I. SUBSTRATE KINETICS

A. Michaelis–Menten Equation

1. Derivation of the Michaelis–Menten Equation

The simplest form of an enzyme-catalyzed reaction is shown in Scheme A.1, where $k_1$ is a second-order rate constant, and $k_{-1}$ and $k_2$ are first-order rate constants. $K_s$ is the dissociation constant for the E·S complex, which equals $k_{-1}/k_1$. We will only be concerned with steady-state kinetics, that is, when the rate of E·S formation equals the rate of E·S breakdown. The pre-steady state is the initial period during which intermediates form until the steady state is reached.

The rates of enzyme-catalyzed reactions show a characteristic dependence on substrate concentration (Figure A.1). At low substrate concentration the initial rate is proportional to both [E]₀ and [S] (a second-order reaction). As the substrate concentration increases, it becomes easier for the enzyme to find the substrate, until when the $[S] \gg [E]_0$, all of the enzyme active sites are occupied with bound substrate (or product). At this point the enzyme is said to be saturated (i.e., all of the

\[ E + S \xrightleftharpoons[k_{-1}]{k_1} E \cdot S \xrightarrow{k_2} E \cdot P \xrightarrow{k_{-2}} E + P \]

E = free enzyme \hspace{1cm} S = substrate \hspace{1cm} P = product

\[ E \cdot S \] enzyme-substrate complex (also called the Michaelis complex)

SCHEME A.1 Simplest form of an enzyme-catalyzed reaction.
[E]₀ is the total enzyme concentration

[E] is free enzyme

[E] = [E]₀ - [E:S]

**FIGURE A.1** Dependence of the rate of an enzyme-catalyzed reaction on the substrate concentration.

Active sites have substrate or product bound, and further increases in the substrate concentration will not increase the rate of the enzyme reaction. This is the maximal rate or $V_{\text{max}}$, which is attained really only at infinite substrate concentration, so it is never fully achieved. At this point, the rate is zeroth order with respect to [S] and is dependent only on the enzyme concentration. The actual rate, then, depends on how much of the total amount of enzyme is in the E:S complex [Eq. (1)].

The rate of formation

$$\nu = k_2[E:S]$$

(1)

of E:S is given in Eq. (2), and the rate of decomposition of E:S back to E + S and to products is given in Eq. (3). During the period that the initial rate is measured,

$$\frac{+d\ [E:S]}{dt} = k_1([E]_0 - [E:S])[S]$$

(2)

$$\frac{-d\ [E:S]}{dt} = k_{-1}[E:S] + k_2[E:S]$$

(3)

[S], ([E]₀ - [E:S]), and [E]₀ remain approximately constant. Therefore, [E:S] can be assumed to remain constant (i.e., it remains in a steady state). If this steady-state assumption of George E. Briggs and J. B. S. Haldane⁴ is made, then the rate of formation of E:S [Eq. (2)] equals the rate of its decomposition [Eq. (3), as shown in Eq. (4)].

$$k_1([E]_0 - [E:S]) [S] = k_{-1}[E:S] + k_2 [E:S]$$

(4)
Rearranging Eq. (4) gives Eq. (5). The $K_m$ is called the Michaelis–Menten constant, which is given in the units of concentration.

$$\frac{([E]_0 - [E:S])[S]}{[E:S]} = \frac{k_{-1} + k_2}{k_1} = K_m$$

(5)

The $K_m$ is the concentration of the substrate required to produce a rate of $V_{\text{max}}/2$. Under certain conditions (see I.A.2), this is a dissociation constant for the E:S complex. Therefore, the smaller the $K_m$, the stronger the interaction between E and S.

Solving Eq. (5) for $[E:S]$ yields Eq. (6).

$$[E:S] = \frac{[E]_0[S]}{K_m + [S]}$$

(6)

Substituting Eq. (6) into Eq. (1) gives Eq. (7).

$$\nu = \frac{k_2[E]_0[S]}{K_m + [S]}$$

(7)

When $k_2 \ll k_{-1}$, then $k_2$ is called $k_{\text{cat}}$, and Eq. (7) becomes Eq. (8), which is the Michaelis–Menten equation, named after Leonor Michaelis and Maud Menten.$^2$

$$\nu = \frac{k_{\text{cat}}[E]_0[S]}{K_m + [S]}$$

(8)

The term $k_{\text{cat}}$ is the rate constant for conversion of the E-S complex to product. It is a measure of the rate at which the enzyme catalyzes the reaction. Because the $k_{\text{cat}}$ can be represented by Eq. (9), the maximum rate divided by the total enzyme concentration, we can substitute this equality for $k_{\text{cat}}$ into Eq. (8), which then can be reduced to Eq. (10). This is an alternative form of the Michaelis–Menten equation. At high $[S]$, all of the enzyme is in the E-S form, and $K_m$ is negligible relative to $[S]$. Consequently, Eq. (10) becomes Eq. (11), indicating that the rate is at its maximum,

$$\nu = \frac{V_{\text{max}}[S]}{[S]} = V_{\text{max}}$$

(11)

which as we saw earlier (Figure A.1) is independent of $[S]$. When $[S] = K_m$, Eq. (10) can be rewritten as Eq. (12),

$$\nu = \frac{V_{\text{max}}K_m}{K_m + K_m} = \frac{V_{\text{max}}}{2}$$

(12)
which shows that the rate is at half the maximum value. At low $[S]$, where $[S] \ll K_m$, $[S]$ is negligible relative to $K_m$, so Eq. (10) becomes Eq. (13).

$$\nu = \frac{V_{\text{max}} [S]}{K_m}$$

(13)

From this equation it can be seen that at low $[S]$, the rate is proportional to $[S]$, where $V_{\text{max}}/K_m$ is the proportionality constant.

