CHAPTER THREE

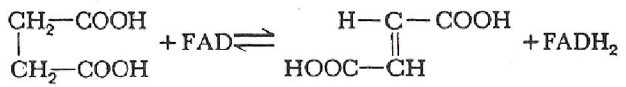
SIMPLE INHIBITION SYSTEMS

Any substance that reduces the velocity of an enzyme-catalyzed reaction can be considered to be an "inhibitor." The inhibition of enzyme activity is one of the major regulatory devices of living cells, and one of the most important diagnostic procedures of the enzymologist. Inhibition studies often tell us something about the specificity of an enzyme, the physical and chemical architecture of the active site, and the kinetic mechanism of the reaction. In our every day life, enzyme inhibitors can be found masquerading as drugs, antibiotics, preservatives, poisons, and toxins. In this chapter we examine three simple types of enzyme inhibitors. We assume that only a single substrate is involved in the reaction, and that only one type of inhibitor is present at any time. The effects of inhibitors on multisubstrate enzymes are discussed in Chapters Six and Nine. The effects of multiple inhibitors are discussed in Chapter Eight.

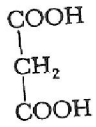
A. COMPETITIVE INHIBITION (SIMPLE INTERSECTING LINEAR COMPETITIVE INHIBITION)

A competitive inhibitor is a substance that combines with free enzyme in a manner that prevents substrate binding. That is, the inhibitor and the substrate are *mutually exclusive*, often because of true competition for the same site. A competitive inhibitor might be a nonmetabolizable analog or derivative of the true substrate, or an alternate substrate of the enzyme, or a product of the reaction.

Malonic acid is a classical example of a true competitive inhibitor. Malonic acid inhibits succinic dehydrogenase, which catalyzes the oxidation of succinic acid to fumaric acid, as shown below.



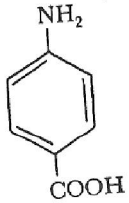
Malonic acid resembles succinic acid sufficiently to combine with the enzyme at the active site.



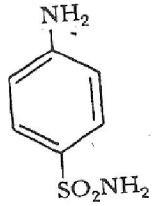
malonic acid

However, because malonic acid has only one methylene group, no oxidation-reduction can take place.

Another classical example of a competitive inhibitor is the sulfa drug sulfanilamide, which interferes with the biosynthesis of folic acid from the precursor *p*-amino-benzoic acid (PABA).



PABA



sulfanilamide

Figure III-1 illustrates several situations that lead to the mutually exclusive binding of S and I, Model 1 represents classical competitive inhibition. Models 2 to 4 yield the same results.

There are many examples of "competitive" inhibition by compounds that bear no structural relationship to the substrate. The inhibitor might be an end-product or near end-product of a metabolic pathway; the enzyme is one that catalyzes an early reaction (or a branch-point reaction) in the pathway. The phenomenon is called feedback inhibition. The inhibitor ("effector," "modulator," or "regulator") combines with the enzyme at a position other than the active (substrate) site. The combination of the inhibitor with the enzyme causes a change in the conformation (tertiary or quaternary structure) of the enzyme that distorts the substrate site and thereby prevents the substrate from binding (Model 5).

The inhibition of the hexokinase-catalyzed reaction between glucose and ATP by fructose or mannose is an example of competitive inhibition by alternate substrates. Glucose, fructose, and mannose are all substrates of hexokinase and can be converted to product (hexose-6-phosphate). All three hexoses combine with the enzyme at the same active site. Consequently, the

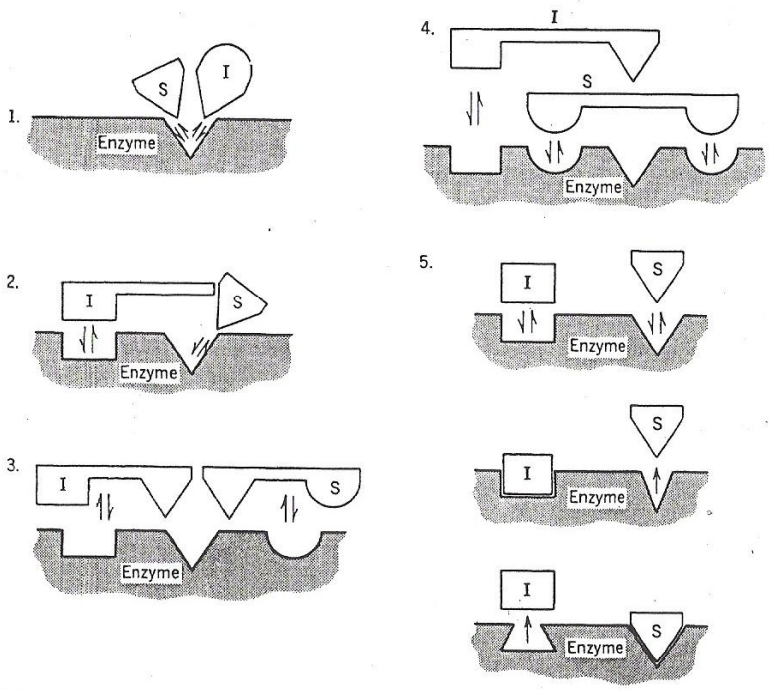


Fig. III-1. Models of competitive inhibition: S and I are mutually exclusive. (1) Classical model. S and I compete for the same binding site. I must resemble S structurally. (2) I and S are mutually exclusive because of steric hinderance. (3) I and S share a common binding group on the enzyme. (4) The binding sites for I and S are distinct, but overlapping. (5) The binding of I to a distinct inhibitor site causes a conformational change in the enzyme that distorts or masks the substrate binding site (and vice versa).

utilization of any one of the hexoses is inhibited in the presence of either of the other two.

The equilibria describing competitive inhibition are shown below. The competition and mutual exclusion of S and I are clearly seen.

$$\begin{array}{ccccccc} \mathrm{E} & + & \mathrm{S} & \overset{K_S}{\rightleftharpoons} & \mathrm{ES} & \overset{k_p}{\longrightarrow} & \mathrm{E} + \mathrm{P} \\ + & & & & & & \\ \mathrm{I} & & & & & & \\ {\scriptstyle K_i}\uparrow\downarrow & & & & & & \\ \mathrm{EI} & & & & & & \end{array}$$

where $K_i = [E][I]/[EI]$, $K_S = [E][S]/[ES]$, and k_p = rate constant for the breakdown of ES to E + P.

The initial velocity of the reaction is proportional to the steady-state concentration of the enzyme-substrate complex, ES. All the enzyme species are reversibly connected. Consequently, we can predict that at any fixed unsaturating concentration of inhibitor (*a*) v_i (the velocity in the presence of a competitive inhibitor) can be made to equal v (the velocity in the absence of the inhibitor), but that a higher substrate concentration will be required (to obtain the same ES concentration), and (*b*) in the presence of an infinitely high (saturating) substrate concentration all the enzyme can be driven to the ES form. Consequently, the maximal initial velocity in the presence of the competitive inhibitor equals V_{max} (the maximum initial velocity in the absence of inhibitor). However, the apparent K_m (measured as [S] required for $\frac{1}{2}V_{max}$) will increase in the presence of a competitive inhibitor because at any inhibitor concentration, a portion of the enzyme exists in the EI form which has no affinity for S.

An expression relating v, V_{max}, [S], K_m, [I], and K_i in the presence of a competitive inhibitor can be derived from either rapid equilibrium or steady-state assumptions. This time we must recognize that the total enzyme $[E]_t$ is present in three forms: free enzyme, [E]; enzyme-substrate complex [ES]; and enzyme-inhibitor complex, [EI].

$$v = k_p[ES]$$

$$\frac{v}{[E]_t} = \frac{k_p[ES]}{[E]+[ES]+[EI]}$$

$$\frac{v}{k_p[E]_t} = \frac{\dfrac{[S]}{K_S}}{1+\dfrac{[S]}{K_S}+\dfrac{[I]}{K_i}}$$

$$\boxed{\frac{v}{V_{max}} = \frac{\dfrac{[S]}{K_S}}{1+\dfrac{[S]}{K_S}+\dfrac{[I]}{K_i}}} \qquad \text{(III-1)}$$

If we compare equation III-1 to the usual velocity equation II-8, we see that the denominator has gained an additional $[I]/K_i$ term representing the

EI complex. The numerator still has one term indicating that there is still only one product-forming complex (ES). To obtain a more familiar form, the numerator and denominator of the right-hand part of equation III-1 can be multiplied by K_S and factored:

$$\frac{v}{V_{max}} = \frac{[S]}{K_S\left(1+\frac{[I]}{K_i}\right)+[S]} \qquad \text{(III-2)}$$

We obtain the same final velocity equation for steady-state conditions; however, K_m replaces K_S. This is not surprising, since the steady-state assumption does not change the form of the velocity equation for the uninhibited reaction while the reaction between E and I to yield EI must be at equilibrium. (There is nowhere for EI to go but back to E + I).

