CHAPTER

7

Enzymes

OUTLINE

What is an enzyme? How do enzymes work? The word "enzyme" was coined in 1877 from the Greek, "in yeast," by German physiologist Wilhelm Kühne. The earliest known enzyme (or more precisely, mixture of enzymes) is *qu* (in Chinese) and bearing the Japanese

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pronunciation koji, or *koh-jee*. Although the making of *qu* can be traced back to the Shang Dynasty (1700–1100 BCE), the first documented mention of *qu* was in Zhouli (The Rites of Zhou Dynasty) in 300 BCE. Qu is a culture prepared by growing either *Aspergillus oryzae* or *Monascus purpureus* mold on cooked grains and/or soybeans in a warm, humid place. It serves as a source of enzymes that breaks down (or hydrolyzes/digests/splits) natural plant constituents into simpler compounds when making miso, soy sauce, sake, amazake, and other fermented foods. Its fragrant white (or red) mycelium, which looks somewhat like the surface of a tennis ball, has a delightful aroma resembling that of mushrooms.

The first enzyme isolated is diastase, which was extracted from malt solution in 1833 by Anselme Payen and Jean-François Persoz, chemists at a French sugar factory. In 1896, amylase was obtained from *Aspergillus oryzae*, in Japan, which is used as digestant. This signifies the first time for a pure enzyme to be produced and applied.

Enzymes are usually proteins of high molecular weight ($15{,}000 < M <$ several million Daltons) that act as catalysts. 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 7.1.

Enzymes have been classified into six main types, depending on the nature of the reaction catalyzed. A four-digit coding (numbering scheme) for enzymes has been developed, in which the classes are distinguished by the first of four digits. The second and third digits describe the type of reaction catalyzed, and the fourth digit is employed to distinguish between the types of the same function on the basis of the actual substrate in the reaction catalyzed. This scheme has proven useful in clearly delineating many enzymes that have similarities. It was developed by the Nomenclature Commission of the International Union of Biochemistry and Molecular Biology, and the prefix EC (stands for Enzyme Code, or Enzyme Commission, as it was originally initiated) is generally employed with the numerical scheme. This classification scheme is useful as it unambiguously identifies the enzyme in question. Earlier nomenclature often resulted in one enzyme being identified by several names if its activity was broad.

TABLE 7.1 International Classification of Enzymes: Code Numbers (EC First Digit.Second Digit.Third Digit. Fourth Digit) and Types of Reactions Catalyzed

First Digit (Class)	Second Digit	Third Digit	Fourth Digit
1 Oxidreductases Transfer of hydrogen or oxygen atoms or electrons from one substrate to another: oxidases or dehydrogenases	Donor of hydrogen or electron 1 alcohol 2 aldehyde or ketone 3 alkene, —CH═CH— 4 primary amine: CH—NH_2 5 secondary amine: CH—NH— 6 NADH or NADPH 7 nitrogenous compound 8 sulfur group 9 heme group 10 diphenols or related 11 peroxide 12 hydrogen 13 single donor with O_2 14 paired donors with O_2 15 acting on superoxide 16 oxidizing metal ion 17 acting on CH or CH_2 groups 18 iron-sulfur-protein 19 reduced flavodoxin 20 phosphorous or arsenic 21 on XH and YH to form XY 97 oxidoreductases 98 other, using H_2 as reductant 99 other, using O_2 as oxidant	Acceptor of hydrogen or electron 1 NAD^+ or $NADP^+$, or peroxidase 2 cytochrome, or heme protein 3 O_2 4 S—S 5 quinone or similar 6 nitrogenous group 7 iron-sulfur-protein 8 flavin 11 incorporating two O 12 incorporating one O 98 other, known 99 other	
2 Transferases Group transfer excluding hydrogen or oxygen: $AX+B \rightarrow BX+A$	General type of group 1 1-carbon group 2 aldehyde or ketone 3 acyl group, COR 4 glycosyl group 5 alkyl or aryl, other than methyl 6 nitrogenous groups 7 phosphate group 8 sulfur-containing groups 9 selenium-containing groups	Nature of group transferred	
3 Hydrolases Catalyzes hydrolysis: A—X+H_2O→X—OH+HA	Type of bond hydrolyzed 1 ester 2 glycosylases 3 ether 4 peptide 5 C—N bonds other than peptides 6 acid anhydrides 7 C—C 8 halide 9 phosphorus-nitrogen		

Continued

TABLE 7.1 International Classification of Enzymes: Code Numbers (EC First Digit.Second Digit.Third Digit. Fourth Digit) and Types of Reactions Catalyzed—cont'd

First Digit (Class)	Second Digit	Third Digit	Fourth Digit
	10 S—N 11 C-phosphorus 12 S—S 13 C—S		
4 Lyases Nonhydrolytic removal of groups with product usually contains double bond	Type of bond broken 1 C—C 2 C—O 3 C—N 4 C—S 5 C—halide 6 P—O 99 Other	Group removed 1 carboxyl 2 aldehyde 3 ketoacid	
5 Isomerases	Type of reaction 1 racemization or epimerization 2 cis-trans isomerizations 3 intramolecular oxidoreductases 4 intramolecular transfer reactions 5 intramolecular lyases 99 Other	Type of molecule 1 amino acids 2 hydroxyacids 3 carbohydrates	
6. Ligases Synthesis of bonds with breaking down of ATP or nucleoside triphosphates	Type of bond formed 1 C—O 2 C—S 3 C—N 4 C—C 5 Phosphoric ester 6 N-metal		

Example: Alcoholdehydrogenase (trivial name)

Systematic name: alcohol: NAD^+ oxidoreductase (an alcohol is the electron donor and NAD^+ is the electron acceptor)

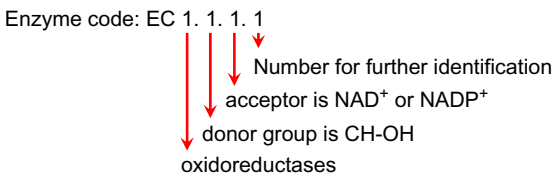


Fig. 7.1 shows the crystallographic structure of one alcoholdehydrogenase. While enzymes are specific in function, the degree of specificity varies. Some may act on closely related substrates (eg, based on a functional group) and are said to exhibit *group specificity*; others are

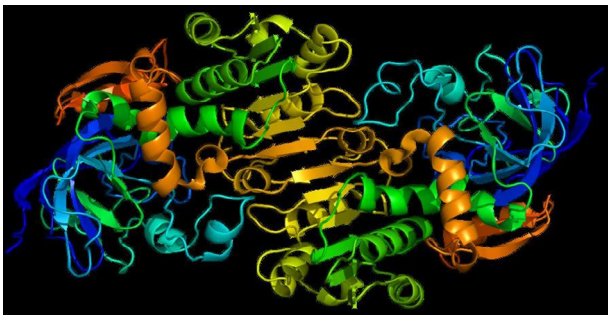


FIG. 7.1 Crystallographic structure of human ADH 5.

more exacting in their substrate requirements, and are said to be *absolutely specific*. The product formed from a particular enzyme and substrate is also unique. Enzymes are able to distinguish between stereochemical forms and only one isomer of a particular substrate may be catalyzed to react. Surprisingly, enzyme reactions may yield stereospecific products from substrates that possess no asymmetric carbon atoms, as long as one carbon is *prochiral*. This chirality is a result of at least a three-point interaction between substrate and enzyme in the active site of the enzyme. In Fig. 7.2, sites 1, 2, and 3 are binding sites on the enzyme. When two of the R groups on the substrate are identical, the molecule has a *prochiral* center, and a chiral center can result from the enzymatic reaction, as the substrate can only "fit" into the active site in one configuration if the site has binding selectivity for three of the R-group substituents. If the substrate has four different R groups, then chirality can be preserved in the reaction as a result of the multipoint attachment.

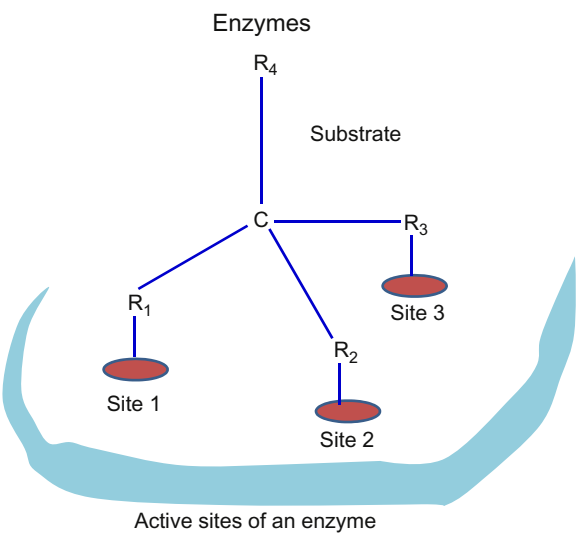


FIG. 7.2 Representation of the three-point interaction of substrate with enzyme.

7.1 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. Fig. 7.3 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 E of the uncatalyzed reaction at 20°C is 75 kJ mol^{-1}, whereas the E values for chemically catalyzed (by colloidal platinum) and enzymatically catalyzed (catalase) decomposition are 54 and 29 kJ mol^{-1}, respectively. That is, catalase accelerates the rate of reaction by a factor of about 10. One 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

$$\frac{e^{-\frac{29{,}000}{8.314\times 293}}}{e^{-\frac{75{,}000}{1.987\times 293}}} = e^{\frac{75{,}000-29{,}000}{8.314\times 293}} = 1.59\times 10^{8}$$ if the preexponent factor remains the same.

The molecular aspects of enzyme-substrate interaction differ for different enzyme and substrate pairs. Various studies using crystallography, 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. When the substrate enters the active site of an enzyme, it will be held initially by noncovalent forces. These noncovalent forces responsible for binding may be employed to lower the activation energy of the reaction as shown in Fig. 7.3. The types of noncovalent forces that are involved can be summarized as:

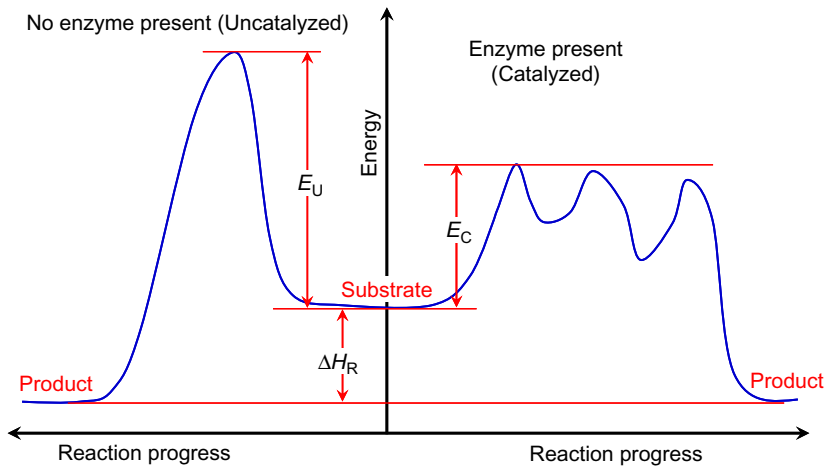


FIG. 7.3 Activation energies of enzymatically catalyzed and uncatalyzed reactions Note that $E_U < E_C$.

(a) *Electrostatic interactions* including charge-charge ($1/d_{\text{S-enz}}$, ie, inversely proportional to the distance between the charged enzyme active site to the charged substrate); dipole-dipole ($d^{-6}_{\text{S-enz}}$); charge-induced dipole ($d^{-4}_{\text{S-enz}}$); and dipole-induced dipole ($d^{-6}_{\text{S-enz}}$) interactions. The magnitude of these forces depends on the distance between molecules, varying with the distance ($d_{\text{S-enz}}$) in the manner indicated above. All depend inversely on the dielectric constant of the solvent between the ions or dipoles.

(b) *van der Waals forces* are comprised of electron cloud repulsion ($d^{-12}_{\text{S-enz}}$) and attractive dispersion forces (London forces) ($d^{-6}_{\text{S-enz}}$). The sum of these is described by the Lennard-Jones 6-12 potential. Dispersion forces are not large, but in an enzyme, the sum of all such forces between substrate and enzyme may be quite significant.

(c) *Hydrogen bonds* are important in biological systems and occur when two electronegative atoms are bound to a common proton. Often oxygen *is* one of the atoms.

(d) *Hydrophobic forces* reflect the tendency of apolar molecules to partition from an aqueous environment to a hydrophobic one. The driving force for such movement can be thought of as a result of the entropy gain when water molecules, which must be structured around an apolar molecule, are able to assume a more random arrangement when the molecule is transferred. The magnitude of this force is found experimentally to depend on the surface area of the molecule.

When the substrate moves from the external aqueous environment to the active site of the enzyme, its salvation shell is lost and one or more of the above forces are important in determining the strength of its binding. Hydrogen bonding and electrostatic interactions are generally most important. The difference in energies between the solvated state and that of the ES complex determines the strength of substrate binding. 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. 7.4.

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*. This is the simplest mechanism by which an enzyme may enhance the rate of a reaction. The reactants were brought at the active sites and would then be present at local concentrations that are much higher than those present in solution. How large a rate enhancement might we expect from such approximation? Some insight into this can be

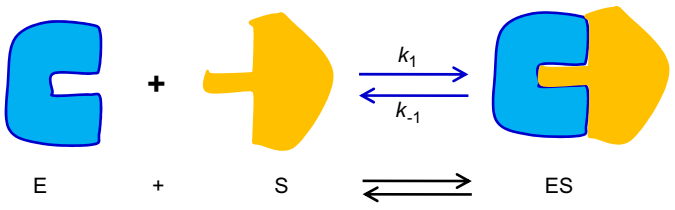
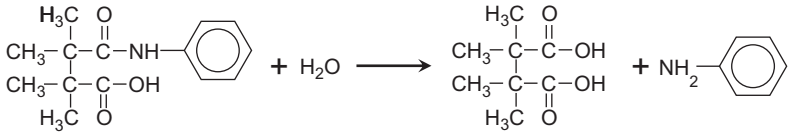


FIG. 7.4 Schematic of the lock-and-key model of enzyme catalysis.

obtained by examining *intramolecular* catalysis. Here one group adjacent to a reacting group provides catalytic assistance in the reaction.

An example of intramolecular catalysis is provided by the hydrolysis of tetramethyl succinanilic acid. This reaction is illustrated as



(7.1)

The carboxylic acid moiety provides intramolecular assistance in the hydrolysis of the amide bond. At pH 5, the reaction occurs with a half-life of 30 min, whereas the corresponding hydrolysis of the unsubstituted acetanilide is some 300 years! This difference in rates corresponds to a rate enhancement of 1.6×10^7.Unsubstituted succinanilic acid is hydrolyzed 1200 times more slowly than the tetramethyl substituted compound; the methyl groups are important in bringing the catalytic group close to the reacting amide bond.

The differences in rates between intramolecular catalysis and the corresponding intermolecular catalysis can be employed to define an apparent concentration of reactant at the reaction site in intramolecular catalysis. In intermolecular catalysis, reactants A and B may react at a rate, which is first order in both A and B, so the overall rate can be expressed as k_2[A][B], where k_2 is a second-order rate constant (L mol^{-1} s^{-1}). In the case of intramolecular catalysis, both A and B are present in the same compound (or complex) and the rate will now be k_1[A-B], with k_1 (s^{-1}) being a first-order rate constant. Thus the ratio k_1/k_2, which has units of molarity, represents the effective concentration of reactant A, which would need to be present at the catalytic site to cause a smaller concentration of B to react at a pseudo-first-order rate equivalent to that of the intramolecularly catalyzed rate. Hence, k_1/k_2 can then be thought of as an "effective" concentration of catalyst A at the reaction site. Such concentrations can be extremely high. When the effective molarities exceed attainable values, then other factors influencing catalysis, in addition to approximation, must be important.

In enzyme-catalyzed reactions, this enhanced local concentration effect can account for some of the rate enhancement, but is generally not sufficiently large. It does provide a lower limit to the rate acceleration that might be expected, however. We must turn to other mechanisms to provide an explanation for the catalytic abilities of enzymes.

Covalent catalysis: *electrophilic and nucleophilic catalysis* is another main route an enzyme may employ to enhance the reaction rate. An enzyme can form a covalent bond with one or more reactants and so alter the reaction path from that observed in the uncatalyzed case. The discovery that enzymes may indeed form covalent intermediates relied on early kinetic observations, including "burst" kinetics and the observation of constant rates of product release from substrates with varying substituents. Today, the crystallographic structures of many enzymes and their substrate-containing intermediates are well known, providing further evidence for the formation of such covalent intermediate compounds.

Covalent catalysis is divided into two types: electrophilic and nucleophilic catalysis. In nucleophilic catalysis, the nucleophilic groups on the enzyme are more electron-donating than the normal attacking groups, and a reactive intermediate is formed that breaks down rapidly

to form the products. Electrophilic catalysis on the other hand involves catalysts that withdraw electrons from the reaction center of the intermediate. We will first consider nucleophilic catalysis.

The most common nucleophiles in enzymes are the serine hydroxyl (found in serine proteases, esterases, lipases, and phosphatases), the cysteine thiol (thiol proteases), the carboxylic group of aspartic acid, the amino group of lysine (aldolase, transaldolase, DNA ligase), the -OH of tyrosine (in topoisomerases), and possibly imidazole (in conjunction with phosphoryl groups in phosphate transfer; otherwise it functions by general base catalysis). A simple example of nucleophilic catalysis by the serine hydroxyl is afforded by acetylcholine esterase. Acetylcholine is found in the nervous tissue and motor nerve tracts; it is an active neurotransmitter. When a nerve impulse travels along the nerve axon to the synapse, acetylcholine is released and diffuses from the nerve ending to the postsynaptic receptor for acetylcholine on the muscle cell membrane. Acetylcholine esterase (EC 3.1.1.7) functions by breaking down acetylcholine, thus ensuring that the nerve signal is of a short, finite duration. If the enzyme is inhibited, tetanic shock and muscle paralysis follow. The enzyme is thus the target for nerve gases and some insecticides. The enzyme has two subsites: one contains the nucleophilic serine, which is involved in the formation of an acetyl-enzyme intermediate (called the esteratic site); and the other is negatively charged and provides a salt bridge to enhance recognition and binding of the trimethylammonium region of acetylcholine. The serine acts as a nucleophile and attacks the ester linkage, presumably via the formation of a tetrahedral intermediate. Then the choline is released and an acyl enzyme intermediate is formed. Water (OH) then releases the acetate from the acyl intermediate. The reaction mechanism is shown in Fig. 7.5.

The enzyme is remarkably efficient; it has a turnover number (k_{cat}, or the first order reaction rate constant) of 25,000 s^{-1}, and thus cleaves one substrate molecule every 40 μs. This rapid rate of cleavage is crucial, as nerve impulses can be carried at a rate of 1000 impulses s^{-1}, necessitating the rapid removal of acetylcholine from the postsynaptic receptor.

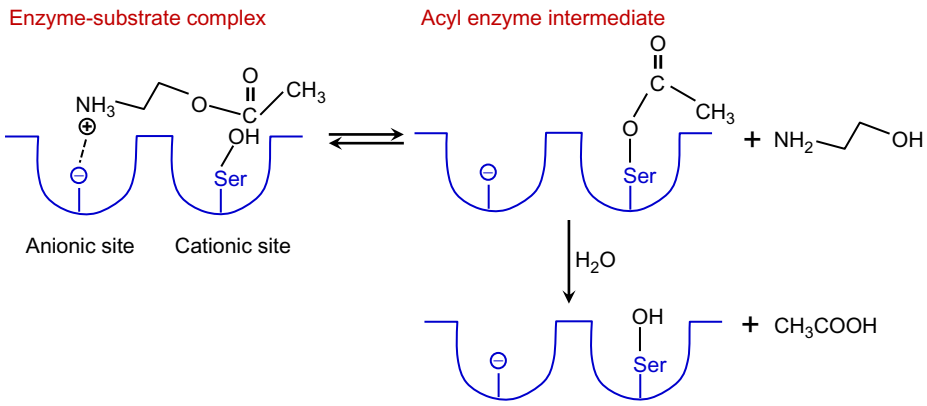


FIG. 7.5 The mechanism of action of acetylcholine esterase (EC 3.1.1.7).

Electrophilic catalysts, in contrast to nucleophilic catalysis, act by withdrawing electrons from the reaction center of the intermediate and are thus *electron sinks*. They stabilize a negative charge. Examples of this mechanism involve coenzymes thiamine pyrophosphate and pyridoxal phosphate. In many cases, including these coenzymes, electrophilic catalysis involves the formation of Schiff bases. For example, acetoacetate decarboxylase (EC 4.1.1.4) catalyzes the decarboxylation of acetoacetate to acetone and CO_2. The mechanism involves the formation of a Schiff base involving a lysine residue. Acetoacetate decarboxylase participates in the production of acetone by fermentation of sugars by anaerobic bacteria, such as *Clostridium acetobutylicum*. This fermentation process was an important route to acetone in World War I, when acetone was employed in the production of the explosive cordite and chemical routes were not available. Other important Schiff base reactions include aldolase and transaldolase reactions.

Acids and bases can catalyze reactions by either donating or accepting a proton that is transferred in the transition state. When such a charged group develops in the transition state, the resulting positive or negative charge makes the transition state unfavorable. The presence of acids or bases results in a stabilization of such a transition state. By providing acid or base groups at the active site, an enzyme is thus able to stabilize the charged transition state. This mechanism is employed by a wide variety of enzymes.

Two types of acid or base catalysis can be distinguished: *general* and *specific*. The distinction between specific and general acids and bases can be best understood by examining experimental observations of catalytic reaction rates. Consider, for example, the hydrolysis of an ester in buffered solutions. The hydrolysis rate can be determined at a constant pH (by maintaining the ratio of acid and base forms of the buffer at a constant value), at several different total concentrations of buffer. If the rate of reaction increases with increasing buffer concentration, then the buffer must be involved in the reaction and act as a catalyst. This is *general* acid or base catalysis. If the rate is unaffected by buffer concentration, then the reaction involves *specific* acid or base catalysis. The reacting species would be only proton (H^+) or hydroxyl anion (OH^-) and the buffer simply serves to maintain these species at constant concentrations.

How does an enzyme obtain the rate enhancements made possible by general acid-base catalysis? The answer lies in the combination of pK_a values for amino acid moieties involved in acid-base catalysis, and the typical values of proton dissociation constant *K*a, describing proton transfer. An acid with a pK_a of 5 is a better general-acid catalyst than one with a pK_a of 7. At pH 7, however, typical of the optimal pH of many enzymes, only 1% of the acid with a pK_a of 5 will be unionized and active in catalysis. The acid with a pK_a of 7 will be 50% unprotonated. The same trend applies to base catalysis. If we consider the amino acids, we see that histidine contains an imidazole moiety, which has a pK_a value around 6–7. Therefore, histidine is widely found in enzymes involved in base catalysis, as it is 50% ionized at neutral pH. The imidazole moiety may thus be considered as the most effective amino acid base existing at neutral pH. In fact, all enzymatic acyl transfers catalyzed by proteases (eg, trypsin, chymotrypsin) involve histidine. The imidazole group of histidine can also function as a nucleophile, and we must be careful to determine whether histidine acts a general base catalyst or a nucleophilic catalyst. In proteases, histidine functions as a base catalyst, but is typically found closely associated with serine. Histidine is thought to deprotonate this neighboring serine

alcohol moiety by general base catalysis. The serine alkoxide ion ($^{-}$O-CH_2CH-Enz) so generated has a pK_a of 13.7 and is thus a stronger base than histidine, but at neutral pH is less reactive. Serine functions primarily as a strong nucleophile in proteases.

In addition to covalent and general acid-base catalysis, enzymes also employ other mechanisms of rate enhancement. One of these is electrostatic catalysis. As we have seen from the transition state analysis described above, electrostatic interactions between substrate and enzyme may stabilize this transition state and thus yield significant rate enhancements. We shall briefly describe this and the other types of catalysis that are found in enzymes.

Electrostatic catalysis: In water, the large dielectric constant results in a small electrostatic interaction energy between charges, and electrostatic catalysis is not generally important in homogeneous catalysis in aqueous systems; however, the active site of a protein is very heterogeneous, and the dielectric constant of the medium between charged groups may be quite different from water. The aromatic and aliphatic amino acid residues present at the active site act to reduce the dielectric constant, and charged amino acid residues act as fixed dipoles, thus stabilizing charge quite effectively. The electrostatic interaction energy depends on the charges and is inversely proportional to the dielectric constant and distance between charges.

