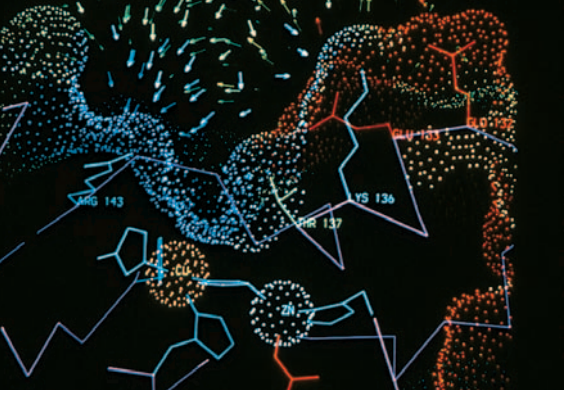


CHAPTER 14 Rates of Enzymatic Reactions

Kinetics is the study of the rates at which chemical reactions occur. A major purpose of such a study is to gain an understanding of a reaction mechanism, that is, a detailed description of the various steps in a reaction process and the sequence with which they occur. Thermodynamics, as we saw in Chapter 3, tells us whether a given process can occur spontaneously but provides little indication as to the nature or even the existence of its component steps. In contrast, *the rate of a reaction and how this rate changes in response to different conditions is intimately related to the path followed by the reaction and is therefore indicative of its reaction mechanism.*

In this chapter, we take up the study of **enzyme kinetics,** a subject that is of enormous practical importance in biochemistry because:

1. It is through kinetic studies that the binding affinities of substrates and inhibitors to an enzyme can be determined and that the maximum catalytic rate of an enzyme can be established.

2. By observing how the rate of an enzymatic reaction varies with the reaction conditions and combining this information with that obtained from chemical and structural studies of the enzyme, the enzyme's catalytic mechanism may be elucidated.

3. Most enzymes, as we shall see in later chapters, function as members of metabolic pathways. The study of the kinetics of an enzymatic reaction leads to an understanding of that enzyme's role in an overall metabolic process.

4. Under the proper conditions, the rate of an enzymatically catalyzed reaction is proportional to the amount of the enzyme present, and therefore most enzyme assays (measurements of the amount of enzyme present) are based on kinetic studies of the enzyme. Measurements of enzymatically catalyzed reaction rates are therefore among the most commonly employed procedures in biochemical and clinical analyses.

We begin our consideration of enzyme kinetics by reviewing chemical kinetics because enzyme kinetics is based on this formalism. Following that, we derive the basic equations of enzyme kinetics, describe the effects of inhibitors on enzymes, and consider how the rates of enzymatic reactions vary with pH. We end by outlining the kinetics of complex enzymatic reactions.

Kinetics is, by and large, a mathematical subject. Although the derivations of kinetic equations are occasionally rather detailed, the level of mathematical skills it requires should not challenge anyone who has studied elementary calculus. Nevertheless, to prevent mathematical detail from obscuring the underlying enzymological principles, the derivations of all but the most important kinetic equations have been collected in the appendix to this chapter. Those who wish to cultivate a deeper understanding of enzyme kinetics are urged to consult this appendix.

1 CHEMICAL KINETICS

Enzyme kinetics is a branch of chemical kinetics and, as such, shares much of the same formalism. In this section we

shall therefore review the principles of chemical kinetics so that, in later sections, we can apply them to enzymatically catalyzed reactions.

A. *Elementary Reactions*

A reaction of overall stoichiometry

$$\mathrm{A} \longrightarrow \mathrm{P}$$

may actually occur through a sequence of **elementary reactions** (simple molecular processes) such as

$$\mathrm{A} \longrightarrow \mathrm{I}_1 \longrightarrow \mathrm{I}_2 \longrightarrow \mathrm{P}$$

Here A represents reactants, P products, and I_1 and I_2 symbolize **intermediates** in the reaction. *The characterization of the elementary reactions comprising an overall reaction process constitutes its mechanistic description.*

a. Rate Equations

At constant temperature, elementary reaction rates vary with reactant concentration in a simple manner. Consider the general elementary reaction:

$$a\mathrm{A} + b\mathrm{B} + \cdots + z\mathrm{Z} \longrightarrow \mathrm{P}$$

The rate of this process is proportional to the frequency with which the reacting molecules simultaneously come together, that is, to the products of the concentrations of the reactants. This is expressed by the following **rate equation**

$$\text{Rate} = k[\mathrm{A}]^a[\mathrm{B}]^b \cdots [\mathrm{Z}]^z \qquad [14.1]$$

where k is a proportionality constant known as a **rate constant.** The **order** of a reaction is defined as $(a + b + \cdots + z)$, the sum of the exponents in the rate equation. *For an elementary reaction, the order corresponds to the* ***molecularity*** *of the reaction, the number of molecules that must simultaneously collide in the elementary reaction.* Thus the elementary reaction $\mathrm{A} \rightarrow \mathrm{P}$ is an example of a **first-order** or **unimolecular** reaction, whereas the elementary reactions $2\mathrm{A} \rightarrow \mathrm{P}$ and $\mathrm{A} + \mathrm{B} \rightarrow \mathrm{P}$ are examples of **second-order** or **bimolecular** reactions. Unimolecular and bimolecular reactions are common. **Termolecular** reactions are unusual and fourth- and higher order elementary reactions are unknown. This is because the simultaneous collision of three molecules is a rare event; that of four or more molecules essentially never occurs.

B. *Rates of Reactions*

We can experimentally determine the order of a reaction by measuring [A] or [P] as a function of time; that is,

$$v = -\frac{d[\mathrm{A}]}{dt} = \frac{d[\mathrm{P}]}{dt} \qquad [14.2]$$

where v is the instantaneous rate or **velocity** of the reaction. For the first-order reaction $\mathrm{A} \rightarrow \mathrm{P}$:

$$v = -\frac{d[\mathrm{A}]}{dt} = k[\mathrm{A}] \qquad [14.3a]$$

For second-order reactions such as $2\mathrm{A} \rightarrow \mathrm{P}$:

$$v = -\frac{d[\mathrm{A}]}{dt} = k[\mathrm{A}]^2 \qquad [14.3b]$$

whereas for $\mathrm{A} + \mathrm{B} \rightarrow \mathrm{P}$, a second-order reaction that is first order in [A] and first order in [B],

$$v = -\frac{d[\mathrm{A}]}{dt} = -\frac{d[\mathrm{B}]}{dt} = k[\mathrm{A}][\mathrm{B}] \qquad [14.3c]$$

The rate constants of first- and second-order reactions must have different units. In terms of units, v in Eq. [14.3a] is expressed as $M \cdot \mathrm{s}^{-1} = kM$. Therefore, k must have units of reciprocal seconds (s^{-1}) in order for Eq. [14.3a] to balance. Similarly, for second-order reactions, $M \cdot \mathrm{s}^{-1} = kM^2$, so that k has the units $M^{-1}\,\mathrm{s}^{-1}$.

The order of a specific reaction can be determined by measuring the reactant or product concentrations as a function of time and comparing the fit of these data to equations describing this behavior for reactions of various orders. To do this we must first derive these equations.

a. First-Order Rate Equation

The equation for [A] as a function of time for a first-order reaction, $\mathrm{A} \rightarrow \mathrm{P}$, is obtained by rearranging Eq. [14.3a]

$$\frac{d[\mathrm{A}]}{[\mathrm{A}]} \equiv d \ln [\mathrm{A}] = -k\,dt$$

and integrating it from $[\mathrm{A}]_\mathrm{o}$, the initial concentration of A, to [A], the concentration of A at time t:

$$\int_{[\mathrm{A}]_\mathrm{o}}^{[\mathrm{A}]} d \ln[\mathrm{A}] = -k \int_0^t dt$$

This results in

$$\ln[\mathrm{A}] = \ln[\mathrm{A}]_\mathrm{o} - kt \qquad [14.4a]$$

or, by taking the antilogs of both sides,

$$[\mathrm{A}] = [\mathrm{A}]_\mathrm{o}\, e^{-kt} \qquad [14.4b]$$

Equation [14.4a] is a linear equation in terms of the

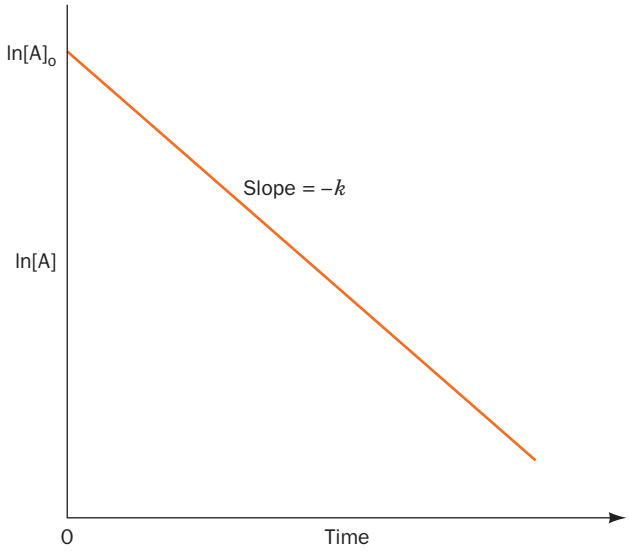


Figure 14-1 **Plot of ln[A] versus time for a first-order reaction.** This illustrates the graphical determination of the rate constant k using Eq. [14.4a].

variables ln[A] and t as is diagrammed in Fig. 14-1. Therefore, if a reaction is first order, a plot of ln[A] versus t will yield a straight line whose slope is $-k$, the negative of the first-order rate constant, and whose intercept on the ln[A] axis is $\ln[A]_o$.

Substances that are inherently unstable, such as radioactive nuclei, decompose through first-order reactions (first-order processes are not just confined to chemical reactions). One of the hallmarks of a first-order reaction is that *the time for half of the reactant initially present to decompose, its* ***half-time*** *or* ***half-life,*** $t_{1/2}$*, is a constant and hence independent of the initial concentration of the reactant*. This is easily demonstrated by substituting the relationship $[A] = [A]_o/2$ when $t = t_{1/2}$ into Eq. [14.4a] and rearranging:

$$\ln\left(\frac{[A]_o/2}{[A]_o}\right) = -kt_{1/2}$$

Thus

$$t_{1/2} = \frac{\ln 2}{k} = \frac{0.693}{k} \qquad [14.5]$$

In order to appreciate the course of a first-order reaction, let us consider the decomposition of ^{32}P, a radioactive isotope that is widely used in biochemical research. It has a half-life of 14 days. Thus, after 2 weeks, one-half of the ^{32}P initially present in a given sample will have decomposed; after another 2 weeks, one-half of the remainder, or three-quarters of the original sample, will have decomposed; etc. The long-term storage of waste ^{32}P therefore presents little problem, since after 1 year (26 half-lives), only 1 part in $2^{26} = 67$ million of the original sample will remain. How much will remain after 2 years? In contrast, ^{14}C, another commonly employed radioactive tracer, has a half-life of 5715 years. Only a small fraction of a given quantity of ^{14}C will decompose over the course of a human lifetime.

b. Second-Order Rate Equation for One Reactant

In a second-order reaction with one type of reactant, $2A \rightarrow P$, the variation of [A] with time is quite different from that in a first-order reaction. Rearranging Eq. [14.3b] and integrating it over the same limits used for the first-order reaction yields

$$\int_{[A]_o}^{[A]} -\frac{d[A]}{[A]^2} = k\int_0^t dt$$

so that

$$\frac{1}{[A]} = \frac{1}{[A]_o} + kt \qquad [14.6]$$

Equation [14.6] is a linear equation in terms of the variables $1/[A]$ and t. Consequently, Eqs. [14.4a] and [14.6] may be used to distinguish a first-order from a second-order reaction by plotting ln[A] versus t and $1/[A]$ versus t and observing which, if any, of these plots is a straight line.

Figure 14-2 compares the different shapes of the progress curves describing the disappearance of A in first- and second-order reactions having the same half-times. Note that before the first half-time, the second-order progress curve descends more steeply than the first-order curve, but after this time the first-order progress curve is the more rapidly decreasing of the two. The half-time for a second-order reaction is expressed $t_{1/2} = 1/(k[A_o])$ and therefore, in contrast to a first-order reaction, is dependent on the initial reactant concentration.

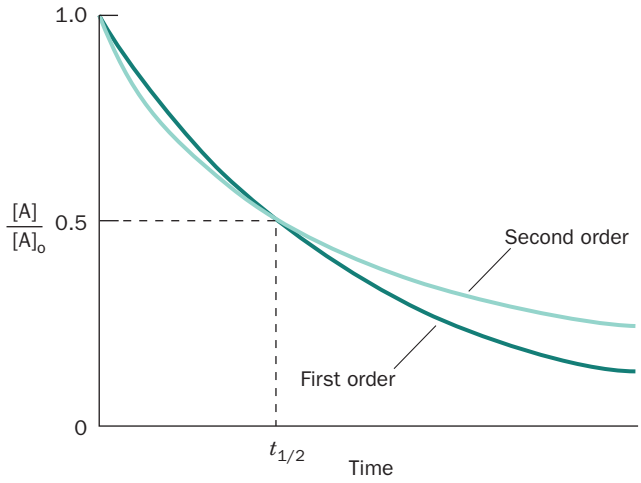


Figure 14-2 **Comparison of the progress curves for first- and second-order reactions that have the same value of $t_{1/2}$.** [After Tinoco, I., Jr., Sauer, K., and Wang, J.C., *Physical Chemistry. Principles and Applications in Biological Sciences* (2nd ed.), *p.* 291, Prentice-Hall (1985).]

C. *Transition State Theory*

The goal of kinetic theory is to describe reaction rates in terms of the physical properties of the reacting molecules. A theoretical framework for doing so, which explicitly considers the structures of the reacting molecules and how they collide, was developed in the 1930s, principally by Henry Eyring. This view of reaction processes, known as **transition state theory** or **absolute rate theory,** is the foundation of much of modern kinetics and has provided an

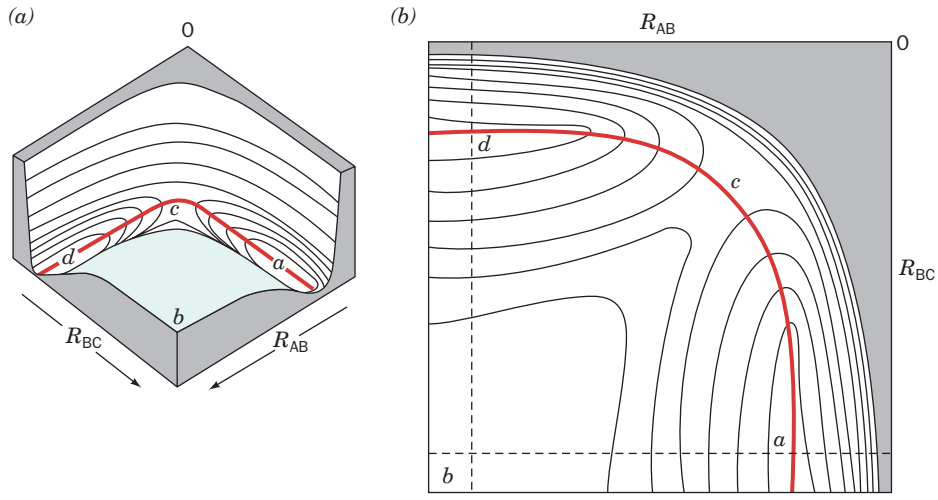


Figure 14-3 **Potential energy of the colinear H + H_2 system as a function of its internuclear distances, R_{AB} and R_{BC}.** The reaction is represented as (*a*) a perspective drawing and (*b*) the corresponding contour diagram. The points *a* and *d* are approaching potential energy minima, *b* is approaching a maximum, and *c* is a saddle point. [After Frost, A.A. and Pearson, R.G., *Kinetics and Mechanism* (2nd ed.), *p.* 80, Wiley (1961).]

extraordinarily productive framework for understanding how enzymes catalyze reactions.

a. The Transition State

Consider a bimolecular elementary reaction involving three atoms A, B, and C:

$$A—B + C \longrightarrow A + B—C$$

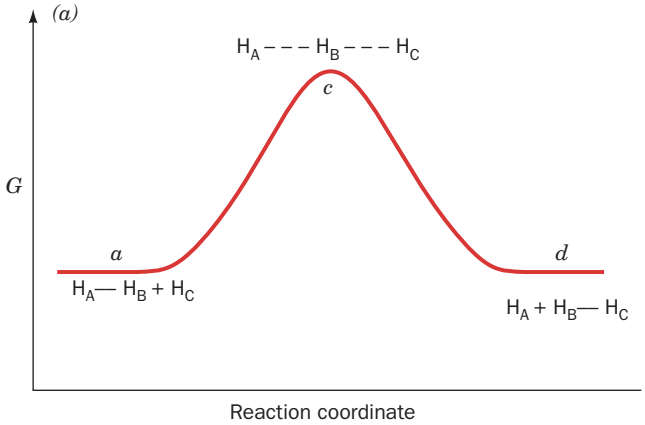
Clearly atom C must approach the diatomic molecule A—B so that, at some point in the reaction, a high-energy (unstable) complex represented as A···B···C exists in which the A—B covalent bond is in the process of breaking while the B—C bond is in the process of forming.