The velocity equation differs from the usual Henri-Michaelis-Menten equation in that the K_m term is multiplied by the factor $(1+[I]/K_i)$. This confirms our original prediction that V_{max} is unaffected by a competitive inhibitor, but that the *apparent* K_m value is increased. The increase in the K_m value does *not* mean that the EI complex has a lower affinity for the substrate. The EI has no affinity at all for the substrate, while the affinity of E (the only form that can bind substrate) is unchanged. The apparent increase in K_m results from a distribution of available enzyme between the "full affinity" and "no affinity" forms. The factor $(1+[I]/K_i)$ may be considered as an [I]-dependent statistical factor describing the distribution of enzyme between the E and EI forms. There are systems in which EI has an altered affinity for S. This type of system, called partial competitive inhibition, is discussed in Chapter Four.

The effect of a competitive inhibitor on the kinetics of an enzyme-catalyzed reaction is illustrated in Figure III-2. The [I] was arbitrarily chosen as $3K_i$; $K_{m_{app}}$ then is $(1+3)K_m = 4K_m$. In the presence of the inhibitor it takes four times as much substrate to attain $0.5\,V_{max}$. In general:

$$\frac{[S]_i}{[S]} = \left(1+\frac{[I]}{K_i}\right) \qquad \text{(III-3)}$$

where $[S]_i/[S]$ represents the ratio of substrate concentration required in the presence of inhibitor to substrate concentration required in the absence of inhibitor for any given velocity. A competitive inhibitor will increase $[S]_{0.9}$ and $[S]_{0.1}$. However, since both concentrations are increased by the same factor, the $[S]_{0.9}/[S]_{0.1}$ ratio is still 81 at all inhibitor concentrations. The

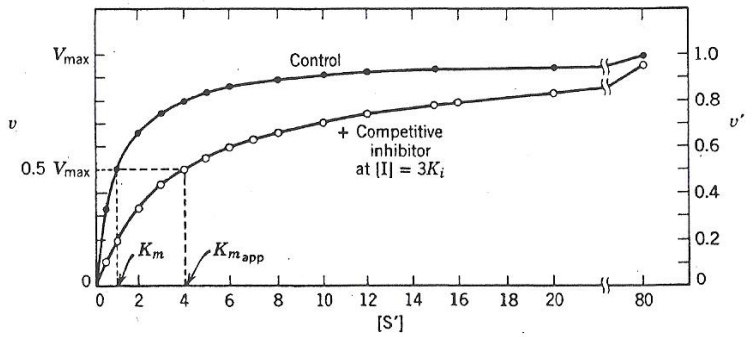


Fig. III-2. The v versus [S] plot in the presence and in the absence of a fixed concentration of a competitive inhibitor.

constant ratio is expected, since the form of the equation is unchanged; only the numerical value of K_m is changed.

An expression for the relative velocity or fractional activity in the presence and absence of a competitive inhibitor can be derived readily:

Let v_i = the initial velocity at a given [S] in the presence of inhibitor

v_0 = the initial velocity at the same [S] in the absence of inhibitor

$\frac{v_i}{v_0} = a$ = the relative activity

$$\frac{v_i}{v_0} = a = \frac{\frac{V_{max}[S]}{K_m\left(1+\frac{[I]}{K_i}\right)+[S]}}{\frac{V_{max}[S]}{K_m+[S]}} \quad \text{or} \quad \boxed{a = \frac{K_m+[S]}{K_m\left(1+\frac{[I]}{K_i}\right)+[S]}} \qquad \text{(III-4)}$$

Relative velocity data are frequently expressed in terms of fractional inhibition (i) or "percent inhibition" ($i_{\%}$) where:

$$i = 1-\frac{v_i}{v_0} = 1-a \qquad \text{and "percent inhibition"} \qquad = 100i$$

$$\boxed{i = \frac{[I]}{[I]+K_i\left(1+\frac{[S]}{K_m}\right)}} \qquad \text{(III-5)}$$

Effect of Concentration Range on Degree of Inhibition

A point not always appreciated is that the degree of inhibition caused by an *n-fold excess* of competitive inhibitor is maximum when both [I] and [S] are very high compared to K_i and K_m, respectively. To put it another way, if [S] is very low compared to K_m, an excess of competitive inhibitor will not cause much inhibition even though K_i is of the same order of magnitude as K_m. A simple example illustrates the point. Suppose $[S]=0.01K_m$ and $K_i=K_m$. What is the degree of inhibition caused by a tenfold excess of inhibitor (i.e., by an $[I]=10[S]$)?

$$i=\frac{0.1K_m}{0.1K_m+K_m(1+0.01)}=\frac{0.1K_m}{1.11K_m}=0.09$$

$$i_{\%}=9\%$$

In other words, the inhibited velocity is 91% of the control velocity; we observe only 9% inhibition. On the other hand, when $[S]=10K_m$, a tenfold excess of inhibitor ($[I]=100K_m$) will inhibit 90% as shown below.

$$i=\frac{100K_m}{100K_m+K_m+10K_m}=\frac{100K_m}{111K_m}$$

$$\therefore \quad i=0.90 \quad \text{or} \quad i_{\%}=90$$

Figure III-3 shows the effect of increasing competitive inhibitor concentration on the initial velocity at three different substrate concentrations. The degree of inhibition depends on the substrate concentration, decreasing as [S] increases, as predicted by equation III-5. To obtain 50% inhibition:

$$\boxed{[I]_{0.5}=\left(1+\frac{[S]}{K_m}\right)K_i} \qquad \text{(III-6)}$$

Similarly, we can show that $[I]_{0.9}$ and $[I]_{0.1}$ (the inhibitor concentrations required for 90% and 10% inhibition, respectively) are:

$$[I]_{0.9}=9\left(1+\frac{[S]}{K_m}\right)K_i, \qquad [I]_{0.1}=\frac{1}{9}\left(1+\frac{[S]}{K_m}\right)K_i$$

Thus the $[I]_{0.9}/[I]_{0.1}$ ratio is always 81, regardless of the substrate concentration or the values of K_m and K_i.

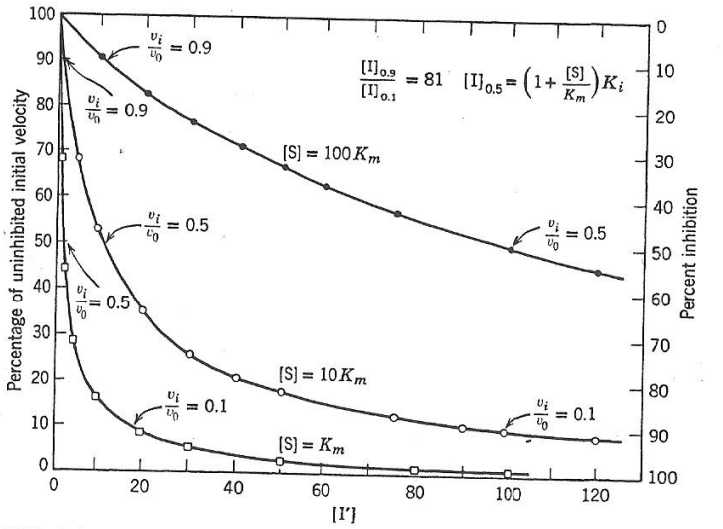


Fig. III-3. Relative activity as a function of competitive inhibitor concentration in the presence of different fixed concentrations of substrate.

Reciprocal Plot for Competitive Inhibition Systems

The velocity equation for competitive inhibition in reciprocal form is:

$$\boxed{\frac{1}{v}=\frac{K_m}{V_{max}}\left(1+\frac{[I]}{K_i}\right)\frac{1}{[S]}+\frac{1}{V_{max}}} \qquad \text{(III-7)}$$

Thus the slope of the plot increases by the factor $(1+[I]/K_i)$ (which multiplied K_m in the original equation), but the $1/v$-axis intercept remains $1/V_{max}$. For each inhibitor concentration, a new reciprocal plot can be drawn. As [I] increases, the "plus inhibitor" curves increase in slope (Fig. III-4) pivoting counterclockwise about the point of intersection with the control curve (at $1/V_{max}$ on the $1/v$-axis). Because the initial velocity can be driven to zero by a saturating inhibitor concentration, the limiting plot will be a vertical line on the $1/v$ axis. As [I] increases, the intercept on the $1/[S]$ axis moves closer to the origin; that is, $K_{m_{app}}$ continually increases. The K_i can be calculated from the slope of any reciprocal plot or from any $K_{m_{app}}$. However, a replot as described below is better.