Lowering the dielectric constant can increase this energy considerably. Proteins may thus use parts of their own structure to solvate transition states and induce electrostatic strain. In fact, enzymes may stabilize charged groups in the transition state better than water, as the amino acids that function as dipoles are rigidly positioned and have a direction in relation to the substrate, whereas in water, this directionality is lost.

The overall significance of electrostatic catalysis in enzymes is still not clear, as determination of the local dielectric constant is difficult. Electrostatic stabilization of charged transition states certainly plays some role, however.

Catalysis involving metal ions: In metalloenzymes, a metal ion is present at the active site, and this ion plays an important role in stabilizing negative charges that are formed in electrophilic catalysis. Zinc, copper, and cobalt are commonly involved in coordination of oxyanions involved as reaction intermediates. The enzyme carboxypeptidase-A (EC 3.4.17), which is a carboxyl-terminus exopeptidase (ie, it acts by hydrolyzing the peptide from the carboxylic acid terminus), contains Zn^{2+} which polarizes the carbonyl oxygen of the terminal peptide bond. The terminal carboxylate is charged paired with the guanidinium cation of Arg 145 leading to polarization of the terminal carboxylic carbonyl group. This polarization increases the electrophilicity of the carbonyl carbon and facilitates nucleophile-mediated hydrolysis of the amide bond. This is illustrated in Fig. 7.6. In addition to stabilizing negative charges, metal ions serve as a source of potent nucleophilic hydroxyl ions. Metal-bound water molecules provide these nucleophilic hydroxyl groups at neutral pH.

An example is the extremely rapid hydration of CO_2 by carbonic anhydrase to produce bicarbonate. The enzyme contains zinc coordinated to the imidazole groups of three histidines, with the fourth ligand being water, which is ionized. The zinc-bound hydroxyl has a pK_a of 7.7 and the reaction mechanism is thought to be that shown in Fig. 7.7.

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

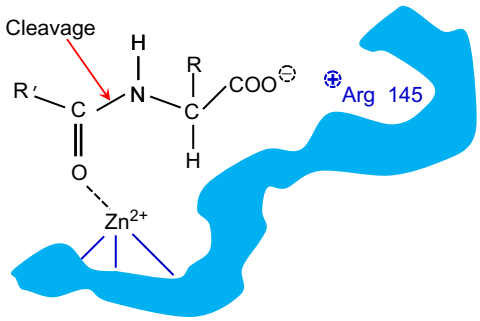


FIG. 7.6 Metalloenzyme carboxypeptidase-A (EC 3.4.17) facilitates nucleophile-mediated hydrolysis of an amide bond.

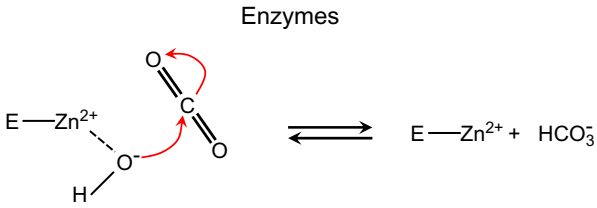


FIG. 7.7 Metalloenzyme carbonic anhydrase (EC 3.4.17) facilitates the hydration of CO_2 to bicarbonate.

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 7.2 lists some enzymes and their cofactors and coenzymes.

7.2 SIMPLE ENZYME KINETICS

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 shown in Fig. 7.8. 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, which will be discussed later in Chapter 10. In this chapter, we will use the simple unimolecular reaction

$$S \rightleftarrows P \tag{7.2}$$

TABLE 7.2 Cofactors (Metal Ions) and Coenzymes of Some Enzymes

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

as an example, if not explicitly specified. Reaction (Eq. 7.2) could also be an approximate reaction, thus not exactly unimolecular.

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.

$$E + S \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} ES \xrightarrow{k_c} E + P \tag{7.3}$$

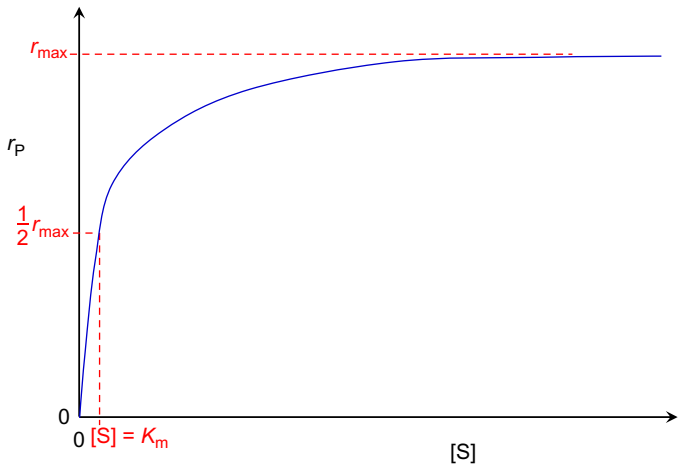


FIG. 7.8 Effect of substrate concentration on the rate of product formation for an enzyme-catalyzed reaction.

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) pseudosteady-state approach.

Both the pseudosteady-state approximation and the assumption of rapid equilibrium share the same few initial steps in deriving a rate expression for the mechanism in Eq. (7.4), where the rate of product formation is

$$r_P = r_2 = k_c[ES] \tag{7.4}$$

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

$$r_{ES} = r_1 - r_2 = k_1[E][S] - k_{-1}[ES] - k_c[ES] \tag{7.5}$$

where [E] is the concentration of free enzyme in the solution, [S] is the concentration of free substrate in the solution, and [ES] is the concentration of enzyme-substrate complex. Since the enzyme is not consumed, the conservation equation on the enzyme yields

$$[E] = E - [ES] \tag{7.6}$$

where E is the enzyme loading or total enzyme concentration.

At this point, an assumption is required to simplify the rate expression, eliminating the requirement of concentrations other than the enzyme loading, substrate and product concentrations.

7.2.1 The Fast Equilibrium Step Assumption

Henri and Michaelis-Menten used essentially this approach—assuming the first step is very fast and equilibrium is reached when one considers the second step. That is to say,

$$0 = r_1 = k_1[E][S] - k_{-1}[ES] \tag{7.7}$$

(Do you see why the rate is zero? This is contrary to what it states, that this first step is very fast.) Eq. (7.7) yields

$$K_m = \frac{k_{-1}}{k_1} = \frac{[E][S]}{[ES]} \tag{7.8}$$

where K_m is the dissociation equilibrium constant of the enzyme-substrate complex. Apply Eq. (7.6) if the enzyme is conserved, then

$$[ES] = \frac{E[S]}{K_m + [S]} \tag{7.9}$$

Substituting Eq. (7.9) into Eq. (7.4) yields

$$r_P = k_c[ES] = \frac{k_c E[S]}{K_m + [S]} = \frac{r_{max}[S]}{K_m + [S]} \tag{7.10}$$

where $r_{max} = k_c E$.

In this case, the maximum forward rate of the reaction is r_{max}. The value of r_{max} changes if more enzyme is added, but the addition of more substrate has no influence on r_{max}. K_m is often called the Michaelis-Menten constant. 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 at which the reaction rate is half of the maximal reaction rate.

An equation of exactly the same form as Eq. (7.10) can be derived with a different, more general assumption applied to the reaction scheme in Eq. (7.3).

7.2.2 The Pseudosteady-State Hypothesis

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 pseudosteady-state assumption in 1925. 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 was small, $r_{ES} \approx 0$. (This logic is flawed. Do you see why?) Exact solutions of the actual time course represented by Eqs. (7.4), (7.5), (7.6) have shown that *in a closed system, the PSSH holds* after a brief transient *if* $S_0 \gg E$. Fig. 7.9 displays one such time course.

By applying PSSH to Eq. (7.5), we find

$$[ES] = \frac{k_1 E[S]}{k_{-1} + k_c} \tag{7.11}$$

Substituting the enzyme conservation Eq. (7.4) in Eq. (7.9) and solve for [ES], we obtain

$$[ES] = \frac{E[S]}{\frac{k_{-1} + k_c}{k_1} + [S]} \tag{7.12}$$

Substituting Eq. (7.12) into Eq. (7.4) yields

$$r_P = k_2[ES] = \frac{k_c E[S]}{\frac{k_{-1} + k_c}{k_1} + [S]} \tag{7.13}$$

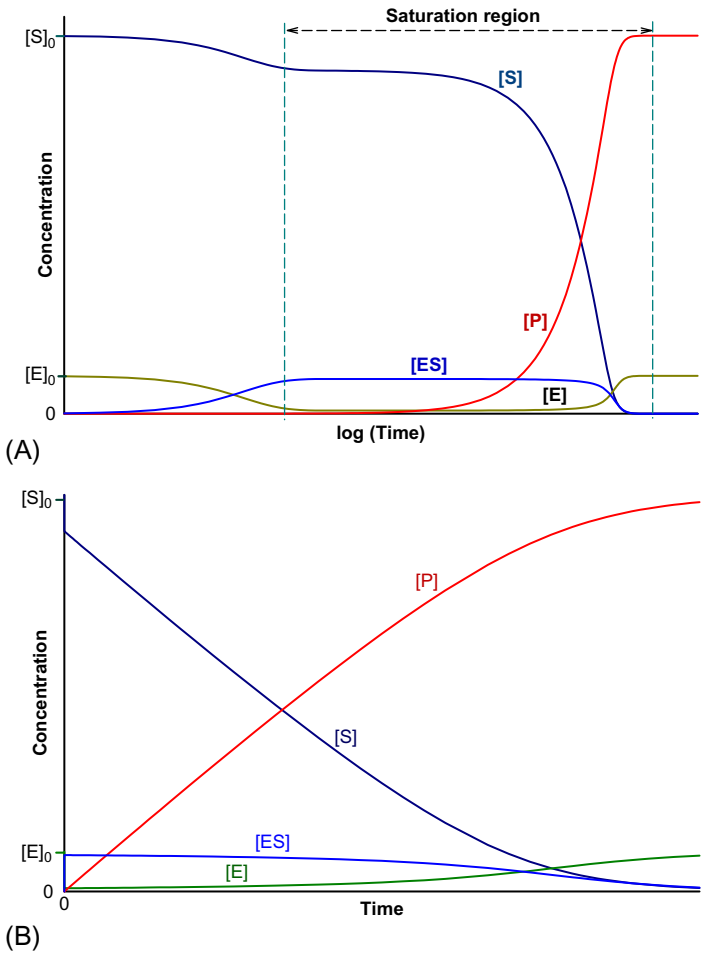


FIG. 7.9 Time course of the formation of an enzyme/substrate complex and initiation of the pseudosteady state and the completion in a batch reactor, as derived from the two kinetic steps on a typical enzyme, Eq. (7.3) with $k_c = 0.01\,k_1$, $k_{-1} = 0.8\,k_1$, and $S_0 = 10$ E. (A) Concentration verse log (time). The pseudo-steady state, i.e. when constant concentrations of [ES] and [E] are reached and beyond, is shown clearly in this plot. (B) Concentration verse time plot. The establishment of the pseudosteady state is accomplished in a very short time period and it nearly shows a step change at time 0 in this linear plot.

Eq (7.13) can be reduced to Eq. (7.10)

$$r_P = \frac{r_{max}[S]}{K_m + [S]} \tag{7.14}$$

However,

$$K_m = \frac{k_{-1} + k_c}{k_1} \tag{7.14}$$

which is not the equilibrium constant as that in Eq. (7.8). Under most circumstances (simple experiments), it is impossible to distinguish the two approaches as both reduced to Eq. (7.10). Table 7.3 shows the range of the Michaelis-Menten rate parameters.

TABLE 7.3 Michaelis-Menten Rate Parameters for Some Enzymes

Enzyme	Substrate	EC Code	K_m, mM	k_c, s^{-1}
Acetylcholinesterase	Acetylcholine	3.1.1.7	9×10^{-2}	1.4×10^{4}
β-Alanine synthase	*N*-carbamoyl-β-alanine	2.5.1.51	9×10^{-3}	0.6
Alliinase	*S*-alkyl-L-cysteine *S*-oxide	4.4.1.4	1.5	0.01
Carbonic anhydrase	CO_2	4.2.1.1	12	10^{6}
Carbonic anhydrase	HCO_3^-	4.2.1.1	26	4×10^{5}
Catalase	H_2O_2	1.11.1.6	1100	4×10^{7}
Chymotrypsin	Polypeptide	3.4.21.1	15	0.14
Fumarase	Fumarate	4.2.1.2	5.0×10^{-3}	8.0×10^{2}
Fumarase	Malate	4.2.1.2	2.5×10^{-2}	9.0×10^{2}
β-Lactamase	Benzylpenicillin	3.5.2.6	0.02	2×10^{3}
Orotate phosphoribosyltransferase	Orotate	2.4.2.10	0.02	4
4-Oxalocrotonase tautomerase	2-Hydroxymuconate	5.3.2.-4-OT	0.19	2.9×10^{6}
Pepsin	Protein	3.4.23.1	0.30	0.50
Superoxide dismutase	O_2^-	1.15.1.1	1.3	10^{4}
Triosephosphate isomerase	G3P	5.3.1.1	0.47	4.3×10^{3}
Tyrosyl-tRNA synthetase	tRNA	6.1.1.1	0.90	7.6
Uridine kinase	Uridine	2.7.1.48	0.04	180

7.2.3 Specific Activity

Enzyme concentrations are often given in terms of "units" rather than in mole or mass concentration. We rarely know the exact mass of the enzyme in a sample, since it is generally prepared via isolation of the enzyme from microorganisms, or animal or plant tissues, and often contains a great deal of noncatalytic protein, the amount of which may vary from sample to sample. Hence a different approach must be adopted, and enzyme concentration is reported in units of *specific activity*. A "unit" is defined as the amount of enzyme (eg, microgram) that gives a certain amount of catalytic activity under specified conditions (eg, producing 1.0 micromole of product per minute in a solution containing a substrate concentration sufficiently high to be in the "saturation" region, as shown in Fig. 7.9 where [ES] and [E] are relatively invariant).

Thus different suppliers of enzymes may have preparations with different units of activity, and care must be taken in analyzing kinetic data. Thus a purified enzyme preparation will have a higher specific activity than a crude preparation; often a protein is considered pure when a constant specific activity is reached during the purification steps.

$$\text{Specific activity} = \frac{\text{Activity}}{\text{mg-protein}} = \frac{\text{mmol-product}}{\text{mg-protein} \times \text{min} \times \text{mL}} \tag{7.15}$$

The activity is given by the amount of product formed or substrate consumed in the reaction mixture, under the conditions specified (temperature, pH, buffer type, substrate and enzyme concentrations, etc.). If the molecular weight of the enzyme is known, the specific activity can also be defined as

$$\text{Specific activity} = \frac{\text{Activity}}{\text{mmol-protein}} = \frac{\text{mmol-product}}{\text{mmol-protein} \times \text{min} \times \text{mL}} \tag{7.16}$$

Example 7.1: Specific Activity

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 that produces 1 μmol of glucose per min in a 4% solution of Linter starch at pH 4.5 and at 60°C. Initial rate experiments show that the reaction produces 0.6 μmol of glucose $(\text{mL min})^{-1}$. What is the specific activity of the crude enzyme preparation?

Solution

The total amount of glucose made is 10 mL × 0.6 μmol glucose $(\text{mL min})^{-1}$ or 6 μmol glucose per min. The specific activity is then

$$\begin{aligned}\text{specific activity} &= \frac{6\,\text{units}}{1\,\text{mL protein solution} \times 8\,\text{mg/mL}} \\ &= 0.75\,\text{units/mg-protein}\end{aligned}$$

The maximum reaction rate r_{max} must have units such as mol-product $(\text{L min})^{-1}$. Since $r_{max} = k_c[E]_0$, the dimensions of k_c must reflect the definition of units in $[E]_0$. In the above example, we had a concentration of enzymes of 8 mg protein/10 mL solution. 0.75 units/mg-protein or 600 units L^{-1}. If, for example, $r_{max} = 1$ mol $(\text{L min})^{-1}$, then $k_c = 1$ mol $(\text{L min})^{-1} \div$ 600 units L^{-1} or $k_c = 1.67$ μmol $(\text{unit min})^{-1}$.

7.3 MULTIPLE-SUBSTRATE AND COMPETITIVE ENZYME KINETICS

In the previous section, we have learned the basic principles of enzyme kinetics. We have been focused on the common type of simple enzymatic reactions: a single substrate and a single active site per enzyme molecule. The analysis can be extended to multiple substrates and irregular situations. We will discuss enzymes having multiple binding sites in Chapter 10. This section shows how more complicated kinetic behaviors involving a single-sited enzymatic reaction can be derived based on the principles we learned in the previous section.

7.3.1 Reversible Reactions

We have so far dealt with the most simplified case of a single substrate acting on an enzyme and the product is not interacting with the enzyme. This case is extremely rare as enzymes can normally catalyze the reactions both ways. Let us continue with this simple case, but allow the reverse reaction to be catalyzed by the enzyme as well

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} E \bullet S \underset{k_{-c}}{\overset{k_c}{\rightleftharpoons}} E \bullet P \underset{K_P}{\rightleftharpoons} E + P \tag{7.17}$$

Comparing with reaction (Eq. 7.3), which we have discussed at length, reaction (Eq. 7.17) allows for the complex of EP to coexist with the substrate-enzyme complex ES. Without loss of generality, let us use the FES approach to examine this simplest of competitive binding kinetics.

The FES of the enzyme complexes

$$[\mathrm{ES}] = \frac{[\mathrm{E}][\mathrm{S}]}{K_\mathrm{S}} \tag{7.18a}$$

$$[\mathrm{EP}] = \frac{[\mathrm{E}][\mathrm{P}]}{K_\mathrm{P}} \tag{7.18b}$$

where K_S and K_P are the dissociation equilibrium constants of the enzyme-substrate and enzyme-product complexes, respectively. The enzyme balance,

$$\mathrm{E} = [\mathrm{E}] + [\mathrm{ES}] + [\mathrm{EP}] \tag{7.19}$$

Substituting Eqs. (7.18a), (7.18b) into (7.19) and rearranging, we obtain

$$[\mathrm{E}] = \frac{\mathrm{E}}{1 + \dfrac{[\mathrm{S}]}{K_\mathrm{S}} + \dfrac{[\mathrm{P}]}{K_\mathrm{P}}} \tag{7.20}$$

The rate of formation of product P is given by

$$r_\mathrm{P} = k_\mathrm{c}[\mathrm{ES}] - k_{-\mathrm{c}}[\mathrm{EP}] \tag{7.21}$$

Substituting Eqs. (7.20), (7.18a), (7.18b) into (7.21), we obtain

$$r_\mathrm{P} = \frac{k_\mathrm{c}\mathrm{E}([\mathrm{S}] - [\mathrm{P}]/K_\mathrm{C})}{K_\mathrm{S} + [\mathrm{S}] + \dfrac{K_\mathrm{S}}{K_\mathrm{P}}[\mathrm{P}]} \tag{7.22}$$

where K_C is the equilibrium constant of the reaction (7.2).

$$K_\mathrm{C} = \frac{k_\mathrm{c}K_\mathrm{S}}{k_{-\mathrm{c}}K_\mathrm{P}} \tag{7.23}$$

Comparing Eq. (7.22) with the well known Michaelis-Menten Eq. (7.10), one can observe the similarity and notice the complication to allow for competitive binding (of the product P with the substrate S) as well as the reversible reaction.

To discuss the effect of competitive binding, let us assume that the reaction is actually irreversible, ie, $K_\mathrm{C} \to \infty$. In this case, the only difference between Eqs. (7.22), (7.10) is that the denominators are different,

$$K_\mathrm{S} + [\mathrm{S}] + \frac{K_\mathrm{S}}{K_\mathrm{P}}[\mathrm{P}] \quad \text{vs} \quad K_\mathrm{S} + [\mathrm{S}]$$

The denominator for the competitive binding has one more term, $K_\mathrm{S}([\mathrm{P}]/K_\mathrm{P})$, reflecting the competition of P-binding on the enzyme. The net effect is a decreased reaction rate for an increase concentration of P. Therefore, we sometimes refer to this effect as product-inhibition (as compared with the simplistic case of Michaelis-Menten equation).

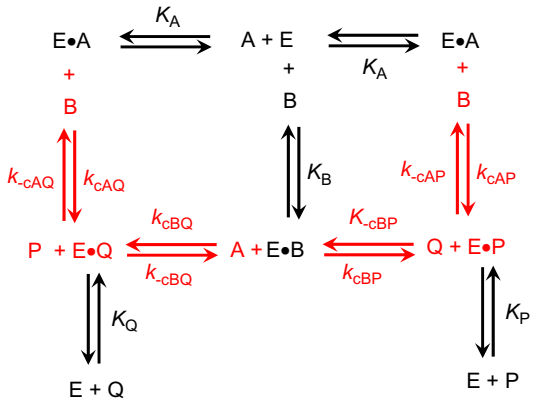
7.3.2 Reactions With Unbound Substrates

The majority of enzymatic reactions do not involve just one substrate, they usually involve two (or more). If two substrates or multisubstrates can bind on the enzyme simultaneously, it will require a more complex enzyme, which will be discussed in Chapter 10. In this section, we assume there is only one substrate molecule that can bind on an enzyme. The second substrate can participate in the reaction without being directly bound on the enzyme. Many of the concepts we have developed to date can be applied to situations where one of the substrates is held at a constant concentration; the kinetics then follow one of the forms described earlier.

A number of possible binding scenarios arise when multiple substrates are involved in the reaction. We will use the simplest case, that of two-substrate (bimolecular) reactions as an example. This reaction is given by

$$A + B \rightarrow P + Q \tag{7.24}$$

For situations where an unbound-bound complex mechanism is involved, the reaction can occur as (1) an unbound B reacts with a bound A, (2) an unbound A reacts with a bound B. The same complication can be said about the products formed as well. These possibilities can be generalized by



(7.25)

We can write the equation below for the total amount of enzyme in the system as

$$E = [E] + [EA] + [EB] + [EQ] + [EP] \tag{7.26}$$

The analysis proceeds by assuming that all enzyme substrate complexes are at fast equilibrium and that the rate of formation of P is given by

$$\begin{aligned} r_P = {} & k_{cAQ}[EA][B] - k_{-cAQ}[EQ][P] + k_{cBQ}[EB][A] - k_{-cBQ}[EQ][P] \\ & + k_{cAP}[EA][B] - k_{-cAP}[EP][Q] + k_{cBP}[EB][A] - k_{-cBP}[EP][Q] \end{aligned} \tag{7.27}$$

Following our convention on the dissociation constants, expressions for each of the enzyme-substrate and enzyme-product complexes can now be written:

$$[EA] = \frac{[E][A]}{K_A} \tag{7.28a}$$

$$[EB] = \frac{[E][B]}{K_B} \tag{7.28b}$$

$$[EP] = \frac{[E][P]}{K_P} \tag{7.28c}$$

$$[EQ] = \frac{[E][Q]}{K_Q} \tag{7.28d}$$

Applying the enzyme balance, Eq. (7.26), we obtain

$$[E] = \frac{E}{1 + \frac{[A]}{K_A} + \frac{[B]}{K_B} + \frac{[P]}{K_P} + \frac{[Q]}{K_Q}} \tag{7.29}$$

The overall rate expression is then

$$r_P = \frac{E\left(\frac{k_{cAP} + k_{cAQ}}{K_A} + \frac{k_{cBP} + k_{cBQ}}{K_B}\right)\left([A][B] - \frac{[P][Q]}{K_C}\right)}{1 + \frac{[A]}{K_A} + \frac{[B]}{K_B} + \frac{[P]}{K_P} + \frac{[Q]}{K_Q}} \tag{7.30}$$

where K_C is the equilibrium constant and is given by

$$K_C = \frac{\frac{k_{cAP} + k_{cAQ}}{K_A} + \frac{k_{cBP} + k_{cBQ}}{K_B}}{\frac{k_{-cAP} + k_{-cBP}}{K_P} + \frac{k_{-cAQ} + k_{-cBQ}}{K_Q}} \tag{7.31}$$

Letting

$$k_{AB} = \frac{k_{cAP} + k_{cAQ}}{K_A} + \frac{k_{cBP} + k_{cBQ}}{K_B} \tag{7.32}$$

The rate expression can be simplified to

$$r_P = \frac{Ek_{AB}\left([A][B] - \frac{[P][Q]}{K_C}\right)}{1 + \frac{[A]}{K_A} + \frac{[B]}{K_B} + \frac{[P]}{K_P} + \frac{[Q]}{K_Q}} \tag{7.33}$$

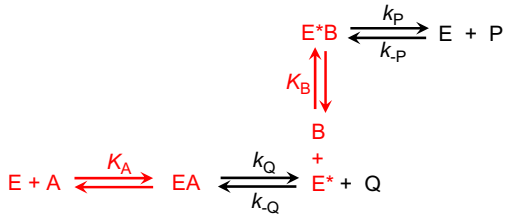
If only one of the substrates is present in excess (let us assume B is in excess) and at initial conditions (product concentration is negligible), Eq. (7.33) is reduced to

$$r_P = \frac{(Ek_{AB}K_A[B])[A]}{\left(K_A + \frac{K_A[B]}{K_B}\right) + [A]} \tag{7.34}$$

which is equivalent to the Michaelis-Menten Eq. (7.10). Therefore, we are able to recover the single-substrate-limiting rate expression.