Let us consider the simplest example of this reaction: that of a hydrogen atom with diatomic hydrogen (H_2) to yield a new H_2 molecule and a different hydrogen atom:

$$H_A—H_B + H_C \longrightarrow H_A + H_B—H_C$$

The potential energy of this triatomic system as a function of the relative positions of its component atoms is plotted in Fig. 14-3. Its shape is of two long and deep valleys parallel to the coordinate axes with sheer walls rising toward the axes and less steep ones rising toward a plateau where both coordinates are large (the region of point *b*). The two valleys are joined by a pass or saddle near the origin of the diagram (point *c*). The minimum energy configuration is that of an H_2 molecule and an isolated atom, that is, with one coordinate large and the other at the H_2 covalent bond distance [near points *a* (the reactants) and *d* (the products)]. During a collision, the reactants generally approach one another with little deviation from the minimum energy reaction pathway (line *a*—*c*—*d*) because other trajectories would require much greater energy. As the atom and molecule come together, they increasingly repel one another (have increasing potential energy) and therefore usually fly apart. *If, however, the system has sufficient kinetic energy to continue its coalescence, it will cause the covalent bond of the H_2 molecule to weaken until ultimately, if the system reaches the saddle point (point c), there is an equal probability that either the reaction will occur or that the system will decompose back to its reactants.* Therefore, at this saddle point, the system is said to be at its **transition state** and hence to be an **activated complex.** Moreover, since the concentration of the activated complex is small, *the decomposition of the activated complex is postulated to be the rate-determining process of this reaction.*

The minimum free energy pathway of a reaction is known as its ***reaction coordinate.*** Figure 14-4*a*, which is



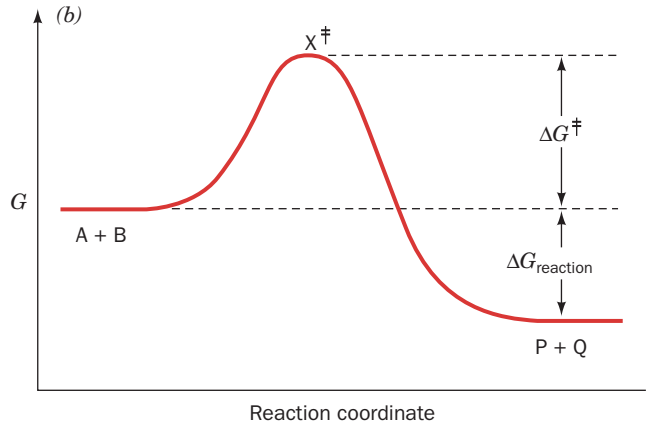


Figure 14-4 **Transition state diagrams.** (*a*) For the H + H_2 reaction. This is a section taken along the *a*—*c*—*d* line in Fig. 14-3. (*b*) For a spontaneous reaction, that is, one in which the free energy decreases.

called a **transition state diagram** or a **reaction coordinate diagram,** shows the free energy of the $H + H_2$ system along the reaction coordinate (line *a*—*c*—*d* in Fig. 14-3). It can be seen that the transition state is the point of highest free energy on the reaction coordinate. If the atoms in the triatomic system are of different types, as is diagrammed in Fig. 14-4*b*, the transition state diagram is no longer symmetrical because there is a free energy difference between reactants and products.

b. Thermodynamics of the Transition State

The realization that the attainment of the transition state is the central requirement in any reaction process led to a detailed understanding of reaction mechanisms. For example, consider a bimolecular reaction that proceeds along the following pathway:

$$A + B \overset{K^{\ddagger}}{\rightleftharpoons} X^{\ddagger} \overset{k'}{\longrightarrow} P + Q$$

where $X^{\ddagger}$ represents the activated complex. Therefore, considering the preceding discussion,

$$\frac{d[P]}{dt} = k[A][B] = k'[X^{\ddagger}] \qquad [14.7]$$

where k is the ordinary rate constant of the elementary reaction and k' is the rate constant for the decomposition of $X^{\ddagger}$ to products.

In contrast to stable molecules, such as A and P, which occur at energy minima, the activated complex occurs at an energy maximum and is therefore only metastable (like a ball balanced on a pin). Transition state theory nevertheless assumes that $X^{\ddagger}$ is in rapid equilibrium with the reactants; that is,

$$K^{\ddagger} = \frac{X^{\ddagger}}{[A][B]} \qquad [14.8]$$

where $K^{\ddagger}$ is an equilibrium constant. *This central assumption of transition state theory permits the powerful formalism of thermodynamics to be applied to the theory of reaction rates.*

If $K^{\ddagger}$ is an equilibrium constant it can be expressed as

$$-RT \ln K^{\ddagger} = \Delta G^{\ddagger} \qquad [14.9]$$

where $\Delta G^{\ddagger}$ is the Gibbs free energy of the activated complex less that of the reactants (Fig. 14-4*b*), T is the absolute temperature, and R ($= 8.3145\ J \cdot K^{-1}\ mol^{-1}$) is the gas constant (this relationship between equilibrium constants and free energy is derived in Section 3-4A). Then combining Eqs. [14.7] through [14.9] yields

$$\frac{d[P]}{dt} = k'\, e^{-\Delta G^{\ddagger}/RT}[A][B] \qquad [14.10]$$

This equation indicates that the rate of a reaction depends not only on the concentrations of its reactants but also decreases exponentially with $\Delta G^{\ddagger}$. *Thus, the larger the difference between the free energy of the transition state and that of the reactants, that is, the less stable the transition state, the slower the reaction proceeds.*

In order to continue, we must now evaluate k', the rate constant for passage of the activated complex over the maximum in the transition state diagram (sometimes referred to as the **activation barrier** or the **kinetic barrier** of the reaction). This transition state model permits us to do so (although the following derivation is by no means rigorous). The activated complex is held together by a bond that is associated with the reaction coordinate and that is assumed to be so weak that it flies apart during its first vibrational excursion. Therefore, k' is expressed

$$k' = \kappa\nu \qquad [14.11]$$

where ν is the vibrational frequency of the bond that breaks as the activated complex decomposes to products and κ, the **transmission coefficient,** is the probability that the breakdown of the activated complex, $X^{\ddagger}$, will be in the direction of product formation rather than back to reactants. For most spontaneous reactions in solution, κ is between 0.5 and 1.0; for the colinear $H + H_2$ reaction, we saw that it is 0.5.

We have nearly finished our job of evaluating k'. All that remains is to determine the value of ν. Planck's law states that

$$\nu = \varepsilon/h \qquad [14.12]$$

where, in this case, ε is the average energy of the vibration that leads to the decomposition of $X^{\ddagger}$, and h ($= 6.6261 \times 10^{-34}\ J \cdot s$) is Planck's constant. Statistical mechanics tells us that at temperature T, the classical energy of an oscillator is

$$\varepsilon = k_B T \qquad [14.13]$$

where k_B ($= 1.3807 \times 10^{-23}\ J \cdot K^{-1}$) is a constant of nature known as the **Boltzmann constant** and $k_B T$ is essentially the available thermal energy. Combining Eqs. [14.11] through [14.13]

$$k' = \frac{\kappa k_B T}{h} \qquad [14.14]$$

Then assuming, as is done for most reactions, that $\kappa = 1$ (κ can rarely be calculated with any confidence), the combination of Eqs. [14.7] and [14.10] with [14.14] yields the expression for the rate constant k of our elementary reaction:

$$k = \frac{k_B T}{h} e^{-\Delta G^{\ddagger}/RT} \qquad [14.15]$$

This equation indicates that *the rate of reaction decreases as its free energy of activation, $\Delta G^{\ddagger}$, increases.* Conversely, as the temperature rises, so that there is increased thermal energy available to drive the reacting complex over the activation barrier, the reaction speeds up. (Of course, enzymes, being proteins, are subject to thermal denaturation, so that the rate of an enzymatically catalyzed reaction falls precipitously with increasing temperature once the enzyme's denaturation temperature has been surpassed.) Keep in mind, however, that transition state theory is an ideal model; real systems behave in a more complicated, although qualitatively similar, manner.

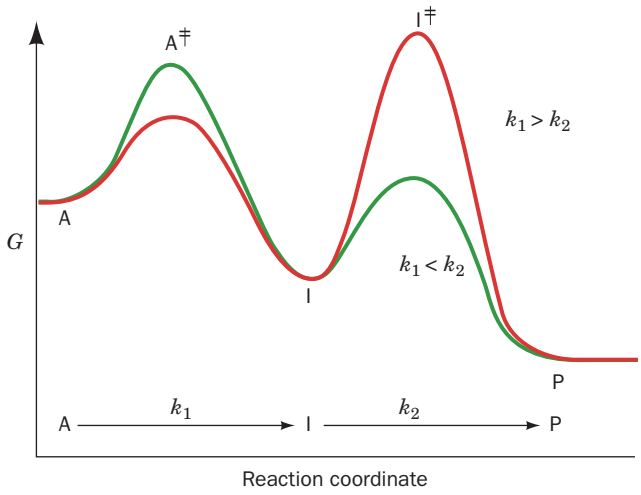


Figure 14-5 **Transition state diagram for the two-step overall reaction A → I → P.** For $k_1 < k_2$ (*green curve*), the first step is rate determining, whereas if $k_1 > k_2$ (*red curve*), the second step is rate determining.

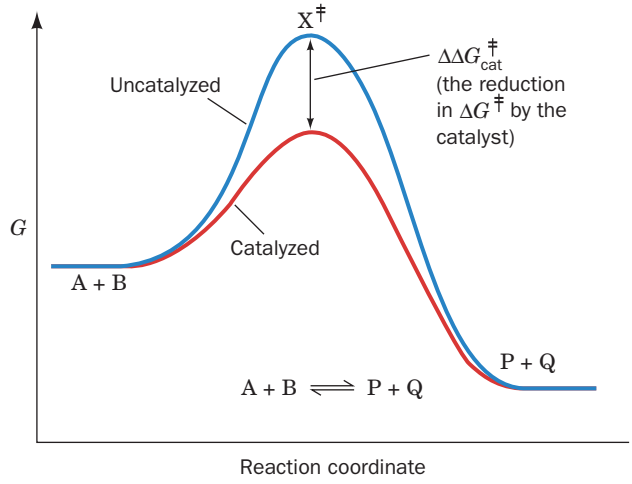


Figure 14-6 **The effect of a catalyst on the transition state diagram of a reaction.** Here $\Delta\Delta G^{\ddagger} = \Delta G^{\ddagger}_{uncat} - \Delta G^{\ddagger}_{cat}$.

c. Multistep Reactions Have Rate-Determining Steps

Since chemical reactions commonly consist of several elementary reaction steps, let us consider how transition state theory treats such reactions. For a multistep reaction such as

$$A \xrightarrow{k_1} I \xrightarrow{k_2} P$$

where I is an intermediate of the reaction, there is an activated complex for each elementary reaction step; the shape of the transition state diagram for such a reaction reflects the relative rates of the elementary reactions involved. For this reaction, if the first reaction step is slower than the second reaction step ($k_1 < k_2$), then the activation barrier of the first step must be higher than that of the second step, and conversely if the second reaction step is the slower (Fig. 14-5). Since the rate of formation of product P can only be as fast as the slowest elementary reaction, *if one reaction step of an overall reaction is much slower than the other, the slow step acts as a "bottleneck" and is therefore said to be the* ***rate-determining step*** *of the reaction.*

d. Catalysis Reduces $\Delta G^{\ddagger}$

Biochemistry is, of course, mainly concerned with enzyme-catalyzed reactions. *Catalysts act by lowering the activation barrier for the reaction being catalyzed* (Fig. 14-6). If a catalyst lowers the activation barrier of a reaction by $\Delta\Delta G^{\ddagger}_{cat}$ then, according to Eq. [14.15], the rate of the reaction is enhanced by the factor $e^{\Delta\Delta G^{\ddagger}_{cat}/RT}$. Thus, a 10-fold rate enhancement requires that $\Delta\Delta G^{\ddagger}_{cat} = 5.71\ kJ \cdot mol^{-1}$, less than half the energy of a typical hydrogen bond; a millionfold rate acceleration occurs when $\Delta\Delta G^{\ddagger}_{cat} = 34.25\ kJ \cdot mol^{-1}$, a small fraction of the energy of most covalent bonds. The rate enhancement is therefore a sensitive function of $\Delta\Delta G^{\ddagger}_{cat}$.

Note that the kinetic barrier is lowered by the same amount for both the forward and the reverse reactions (Fig. 14-6). Consequently, a catalyst equally accelerates the forward and the reverse reactions so that the equilibrium constant for the reaction remains unchanged. The chemical mechanisms through which enzymes lower the activation barriers of reactions are the subject of Section 15-1. There we shall see that the most potent such mechanism often involves the enzymatic binding of the transition state of the catalyzed reaction in preference to the substrate.

2 ENZYME KINETICS

See Guided Exploration 12: Michaelis–Menten kinetics, Lineweaver–Burk plots, and enzyme inhibition The chemical reactions of life are mediated by enzymes. These remarkable catalysts, as we saw in Chapter 13, are individually highly specific for particular reactions. Yet collectively they are extremely versatile in that the many thousand enzymes now known carry out such diverse reactions as hydrolysis, polymerization, functional group transfer, oxidation–reduction, dehydration, and isomerization, to mention only the most common classes of enzymatically mediated reactions. Enzymes are not passive surfaces on which reactions take place but, rather, are complex molecular machines that operate through a great diversity of mechanisms. For instance, some enzymes act on only a single substrate molecule; others act on two or more different substrate molecules whose order of binding may or may not be obligatory. Some enzymes form covalently bound intermediate complexes with their substrates; others do not.

Kinetic measurements of enzymatically catalyzed reactions are among the most powerful techniques for elucidating the catalytic mechanisms of enzymes. The remainder of this chapter is therefore largely concerned with the development of the kinetic tools that are most useful in the determination of enzymatic mechanisms. We begin, in this section, with a presentation of the basic theory of enzyme kinetics.