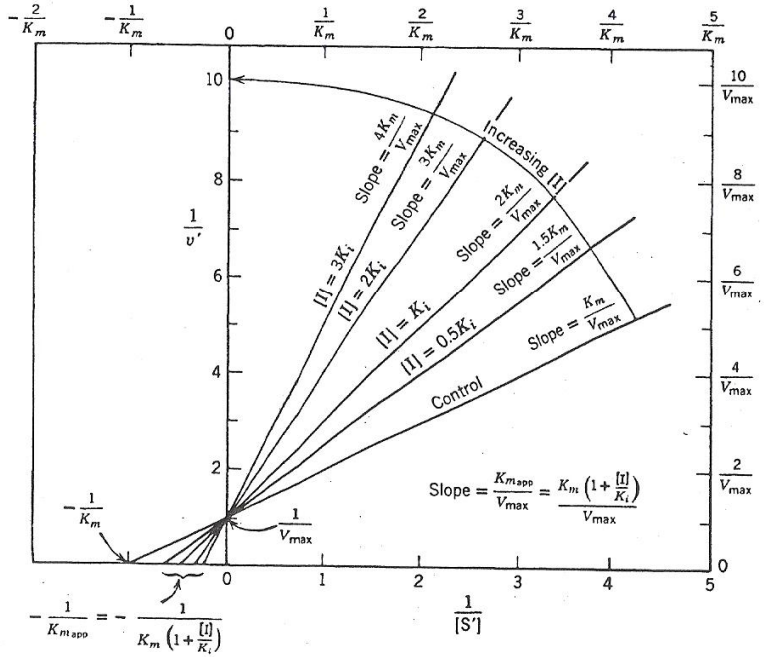


Fig. III-4. The $1/v$ versus $1/[S]$ plot in the presence of different fixed concentrations of a competitive inhibitor.

Replots of Slope and $K_{m_{app}}$ Versus [I]

The slope of the reciprocal plot in the presence of a competitive inhibitor is given by:

$$slope_{1/S} = \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_i}\right) \quad \text{or} \quad \boxed{slope_{1/S} = \frac{K_m}{V_{max}K_i}[I] + \frac{K_m}{V_{max}}} \tag{III-8}$$

A replot of the slope of each reciprocal plot versus the corresponding inhibitor concentration will be a straight line with a slope of $K_m/V_{max}K_i$ and an intercept on the $slope_{1/S}$-axis of K_m/V_{max} (i.e., control slope at $[I]=0$) (Fig. III-5*a*). When $slope_{1/S}=0$, the intercept on the [I]-axis gives $-K_i$. For

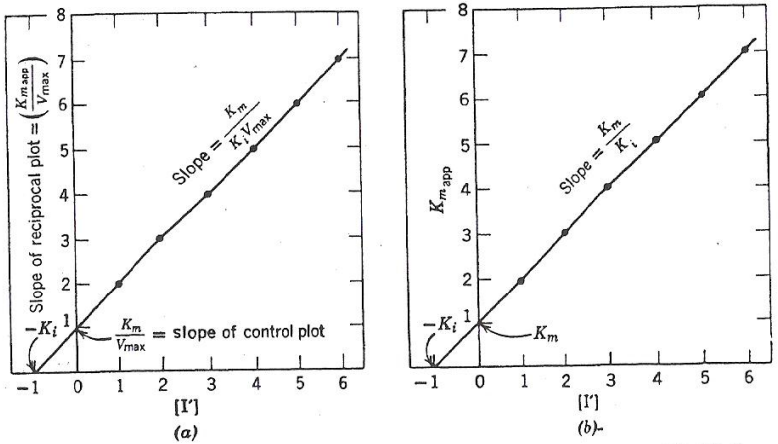


Fig. III-5. Replots of data taken from the reciprocal plot. (*a*) $Slope_{1/S}$ versus [I]. (*b*) $K_{m_{app}}$ versus [I].

convenience the slope of the reciprocal plots can be read off directly as the ratio (absolute values) of the vertical axis intercept to horizontal axis intercept. A linear $slope_{1/S}$ versus [I] replot distinguishes pure competitive inhibition from partial competitive inhibition. The latter gives hyperbolic $slope_{1/S}$ versus [I] replots (Chapter Four). The $K_{m_{app}}$ is also a linear function of the inhibitor concentration in pure competitive systems:

$$\boxed{K_{m_{app}} = \frac{K_m}{K_i}[I] + K_m} \tag{III-9}$$

A replot of $K_{m_{app}}$ versus [I] has intercepts of K_m (on the $K_{m_{app}}$-axis) and $-K_i$ (on the [I]-axis) (Fig. III-5*b*).

Dixon Plot for Competitive Inhibition: $1/v$ Versus [I]

The Dixon plot is used frequently to identify the type of inhibition and to determine the K_i value. The velocity equation for competitive inhibition may be converted to a linear form in which the varied ligand is [I]. Starting

with the reciprocal equation:

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

$$\boxed{\frac{1}{v} = \frac{K_m}{V_{max}[S]K_i}[I]+\frac{1}{V_{max}}\left(1+\frac{K_m}{[S]}\right)} \qquad \text{(III-10)}$$

A plot of $1/v$ versus [I] at some unsaturating [S] will yield a straight line with a positive slope as shown in Figure III-6*a*. If the inhibition is known to be competitive and V_{max} is known, a horizontal line at a height of $1/V_{max}$ can be drawn. The $-[I]$ value at the intersection of two lines gives K_i as shown below. When $1/v = 1/V_{max}$:

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

$$1 = \frac{K_m[I]}{[S]K_i}+1+\frac{K_m}{[S]}$$

$$-\frac{K_m[I]}{[S]K_i} = \frac{K_m}{[S]} \qquad \text{or} \qquad [I] = -K_i$$

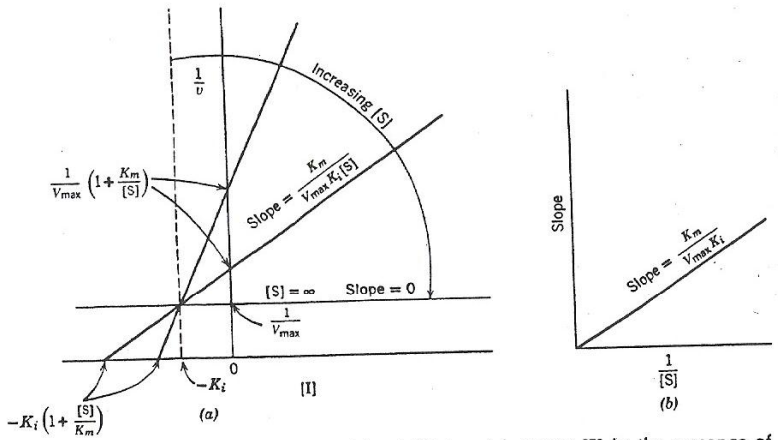


Fig. III-6. (*a*) Dixon plot for a competitive inhibitor: $1/v$ versus [I] in the presence of different fixed concentrations of substrate. If V_{max} is known, a horizontal line at a height of $1/V_{max}$ can be drawn directly. (*b*) Replot of the slopes of the Dixon plot.

The horizontal line at $1/V_{max}$ also signifies that at an infinitely high [S], increasing the inhibitor concentration will have no effect on v. If the inhibition is not known for sure to be competitive, or if V_{max} is unknown, another series of experiments at a different unsaturating [S] will yield a second line with a different positive slope. The intersection of the $[S]_1$ and $[S]_2$ lines, where $1/v_1 = 1/v_2$, gives K_i as shown below. When $1/v_1 = 1/v_2$:

$$\frac{K_m[I]}{V_{max}[S]_1K_i}+\frac{[S]_1+K_m}{V_{max}[S]_1} = \frac{K_m[I]}{V_{max}[S]_2K_i}+\frac{[S]_2+K_m}{V_{max}[S]_2} \quad \text{or} \quad \frac{[I]+K_i}{[S]_1} = \frac{[I]+K_i}{[S]_2}$$

The equation above holds true only if $[S]_1 = [S]_2$, which is not the case, or when both sides equal zero; that is, when $[I] = -K_i$.