7.3.3 Enzyme-Substituted Reactions—the Ping-Pong Mechanism

In enzyme-substituted or double-displacement mechanisms, both substrates need not be bound together at the active site of the enzyme. There is thus a required order of substrate binding (ie, a *sequential* mechanism). This mechanism occurs when, for example, a phosphate-transferring enzyme, such as phosphoglycerate mutase, is phosphorylated. One substrate (A) reacts with the enzyme to give E* (eg, a phosphorylenzyme), which then transfers the phosphoryl group to the second substrate B.



(7.35)

Kinetics of this type thus provide information about the existence of a covalent intermediate. Often the finding of double displacement (or "ping-pong") kinetics is used as evidence for the existence of this intermediate, but other confirming information should be sought. The kinetic pattern of this type of mechanism is unique. Using FES on the enzyme-substrate complex formations, the rate expression can be derived as

$$r_P = r_Q = \frac{E k_P k_Q ([A][B] - [P][Q]/K_C)}{(K_A + [A])(k_{-Q} K_B [Q] + k_P [B]) + (K_B + [B])(k_Q [A] + K_A k_{-P} [P])} \tag{7.36}$$

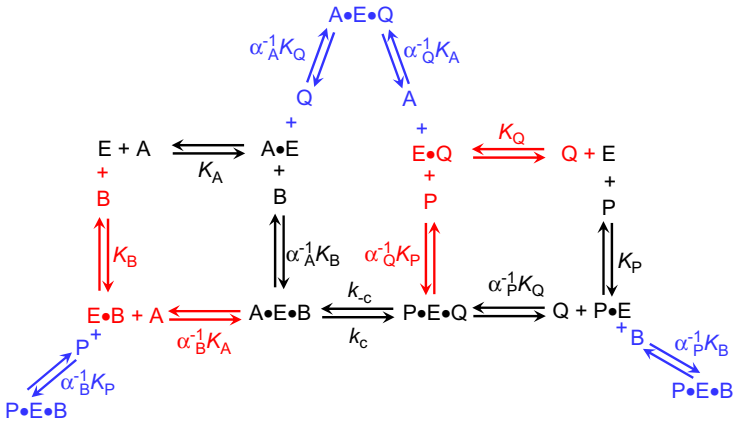
where K_C is the equilibrium constant and is given by

$$K_C = \frac{k_P k_Q}{k_{-P} k_{-Q} K_A K_B} \tag{7.37}$$

7.3.4 Allosteric Enzymes

Some enzymes have additional binding sites, although not accessible to the substrate, but accessible to effectors or cofactors that can affect the kinetics. When an effector molecule is bound on the enzyme, the enzyme could undergo conformational change and result in the affinity as well as reactivity change on the catalytic site of the enzyme.

It is the additional binding sites that also make the multisubstrate reactions possible. Often the second substrate is a cofactor, such as NAD^+ in oxidoreductases. Commonly, the cofactor is also bound on the enzyme, but on the allosteric site that is not active to the substrate. In general, when one substrate is bound on the enzyme, the conformal structure of the enzyme will change and result in the change of the binding coefficient. Let us re-examine the case shown exemplified by reaction (Eq. 7.24) in the previous subsection.



(7.38)

which is the general mechanistic model for reaction (Eq. 7.24) when two binding sites are available, one for A and one for B, and they respectively can bind P and Q as well.

Without loss of generality, reaction scheme (Eq. 7.38) shows three possible scenarios: 1) A binds on E first before B binds to induce the reaction to occur (when alternatives present, the black-colored route is chosen); 2) B binds on E first before A binds to induce the reaction (when alternatives present, the red-colored route is chosen); and 3) There is no sequence in the binding, or the binding is random.

(1) A Binding before B

In case (1) where A binds before B, the enzyme balance can be written as

$$E = [E]_A + [AE] + [AEB] + [PEQ] + [PE] + [PEB] + [AEQ] \tag{7.39}$$

where the subscript A stands for A-free (or more precisely A and P free). The FES of the enzyme complexes

$$[AE] = \frac{[E]_A[A]}{K_A} \tag{7.40a}$$

$$[AEB] = \alpha_A \frac{[AE][B]}{K_B} = \alpha_A \frac{[E]_A[A][B]}{K_A K_B} \tag{7.40b}$$

$$[AEQ] = \alpha_A \frac{[AE][Q]}{K_Q} = \alpha_A \frac{[E]_A[A][Q]}{K_A K_Q} \tag{7.40c}$$

$$[PE] = \frac{[E]_A[P]}{K_P} \tag{7.40d}$$

$$[PEQ] = \alpha_P \frac{[E]_A[P][Q]}{K_P K_Q} \tag{7.40e}$$

$$[PEB] = \alpha_P \frac{[E]_A[P][B]}{K_P K_B} \tag{7.40f}$$

Substituting Eqs. (7.40a)–(7.40f) into Eq. (7.39) and rearranging, we obtain

$$[\mathrm{E}]_{\mathrm{A}} = \frac{\mathrm{E}}{1 + \dfrac{[\mathrm{A}]}{K_{\mathrm{A}}} + \dfrac{[\mathrm{P}]}{K_{\mathrm{P}}} + \alpha_{\mathrm{A}}\dfrac{[\mathrm{A}][\mathrm{B}]}{K_{\mathrm{A}}K_{\mathrm{B}}} + \alpha_{\mathrm{A}}\dfrac{[\mathrm{A}][\mathrm{Q}]}{K_{\mathrm{A}}K_{\mathrm{Q}}} + \alpha_{\mathrm{P}}\dfrac{[\mathrm{P}][\mathrm{Q}]}{K_{\mathrm{P}}K_{\mathrm{Q}}} + \alpha_{\mathrm{P}}\dfrac{[\mathrm{P}][\mathrm{B}]}{K_{\mathrm{P}}K_{\mathrm{B}}}} \tag{7.41}$$

The rate of reaction is governed by the rate-limiting step,

$$r_{\mathrm{P}} = r_{\mathrm{Q}} = k_{\mathrm{c}}[\mathrm{EAB}] - k_{-\mathrm{c}}[\mathrm{PEQ}] \tag{7.42}$$

Substituting Eqs. (7.41), (7.40b), (7.40e) into Eq. (7.42), we obtain

$$r_{\mathrm{P}} = r_{\mathrm{Q}} = \frac{\alpha_{\mathrm{A}}k_{\mathrm{c}}\mathrm{E}([\mathrm{A}][\mathrm{B}] - [\mathrm{P}][\mathrm{Q}]/K_{\mathrm{C}})}{K_{\mathrm{A}}K_{\mathrm{B}}\left\{1 + \left(\dfrac{[\mathrm{B}]}{K_{\mathrm{B}}} + \dfrac{[\mathrm{Q}]}{K_{\mathrm{Q}}}\right)\left(1 + \alpha_{\mathrm{A}}\dfrac{[\mathrm{A}]}{K_{\mathrm{A}}} + \alpha_{\mathrm{P}}\dfrac{[\mathrm{P}]}{K_{\mathrm{P}}}\right)\right\}} \tag{7.43}$$

where K_{C} is the equilibrium constant and is given by

$$K_{\mathrm{C}} = \frac{k_{\mathrm{c}}\alpha_{\mathrm{P}}K_{\mathrm{A}}K_{\mathrm{B}}}{k_{-\mathrm{c}}\alpha_{\mathrm{A}}K_{\mathrm{P}}K_{\mathrm{Q}}} \tag{7.44}$$

(2) B Binding before A

Similar to the case (1), the rate expression can be derived as

$$r_{\mathrm{P}} = r_{\mathrm{Q}} = \frac{\alpha_{\mathrm{B}}k_{\mathrm{c}}\mathrm{E}([\mathrm{A}][\mathrm{B}] - [\mathrm{P}][\mathrm{Q}]/K_{\mathrm{C}})}{K_{\mathrm{A}}K_{\mathrm{B}}\left\{1 + \left(\dfrac{[\mathrm{A}]}{K_{\mathrm{A}}} + \dfrac{[\mathrm{P}]}{K_{\mathrm{P}}}\right)\left(1 + \alpha_{\mathrm{B}}\dfrac{[\mathrm{B}]}{K_{\mathrm{B}}} + \alpha_{\mathrm{Q}}\dfrac{[\mathrm{Q}]}{K_{\mathrm{Q}}}\right)\right\}} \tag{7.45}$$

(3) Random order

The rate of formation of product P is given by

$$r_{\mathrm{P}} = r_{\mathrm{Q}} = \frac{\dfrac{(\alpha_{\mathrm{A}} + \alpha_{\mathrm{B}})k_{\mathrm{c}}\mathrm{E}}{K_{\mathrm{A}}K_{\mathrm{B}}}\left([\mathrm{A}][\mathrm{B}] - \dfrac{[\mathrm{P}][\mathrm{Q}]}{K_{\mathrm{C}}}\right)}{\left\{1 + \left(\dfrac{[\mathrm{A}]}{K_{\mathrm{A}}} + \dfrac{[\mathrm{P}]}{K_{\mathrm{P}}}\right)\left(1 + \alpha_{\mathrm{B}}\dfrac{[\mathrm{B}]}{K_{\mathrm{B}}} + \alpha_{\mathrm{Q}}\dfrac{[\mathrm{Q}]}{K_{\mathrm{Q}}}\right) + \left(\dfrac{[\mathrm{B}]}{K_{\mathrm{B}}} + \dfrac{[\mathrm{Q}]}{K_{\mathrm{Q}}}\right)\left(1 + \alpha_{\mathrm{A}}\dfrac{[\mathrm{A}]}{K_{\mathrm{A}}} + \alpha_{\mathrm{P}}\dfrac{[\mathrm{P}]}{K_{\mathrm{P}}}\right)\right\}} \tag{7.46}$$

7.3.5 Enzyme Inhibition

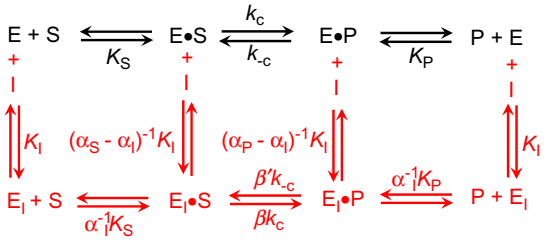
Certain compounds may bind to enzymes and reduce their activity. These compounds are known to be enzyme inhibitors. Enzyme inhibitions may be irreversible or reversible. Irreversible inhibitors such as heavy metals (lead, cadmium, mercury, and others) form a stable complex with the enzyme and reduce 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.

As we are aware, allosteric enzymes can accommodate multiple substrates, and binding of one substrate can change the conformal structure of the enzyme. The change of enzyme structure can affect the catalytic behavior of the enzyme. Therefore, there are two scenarios for

enzyme inhibition: (1) Allosteric inhibition, and (2) Competitive binding on the single site. We will discuss these two cases in the following.

7.3.5.1 *Allosteric Inhibition*

As an effector can regulate the enzyme, let us use our simplest case of a single substrate reaction,



(7.47)

Eq. (7.47) illustrates the mechanism of a double-sited (allosteric) enzyme. Enzyme E functions normally when no effector (identified as I) is present. When an effector is bound with the enzyme, the enzyme changes shape and the new enzyme is now shown as E_I (with the effector bound on). The affinity of the enzyme bound with an effector I (E_I) is different from the virgin enzyme (E). The rate of the enzymatic catalytic reaction with the allosteric effector can be derived based on the fast-binding equilibrium

$$K_S = \frac{[E][S]}{[ES]} \tag{7.48a}$$

$$K_P = \frac{[E][P]}{[EP]} \tag{7.48b}$$

$$K_I = \frac{[E][I]}{[E_I]} \tag{7.48c}$$

$$[E_IS] = \frac{[E_I][S]}{\alpha_I^{-1}K_S} + \frac{[ES][I]}{(\alpha_S - \alpha_I)^{-1}K_I} = \alpha_S \frac{[E][S][I]}{K_S K_I} \tag{7.48d}$$

$$[E_IP] = \frac{[E_I][P]}{\alpha_I^{-1}K_P} + \frac{[EP][I]}{(\alpha_P - \alpha_I)^{-1}K_I} = \alpha_P \frac{[E][P][I]}{K_P K_I} \tag{7.48e}$$

Enzyme balance

$$E = [E] + [ES] + [EP] + [E_I] + [E_IS] + [E_IP] \tag{7.49}$$

and catalytic rate determined by the rate-limiting steps

$$r_P = k_c[ES] - k_{-c}[EP] + (\beta k_c[E_IS] - \beta' k_{-c}[E_IP]) \tag{7.50}$$

where K_I is the (reciprocal) binding affinity of the effector on the enzyme (at a site not being used by substrate S), [E] is the concentration of the free enzyme, [E_I] is the concentration of the enzyme bound with the effector I, [E_IS] is the concentration of the enzyme that is bound with

both I and S, [ES] is the concentration of the enzyme that is bound with the substrate S only. Eq. (7.50) can be reduced to

$$r_P = \frac{k_c E}{K_S}\left(1+\alpha_S\beta\frac{[I]}{K_I}\right)\frac{[S]-\dfrac{[P]}{K_C}}{1+\dfrac{[S]}{K_S}+\dfrac{[P]}{K_P}+\dfrac{[I]}{K_I}\left(1+\alpha_S\dfrac{[S]}{K_S}+\alpha_P\dfrac{[P]}{K_P}\right)} \tag{7.51}$$

where K_C is the equilibrium constant and is given by

$$K_C = \frac{k_c K_P}{k_{-c} K_S} = \frac{\beta\alpha_S k_c K_P}{\beta'\alpha_P k_{-c} K_S} \tag{7.52}$$

Clearly, when no effector is present or $[I]=0$, Eq. (7.51) is reduced to Eq. (7.22).

Eq. (7.51) can also be written as

$$r_P = \frac{K_I+\alpha_S\beta[I]}{K_I+\alpha_S[I]}k_c E\frac{[S]-\dfrac{[P]}{K_C}}{K_S\dfrac{K_I+[I]}{K_I+\alpha_S[I]}+[S]+\dfrac{K_S}{K_P}\dfrac{K_I+\alpha_P[I]}{K_I+\alpha_S[I]}[P]} \tag{7.53}$$

When the product concentration is negligible, Eq. (7.53) is reduced to (initial rate),

$$r_{P_0} = \frac{K_I+\alpha_S\beta[I]}{K_I+\alpha_S[I]}k_c E\frac{[S]}{K_S\dfrac{K_I+[I]}{K_I+\alpha_S[I]}+[S]} \tag{7.54}$$

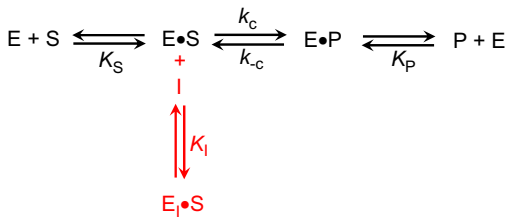
Fig. 7.10 shows a comparison of the allosteric enzyme kinetics and the Michaelis-Menten kinetics. As is noticeable from Eq. (7.54), the reaction rate of the allosteric enzyme still shows saturation kinetics (the same as the Michaelis-Menten kinetics), and the saturation rate is different from that of Michaelis-Menten kinetics due to the change of reactivity for the effector-bound site (β). The half maximum rate may not necessarily be reached at $[S]=K_S$ for allosteric enzyme.

When $\beta=0$ and $\alpha_S=1$, Eq. (7.54) is reduced to

$$r_{P_0} = \frac{r_{max}K_I}{K_I+[I]}\frac{[S]}{K_S+[S]} \tag{7.55}$$

which is known as noncompetitive inhibition. Only the maximum rate is modified due to the noncompetitive inhibition.

Before we leave this section, let us look at a rare case, where the effector can only bind if the substrate is bound. That is,



(7.56)

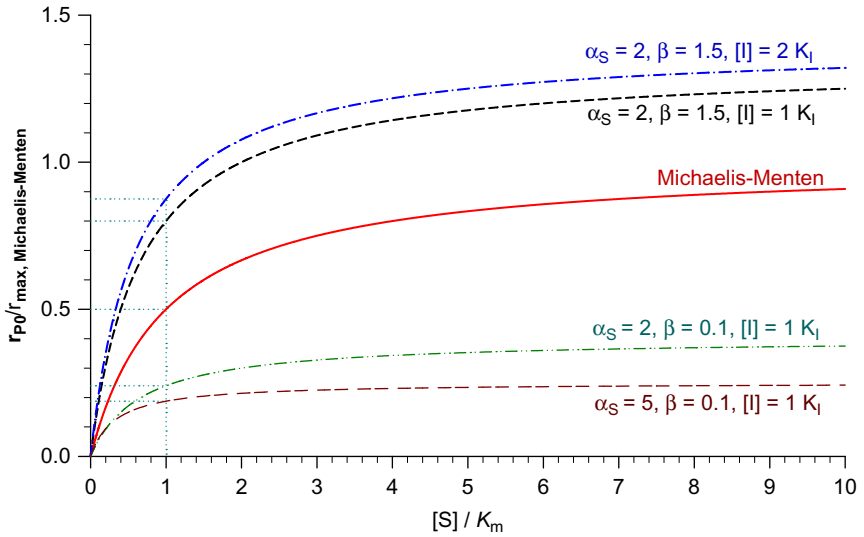


FIG. 7.10 Comparison of allosteric enzyme kinetics and Michaelis-Menten kinetics; all lines are plotted using Eq. (7.54); Michaelis-Menten kinetics is equivalent to [I] = 0.

which is known in the literature as the uncompetitive inhibition. In this case, the enzyme balance is given by

$$\mathrm{E} = [\mathrm{E}] + [\mathrm{ES}] + [\mathrm{EP}] + [\mathrm{E_IS}] \tag{7.57}$$

and the rate of enzymatic conversion with the uncompetitive inhibition

$$r_\mathrm{P} = \frac{k_\mathrm{c}\mathrm{E}\left([\mathrm{S}] - \dfrac{[\mathrm{P}]}{K_\mathrm{C}}\right)}{K_\mathrm{S} + [\mathrm{S}]\left(1 + \dfrac{[\mathrm{I}]}{K_\mathrm{I}}\right) + [\mathrm{P}]\dfrac{K_\mathrm{S}}{K_\mathrm{P}}} \tag{7.58}$$

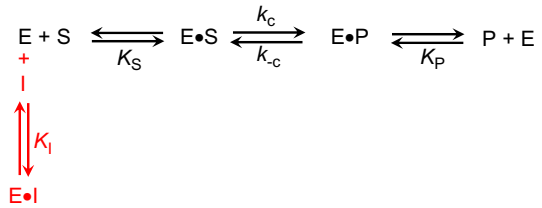
or

$$r_{\mathrm{P}_0} = \frac{k_\mathrm{c}\mathrm{E}\left(1 + \dfrac{[\mathrm{I}]}{K_\mathrm{I}}\right)^{-1}[\mathrm{S}]}{K_\mathrm{S}\left(1 + \frac{[\mathrm{I}]}{K_\mathrm{I}}\right)^{-1} + [\mathrm{S}]} \tag{7.59}$$

when the product concentration is negligible. Therefore, both the maximum rate and the saturation coefficient are modified due to the uncompetitive inhibition.

7.3.5.2 *Competitive Inhibition*

When the inhibitor is competing with the substrate for the active sites,



(7.60)

This case can also result from allosteric inhibition—when the inhibitor is bound, no other molecule can bind.

The enzyme balance is given by

$$E = [E] + [ES] + [EP] + [EI] \tag{7.61}$$

and the rate of enzymatic conversion with the uncompetitive inhibition:

$$r_P = \frac{k_c E\left([S] - \dfrac{[P]}{K_C}\right)}{K_S + [S] + [P]\dfrac{K_S}{K_P} + [I]\dfrac{K_S}{K_I}} \tag{7.62}$$

or,

$$r_{P_0} = \frac{k_c E[S]}{K_S + \dfrac{K_S}{K_I}[I] + [S]} \tag{7.63}$$

when the product concentration is negligible. Therefore, only the saturation coefficient is modified due to the competitive inhibition.

Fig. 7.11 shows a comparison of the initial rates for the three inhibition kinetics with the uninhibited Michaelis-Menten rate. One can observe that competitive inhibition changes

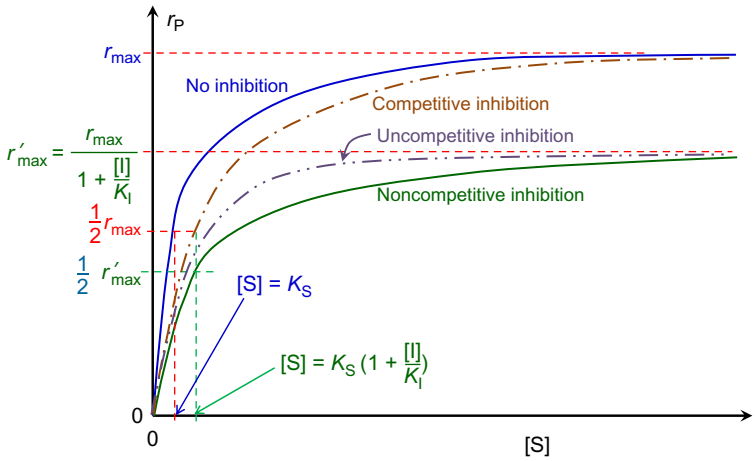


FIG. 7.11 Comparison of Michaelis-Menten, competitive, uncompetitive and noncompetitive inhibition kinetics.

the saturation coefficient or effective binding affinity K_S, while noncompetitive inhibition changes the saturation rate. The uncompetitive inhibition lowers both the saturation coefficient and the maximum rate. In all cases, the catalytic rate is lower as compared with the Michaelis-Menten rate.

7.3.6 Substrate Inhibition

When substrate concentration is high, the substrate-enzyme interaction can be more complicated. In this case, substrates are competing for the active site or enzyme. Depending on the molecular structure of the enzyme, the catalytic rate can be influenced by the substrate concentration in different ways. Enzymes are specific, and the active site can accommodate only one specific substrate molecule. If there are multiple substrate molecules approaching the enzyme at the active site at the same time, there could be different outcomes. In this section, we deal with the case when two substrate molecules approach the active site on an enzyme molecule simultaneously, and both the substrate molecules and enzyme get entangled, creating an inactive complex. This can be the case when the active site is sterically accessible to one molecule, but barely enough to accommodate two substrate molecules located nearby. The approaching of two substrate molecules in the same time prevents either from accessing the active site, but "jammed at the entrance." Stoichiometrically,

$$\mathrm{E} + 2\mathrm{S} \underset{}{\overset{K_m K_{S_2}}{\rightleftharpoons}} \mathrm{S_E S} \tag{7.64}$$

with the catalytic reaction unchanged, Eq. (7.3). The fast equilibrium steps give rise to

$$K_m = \frac{[\mathrm{E}][\mathrm{S}]}{[\mathrm{ES}]} \tag{7.65}$$

$$K_m K_{S_2} = \frac{[\mathrm{E}][\mathrm{S}]^2}{[\mathrm{SES}]} \tag{7.66}$$

$$\mathrm{E} = [\mathrm{E}] + [\mathrm{ES}] + [\mathrm{SES}] \tag{7.67}$$

and the catalytic rate

$$r_P = k_c[\mathrm{ES}] \tag{7.68}$$

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

$$r_P = \frac{r_{max}[\mathrm{S}]}{K_m + [\mathrm{S}] + \dfrac{[\mathrm{S}]^2}{K_{S_2}}} \tag{7.69}$$

At low substrate concentrations, $[\mathrm{S}]^2 \ll K_{S_2}$ and inhibition is negligible. The rate in this case is reduced to

$$r_P = \frac{r_{max}[\mathrm{S}]}{K_m + [\mathrm{S}]} \tag{7.10}$$

At high substrate concentrations, $[S] \gg K_M$ and inhibition is dominant. The rate in this case is

$$r_P = \frac{r_{max}}{1 + \frac{[S]}{K_{S_2}}} \tag{7.70}$$

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

$$S_{max} = \sqrt{K_m K_{S_2}} \tag{7.71}$$

Fig. 7.12 shows a comparison of substrate inhibition and normal Michaelis-Menten kinetics. One can observe that the catalytic decreases with increasing substrate concentration, instead of reaching the maximum rate, at high substrate concentrations.