A. *The Michaelis–Menten Equation*

The study of enzyme kinetics began in 1902 when Adrian Brown reported an investigation of the rate of hydrolysis of sucrose as catalyzed by the yeast enzyme **invertase** (now known as **β-fructofuranosidase**):

$$\text{Sucrose} + H_2O \longrightarrow \text{glucose} + \text{fructose}$$

Brown demonstrated that when the sucrose concentration is much higher than that of the enzyme, the reaction rate becomes independent of the sucrose concentration; that is, the rate is **zero order** with respect to sucrose. He therefore proposed that the overall reaction is composed of two elementary reactions in which the substrate forms a complex with the enzyme that subsequently decomposes to products and enzyme:

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

Here E, S, ES, and P symbolize the enzyme, substrate, **enzyme–substrate complex,** and products, respectively (for enzymes composed of multiple identical subunits, E refers to active sites rather than enzyme molecules). According to this model, *when the substrate concentration becomes high enough to entirely convert the enzyme to the ES form, the second step of the reaction becomes rate limiting and the overall reaction rate becomes insensitive to further increases in substrate concentration.*

The general expression for the **velocity** (rate) of this reaction is

$$v = \frac{d[P]}{dt} = k_2[ES] \qquad [14.16]$$

The overall rate of production of ES is the difference between the rates of the elementary reactions leading to its appearance and those resulting in its disappearance:

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_2[ES] \qquad [14.17]$$

This equation cannot be explicitly integrated, however, without simplifying assumptions. Two possibilities are

1. Assumption of equilibrium: In 1913, Leonor Michaelis and Maud Menten, building on earlier work by Victor Henri, assumed that $k_{-1} >> k_2$, so that the first step of the reaction achieves equilibrium.

$$K_S = \frac{k_{-1}}{k_1} = \frac{[E][S]}{[ES]} \qquad [14.18]$$

Here K_S is the dissociation constant of the first step in the enzymatic reaction. With this assumption, Eq. [14.17] can be integrated. Although this assumption is not often correct, in recognition of the importance of this pioneering work, the noncovalently bound enzyme–substrate complex ES is known as the **Michaelis complex.**

2. Assumption of steady state: Figure 14-7 illustrates the progress curves of the various participants in the preceding reaction model under the physiologically common

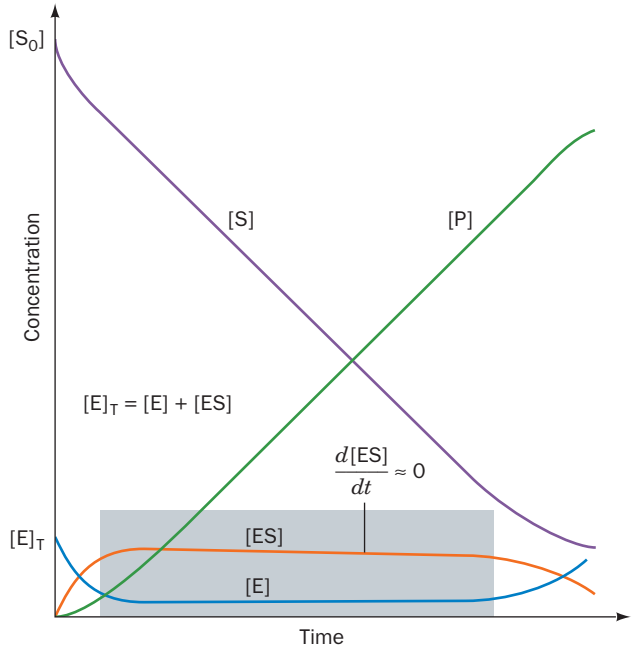


Figure 14-7 **Progress curves for the components of a simple Michaelis–Menten reaction.** Note that with the exception of the transient phase of the reaction, which occurs before the shaded block, the slopes of the progress curves for [E] and [ES] are essentially zero so long as $[S] >> [E]_T$ (within the shaded block). [After Segel, I.H., *Enzyme Kinetics, p.* 27, Wiley (1975).] **See the Animated Figures**

condition that substrate is in great excess over enzyme. With the exception of the initial stage of the reaction, the so-called **transient phase,** which is usually over within milliseconds of mixing the enzyme and substrate, [ES] remains approximately constant until the substrate is nearly exhausted. Hence, the rate of synthesis of ES must equal its rate of consumption over most of the course of the reaction; that is, [ES] maintains a **steady state.** One can therefore assume with a reasonable degree of accuracy that [ES] is constant; that is,

$$\frac{d[ES]}{dt} = 0 \qquad [14.19]$$

This so-called **steady-state assumption** was first proposed in 1925 by George E. Briggs and John B.S. Haldane.

In order to be of use, kinetic expressions for overall reactions must be formulated in terms of experimentally measurable quantities. The quantities [ES] and [E] are not, in general, directly measurable but the total enzyme concentration

$$[E]_T = [E] + [ES] \qquad [14.20]$$

is usually readily determined. The rate equation for our enzymatic reaction is then derived as follows. Combining Eq. [14.17] with the steady-state assumption, Eq. [14.19], and the conservation condition, Eq. [14.20], yields

$$k_1([E]_T - [ES])[S] = (k_{-1} + k_2)[ES]$$

which on rearrangement becomes

$$[\mathrm{ES}](k_{-1} + k_2 + k_1[\mathrm{S}]) = k_1[\mathrm{E}]_\mathrm{T}[\mathrm{S}]$$

Dividing both sides by k_1 and solving for [ES],

$$[\mathrm{ES}] = \frac{[\mathrm{E}]_\mathrm{T}[\mathrm{E}]}{K_M + [\mathrm{S}]}$$

where K_M, which is known as the **Michaelis constant,** is defined

$$K_M = \frac{k_{-1} + k_2}{k_1} \qquad [14.21]$$

The meaning of this important constant is discussed below.

The **initial velocity** of the reaction from Eq. [14.16] can then be expressed in terms of the experimentally measurable quantities $[\mathrm{E}]_\mathrm{T}$ and [S]:

$$v_\mathrm{o} = \left(\frac{d[\mathrm{P}]}{dt}\right)_{t=t_\mathrm{s}} = k_2[\mathrm{ES}] = \frac{k_2[\mathrm{E}]_\mathrm{T}[\mathrm{S}]}{K_M + [\mathrm{S}]} \qquad [14.22]$$

where t_s is the time when the steady state is first achieved (usually milliseconds after $t = 0$). The use of the initial velocity (operationally taken as the velocity measured before more than ~10% of the substrate has been converted to product) rather than just the velocity minimizes such complicating factors as the effects of reversible reactions, inhibition of the enzyme by product, and progressive inactivation of the enzyme.

The **maximal velocity** of a reaction, V_max, occurs at high substrate concentrations when the enzyme is **saturated,** that is, when it is entirely in the ES form:

$$V_\mathrm{max} = k_2[\mathrm{E}]_\mathrm{T} \qquad [14.23]$$

Therefore, combining Eqs. [14.22] and [14.23], we obtain

$$v_\mathrm{o} = \frac{V_\mathrm{max}[\mathrm{S}]}{K_M + [\mathrm{S}]} \qquad [14.24]$$

*This expression, the **Michaelis–Menten equation,** is the basic equation of enzyme kinetics.* It describes a rectangular hyperbola such as is plotted in Fig. 14-8 (although this curve is rotated by 45° and translated to the origin with respect to the examples of hyperbolas seen in most elementary algebra texts). The saturation function for oxygen binding to myoglobin, Eq. [10.4], has the same functional form.

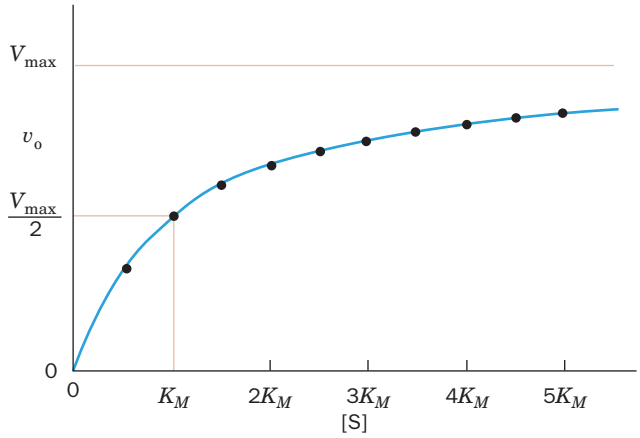


Figure 14-8 **Plot of the initial velocity v_o of a simple Michaelis–Menten reaction versus the substrate concentration [S].** Points are plotted in 0.5-K_M intervals of substrate concentration between 0.5 K_M and 5 K_M. **See the Animated Figures**

a. Significance of the Michaelis Constant

The Michaelis constant, K_M, has a simple operational definition. At the substrate concentration where $[\mathrm{S}] = K_M$, Eq. [14.24] yields $v_\mathrm{o} = V_\mathrm{max}/2$ so that *K_M is the substrate concentration at which the reaction velocity is half-maximal.* Therefore, if an enzyme has a small value of K_M, it achieves maximal catalytic efficiency at low substrate concentrations.

The magnitude of K_M varies widely with the identity of the enzyme and the nature of the substrate (Table 14-1). It is also a function of temperature and pH (see Section 14-4). The Michaelis constant (Eq. [14.21]) can be expressed as

$$K_M = \frac{k_{-1}}{k_1} + \frac{k_2}{k_1} = K_\mathrm{S} + \frac{k_2}{k_1} \qquad [14.25]$$

Since K_S is the dissociation constant of the Michaelis complex, as K_S decreases, the enzyme's affinity for substrate

Table 14-1 **Values of K_M, k_cat, and k_cat/K_M for Some Enzymes and Substrates**

Enzyme	Substrate	K_M (M)	k_cat (s^{-1})	k_cat/K_M ($M^{-1} \cdot s^{-1}$)
Acetylcholinesterase	Acetylcholine	9.5×10^{-5}	1.4×10^{4}	1.5×10^{8}
Carbonic anhydrase	CO_2	1.2×10^{-2}	1.0×10^{6}	8.3×10^{7}
	HCO_3^-	2.6×10^{-2}	4.0×10^{5}	1.5×10^{7}
Catalase	H_2O_2	2.5×10^{-2}	1.0×10^{7}	4.0×10^{8}
Chymotrypsin	*N*-Acetylglycine ethyl ester	4.4×10^{-1}	5.1×10^{-2}	1.2×10^{-1}
	N-Acetylvaline ethyl ester	8.8×10^{-2}	1.7×10^{-1}	1.9
	N-Acetyltyrosine ethyl ester	6.6×10^{-4}	1.9×10^{2}	2.9×10^{5}
Fumarase	Fumarate	5.0×10^{-6}	8.0×10^{2}	1.6×10^{8}
	Malate	2.5×10^{-5}	9.0×10^{2}	3.6×10^{7}
Superoxide dismutase	Superoxide ion ($O_2^{\cdot -}$)	3.6×10^{-4}	1.0×10^{6}	2.8×10^{9}
Urease	Urea	2.5×10^{-2}	1.0×10^{4}	4.0×10^{5}

increases. K_M is therefore also a measure of the affinity of the enzyme for its substrate providing k_2/k_1 is small compared with K_S, that is, $k_2 < k_{-1}$.

B. *Analysis of Kinetic Data*

There are several methods for determining the values of the parameters of the Michaelis–Menten equation. At very high values of [S], the initial velocity v_o asymptotically approaches V_{max}. In practice, however, it is very difficult to assess V_{max} accurately from direct plots of v_o versus [S] such as Fig. 14-8. Even at such high substrate concentrations as [S] = 10 K_M, Eq. [14.24] indicates that v_o is only 91% of V_{max}, so that the extrapolated value of the asymptote will almost certainly be underestimated.

A better method for determining the values of V_{max} and K_M, which was formulated by Hans Lineweaver and Dean Burk, uses the reciprocal of Eq. [14.24]:

$$\frac{1}{v_o} = \left(\frac{K_M}{V_{max}}\right)\frac{1}{[S]} + \frac{1}{V_{max}} \qquad [14.26]$$

This is a linear equation in $1/v_o$ and $1/[S]$. If these quantities are plotted, in the so-called **Lineweaver–Burk** or **double-reciprocal plot,** the slope of the line is K_M/V_{max}, the $1/v_o$ intercept is $1/V_{max}$, and the extrapolated $1/[S]$ intercept is $-1/K_M$ (Fig. 14-9). A disadvantage of this plot is that most experimental measurements involve relatively high [S] and are therefore crowded onto the left side of the graph. Furthermore, for small values of [S], small errors in v_o lead to large errors in $1/v_o$ and hence to large errors in K_M and V_{max}.

Several other types of plots, each with its advantages and disadvantages, have been formulated for the determination of V_{max} and K_M from kinetic data. With the advent of conveniently available computers, however, kinetic data are commonly analyzed by mathematically sophisticated statistical treatments. Nevertheless, Lineweaver–Burk plots are valuable for the visual presentation of kinetic data as well as being useful in the analysis of kinetic data from enzymes requiring more than one substrate (Section 14-5C).

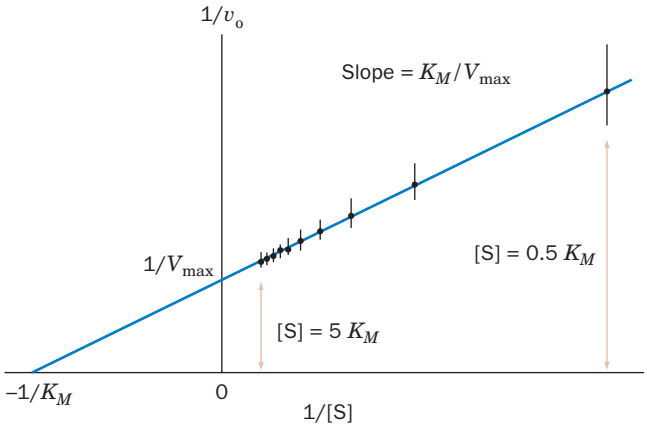


Figure 14-9 **A double-reciprocal (Lineweaver–Burk) plot.** Error bars are ±0.05 V_{max}. The indicated points are the same as those in Fig. 14-8. Note the large effect of small errors at small [S] (large 1/[S]) and the crowding together of points at large [S].
See the Animated Figures

a. k_{cat}/K_M Is a Measure of Catalytic Efficiency

An enzyme's kinetic parameters provide a measure of its catalytic efficiency. We may define the catalytic constant of an enzyme as

$$k_{cat} = \frac{V_{max}}{[E]_T} \qquad [14.27]$$

This quantity is also known as the **turnover number** of an enzyme because it is the number of reaction processes (turnovers) that each active site catalyzes per unit time. The turnover numbers for a selection of enzymes are given in Table 14-1. Note that these quantities vary by over eight orders of magnitude depending on the identity of the enzyme as well as that of its substrate. Equation [14.23] indicates that for the Michaelis–Menten model, $k_{cat} = k_2$. For enzymes with more complicated mechanisms, k_{cat} may be a function of several rate constants.