2. Difference between $K_m$ and $K_s$

As noted, when $k_{-1}$ and $k_1$ are large relative to $k_2$, then $k_2$ is referred to as $k_{\text{cat}}$. If $k_2$ is negligible, then Eq. (5) reduces to Eq. (14), which is the $K_s$ (the dissociation constant for E-S). Only under these special conditions—that is, $k_2 \ll k_{-1}$—is $K_m = K_s$.

$$K_m = \frac{k_{-1} + k_2}{k_1}$$

(5)

$$K_m \approx \frac{k_{-1}}{k_1}$$

(14)

When $k_2$ is not $\ll k_{-1}$, then Eq. (5) holds. Substitution of $K_s = k_{-1}/k_1$ (actually, we are substituting $k_{-1} = k_1 K_s$), into Eq. (5) gives Eq. (15).

$$K_m = K_s + \frac{k_2}{k_1}$$

(15)

In this case, $K_m > K_s$. When intermediates occur after the E-S complex, as shown in Scheme A.2, $K_m$ and $k_{\text{cat}}$ are a combination of various rate and equilibrium constants, but $K_m$ is always less than or equal to $K_s$.

3. $k_{\text{cat}}$

The term $k_{\text{cat}}$ represents the maximum number of substrate molecules converted to product molecules per active site per unit time, the number of times the enzyme "turns over" substrate to product per unit time (called the turnover number). Values for $k_{\text{cat}}$ on the order of $10^3 \text{ s}^{-1}$ are typical.

$$E + S \xrightleftharpoons[K_s]{k_{\text{cat}}} E \cdot S \xrightleftharpoons[K_{\text{II}}]{K_{\text{II}}} E \cdot S_{\text{II}} \xrightarrow{[k_4]_{\text{slow}}} E + P$$

SCHEME A.2 Form of an enzyme-catalyzed reaction when there are intermediates between the E-S and E-P complexes.
4. \( k_{cat} / K_m \)

The term \( k_{cat} / K_m \) is called the **specificity constant**. At low [S] the Michaelis–Menten equation [Eq. (8)] reduces to Eq. (16). Therefore,

\[
\nu = \frac{k_{cat} [E]_0 [S]}{K_m + [S]} \tag{8}
\]

\[
\nu = \frac{k_{cat} [E]_0 [S]}{K_m} \tag{16}
\]

\( k_{cat} / K_m \) is a second-order proportionality constant for the reaction of free E+S to give product (at low [S], [E]₀ is approximately the same as [E]). The value of the term \( k_{cat} / K_m \) allows you to rank an enzyme according to how good it is with different substrates. It contains information about how fast the reaction of a given substrate would be when bound to the enzyme \( (k_{cat}) \) and how much of the substrate is required to reach \( V_{max} \). The value of \( k_{cat} / K_m \) can approach the rate of diffusion (about \( 10^9 \text{ M}^{-1} \text{ s}^{-1} \)), which is when a reaction occurs with every collision between molecules.

Substituting \( k_2 \) for \( k_{cat} \) and \( (k_{-1} + k_2)/k_1 \) for \( K_m \) [Eq. (5)], then it can be seen in Eq. (17) that when \( k_2 >> k_{-1} \), then \( k_{cat} / K_m = k_1 \), the rate of encounter between E and S.

\[
k_{cat} / K_m = \frac{k_2}{(k_{-1} + k_2)/k_1} = \frac{k_1 k_2}{k_{-1} + k_2} = \frac{k_1 k_2}{k_2} = k_1 \tag{17}
\]

B. Graphical Representations

Because the \( V_{max} \) is attained only at infinite substrate concentration, it can never really be achieved. The difficulty in obtaining \( V_{max} \) experimentally and the curved nature of the \( \nu \) versus [S] plot (Figure A.1) led to the transformation of the Michaelis–Menten equation into linear forms. This allows for more accurate determination of \( K_m \) and \( V_{max} \) values. We will consider three graphical representations of the kinetic data:

\[
\frac{1}{\nu} \text{ versus } \frac{1}{[S]} \quad \text{Lineweaver–Burk plot}
\]

\[
\nu \text{ versus } \frac{\nu}{[S]} \quad \text{Eadie–Hofstee plot}
\]

\[
\frac{S}{\nu} \text{ versus } [S] \quad \text{Hanes–Woolf plot}
\]

The most commonly used is the **Lineweaver–Burk plot**, but this is the least accurate for linear regression analysis if there is significant experimental error in the
initial rate measurements. Taking the reciprocal of the Michaelis–Menten equation [Eq. (10)] gives Eq. (18). A plot of $1/\nu$ versus $1/[S]$ gives a straight line ($a = b + mx$), and this is called a Lineweaver–Burk plot (Figure A.2). The interception of the x-axis is $-1/K_m$, and the interception of the y-axis is $1/V_{\text{max}}$. The slope

$$\nu = \frac{V_{\text{max}} [S]}{K_m + [S]} \quad (10)$$

$$\frac{1}{\nu} = \frac{1}{V_{\text{max}}} + \frac{K_m}{V_{\text{max}} [S]} \quad (18)$$

defines $K_m/V_{\text{max}}$. As the [S] increases, the rate approaches $V_{\text{max}}$. The Lineweaver–Burk plot, however, has the disadvantages of compressing the data points at high concentrations into a small region and emphasizing the points at low concentrations. Also, small errors in the determination of $\nu$ are magnified when the reciprocals are taken. Because of these problems, very careful choice of substrate concentrations and a large number of data points are required to increase the accuracy.