Fig. 7.13 shows the effect of glucose concentration on the initial glucose phosphorylation rate with hexokinase III. One can observe that the catalytic rate decreases with an increasing substrate concentration at high glucose concentrations. The catalytic rate increases sharply with glucose concentration at low concentrations and it reaches maximum at about 1 mM. Increasing glucose concentration beyond 1 mM results in a decrease in the catalytic rate.

7.3.7 Substrate Push

In Section 7.3.6, we examined one scenario of high substrate concentration. If the enzyme has two active sites nearby, only one of them can be active due to steric interactions. In other cases, the reaction can be enhanced when another molecule is approaching an already occupied active site. The approaching of one substrate molecule toward a bound enzyme may result in the availability of the active site (by releasing the product formed) to the approaching substrate molecule. Stoichiometrically,

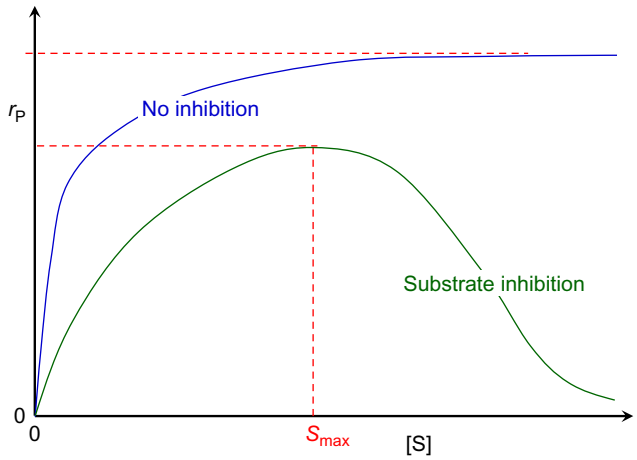


FIG. 7.12 Comparison of substrate inhibited and uninhibited enzymatic reactions.

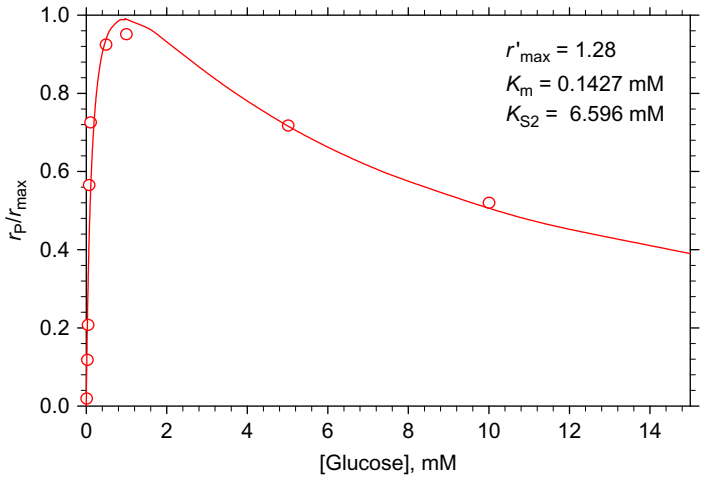


FIG. 7.13 Effect of substrate concentration on the initial rate of Hexokinase III. The line is based on Eq. (7.69) with parameters shown in the graph. The symbols represent data from Palma F et al. Mol. Cell. Biochem. 1996; 155:23–29.

$$S + ES \xrightarrow{\beta k_c} P + ES \tag{7.72}$$

with the rest of the catalytic reaction unchanged from Eq. (7.3). The catalytic rate is given by

$$r_P = k_c[ES] + \beta k_c[ES][S] \tag{7.73}$$

which can be yielded to

$$r_P = r_{max}[S]\frac{1+\beta[S]}{K_m + [S]} \tag{7.74}$$

At low substrate concentrations, $\beta[S] \ll 1$, the effect of concentration push is negligible. The rate in this case is reduced to

$$r_P = \frac{r_{max}[S]}{K_m + [S]} \tag{7.10}$$

At high substrate concentrations, $[S] \gg K_M$

$$r_P = r_{max}(1 + \beta[S]) \tag{7.75}$$

That is, there is no maximum reaction rate and it is atypical of enzymatic reaction. The catalytic rate continues to rise, although at a slow rate. Fig. 7.14 shows the effect of glucose on the glycolysis rate of hexokinase II. One can observe that, while a saturation rate is expected from Michaelis-Menten kinetics, the catalytic rate continues to rise at high glucose concentrations.

Example 7.2: Allosteric Inhibition

The conversion of glutamate to glutamine is catalyzed by glutamine synthetase as shown below

$$\text{Glutamate} + NH_3 \xrightarrow[\text{ATP}]{\text{Glutamine synthetase}} \text{Glutamine} \tag{E7.2.1}$$

Alanine is an inhibitor to the reaction. A set of rate data is shown in Table E7.2.1. Determine the kinetic parameters.

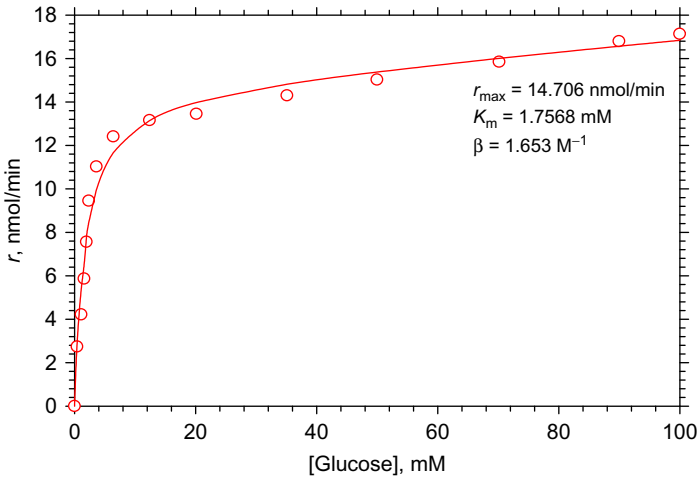


FIG. 7.14 Effect of glucose concentration on the initial rate of hexokinase II. The line shown is based on Eq. (7.74) with parameters shown. The symbols represent data taken from Ardehali H et al., J. Biol. Chem. 1996; 271: 1849–1852.

TABLE E7.2.1 Initial Reaction Rate for the Alanine Inhibition of Glutamate Synthetase Conversion of Glutamate.

Alanine [I], μM	0	1	2	3	4	5
Glutamate[S], μM			Initial rate (μM min^{-1})			
2	0.242	0.213	0.195	0.182	0.172	0.165
2.5	0.271	0.242	0.223	0.209	0.199	0.191
3.33	0.308	0.279	0.26	0.246	0.235	0.227
5	0.356	0.33	0.312	0.298	0.288	0.279

Data of Whiteley C.G., 1997. Biochem. Educ. 23(3), 144–146, 1997

Solution

Let us use the allosteric inhibition, Eq. (E7.2.2),

$$r_{P_0} = \frac{K_I + \alpha_S \beta [I]}{K_I + \alpha_S [I]} k_c E \frac{[S]}{K_S \frac{K_I + [I]}{K_I + \alpha_S [I]} + [S]} \tag{E7.2.2}$$

to treat the data. The data fitting is best conducted by minimizing the standard deviation (or least square) of the data from the kinetic model by changing the kinetic parameters. This can be done in Microsoft Excel, as shown in Table E7.2.1. The minimization can be carried out with the Solver® in Excel. The final parameters are determined as $r_{max} = 0.5193$ μM min^{-1}, $K_S = 2.2913$ μM, $K_I = 2.4847$ μM, $\alpha_S = 0.3004$ and $\beta = 1.0019$.

The quality of fit is shown in Figs. E7.2.1 and E7.2.2. One can observe that the fit is perfect. This example shows that minimization of "errors" can be applied to estimate the kinetic parameters despite the complex form of the kinetic equation. Fitting of this type of kinetic equation,

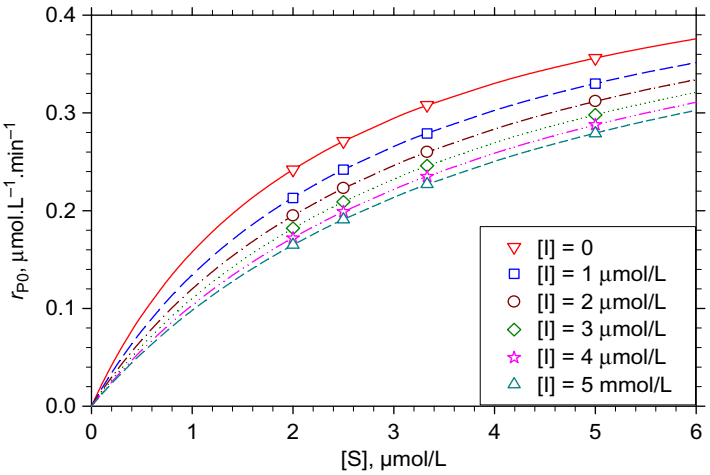


FIG. E7.2.1 The quality of allosteric inhibition kinetic model fit to data fit as shown in Table E7.2.2 for the enzymatic conversion rate vs the substrate concentration; the symbols are experimental data, while the curves are model fit.

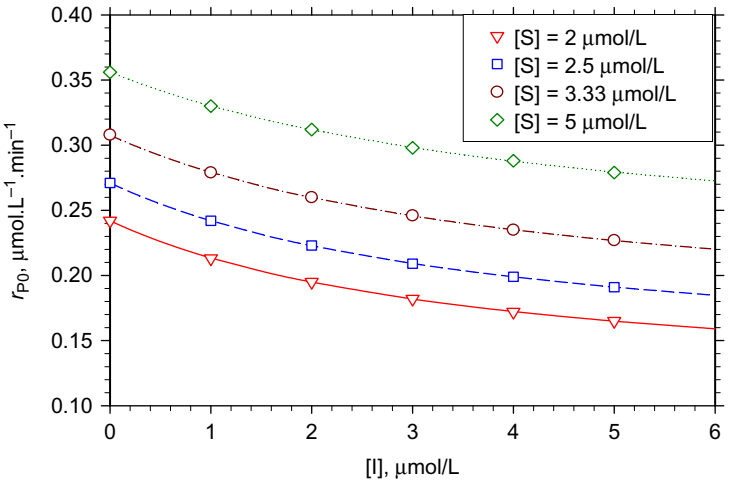


FIG. E7.2.2 The quality of allosteric kinetic model fit to data as shown in Table E7.2.2 for the enzymatic conversion rate vs the inhibitor concentration; the symbols are experimental data, while the curves are model fit.

although simple shown here, can be tedious and requires elaborate manipulation to accomplish in the age prior to when computers were easily accessible, which is evident from the literature.

7.4 pH EFFECTS

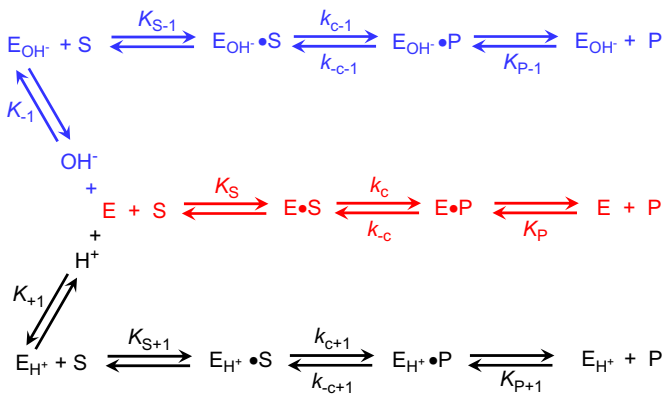
Certain enzymes have ionic groups on their active sites, and these ionic groups must be in a suitable form (acid or base) to function. Variations in the pH of the medium result in changes in the ionic form of the active site and changes in the activity of the enzyme and hence the reaction rate. Changes in pH may also alter the three-dimensional shape of the enzyme.

TABLE E7.2.2 Allosteric Inhibition Kinetic Model-Fitting Results of the Data in Table E7.2.1

Alanine [I], μM	0	1	2	3	4	5	Parameters	
Glutamate[S], μM	Initial rate (μM min^{-1})						r_{max}, μM min^{-1}	0.5193
2	0.242	0.213	0.195	0.182	0.172	0.165	K_S, μM	2.2913
2.5	0.271	0.242	0.223	0.209	0.199	0.191	K_I, μM	2.4847
3.33	0.308	0.279	0.26	0.246	0.235	0.227	α_S	0.3004
5	0.356	0.33	0.312	0.298	0.288	0.279	β	1.0019
Model, Eq. (E7.2.2)								Σ $error^2$
2	0.2420	0.2135	0.1949	0.1819	0.1723	0.1649		3.427×10^{-7}
2.5	0.2710	0.2420	0.2228	0.2091	0.1989	0.1910		7.443×10^{-8}
3.33	0.3077	0.2791	0.2598	0.2457	0.2351	0.2268		3.370×10^{-7}
5	0.3561	0.3302	0.3118	0.2982	0.2877	0.2794		3.328×10^{-7}
							σ	0.00021739

For these reasons, enzymes are only active over a certain range of pH. The pH of the medium may affect the maximum reaction rate r_{max}, saturation coefficient 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.



(7.76)

With the FES on the enzyme complexes

$$K_S = \frac{[E][S]}{[ES]} \tag{7.77a}$$

$$K_{\mathrm{P}} = \frac{[\mathrm{E}][\mathrm{P}]}{[\mathrm{EP}]} \tag{7.77b}$$

$$K_{+1} = \frac{[\mathrm{E}][\mathrm{H}^+]}{[\mathrm{E}_{\mathrm{H}^+}]} \tag{7.77c}$$

$$K_{\mathrm{S}+1} = \frac{[\mathrm{E}_{\mathrm{H}^+}][\mathrm{S}]}{[\mathrm{E}_{\mathrm{H}^+}\mathrm{S}]} \tag{7.77d}$$

$$K_{\mathrm{P}} = \frac{[\mathrm{E}][\mathrm{P}]}{[\mathrm{EP}]} \tag{7.77e}$$

$$K_{-1} = \frac{[\mathrm{E}][\mathrm{OH}^-]}{[\mathrm{E}_{\mathrm{OH}^-}]} \tag{7.77f}$$

$$K_{\mathrm{S}-1} = \frac{[\mathrm{E}_{\mathrm{OH}^-}][\mathrm{S}]}{[\mathrm{E}_{\mathrm{OH}^-}\mathrm{S}]} \tag{7.77g}$$

$$K_{\mathrm{P}} = \frac{[\mathrm{E}_{\mathrm{OH}^-}][\mathrm{P}]}{[\mathrm{E}_{\mathrm{OH}^-}\mathrm{P}]} \tag{7.77h}$$

and the overall reaction rate,

$$r_{\mathrm{P}} = k_{\mathrm{c}}[\mathrm{ES}] - k_{-\mathrm{c}}[\mathrm{EP}] + k_{\mathrm{c}+1}[\mathrm{E}_{\mathrm{H}^+}\mathrm{S}] - k_{-\mathrm{c}+1}[\mathrm{E}_{\mathrm{H}^+}\mathrm{P}] + k_{\mathrm{c}-1}[\mathrm{E}_{\mathrm{OH}^-}\mathrm{S}] - k_{-\mathrm{c}-1}[\mathrm{E}_{\mathrm{OH}^-}\mathrm{P}] \tag{7.78}$$

The enzyme balance

$$\mathrm{E} = [\mathrm{E}] + [\mathrm{E}_{\mathrm{H}^+}] + [\mathrm{E}_{\mathrm{OH}^-}] + [\mathrm{E}_{\mathrm{H}^+}\mathrm{S}] + [\mathrm{E}_{\mathrm{H}^+}\mathrm{P}] + [\mathrm{E}_{\mathrm{OH}^-}\mathrm{S}] + [\mathrm{E}_{\mathrm{OH}^-}\mathrm{P}] \tag{7.89}$$

We can derive the following rate expression

$$r_{\mathrm{P}} = \frac{\dfrac{k_{\mathrm{c}} + \dfrac{k_{\mathrm{c}+1}K_{\mathrm{S}}[\mathrm{H}^+]}{K_{\mathrm{S}+1}K_{+1}} + \dfrac{k_{\mathrm{c}-1}K_{\mathrm{S}}[\mathrm{OH}^-]}{K_{\mathrm{S}-1}K_{-1}}}{1 + \dfrac{K_{\mathrm{S}}[\mathrm{H}^+]}{K_{\mathrm{S}+1}K_{+1}} + \dfrac{K_{\mathrm{S}}[\mathrm{OH}^-]}{K_{\mathrm{S}-1}K_{-1}}} \mathrm{E}\left([\mathrm{S}] - \dfrac{[\mathrm{P}]}{K_{\mathrm{C}}}\right)}{K_{\mathrm{S}} \dfrac{1 + \dfrac{[\mathrm{H}^+]}{K_{+1}} + \dfrac{[\mathrm{OH}^-]}{K_{-1}}}{1 + \dfrac{K_{\mathrm{S}}[\mathrm{H}^+]}{K_{\mathrm{S}+1}K_{+1}} + \dfrac{K_{\mathrm{S}}[\mathrm{OH}^-]}{K_{\mathrm{S}-1}K_{-1}}} + [\mathrm{S}] + [\mathrm{P}]\dfrac{K_{\mathrm{S}}}{K_{\mathrm{P}}} \dfrac{1 + \dfrac{K_{\mathrm{P}}[\mathrm{H}^+]}{K_{\mathrm{P}+1}K_{+1}} + \dfrac{K_{\mathrm{P}}[\mathrm{OH}^-]}{K_{\mathrm{P}-1}K_{-1}}}{1 + \dfrac{K_{\mathrm{S}}[\mathrm{H}^+]}{K_{\mathrm{S}+1}K_{+1}} + \dfrac{K_{\mathrm{S}}[\mathrm{OH}^-]}{K_{\mathrm{S}-1}K_{-1}}}} \tag{7.80}$$

where

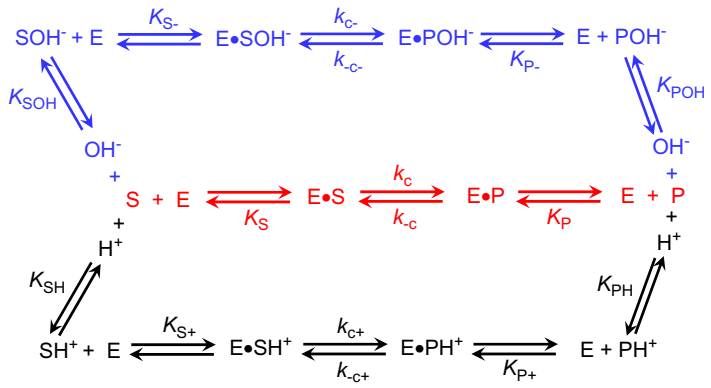
$$K_C = \frac{k_c K_P}{k_{-c} K_S} = \frac{k_{c+1} K_{P+1}}{k_{-c+1} K_{S+1}} = \frac{k_{c-1} K_{P-1}}{k_{-c-1} K_{S-1}} \tag{7.81}$$

is the equilibrium constant for the overall reaction. Note that

$$[OH^-] = \frac{K_w}{[H^+]} \tag{7.82}$$

where K_W is the ionic dissociation equilibrium constant of water. At 25°C, $K_W = 10^{-14}$. As a result of this behavior, the pH optimum of the enzyme is between pK_{+1} and $14 - pK_{-1}$.

For the case of ionizing substrate, the resulting rate expression is similar to the case of ionizing enzymes. Consider the scheme



(7.83)

The rate expression can be developed

$$r_P = \frac{\dfrac{k_c + k_{c+}\dfrac{K_S[H^+]}{K_{S+}K_{SH}} + k_{c-}\dfrac{K_S[OH^-]}{K_{S-}K_{SOH}}}{1 + \dfrac{K_S[H^+]}{K_{S+}K_{SH}} + \dfrac{K_S[OH^-]}{K_{S-}K_{SOH}}} E\left([S] - \dfrac{[P]}{K_C}\right)}{\dfrac{K_S}{1 + \dfrac{K_S[H^+]}{K_{S+}K_{SH}} + \dfrac{K_S[OH^-]}{K_{S-}K_{SOH}}} + [S] + \dfrac{K_S}{K_P}\dfrac{1 + \dfrac{K_P[H^+]}{K_{P+}K_{PH}} + \dfrac{K_P[OH^-]}{K_{P-}K_{POH}}}{1 + \dfrac{K_S[H^+]}{K_{S+}K_{SH}} + \dfrac{K_S[OH^-]}{K_{S-}K_{SOH}}}[P]} \tag{7.84}$$

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. Fig. 7.15 depicts the variation of enzymatic activity with pH for two different enzymes.

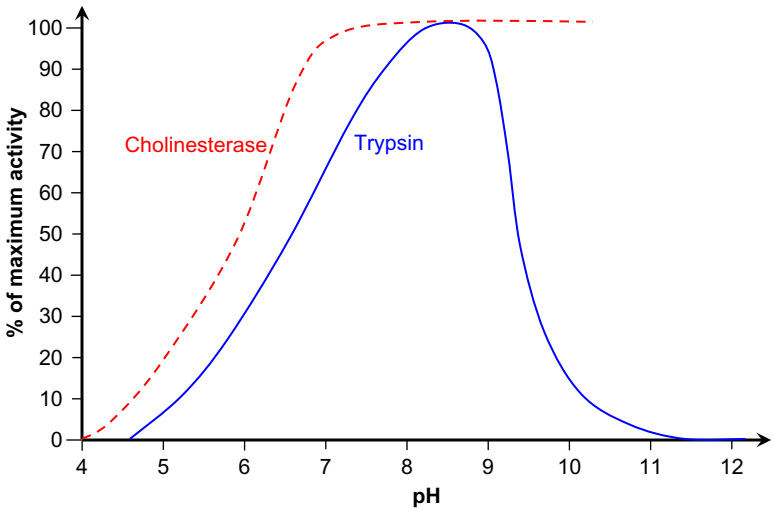


FIG. 7.15 The pH-activity profiles of two enzymes.

7.5 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. Fig. 7.16 depicts the variation of reaction rate with temperature and the presence of an optimal temperature. The ascending part of Fig. 7.16 is known as *temperature activation*. The rate varies according to the Arrhenius equation in this region

$$r_{max} = k_c[E] \tag{7.85}$$

$$k_c = k_{c_0} \exp\left(-\frac{E_a}{RT}\right) \tag{7.86}$$

where E_a is the activation energy (kJ mol^{-1}) and E is the active enzyme concentration.

The descending part of Fig. 7.16 is known as *temperature inactivation* or *thermal denaturation*. The kinetics of thermal denaturation can be expressed as

$$-\frac{dE}{dt} = k_d E \tag{7.87}$$

$$E = E_0 e^{-k_d t} \tag{7.88}$$

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.

$$k_d = k_{d_0} \exp\left(-\frac{E_d}{RT}\right) \tag{7.89}$$

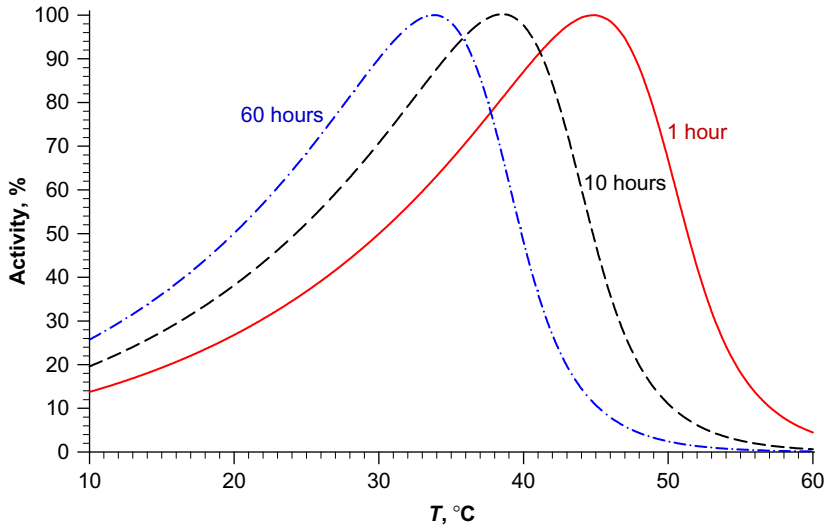


FIG. 7.16 Effect of temperature on the activity of an enzyme. Here we have assumed a value of $E_a = 46$ kJ mol^{-1}, $E_d = 301$ kJ mol^{-1} and $k_{d_0} = 1.0 \times 10^{49}$h^{-1}. The increase in maximum rate is due to the increase in the activity via Arrhenius law, while the descending of the curve is due to the dominance of the thermal denaturation. The enzyme activity or relative maximum rate is averaged for a total exposure of 1-h, 10-h, and 60-h to the temperature, which is shown in Eq. (7.91).

where E_d is the deactivation energy (kJ mol^{-1}). Consequently,

$$r_{max} = k_{c_0} E_0 \exp\left(-\frac{E_a}{RT}\right) e^{-k_d t} \tag{7.90}$$

The activity or average maximum rate for a total exposure time of t is thus given by

$$\bar{r}_{max} = \frac{\int_0^t r_{max} dt}{t} = k_{c_0} [E]_0 \exp\left(-\frac{E_a}{RT}\right) \frac{1 - e^{-k_d t}}{k_d t} \tag{7.91}$$

The activation energies of enzyme-catalyzed reactions are within the 15–85 kJ mol^{-1} range (mostly about 46 kJ mol^{-1}). Deactivation energies E_d vary between 170 and 550 kJ mol^{-1} (mostly about 300 kJ mol^{-1}). That is, enzyme denaturation by temperature is much faster than enzyme activation. A rise in temperature from 30° to 40°C results in a 1.7-fold increase in enzyme activity, but a 45-fold increase in enzyme denaturation. Variations in temperature may affect both r_{max} and K_m values of enzymes. Fig. 7.16 is plotted using Eq. (7.91).