The slope of the Dixon plot is given by:

$$slope = \frac{K_m}{V_{max}K_i}\frac{1}{[S]} \qquad \text{(III-11)}$$

Thus a replot of *slope* versus the corresponding $1/[S]$ (Fig. III-6*b*) will be a straight line through the origin with a slope of $K_m/V_{max}K_i$.

The family of Dixon plots for pure competitive inhibition intersects above the [I]-axis at $[I] = -K_i$ and $1/v = 1/V_{max}$. Certain types of mixed inhibition systems also yield lines that intersect above the [I]-axis. Consequently, a Dixon plot such as that shown in Figure III-6*a* establishes only that the inhibition is neither noncompetitive nor uncompetitive. Other plots for competitive inhibition systems are described in Chapter Four.

General Principles

A competitive inhibitor acts only to increase the apparent K_m for the substrate. As [I] *increases, $K_{m_{app}}$ increases. The V_{max} remains unchanged, but in the presence of a competitive inhibitor a much greater substrate concentration is required to attain any given fraction of V_{max}. The v_i may be considered equal to V_{max} when $[S] \geq 100K_{m_{app}}$.*

The degree of inhibition caused by a competitive inhibitor depends on [S], [I], K_m, *and K_i. An increase in* [S] *at constant* [I] *decreases the degree of inhibition. An increase in* [I] *at constant* [S] *increases the degree of inhibition. The lower the value of K_i, the greater is the degree of inhibition at any given* [S] *and* [I]. *The K_i is equivalent to the concentration of* I *that doubles the slope of the $1/v$ versus $1/[S]$ plot. (K_i is not equivalent to the* [I] *that yields* 50% *inhibition*).

Integrated Rate Equation in the Presence of a Competitive Inhibitor

If the reaction has a very large K_{eq} and none of the products have an appreciable affinity for the enzyme, then the integrated Henri-Michaelis-Menten equation in the presence of a competitive inhibitor can be written as:

$$\frac{2.3}{t}\log\frac{[S]_0}{[S]} = -\frac{1}{K_m\left(1+\frac{[I]}{K_i}\right)}\frac{[P]}{t} + \frac{V_{max}}{K_m\left(1+\frac{[I]}{K_i}\right)} \qquad \text{(III-12)}$$

where $[P]=[S]_0-[S]$. The equation assumes that [I] remains constant as [S] decreases. Consequently, I cannot be an alternate substrate. The determination of [P] at various times during the course of the reaction will permit $K_{m_{app}}$ and V_{max} to be determined. A family of curves can be obtained for different inhibitor concentrations (Fig. III-7). The values of K_m and K_i can be determined from appropriate replots of the slopes or vertical axis intercepts.

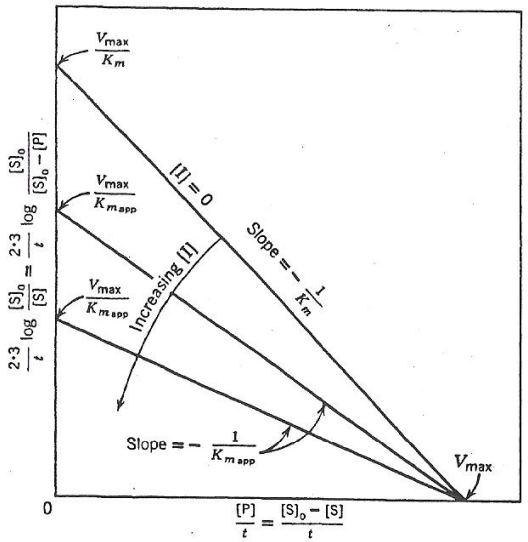


Fig. III-7. Plot of the integrated velocity equation in the presence of a competitive inhibitor.

Competitive Inhibition and Total Velocity with Mixed Alternative Substrates

When a single enzyme acts on two different substrates, and both are present simultaneously, each will act as a competitive inhibitor with respect to the other. If the products of the two substrates can be distinguished from each other, the system may be treated by the usual competitive inhibition relationships. If, on the other hand, the products are identical, or appear so by the assay method used, then the situation becomes more complex. For example, suppose the enzyme is a nonspecific phosphatase that catalyzes the reactions A→Q+P and B→R+P where A and B are two phosphate esters, Q and R are two distinct alcohols, and P is inorganic phosphate. In the presence of A and B the equilibria are:

$$\begin{array}{ccccccccc} E & + & A & \overset{K_A}{\rightleftharpoons} & EA & \xrightarrow{k_{p_A}} & E & + & Q & + & P \\ + & & & & & & & & & & \\ B & & & & & & & & & & \\ {\scriptstyle K_B}\uparrow\downarrow & & & & & & & & & & \\ EB & & & \xrightarrow{k_{p_B}} & E & + & R & + & P & & \end{array}$$

If the rate of P formation is measured, the observed velocity, v_t, is the sum of two reactions:

$$v_t = k_{p_A}[EA] + k_{p_B}[EB] \qquad \text{and} \qquad \frac{v_t}{[E]_t} = \frac{k_{p_A}[EA]+k_{p_B}[EB]}{[E]+[EA]+[EB]}$$

Under rapid equilibrium or steady-state conditions:

$$v_t = \frac{V_{max_A}\frac{[A]}{K_{m_A}} + V_{max_B}\frac{[B]}{K_{m_B}}}{1+\frac{[A]}{K_{m_A}}+\frac{[B]}{K_{m_B}}} \qquad \text{(III-13)}$$

where $V_{max_A} = k_{p_A}[E]_t$ = the maximal velocity with A as a substrate
$V_{max_B} = k_{p_B}[E]_t$ = the maximal velocity with B as the substrate

Equation III-13 can be rearranged to:

$$v_t = \frac{V_{\max_A}[A]}{K_{m_A}\left(1+\frac{[B]}{K_{m_B}}\right)+[A]} + \frac{V_{\max_B}[B]}{K_{m_B}\left(1+\frac{[A]}{K_{m_A}}\right)+[B]} \qquad \text{(III-14)}$$

Thus one enzyme that accepts two substrates will yield results identical to two enzymes, each of which is specific for one substrate but is competitively inhibited by the other's substrate.

The v_t can be expressed in terms of v_A (the velocity observed at the given [A] in the absence of B) and v_B (the velocity observed at the given [B] in the absence of A).

Substituting:

$$V_{\max_A} = v_A\left(1+\frac{K_{m_A}}{[A]}\right) \quad \text{and} \quad V_{\max_B} = v_B\left(1+\frac{K_{m_B}}{[B]}\right)$$

into equation III-13 and simplifying:

$$v_t = \frac{v_A\left(1+\frac{[A]}{K_{m_A}}\right)+v_B\left(1+\frac{[B]}{K_{m_B}}\right)}{1+\frac{[A]}{K_{m_A}}+\frac{[B]}{K_{m_B}}} \qquad \text{(III-15)}$$

If A and B are equally acceptable substrates (i.e., same K_m and $V_{\max}$), then the v_t observed in the presence of any given mixture of A and B will be the same as the v observed with either A or B alone at the same total specific concentration. For example, if $V_{\max_A}=1$ and $V_{\max_B}=1$, then at $[A]=0.2K_{m_A}$ plus $[B]=0.6K_{m_B}=0.6K_{m_A}$, v_t equals 0.444. This is the same v observed at $[A]=0.8K_{m_A}$ and $[B]=0$, or $[B]=0.8K_{m_B}$ and $[A]=0$. On the other hand, v_t is *always less than* the sum of the velocities observed with each substrate alone at a given concentration. In the example above, $v_A=0.167$ at $[A]=0.2K_{m_A}$ and $[B]=0$. The $v_B=0.375$ at $[B]=0.6K_{m_B}$ and $[A]=0$. The sum, $v_A+v_B=0.542$, is greater than the observed v_t of 0.444 at $[A]=0.2K_{m_A}$ plus $[B]=0.6K_{m_B}$. The fact that v_t is always less than v_A+v_B may seem odd at first. But suppose A and B are really the same compound and $V_{\max}=1$. At $[A]=K_{m_A}$, $v_A=0.5$. At $[B]=K_{m_B}$, $v_B=0.5$. $v_A+v_B=1$, yet we know that at $[A]=2K_{m_A}$ or $[B]=2K_{m_B}$, v is only 0.667. Note that $v_t<v_A+v_B$ holds regardless of the relative values of K_{m_A}, K_{m_B}, $V_{\max_A}$ and $V_{\max_B}$. As with ordinary competitive inhibition, the degree of inhibition (in this case, the difference between v_t and v_A+v_B)

decreases as the concentration of either A or B becomes small compared to the respective K_m values. The maximum difference between v_t and v_A+v_B is observed when both A and B are present at concentrations that are very high compared to their respective K_m values.