Rearranging the Michaelis–Menten equation [Eq. (10)] a different way, that is, by multiplying the Lineweaver–Burk equation [Eq. (18)] by $V_{\text{max}} \cdot \nu$ gives Eq. (19) or (20). A plot of $\nu$ versus $\nu/[S]$, an Eadie–Hofstee plot (Figure A.3), gives a straight line with the intercept at the x-axis equal to

$$V_{\text{max}} = K_m \frac{\nu}{[S]} + \nu \quad (19)$$

$$\nu = V_{\text{max}} - K_m \frac{\nu}{[S]} \quad (20)$$

**FIGURE A.2** Lineweaver–Burk plot for determination of substrate kinetic constants.
$V_{\text{max}}/K_m$, the $y$-axis intercept equal to $V_{\text{max}}$, and the slope $= -K_m$. The Eadie–Hofstee plot does not compress the higher substrate concentration values. However, the fact that $\nu$ appears on both the $x$- and $y$-axis means that errors in $\nu$ affect both axes and cause deviations toward or away from the origin. Therefore, instead of making poor data look better, as in the case of the Lineweaver–Burk plot, it tends to make good data look worse. Because of the exaggerated deviations from linearity, the data must be excellent to obtain a straight line with this kind of a plot. If you want to detect small deviations from Michaelis–Menten kinetics, this is the plot to use.

Multiplication of the Lineweaver–Burk equation [Eq. (18)] by $[S]$ gives a third transformation [Eq. (21)]. A plot of $[S]/\nu$ versus $S$, a Hanes–Woolf plot\(^5\) (Figure A.4), gives a straight line where the $x$-axis intercept corresponds to $-K_m$, the $y$-axis intercept is $K_m/V_{\text{max}}$, and the slope is $1/V_{\text{max}}$. This is a popular way to represent the
kinetic data graphically; over a wide range of [S] values, the errors in [S]/ν provide a fair reflection of the errors in ν, and therefore, this should be the preferred plot for most purposes.

\[
\frac{[S]}{\nu} = \frac{K_m}{V_{\text{max}}} + [S] \left( \frac{1}{V_{\text{max}}} \right)
\]  

(21)

II. KINETICS OF ENZYME INHIBITION

A. Reversible Enzyme Inhibition

A reversible enzyme inhibitor is a molecule that binds reversibly to the enzyme in such a way as to slow down or prevent enzyme turnover. There are naturally occurring inhibitors that control metabolism and synthetic inhibitors that are used as drugs or for agricultural purposes (e.g., insecticides, fungicides, and weed-killers). There are three simple types of inhibition: competitive, noncompetitive/mixed, and uncompetitive inhibition.

1. Competitive Inhibition

Competitive inhibitors (I) bind to the active site of the free enzyme and prevent the substrate from binding (and vice versa). Therefore, I and S compete for the active site (Scheme A.3). The term \( K_i \) is the dissociation constant for the E·I complex. The rate of the reaction depends on [I], \( K_i \), [S], and \( K_m \). By solving the equilibrium and rate equations, Eq. (22) can be derived (but not here).

\[
\nu = \frac{V_{\text{max}} [S]}{K_m \left( 1 + \frac{[I]}{K_i} \right) + [S]}
\]

(22)

Eq. (22) is identical to the Michaelis–Menten equation [Eq. (10)] except that the presence of the inhibitor results in \( K_m \) being multiplied by the factor \( 1 + ([I]/K_i) \).

\[
E + S \underset{K_m}{\xrightarrow{k_{\text{cat}}}} E \cdot S \xrightarrow{\text{[E]}_0 = [E \cdot S] + [E \cdot I] + [E]} E + P
\]

+ I

\[
K_i
\]

E·I

SCHEME A.3 Competitive inhibition.
II. Kinetics of Enzyme Inhibition

![Lineweaver–Burk plot for determination of competitive inhibitor kinetic constants.](image)

Consequently, the Lineweaver–Burk equation becomes Eq. (23).

\[
\frac{1}{\nu} = \frac{K_m}{V_{\text{max}}} \left( \frac{1}{[S]} \right) \left( 1 + \frac{[I]}{K_i} \right) + \frac{1}{V_{\text{max}}}
\]  

This equation is plotted graphically in Figure A.5. The rate is determined as a function of [S] in the absence of inhibitor (line a), then the experiment is repeated except at each [S] a constant amount of inhibitor is added (line b). The experiment is repeated with more inhibitor added at each [S] (lines c, d, and e). As more I is added, the slope increases, but there is no effect on \( V_{\text{max}} \). As 1/[S] approaches 0 (infinite [S]), all of the inhibitor is displaced by the substrate, and the enzyme is present only as the E·S complex, so the same \( V_{\text{max}} \) is observed regardless of whether I is present or not.

At low [S], the enzyme is predominantly in the E form. The competitive inhibitor can bind to E, and this decreases the rate (there are then fewer E molecules available to bind to S). Because the rate is proportional to \( V_{\text{max}}/K_m \), the slopes of the lines are affected. Increasing [I] causes the \( K_m/V_{\text{max}} \) to increase. This is because it now requires more substrate to give the same \( V_{\text{max}} \) than would be obtained in the absence of the inhibitor, thereby making it appear that the \( K_m \) is larger (i.e., the apparent \( K_m \) or \( K_{m,\text{app}} \) is larger), and the \( V_{\text{max}} \) is smaller. A larger \( K_m/V_{\text{max}} \) means a steeper slope (a < b < c < d). At each [I] the apparent \( K_m \) (\( K_{m,\text{app}} \)) can be calculated from each x-axis intercept using Eq. (24).

\[
\frac{1}{K_m} \left( 1 + \frac{[I]}{K_i} \right) = \frac{1}{K_{m,\text{app}}}
\]  

(24)
To obtain the $K_i$ values, the data from the Lineweaver–Burk plot (Figure A.5) are replotted as $K_{m,\text{app}}$ versus [I] (Figure A.6) or as $K_{m,\text{app}}/V_{\text{max}}$ (the slope) versus [I] (Figure A.7).