7.6 INSOLUBLE SUBSTRATES

Enzymes are often used to attack large, insoluble substrates such as woodchips (in biopulping for paper manufacture) or cellulosic residues from agriculture (eg, 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 to that of the typical situation with soluble substrates, where access to the enzyme's active site limits reaction. For example, considering the equilibrium adsorption of enzyme (E) onto substrate (S)

$$\mathrm{E} + \mathrm{S} \underset{k_{\mathrm{des}}}{\overset{k_{\mathrm{ads}}}{\rightleftharpoons}} \mathrm{ES} \tag{7.92}$$

If the total concentration of adsorption sites on the substrate surface is S_0, expressed per liquid volume, then we can write

$$S_0 = [\mathrm{S}] + [\mathrm{ES}] \tag{7.93}$$

and the equilibrium rate expression

$$0 = r_1 = k_{\mathrm{ads}}[\mathrm{E}][\mathrm{S}] - k_{\mathrm{des}}[\mathrm{ES}] \tag{7.94}$$

The rate of reaction for the product formation is now assumed to be first order in [ES], with a constant rate constant k_2. Thus,

$$r_{\mathrm{P}} = \frac{r_{\max}\mathrm{E}}{K_{\mathrm{eq}} + \mathrm{E}} \tag{7.95}$$

where

$$r_{\max} = k_2 S_0 \tag{7.96}$$

and

$$K_{\mathrm{eq}} = \frac{k_{\mathrm{des}}}{k_{\mathrm{ads}}} \tag{7.97}$$

The previous equation assumes slow binding of enzyme, ie,

$$[\mathrm{E}] \approx \mathrm{E} \tag{7.98}$$

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.

7.7 IMMOBILIZED ENZYME SYSTEMS

The restriction of enzyme mobility in a fixed space is known as *enzyme immobilization*. In many applications, 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. Immobilized enzymes are typically macroscopic catalysts that are retained in the reactor; therefore, continuous replacement of the enzyme is not necessary, and separation of the enzyme from other components in the reaction mixture is simplified. Immobilized enzymes can be employed in a wide range of different reactor

configurations and, because high concentrations of catalyst can be obtained, correspondingly high volumetric productivities are possible. Higher reactor productivities lead to lower capital costs. Moreover, immobilized enzymes are often more stable than enzymes in solution. It is also important to note that the properties of the support, for example, its ionic charge, can in some cases be exploited to modify the behavior of the enzyme. 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.

7.7.1 Methods of Immobilization

Major methods of immobilization are summarized in Fig. 7.17. The two major categories are entrapment and surface immobilization.

7.7.1.1 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 in a polymer matrix, the enzyme solution is mixed with the 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.

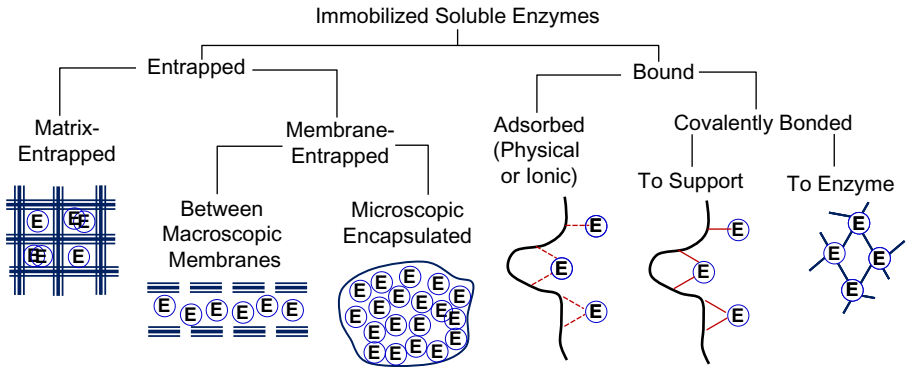


FIG. 7.17 Major immobilization methods.

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 micro drop.

Despite the aforementioned advantages, enzyme entrapment may have its inherent problems, such as enzyme leakage into solution, significant diffusion limitations, reduced enzyme activity and stability, and lack of control of microenvironmental conditions. Enzyme leakage can be overcome by reducing the molecular weight 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.

7.7.1.2 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.

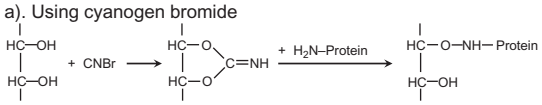
Covalent binding is the retention of enzyme on support surfaces by covalent bond formation. Enzyme molecules bind to support material via certain functional groups, such as amino, carboxyl, hydroxyl, and sulfhydryl groups. These functional groups must not be in the active site. One common trick is to 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 7.4.

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.

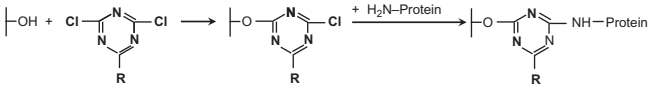
TABLE 7.4 Methods of covalent binding of enzymes to supports

Supports with –OH end group

a). Using cyanogen bromide

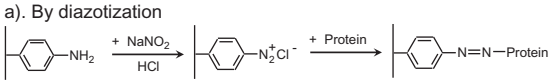


b). Using S-triazine derivatives

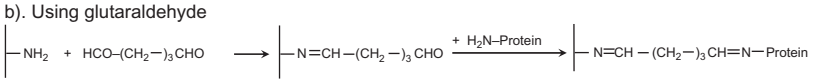


Supports with $-NH_2$ end group

a). By diazotization

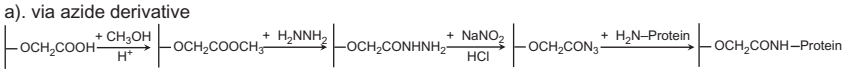


b). Using glutaraldehyde

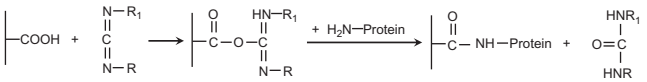


Supports with –COOH end group

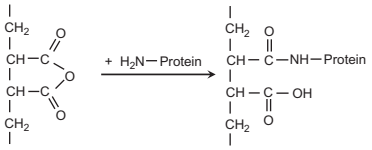
a). via azide derivative



b). Using a carbodiimide



Supports containing anhydrides



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.

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

TABLE 7.5 Effects of Immobilization Methods on the Retention of Enzymatic Activity of Aminoacylase

Support	Method	Observed Activity, Units	Immobilized Enzyme Activity, %
Polyacrylamide	Entrapment	526	52.6
Nylon	Encapsulation	360	36.0
DEAE-cellulose	Ionic binding	668	55.2
DEAE-Spephadex A-50	Ionic binding	680	56.2
CM-Sephadex C-50	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

(Source: Chibata I., Tosa T., Sato T., Mori T. and Matuo Y., 1972. Proc. of the 4th Int. Fermentation Symp.: Fermentation Technology Today. p. 383–389.)

the method used. Table 7.5 summarizes the retention of activity of arninoacylase immobilized by different methods.

7.7.2 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

$$\Delta\text{pH} = \text{pH}_\text{i} - \text{pH}_\text{e} = \frac{zN_\text{F}\Psi}{RT} \tag{7.99}$$

where pH_i and pH_e are internal and external pH values, respectively; z is the charge (valence) on the substrate; N_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. (7.99) 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.

7.8 ANALYSIS OF BIOPROCESS WITH ENZYMATIC REACTIONS

Enzymatic reactions are normally carried out in batch reactors. They can be carried out in flow reactors, especially when enzymes are immobilized. Analysis with enzymatic reactions can be performed in the same manner, as we have learned in Chapters 4 and 5.

Example 7.3: Batch Enzyme Reaction

Urease catalyzed urea decomposition reaction

$$NH_2CONH_2 \rightarrow 2NH_3 + CO_2$$

is to be carried out in a batch reactor. The Michaelis-Menten rate parameters determined from experimentation with an enzyme loading of 5 g L^{-1} are given by $r_{max} = 1.35$ mol L^{-1} s^{-1} and $K_m = 0.0265$ mol L^{-1}. Determine the time needed for 90% conversion of urea to ammonia and carbon dioxide of a solution containing 0.2 mol L^{-1} of urea in a 2 L reactor. The enzyme loading is 0.001 g L^{-1}.

Solution

The Michaelis-Menten rate law is given by

$$r = \frac{r_{max} S}{K_m + S} \tag{E7.3.1}$$

We know that $K_m = 0.0265$ mol L^{-1}. Since r_{max} is proportional to the enzyme loading, we have $r_{max} = 1.35 \times 0.001/5$ mol L^{-1} $s^{-1} = 2.7 \times 10^{-4}$ mol L^{-1} s^{-1}.

Mole balance of substrate (urea) in the reactor gives

$$0 - 0 + r_S V = \frac{dn_S}{dt} \tag{E7.3.2}$$

The reactor volume is constant (isothermal liquid/suspended phase reaction). Eq. (E7.3.2) is reduced to

$$-\frac{dS}{dt} = \frac{r_{max} S}{K_m + S} \tag{E7.3.3}$$

Separation of variable leads to

$$\left(\frac{K_m}{S} + 1\right) dS = -r_{max} dt \tag{E7.3.4}$$

Integrating Eq. (E7.3.4) from $t = 0$ and $S = S_0$, we obtain

$$K_m \ln \frac{S}{S_0} + S - S_0 = -r_{max} t \tag{E7.3.5}$$

The conversion of substrate is defined as

$$f_S = \frac{S_0 - S}{S_0} \tag{E7.3.6}$$

Substitute Eq. (E7.3.6) into Eq. (E7.3.5), and we obtain

$$t = \frac{S_0 f_S - K_m \ln(1 - f_S)}{r_{max}} \tag{E7.3.7}$$

Thus, the time required for 90% conversion is

$$t = \frac{S_0 f_S - K_m \ln(1 - f_S)}{r_{max}} = \frac{0.1 \times 0.9 - 0.0265 \ln(1 - 0.9)}{2.7 \times 10^{-4}} \text{ s} = 559.3, \text{ s} = 9.322 \text{ min}$$

Example 7.4: Batch Enzymatic Reactor Optimization
Consider the reaction

$$S \rightarrow P$$

catalyzed by enzyme E. Michaelis-Menten rate parameters are $r_{max} = 0.15 \text{ min}^{-1}$ and $K_m = 0.01 \text{ mol L}^{-1}$ when the enzyme loading 0.1 g L^{-1}. A continuous stirred-tank reactor (CSTR) has been selected to produce 100 mol of P per hour from a stream of substrate containing 1 mol S L^{-1}. S costs $\$0.1 \text{ mol}^{-1}$, P sells for $\$1 \text{ mol}^{-1}$, and the enzyme E cost $\$100 \text{ g}^{-1}$. The cost of operating the reactor is $\$0.1 \text{ L}^{-1} \text{ h}^{-1}$. Assume no value or cost of disposal of unreacted S (ie, separation or recovery cost to S is identical to the fresh S cost).

(a) What is the relationship between the rate of formation of P and enzyme loading?
(b) Perform a mole balance on the reactor to relate the concentration of exiting S with reactor size.
(c) What is the optimal concentration of S at the outlet of the reactor? What is the optimal enzyme loading?
(d) What is the cash flow per mole of product from the process?

Solution

A sketch of the reactor system is shown in Fig. E7.4. Substrate and enzyme are fed to the CSTR. The enzyme is let out unchanged, while some substrate is turned into product P.

(a) The maximum rate is proportional to the enzyme loading, that is,

$$r_{max} = kE_0 \tag{E7.4.1}$$

Thus the rate of formation of P is given by

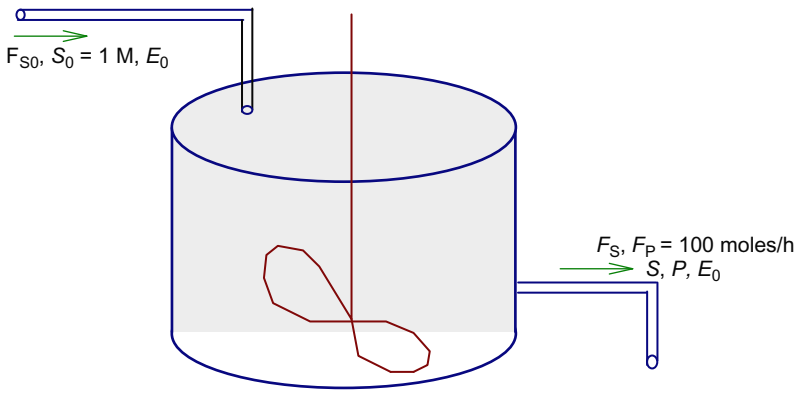


FIG. E7.4 Well mixed reaction vessel.

$$r_P = \frac{kE_0S}{K_m + S} \tag{E7.4.2}$$

$K_m = 0.01$ mol L^{-1} and $k = 0.15\ min^{-1}/(0.1\ g\ L^{-1}) = 1.5\ L\ min^{-1}\ g^{-1}$.

(b) It is understood that the CSTR is operating under constant temperature (isothermal) and steady-state conditions, ie, no accumulation of any sort of materials inside the reactor. Mole balance around the reactor for P yields

$$0 - F_P + r_P V = 0 \tag{E7.4.3}$$

Substituting Eq. (E7.4.2) into Eq. (E7.4.3), we obtain

$$V = \frac{F_P}{r_P} = F_P \frac{K_m + S}{kE_0S} \tag{E7.4.4}$$

(c) Based on the economic information at hand, we can estimate the gross profit for the reaction process as

$$GP\$ = \$_P F_P - \$_S F_{S_0} - \$_E F_{E_0} - \$_V V \tag{E7.4.5}$$

where $\$_P$, $\$_S$, $\$_E$ and $\$_V$ are the molar value of product P, molar cost of substrate S, unit cost of enzyme, and the unit operating cost of reactor based on the reactor volume and time; respectively.

Based on the stoichiometry, we have

$$Q = \frac{F_P}{P} = \frac{F_P}{S_0 - S} \tag{E7.4.6}$$

which leads to

$$F_{S_0} = \frac{F_P S_0}{S_0 - S} \tag{E7.4.7a}$$

$$F_{E_0} = \frac{F_P E_0}{S_0 - S} \tag{E7.4.7b}$$

Substituting Eqs. (E7.4.7a, E7.4.7b), (E7.4.4) into Eq. (E7.4.5), we obtain

$$GP\$ = \$_P F_P - \$_S \frac{F_P S_0}{S_0 - S} - \$_E \frac{F_P E_0}{S_0 - S} - \$_V \frac{F_P (K_m + S)}{kE_0S} \tag{E7.4.8}$$

To find the optimal reactor conditions, we maximize the gross profit by varying the effluent substrate concentration and enzyme loading. This task can easily be achieved by an optimizer like the Excel Solver. Substituting the known parameters into Eq. (E7.4.8), we obtain

$$GP\$ = 1 \times 100 - 0.1 \times \frac{100 \times 1}{1 - S} - 100 \frac{100E_0}{1 - S} - 0.1 \times \frac{100 \times (0.01 + S)}{1.5 \times 60E_0S} \$/h$$

which is reduced to

$$GP\$ = 100 - \frac{10}{1 - S} - \frac{10000E_0}{1 - S} - \frac{0.01 + S}{9E_0S} \$/h \tag{E7.4.9}$$

Maximizing the gross profit GP$, we obtain: $S = 0.075969$ mol L^{-1}, $E_0 = 0.0024102$ g L^{-1}, and GP$ = \$10.9256\ h^{-1}$

Therefore, we have found the optimal enzyme loading and the effluent substrate concentration.

(d) The cash flow based on Eq. (E7.4.8),

$$\frac{GP\$}{F_P} = \$_P - \$_S\frac{S_0}{S_0 - S} - \$_E\frac{E_0}{S_0 - S} - \$_V\frac{(K_m + S)}{kE_0S} \tag{E7.4.14}$$

Or since we have a GP$ value already,

$$\frac{GP\$}{F_P} = \frac{10.9256}{100}\ \$/\text{mol-P} = \$0.109256/\text{mol-P}$$

This concludes the example. One must be careful of the units whenever numbers are inserted into the equation.

Example 7.4 shows that enzyme costs can be significant. Therefore, separating and reusing the enzyme is very important for bioprocess systems.

Example 7.5: Enzymatic Reaction in a CSTR

It has been observed that substrate inhibition occurs in the following enzymatic reaction

$$E + S \rightarrow P + E$$

(a) *Show that the rate law for substrate inhibition is consistent with the data* shown in the Fig. E7.5.1 for r_P versus the substrate concentration of S.

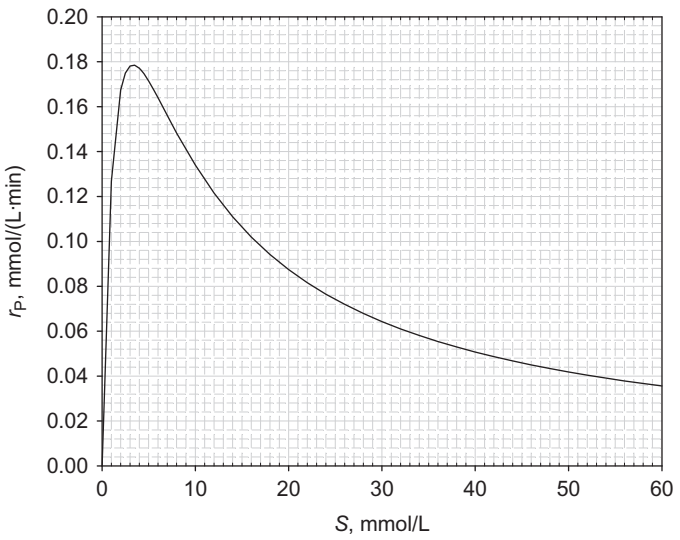


FIG. E7.5.1 Enzymatic reaction rate as a function of substrate concentration.

(b) If this reaction is carried out in a fluidized CSTR that has a working volume of 5000 L, to which the volumetric flow rate is 15 L min^{-1} and the feed concentration of the substrate is 60 mmol L^{-1}, determine the possible conversion of substrate to the desired product. Since the enzyme is immobilized on the solid particle, assume that there is no loss of the enzyme to the effluent.

(c) What would be the effluent substrate concentration if the enzyme loading is 25% lower?

Solution

For substrate inhibition, the mechanism is given by

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E\bullet S \tag{E7.5.1}$$

$$E\bullet S \xrightarrow{k_2} P + E \tag{E7.5.2}$$

$$E\bullet S + S \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} S\bullet E\bullet S \tag{E7.5.3}$$

The inhibition step, reaction Eq. (E7.5.3), renders the enzyme ineffective and thus slows down the overall reaction rate of utilizing the substrate. The rate of formation for P is given by

$$r_P = k_2[E\bullet S] \tag{E7.5.4}$$

where the intermediate concentration can be obtained through PSSH for the intermediates

$$0 = r_{E\bullet S} = k_1[E][S] - k_{-1}[E\bullet S] - k_2[E\bullet S] - k_3[E\bullet S] + k_{-3}[S\bullet E\bullet S] \tag{E7.5.5}$$

$$0 = r_{S\bullet E\bullet S} = k_3[E\bullet S][S] - k_{-3}[S\bullet E\bullet S] \tag{E7.5.6}$$

Eqs. (E7.5.5), (E7.5.6) lead to

$$[E\bullet S] = \frac{k_1[S]}{k_{-1} + k_2}[E] \tag{E7.5.7}$$

$$[S\bullet E\bullet S] = \frac{k_3}{k_{-3}}[E\bullet S][S] = \frac{k_3}{k_{-3}}\frac{k_1[S]}{k_{-1} + k_2}[E][S] \tag{E7.5.8}$$

The total amount of enzyme in the reactor is the sum of free enzyme [E] and those associated with the substrate, [E•S] and [S•E•S]. Thus,

$$\begin{aligned}[E]_0 &= [E] + [S\bullet E\bullet S] + [E\bullet S] \\ &= [E] + \frac{k_3}{k_{-3}}\frac{k_1[S]}{k_{-1} + k_2}[E][S] + \frac{k_1[S]}{k_{-1} + k_2}[E]\end{aligned} \tag{E7.5.9}$$

which leads to

$$[\mathrm{E}] = \frac{[\mathrm{E}]_0}{1 + \dfrac{k_3}{k_{-3}}\dfrac{k_1[\mathrm{S}]}{k_{-1}+k_2}[\mathrm{S}] + \dfrac{k_1[\mathrm{S}]}{k_{-1}+k_2}} \tag{E7.5.10}$$

and

$$[\mathrm{E}\bullet\mathrm{S}] = \frac{\dfrac{k_1[\mathrm{S}]}{k_{-1}+k_2}[\mathrm{E}]_0}{1 + \dfrac{k_3}{k_{-3}}\dfrac{k_1[\mathrm{S}]}{k_{-1}+k_2}[\mathrm{S}] + \dfrac{k_1[\mathrm{S}]}{k_{-1}+k_2}} \tag{E7.5.11}$$

Substitute Eqs. (E7.5.10), (E7.5.11) into Eq. (E7.5.4), and we obtain

$$r_\mathrm{P} = \frac{k_2[\mathrm{E}]_0[\mathrm{S}]}{\dfrac{k_{-1}+k_2}{k_1} + [\mathrm{S}] + \dfrac{k_3}{k_{-3}}[\mathrm{S}]^2} \tag{E7.5.12}$$

(a) Eq. (E7.5.12) leads to

(1) When $[\mathrm{S}]=0$, $r_\mathrm{P}=0$
(2) When [S] is low, r_P increases with S
(3) When [S] is very high, r_P decreases with increasing [S] because of the $[\mathrm{S}]^2$ term in the denominator
(4) There is only one maximum (peak) value for r_P as only [S] in the numerator and [S] and $[\mathrm{S}]^2$ in the denominator

Therefore, the data or curve shown in Fig. E7.5.1 is consistent with Eq. (E7.5.12).

(b) Mole balance on the substrate around the CSTR (Fig. E7.5.2) leads to:

$$\mathrm{QS}_0 - \mathrm{QS} + r_\mathrm{S}\mathrm{V} = 0$$

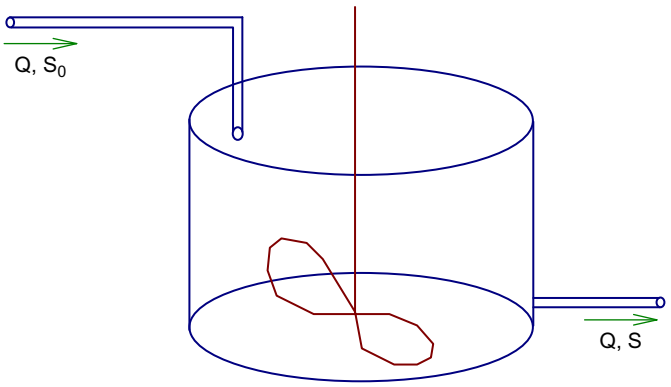


FIG. E7.5.2 A schematic of a CSTR

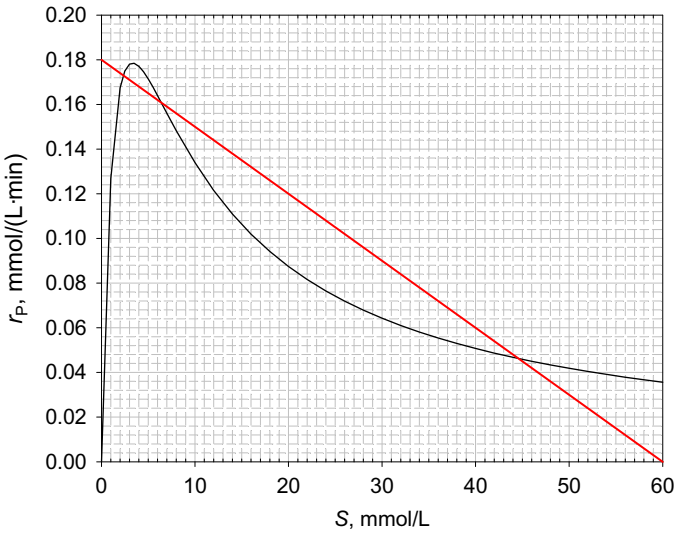


FIG. E7.5.3 Superimposing mass balance equation on the plot of reaction rate vs substrate concentration in the reactor.