If the specific concentrations of A and B are equal:

$$v_t = \frac{v_A\left(1+\frac{[A]}{K_{m_A}}\right)+v_B\left(1+\frac{[A]}{K_{m_A}}\right)}{1+\frac{[A]}{K_{m_A}}+\frac{[A]}{K_{m_A}}} = (v_A+v_B)\frac{1+\frac{[A]}{K_{m_A}}}{1+\frac{2[A]}{K_{m_A}}} \qquad \text{(III-16)}$$

Thus when $[A]/K_{m_A}$ and $[B]/K_{m_B}$ are very small, $v_t \cong v_A+v_B$. As $[A]/K_{m_A}$ and $[B]/K_{m_B}$ increase (but remain equal), v_t increases to a limit of $(v_A+v_B)/2$.

An interesting relationship can be derived for the special case where A and B are present at equimolar concentrations (not equal specific concentrations). If $[A]=[B]$:

$$v_t = \frac{V_{\max_A}\frac{[A]}{K_{m_A}}+V_{\max_B}\frac{[A]}{K_{m_B}}}{1+\frac{[A]}{K_{m_A}}+\frac{[A]}{K_{m_B}}}$$

Dividing numerator and denominator by [A]:

$$v_t = \frac{\frac{V_{\max_A}}{K_{m_A}}+\frac{V_{\max_B}}{K_{m_B}}}{\frac{1}{[A]}+\frac{1}{K_{m_A}}+\frac{1}{K_{m_B}}}$$

When [A] and [B] are very high compared to their respective K_m values, the $1/[A]$ term becomes negligible and the observed combined velocity is maximal.

$$v_t \cong \frac{\frac{V_{\max_A}}{K_{m_A}}+\frac{V_{\max_B}}{K_{m_B}}}{\frac{1}{K_{m_A}}+\frac{1}{K_{m_B}}} = V_{\max_t}$$

or

$$\frac{K_{m_B}}{K_{m_A}} = \frac{V_{\max_B}-V_{\max_t}}{V_{\max_t}-V_{\max_A}} \qquad \text{(III-17)}$$

Thus the relative K_m values can be determined from three measurements, namely, V_{max_A}, V_{max_B}, and V_{max_i} (maximal mixed velocity with an equimolar mixture of A and B).

If two specific enzymes are present and each is unaffected by the other's substrate, then v_i will equal $v_A + v_B$. However, there are at least five conditions where a mixture of two enzymes yields $v_i < v_A + v_B$: (*a*) each enzyme is catalytically active with only one of the substrates, but is competitively inhibited by the other substrate (equation III-14); (*b*) each enzyme is catalytically active with only one of the substrates but one of the enzymes (e.g., the A-specific enzyme) is competitively inhibited by the other's substrate (B); (*c*) one enzyme is catalytically active with only one of the substrates (e.g., A) and is unaffected by the other substrate (B), and the second enzyme is nonspecific; (*d*) one enzyme is catalytically active with only one of the substrates (e.g., A), but is competitively inhibited by the other substrate, and the second enzyme is nonspecific; (*e*) two nonspecific enzymes. Under conditions *c* and *d*, nonlinear reciprocal and Eadie-Scatchard plots may be seen when the varied substrate is the one acted on by both enzymes. Under condition *e*, nonlinear plots may be seen for both substrates. Conditions *a* and *b* yield linear plots for both substrates. (In both cases, there is only one enzyme active on a given substrate.) Additional distinctions may be made if v is measured as the rate of unique product (Q and R) formation. Under conditions *a*, *d*, and *e*, a saturating concentration of either substrate will inhibit completely the formation of the unique product of the other substrate. Under condition *b*, a saturating concentration of one substrate (e.g., B) will inhibit completely the formation of the unique product of the other substrate (e.g., Q from A), but not vice versa (i.e., [A] will have no effect on R formation from B). Under condition *c*, a saturating concentration of one substrate (e.g., A) will inhibit completely the formation of the unique product of the other substrate (e.g., R from B). The reverse experiment yields partial inhibition. A saturating concentration of B will inhibit only the activity of the nonspecific enzyme on A. The activity of the A-specific enzyme is unaffected.

The mixed substrate phenomenon was applied in an interesting way to a study of NH_4^+ transport by *Penicillium chrysogenum*. In this study methylammonium-^{14}C was used as an NH_4^+ analog. The K_m for methylammonium-^{14}C transport was 10^{-5} M, and the V_{max} was 10 μmoles$\times$g dry weight cells$^{-1}\times$min^{-1}. The physiologically important substrate of the transport system, NH_4^+ was a potent inhibitor of methylammonium-^{14}C transport ($K_{i_{NH_4^+}} = 2.5\times10^{-7}$ M). The K_i value for NH_4^+ as an inhibitor was assumed to be equivalent to the K_m value for NH_4^+ as a substrate. The mixed substrate method was used to estimate V_{max} for NH_4^+ transport. Varying concentrations of NH_4^+ (10^{-5} to 10^{-4} M) were mixed with a constant concentration (10^{-4} M) of methylammonium-^{14}C and the uptake of the methyl ammonium-^{14}C from each mixture was determined over a period of time. The results are shown in Figure III-8. During the early stages of the assay, NH_4^+ almost completely displaced methylammonium-^{14}C from the transport system. During the same time period, NH_4^+ was transported into the cells thereby reducing its external concentration. This resulted in a progressive decrease in the inhibition of methylammonium-^{14}C transport with time. When the NH_4^+ had been depleted, the methylammonium-^{14}C transport rate attained the control rate. The lag period (between zero-time and the time when the methylammonium-^{14}C transport rate attained the control rate) was taken as the time required to transport the NH_4^+ present. The length of the lag period was proportional to the initial NH_4^+ concentration.

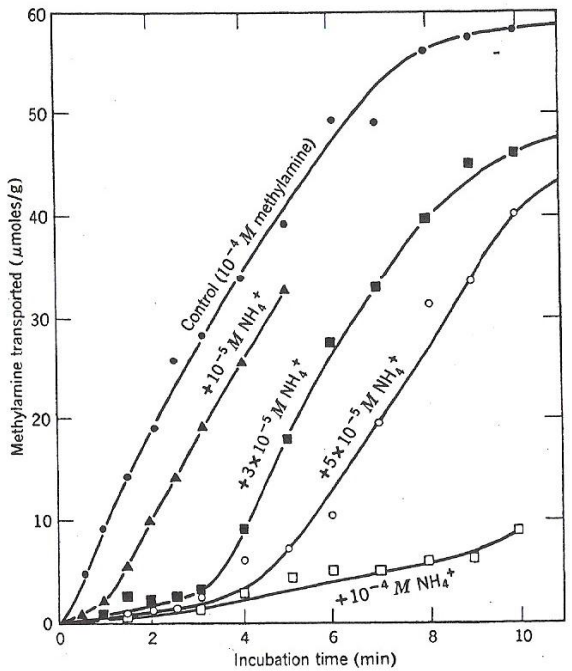


Fig. III-8. Effect of NH_4^+ on methylamine-^{14}C transport by *Penicillium chrysogenum*. Methylamine-^{14}C is excluded from the transport system as long as NH_4^+ is present in the medium. [Redrawn from Hackette, S. L., Skye, G. E., Burton, C. and Segel, I. H., *J. Biol. Chem.* 245, 4241, (1970).] (See Dixon, M. and Webb, E. C., *Enzymes*, 2nd ed., Ch. 4, p. 88 for another example observed by Willstätter *et al.*, in 1927.)

The rate thus calculated was estimated as V_{max} for NH_4^+ transport. The estimate assumes that (*a*) 10^{-4} *M* methylammonium had little effect on the rate of NH_4^+ transport from solutions containing 10^{-5} to 10^{-4} *M* NH_4^+, and (*b*) the rate of NH_4^+ transport was essentially constant over the lag period. Assumption *a* is valid because the affinity of the transport system for NH_4^+ is 40-fold greater than the affinity for methylammonium. Assumption *b* is also valid because more than 97% of the NH_4^+ would have been transported before its concentration decreased to the K_m value.

Apparent Competitive Inhibition by Carrier Dilution (Isotope Competition)

In assays employing radioactive substrates, the addition of unlabeled substrate will produce the same *apparent* degree of inhibition as an equivalent amount of an alternative substrate with the same K_m value, or a nonsubstrate competitive inhibitor whose K_i value equals the K_m of the radioactive substrate. This method can be used to obtain a rapid comparison of the relative affinities of a variety of alternative substrates or nonsubstrate inhibitors. The carrier dilution method is illustrated below.