The other graphical representation we will consider for competitive inhibition is the Dixon plot\(^6\) (Figure A.8), a plot of Eq. (25). The [I] is plotted against $1/\nu$, varying the amount of substrate. The negative $K_i$ value can be read directly from the plot at the intersection of the lines.

$$
\frac{1}{\nu} = \frac{K_m}{V_{\text{max}}[S]K_i} [I] + \frac{1}{V_{\text{max}}} \left( 1 + \frac{K_m}{[S]} \right)
$$

(25)

To determine what kind of inhibition has occurred, the data from the Dixon plot must be replotted in a *Cornish-Bowden plot*\(^7\) (Figure A.9), that is, $S/\nu$ versus [I]. Parallel lines indicate competitive inhibition.
FIGURE A.8 Dixon plot for determination of competitive inhibitor kinetic constants.

\[ \frac{1}{V_{\text{max}}} \left( 1 + \frac{K_m}{[S]} \right) \]

slope = \[ \frac{K_m}{V_{\text{max}}[S] K_i} \]

FIGURE A.9 Cornish-Bowden replot of the data from the Dixon plot for determination of the type of inhibition (in this case, competitive).
2. Noncompetitive Inhibition

When the inhibitor binds to both E and E·S, noncompetitive inhibition results. This is depicted in Scheme A.4. It is rare for single-substrate reactions to exhibit this inhibition, but it is common in multiple-substrate systems. So that the ternary complex, E·S·I, can form, the inhibitor must bind at a site other than the active site. If you assume that the dissociation of S from E·S is the same as from E·S·I (i.e., $K_m = K'_m$), then the rate is described by Eq. (26), derived from the Michaelis–Menten equation.

$$
\nu = \frac{V_{\text{max}}[S]}{K_m + [S]} \left(1 + \frac{[I]}{K_i}\right)
$$

(26)

This is pure noncompetitive inhibition. The Lineweaver–Burk transformation of this equation is shown in Eq. (27);

$$
\frac{1}{\nu} = \left(\frac{K_m}{V_{\text{max}}[S]} + \frac{1}{V_{\text{max}}}\right) \left(1 + \frac{[I]}{K_i}\right)
$$

(27)

Figure A.10 gives a graphical representation. As is apparent from Figure A.10, noncompetitive inhibitors affect the $V_{\text{max}}$ (note that the interception on the y-axis varies) but do not affect the $K_m$ (all of the lines intersect at the same point on the x-axis). The $K_i$ value can be obtained by a replot of the data from Figure A.10, either as a plot of the slope versus $[I]$ (Figure A.11A) or $1/V_{\text{max,app}}$ versus $[I]$ (Figure A.11B).

Equation 27 can be rearranged to Eq. (28). A plot of $1/\nu$ versus $[I]$, a Dixon plot, gives a straight line (Figure A.12) from which the $K_i$ can be read directly at the $-x$-axis intercept.

$$
\frac{1}{\nu} = \left(\frac{1 + K_m}{[S]}\right) \left[\frac{1}{V_{\text{max}}K_i} + \frac{1}{V_{\text{max}}} \left(1 + \frac{K_m}{[S]}\right)\right]
$$

(28)
II. Kinetics of Enzyme Inhibition

\[
\frac{1}{V_{\text{max, app}}} = \frac{1}{V_{\text{max}}} \left( 1 + \frac{[I]}{K_i} \right)
\]

\[
\text{slope} = \frac{K_m}{V_{\text{max}}} \left( 1 + \frac{[I]}{K_i} \right)
\]

FIGURE A.10 Lineweaver–Burk plot for determination of noncompetitive inhibitor kinetic constants.

---

FIGURE A.11 Replot of data from Lineweaver–Burk plots for determination of \(K_i\) values for non-competitive inhibitors. **A**, plot of the slope of lines from the Lineweaver–Burk plot versus inhibitor concentration; **B**, plot of \(1/V_{\text{max, app}}\) from the Lineweaver–Burk plot versus inhibitor concentration.

To determine what kind of inhibition has occurred, the data from the Dixon plot must be replotted in a Cornish-Bowden plot (Figure A.13), that is, \(S/\nu\) versus \([I]\). Lines that intersect on the \(-x\)-axis indicate a noncompetitive inhibitor.
\[ \frac{1}{V_{\text{max, app}}} = \frac{1}{V_{\text{max}}} \left(1 + \frac{K_m}{[S]} \right) \]

\[ \text{slope} = \frac{1 + \frac{K_m}{[S]}}{V_{\text{max}} K_i} \]

FIGURE A.12 Dixon plot for determination of noncompetitive inhibitor kinetic constants.

\[ \frac{S}{v} \]

FIGURE A.13 Cornish-Bowden replot of the data from the Dixon plot for determination of the type of inhibition (in this case, noncompetitive).