From stoichiometry, $\frac{r_S}{\nu_S}=\frac{r_P}{\nu_P}$. Thus,

$$r_P=-r_S=\frac{QS_0-QS}{V}=D(S_0-S) \tag{E7.5.13}$$

$D=Q/V=15/5000\ \text{min}^{-1}=3\times10^{-3}\ \text{min}^{-1}$

Therefore, the mole balance Eq. (E7.5.13) is a straight line on the (S, r_P) plane with a negative slope of $-D$ ($-3\times10^{-3}\ \text{min}^{-1}$) and intercept on the S-axis of $S_0=60$ mmol L^{-1}. Plot the mole balance line (red line) on to the Fig. E7.5.1 yields Fig. E7.5.3.

The mole balance line intercepts with the consumption rate of substrate (=generation rate of product) three times. Therefore, there are three possible steady state solutions: $S=44$, 6.5, and 2.3 mmol L^{-1}. Correspondingly, the conversions are given by

$$f_S=\frac{S_0-S}{S_0}=1-\frac{S}{S_0} \tag{E7.5.14}$$

The three possible fractional conversions are: 0.267, 0.892, and 0.962.

(c) If the enzyme loading is reduced by 25%, ie, $[E]_0=0.75[E]_{0\text{previous}}$, based on Eq. (E7.5.12), the reaction rate is going to be reduced by 25%. Therefore, we can rescale the vertical axis of Fig. E7.5.1 (see the scale on the right on Fig. E7.5.4), while plotting the mole balance line, Eq. (E7.5.13) based on the new scale to find the possible steady-state solutions.

Now only one steady-state solution is obtained. $S=48.5$ mmol L^{-1} or the conversion $f_S=0.192$.

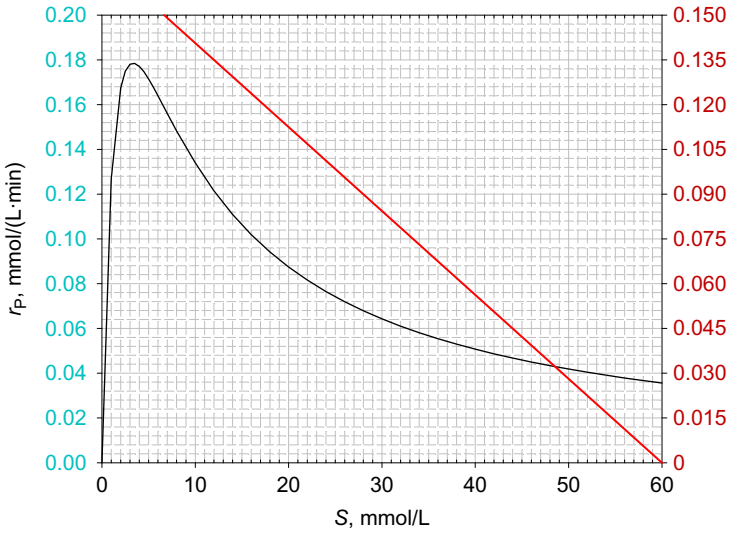


FIG. E7.5.4 Change of enzyme loading changes reaction rate as the enzymatic rate is proportional to enzyme loading.

7.9 LARGE-SCALE PRODUCTION OF ENZYMES

Among various enzymes produced on a 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 later in the text.

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 *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. Fig. 7.18 depicts a schematic of an enzyme plant producing extracellular enzymes.

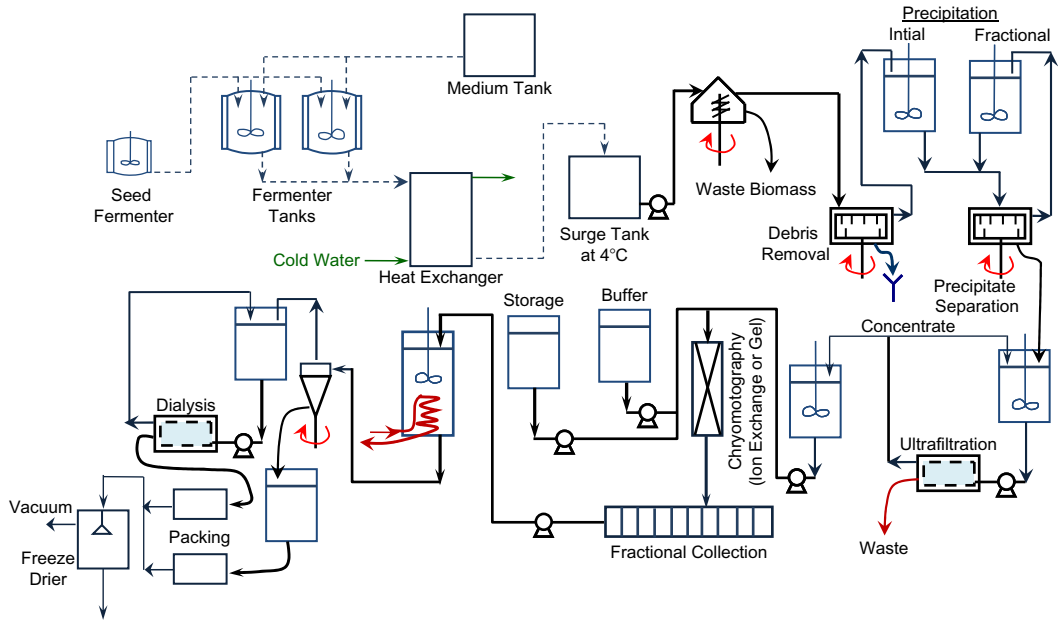


FIG. 7.18 A flowsheet for the production of an extracellular enzyme.

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 protein from recombinant DNA.

7.10 MEDICAL AND INDUSTRIAL UTILIZATION OF ENZYMES

Enzymes have been significant industrial products for over a century. The range of potential applications is still 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 manufacturing, 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 (eg, deep oceans, salt lakes, hot springs, and industrial waste sites) 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 (Chapter 13). We no longer need to depend solely on natural sources for enzymes.

While there are many reasons to be optimistic about increasing the use of enzymes, the number of enzymes made at high volume for industrial purposes evolves more slowly. In 2014, worldwide sales of industrial enzymes were at $4.2 billion, and the sales are projected to grow at 7.0% annually from 2015 to 2020. The products made in enzyme processes are worth billions of dollars. Table 7.6 lists some industrially important enzymes.

TABLE 7.6 Some Industrially Important Enzymes

Name	Example of Sources	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 and/or 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

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 pancreases, 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); in tanning; and the medical treatment of wounds.

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, Inter-esterification of oils and fats may be catalyzed by lipase. 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-l,4 glycosidic bonds randomly on the amylose chain and solubilizes amylase. For this reason, α-amylase is known as the starch-liquefying enzyme. β-Amylase hydrolyzes α-l,4 glycosidic bonds on the nonreducing ends of amylose and produces maltose residues. β-Amylase is known as a saccharifying enzyme. α-l,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 average, nearly 5.9×10^8 kg year^{-1} of glucose is produced by the enzymatic hydrolysis of starch. The enzyme pullulase also hydrolyzes α-l,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* cellulose 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 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 filtration efficiency 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. The 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-Acylaminoacids are converted to a mixture of L- and D-aminoacids by immobilized aminoacylase. L-Aminoacids are separated from D-acylaminoacid, which is recycled back to the column. L-Aminoacids 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 anti-inflammatory 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 (eg, 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.

7.11 KINETIC APPROXIMATION: WHY MICHAELIS-MENTEN EQUATION WORKS

In Section 7.2, we have discussed the kinetic models of enzyme-catalyzed reactions via two approximations: (1) rapid equilibrium steps; and (2) PSSH. Either approach leads to an

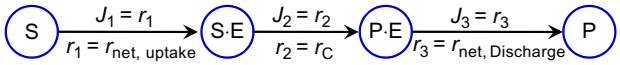


FIG. 7.19 A schematic of reaction pathway showing the rates and fluxes between each adjacent intermediate or substance for the enzymatic isomerization of substrate S to product P.

equation that looks similar, and when the parameters are lumped, the two equations become identical. The resulting equation is commonly referred to as Michaelis-Menten equation. The success or usefulness of this equation cannot be underestimated, as it has been applied to cases where the simple mechanistic model it implies is not nearly close to the real case. We shall examine why this is the case in this section.

Before we proceed to the discussion, let us consider a simple bioreaction network or pathway that is illustrated in Fig. 7.19 to be carried out in a batch reactor

$$\begin{aligned} \mathrm{S} + \mathrm{E} &\rightleftharpoons \mathrm{S{\cdot}E} \\ \mathrm{S{\cdot}E} &\rightleftharpoons \mathrm{P{\cdot}E} \\ \mathrm{P{\cdot}E} &\rightleftharpoons \mathrm{P} + \mathrm{E} \\ \hline +) = \text{overall} \qquad \mathrm{S} &\rightleftharpoons \mathrm{P} \end{aligned}$$

The uptake of substrate by the enzyme (or substrate-enzyme complexing) is described as a reversible reaction, ie,

$$r_1 = k_1 C_S C_E - k_{-1} C_{SE} \tag{7.102}$$

The catalytic reaction occurring or assisted by the enzyme is governed by

$$r_2 = k_c C_{SE} - k_{-c} C_{PE} \tag{7.103}$$

and finally, the discharging of product is governed by

$$r_3 = k_3 C_{PE} - k_{-3} C_P C_E \tag{7.104}$$

Since all the steps are already assumed to be reversible and the rates are net rates, the fluxes passing from one "node" to another "node" as shown in Fig. 7.19 are identical to the net reaction rates. As the pathway is still simple enough for attempting on full solution, we shall show how this problem can be solved without further approximation.Mole balances of all the species in the batch reactor lead to

$$\frac{dC_S}{dt} = -k_1 (C_S C_E - C_{SE}/K_S) \tag{7.105}$$

$$\frac{dC_P}{dt} = k_{-3} (C_{PE}/K_P - C_P C_E) \tag{7.106}$$

$$\frac{dC_{SE}}{dt} = k_1 (C_S C_E - C_{SE}/K_S) - k_c (C_{SE} - C_{PE} K_S/K_P/K_C) \tag{7.107}$$

$$\frac{dC_{PE}}{dt} = k_c (C_{SE} - C_{PE} K_S/K_P/K_C) - k_{-3} (C_{PE}/K_P - C_P C_E) \tag{7.108}$$

$$C_{E_0} = C_E + C_{SE} + C_{PE} \tag{7.109}$$

where K_S and K_P are association (or saturation) constants of S and P with the enzyme; respectively, and K_C is the equilibrium constant of the overall reaction (Eq. 7.108). That is,

$$K_S = \frac{k_1}{k_{-1}} \tag{7.110a}$$

$$K_P = \frac{k_{-3}}{k_3} \tag{7.110b}$$

$$K_C = \frac{k_c}{k_{-c}} \times \frac{K_S}{K_P} \tag{7.110c}$$

The five Eqs. (7.105)–(7.109) can be solved simultaneously with the initial conditions (in the reactor at time 0)

$$C_S = C_{S_0};\ C_E = C_{E_0};\ C_P = 0;\ C_{SE} = 0;\ C_{PE} = 0 \quad \text{when } t = 0 \tag{7.111}$$

That is to say, the batch reactor is loaded with the substrate *S* and the free enzyme *E* at time 0.

We shall use an integrator to show how this problem can be solved on computer. In this case, we use the ODExLIMS to integrate Eqs. (7.105)–(7.108). Let

$y_1 = C_S; y_2 = C_P; y_3 = C_{SE}; y_4 = C_{PE}$; and the independent variable $x = t$.

Parameters $c_1 = k_1 C_{E_0}; c_2 = K_S C_{E_0}; c_3 = k_c; c_4 = K_C; c_5 = K_P C_{E_0}; c_6 = k_{-3} C_{E_0}$.

The kernel functions can then be input into the visual basic module as shown in Fig. 7.20. The setup and solution on Excel worksheet for one set of parameters is shown in Fig. 7.21. Some of the results for selected values of the parameters are shown in Figs. 7.22–7.24.

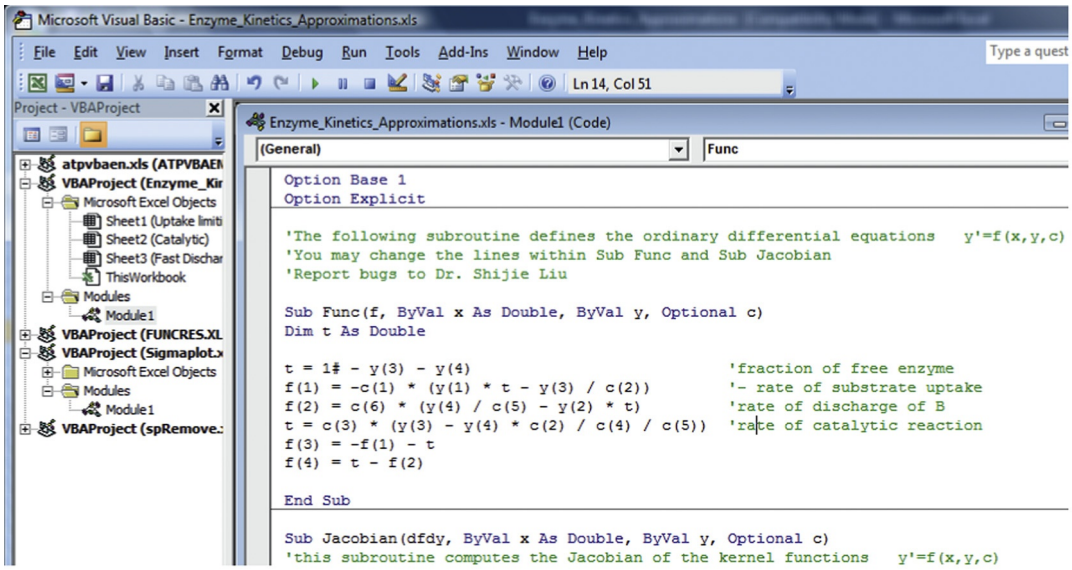
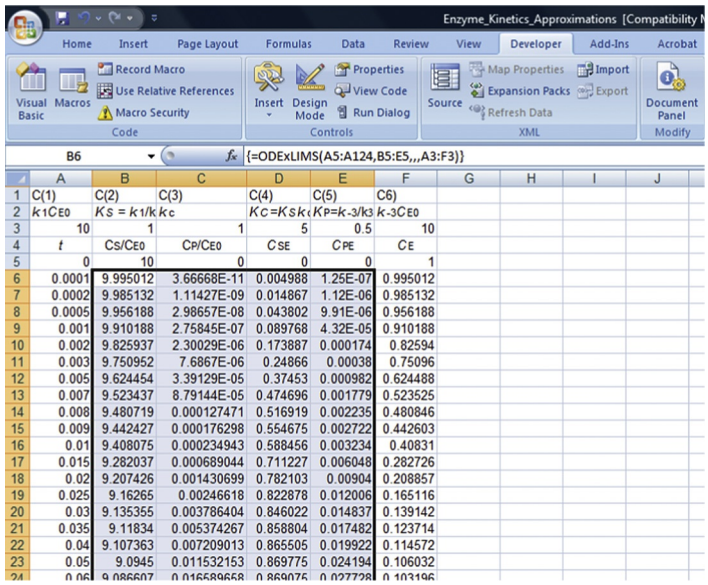


FIG. 7.20 Visual basic module shown the integral kernels for the enzyme-catalyzed reaction.

FIG. 7.21 Excel worksheet for the enzyme-catalyzed reactions Eqs. (7.105)–(7.107).



	A	B	C	D	E	F
1	C(1)	C(2)	C(3)	C(4)	C(5)	C6)
2	k_1C_{E0}	$K_S = k_1/k$	k_C	$K_C=K_Sk$	$K_P=k_{-3}/k$	$k_{-3}C_{E0}$
3	10	1	1	5	0.5	10
4	t	C_S/C_{E0}	C_P/C_{E0}	C_{SE}	C_{PE}	C_E
5	0	10	0	0	0	1
6	0.0001	9.995012	3.66668E-11	0.004988	1.25E-07	0.995012
7	0.0002	9.985132	1.11427E-09	0.014867	1.12E-06	0.985132
8	0.0005	9.956188	2.98657E-08	0.043802	9.91E-06	0.956188
9	0.001	9.910188	2.75845E-07	0.089768	4.32E-05	0.910188
10	0.002	9.825937	2.30029E-06	0.173887	0.000174	0.82594
11	0.003	9.750952	7.6867E-06	0.24866	0.00038	0.75096
12	0.005	9.624454	3.39129E-05	0.37453	0.000982	0.624488
13	0.007	9.523437	8.79144E-05	0.474696	0.001779	0.523525
14	0.008	9.480719	0.000127471	0.516919	0.002235	0.480846
15	0.009	9.442427	0.000176298	0.554675	0.002722	0.442603
16	0.01	9.408075	0.000234943	0.588456	0.003234	0.40831
17	0.015	9.282037	0.000689044	0.711227	0.006048	0.282726
18	0.02	9.207426	0.001430699	0.782103	0.00904	0.208857
19	0.025	9.16265	0.00246618	0.822878	0.012006	0.165116
20	0.03	9.135355	0.003786404	0.846022	0.014837	0.139142
21	0.035	9.11834	0.005374267	0.858804	0.017482	0.123714
22	0.04	9.107363	0.007209013	0.865505	0.019922	0.114572
23	0.05	9.0945	0.011532153	0.869775	0.024194	0.106032
24	0.06	9.086607	0.016689668	0.869075	0.027728	0.103196

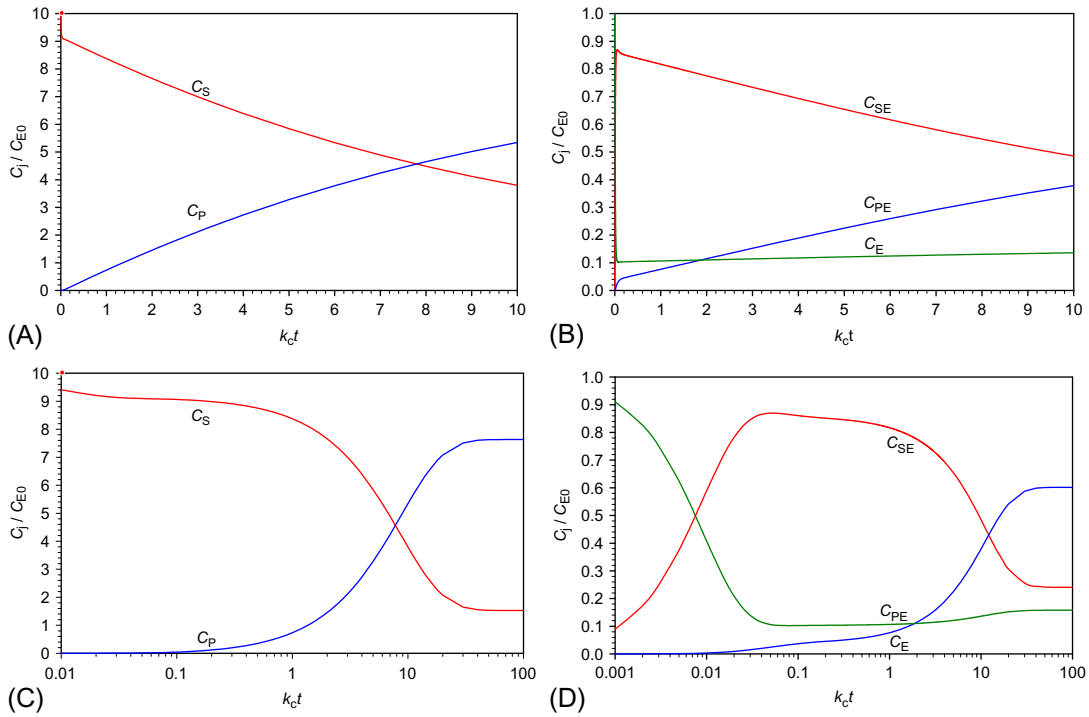


FIG. 7.22 Variations of free substrate and free product (A, C), and enzyme concentrations (B, D) with time for $k_1C_{E_0} = 10k_c$; $K_S = 1/C_{E_0}$; $K_C = 5$; $K_P = 0.5/C_{E_0}$; and $k_{-3}C_{E_0} = 10k_c$.

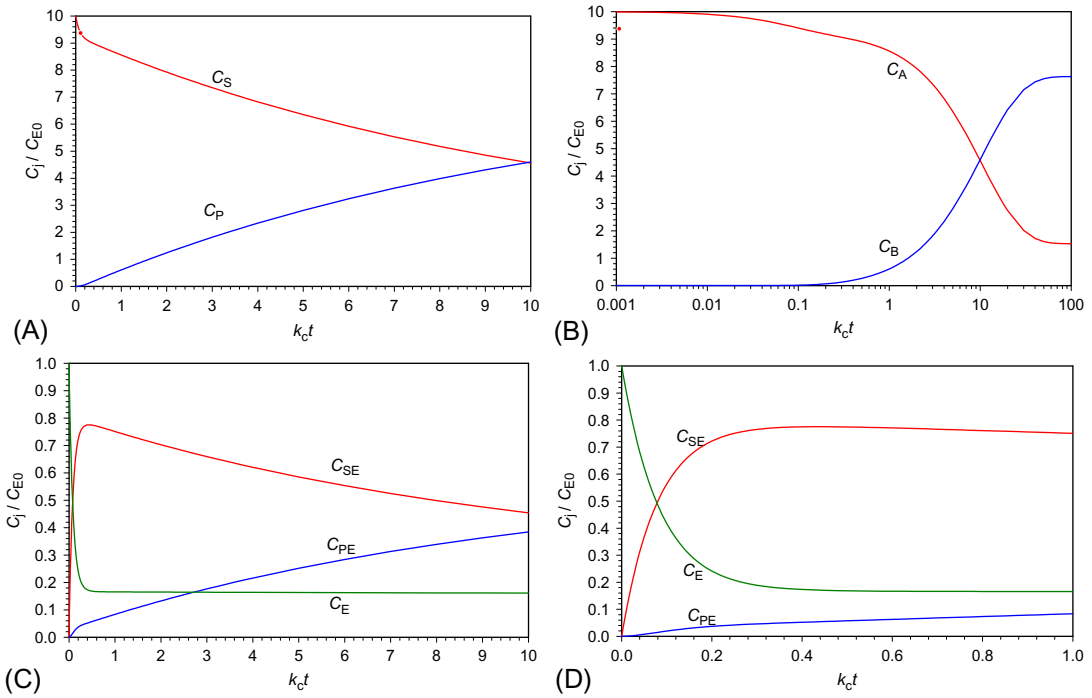


FIG. 7.23 Variations of free substrate and free product concentrations, (A, B), and enzyme distributions, (C, D), as function of time for $k_1 C_{E_0} = k_c$; $K_S = 1/C_{E_0}$; $K_C = 5$; $K_P = 0.5/C_{E_0}$; and $k_{-3} C_{E_0} = 10 k_c$.

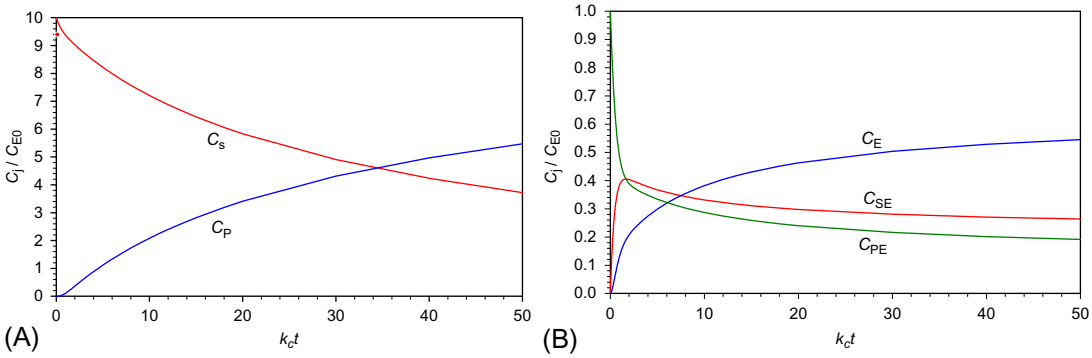


FIG. 7.24 Variations of free substrate and product concentrations (A) and enzyme distributions (B) with time for $k_1 C_{E_0} = 0.1 k_c$; $K_S = 1/C_{E_0}$; $K_C = 5$; $K_P = 0.5/C_{E_0}$; and $k_{-3} C_{E_0} = k_c$.