Suppose an enzyme catalyzes a reaction with a certain substrate, S, where $K_m = 2\times10^{-5}$ *M* and $V_{max} = 25$ μmoles/min. If radioactive S is used with a specific activity of 300,000 cpm/μmole, then at $[S] = K_m$, for example, the initial velocity will be $0.5\,V_{max}$, or 12.5 μmoles/min. The experimental raw data value of v would be (12.5 μmoles/min)×(3×10^5 cpm/μmole)=37.5×10^5 cpm/min. If a tenfold excess of unlabeled substrate (2×10^{-4} *M*) is added to the assay mixture together with the radioactive substrate, the specific activity of the substrate will be reduced to 1/11 of the original specific activity. The new specific activity will be 27,272 cpm/μmole. The new [S] will be $11K_m$ and the new velocity will be $11/12\,V_{max}$, or 22.9 μmoles/min. The raw data value of v will be (22.9 μmoles/min)×(27,272 cpm/μmole)=6.25×10^5 cpm/min. Compared to the original rate of 37.5×10^5 cpm/min, we observe an *apparent* 83.3% inhibition.

$$a = \frac{6.25\times10^5}{37.5\times10^5} = 0.167 \qquad i = 1 - 0.167 = 0.833$$

This corresponds to an *apparent* v of 2.08 μmoles/min. The true velocity, of course, has not decreased. It has increased on adding the additional substrate. However, v will appear to decrease if the raw data rate in terms of cpm/min are compared to the original raw data rate, or if the rate in terms of μmoles/min is calculated using the original, undiluted specific activity.

Now, let us calculate the degree of inhibition caused either by a tenfold excess of an alternate substrate where the K_m value equals the K_m of the radioactive substrate, or by a tenfold excess of a nonsubstrate competitive inhibitor where K_i equals the K_m of the substrate.

$$v = \frac{(25)(K_m)}{K_m\left(1+\dfrac{10K_i}{K_i}\right)+K_m} = \frac{25K_m}{12K_m} = 2.08\ \mu\text{moles/min}$$

The specific activity of the substrate is unchanged, so the observed raw data value of v_0 would be (2.08 μmoles/min)×(3×10^5 cpm/μmole)=6.25×10^5 cpm/min. Thus whether we compare the raw data velocities in terms of cpm/min, or velocities in terms of μmoles/min, the inhibitor and the unlabeled substrate produced the same degree of inhibition—real in the presence of the inhibitor, but only apparent in the presence of the excess unlabeled substrate. If the inhibitor produced a lower degree of inhibition than an equivalent amount of excess substrate, then we could conclude that $K_i > K_m$. If the inhibitor produced a greater degree of inhibition, then K_i must be less than K_m. It is not necessary that K_m and V_{max} be known to compare affinities by this method. However, the degree of inhibition by an *n*-fold excess of unlabeled substrate or inhibitor will be maximum when the concentration of radioactive substrate is high compared to K_m.

Isotope competition can be used to determine unknown concentrations of unlabeled substrate in solutions known to be free of real inhibitors. If we substitute $[S]/K_m$ for $[I]/K_i$ in equation III-4 we obtain:

$$\boxed{\frac{v_i}{v_0} = a = \frac{K_m + [S^*]}{K_m + [S] + [S^*]}} \qquad \text{(III-17a)}$$

where $[S^*]$ = the known concentration of radioactive substrate in the assay mixture

$[S]$ = the added unknown concentration of unlabeled substrate

$v_i/v_0 = a$ = the relative activity in terms of cpm/min or velocities (μmoles/min) if the original, undiluted specific activity of the radioactive substrate is used to calculate v_i

Equation III-17a can be solved for the concentration of unlabeled substrate present:

$$\boxed{[S] = (K_m + [S^*])\frac{1-a}{a}} \qquad \text{(III-17b)}$$

(See also Chapter Two, Section O. The concentration of unlabeled S can also be calculated from $K_{m_{app}}$ as given by equation II-84, provided the true K_m is known).

Competitive Product Inhibition Where [S]+[P] is Constant (Regulation Via "Energy Charge")

Consider a system in which the substrate and product are interconvertible, but the total pool of [S]+[P] remains essentially constant. The rate of the S→P reaction will depend on the relative concentrations of the substrate and the product which competes with the substrate for the enzyme. Because [S]+[P] is constant, an increase in [S] automatically means that [P] must decrease. Consequently, any increase in [S] is accompanied by a decrease in the degree of competitive product inhibition. The velocity curve can be concave (decreasing slope), convex (increasing slope), or linear, depending on the relative affinities of the enzyme for S and P. To simplify matters, we will assume that K_{eq} is very large (because V_{max_r} is very small) so that even at the lowest [S]/[P] ratio the observed initial velocity is the true forward velocity of the S→P reaction, uncomplicated by the P→S reaction. This assumption eliminates the $[P]/K_{eq}$ term from the numerator of equation II-20. Alternately, we can assume that the reaction yields two products, one of which is removed in a subsequent reaction. Under either of these conditions the velocity equation is:

$$\frac{v}{V_{max}} = \frac{[S]}{K_S\left(1+\frac{[P]}{K_P}\right)+[S]} \qquad \text{(III-18)}$$

Figure III-9 shows the velocity curves for a system where $[S]+[P]=10K_S$. Note that it is possible to obtain very steep (convex) curves if $K_P < K_S$. Steep velocity curves are usually associated with multisite enzymes that display cooperative binding and possess specific effector sites. In the present system, the [S]/[P] ratio exerts a very sensitive control over the velocity when $K_P < K_S$, yet only a single binding site for S and P is involved. Atkinson and co-workers (1970) have shown that a number of ATP-utilizing enzymes are strongly inhibited by their product, ADP or AMP. The initial velocities of these reactions are markedly influenced by the ATP-ADP-AMP

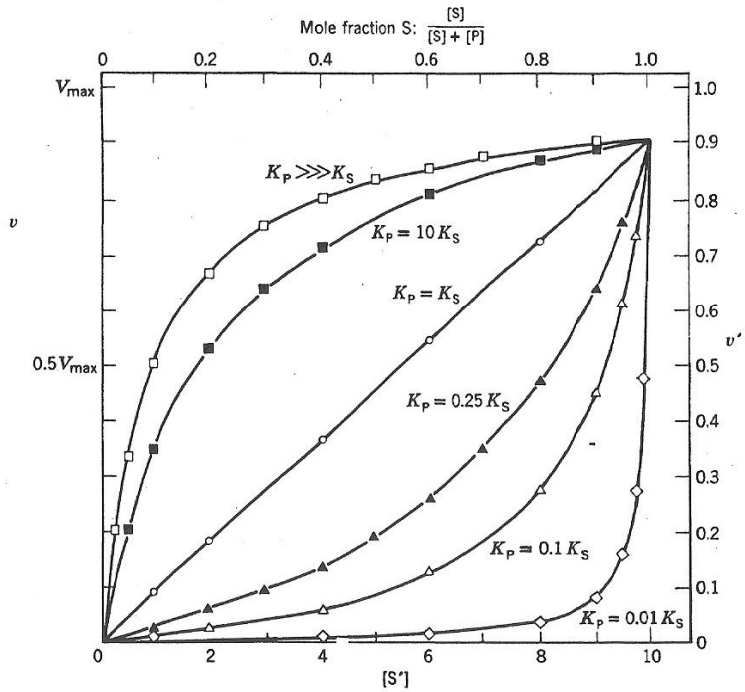


Fig. III-9. The v versus [S] plots where the total pool of [S]+[P] is constant at $10K_S$ and P has some affinity for E.

balance, called the "energy charge" of the system, where:

$$\text{"energy charge"} = \frac{[ATP]+\frac{1}{2}[ADP]}{[ATP]+[ADP]+[AMP]} \qquad \text{(III-19)}$$

The use of "energy charge" in place of the [ATP]/[ADP] or [ATP]/[AMP] ratios represents an attempt to simulate a given total adenine nucleotide pool under conditions that would exist *in vivo* where (presumably) adenylate kinase maintains the three nucleotides at equilibrium:

$$ATP + AMP \underset{}{\overset{\text{adenylate kinase}}{\rightleftharpoons}} 2ADP$$