3. Mixed Inhibition

It is more common that the dissociation constant of S from the E⋅S complex \((K_m)\) in Scheme A.4 is different from that of S from the E⋅S⋅I complex \((K'_m)\). When E⋅I has a lower affinity for S than does E, the E⋅S⋅I complex is nonproductive. In this case, the inhibition is a mixture of partial competitive and pure noncompetitive inhibition, called mixed inhibition. As long as the inhibitor is present, some of the enzyme will always be in the nonproductive E⋅S⋅I form, even at infinite substrate concentration. In effect, this lowers the concentration of free enzyme. Therefore, the \(V_{\text{max}}\) will be less than that of the free enzyme. Also, because a portion of the
enzyme available for substrate binding will exist in the lower affinity form, E–I, the
$K_m$ will be greater than that for the free enzyme. The reciprocal form of the rate
equation is shown in Eq. (29) (note that in the Cleland nomenclature $K_i$ would be
$K_i$ and $K'_i$ would be $K_{II}$).

\[
\frac{1}{\nu} = \frac{K_m}{V_{max}} \left( \frac{1}{[S]} \right) \left( 1 + \frac{[I]}{K_i} \right) + \frac{1}{V_{max}} \left( 1 + \frac{[I]}{K'_i} \right)
\]  
(29)

With this type of inhibition, the interception of the lines in a Lineweaver–Burk
plot does not occur on the x-axis; it may occur either above the x-axis (Figure
A.14A) or below the x-axis (Figure A.14B).

To obtain the $K_i$ values, the data from the Lineweaver–Burk plots are replotted
as $K_m/V_{max} (1 + ([I]/K_i))$ (the slope) versus [I] (Figure A.15A) or as $1/V_{max,app}$ (the
y-axis intercept) versus [I] (Figure A.15B). The x-axis intercept for the slope versus [I] plot is $-K_i$; the x-axis intercept for the $1/V_{max,app}$ versus [I] plot is $-K'_i$.

Dixon plots, $1/\nu$ versus [I], for most mixed inhibitors are curved; however, when
the E·S·I complex is not catalytically active, the plot is linear (Figure A.16). Eq. (30)
is the equation from which the Dixon plot is derived.

\[
\frac{1}{\nu} = \frac{\left(1 + \frac{K'_m}{[S]}\right)}{V_{max}K'_i} [I] + \frac{1}{V_{max}} \left( 1 + \frac{K_m}{[S]} \right)
\]  
(30)

For a Dixon plot in which the lines intersect above the x-axis, the corresponding
Cornish-Bowden plot lines intersect below the x-axis (Figure A.17) and vice versa.

4. Uncompetitive Inhibition

When the inhibitor binds to the E·S complex, but not to free enzyme, thereby pro-
ducing an inactive E·S·I complex, uncompetitive inhibition results (Scheme A.5). This
is rare in single-substrate reactions, but common in multiple-substrate systems. Of-
ten an inhibitor of a two-substrate enzyme that is competitive against one of the
substrates is found to give uncompetitive inhibition when the other substrate is var-
ied. The inhibitor combines at the active site but only prevents the binding of one of
the substrates. The form of the Michaelis–Menten equation that describes this
behavior is given in Eq. (31).

\[
\nu = \frac{V_{max}[S]}{\left(1 + \frac{[I]}{K_i}\right)} - \frac{K_m}{\left(1 + \frac{[I]}{K_i}\right)} + [S]
\]  
(31)
FIGURE A.14 Two possible Lineweaver–Burk plots for determination of mixed inhibitor kinetic constants. (A), intersection of lines above the x-axis; (B), intersection of lines below the x-axis.

The Lineweaver–Burk transformation of this equation is shown in Eq. (32),

\[
\frac{1}{v} = \frac{K_m}{V_{max}} \left( \frac{1}{[S]} \right) + \frac{1}{V_{max}} \left( 1 + \frac{[I]}{K_i} \right)
\] (32)

which is depicted graphically in Figure A.18. Because an uncompetitive inhibitor does not bind to free enzyme, the inhibitor has no effect on the \( V_{max}/K_m \), and the slopes are independent of inhibitor concentration. The \( K_i \) values can be obtained
from replots of either $1/V_{\text{max,app}}$ (the $y$-axis intercepts) versus [I] (Figure A.19A) or $1/K_{m,\text{app}}$ (the $x$-axis intercepts) versus [I] (Figure A.19B).

Equation 33 is the equation for a Dixon plot of uncompetitive inhibition, which is represented graphically in Figure A.20.

$$\frac{1}{\nu} = \frac{1}{V_{\text{max}}K_i}[I] + \frac{1}{V_{\text{max}}} \left(1 + \frac{K_m}{[S]}\right)$$ (33)
\[
\frac{1}{V_{\text{max, app}}} = \frac{1}{V_{\text{max}}} \left(1 + \frac{K_m}{[S]}\right)
\]

\[
\text{slope} = \frac{1}{V_{\text{max}}} \frac{K_m}{K_i'} \frac{[S]}{V_{\text{max}} K_i'}
\]

\[
[S] = -K_i' \left(1 + \frac{K_m}{[S]}\right) \left(1 + \frac{K_m}{[S]}\right)
\]

**FIGURE A.16** Dixon plot for determination of mixed inhibitor kinetic constants when the E-S-I complex is not catalytically active.

**FIGURE A.17** Cornish-Bowden replot of the data from the Dixon plot for determination of the type of inhibition (in this case, mixed, when the lines of the Dixon plot intersect above the x-axis).

Because the slope expression does not contain a [S] term, the lines are parallel for all substrate concentrations. The Cornish-Bowden replot of these data is shown in Figure A.21.
5. **Slow-Binding and Slow, Tight-Binding Inhibition**

Some reversible inhibitors attain the equilibrium between enzyme, inhibitor, and E-I complex slowly (with a steady-state time scale of seconds to minutes instead of milliseconds, as for the classical competitive inhibitor). These inhibitors are called **slow-binding inhibitors**. In some cases the ratio of total inhibitor to total enzyme must be high, as in the case of the classical competitive inhibitors, but in other cases, the attainment of the equilibrium of E, I, and the E-I complex occurs when the [I] is approximately the same as the [E], in which case the inhibitors are called **slow, tight-binding inhibitors**. The lifetimes of enzyme complexes with slow-binding and slow, tight-binding inhibitor exhibit slow off rates \(k_{\text{off}}\), but the on rates \(k_{\text{on}}\) may be
fast or slow. As the rate of release of the inhibitor from the E:I complex becomes vanishing slow, the inhibition approaches irreversible (see II.B). Inhibition by both of these types of inhibitors cannot be described by Michaelis–Menten kinetics.