We first examine a case where the catalytic reaction rate is limiting. For this case, we scale the time with the catalytic reaction rate constant. The solutions for $C_{S_0} = 10 C_{E_0}$; $k_1 C_{E_0} = 10 k_c$; $K_S = 1/C_{E_0}$; $K_C = 5$; $K_P = 0.5/C_{E_0}$; and $k_{-3} C_{E_0} = 10 k_c$ are shown in Fig. 7.22. One can observe that in the batch operation, the free substrate concentration (Fig. 7.22A) experiences a quick

drop before the free product in the reaction mixture is observed. The decrease is simply due to the complexing of substrate with enzyme, and thus is dependent on the amount of enzyme or total enzyme activity. Since the product P is also associated with enzyme, the fraction of enzyme associated with substrate S increases sharply at the start (Fig. 7.22B and D), reaches a maximum, and then decreases as the product P is being formed. The fraction of enzyme associated with product P increases with time, and as more product P is formed, it also discharged to the reaction mixture and causes the concentration of free product P to increase. The concentration of free product shows almost a delay initially before graduate increase with time (Fig. 7.22A and C).

In the second case, we examine the situation where the uptake of substrate or association of substrate S with enzyme and catalytic reaction are of similar rates, but the discharge of product from the enzyme is fast. The results are shown in Fig. 7.23. One can observe that there is a drop in the concentration of free substrate S initially, but the drop is gradually at the time scale of reaction (Fig. 7.23A and B). C_{SE} reached maximum gradually and not as obviously as compared Fig. 7.23C with Fig. 7.23B. After C_{SE} reached maximum, and the fraction of free enzyme became "steady" at about $k_c t = 0.25$ (Fig. 7.23D).

We then examine the case when uptake of substrate S is the rate-limiting step as shown in Fig. 7.24. In this case, the decrease in C_S initially is not as apparent in the time scale of reaction (and as the previous two cases). The increase in the concentration of free product P showed almost a "delay." Therefore, the effect of substrate uptake rate is still important.

The effects of the amount of enzyme, substrate and product saturation constants are important if there is a significant amount of enzyme being added to a batch system.

7.11.1 Pseudosteady State Hypothesis

We next examine the approximation by PSSH based on the same set of parameters. The PSSH assumes that the rate of change of intermediates be zero. In this case,

$$0 = r_{SE} = r_1 - r_2 = k_1(C_E C_S - C_{SE}/K_S) - k_c(C_{SE} - C_{PE}K_S/K_P/K_C) \tag{7.112}$$

$$0 = r_{PE} = r_2 - r_3 = k_c(C_{SE} - C_{PE}K_S/K_P/K_C) - k_{-3}(C_{PE}/K_P - C_E C_B) \tag{7.113}$$

This is equivalent to saying that $r_1 = r_2 = r_3$. Referring to Fig. 7.18, the implication is that at PSSH, the fluxes passing through all the nodes are equal. In this case, the overall reaction rate or total flux is the same as any of the individual ones (in series).

Eqs. (7.112), (7.113) can be rearranged to yield

$$-C_{SE}(k_1/K_S + k_c) + C_{PE}k_c K_S/K_P/K_C + C_E k_1 C_S = 0 \tag{7.114}$$

$$C_{SE}k_c - C_{PE}(k_c K_S/K_P/K_C + k_{-3}/K_P) + C_E k_{-3} C_P = 0 \tag{7.115}$$

Eqs. (7.114), (7.115) can be solved to give

$$C_{SE} = \frac{\left(K_C^{-1}k_{-3}^{-1} + K_S^{-1}k_c^{-1}\right)C_S + K_C^{-1}k_1^{-1}C_P}{K_C^{-1}k_{-3}^{-1} + K_S^{-1}k_c^{-1} + k_1^{-1}} K_S C_E \tag{7.116}$$

$$C_{PE} = \frac{\left(k_1^{-1} + K_S^{-1}k_c^{-1}\right)C_P + k_{-3}^{-1}C_S}{K_C^{-1}k_{-3}^{-1} + K_S^{-1}k_c^{-1} + k_1^{-1}} K_P C_E \tag{7.117}$$

Total enzyme balance leads to

$$\begin{aligned} C_{E_0} &= C_E + C_{SE} + C_{PE} \\ &= C_E + \frac{(K_C^{-1}k_{-3}^{-1} + K_S^{-1}k_c^{-1})C_S + K_C^{-1}k_1^{-1}C_P}{K_C^{-1}k_{-3}^{-1} + K_S^{-1}k_c^{-1} + k_1^{-1}} K_S C_E + \frac{(k_1^{-1} + K_S^{-1}k_c^{-1})C_P + k_{-3}^{-1}C_A}{K_C^{-1}k_{-3}^{-1} + K_S^{-1}k_c^{-1} + k_1^{-1}} K_P C_E \end{aligned} \tag{7.118}$$

Thus,

$$C_E = \frac{C_{E_0}}{1 + \dfrac{(K_S K_C^{-1}k_{-3}^{-1} + k_c^{-1} + k_{-3}^{-1}K_P)C_S + (K_C^{-1}k_1^{-1}K_S + k_1^{-1}K_P + K_S^{-1}k_c^{-1}K_P)C_P}{K_C^{-1}k_{-3}^{-1} + K_S^{-1}k_c^{-1} + k_1^{-1}}} \tag{7.119}$$

Substituting Eq. (7.119) into Eqs. (7.116), (7.117), we obtain the concentration of enzyme associated with substrate or product.

The overall rate of reaction

$$r = -r_S = r_1 = k_1 C_S C_E - k_{-1} C_{SE} \tag{7.120}$$

That is

$$r = k_1 (C_S C_E - C_{SE}/K_S) \tag{7.121}$$

Substituting the concentration of enzyme-substrate complex A and the concentration of free enzyme in the reaction mixture, Eq. (7.121) renders

$$\begin{aligned} r &= k_1 \left[C_S - \frac{(K_C^{-1}k_{-3}^{-1} + K_S^{-1}k_c^{-1})C_S + K_C^{-1}k_1^{-1}C_P}{K_C^{-1}k_{-3}^{-1} + K_S^{-1}k_c^{-1} + k_1^{-1}} \right] C_E \\ &= \frac{C_S - K_C^{-1}C_P}{K_C^{-1}k_{-3}^{-1} + K_S^{-1}k_c^{-1} + k_1^{-1}} C_E \end{aligned} \tag{7.122}$$

or

$$r = \frac{\dfrac{C_S - K_C^{-1}C_P}{K_C^{-1}k_{-3}^{-1} + K_S^{-1}k_C^{-1} + k_1^{-1}} C_{E_0}}{1 + \dfrac{(K_S K_C^{-1}k_{-3}^{-1} + k_c^{-1} + k_{-3}^{-1}K_P)C_S + (K_C^{-1}k_1^{-1}K_S + k_1^{-1}K_P + K_S^{-1}k_c^{-1}K_P)C_P}{K_C^{-1}k_{-3}^{-1} + K_S^{-1}k_c^{-1} + k_1^{-1}}} \tag{7.123}$$

Based on Fig. 7.19, one can think of the reaction network analogously to the electric conduction: (1) the fluxes must be equal at any given point; and (2) the total resistance is the summation of all the resistors in series. If one were to look carefully, these statements could be detected, as both are embedded in Eq. (7.123). Therefore, not only is the rate expression unique for PSSH, but it brings the reaction network to be directly in analog to the electric circuit.

At this point, one may look back at the full solutions as illustrated in Figs. 7.22–7.24, especially with Figs. 7.20B and D, 7.21C and D, and 7.23B, that the concentrations of intermediates (in this case, the concentrations of enzyme associated with S and with P) are hardly constant or at "steady state" in any reasonably wide regions where we would like to have the solutions meaningful. Therefore, the assumption is rather strong for the directly involved species that

we have eliminated from the rate expression. How do the solutions actually measure up with the full solutions?

To apply the PSSH expression (7.122) or (7.123), we must first ensure that the reaction mixture is already in pseudosteady state to minimize the error may cause in the solution. This error can be negligible if the amount of catalyst is negligible or for steady flow reactors where steady-state is already reached.

Let us consider again the case where no P is present in the reaction mixture at the start of the reaction. The concentrations of S and P charged into the batch reactor are C_{ST_0} and $C_{ST_0} = 0$. Since there is no P present in the initial reaction mixture, $C_{P_0} = 0$. Overall mole balance at the onset of the reaction leads to

$$C_{ST_0} = C_{S_0} + C_{SE_0} + C_{PE_0} \tag{7.124}$$

where C_{SE_0} and C_{PE_0} satisfy Eqs. (7.116), (7.117). Since $C_{P_0} = 0$, substituting Eqs. (7.116), (7.117) into Eq. (7.124), we obtain

$$C_{ST_0} = C_{S_0} + \frac{\left(K_S K_C^{-1} k_{-3}^{-1} + k_c^{-1} + k_{-3}^{-1} K_P\right) C_{S_0} C_{E_0}}{K_C^{-1} k_{-3}^{-1} + K_S^{-1} k_c^{-1} + k_1^{-1} + \left(K_S K_C^{-1} k_{-3}^{-1} + k_c^{-1} + k_{-3}^{-1} K_P\right) C_{S_0}} \tag{7.125}$$

This quadratic equation can be solved to give

$$C_{S_0} = \frac{C_{ST_0} - C_{E_0} - a + \sqrt{(C_{ST_0} - C_{E_0} - a)^2 + 4aC_{ST_0}}}{2} \tag{7.126}$$

where

$$a = \frac{K_C^{-1} k_{-3}^{-1} + K_S^{-1} k_c^{-1} + k_1^{-1}}{K_S K_C^{-1} k_{-3}^{-1} + k_c^{-1} + k_{-3}^{-1} K_P} \tag{7.127}$$

Fig. 7.25 shows the comparison between the full solutions and those from PSSH treatment for the particular case of $k_1 C_{E_0} = 10k_c$ and $k_{-3} C_{E_0} = 10k_c$. One can observe that solutions based on PSSH are surprisingly close to the full solutions. Although we have used the argument that

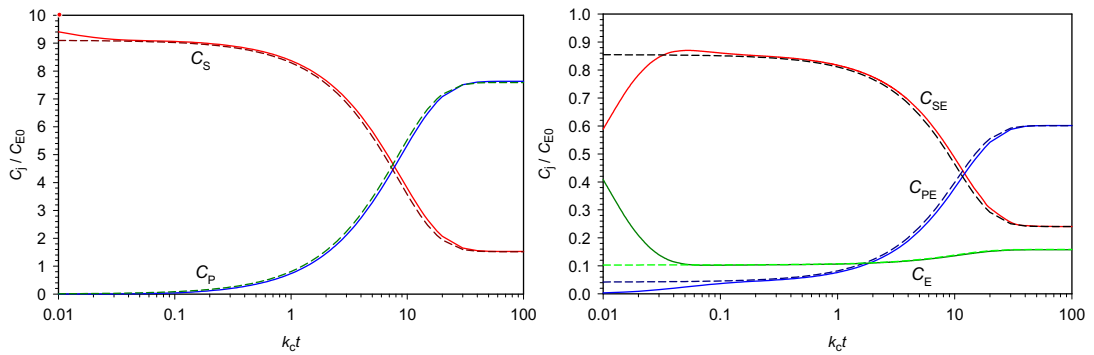


FIG. 7.25 Variations of free substrate, free product and enzyme concentrations with time for $k_1 C_{E_0} = 10k_c$; $K_S = 1/C_{E_0}$; $K_C = 5$; $K_P = 0.5/C_{E_0}$; and $k_{-3} C_{E_0} = 10k_c$. The dashed lines are the predictions from PSSH kinetics, whereas the solid lines are from the full solutions.

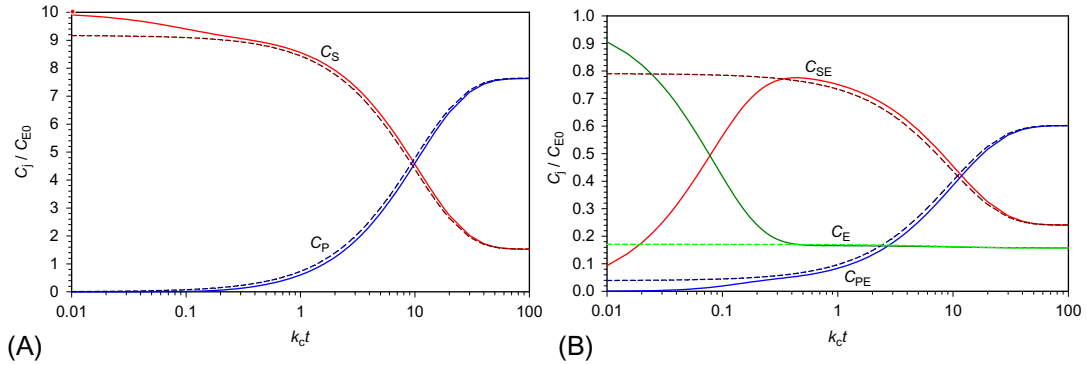


FIG. 7.26 Variations of free substrate, free product and enzyme concentrations with time for $k_1 C_{E_0} = k_c$; $K_S = 1/C_{E_0}$; $K_C = 5$; $K_P = 0.5/C_{E_0}$; and $k_{-3} C_{E_0} = 10 k_c$. The dashed lines are the predictions from PSSH kinetics, whereas the solid lines are from the full solutions.

all the intermediates remain at steady state (pseudosteady state) and the solutions do not appear to be the case, the computed enzyme distributions (dashed lines) agree quite well with the full solutions (solid lines) when $k_c t > 0.05$.

To show the reasonable agreement with the solutions from the PSSH approximation to the process, Fig. 7.26 shows a comparison between the full solutions (solid lines) and those from PSSH treatment for the case of $k_1 C_{E_0} = k_c$ and $k_{-3} C_{E_0} = 10 k_c$ (dashed lines). One can observe that the agreement between the full solutions and the PSSH solutions is rather good at "long" times when $k_c t > 0.3$.

7.11.2 Fast Equilibrium Step Approximation

We next examine how FES assumption is applied to approximate the enzyme reaction network. If the catalytic reaction is the rate-limiting step (Michaelis-Menten), we have the overall rate for reaction (Eq. 7.108):

$$r = r_2 = k_c C_{SE} - k_{-c} C_{PE} \tag{7.128}$$

The other two steps (Eqs. 7.107, 7.102) are in equilibrium,

$$0 = r_1 = k_1 C_S C_E - k_{-1} C_{SE} \tag{7.129}$$

$$0 = r_3 = k_3 C_{PE} - k_{-3} C_P C_E \tag{7.130}$$

which can be rearranged to give

$$C_{SE} = \frac{k_1}{k_{-1}} C_S C_E = K_S C_S C_E \tag{7.131}$$

and

$$C_{PE} = \frac{k_{-3}}{k_3} C_B C_E = K_P C_P C_E \tag{7.132}$$

Total enzyme balance

$$C_{E_0} = C_E + C_{SE} + C_{PE} = C_E + K_S C_S C_E + K_P C_P C_E \tag{7.133}$$

Thus,

$$C_E = \frac{C_{E_0}}{1 + K_S C_S + K_P C_P} \tag{7.134}$$

Substituting Eq. (7.130) into Eqs. (7.131), (7.132) and then (7.128), we obtain

$$C_{SE} = \frac{K_S C_S}{1 + K_S C_S + K_P C_P} C_{E_0} \tag{7.135}$$

$$C_{PE} = \frac{K_P C_P}{1 + K_S C_S + K_P C_P} C_{E_0} \tag{7.136}$$

$$r = k_c C_{E_0} K_S \frac{C_S - C_P/K_C}{1 + K_S C_S + K_P C_P} \tag{7.137}$$

Eqs. (7.123), (7.137) are quite similar when the rate constants are lumped together. If one were to use Eq. (7.123) to correlate experimental data, one would not be able to distinguish whether it is PSSH model or Michaelis-Menten model. The appeal of the PSSH approach is that it may be able to approximate the reaction rate in general (ie, all the fluxes are considered), without imposing a particular step as the rate-limiting step, as illustrated in Fig. 7.18. This approximation is particularly useful when the beginning (reactants) and end (products) are only of concern. Still, Eq. (7.137) looks simpler to use.

At time $t = 0$ in the batch reactor, the reaction is not started yet; however, the rapid-equilibrium-approximated expression (Eq. 7.137) requires that the uptake of substrate is already in equilibrium. While this requirement is not of issue for reactions carried out in flow reactors (after the transient period) or when the amount of enzyme is negligible, it becomes important when a noticeable amount of enzyme is employed in a batch reactor. For example, the concentration of free substrate S can be obtained via mole balance,

$$C_{ST_0} = C_{S_0} + C_{SE_0} = C_{S_0} + \frac{K_S C_{S_0}}{1 + K_S C_{S_0} + K_P C_{P_0}} C_{E_0} \tag{7.138}$$

The concentrations of free substrate S and product P charged into the batch reactor are C_{ST_0} and $C_{PT_0} = 0$, assuming only substrate S was loaded. Since there is no P present in the initial reaction mixture, $C_{P_0} = 0$. From Eq. (7.138), we can solve for the free substrate concentration in the batch reactor as

$$C_{S_0} = \frac{C_{ST_0} - C_{E_0} - K_S^{-1} + \sqrt{\left(C_{ST_0} - C_{E_0} - K_S^{-1}\right)^2 + 4K_S^{-1} C_{ST_0}}}{2} \tag{7.139}$$

Mole balances on substrate S and product P in the reactor leads to

$$\frac{dC_S}{dt} = -r = k_c C_{E_0} K_S \frac{C_S - C_P/K_C}{1 + K_S C_S + K_P C_P} \tag{7.140}$$

$$\frac{dC_P}{dt} = r = k_c C_{E_0} K_S \frac{C_S - C_P/K_C}{1 + K_S C_S + K_P C_P} \tag{7.141}$$

The solutions from the rapid equilibrium (Michaelis-Menten) model can be obtained by solving Eqs. (7.140), (7.141) with initial conditions given by C_{S_0} (Eq. 7.139) and $C_{P_0} = 0$ at $t = 0$. Subsequently, the enzyme distributions can be obtained from Eqs. (7.135), (7.136). One should note that the initial conditions set for Michaelis-Menten model must have taken the substrate uptake and product discharge equilibria into consideration.

Instead of having four equations to solve, we now have only two equations to solve. The solutions to one case consistent with the approximation to the first case discussed earlier are shown in Fig. 7.27. For comparison purposes, we have also plotted the full solutions as shown by dashed lines. One can observe that the enzyme distributions as predicted by the Michaelis-Menten approximation are quite similar to the full solutions. Michaelis-Menten approximation becomes suitable once the free enzyme concentration becomes nearly constant.

Fig. 7.27 shows the variations of free substrate, free product, and enzyme concentrations with reaction time for a case where the catalytic reaction rate is one-tenth of those of the other two steps. The Michaelis-Menten approximation are shown as solid lines, whereas the dashed lines are full solutions. One can observe that the Michaelis-Menten approximation agrees reasonably well with the actual system, although there is a noticeable time shift due to the small difference among the rates of the three steps. In deriving at Michaelis-Menten approximation, we have assumed that the rates of the other two steps are very fast. The 10-fold difference in the rate constant is still noticeable.

One can observe from Fig. 7.27 that while the variation of free substrate concentrations for Michaelis-Menten approximation looks similar to the full solutions, there is a shift log t (to the right) that could make the agreement closer. This is due to the fact that the finite rates of uptake of substrate S (or enzyme complexing of S) and discharge of P contributes to the decline of overall rate as used by Michaelis-Menten approximation. As a result, the Michaelis-Menten approximations overpredicted the reaction rate and thus led to a quicker change in the bulk phase concentrations. Overall, the shape of the curve (or how the concentrations change with time) is remarkably similar. Therefore, if one were to correlate the experimental data, the

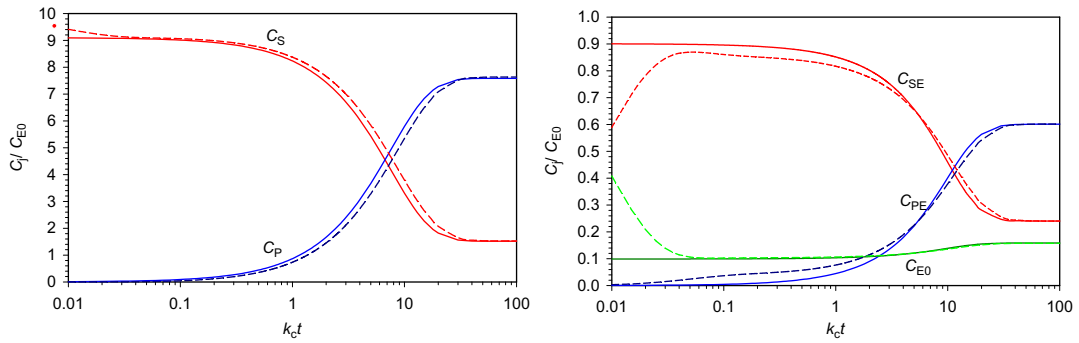


FIG. 7.27 Variations of free substrate, free product, and enzyme concentrations with time for $K_S = 1/C_{E_0}$; $K_C = 5$; $K_P = 0.5/C_{E_0}$. The solid lines are the predictions from Michaelis-Menten kinetics assuming the catalytic reaction is the rate-limiting step, whereas the dashed lines are for $k_1 C_{E_0} = 10k_c$ and $k_{-3} C_{E_0} = 10k_c$, as shown earlier.

difference between the quality of full solutions and the quality of fit from Michaelis-Menten approximations would not be noticeable.

7.11.3 Modified Fast Equilibrium Approximation

Comparing Figs. 7.25 and 7.27, one can observe that the PSSH approximation is closer to the full solutions. Also, PSSH approximation can be applied to a variety of systems, whereas the rapid equilibrium approximation is more specific. Therefore, the PSSH approximations are better suited as kinetic models than the equilibrium step assumptions when true kinetic constants are employed; however, the rate expressions from PSSH are quite similar to those of Michaelis-Menten or equilibrium step assumptions. When utilized to correlate experimental data, one would not be able to distinguish the two treatments.

To this point, we have learned now that PSSH approximation is easily visualized and implemented to approximate reaction networks; however, the steps involved in mathematical derivation are tedious and one would almost want to solve the rates via computer (algebraic equations or matrices). The resulting rate expressions can be simplified, though, when the rate constants are properly lumped. On the other hand, the rapid equilibrium assumption is much easier to deal with, as only one rate is specified as the limiting rate, equilibria hold for other steps. The resulting rate expressions are simpler than those from PSSH. Still, one needs to identify the right rate-limiting step. Comparing the PSSH and rapid equilibrium approximations with the full solutions, one can infer that PSSH approximation is closer to the full solution. When the rates are significantly different, ie, there is a truly one rate-limiting step, one may not find any difference between the three solutions when the initial moments were ignored.

To accommodate the rate difference and making rapid equilibrium approximations more useful, we can make further approximations to recover some of the error incurred due to the assumption of rapid equilibrium steps. This can be accomplished by

$$r = \left(r_1^{-1} + r_2^{-1} + r_3^{-1}\right)^{-1} \approx \frac{r_1|_{r_2=0, r_3=0}}{1 + \dfrac{k_1 C_{E_0}}{k_c} + \dfrac{K_P}{k_{-3} K_C} k_1 C_{E_0}} \tag{7.142a}$$

$$r = \left(r_1^{-1} + r_2^{-1} + r_3^{-1}\right)^{-1} \approx \frac{r_2|_{r_1=0, r_3=0}}{1 + \dfrac{k_c}{k_1 C_{E_0}} + \dfrac{K_P}{k_{-3} K_C} k_c} \tag{7.142b}$$

$$r = \left(r_1^{-1} + r_2^{-1} + r_3^{-1}\right)^{-1} \approx \frac{r_3|_{r_1=0, r_2=0}}{1 + \dfrac{k_{-3} K_C}{k_1 C_{E_0} K_P} + \dfrac{k_{-3} K_C}{k_c K_P}} \tag{7.142c}$$

Fig. 7.28 shows the variations of free substrate and product concentrations with reaction time comparing the full solutions with the approximate kinetics. One can observe that the rescaled Michaelis-Menten approximation agrees with full solutions reasonably well.