When [S] << K_M, very little ES is formed. Consequently, [E] ≈ $[E]_T$, so that Eq. [14.22] reduces to a second-order rate equation:

$$v_o \approx \left(\frac{k_2}{K_M}\right)[E]_T[S] \approx \left(\frac{k_{cat}}{K_M}\right)[E][S] \qquad [14.28]$$

k_{cat}/K_M is the apparent second-order rate constant of the enzymatic reaction; the rate of the reaction varies directly with how often enzyme and substrate encounter one another in solution. *The quantity k_{cat}/K_M is therefore a measure of an enzyme's catalytic efficiency.*

b. Some Enzymes Have Attained Catalytic Perfection

Is there an upper limit on enzymatic catalytic efficiency? From Eq. [14.21] we find

$$\frac{k_{cat}}{K_M} = \frac{k_2}{K_M} = \frac{k_1k_2}{k_{-1} + k_2} \qquad [14.29]$$

This ratio is maximal when $k_2 >> k_{-1}$, that is, when the formation of product from the Michaelis complex, ES, is fast compared to its decomposition back to substrate and enzyme. Then $k_{cat}/K_M = k_1$, the second-order rate constant for the formation of ES. The term k_1, of course, can be no greater than the frequency with which enzyme and substrate molecules collide with each other in solution. This **diffusion-controlled limit** is in the range of 10^8 to $10^9\ M^{-1} \cdot s^{-1}$. Thus, enzymes with such values of k_{cat}/K_M must catalyze a reaction almost every time they encounter a substrate molecule. Table 14-1 indicates that several enzymes, namely, catalase, superoxide dismutase, fumarase, acetylcholinesterase, and possibly carbonic anhydrase, have achieved this state of virtual catalytic perfection.

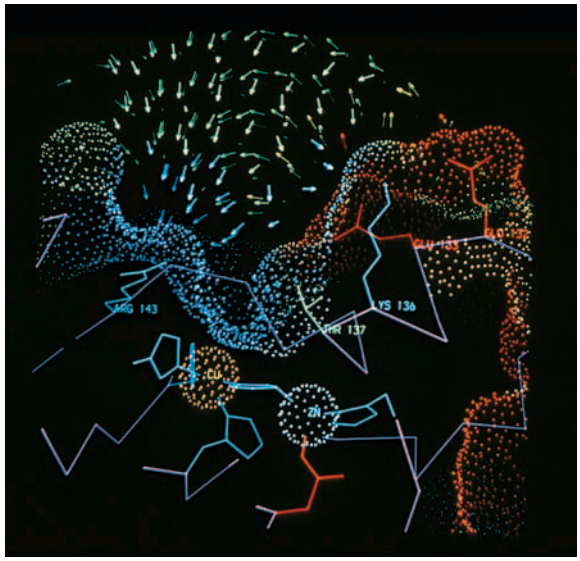


Figure 14-10 **Cross section through the active site of human superoxide dismutase (SOD).** The enzyme binds both a Cu^{2+} and a Zn^{2+} ion (*orange and cyan spheres*). SOD's molecular surface is represented by a dot surface that is colored according to its electrostatic charge, with red most negative, yellow negative, green neutral, cyan positive, and blue most positive. The electrostatic field vectors are represented by similarly colored arrows. Note how this electrostatic field would draw the negatively charged superoxide ion into its binding site, which is located between the Cu^{2+} ion and Arg 143. [Courtesy of Elizabeth Getzoff, The Scripps Research Institute, La Jolla, California.]

Since the active site of an enzyme generally occupies only a small fraction of its total surface area, how can any enzyme catalyze a reaction every time it encounters a substrate molecule? In the case of **superoxide dismutase (SOD),** it appears that the arrangement of charged groups on the enzyme's surface serves to electrostatically guide the charged substrate to the enzyme's active site (Fig. 14-10). [SOD, which is present in nearly all cells, functions to inactivate the highly reactive and therefore destructive **superoxide radical** $O_2^{\cdot -}$ by catalyzing the reaction $2\,O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$; Section 22-4Ch]. Other enzymes, including **acetylcholinesterase** (Section 20-5C), have similar mechanisms to funnel polar substrates to their active sites.

C. *Reversible Reactions*

The Michaelis–Menten model implicitly assumes that enzymatic reverse reactions may be neglected. Yet many enzymatic reactions are highly reversible (have a small free energy of reaction) and therefore have products that back react to form substrates at a significant rate. In this section we therefore relax the Michaelis–Menten restriction of no back reaction and, by doing so, discover some interesting and important kinetic principles.

a. The One-Intermediate Model

Modification of the Michaelis–Menten model to incorporate a back reaction yields the following reaction scheme:

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

(Here ES might just as well be called EP because this model does not specify the nature of the intermediate complex.) The equation describing the kinetic behavior of this model, which is derived in Appendix A of this chapter, is expressed

$$v = \frac{\dfrac{V^f_{\max}[S]}{K^S_M} - \dfrac{V^r_{\max}[P]}{K^P_M}}{1 + \dfrac{[S]}{K^S_M} + \dfrac{[P]}{K^P_M}} \qquad [14.30]$$

where

$$V^f_{\max} = k_2[E]_T \qquad V^r_{\max} = k_{-1}[E]_T$$

$$K^S_M = \frac{k_{-1} + k_2}{k_1} \qquad K^P_M = \frac{k_{-1} + k_2}{k_{-2}}$$

and

$$[E]_T = [E] + [ES]$$

This is essentially a Michaelis–Menten equation that works backwards as well as forwards. Indeed, at $[P] = 0$, that is, when $v = v_o$, this equation becomes the Michaelis–Menten equation.

b. The Haldane Relationship

At equilibrium (which occurs after the reaction has run its course), $v = 0$, so Eq. [14.30], which holds at equilibrium as well as at steady state, can be solved to yield

$$K_{eq} = \frac{[P]_{eq}}{[S]_{eq}} = \frac{V^f_{\max} K^P_M}{V^r_{\max} K^S_M} \qquad [14.31]$$

where $[P]_{eq}$ and $[S]_{eq}$ are the concentrations of P and S at equilibrium. This so-called **Haldane relationship** demonstrates that *the kinetic parameters of a reversible enzymatically catalyzed reaction are not independent of one another. Rather, they are related by the equilibrium constant for the overall reaction, which, of course, is independent of the presence of the enzyme.*

c. Kinetic Data Cannot Unambiguously Establish a Reaction Mechanism

An enzyme that forms a reversible complex with its substrate should likewise form one with its product; that is, it should have a mechanism such as

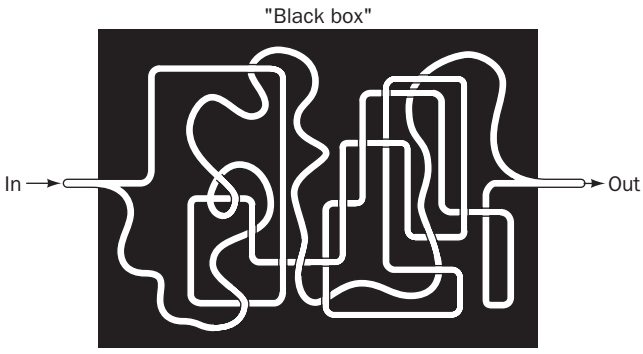
$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} P + E$$

The equation describing the kinetic behavior of this two-intermediate model, whose derivation is analogous to that described in Appendix A for the one-intermediate model,

has a form identical to that of Eq. [14.30]. However, its parameters V^f_{max}, V^r_{max}, K^S_M, and K^P_M are defined in terms of the six kinetic constants of the two-intermediate model rather than the four of the one-intermediate model. In fact, the steady-state rate equations for reversible reactions with three or more intermediates also have this same form but with yet different definitions of the four parameters.

The values of V^f_{max}, V^r_{max}, K^S_M, and K^P_M in Eq. [14.30] can be determined by suitable manipulations of the initial substrate and product concentrations under steady-state conditions. This, however, will not yield the values of the rate constants for our two-intermediate model because there are six such constants and only four equations describing their relationships. Moreover, steady-state kinetic measurements are incapable of distinguishing the number of intermediates in a reversible enzymatic reaction because the form of Eq. [14.30] does not change with the number of intermediates.

The functional identities of the equations describing these reaction schemes may be understood in terms of an analogy between our *n*-intermediate reversible reaction model and a "black box" containing a system of water pipes with one inlet and one drain:



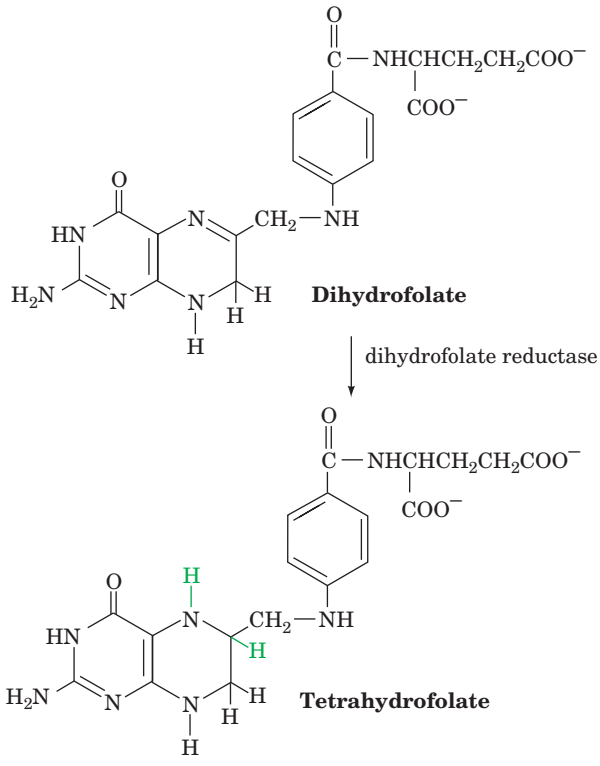
At steady state, that is, after the pipes have filled with water, one can measure the relationship between input pressure and output flow. However, such measurements yield no information concerning the detailed construction of the plumbing connecting the inlet to the drain. This would require additional observations such as opening the black box and tracing the pipes. *Likewise, steady-state kinetic measurements can provide a phenomenological description of enzymatic behavior, but the nature of the intermediates remains indeterminate. Rather, these intermediates must be detected and characterized by independent means such as by spectroscopic analysis.*

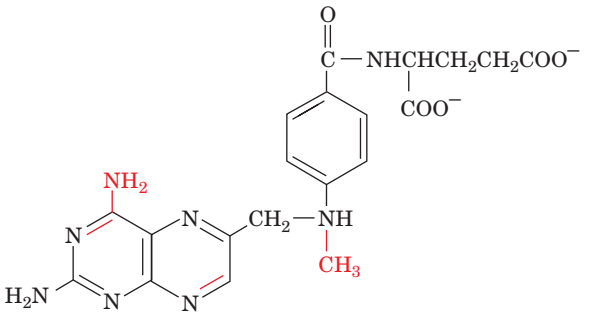
The foregoing discussion brings to light a central principle of kinetic analysis: *The steady-state kinetic analysis of a reaction cannot unambiguously establish its mechanism.* This is because no matter how simple, elegant, or rational a mechanism one postulates that fully accounts for kinetic data, there are an infinite number of alternative mechanisms, perhaps complicated, awkward, and seemingly irrational, that can account for these kinetic data equally well. Usually it is the simpler and more elegant mechanism that turns out to be correct, but this is not always the case. *If, however, kinetic data are not compatible with a given mechanism, then the mechanism must be rejected.* Therefore, although kinetics cannot be used to establish a mechanism unambiguously without confirming data, such as the physical demonstration of an intermediate's existence, the steady-state kinetic analysis of a reaction is of great value because it can be used to eliminate proposed mechanisms.

3 INHIBITION

Many substances alter the activity of an enzyme by combining with it in a way that influences the binding of substrate and/or its turnover number. Substances that reduce an enzyme's activity in this way are known as **inhibitors.**

Many inhibitors are substances that structurally resemble their enzyme's substrate but either do not react or react very slowly compared to substrate. Such inhibitors are commonly used to probe the chemical and conformational nature of a substrate-binding site as part of an effort to elucidate the enzyme's catalytic mechanism. In addition, many enzyme inhibitors are effective chemotherapeutic agents, since an "unnatural" substrate analog can block the action of a specific enzyme. For example, **methotrexate** (also called **amethopterin**) chemically resembles **dihydrofolate.** Methotrexate binds tightly to the enzyme **dihydrofolate reductase,** thereby preventing it from carrying out its normal function, the reduction of dihydrofolate to **tetrahydrofolate,** an essential cofactor in the biosynthesis of the DNA precursor dTMP (Section 28-3Bd):





Methotrexate

dihydrofolate reductase

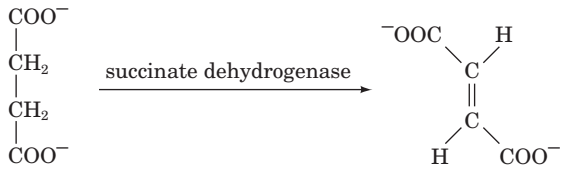
NO REACTION

Rapidly dividing cells, such as cancer cells, which are actively engaged in DNA synthesis, are far more susceptible to methotrexate than are slower growing cells such as those of most normal mammalian tissues. Hence, methotrexate, when administered in proper dosage, kills cancer cells without fatally poisoning the host.

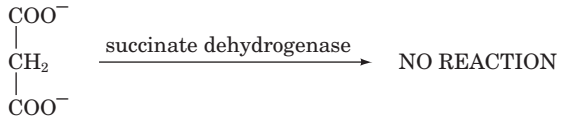
There are various mechanisms through which enzyme inhibitors can act. In this section, we discuss several of the simplest such mechanisms and their effects on the kinetic behavior of enzymes that follow the Michaelis–Menten model.

A. *Competitive Inhibition*

A substance that competes directly with a normal substrate for an enzymatic binding site is known as a **competitive inhibitor.** Such an inhibitor usually resembles the substrate to the extent that it specifically binds to the active site but differs from it so as to be unreactive. Thus methotrexate is a competitive inhibitor of dihydrofolate reductase. Similarly, **succinate dehydrogenase,** a citric acid cycle enzyme that functions to convert **succinate** to **fumarate** (Section 21-3F), is competitively inhibited by **malonate,** which structurally resembles succinate but cannot be dehydrogenated:



Succinate **Fumarate**



Malonate

The effectiveness of malonate in competitively inhibiting succinate dehydrogenase strongly suggests that the enzyme's substrate-binding site is designed to bind both of the substrate's carboxylate groups, presumably through the influence of two appropriately placed positively charged residues.