The principal cause for slow-binding inhibition is believed to be a slow isomerization of the initially formed E:I complex, as the result of a conformational change, to another E:I complex (E:I\textsuperscript{8}), in which the enzyme is in a tighter complex with I than it was in the E:I complex (Scheme A.6).\textsuperscript{8} In this case the overall
FIGURE A.20 Dixon plot for determination of uncompetitive inhibitor kinetic constants.

FIGURE A.21 Cornish-Bowden replot of the data from the Dixon plot for determination of the type of inhibition (in this case, uncompetitive).

\[ \frac{S}{v} \]

\[ -K_i \]

\[ 0 \]

\[ [I] \]

\[ \frac{1}{v} \]

\[ \frac{1}{V_{max}} \]

\[ \frac{1}{V_{max} K_i} \]

\[ \frac{1}{V_{max}} \left( 1 + \frac{K_m}{[S]} \right) \]

\[ -K_i \left( 1 + \frac{K_m}{[S]} \right) \]

\[ [S] = \infty \]

\[ \text{Slope} = \frac{1}{V_{max} K_i} \]

\[ \text{E} + I \xrightleftharpoons[k_1]{k_{-1}} \text{E} \cdot I \xrightleftharpoons[k_2]{k_1} \text{E} \cdot I^* \]

\[ \text{slow} \]

SCHEME A.6 Slow-binding and slow, tight-binding inhibition.
dissociation constant \( (K_{i^*}) \) is defined by Eq. (34).

\[
K_{i^*} = \frac{(E)(I)}{(E\cdot I) + (E\cdot I^*)} = \frac{K_i k_{-2}}{k_2 + k_{-2}}
\]  

(34)

In addition to a slow conversion of E-I to E-I*, the reverse isomerization rate \( (k_{-2}) \) must be less than the forward isomerization rate \( (k_2) \).

Progress curves for the attainment of a slow-binding or slow, tight-binding inhibitor are described by the general integrated Eq. (35),

\[
P = \nu_o t + \frac{(\nu_o - \nu_i)(1 - e^{-kt})}{k}
\]  

(35)

where \( \nu_o, \nu_i, \) and \( k \) represent, respectively, the initial rate, the final steady-state rate, and the apparent first-order rate constant for establishment of the equilibrium between E-I and E-I*. The initial rate is obtained from Eq. (36),

\[
\nu_o = \frac{V_{max}[S]}{K_m(1 + [I]/K_i) + [S]}
\]  

(36)

where \( V_{max} \) is the maximum rate, \( [S] \) is the concentration of the substrate for which I is an inhibitory analogue, \( K_m \) is the Michaelis constant for S, and \( K_i \) is the dissociation constant for the E-I complex. Note that Eq. (36) is the same as the rate equation for a competitive reversible inhibitor (Eq. (22)). The final steady-state rate is obtained from Eq. (37),

\[
\nu_i = \frac{V_{max}[S]}{K_m(1 + [I]/K_{i^*}) + [S]}
\]  

(37)

where \( K_{i^*} \) is the overall inhibition constant as defined in Eq. (34). The apparent first-order rate constant \( (k) \) for the interconversion of E-I and E-I* in the presence of substrate S is expressed in equation 38. Figure A.22 gives an example of a progress curve for a slow-binding inhibitor. For each curve with inhibitor present, there is an initial burst followed by a slower steady-state rate.

\[
k = k_{-2} + k_2 \left( \frac{[I]/K_i}{1 + ([S]/K_m) + ([I]/K_i)} \right)
\]  

(38)

**B. Irreversible Enzyme Inhibition**

The two types of irreversible inhibition that will be discussed are affinity labeling and mechanism-based inactivation.

**1. Affinity Labeling**

These inactivators are reactive compounds that initially form a reversible E-I complex, then an active-site nucleophile reacts with the enzyme-bound inactivator to
form a covalent adduct (Scheme A.7). Loss of enzyme activity is time dependent, a measure of the rate of the reaction of the enzyme with the inactivator. To determine the kinetic constants, $K_1$ and $k_{\text{inact}}$, a plot of the log of the enzyme activity versus time is constructed (Figure A.23). From this plot, the half-lives for inactivation ($t_{1/2}$) at each inactivator concentration ($a-e$) can be determined. A replot of the half-lives versus the inverse of the inactivator concentration (Figure A.24), known as a Kitz and Wilson replot,\(^9\) is then made to determine the kinetic constants for inactivation.

The term $K_1$ represents the concentration of the inactivator that gives half-maximal inactivation, just like $K_m$ represents the concentration of substrate that gives half-maximal rate. Likewise, $k_{\text{inact}}$ is the maximal rate constant for inactivation.

### 2. Mechanism-Based Inactivation

These inactivators are unreactive compounds that have a structural similarity to the substrate or product for the target enzyme and are converted by the target enzyme
FIGURE A.23 Time-dependent inactivation by affinity labeling agents or mechanism-based inactivators.

FIGURE A.24 Kitz and Wilson replot of the data from Figure A.22 for determination of the kinetic constants of inactivation by affinity labeling agents or mechanism-based inactivators.

into species that inactivate the enzyme prior to its release from the active site. Because of this additional step of converting the inactivator into the active form, an additional step(s) has to be added to the kinetic expression (Scheme A.8). The ki-
Kinetic constants ($K_1$ and $k_{\text{inact}}$) are determined the same way as for affinity labeling agents (Figures A.23 and A.24).

