upon immobilization due to the presence of thermal diffusion barriers and the constraints on protein unfolding. However, decreases in thermal stability have been noted in a few cases. The pH stability of enzymes usually increases upon immobilization, too.

3.5. LARGE-SCALE PRODUCTION OF ENZYMES

Among various enzymes produced at large scale are proteases (subtilisin, rennet), hydrolases (pectinase, lipase, lactase), isomerases (glucose isomerase), and oxidases (glucose oxidase). These enzymes are produced using overproducing strains of certain organisms. Separation and purification of an enzyme from an organism require disruption of cells, removal of cell debris and nucleic acids, precipitation of proteins, ultrafiltration of the desired enzyme, chromatographic separations (optional), crystallization, and drying. The process scheme varies depending on whether the enzyme is intracellular or extracellular. In some cases, it may be more advantageous to use inactive (dead or resting) cells with the desired enzyme activity in immobilized form. This approach eliminates costly enzyme separation and purification steps and is therefore economically more feasible. Details of protein separations are covered in Chapter 11.

The first step in the large-scale production of enzymes is to cultivate the organisms producing the desired enzyme. Enzyme production can be regulated and fermentation conditions can be optimized for overproduction of the enzyme. Proteases are produced by using overproducing strains of *Bacillus*, *Aspergillus*, *Rhizopus*, and *Mucor*; pectinases are produced by

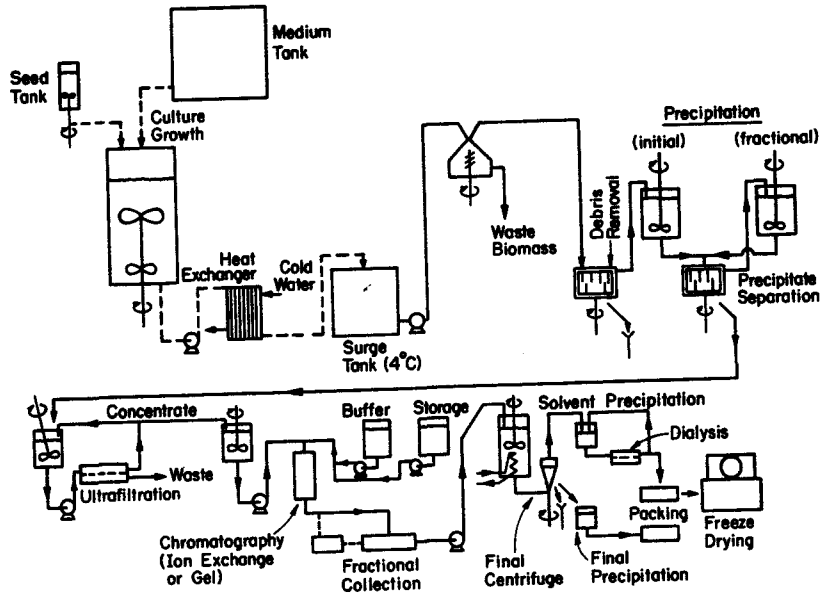

Figure 3.23. A flowsheet for the production of an extracellular enzyme.

Aspergillus niger; lactases are produced by yeast and *Aspergillus;* lipases are produced by certain strains of yeasts and fungi; glucose isomerase is produced by *Flavobacterium arborescens* or *Bacillus coagulans*. After the cultivation step, cells are separated from the media usually by filtration or sometimes by centrifugation. Depending on the intracellular or extracellular nature of the enzyme, either the cells or the fermentation broth is further processed to separate and purify the enzyme. The recovery of intracellular enzymes is more complicated and involves the disruption of cells and removal of cell debris and nucleic acids. Figure 3.23 depicts a schematic of an enzyme plant producing intracellular enzymes.

In some cases, enzyme may be both intracellular and extracellular, which requires processing of both broth and cells. Intracellular enzymes may be released by increasing the permeability of cell membrane. Certain salts such as $CaCl_2$ and other chemicals such as dimethylsulfoxide (DMSO) and pH shift may be used for this purpose. If enzyme release is not complete, then cell disruption may be essential.

The processes used to produce these industrial enzymes have much in common with our later discussions on processes to make proteins from recombinant DNA.