Thus "energy charge" represents the mole fraction of adenine nucleotides represented by ATP or its equivalent ($\frac{1}{2}$ADP). The adenylate system is analogous to an electrochemical storage battery. The system is fully charged when all adenylate is present as ATP ("energy charge" = 1.0), and completely discharged when all adenylate is present as AMP ("energy charge" = 0). A system in which all the ATP had been converted to ADP would have an "energy charge" of 0.5. After equilibration via adenylate kinase, the "energy charge" would still be 0.5, since the total concentration of phosphate anhydride bonds would be unchanged. (Energy charge can also be defined as one half the phosphate anhydride bonds per adenosine.) The distribution of adenylates as a function of the "energy charge" is shown in Figure III-10. Many biosynthetic (i.e., energy utilizing) reactions are promoted by a high energy charge and inhibited by a low energy charge while the converse is true for energy-producing reactions. For example, Figure III-11 shows the velocity response to the [S]/[P] ratio for two enzymes, one of which catalyzes an essentially irreversible ATP-utilizing reaction (indicated as S→P) while the other catalyzes an essentially irreversible ATP-generating reaction (indicated as P→S). In both cases, $K_P = 0.1K_S$. The pool of [S]+[P] is fixed at $10K_S$. The velocities are given by the usual equations taking into account the product inhibition by P in the S→P reaction and the product inhibition by S in the P→S reaction:

$$\frac{v}{V_{max}} = \frac{[S]}{K_S\left(1+\frac{[P]}{K_P}\right)+[S]} \qquad \text{(for S}\longrightarrow\text{P)}$$

$$\frac{v}{V_{max}} = \frac{[P]}{K_P\left(1+\frac{[S]}{K_S}\right)+[P]} \qquad \text{(for P}\longrightarrow\text{S)}$$

If, *in vivo*, the mole fraction of [S] is poised at about 0.9 (for the particular parameters chosen), then the velocities of the S-utilizing and S-generating reactions will proceed at about $0.5V_{max}$ and small changes in the [S]/[P] ratio will tend to reestablish the original [S]/[P] ratio. (When [S] decreases, the velocity of the S→P reaction slows up while that of the P→S reaction increases.) If we consider only the S→P reaction, we see that the "energy charge" model can provide an effective "off-on" switch; that is, for a wide range of S concentrations, the velocity of the reaction can be relatively low and insensitive to increasing [S]. Then, for a relatively small increase in [S], v can increase markedly. To be an effective control system, K_P must be significantly less than K_S and the total concentration of [S]+[P] must be

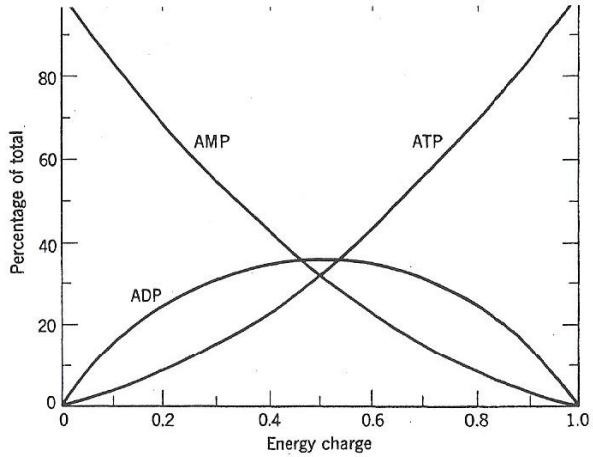


Fig. III-10. Relative concentrations of adenine nucleotides as a funtion of energy charge when the adenylate kinase reaction is at equilibrium ($K'_{eq} = 0.8$). [Redrawn with permission from Atkinson, D.E., *Biochemistry* 7 4030 (1968). Copyright by the American Chemical Society.]

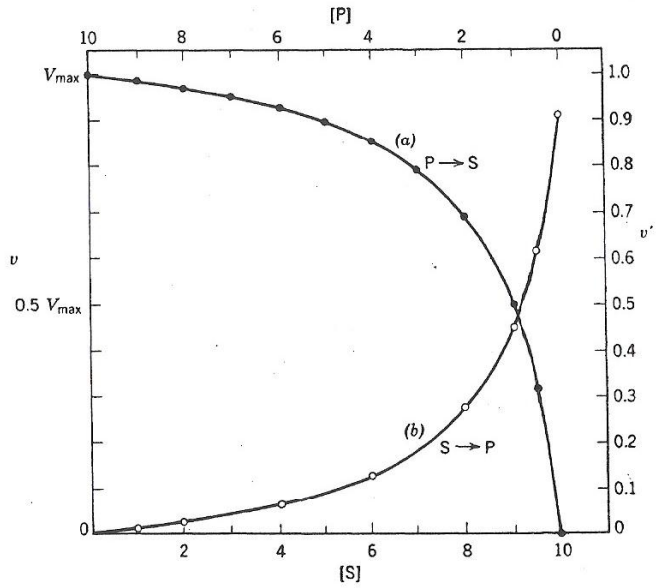


Fig. III-11. Velocity response to changing [S]/[P] ratio where [S]+[P] is constant. (*a*) A P-utilizing enzyme. (*b*) An S-utilizing enzyme. The product of one enzyme is the substrate of the other and vice versa; $K_P = 0.1$, $K_S = 1.0$, [S]+[P] = 10.

large compared to K_S. (If $[S]+[P]=100K_S$, the velocity curves would be essentially the same as those shown in Figure III-11 for the same K_S and K_P values.) In this respect, it is noteworthy that the intracellular levels of adenine nucleotides are quite high compared to their K_m values. It seems likely that many biological oxidation-reduction reactions will be regulated by an analogous "reduction charge," that is, the [NADH]/[NADH+NAD$^+$] or [NADPH]/[NADPH+NADP$^+$] ratio. For most dehydrogenases involved in energy metabolism, K_{NADH} is less than K_{NAD^+}, which is exactly the condition necessary for a steep velocity response to the "reduction charge." Furthermore, the intracellular concentration of pyridine nucleotides is high compared to their K_m values (another required condition). The pyruvic dehydrogenase of *E. coli* responds to both the adenylate "energy charge" and the oxidation level of the NADH+NAD$^+$ pool. The response of pyruvic dehydrogenase to the adenylate "energy charge" can be treated as described in activation system A-5 (Chapter Five) where the enzyme activity is regulated by the [I]/[A] ratio and neither effector is a substrate or product of the reaction. Other factors, in addition to the [S]+[P] concentration and the relative K_S and K_P values, can influence the velocity response to "energy charge" or "reduction charge." These factors include (*a*) the concentrations of effectors which alter K_S or K_P, (*b*) the concentration of Mg^{2+} (when the true S and P species binding to the enzyme are the Mg complexes), and, for reactions involving two or more substrates and/or products, (*c*) the kinetic mechanism, and (*d*) the number and nature of dead-end complexes that can form. Regulation via "energy charge" may have been one of the first control devices evolved by living cells. In its simplest form, the model requires only effective competition between P and S for a single binding site. Examples of enzymes regulated by the energy charge are (*a*) citrate cleavage enzyme from rat liver, which produces extramitochondrial acetyl-*S*-CoA for fatty acid biosynthesis via the reaction:

$$\text{ATP} + \text{citrate} + \text{CoASH} \rightleftharpoons \text{acetyl-}S\text{-CoA} + \text{oxalacetate} + \text{ADP} + \text{P}_i$$

and (*b*) phosphoribosylpyrophosphate (PRPP) synthetase of *E. coli*, which furnishes PRPP for histidine, trytophan, and purine and pyrimidine nucleotides via the reaction:

$$\text{ATP} + \text{ribose-5-phosphate} \rightleftharpoons \text{PRPP} + \text{AMP}$$

It is noteworthy that ADP is much better than AMP as an inhibitor of PRPP synthetase. Thus ADP must have a higher affinity than AMP for the AMP-ATP site, or ADP binds to a distinct regulatory site. In either case, the "energy charge" of the system controls the velocity of the reaction, since a high ADP or high AMP level signifies a low ATP level, and vice versa. Another example of a biosynthetic enzyme regulated by the "energy charge" is the adenosinediphosphoglucose (ADPG) synthetase (pyrophosphorylase) of bacteria. This enzyme catalyzes the production of ADPG from ATP and glucose-1-phosphate.

$$\text{ATP} + \text{glucose-1-phosphate} \rightleftharpoons \text{ADPG} + \text{PP}_i$$

In this case neither ADP nor AMP are products of the reaction, yet both are inhibitors, with AMP being the more potent. ADP is produced in the subsequent reaction where ADPG serves as the glucosyl donor for the bacterial glycogen synthetase:

$$\text{ADPG} + (\text{glycogen})_n \longrightarrow \text{ADP} + (\text{glycogen})_{n+1}$$

With ADPG synthetase, we do not have a simple case of competitive product inhibition, yet the response of the system to the "energy charge" still serves to insure that glycogen synthesis will proceed only when the cell is energy-sufficient.