Therefore, one can conclude that Michaelis-Menten equation can be applied to model enzyme reactions after the initial "mixing" period without knowing if there is a rate-limiting

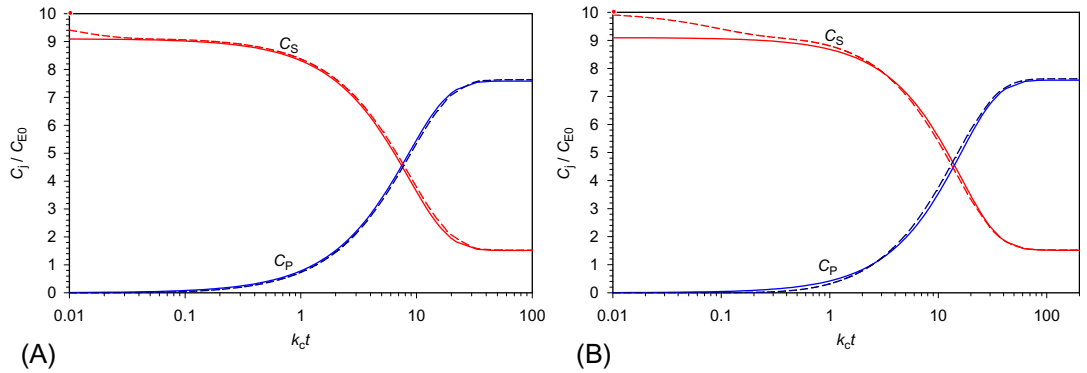


FIG. 7.28 Variations of free substrate, free product and enzyme concentrations with time for $K_S = 1/C_{E_0}$; $K_C = 5$; $K_P = 0.5/C_{E_0}$, $k_1 C_{E_0} = 10k_c$ and $k_{-3} C_{E_0} = 10k_c$. Two cases are shown: (A) $k_1 C_{E_0} = 10k_c$ as shown in Fig. 7.27; and (B) $k_1 C_{E_0} = k_c$, which does not qualify for single rate-liming step assumption. The solid lines are the predictions from Michaelis-Menten kinetics with the rate coefficient k_c replaced by $\left(k_c^{-1} + k_1^{-1} C_{E_0}^{-1} + K_P k_{-3}^{-1} C_{E_0}^{-1}\right)^{-1}$ or the time rescaled.

step. The rate constants: r_{max} and K_m are functions of the rate constants of all the steps involved. In reality, Michaelis-Menten equation is the "asymptote" of simple enzyme reactions.

7.12 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.

The kinetics of enzyme-catalyzed reactions are usually modeled after FES: the catalytic reaction step is the rate-limiting step, while other steps are in equilibrium or their rates are zero. The resulting rate expression is usually referred to as the Michaelis-Menten equation. The rapid equilibrium assumption produces simplest rate expression and is easy to apply; however, it has the limitation requiring other steps to be much faster. PSSH is more general, and it puts reaction networks in direct analogy with electric circuit. The final rate expression, on the other hand, is quite similar to Michaelis-Menten equation when the kinetic parameters are lumped together. Finally, the Michaelis-Menten rate expression is the asymptote of kinetic behaviors of simple enzymes. As such, it can be employed to correlate experimental data, without a true rate-limiting step.

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. Allosteric enzymes can have activity altered by cofactors

and without affecting the number of available active centers for the substrate. High substrate and product concentrations may be inhibitory and, in rare situations, high substrate concentration can have a higher rate, 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 16 to 84 kJ mol^{-1}. Above the optimal temperature, enzymes lose their activity, and the inactivation energy is on the order of 170 to 540 kJ mol^{-1}.

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, 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 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 multibillion-dollar businesses with a great potential for expansion.

Reactor performance for enzymatic reactions can be carried out in the same manner as other chemical reactors. Enzymatic reactions are commonly carried out in batch reactors, while flow reactors can be used when enzymes are immobilized.

Bibliography

Adams, M.W.W., Kelly, R.M., 1995. Enzymes from microorganisms in extreme environments. Chem. Eng. News 75, 32–42.

Bailey, J.E., Ollis, D.F., 1986. Biochemical Engineering Fundamentals, second ed. McGraw-Hill Book Co., New York.

Blanch, H.W., Clark, D.S., 1996. Biochemical Engineering. Marcel Dekker, Inc., New York.

Nielsen, J., Villadsen, J., Liden, G., 2003. Bioreaction Engineering Principles, second ed. Kluwer Academic/Plenum Publishers, New York.

Katchalski-Katzir, E., 1993. Immobilized enzymes-learning from past successes and failures. Trends Biotechnol. 11, 471–478.

Moran, L.A., Scrimgeourh, K.G., Horton, R., Ochs, R.S., Rawn, J.D., 1994. Biochemistry. Prentice-Hall, Inc., Upper Saddle River, NJ.

Sellek, G.A., Chaudhuri, J.B., 1999. Biocatalysis in organic media using enzymes from extremophiles. Enzyme Microb. Technol. 25, 471–482.

Stinson, S.C., 1997. Counting on chiral drugs. Chem. Eng. News. 83–104.

Walsh, G., 2002. Proteins—Biochemistry and Biotechnology. John Wiley & Sons, New York.

PROBLEMS

7.1. Chymotrypsin is a serine protease that cleaves the amide linkages in proteins and peptides. It has a binding pocket that is selective for the aromatic residues of amino acids. The reaction occurs by the reversible formation of a Michaelis-Menten complex, followed by acylation of Ser-195 to give a tetrahedral acyl enzyme intermediate. Chymotrypsin will also act as an esterase; we can write the elementary reaction steps in the following form, where RCO-X is an amide or an ester

$$E + RCO\text{-}X \rightleftarrows RCO\text{-}X \cdot E \rightarrow RCO\text{-}E' + XH$$

$$RCO\text{-}E' + H_2O \rightarrow RCOOH + E$$

Where X = NH—R′ (amide) or X = O—R′ (ester) and RCO-E is the acyl-enzyme intermediate. This can be written more simply as

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES_1 \xrightarrow{k_2} ES_2 \xrightarrow{k_3} E + P$$

Derive the rate of formation of P that is consistent with this mechanism.

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

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

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

$$r_P = \frac{r_{max}}{K_m} \frac{[S] - K_C^{-1}[P]}{1 + K_m^{-1}[S] + K_P^{-1}[P]}$$

where $K_m = \dfrac{k_{-1} + k_2}{k_1}$, $K_P = \dfrac{k_{-1} + k_2}{k_{-2}}$, $K_C = \dfrac{k_1 k_2}{k_{-1} k_{-2}}$ and $r_{max} = k_2[E]_0$

7.3. Consider the inosine monophosphate hydrolysis as catalyzed by inosine nucleosidase

$$\text{Inosine monophosphate} + H_2O \xrightarrow{\text{Inosine nucleosidase}} \text{Hypoxanthine} + \text{ribose phosphate}$$

which is inhibited by adenine. Table P7.3 shows the initial rate as functions of adenine and inosine concentrations.

TABLE P7.3 Adenine Inhibition on Inosine Nucleosidase

Adenine [I], μM	0	5	10	15	20	25
Inosine [S], μM	Initial rate, μM min^{-1}					
1.5	0.31	0.28	0.26	0.25	0.23	0.22
2	0.37	0.33	0.31	0.29	0.28	0.27
3	0.45	0.41	0.38	0.36	0.34	0.33
4	0.51	0.47	0.43	0.41	0.39	0.37

Determine the rate expression when the product concentrations are negligible.

7.4. Consider the conversion of acetaldehyde to alcohol as catalyzed by alcohol dehydrogenase:

$$\text{Acetaldehyde} + \text{NADH} + \text{H}^+ \xrightarrow{\text{alcohol dehydrogenase}} \text{ethanol} + \text{NAD}^+$$

which is inhibited by adenosine monophosphate (AMP). Table P7.4 shows the initial rate as functions of AMP and acetaldehyde concentrations.

TABLE P7.4 Adenine Monophosphate Inhibition on Alcohol Dehydrogenase

AMP [I] μM	0	1	2	3	4	5
Acetaldehyde [S] μM	Initial rate, μM min^{-1}					
1	0.096	0.049	0.033	0.024	0.02	0.016
1.5	0.128	0.065	0.043	0.033	0.026	0.022
2.5	0.175	0.089	0.059	0.045	0.036	0.03
5	0.238	0.122	0.082	0.061	0.049	0.041

Determine the rate expression when the product concentrations are negligible.

7.5. Consider the hydrolysis of glucose-6-phosphate (G6P) to glucose (G) and inorganic phosphate (Pi) as catalyzed by a glucose-6-phosphatase (EC 3.1.3.9) purified from an extract of rat liver. Table P7.5 shows the initial rate a function of G6P and G.

Determine the rate expression.

TABLE P7.5 Initial Rate of Glucose-6-Phosphate Hydrolysis, mmol-G/min/g-Enzyme

	[G], mM			
[G6P], mM	**0**	**20**	**40**	**80**
10	57.1	42.6	33.9	24.1
20	88.9	67.8	54.8	39.6
40	123.1	96.4	79.2	58.4
80	152.4	122.1	101.9	76.6

7.6. Consider the synthesis of ascorbyl oleate as catalyzed by *Candida rugose* lipase (EC 3.1.1.3) immobilized on γ-Fe_2O_3 magnetic nanoparticles:

$$\text{Ascorbic acid} + \text{Oleic acid} \rightarrow \text{Ascorbyl oleate} + H_2O$$

Table P7.6 shows the initial rate as a function of Ascorbic acid concentration [A] and Oleic acid concentration [O].

TABLE P7.6 Initial Rate of Ascorbyl Oleate Synthesis, mmol/min/g-Enzyme

	[O], mM				
[A], mM	**0.1**	**0.25**	**0.5**	**0.75**	**1**
0.5	2.432	5.562	9.742	12.997	15.605
1	3.626	7.961	13.234	16.984	19.788
1.5	4.336	9.298	15.031	18.919	21.73
3	5.392	11.175	17.391	21.351	24.093
6	6.139	12.429	18.873	22.817	25.479

Determine the rate expression.

7.7. Effect of pH on enzyme. Table P7.7 shows the Michaelis-Menten rate parameters as functions of pH for the hydrolysis of maltose with a glucoamylase from *Aspergillus awamori*. Determine explicit kinetic expression with pH effect.

TABLE P7.7 Michaelis-Menten Rate Constants as Functions of pH

pH	r_{max}, **mmol min^{-1} L^{-1}**	K_m, **mM**
0.5	0.063	31.62
1	0.20	31.60
1.5	0.63	31.58
2	1.98	31.41
3	18.18	29.66
4	100.0	20.82
5	180.0	12.14
5.5	188.0	11.29
5.8	185.4	11.58
6.8	122.5	18.37
7.8	27.36	28.66
8.8	3.12	31.29
9.3	1.0	31.52
9.8	0.316	31.59
10.3	0.10	31.61
10.8	0.032	31.62

7.8. The enzyme catalyzed aqueous reaction

$$\mathrm{A} \rightarrow \text{products}$$

has a rate $r = 1.2\, C_A/(0.01 + C_A)$ with rate in mol (L min)$^{-1}$ and concentration in mol L^{-1}. We need to process a stream consisting of 100 mol A h^{-1} at 2 mol L^{-1} substrate A to 95% conversion. Batch operations were chosen. For each batch of operations, the shifting time (loading, raising temperature, unloading after reaction, and cleaning) is 5 h. Calculate the reactor volumes required.

7.9. We wish to treat 10 L min^{-1} of liquid feed containing 1 mol A L^{-1} to 99% conversion. The reaction stoichiometry is given by

$$2\mathrm{A} \rightarrow \mathrm{R} + \mathrm{U}, \quad r = \frac{k_1 C_A}{K_m + C_A}$$

where $k_1 = 0.5$ mol L^{-1} min^{-1} and $K_m = 0.2$ mol L^{-1}. Suggest a good arrangement for doing this using two CSTRs. Find the sizes of the two units needed.

7.10. Enzyme E catalyzes the aqueous decomposition of substrate A to products R and B as follows:

$$\mathrm{A} \xrightarrow{\text{enzyme}} \mathrm{R} + \mathrm{B}, \quad r = \frac{200 C_A C_{E_0}}{0.1 + C_A}$$

where r is in mol L^{-1} min^{-1}, C_A and C_{E_0} are in mol L^{-1}. The reaction is to carry out in a plug flow reactor. In the feed stream, the concentrations of the enzyme and substrate A are 0.001 mol L^{-1} and 10 mol L^{-1}, respectively. How does density change in the reactor? Find the space time and mean residence time required to drop the substrate concentration to 0.025 mol L^{-1}.

7.11. The enzyme catalyzed aqueous reaction

$$\mathrm{A} \rightarrow \text{products}$$

has a rate $r = 2\, C_A/(0.01 + C_A)$ with rate in mol (L min)$^{-1}$ and concentration in mol L^{-1}. We need to process 100 mol h^{-1} of 2 mol L^{-1} feed to 95% conversion. Calculate the reactor volumes required if the reactor system were consisted of

(a) a PFR,
(b) a CSTR,
(c) two equal-volume CSTRs.
(d) an ideal combination of reactors for total minimum volume for two CSTR's.

7.12. It has been observed that substrate inhibition occurs in the following enzymatic reaction:

$$\mathrm{E} + \mathrm{S} \rightarrow \mathrm{P} + \mathrm{E}$$

(a) Show that the rate law for substrate inhibition is consistent with the data shown in the Fig. P7.12 below for r_P, mmol L^{-1} min^{-1}, versus the substrate concentration of S, mmol L^{-1}.

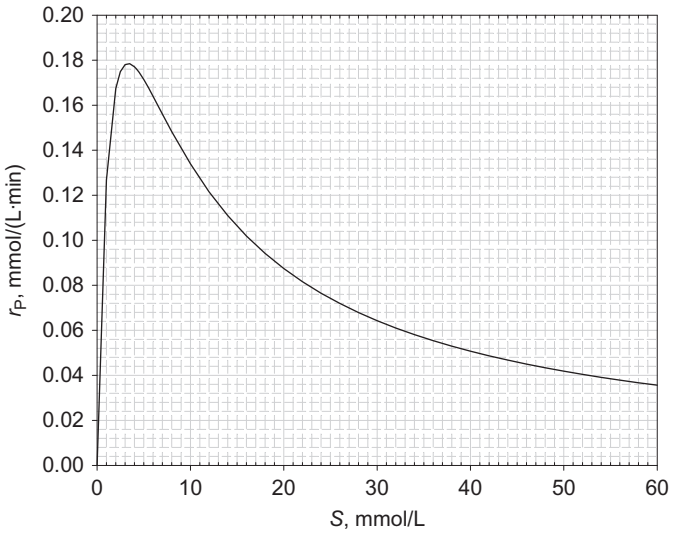


FIG. P7.12 Variation of enzymatic rate with substrate concentration at a fixed enzyme loading.

(b) *If this reaction is carried out in a fluidized CSTR with an immobilized enzyme system*. The reactor has a working volume of 1000 L, to which the volumetric flow rate is 3.2 L min^{-1} and the feed concentration of the substrate is 50 mmol L^{-1}, determine the possible conversion of substrate to the desired product. Assume that there is no loss of the enzyme to the effluent and the effectiveness factor of the immobilized enzymes is 100%, i.e. no impact of immobilization on the enzyme on catalytic activity.

(c) How many possible solutions have you obtained in part (b)? If more than one operation outputs are possible, which one would you like to choose to operate?

(d) What is the dilution rate in part (b)? What would be the effluent substrate concentration if the dilution rate is increased by 25%?

7.13. The enzyme catalyzed reaction:

$$E + S \rightleftarrows P + E$$

The rate of formation P is observed in the lab as shown in Fig. P7.12 with an enzyme loading of $E_0 = 50$ Units L^{-1}. In an industrial application, it is decided that enzyme loading can be raised to 75 Units L^{-1}. This reaction is to be carried out in a fluidized CSTR with an immobilized enzyme system. The volumetric flow rate is 380 L h^{-1} and the feed concentration of the substrate is 60 mmol L^{-1}. Assume that there is no loss of the enzyme to the effluent, and the effectiveness factor of the immobilized enzymes is 100%, ie, no impact of immobilization on the enzyme on catalytic activity. Determine the conversion of substrate if the reactor has a working volume of 1000 L.

7.14. In an enzymatic reaction, substrate S is converted irreversibly to produce P. It is suspected that the product inhibits the reaction, and consequently the rate of the reaction is determined for four values of S at each of four levels of the product concentration P. The 16 rate measurements are collected in Table P7.14. Determine the rate constants and type of product inhibition.

TABLE P7.14 Enzymatic Rate Data r_P at Four Levels of S and P

	P, mg L^{-1}			
S, g L^{-1}	**3**	**9**	**27**	**81**
0.1	0.073	0.058	0.036	0.017
0.4	0.128	0.102	0.064	0.030
1.6	0.158	0.126	0.079	0.037
6.4	0.168	0.134	0.084	0.039

7.15. Decarboxylation of glyoxalate (S) by mitochondria is inhibited by malonate (I) Using data in Table P7.15 obtained in batch experiments, determine the following:
(a) What type of inhibition is this?
(b) Determine the constants r_{max}, K_m, and K_I

TABLE P7.15 Initial Glyoxalate Decarboxylation Rate as Functions of Substrate and Inhibitor Concentrations

	Rate of CO_2 evolution, r_{CO_2}, mmol (L h)$^{-1}$		
Glyox S, mmol L^{-1}	**$I = 0$**	**$I = 1.26$ mmol L^{-1}**	**$I = 1.95$ mmol L^{-1}**
0.25	1.02	0.73	0.56
0.33	1.39	0.87	0.75
0.40	1.67	1.09	0.85
0.50	1.89	1.30	1.00
0.60	2.08	1.41	1.28
0.75	2.44	1.82	1.39
1.00	2.50	2.17	1.82

7.16. We wish to treat 50 L min^{-1} of liquid feed containing 1 mol AL^{-1} to 98% conversion with a batch reactor system. The overall reaction stoichiometry is given by

$$2A \rightarrow R + U, \quad r = \frac{k_1 C_A}{K_m + C_A}$$

where $k_1 = 0.8$ mol L^{-1} min^{-1} and $K_m = 0.025$ mol L^{-1}. For each batch of operation, 6 h of shifting time is needed. Determine the reactor size needed to complete the task.

7.17. We wish to treat a stream containing 150 mol A min^{-1} at 1.2 mol A L^{-1} to 99% conversion with a batch reactor system. The overall reaction stoichiometry is given by

$$1.2A \rightarrow R + U, \quad r = \frac{k_1 C_A}{K_m + C_A}$$

where $k_1 = 0.8$ mol L^{-1} min^{-1} and $K_m = 0.012$ mol L^{-1}. For each batch of operation, 3 h of shifting time is needed. Determine the reactor size needed to complete the task.

7.18. We wish to treat a stream containing 250 mol A min^{-1} at 2 mol A L^{-1} to 99.9% conversion with a flow reactor system. The overall reaction stoichiometry is given by

$$A \rightarrow R + U, \quad r = \frac{k_1 C_A}{K_m + C_A}$$

where $k_1 = 0.75$ mol L^{-1} min^{-1} and $K_m = 0.012$ mol L^{-1}. It is decided that one PFR and one CSTR are to be chosen to carry out the task. Determine the optimal arrangement and reactor sizes needed.

7.19. We wish to treat a liquid stream of 360 L min^{-1} containing at 1.5 mol A L^{-1} to 98% conversion with a flow reactor system. The overall reaction stoichiometry is given by

$$A \rightarrow R + U, r = \frac{k_1 C_A}{K_m + C_A}$$

where $k_1 = 0.9$ mol L^{-1} min^{-1} and $K_m = 0.001$ mol L^{-1}. It is decided that two CSTRs are to be chosen to carry out the task. Determine the reactor sizes needed.

7.20. The enzymatic hydrolization of fish oil extracted from crude eel oil has been carried out using lipase L. One of the desired products is docosahexaenoic acid, which is used as a medicine in China. For 40 mg of enzyme, the Michaelis-Menten saturation constant is 6.2×10^{-2} mmol L^{-1} and r_{max} is 5.6 mmol L^{-1} min^{-1}.

(a) Calculate the time necessary to reduce the concentration of fish oil from 1.4% to 0.2% (wt/vol).

(b) Calculate the time necessary to reduce the concentration of fish oil from 1.2% to 0.1% (wt/vol) if the enzyme loading is 60 mg.

7.21. Beef catalase has been used to accelerate the decomposition of hydrogen peroxide to yield water and oxygen. The concentration of hydrogen peroxide is given as a function of time for a reaction mixture with a pH of 6.76 maintained at 30°C as shown in Table P7.21.

TABLE P7.21 Concentration of Peroxide as a Function of Time

t, min	0	10	20	50	100
$C_{H_2O_2}$, mol L^{-1}	0.02	0.01775	0.0158	0.0106	0.005

(a) Determine the Michaelis-Menten parameters: r_{max} and K_m.
(b) If the enzyme loading is tripled, what will be the substrate concentration after 20 min?

7.22. The production of L-malic acid (used in medicines and food additives) was produced over immobilized cells of *Bacillus flavum* MA-3.

$$HOOCCH=CHCOOH + H_2O \xrightarrow{\text{fumarase}} HOOCCH_2CH(OH)COOH$$

The following rate law was obtained for the rate of formation of product:

$$r_P = \frac{r_{max}S}{K_m + S}\left(1 - \frac{P}{P_\infty}\right)$$

where $r_{max} = 76$ mol L^{-1} day^{-1}, $K_m = 0.048$ mol L^{-1}, and $P_\infty = 1.69$ mol L^{-1}. Design a reactor to process 10 m^3 day^{-1} of 1.2 mol L^{-1} of fumaric acid (S).
(a) A batch reactor is chosen. The required conversion of fumaric acid is 99%. The shifting time (loading, unloading and cleaning) is 3 h for each batch.
(b) A CSTR is chosen. The required fumaric acid conversion is 99%.

7.23. One hundred fifty moles of S per hour are available in concentration of 0.5 mol L^{-1} by a previous process. This stream is to be reacted with B to produce P and D. The reaction proceeds by the aqueous-phase reaction and catalyzed by an enzyme E,

$$S + B \rightarrow P + D$$

S is the limiting substrate and B is in excess. Michaelis-Menten rate parameters are $r_{max} = 2.0$ mol L^{-1} min^{-1} and $K_m = 0.02$ mol L^{-1} when the enzyme loading 0.001 g L^{-1}. CSTR has been selected to carry out the process. S costs \$0.1 mol^{-1}, P sells for \$0.8 mol^{-1}, and the enzyme E cost \$50 g^{-1}. The cost of operating the reactor is \$0.1 L^{-1} - h^{-1}. Assume no value or cost of disposal of unreacted S and entrained enzyme E (i.e., separation or recovery cost to S is identical to the fresh S cost).
(a) What is the relationship between rate of formation of P and enzyme loading?
(b) Perform a mole balance on the reactor to relate the concentration of exiting S with reactor size.
(c) What is the optimal concentration of S at the outlet of the reactor? What is the optimum enzyme loading? What is the optimal reactor size?
(d) What is the cash flow per mole of product from the process?
(e) What is the cost of enzyme per unit cost of substrate?

7.24. We have an opportunity to supply 500 mol of P per hour to enter the market. P is to be produced by

$$S \rightarrow P$$

Catalyzed by an enzyme E. Michaelis-Menten rate parameters are $r_{max} = 0.1$ mol L^{-1} min^{-1} and $K_m = 0.01$ mol L^{-1} when the enzyme loading 0.001 g L^{-1}. CSTR has been selected to carry out the process. There is a stream containing S at 2 mol L^{-1} is available for use. S costs \$0.2 mol^{-1}, P sells for \$1.5 mol^{-1},

and the enzyme E cost \$80 g^{-1}. The cost of operating the reactor is \$0.1 L^{-1} h^{-1}. Assume no value or cost of disposal of unreacted S and entrained enzyme E (i.e. separation or recovery cost to S is identical to the fresh S cost).

$$S \rightarrow P$$

(a) What is the relationship between rate of formation of P and enzyme loading?
(b) Perform a mole balance on the reactor to relate the concentration of exiting S with reactor size.
(c) What is the optimum concentration of S at the outlet of the reactor? What is the optimum enzyme loading? What is the optimum reactor size?
(d) What is the cash flow per mole of product from the process?
(e) What is the cost of enzyme per unit cost of substrate?