3.6. MEDICAL AND INDUSTRIAL UTILIZATION OF ENZYMES

Enzymes have been significant industrial products for more than a hundred years. However, the range of potential application is increasing rapidly. With the advent of recombinant DNA technology it has become possible to make formerly rare enzymes in large

quantities and, hence, reduce cost. Also, in pharmaceutical manufacture the desire to make chirally pure compounds is leading to new opportunities. Chirality is important in a product; in a racemic mixture one enantiomer is often therapeutically useful while the other may cause side effects and add no therapeutic value. The ability of enzymes to recognize chiral isomers and react with only one of them can be a key component in pharmaceutical synthesis. Processes that depend on a mixture of chemical and enzymatic synthesis are being developed for a new generation of pharmaceuticals.

Technological advances have facilitated the use of enzymes over an increasingly broad range of process conditions. Enzymes from organisms that grow in unusual environments (e.g., deep ocean, salt lakes, and hot springs) are increasingly available for study and potential use. New enzymes and better control of reaction conditions allow the use of enzymes in the presence of high concentrations of organics, in high-salt aqueous environments, or at extreme temperatures, pH, or pressures. As we couple new insights into the relationship of enzyme structure to biological function with recombinant DNA technology, we are able to produce enzymes that are human designed or manipulated (see Section 14.9 on protein engineering). We no longer need to depend solely on natural sources for enzymes.

While there are many reasons to be optimistic about increasing use of enzymes, the number of enzymes made at high volume for industrial purposes evolves more slowly. In 1996 the U.S. sales of industrial enzymes were $372 million, and sales are projected to grow to $686 million by 2006. The products made in enzyme processes are worth billions of dollars. Table 3.5 provides a breakdown of projected enzyme sales by industrial sector. Table 3.6 lists some industrially important enzymes.

Proteases hydrolyze proteins into smaller peptide units and constitute a large and industrially important group of enzymes. Proteases constitute about 60% of the total enzyme market. Industrial proteases are obtained from bacteria (*Bacillus*), molds (*Aspergillus, Rhizopus,* and *Mucor*), animal pancreas, and plants. Most of the industrial proteases are endoproteases. Proteases are used in food processing, such as cheese making (rennet), baking, meat tenderization (papain, trypsin), and brewing (trypsin, pepsin); in detergents for the hydrolysis of protein stains (subtilisin Carlsberg); and in tanning and the medical treatment of wounds.

TABLE 3.5. Industrial Enzyme Market*

Application	1996 Sales (U.S. $ in millions)	2006 Projected Sales (U.S. $ in millions)
Food	170	214
Detergent	160	414
Textiles	27	32
Leather	11	13
Paper & Pulp	1	5
Other	3	8
TOTAL	372	686

*Data from C. Wrotnowski, *Genetic & Engineering News,* pp. 14 and 30, Feb. 1, 1997.

TABLE 3.6 Some Industrially Important Enzymes

Name	Example of Source	Application
Amylase	*Bacillus subtilis, Aspergillus niger*	Starch hydrolysis, glucose production
Glucoamylase	*A. niger, Rhizopus niveus, Endomycopsis*	Saccharification of starch, glucose production
Trypsin	Animal pancreas	Meat tenderizer, beer haze removal
Papain	Papaya	Digestive aid, meat tenderizer, medical applications
Pepsin	Animal stomach	Digestive aid, meat tenderizer
Rennet	Calf stomach/recombinant *E. coli*	Cheese manufacturing
Glucose isomerase	*Flavobacterium arborescens, Bacillus coagulans, Lactobacillus brevis*	Isomerization of glucose to fructose
Penicillinase	*B. subtilis*	Degradation of penicillin
Glucose oxidase	*A. niger*	Glucose → gluconic acid, dried-egg manufacture
Lignases	Fungal	Biopulping of wood for paper manufacture
Lipases	*Rhizopus*, pancreas	Hydrolysis of lipids, flavoring and digestive aid
Invertase	*S. cerevisiae*	Hydrolysis of sucrose for further fermentation
Pectinase	*A. oryzae, A. niger, A. flavus*	Clarification of fruit juices, hydrolysis of pectin
Cellulase	*Trichoderma viride*	Cellulose hydrolysis

Pectinases are produced mainly by *A. niger*. The major components in pectinases are pectin esterase, polygalacturonase, and polymethylgalacturonatelyase. Pectinases are used in fruit juice processing and wine making to increase juice yield, reduce viscosity, and clear the juice.