### III. SUBSTRATE INHIBITION

Occasionally, the dependence of rate on substrate concentration does not give a hyperbolic curve, as in Figure A.1; instead, the rate may reach a maximum, then diminish as [S] increases further (Figure A.25). This is known as substrate inhibition (Figure A.25A demonstrates complete substrate inhibition, and Figure A.25B shows partial substrate inhibition), depicted in Scheme A.9. At high [S], an inactive E·S·S complex forms, which decreases the rate. A possible physical representation for this phenomenon is shown in Figure A.26. One substrate molecule is bound to the active site in A, and two substrate molecules are bound in B.

\[
\begin{align*}
E + I & \xrightarrow{K_1} E \cdot I \\
E \cdot I & \xrightarrow{k_{\text{inact}}} E \cdot I' \\
E + I' & \\
\end{align*}
\]

SCHEME A.8  Mechanism-based inactivation.

\[
E + S \xrightarrow{-S} E \cdot S \xrightarrow{+S, K_1} E + P
\]

\[
E \cdot S \xrightarrow{-S} E \cdot S \cdot S
\]

SCHEME A.9  Substrate inhibition.
The rate of an enzyme reaction that shows substrate inhibition is described by Eq. (39).

\[
\nu = \frac{V_{\text{max}}[S]}{K'_m + [S] + K_i[S]^2}
\]  

(39)

\(K_i\), in this case, is the equilibrium constant for formation of \(E\cdot S\cdot S\) from \(E\cdot S\) and \(S\). \(K'_m\) is a modified Michaelis constant. The reciprocal of Eq. (39) gives Eq. (40), which is analogous to the Lineweaver–Burk equation and is depicted graphically in Figure A.27.

\[
\frac{1}{\nu} = \frac{K'_m}{V_{\text{max}}[S]} + \frac{1}{V_{\text{max}}} + \frac{K_i[S]}{V_{\text{max}}}
\]  

(40)

At low \([S]\) (large \(1/[S]\)), the term \(K_i[S]/V_{\text{max}}\) becomes negligible, and Eq. (40) reduces to the Lineweaver–Burk equation [Eq. (18)], which gives a straight line (the linear part of Figure A.27).
The two principal reasons for the failure of the Michaelis–Menten equation are substrate inhibition, as described earlier, and substrate activation, that is, an E·S·S complex that is more active than E·S.

IV. NONPRODUCTIVE BINDING

When a substrate binds at the active site in an alternative unreactive mode in competition with its productive mode of binding, it is called nonproductive binding (Scheme A.10). The effect is to lower both \( k_{\text{cat}} \) and \( K_m \). The \( k_{\text{cat}} \) is lowered because, at saturation, only a fraction of the enzyme molecules has substrate bound productively; therefore, fewer enzyme molecules are active. The \( K_m \) is lower than the \( K_s \) because the existence of additional binding modes apparently leads to tighter binding. Eq. (41) describes this behavior.

\[
\nu = \frac{[E]_0 [S] k_2}{K_s + [S] (1 + K_s/K'_s)}
\]  

(41)

By a comparison of Eq. (41) with the Michaelis–Menten equation [Eq. (8)], \( k_{\text{cat}} \) and \( K_m \) can be redefined under these conditions as Eq. (42) and Eq. (43), respectively.

\[
\begin{align*}
K_s & \quad E \cdot S \xrightleftharpoons[k_2]{E + P} \\
E + S & \quad K'_s \quad E \cdot S'
\end{align*}
\]

SCHEME A.10  Nonproductive binding.
Dividing $k_{\text{cat}}$ by $K_m$ gives Eq. (44). Therefore,

$$k_{\text{cat}} = \frac{k_2}{1 + K_s/K'_s}$$  \hspace{1cm} (42)

$$K_m = \frac{K_s}{1 + K_s/K'_s}$$  \hspace{1cm} (43)

$$k_{\text{cat}}/K_m = k_2/K_s$$  \hspace{1cm} (44)

$k_{\text{cat}}/K_m$ is unaffected by the presence of the additional binding mode, because $k_{\text{cat}}$ and $K_m$ are altered in a compensating manner. For example, if the nonproductive site binds the substrate 1000 times more strongly than the productive site, $K_m$ will be 1000 times lower than $K_s$, but because only 1 molecule in 1000 is productively bound, $k_{\text{cat}}$ is 1000 times lower than $k_2$.

V. COMPETING SUBSTRATES

Suppose two different substrates compete for the active site of an enzyme (Scheme A.11). An alternative way to calculate reaction rates besides making the Michaelis–Menten assumptions is from Eq. (45). The consumption of substrate A is described by Eq. (46) and consumption of substrate B is described by Eq. (47).

$$\nu = \frac{k_{\text{cat}}}{K_m} [E] [S]$$ \hspace{1cm} (45)

$$-\frac{d [A]}{dt} = \nu_A = \left( \frac{k_{\text{cat}}}{K_m} \right)_A [E][A]$$ \hspace{1cm} (46)

$$-\frac{d [B]}{dt} = \nu_B = \left( \frac{k_{\text{cat}}}{K_m} \right)_B [E][B]$$ \hspace{1cm} (47)

The ratio of the rate with substrate A to substrate B is given by Eq. (48).

$$\frac{\nu_A}{\nu_B} = \left( \frac{k_{\text{cat}}/K_m}{K_m} \right)_A [A] \frac{[B]}{[B]}$$ \hspace{1cm} (48)

SCHEME A.11 Competing substrates.
Because the reaction specificity is determined by the ratios of $k_{cat}/K_m$, not just by $K_m$, nonproductive binding, which we have already learned has no effect on $k_{cat}/K_m$, does not affect substrate specificity.