The general model for competitive inhibition is given by the following reaction scheme:

$$\begin{array}{ccccc} \mathrm{E} + \mathrm{S} & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & \mathrm{ES} & \xrightarrow{k_2} & \mathrm{P} + \mathrm{E} \\ + & & & & \\ \mathrm{I} & & & & \\ K_\mathrm{I} \Big\Updownarrow & & & & \\ \mathrm{EI} + \mathrm{S} & \longrightarrow & \text{NO REACTION} & & \end{array}$$

Here it is assumed that I, the inhibitor, binds reversibly to the enzyme and is in rapid equilibrium with it so that

$$K_\mathrm{I} = \frac{[\mathrm{E}][\mathrm{I}]}{[\mathrm{EI}]} \qquad [14.32]$$

and EI, the enzyme–inhibitor complex, is catalytically inactive. *A competitive inhibitor therefore acts by reducing the concentration of free enzyme available for substrate binding.*

Our goal, as before, is to express v_o in terms of measurable quantities, in this case $[\mathrm{E}]_\mathrm{T}$, [S], and [I]. We begin, as in the derivation of the Michaelis–Menten equation, with the expression for the conservation condition, which must now take into account the existence of EI.

$$[\mathrm{E}]_\mathrm{T} = [\mathrm{E}] + [\mathrm{EI}] + [\mathrm{ES}] \qquad [14.33]$$

The enzyme concentration can be expressed in terms of [ES] by rearranging Eq. [14.17] under the steady-state condition:

$$[\mathrm{E}] = \frac{K_M[\mathrm{ES}]}{[\mathrm{S}]} \qquad [14.34]$$

That of the enzyme–inhibitor complex is found by rearranging Eq. [14.32] and substituting Eq. [14.34] into it:

$$[\mathrm{EI}] = \frac{[\mathrm{E}][\mathrm{I}]}{K_\mathrm{I}} = \frac{K_M[\mathrm{ES}][\mathrm{I}]}{[\mathrm{S}]K_\mathrm{I}} \qquad [14.35]$$

Substituting the latter two results into Eq. [14.33] yields

$$[\mathrm{E}]_\mathrm{T} = [\mathrm{ES}]\left\{\frac{K_M}{[\mathrm{S}]}\left(1 + \frac{\mathrm{I}}{K_\mathrm{I}}\right) + 1\right\}$$

which can be solved for [ES] by rearranging it to

$$[\mathrm{ES}] = \frac{[\mathrm{E}]_\mathrm{T}[\mathrm{S}]}{K_M\left(1 + \dfrac{[\mathrm{I}]}{K_\mathrm{I}}\right) + [\mathrm{S}]}$$

so that, according to Eq. [14.22], the initial velocity is expressed

$$v_\mathrm{o} = k_2[\mathrm{ES}] = \frac{k_2[\mathrm{E}]_\mathrm{T}[\mathrm{S}]}{K_M\left(1 + \dfrac{[\mathrm{I}]}{K_\mathrm{I}}\right) + [\mathrm{S}]} \qquad [14.36]$$

Then defining

$$\alpha = \left(1 + \frac{[\mathrm{I}]}{K_\mathrm{I}}\right) \qquad [14.37]$$

and $V_{max} = k_2[\mathrm{E}]_\mathrm{T}$ as in Eq. [14.23],

$$v_\mathrm{o} = \frac{V_{max}[\mathrm{S}]}{\alpha K_M + [\mathrm{S}]} \qquad [14.38]$$

This is the Michaelis–Menten equation with K_M modulated by α, a function of the inhibitor concentration (which, according to Eq. [14.37], must always be ≥ 1). The value of [S] at $v_\mathrm{o} = V_{max}/2$ is therefore αK_M.

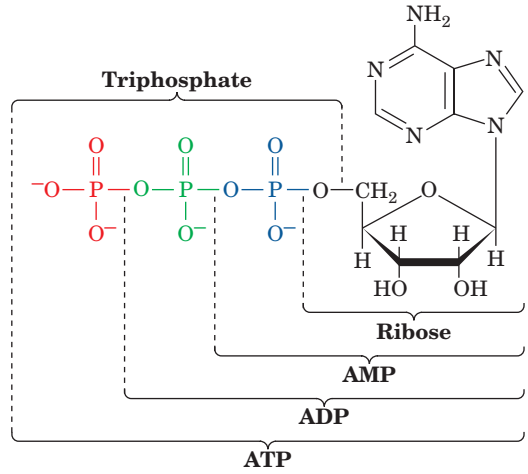
Figure 14-11 shows the hyperbolic plot of Eq. [14.38] for various values of α. Note that as $[\mathrm{S}] \to \infty$, $v_\mathrm{o} \to V_{max}$ for any value of α. The larger the value of α, however, the greater [S] must be to approach V_{max}. Thus, the inhibitor does not affect the turnover number of the enzyme. Rather, the presence of I has the effect of making [S] appear more dilute than it actually is, or alternatively, making K_M appear larger than it really is. Conversely, increasing [S] shifts the substrate-binding equilibrium toward ES. Hence, there is true competition between I and S for the enzyme's substrate-binding site; their binding is mutually exclusive.

Recasting Eq. [14.38] in the double-reciprocal form yields

$$\frac{1}{v_\mathrm{o}} = \left(\frac{\alpha K_M}{V_{max}}\right)\frac{1}{[\mathrm{S}]} + \frac{1}{V_{max}} \qquad [14.39]$$

A plot of this equation is linear and has a slope of $\alpha K_M/V_{max}$, a $1/[\mathrm{S}]$ intercept of $-1/\alpha K_M$, and a $1/v_\mathrm{o}$ intercept of $1/V_{max}$ (Fig. 14-12). *The double-reciprocal plots for a competitive inhibitor at various concentrations of I intersect at $1/V_{max}$ on the $1/v_\mathrm{o}$ axis; this is diagnostic for competitive inhibition as compared with other types of inhibition (Sections 14-3B and 14-3C).*

By determining the values of α at different inhibitor concentrations, the value of K_I can be found from Eq. [14.37]. In this way, competitive inhibitors can be used to probe the structural nature of an active site. For example, to ascertain the importance of the various segments of an ATP molecule



for binding to the active site of an ATP-requiring enzyme, one might determine the K_I, say, for ADP, AMP (adenosine monophosphate), ribose, triphosphate ion, etc. Since many of these ATP components are catalytically inactive, inhibition studies are the most convenient means of monitoring their binding to the enzyme.

If the inhibitor binds irreversibly to the enzyme, the inhibitor is classified as an **inactivator,** as is any agent that somehow inactivates the enzyme. Inactivators truly reduce the effective level of $[\mathrm{E}]_\mathrm{T}$ at all values of [S]. Reagents that modify specific amino acid residues can act in this manner.

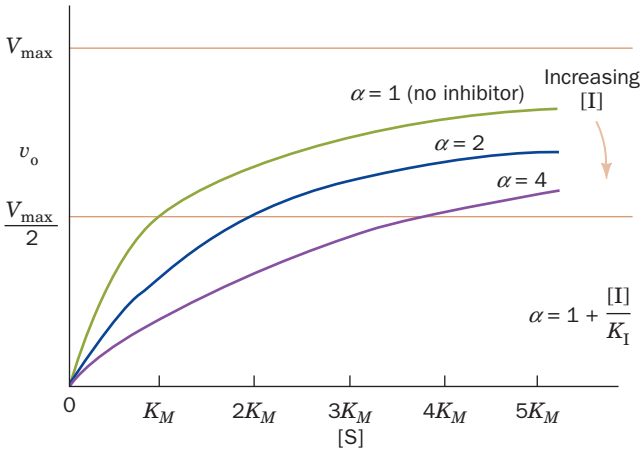


Figure 14-11 **Competitive inhibition.** Plot of the initial velocity v_o of a simple Michaelis–Menten reaction versus the substrate concentration [S] in the presence of different concentrations of a competitive inhibitor.

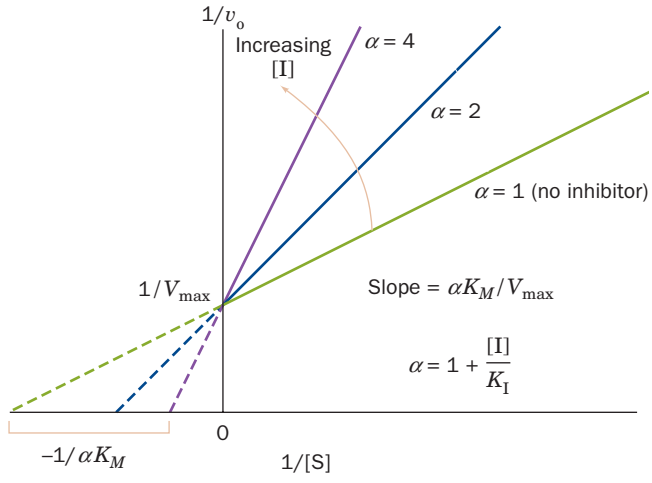


Figure 14-12 **Lineweaver–Burk plot of the competitively inhibited Michaelis–Menten enzyme described by Fig. 14-11.** Note that all lines intersect on the $1/v_\mathrm{o}$ axis at $1/V_{max}$. **See the Animated Figures**

B. *Uncompetitive Inhibition*

In **uncompetitive inhibition,** the inhibitor binds directly to the enzyme–substrate complex but not to the free enzyme:

$$\begin{array}{ccccc} \mathrm{E} + \mathrm{S} & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & \mathrm{ES} & \xrightarrow{k_2} & \mathrm{P} + \mathrm{E} \\ & & + & & \\ & & \mathrm{I} & & \\ & & K_\mathrm{I}' \Big\| & & \\ & & \mathrm{ESI} & \longrightarrow & \text{NO REACTION} \end{array}$$

The inhibitor-binding step, which has the dissociation constant

$$K_\mathrm{I}' = \frac{[\mathrm{ES}][\mathrm{I}]}{[\mathrm{ESI}]} \qquad [14.40]$$

is assumed to be at equilibrium. The binding of the uncompetitive inhibitor, which need not resemble the substrate, is envisioned to cause structural distortion of the active site, thereby rendering the enzyme catalytically inactive. (If the inhibitor binds to enzyme alone, it does so without affecting its affinity for substrate.)

The Michaelis–Menten equation for uncompetitive inhibition, which is derived in Appendix B of this chapter, is

$$v_\mathrm{o} = \frac{V_\mathrm{max}[\mathrm{S}]}{K_M + \alpha'[\mathrm{S}]} \qquad [14.41]$$

where

$$\alpha' = 1 + \frac{[\mathrm{I}]}{K_\mathrm{I}'} \qquad [14.42]$$

Inspection of this equation indicates that *at high values of [S], v_o asymptotically approaches V_{max}/α', so that, in contrast to competitive inhibition, the effects of uncompetitive inhibition on V_{max} are not reversed by increasing the substrate concentration.* However, at low substrate concentrations, that is, when [S] << K_M, the effect of an uncompetitive inhibitor becomes negligible, again the opposite behavior of a competitive inhibitor.

When cast in the double-reciprocal form, Eq. [14.41] becomes

$$\frac{1}{v_\mathrm{o}} = \left(\frac{K_M}{V_\mathrm{max}}\right)\frac{1}{[\mathrm{S}]} + \frac{\alpha'}{V_\mathrm{max}} \qquad [14.43]$$

The Lineweaver–Burk plot for uncompetitive inhibition is linear with slope K_M/V_{max}, as in the uninhibited reaction, and with $1/v_o$ and 1/[S] intercepts of α'/V_{max} and $-\alpha'/K_M$, respectively. *A series of Lineweaver–Burk plots at various uncompetitive inhibitor concentrations consists of a family of parallel lines* (Fig. 14-13). *This is diagnostic for uncompetitive inhibition.*

Uncompetitive inhibition requires that the inhibitor affect the catalytic function of the enzyme but not its substrate binding. For single-substrate enzymes it is difficult to conceive of how this could happen with the exception of small inhibitors such as protons (see Section 14-4) or metal ions. As we discuss in Section 14-5C, however, uncompetitive inhibition is important for multisubstrate enzymes.

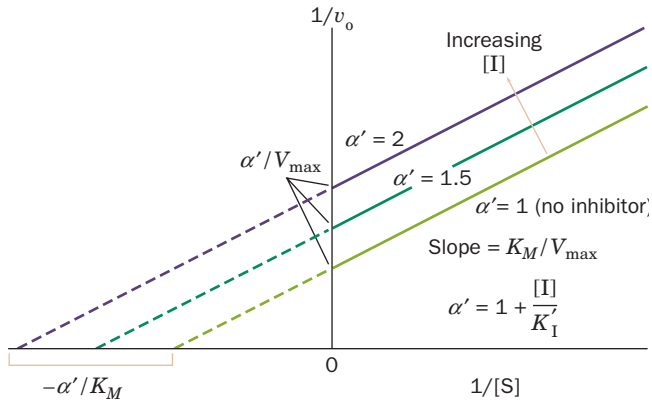


Figure 14-13 **Lineweaver–Burk plot of a simple Michaelis–Menten enzyme in the presence of uncompetitive inhibitor.** Note that all lines have identical slopes of K_M/V_{max}.
See the Animated Figures

C. *Mixed Inhibition*

If both the enzyme and the enzyme–substrate complex bind inhibitor, the following model results:

$$\begin{array}{ccccc} \mathrm{E} + \mathrm{S} & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & \mathrm{ES} & \xrightarrow{k_2} & \mathrm{P} + \mathrm{E} \\ + & & + & & \\ \mathrm{I} & & \mathrm{I} & & \\ K_\mathrm{I} \Big\| & & K_\mathrm{I}' \Big\| & & \\ \mathrm{EI} & & \mathrm{ESI} & \longrightarrow & \text{NO REACTION} \end{array}$$

Both of the inhibitor-binding steps are assumed to be at equilibrium but with different dissociation constants:

$$K_\mathrm{I} = \frac{[\mathrm{E}][\mathrm{I}]}{[\mathrm{EI}]} \quad \text{and} \quad K_\mathrm{I}' = \frac{[\mathrm{ES}][\mathrm{I}]}{[\mathrm{ESI}]} \qquad [14.44]$$

This phenomenon is alternatively known as **mixed inhibition** or **noncompetitive inhibition.** Presumably a mixed inhibitor binds to enzyme sites that participate in both substrate binding and catalysis.

The Michaelis–Menten equation for mixed inhibition, which is derived in Appendix C of this chapter, is

$$v_\mathrm{o} = \frac{V_\mathrm{max}[\mathrm{S}]}{\alpha K_M + \alpha'[\mathrm{S}]} \qquad [14.45]$$

where α and α' are defined in Eqs. [14.37] and [14.42], respectively. It can be seen from Eq. [14.45] that the name "mixed inhibition" arises from the fact that the denominator has the factor α multiplying K_M as in competitive inhibition (Eq. [14.38]) and the factor α' multiplying [S] as in uncompetitive inhibition (Eq. [14.41]). Mixed inhibitors

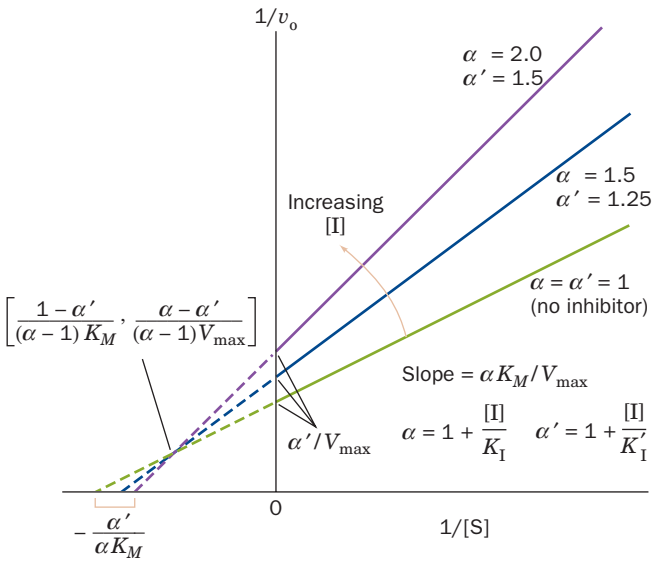


Figure 14-14 **Lineweaver–Burk plot of a simple Michaelis–Menten enzyme in the presence of a mixed inhibitor.** Note that the lines all intersect to the left of the $1/v_o$ axis. The coordinates of this intersection point are given in brackets. When $K_I = K'_I$, $\alpha = \alpha'$ and the lines intersect on the 1/[S] axis at $-1/K_M$.
See the Animated Figures

are therefore effective at both high and low substrate concentrations.

The Lineweaver–Burk equation for mixed inhibition is

$$\frac{1}{v_o} = \left(\frac{\alpha K_M}{V_{max}}\right)\frac{1}{[S]} + \frac{\alpha'}{V_{max}} \qquad [14.46]$$

The plot of this equation consists of lines that have slope $\alpha K_M/V_{max}$ with a $1/v_o$ intercept of α'/V_{max} and a 1/[S] intercept of $-\alpha'/\alpha K_M$ (Fig. 14-14). Algebraic manipulation of Eq. [14.46] for different values of [I] reveals that this equation describes a family of lines that intersect to the left of the $1/v_o$ axis (Fig. 14-14). For the special case in which $K_I = K'_I$ ($\alpha = \alpha'$), the intersection is, in addition, on the 1/[S] axis, a situation which, in an ambiguity of nomenclature, is sometimes described as noncompetitive inhibition.