Lipases hydrolyze lipids into fatty acids and glycerol and are produced from animal pancreas, some molds, and yeasts. Lipases may be used to hydrolyze oils for soap manufacture and to hydrolyze the lipid-fat compounds present in waste-water streams. Interesterification of oils and fats may be catalyzed by lipases. Lipases may also be used in the cheese and butter industry to impart flavor as a result of the hydrolysis of fats. Lipase-containing detergents are an important application of lipases.

Amylases are used for the hydrolysis of starch and are produced by many different organisms, including *A. niger* and *B. subtilis*. Three major types of amylases are α-amylase, β-amylase, and glucoamylase. α-amylase breaks α-1,4 glycosidic bonds randomly on the amylose chain and solubilizes amylose. For this reason, α-amylase is known as the starch-liquefying enzyme. β-amylase hydrolyzes α-1,4 glycosidic bonds on the nonreducing ends of amylose and produces maltose residues. β-amylase is known as a saccharifying enzyme. α-1,6 glycosidic linkages in the amylopectin fraction of starch are hydrolyzed by glucoamylase, which is also known as a saccharifying enzyme. In the United States on the average, nearly 1.3×10^9 lb/yr of glucose is produced by the enzymatic hydrolysis of starch. The enzyme pullulanase also hydrolyzes α-1,6 glycosidic linkages in starch selectively.

Cellulases are used in the hydrolysis of cellulose and are produced by some *Trichoderma* species, such as *Trichoderma viride* or *T. reesei*; and by some molds, such as *Aspergillus niger* and *Thermomonospora;* and by some *Clostridium* species. Cellulase is an enzyme complex and its formation is induced by cellulose. *Trichoderma* cellulase hydrolyzes crystalline cellulose, but *Aspergillus* cellulase does not. Cellulose is first hydrolyzed to cellobiose by cellulase, and cellobiose is further hydrolyzed to glucose by β-glucosidase. Both of these enzymes are inhibited by their end products, cellobiose and glucose. Cellulases are used in cereal processing, alcohol fermentation from biomass, brewing, and waste treatment.

Hemicellulases hydrolyze hemicellulose to five-carbon sugar units and are produced by some molds, such as white rot fungi and *A. niger*. Hemicellulases are used in combination with other enzymes in baking doughs, brewing mashes, alcohol fermentation from biomass, and waste treatment.

Lactases are used to hydrolyze lactose in whey to glucose and galactose and are produced by yeast and some *Aspergillus* species. Lactases are used in the fermentation of cheese whey to ethanol.

Other microbial β-1,4 glucanases produced by *Bacillus amyloliquefaciens*, *A. niger*, and *Penicillium emersonii* are used in brewing mashes containing barley or malt. These enzymes improve wort filtration and extract yield.

Penicillin acylase is used by the antibiotic industry to convert penicillin G to 6-aminopenicillanic acid (6-APA), which is a precursor for semisynthetic penicillin derivatives.

Among other important industrial applications of enzymes are the conversion of fumarate to L-aspartate by aspartase. In industry, this conversion is realized in a packed column of immobilized dead *E. coli* cells with active aspartase enzyme. Fumarate solution is passed through the column, and aspartate is obtained in the effluent stream. Aspartate is further coupled with L-phenylalanine to produce aspartame, which is a low-calorie sweetener known as "Nutrasweet®."

The conversion of glucose to fructose by immobilized glucose isomerase is an important industrial process. Fructose is nearly 1.7 times sweeter than glucose and is used as a sweetener in soft drinks. Glucose isomerase is an intracellular enzyme and is produced by different organisms, such as *Flavobacterium arborescens*, *Bacillus licheniformis*, and some *Streptomyces* and *Arthrobacter* species. Immobilized inactive whole cells with glucose isomerase activity are used in a packed column for fructose formation from glucose. Cobalt (Co^{2+}) and magnesium (Mg^{2+}) ions (4×10^{-4} *M*) enhance enzyme activity. Different immobilization methods are used by different companies. One uses flocculated whole cells of *F. arborescens* treated with glutaraldehyde in the form of dry spherical particles. Entrapment of whole cells in gelatin treated with glutaraldehyde, the use of glutaraldehyde-treated lysed cells in the form of dry particles, and immobilization of the enzyme on inorganic support particles such as silica and alumina are methods used by other companies.

DL-Acylamino acids are converted to a mixture of L- and D-amino acids by immobilized aminoacylase. L-Amino acids are separated from D-acylaminoacid, which is recycled back to the column. L-Amino acids have important applications in food technology and medicine.