VI. MULTISUBSTRATE SYSTEMS

For the most part, the preceding discussions dealt with single-substrate enzymes. The equations become much more complex and difficult to solve when multiple substrates are involved. Therefore, if you are interested in these systems, I recommend you read an advanced text on enzyme kinetics (see General References at the end of this appendix). Here, however, I have described, in a nonmathematical way, some of the standard multisubstrate pathways, so you can get, at least, a physical understanding of their meaning.

A. Sequential Mechanisms

1. Random Sequential Mechanism

In this mechanism there is no obligatory order of combination of the substrates and enzyme or release of products (Scheme A.12).

2. Ordered Sequential Mechanism

In this mechanism there is an obligatory order for how substrates combine with the enzyme and how products dissociate (Scheme A.13). If substrate A has a lower $K_m$ for the enzyme than does substrate B, A will bind first. This may cause a conformational change in the enzyme that increases the affinity of the enzyme for substrate B, producing the ternary complex $E\cdot A\cdot B$.

![Scheme A.12](image1)

**SCHEME A.12** Random sequential mechanism.

![Scheme A.13](image2)

**SCHEME A.13** Ordered sequential mechanism.
3. Theorell–Chance Sequential Mechanism

This is a type of ordered mechanism in which the ternary complex does not accumulate (Scheme A.14; P is one product and Q is the second product).

4. Ping Pong Mechanism

Reactions in which one or more products are released before all the substrates are added are called Ping Pong reactions (Scheme A.15). When two substrates are involved and two products are formed, this is referred to as a Ping Pong Bi-Bi mechanism, such as that shown in Scheme A.15. If one substrate is cleaved into two products, it would be a Ping Pong Uni-Bi mechanism, and so forth. An example of a Ping Pong mechanism is phosphoglycerate mutase. The enzyme is phosphorylated by one substrate, and then the phosphoryl group is transferred to a second substrate. This reaction is depicted in Figure A.28 in the shorthand notation for enzyme mechanisms that was described by Cleland (except using A-PO$_3^{2-}$ as the phosphorylated substrate instead of A, E-PO$_3^{2-}$ as the phosphorylated enzyme instead of F, and B-PO$_3^{2-}$ as the phosphorylated product instead of P)$^{10}$ The horizontal line represents the enzyme. Substrate A-PO$_3^{2-}$ binds to the enzyme (downward vertical arrow), forming an E·A-PO$_3^{2-}$ complex, which forms phosphorylated en-

---

![Diagram](attachment:image.png)
zyme (E-PO$_3^{2-}$) and the bound dephosphorylated substrate Q (E-PO$_3^{2-}$·Q). Q is released from the enzyme (upward vertical arrow), leaving phosphorylated enzyme (E-PO$_3^{2-}$). Then the second substrate B binds to the enzyme (downward vertical arrow) to give the E-PO$_3^{2-}$·B complex. The phosphate is transferred to B (E·B-PO$_3^{2-}$), and B·PO$_3^{2-}$ is released from the enzyme (upward vertical arrow), leaving free enzyme (E).

A plot of $1/\nu$ versus $1/[S]$ for a Ping Pong reaction gives parallel lines (Figure A.29). As the [B] increases, $V_{\text{max}}$ increases, as does the $K_m$ for A-PO$_3^{2-}$, but $V_{\text{max}}/K_m$ remains constant.

VII. ALLOSTERISM AND COOPERATIVITY

A. General

The terms *allosterism* and *cooperativity* generally apply to multisubunit enzymes. Binding (and catalytic) events at one active site can influence binding (and catalytic) events at another active site in a multimeric protein. Generally, the active sites are located on different subunits. The binding of the *effector molecule* (the one causing the effect) causes a conformational change in the protein in such a way that signals the other subunits that it has been bound. If the effector acts at another site and is not the substrate, the effect is called *allosteric* and *heterotropic*. If the effector is the substrate, the effect is termed *cooperative* and *homotropic*.

Positive cooperativity means that binding of the substrate at one site makes it easier for another substrate molecule to bind to the active site. Negative cooperativity means binding at one site causes a decrease in affinity of the substrate for the active site. Cooperative enzymes show sigmoidal kinetics, not Michaelis–Menten-like kinetics (Figure A.30).
FIGURE A.30  Rate versus substrate concentration plots for enzyme-catalyzed reactions that exhibit (A), no cooperativity; (B), positive cooperativity; (C), negative cooperativity.

B. Monod–Wyman–Changeux (MWC) Concerted Model

This is one of the models that is used to rationalize the effects of cooperativity. It proposes that the protein is in a dynamic equilibrium between a state that has a low affinity for the substrate (called the T state, the tense state) and much less of a state
that has a high affinity for the substrate (the R state, the relaxed state). According to this model, in a multisubunit protein all subunits of an enzyme molecule are either in the T conformation or in the R conformation. The T and R states coexist in the absence of substrate (Figure A.31). When the substrate is added, it binds to the R state and drives the equilibrium toward R.

*Allosteric (or heterotropic) inhibitors* bind specifically to the T state and make it harder for the substrate to switch the enzyme into the R state (Figure A.32).

*Allosteric (or heterotropic) activators* bind specifically to the R state and pull more of the enzyme into the more active R state, thereby activating the enzyme. A substrate or effector that binds preferentially to the R state increases the concentration of the R state, giving positive cooperativity. However, the MWC model cannot account for negative cooperativity (this is rare), because binding of the first ligand can only stabilize the R state and cannot increase the proportion of the T state.

C. Koshland–Némethy–Filmer (KNF) Sequential Model

This model states that the progress from the T state to the R state is a sequential process, not an either T or R process (Figure A.33). The conformation of each