Table 14-2 provides a summary of the preceding results concerning the inhibition of simple Michaelis–Menten enzymes. The quantities K_M^{app} and V_{max}^{app} are the "apparent" values of K_M and V_{max} that would actually be observed in the presence of inhibitor for the Michaelis–Menten equation describing the inhibited enzymes.

4 EFFECTS OF pH

Enzymes, being proteins, have properties that are quite pH sensitive. Most proteins, in fact, are active only within a narrow pH range, typically 5 to 9. This is a result of the effects of pH on a combination of factors: (1) the binding of substrate to enzyme, (2) the catalytic activity of the enzyme, (3) the ionization of substrate, and (4) the variation of protein structure (usually significant only at extremes of pH).

a. pH Dependence of Simple Michaelis–Menten Enzymes

The initial rates for many enzymatic reactions exhibit bell-shaped curves as a function of pH (e.g., Fig. 14-15). These curves reflect the ionizations of certain amino acid residues that must be in a specific ionization state for enzyme activity. The following model can account for such pH effects.

$$\begin{array}{ccc} E^- & & ES^- \\ K_{E2}\Big\| H^+ & & K_{ES2}\Big\| H^+ \\ EH + S & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & ESH \xrightarrow{k_2} P + EH \\ K_{E1}\Big\| H^+ & & K_{ES1}\Big\| H^+ \\ EH_2^+ & & ESH_2^+ \end{array}$$

In this expansion of the simple one substrate–no back reaction mechanism, it is assumed that only EH and ESH are catalytically active.

The Michaelis–Menten equation for this model, which is derived in Appendix D, is

$$v_o = \frac{V'_{max}[S]}{K'_M + [S]} \qquad [14.47]$$

Table 14-2 **Effects of Inhibitors on the Parameters of the Michaelis–Menten Equation[a]**

Type of Inhibition	V_{max}^{app}	K_M^{app}
None	V_{max}	K_M
Competitive	V_{max}	αK_M
Uncompetitive	V_{max}/α'	K_M/α'
Mixed	V_{max}/α'	$\alpha K_M/\alpha'$

[a] $\alpha = 1 + \frac{[I]}{K_I}$ and $\alpha' = 1 + \frac{[I]}{K'_I}$.

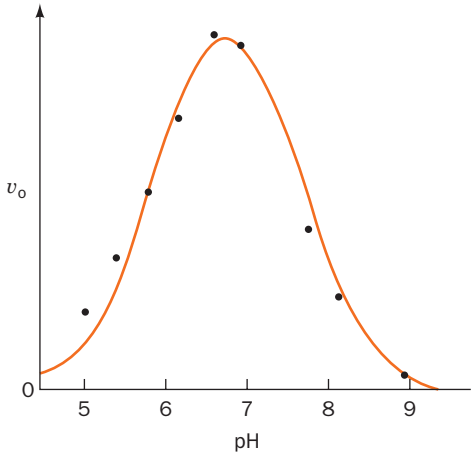


Figure 14-15 **Effect of pH on the initial rate of the reaction catalyzed by the enzyme fumarase.** [After Tanford, C., *Physical Chemistry of Macromolecules, p.* 647, Wiley (1961).]

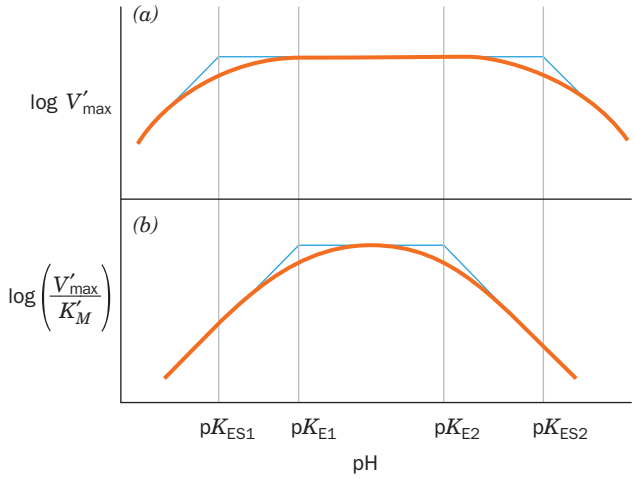


Figure 14-16 **The pH dependence of (*a*) log V'_{max} and (*b*) log (V'_{max}/K'_M).** The light blue lines indicate how the values of the molecular ionization constants can be determined by graphical extrapolation.

Here the apparent Michaelis–Menten parameters are defined

$$V'_{max} = V_{max}/f_2 \qquad \text{and} \qquad K'_M = K_M(f_1/f_2)$$

where

$$f_1 = \frac{[H^+]}{K_{E1}} + 1 + \frac{K_{E2}}{[H^+]}$$

$$f_2 = \frac{[H^+]}{K_{ES1}} + 1 + \frac{K_{ES2}}{[H^+]}$$

and V_{max} and K_M refer to the active forms of the enzyme, EH and ESH. Note that at any given pH, Eq. [14.47] behaves as a simple Michaelis–Menten equation, but because of the pH dependence of f_1 and f_2, v_o varies with pH in a bell-shaped manner (e.g., Fig. 14-15).

b. Evaluation of Ionization Constants

The ionization constants of enzymes that obey Eq. [14.47] can be evaluated by the analysis of the curves of log V'_{max} versus pH, which provides values of K_{ES1} and K_{ES2} (Fig. 14-16*a*), and of log (V'_{max}/K'_M) versus pH, which yields K_{E1} and K_{E2} (Fig. 14-16*b*). This, of course, entails the determination of the enzyme's Michaelis–Menten parameters at each of a series of different pH's.

The measured pK's often provide valuable clues as to the identities of the amino acid residues essential for enzymatic activity. For example, a measured pK of ~4 suggests that an Asp or Glu residue is essential to the enzyme. Similarly, pK's of ~6 or ~10 suggest the participation of a His or a Lys residue, respectively. However, a given acid–base group may vary by as much as several pH units from its expected value as a consequence of the electrostatic influence of nearby charged groups, as well as of the proximity of regions of low polarity. For example, the carboxylate group of a Glu residue forming a salt bridge with a Lys residue is stabilized by the nearby positive charge and therefore has a lower pK than it would otherwise have; that is, it is more difficult to protonate. Conversely, a carboxylate group immersed in a region of low polarity is less acidic than normal because it attracts protons more strongly than if it were in a region of higher polarity. The identification of a kinetically characterized pK with a particular amino acid residue must therefore be verified by other types of measurements such as the use of group-specific reagents to inactivate a putative essential residue.

5 BISUBSTRATE REACTIONS

We have heretofore been concerned with reactions involving enzymes that require only a single substrate. Yet enzymatic reactions involving two substrates and yielding two products

$$A + B \overset{E}{\rightleftharpoons} P + Q$$

account for ~60% of known biochemical reactions. Almost all of these so-called **bisubstrate reactions** are either **transferase** reactions in which the enzyme catalyzes the transfer of a specific functional group, X, from one of the substrates to the other:

$$P{-}X + B \overset{E}{\rightleftharpoons} P + B{-}X$$

or oxidation–reduction reactions in which reducing equivalents are transferred between the two substrates. For example, the hydrolysis of a peptide bond by trypsin (Section 7-1Da) is the transfer of the peptide carbonyl group from

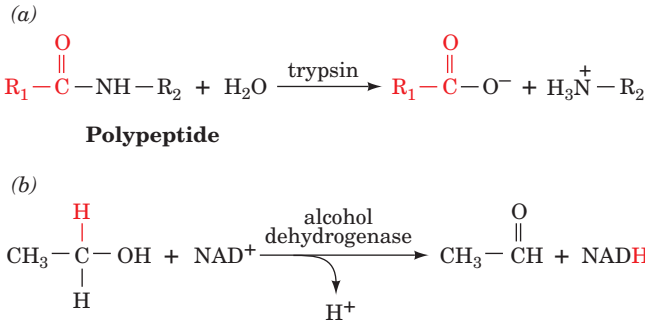


Figure 14-17 **Some bisubstrate reactions.** (*a*) In the peptide hydrolysis reaction catalyzed by trypsin, the peptide carbonyl group, with its pendent polypeptide chain, is transferred from the peptide nitrogen atom to a water molecule. (*b*) In the alcohol dehydrogenase reaction, a hydride ion is formally transferred from ethanol to NAD^+.

the peptide nitrogen atom to water (Fig. 14-17*a*). Similarly, in the alcohol dehydrogenase reaction (Section 13-2A), a hydride ion is formally transferred from ethanol to NAD^+ (Fig. 14-17*b*). Although such bisubstrate reactions could, in principle, occur through a vast variety of mechanisms, only a few types are commonly observed.

A. *Terminology*

We shall follow the nomenclature system introduced by W.W. Cleland for representing enzymatic reactions:

1. Substrates are designated by the letters A, B, C, and D *in the order that they add to the enzyme.*

2. Products are designated P, Q, R, and S *in the order that they leave the enzyme.*

3. Stable enzyme forms are designated E, F, and G with E being the free enzyme, if such distinctions can be made. A stable enzyme form is defined as one that by itself is incapable of converting to another stable enzyme form (see below).

4. The numbers of reactants and products in a given reaction are specified, in order, by the terms **Uni** (one), **Bi** (two), **Ter** (three), and **Quad** (four). A reaction requiring one substrate and yielding three products is designated a Uni Ter reaction. In this section, we shall be concerned with reactions that require two substrates and yield two products, that is, Bi Bi reactions. Keep in mind, however, that there are numerous examples of even more complex reactions.

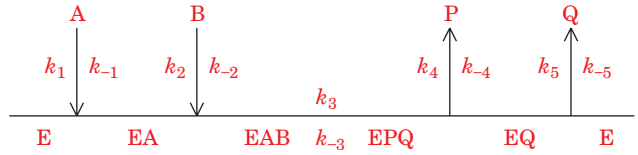
a. Types of Bi Bi Reactions

Enzyme-catalyzed group-transfer reactions fall under two major mechanistic classifications:

1. Sequential Reactions: *Reactions in which all substrates must combine with the enzyme before a reaction can occur and products can be released are known as* ***Sequential reactions.*** In such reactions, the group being transferred, X, is directly passed from A (= P—X) to B, yielding P and Q (= B—X). Hence, such reactions are also called **single-displacement reactions.**

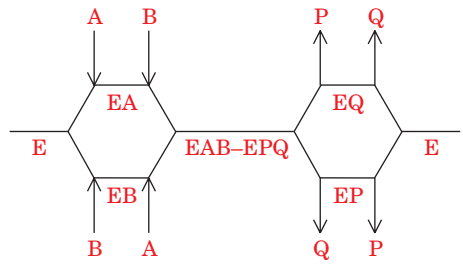
Sequential reactions can be subclassified into those with a compulsory order of substrate addition to the enzyme, which are said to have an **Ordered mechanism,** and those with no preference for the order of substrate addition, which are described as having a **Random mechanism.** In the Ordered mechanism, the binding of the first substrate is apparently required for the enzyme to form the binding site for the second substrate, whereas for the Random mechanism, both binding sites are present on the free enzyme.

Let us describe enzymatic reactions using Cleland's shorthand notation. The enzyme is represented by a horizontal line and successive additions of substrates and release of products are denoted by vertical arrows. Enzyme forms are placed under the line and rate constants, if given, are to the left of the arrow or on top of the line for forward reactions. An **Ordered Bi Bi** reaction is represented:



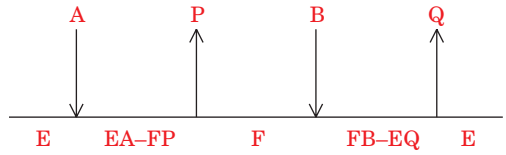
where A and B are said to be the **leading** and **following** substrates, respectively. Here, only minimal details are given concerning the interconversions of intermediate enzyme forms because, as we have seen for reversible single-substrate enzymes, steady-state kinetic measurements provide no information concerning the number of intermediates in a given reaction step. Many NAD^+- and $NADP^+$-requiring dehydrogenases follow an Ordered Bi Bi mechanism in which the coenzyme is the leading reactant.

A **Random Bi Bi** reaction is diagrammed:



Some dehydrogenases and kinases operate through Random Bi Bi mechanisms.

2. Ping Pong Reactions: *Mechanisms in which one or more products are released before all substrates have been added are known as* ***Ping Pong reactions.*** The **Ping Pong Bi Bi** reaction is represented by



In it, a functional group X of the first substrate A (= P—X) is displaced from the substrate by the enzyme E to yield the first product P and a stable enzyme form F (= E—X) in which X is tightly (often covalently) bound to the enzyme (Ping). In the second stage of the reaction, X is displaced from the enzyme by the second substrate B to yield the second product Q (= B—X), thereby regenerating the original form of the enzyme, E (Pong). Such reactions are therefore also known as **double-displacement reactions.** *Note that in Ping Pong Bi Bi reactions, the substrates A and B do not encounter one another on the surface of the enzyme.* Many enzymes, including chymotrypsin (Section 15-3), transaminases (Section 26-1A), and some flavoenzymes, react with Ping Pong mechanisms.

B. *Rate Equations*

Steady-state kinetic measurements can be used to distinguish among the foregoing bisubstrate mechanisms. In order to do so, one must first derive their rate equations. This can be done in much the same manner as for single-substrate enzymes, that is, solving a set of simultaneous linear equations consisting of an equation expressing the steady-state condition for each kinetically distinct enzyme complex plus one equation representing the conservation condition for the enzyme. This, of course, is a more complex undertaking for bisubstrate enzymes than it is for single-substrate enzymes.

The rate equations for the above described bisubstrate mechanisms in the absence of products are given below in double-reciprocal form.

a. Ordered Bi Bi

$$\frac{1}{v_o} = \frac{1}{V_{max}} + \frac{K_M^A}{V_{max}[A]} + \frac{K_M^B}{V_{max}[B]} + \frac{K_S^A K_M^B}{V_{max}[A][B]} \qquad [14.48]$$

b. Rapid Equilibrium Random Bi Bi

The rate equation for the general Random Bi Bi reaction is quite complicated. However, in the special case that both substrates are in rapid and independent equilibrium with the enzyme, that is, when the EAB–EPQ interconversion is rate determining, the initial rate equation reduces to the following relatively simple form. This mechanism is known as the **Rapid Equilibrium Random Bi Bi** mechanism:

$$\frac{1}{v_o} = \frac{1}{V_{max}} + \frac{K_S^A K_M^B}{V_{max} K_S^B [A]} + \frac{K_M^B}{V_{max}[B]} + \frac{K_S^A K_M^B}{V_{max}[A][B]} \qquad [14.49]$$

c. Ping Pong Bi Bi

$$\frac{1}{v_o} = \frac{K_M^A}{V_{max}[A]} + \frac{K_M^B}{V_{max}[B]} + \frac{1}{V_{max}} \qquad [14.50]$$

d. Physical Significance of the Bisubstrate Kinetic Parameters

The kinetic parameters in the equations describing bisubstrate reactions have meanings similar to those for single-substrate reactions. V_{max} is the maximal velocity of the enzyme obtained when both A and B are present at saturating concentrations, K_M^A and K_M^B are the respective concentrations of A and B necessary to achieve $\frac{1}{2}V_{max}$ in the presence of a saturating concentration of the other, and K_S^A and K_S^B are the respective dissociation constants of A and B from the enzyme, E.