Enzymes are commonly used in medicine for diagnosis, therapy, and treatment purposes. Trypsin can be used as an antiinflammatory agent; lysozyme, which hydrolyzes the

cell wall of gram-positive bacteria, is used as an antibacterial agent; streptokinase is used as an antiinflammatory agent; urokinase is used in dissolving and preventing blood clots. Asparaginase, which catalyzes the conversion of L-asparagine to L-aspartate, is used as an anticancer agent. Cancer cells require L-asparagine and are inhibited by asparaginase. Asparaginase is produced by *E. coli*. Glucose oxidase catalyzes the oxidation of glucose to gluconic acid and hydrogen peroxide, which can easily be detected. Glucose oxidase is used for the determination of glucose levels in blood and urine. Penicillinases hydrolyze penicillin and are used to treat allergic reactions against penicillin. Tissue plasminogen activator (TPA) and streptokinase are used in the dissolution of blood clots (particularly following a heart attack or stroke).

The development of biosensors using enzymes as integral components is proceeding rapidly. Two examples of immobilized enzyme electrodes are those used in the determination of glucose and urea by using glucose oxidase and urease immobilized on the electrode membrane, respectively. Scarce enzymes (e.g., tissue plasminogen activator) are finding increasing uses, as the techniques of genetic engineering now make it possible to produce usable quantities of such enzymes.

The preceding list of enzymes and uses is not exhaustive, but merely illustrative.

3.7. SUMMARY

Enzymes are protein, glycoprotein, or RNA molecules that catalyze biologically important reactions. Enzymes are very effective, specific, and versatile biocatalysts. Enzymes bind substrate molecules and reduce the activation energy of the reaction catalyzed, resulting in significant increases in reaction rate. Some protein enzymes require a nonprotein group for their activity as a cofactor.

Simple single-enzyme-catalyzed reaction kinetics can be described by Michaelis-Menten kinetics, which has a hyperbolic form in terms of substrate concentration. The activity of some enzymes can be altered by inhibitory compounds, which bind the enzyme molecule and reduce its activity. Enzyme inhibition may be competitive, noncompetitive, and uncompetitive. High substrate and product concentrations may be inhibitory, too.

Enzymes require optimal conditions (pH, temperature, ionic strength) for their maximum activity. Enzymes with an ionizing group on their active site show a distinct optimal pH that corresponds to the natural active form of the enzyme. The activation energy of enzyme-catalyzed reactions is within 4 to 20 kcal/g mol. Above the optimal temperature, enzymes lose their activity, and the inactivation energy is on the order of 40 to 130 kcal/g mol.

Enzymes can be used in suspension or in immobilized form. Enzymes can be immobilized by entrapment in a porous matrix, by encapsulation in a semipermeable membrane capsule or between membranes, such as in a hollow-fiber unit, or by adsorption onto a solid support surface. Enzyme immobilization provides enzyme reutilization, eliminates costly enzyme recovery and purification processes, and may result in increased activity by providing a more suitable microenvironment for the enzyme. Enzyme immobilization may result in diffusion limitations within the matrix. Immobilization may also cause enzyme instability, loss of activity, and a shift in optimal conditions (pH, ionic strength). To obtain maximum reaction rates, the particle size of the support material and enzyme loading need to be optimized, and a support material with the correct surface characteristics must be selected.

Enzymes are widely used in industry and have significant medical applications. Among the most widely used enzymes are proteases (papain, trypsin, subtilisin); amylases (starch hydrolysis); rennet (cheese manufacturing); glucose isomerase (glucose-to-fructose conversion); glucose oxidase (glucose-to-gluconic acid conversion); lipases (lipid hydrolysis), and pectinases (pectin hydrolysis). Enzyme production and utilization are a multibillion-dollar business with a great potential for expansion.

SUGGESTIONS FOR FURTHER READING

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PROBLEMS

3.1. Consider the following reaction sequence:

$$S + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (ES)_1 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} (ES)_2 \xrightarrow{k_5} P + E$$

Develop a suitable rate expression for production formation [$v = k_5(ES)_2$] by using (a) the equilibrium approach, and (b) the quasi-steady-state approach.

3.2. Consider the reversible product-formation reaction in an enzyme-catalyzed bioreaction:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (ES) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P$$

Develop a rate expression for product-formation using the quasi-steady-state approximation and show that

$$v = \frac{d[P]}{dt} = \frac{(v_r/K_m)[S] - (v_p/K_p)[P]}{1 + \frac{[S]}{K_m} + \frac{[P]}{K_p}}$$