C. *Differentiating Bisubstrate Mechanisms*

One can discriminate between Ping Pong and Sequential mechanisms from their contrasting properties in linear plots such as those of the Lineweaver–Burk type.

a. Diagnostic Plot for Ping Pong Bi Bi Reactions

A plot of $1/v_o$ versus 1/[A] at constant [B] for Eq. [14.50] yields a straight line of slope K_M^A/V_{max} and an intercept on the $1/v_o$ axis equal to the last two terms in Eq. [14.50]. Since the slope is independent of [B], such plots for different values of [B] yield a family of parallel lines (Fig. 14-18). A plot of $1/v_o$ versus 1/[B] for different values of [A] likewise yields a family of parallel lines. *Such parallel lines are diagnostic for a Ping Pong mechanism.*

b. Diagnostic Plot for Sequential Bi Bi Reactions

The equations representing the Ordered Bi Bi mechanism (Eq. [14.48]) and the Rapid Equilibrium Random Bi

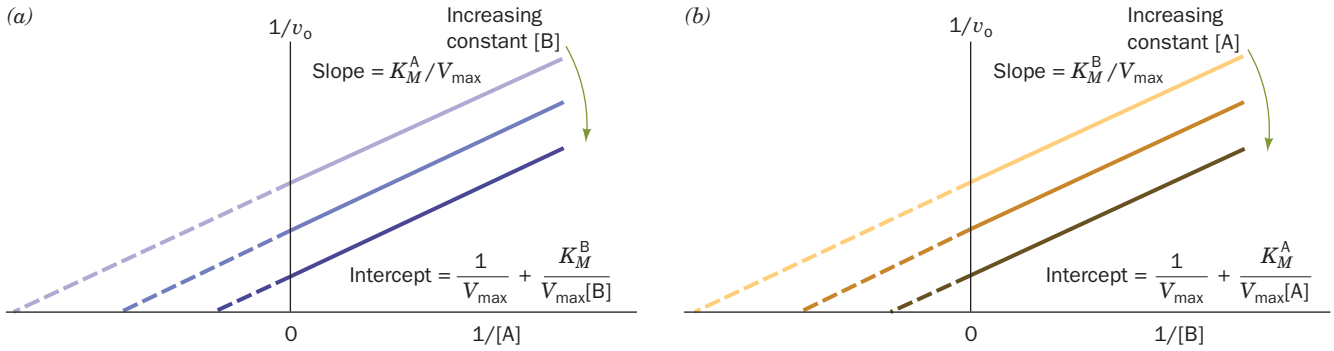


Figure 14-18 **Double-reciprocal plots for an enzymatic reaction with a Ping Pong Bi Bi mechanism.** (*a*) Plots of $1/v_o$ versus 1/[A] at various constant concentrations of B. (*b*) Plots of $1/v_o$ versus 1/[B] at various constant concentrations of A.

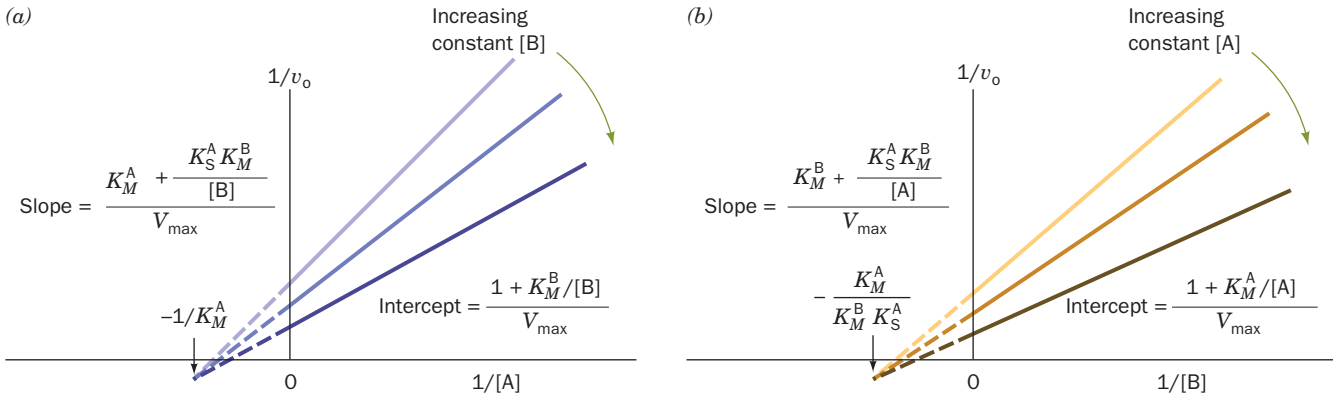


Figure 14-19 **Double-reciprocal plots of an enzymatic reaction with a Sequential Bi Bi mechanism.** (*a*) Plots of $1/v_o$ versus 1/[A] at various constant concentrations of B. (*b*) Plots of $1/v_o$ versus 1/[B] at various constant concentrations of A. The corresponding plots for Rapid Equilibrium Random Bi Bi reactions have identical appearances; their lines all intersect to the left of the $1/v_o$ axis.

Bi mechanism (Eq. [14.49]) have identical functional dependence on [A] and [B].

Equation [14.48] can be rearranged to

$$\frac{1}{v_o} = \frac{K_M^A}{V_{max}}\left(1 + \frac{K_S^A K_M^B}{K_M^A[B]}\right)\frac{1}{[A]} + \frac{1}{V_{max}}\left(1 + \frac{K_M^B}{[B]}\right) \qquad [14.51]$$

Thus plotting $1/v_o$ versus 1/[A] for constant [B] yields a linear plot with a slope equal to the coefficient of 1/[A] and an intercept on the $1/v_o$ axis equal to the second term of Eq. [14.51] (Fig. 14-19*a*). Alternatively, Eq. [14.48] can be rearranged to

$$\frac{1}{v_o} = \frac{K_M^B}{V_{max}}\left(1 + \frac{K_S^A}{[A]}\right)\frac{1}{[B]} + \frac{1}{V_{max}}\left(1 + \frac{K_M^A}{[A]}\right) \qquad [14.52]$$

which yields a linear plot of $1/v_o$ versus 1/[B] for constant [A] with a slope equal to the coefficient of 1/[B] and an intercept on the $1/v_o$ axis equal to the second term of Eq. [14.52] (Fig. 14-19*b*). *The characteristic feature of these plots, which is indicative of a Sequential mechanism, is that the lines intersect to the left of the $1/v_o$ axis.*

C. Differentiating Random and Ordered Sequential Mechanisms

The Ordered Bi Bi mechanism may be experimentally distinguished from the Random Bi Bi mechanism through **product inhibition studies.** If only one product of the reaction, P or Q, is added to the reaction mixture, the reverse reaction still cannot occur. Nevertheless, by binding to the enzyme, this product will inhibit the forward reaction. For an Ordered Bi Bi reaction, Q (= B—X, the second product to be released) directly competes with A (= P—X, the leading substrate) for binding to E and hence is a competitive inhibitor of A when [B] is fixed (the presence of X in Q = B—X interferes with the binding of A = P—X). However, since B combines with EA, not E, Q is a mixed inhibitor of B when [A] is fixed (Q interferes with both the binding of B to enzyme and with the catalysis of the reaction). Similarly, P, which combines only with EQ, is a mixed inhibitor of A when [B] is held constant and of B when [A] is held constant. In contrast, in a Rapid Equilibrium Bi Bi reaction, since both products as well as both substrates can combine directly with E, both P and Q are competitive inhibitors of A when [B] is constant and of B when [A] is constant. These product inhibition patterns are summarized in Table 14-3.

D. *Isotope Exchange*

Mechanistic conclusions based on kinetic analyses alone are fraught with uncertainties and are easily confounded by inaccurate experimental data. A particular mechanism for an enzyme is therefore greatly corroborated if the mechanism can be shown to conform to experimental criteria other than kinetic analysis.

Sequential (single-displacement) and Ping Pong (double-displacement) bisubstrate mechanisms may be differentiated through the use of ***isotope exchange*** *studies.* Double-displacement reactions are capable of exchanging an isotope from the first product P back to the first substrate A in

Table 14-3 **Patterns of Product Inhibition for Sequential Bisubstrate Mechanisms**

Mechanism	Product Inhibitor	[A] Variable	[B] Variable
Ordered Bi Bi	P	Mixed	Mixed
	Q	Competitive	Mixed
Rapid Equilibrium Random Bi Bi	P	Competitive	Competitive
	Q	Competitive	Competitive

the absence of the second substrate. Consider an overall Ping Pong reaction catalyzed by the bisubstrate enzyme E

$$P{-}X + B \overset{E}{\rightleftharpoons} P + B{-}X$$

in which, as usual, A = P—X, Q = B—X, and X is the group that is transferred from one substrate to the other in the course of the reaction. Only the first step of the reaction can take place in the absence of B. If a small amount of isotopically labeled P, denoted P*, is added to this reaction mixture then, in the reverse reaction, P*—X will form:

Forward reaction $\quad E + P{-}X \longrightarrow E{-}X + P$

Reverse reaction $\quad E{-}X + P^* \longrightarrow E + P^*{-}X$

that is, isotopic exchange will occur.

In contrast, let us consider the first step of a Sequential reaction. Here a noncovalent enzyme–substrate complex forms:

$$E + P{-}X \rightleftharpoons E \cdot P{-}X$$

Addition of P* cannot result in an exchange reaction because no covalent bonds are broken in the formation of E · P—X; that is, there is no P released from the enzyme to exchange with P*. The demonstration of isotopic exchange for a bisubstrate enzyme is therefore convincing evidence favoring a Ping Pong mechanism.

a. Isotope Exchange in Sucrose Phosphorylase and Maltose Phosphorylase

The enzymes **sucrose phosphorylase** and **maltose phosphorylase** provide two clear-cut examples of how enzymatically catalyzed isotopic exchange reactions are used to differentiate kinetic mechanisms. Sucrose phosphorylase catalyzes the overall reaction

Glucose—fructose + phosphate
Sucrose
⇅ E
Glucose-1-phosphate + fructose

If the enzyme is incubated with sucrose and isotopically labeled fructose in the absence of phosphate, it is observed that the label passes into the sucrose:

Glucose—fructose + fructose*
Sucrose
⇅ E
Glucose—fructose* + fructose

For the reverse reaction, if the enzyme is incubated with glucose-1-phosphate and ^{32}P-labeled phosphate, this label exchanges into the glucose-1-phosphate:

Glucose-1-phosphate + phosphate*
⇅ E
Glucose-1-phosphate* + phosphate

These observations indicate that a tight glucosyl–enzyme complex is formed with the release of fructose, thereby establishing that the sucrose phosphorylase reaction occurs via a Ping Pong mechanism. This finding has been conclusively corroborated by the isolation and characterization of the glucosyl–enzyme complex.

The enzyme **maltose phosphorylase** catalyzes a similar overall reaction:

Glucose—glucose + phosphate
Maltose
⇅ E
Glucose-1-phosphate + glucose

In contrast to sucrose phosphorylase, however, it does not catalyze isotopic exchange between glucose-1-phosphate and [^{32}P]phosphate or between maltose and [^{14}C]glucose. Likewise, a glucosyl–enzyme complex has not been detected. This evidence is consistent with maltose phosphorylase having a sequential mechanism.

APPENDIX Derivations of Michaelis–Menten Equation Variants

A. *The Michaelis–Menten Equation for Reversible Reactions–Equation [14.30]*

The conservation condition for the reversible reaction with one intermediate (Section 14-2Ca) is

$$[E]_T = [E] + [ES] \qquad [14.A1]$$

The steady-state condition (as well as the equilibrium condition) is

$$\frac{d[ES]}{dt} = k_1[E][S] + k_{-2}[E][P] - (k_{-1} + k_2)[ES] = 0 \qquad [14.A2]$$

so that

$$[E] = \left(\frac{k_{-1} + k_2}{k_1[S] + k_{-2}[P]}\right)[ES] \qquad [14.A3]$$

Substituting this result into Eq. [14.A1] yields

$$[E]_T = \left(\frac{k_{-1} + k_2}{k_1[S] + k_{-2}[P]} + 1\right)[ES] \qquad [14.A4]$$

The velocity of the reaction is expressed

$$v = -\frac{d[S]}{dt} = k_1[E][S] - k_{-1}[ES] \qquad [14.A5]$$

which can be combined with Eq. [14.A3] to give

$$v = \left(\frac{k_1[\mathrm{S}](k_{-1} + k_2)}{k_1[\mathrm{S}] + k_{-2}[\mathrm{P}]} - k_{-1}\right)[\mathrm{ES}] \qquad [14.A6]$$

which, in turn, is combined with Eq. [14.A4] to yield

$$v = \left(\frac{k_1k_2[\mathrm{S}] - k_{-1}k_{-2}[\mathrm{P}]}{k_{-1} + k_2 + k_1[\mathrm{S}] + k_{-2}[\mathrm{P}]}\right)[\mathrm{E}]_\mathrm{T} \quad [14.A7]$$

Dividing the numerator and denominator of this equation by $(k_{-1} + k_2)$ results in

$$v = \left(\frac{k_2\left(\frac{k_1}{k_{-1} + k_2}\right)[\mathrm{S}] - k_{-1}\left(\frac{k_{-2}}{k_{-1} + k_2}\right)[\mathrm{P}]}{1 + \left(\frac{k_1}{k_{-1} + k_2}\right)[\mathrm{S}] + \left(\frac{k_{-2}}{k_{-1} + k_2}\right)[\mathrm{P}]}\right)[\mathrm{E}]_\mathrm{T} \quad [14.A8]$$

Then, if we define the following parameters analogously with the constants of the Michaelis–Menten equation (Eqs. [14.23] and [14.21]),

$$V^f_{\max} = k_2[\mathrm{E}]_\mathrm{T} \qquad V^r_{\max} = k_{-1}[\mathrm{E}]_\mathrm{T}$$

$$K^\mathrm{S}_M = \frac{k_{-1} + k_2}{k_1} \qquad K^\mathrm{P}_M = \frac{k_{-1} + k_2}{k_{-2}}$$

we obtain the Michaelis–Menten equation for a reversible one-intermediate reaction:

$$v = \frac{\frac{V^f_{\max}[\mathrm{S}]}{K^\mathrm{S}_M} - \frac{V^r_{\max}[\mathrm{P}]}{K^\mathrm{P}_M}}{1 + \frac{[\mathrm{S}]}{K^\mathrm{S}_M} + \frac{[\mathrm{P}]}{K^\mathrm{P}_M}} \qquad [14.30]$$

B. *Michaelis–Menten Equation for Uncompetitive Inhibition–Equation [14.41]*

For uncompetitive inhibition (Section 14-3B), the inhibitor binds to the Michaelis complex with dissociation constant

$$K'_\mathrm{I} = \frac{[\mathrm{ES}][\mathrm{I}]}{[\mathrm{ESI}]} \qquad [14.A9]$$

The conservation condition is

$$[\mathrm{E}]_\mathrm{T} = [\mathrm{E}] + [\mathrm{ES}] + [\mathrm{ESI}] \qquad [14.A10]$$

Substituting in Eqs. [14.34] and [14.A9] yields

$$[\mathrm{E}]_\mathrm{T} = [\mathrm{ES}]\left(\frac{K_M}{[\mathrm{S}]} + 1 + \frac{[\mathrm{I}]}{K'_\mathrm{I}}\right) \qquad [14.A11]$$

Defining α' analogously to Eq. [14.37] as

$$\alpha' = 1 + \frac{[\mathrm{I}]}{K'_\mathrm{I}} \qquad [14.A12]$$

and v_o and $V_{\max}$ as in Eqs. [14.22] and [14.23], respectively,

$$v_\mathrm{o} = k_2[\mathrm{ES}] = \frac{V_{\max}}{\frac{K_M}{[\mathrm{S}]} + \alpha'} \qquad [14.A13]$$

which on rearrangement yields the Michaelis–Menten equation for uncompetitive inhibition:

$$v_\mathrm{o} = \frac{V_{\max}[\mathrm{S}]}{K_M + \alpha'[\mathrm{S}]} \qquad [14.41]$$

C. *The Michaelis–Menten Equation for Mixed Inhibition–Equation [14.45]*

In mixed inhibition (Section 14-3C), the inhibitor-binding steps have different dissociation constants:

$$K_\mathrm{I} = \frac{[\mathrm{E}][\mathrm{I}]}{[\mathrm{EI}]} \qquad \text{and} \qquad K'_\mathrm{I} = \frac{[\mathrm{ES}][\mathrm{I}]}{[\mathrm{ESI}]} \qquad [14.A14]$$

(Here, for the sake of mathematical simplicity, we are making the thermodynamically unsupportable assumption that EI does not react with S to form ESI. Inclusion of this reaction requires a more complex derivation than that given here but leads to results that are substantially the same.) The conservation condition for this reaction scheme is

$$[\mathrm{E}]_\mathrm{T} = [\mathrm{E}] + [\mathrm{EI}] + [\mathrm{ES}] + [\mathrm{ESI}] \qquad [14.A15]$$

so that substituting in Eqs. [14.A14]

$$[\mathrm{E}]_\mathrm{T} = [\mathrm{E}]\left(1 + \frac{[\mathrm{I}]}{K_\mathrm{I}}\right) + [\mathrm{ES}]\left(1 + \frac{[\mathrm{I}]}{K'_\mathrm{I}}\right) \qquad [14.A16]$$

Defining α and α' as in Eqs. [14.37] and [14.A12], respectively, Eq. [14.A16] becomes

$$[\mathrm{E}]_\mathrm{T} = [\mathrm{E}]\alpha + [\mathrm{ES}]\alpha' \qquad [14.A17]$$

Then substituting in Eq. [14.34]

$$[\mathrm{E}]_\mathrm{T} = [\mathrm{ES}]\left(\frac{\alpha K_M}{[\mathrm{S}]} + \alpha'\right) \qquad [14.A18]$$

Defining v_o and $V_{\max}$ as in Eqs. [14.22] and [14.23] results in the Michaelis–Menten equation for mixed inhibition:

$$v_\mathrm{o} = \frac{V_{\max}[\mathrm{S}]}{\alpha K_M + \alpha'[\mathrm{S}]} \qquad [14.45]$$

D. *The Michaelis–Menten Equation for Ionizable Enzymes–Equation [14.47]*

In the model presented in Section 14-4a to account for the effect of pH on enzymes, the dissociation constants for the ionizations are

$$K_{\mathrm{E2}} = \frac{[\mathrm{H}^+][\mathrm{E}^-]}{[\mathrm{EH}]} \qquad K_{\mathrm{ES2}} = \frac{[\mathrm{H}^+][\mathrm{ES}^-]}{[\mathrm{ESH}]}$$

$$K_{\mathrm{E1}} = \frac{[\mathrm{H}^+][\mathrm{EH}]}{[\mathrm{EH}_2^+]} \qquad K_{\mathrm{ES1}} = \frac{[\mathrm{H}^+][\mathrm{ESH}]}{[\mathrm{ESH}_2^+]} \qquad [14.A19]$$

Protonation and deprotonation are among the fastest known reactions, so that, with the exception of the few enzymes with extremely high turnover numbers, it can be reasonably assumed that all acid–base reactions are at

equilibrium. The conservation condition is

$$[E]_T = [EH]_T + [ESH]_T \qquad [14.A20]$$

where $[E]_T$ is the total enzyme present in any form,

$$\begin{aligned}[EH]_T &= [EH_2^+] + [EH] + [E^-] \\ &= [EH]\left(\frac{[H^+]}{K_{E1}} + 1 + \frac{K_{E2}}{[H^+]}\right) \\ &= [EH]f_1 \qquad [14.A21]\end{aligned}$$

and

$$\begin{aligned}[ESH]_T &= [ESH_2^+] + [ESH] + [ES^-] \\ &= [ESH]\left(\frac{[H^+]}{K_{ES1}} + 1 + \frac{K_{ES2}}{[H^+]}\right) \\ &= [ESH]f_2 \qquad [14.A22]\end{aligned}$$

Then making the steady-state assumption

$$\frac{d[ESH]}{dt} = k_1[EH][S] - (k_{-1} + k_2)[ESH] = 0 \qquad [14.A23]$$

and solving for [EH]

$$[EH] = \frac{(k_{-1} + k_2)[ESH]}{k_1[S]} = \frac{K_M[ESH]}{[S]} \qquad [14.A24]$$

Therefore, from Eq. [14.A21],

$$[EH]_T = \frac{K_M[ESH]f_1}{[S]} \qquad [14.A25]$$

which, together with Eqs. [14.A20] and [14.A22], yields

$$[E]_T = [ESH]\left(\frac{K_M f_1}{[S]} + f_2\right) \qquad [14.A26]$$

As in the simple Michaelis–Menten derivation, the initial rate is

$$v_o = k_2[ESH] = \frac{k_2[E]_T}{\left(\frac{K_M f_1}{[S]}\right) + f_2} = \frac{(k_2/f_2)[E]_T[S]}{K_M(f_1/f_2) + [S]} \qquad [14.A27]$$

Then defining the "apparent" values of K_M and $V_{max} = k_2[E]_T$ at a given pH:

$$K'_M = K_M(f_1/f_2) \qquad [14.A28]$$

and

$$V'_{max} = V_{max}/f_2 \qquad [14.A29]$$

the Michaelis–Menten equation modified to account for pH effects is

$$v_o = \frac{V'_{max}[S]}{K'_M + [S]} \qquad [14.47]$$

CHAPTER SUMMARY

1 Chemical Kinetics Complicated reaction processes occur through a series of elementary reaction steps defined as having a molecularity equal to the number of molecules that simultaneously collide to form products. The order of a reaction can be determined from the characteristic functional form of its progress curve. Transition state theory postulates that the rate of a reaction depends on the free energy of formation of its activated complex. This complex, which occurs at the free energy maximum of the reaction coordinate, is poised between reactants and products and is therefore also known as the transition state. Transition state theory explains that catalysis results from the reduction of the free energy difference between the reactants and the transition state.

2 Enzyme Kinetics In the simplest enzymatic mechanism, the enzyme and substrate reversibly combine to form an enzyme–substrate complex known as the Michaelis complex, which may irreversibly decompose to form product and the regenerated enzyme. The rate of product formation is expressed by the Michaelis–Menten equation, which is derived under the assumption that the concentration of the Michaelis complex is constant, that is, at a steady state. The Michaelis–Menten equation, which has the functional form of a rectangular hyperbola, has two parameters: V_{max}, the maximal rate of the reaction, which occurs when the substrate concentration is saturating, and K_M, the Michaelis constant, which has the value of the substrate concentration at the half-maximal reaction rate. These parameters may be graphically determined using the Lineweaver–Burk plot. Physically more realistic models of enzyme mechanisms than the Michaelis–Menten model assume the enzymatic reaction to be reversible and to have one or more intermediates. The functional form of the equations describing the reaction rates for these models is independent of their number of intermediates, so that the models cannot be differentiated using only steady-state kinetic measurements.

3 Inhibition Enzymes may be inhibited by competitive inhibitors, which compete with the substrate for the enzymatic binding site. The effect of a competitive inhibitor may be reversed by increasing the substrate concentration. An uncompetitive inhibitor inactivates a Michaelis complex on binding to it. The maximal rate of an uncompetitively inhibited enzyme is a function of inhibitor concentration, and therefore the effect of an uncompetitive inhibitor cannot be reversed by increasing substrate concentration. In mixed inhibition, the inhibitor binds to both the enzyme and the enzyme–substrate complex to form a complex that is catalytically inactive. The rate equation describing this situation has characteristics of both competitive and uncompetitive reactions.

4 Effects of pH The rate of an enzymatic reaction is a function of hydrogen ion concentration. At any pH, the rate of a simple enzymatic reaction can be described by the Michaelis–Menten equation. However, its parameters V_{max} and K_M vary with pH. By the evaluation of kinetic rate curves

as a function of pH, the p*K*'s of an enzyme's ionizable binding and catalytic groups can be determined, which may help identify these groups.

5 Bisubstrate Reactions The majority of enzymatic reactions are bisubstrate reactions in which two substrates react to form two products. Bisubstrate reactions may have Ordered or Random Sequential mechanisms or Ping Pong Bi Bi mechanisms, among others. The initial rate equations for any of these mechanisms involve five parameters, which are analogous to either Michaelis–Menten equation parameters or equilibrium constants. The various bisubstrate mechanisms may be experimentally differentiated according to the forms of their double-reciprocal plots and from the nature of their product inhibition patterns. Isotope exchange reactions provide an additional, nonkinetic method of differentiating bisubstrate mechanisms.

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PROBLEMS

1. The hydrolysis of sucrose:

$$\text{Sucrose} + H_2O \longrightarrow \text{glucose} + \text{frutose}$$

takes the following time course.

Time (min)	[Sucrose] (M)
0	0.5011
30	0.4511
60	0.4038
90	0.3626
130	0.3148
180	0.2674

Determine the first-order rate constant and the half-life of the reaction. Why does this bimolecular reaction follow a first-order rate law? How long will it take to hydrolyze 99% of the sucrose initially present? How long will it take if the amount of sucrose initially present is twice that given in the table?

2. By what factor will a reaction at 25°C be accelerated if a catalyst reduces the free energy of its activated complex by 1 kJ · mol^{-1}; by 10 kJ · mol^{-1}?

3. For a Michaelis–Menten reaction, $k_1 = 5 \times 10^7\ M^{-1} \cdot s^{-1}$, $k_{-1} = 2 \times 10^4\ s^{-1}$, and $k_2 = 4 \times 10^2\ s^{-1}$. Calculate K_S and K_M for this reaction. Does substrate binding achieve equilibrium or the steady state?

***4.** The following table indicates the rates at which a substrate reacts as catalyzed by an enzyme that follows the Michaelis–Menten mechanism: (1) in the absence of inhibitor; (2) and (3) in the presence of 10 mM concentration, respectively, of either of two inhibitors. Assume $[E]_T$ is the same for all reactions.

[S] (mM)	(1) v_o ($\mu M \cdot s^{-1}$)	(2) v_o ($\mu M \cdot s^{-1}$)	(3) v_o ($\mu M \cdot s^{-1}$)
1	2.5	1.17	0.77
2	4.0	2.10	1.25
5	6.3	4.00	2.00
10	7.6	5.7	2.50
20	9.0	7.2	2.86

(a) Determine K_M and V_{max} for the enzyme. For each inhibitor determine the type of inhibition and K_I and/or K'_I. What additional information would be required to calculate the turnover number

of the enzyme? (b) For [S] = 5 mM, what fraction of the enzyme molecules have a bound substrate in the absence of inhibitor, in the presence of 10 mM inhibitor of type (2), and in the presence of 10 mM inhibitor of type (3)?

***5.** Ethanol in the body is oxidized to acetaldehyde (CH_3CHO) by liver alcohol dehydrogenase (LADH). Other alcohols are also oxidized by LADH. For example, methanol, which is mildly intoxicating, is oxidized by LADH to the quite toxic product formaldehyde (CH_2O). The toxic effects of ingesting methanol (a component of many commercial solvents) can be reduced by administering ethanol. The ethanol acts as a competitive inhibitor of the methanol by displacing it from LADH. This provides sufficient time for the methanol to be harmlessly excreted by the kidneys. If an individual has ingested 100 mL of methanol (a lethal dose), how much 100 proof whiskey (50% ethanol by volume) must he imbibe to reduce the activity of his LADH toward methanol to 5% of its original value? The adult human body contains ~40 L of aqueous fluids throughout which ingested alcohols are rapidly and uniformly mixed. The densities of ethanol and methanol are both 0.79 g · cm^{-3}. Assume the K_M values of LADH for ethanol and methanol to be 1.0×10^{-3} M and 1.0×10^{-2} M, respectively, and that $K_I = K_M$ for ethanol.

6. The K_M of a Michaelis–Menten enzyme for a substrate is 1.0×10^{-4} M. At a substrate concentration of 0.2M, $v_o = 43$ μM · min^{-1} for a certain enzyme concentration. However, with a substrate concentration of 0.02M, v_o has the same value. (a) Using numerical calculations, show that this observation is accurate. (b) What is the best range of [S] for measuring K_M?

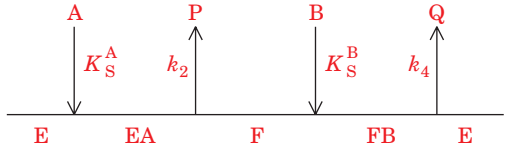
7. Why are uncompetitive and mixed inhibitors generally considered to be more effective *in vivo* than competitive inhibitors?

8. Explain why an exact fit to a kinetic model of the experimental parameters describing a reaction does not prove that the reaction follows the model.

9. An enzyme that follows the model for pH effects presented in Section 14-4a has $pK_{ES1} = 4$ and $pK_{ES2} = 8$. What is the pH at which V'_{max} is a maximum for this enzyme? What fraction of V_{max} does V'_{max} achieve at this pH?

10. Derive the initial rate equation for a Rapid Equilibrium Random Bi Bi reaction. Assume the equilibrium constants K_S^A and K_S^B for binding A and B to the enzyme are independent of whether the other substrate is bound (an assumption that constrains $K_M^B = K_S^B$ in Eq. [14.49]).

***11.** Consider the following variation of a Ping Pong Bi Bi mechanism.



Assume that the substrate-binding reactions are in rapid equilibrium,

$$K_S^A = \frac{[E][A]}{[EA]} \quad \text{and} \quad K_S^B = \frac{[F][B]}{[FB]}$$

that both [A] >> $[E]_T$ and [B] >> $[E]_T$, that neither product release reaction is reversible, and that the steady-state approximation is valid. (a) Derive an expression for v_o in terms of K_S^A, K_S^B, k_2, and k_4. (b) Indicate the form of the double-reciprocal plots for $1/v_o$ versus 1/[A] for various values of [B]. (c) Indicate the form of the double-reciprocal plots for $1/v_o$ versus 1/[B] for various values of [A].

12. Creatine kinase catalyzes the reaction

$$MgADP^- + \text{phosphocreatine} \rightleftharpoons MgATP^{2-} + \text{creatine}$$

which functions to regenerate ATP in muscle. Rabbit muscle creatine kinase exhibits the following kinetic behavior. In the absence of both products, plots of $1/v_o$ versus 1/[$MgADP^-$] at different fixed concentrations of phosphocreatine yield lines that intersect to the left of the $1/v_o$ axis. Similarly, plots of $1/v_o$ versus 1/[phosphocreatine] in the absence of product at different fixed concentrations of $MgADP^-$ yield lines that intersect to the left of the $1/v_o$ axis. In the absence of one of the reaction products, $MgATP^{2-}$ or creatine, plots of $1/v_o$ versus 1/[$MgADP^-$] at different fixed concentrations of the other product intersect on the $1/v_o$ axis. The same is true of the plots of $1/v_o$ versus 1/[phosphocreatine]. Indicate a kinetic mechanism that is consistent with this information.