B. NONCOMPETITIVE INHIBITION (SIMPLE INTERSECTING LINEAR NONCOMPETITIVE INHIBITION)

A classical noncompetitive inhibitor has no effect on substrate binding, and vice versa. The inhibitor and the substrate bind reversibly, randomly, and independently at different sites. That is, I binds to E and to ES; S binds to E and to EI. The binding of one ligand has no effect on the dissociation constant of the other. However, the resulting ESI complex is inactive. Noncompetitive inhibition is common in steady-state multireactant systems (Chapter Nine), but for reasons somewhat different than those presented here. A model for classical noncompetitive inhibition is shown in Figure III-12. It is assumed that I distorts the enzyme sufficiently to prevent the proper positioning of the catalytic center and thus ESI is nonproductive. A similar situation is shown in Figure III-13. Here, there is no direct path from ES to ESI, but the same four enzyme species are at equilibrium. If ES is assumed to exist in two forms, one "open" (and able to bind I) and one "closed" (as shown), the properties of the system would be unchanged since multiple central complexes (e.g., ES, ES′, EP, EP′) do not affect the velocity

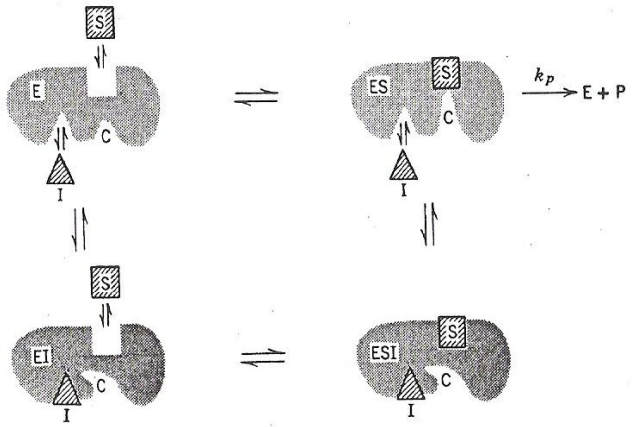


Fig. III-12. Noncompetitive inhibition; S and I are not mutually exclusive but ESI is catalytically inactive. When S binds, the enzyme undergoes a conformational change which aligns the catalytic center, C, with the susceptible bonds of S; I interferes with the conformational change, but has no effect on S binding.

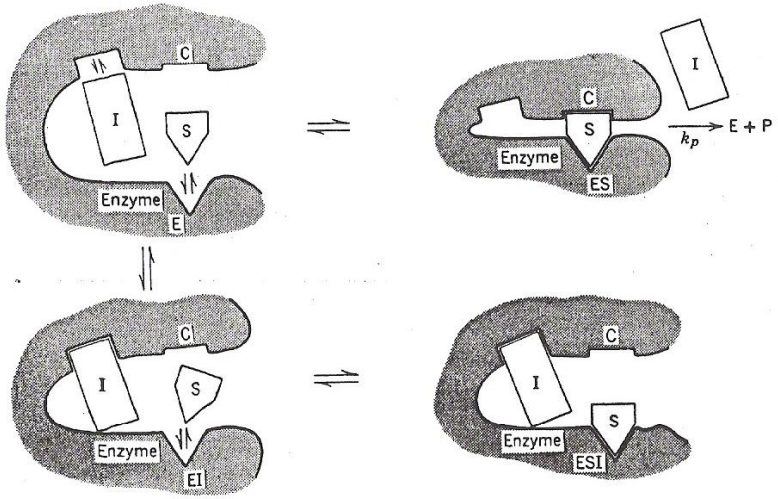


Fig. III-13. Noncompetitive inhibition. In this model, I cannot bind to ES, but the properties of the system are identical to that shown in Fig. III-12 because the same four enzyme species are at equilibrium. In steady-state conditions substrate inhibition is observed.

equations. The equilibria are:

$$\begin{array}{ccccc} E + S & \overset{K_S}{\rightleftharpoons} & ES & \overset{k_p}{\longrightarrow} & E + P \\ + & & + & & \\ I & & I & & K_S = \dfrac{[E][S]}{[ES]} = \dfrac{[EI][S]}{[ESI]} \\ K_i \uparrow\downarrow & & K_i \uparrow\downarrow & & \\ EI + S & \overset{K_S}{\rightleftharpoons} & ESI & & K_i = \dfrac{[E][I]}{[EI]} = \dfrac{[ES][I]}{[ESI]} \end{array}$$

We can see from the equilibria that at any inhibitor concentration, an infinitely high substrate concentration cannot drive all the enzyme to the productive ES form. At any [I] a portion of the enzyme will remain as the nonproductive ESI complex. Consequently, we can predict that the V_{max} in the presence of a noncompetitive inhibitor (V_{max_i}) will be less than the V_{max} observed in the absence of inhibitor. The K_m value (measured as the [S] required for $\frac{1}{2}V_{max_i}$) will be unchanged by a noncompetitive inhibitor because at any inhibitor concentration the enzyme forms which can combine with S (E and EI) have equal affinities for S. The net effect of a reversibly bound noncompetitive inhibitor is to make it appear as if less total enzyme is present.

A substance that *irreversibly* inactivates an enzyme is sometimes (incorrectly) called a noncompetitive inhibitior because V_{max} is decreased. Irreversible inhibition and reversible noncompetitive inhibition may be distinguished by plotting V_{max} versus $[E]_t$, where $[E]_t$ represents total units of enzyme activity added to the assay (Fig. III-14). For a reversible noncompetitive inhibitor, the "plus inhibitor" curve will have a smaller slope than the control curve and will go through the origin. If an irreversible inhibitor is present, the "plus inhibitor" curve will have the same slope as the control curve, but will intersect the horizontal axis at a position equivalent to the amount of enzyme that is irreversibly inactivated.

An expression relating v, V_{max}, [S], K_S, [I], and K_i in the presence of a noncompetitive inhibitor can be derived easily from rapid equilibrium assumptions. This time we must recognize that the total enzyme, $[E]_t$, is present in four forms: free enzyme, [E]; enzyme-substrate complex, [ES];

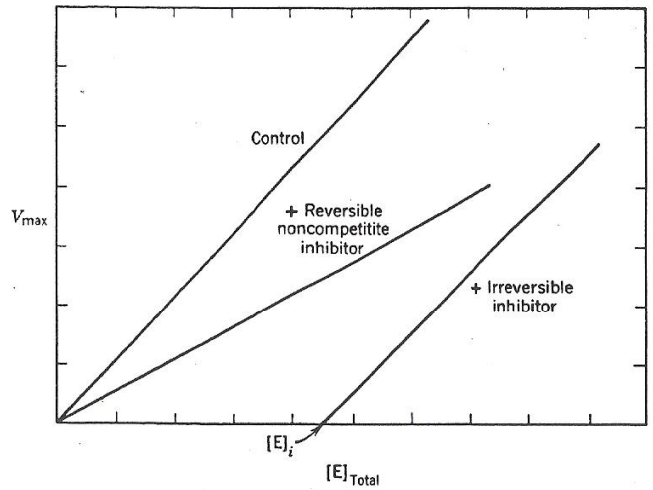


Fig. III-14. A plot of V_{max} versus amount of enzyme added will distinguish between a reversible and an irreversible noncompetitive inhibitor. $[E]_i$ represents the amount of enzyme titrated by the irreversible inhibitor.

enzyme-inhibitor complex, [EI]; and enzyme-substrate-inhibitor complex, [ESI].

$$v = k_p[ES]$$

$$\frac{v}{[E]_t} = \frac{k_p[ES]}{[E]+[ES]+[EI]+[ESI]}$$

$$\boxed{\frac{v}{V_{max}} = \frac{\dfrac{[S]}{K_S}}{1+\dfrac{[S]}{K_S}+\dfrac{[I]}{K_i}+\dfrac{[I][S]}{K_S K_i}}} \qquad \text{(III-20)}$$

where $V_{max} = k_p[E]_t$. In this form, we see that the denominator has two additional terms compared to the normal velocity equation. The $[I]/K_i$ term is contributed by the EI complex, while the $[I][S]/K_S K_i$ term results from the ESI complex. The latter term does not appear in the velocity equation for competitive inhibition because there is no ESI complex. The numerator

still contains only one term as there is only one product-forming complex (ES). To obtain a more familiar form, we can multiply the numerator and denominator of equation III-20 by K_S and factor:

$$\boxed{\frac{v}{V_{max}} = \frac{[S]}{K_S\left(1+\dfrac{[I]}{K_i}\right)+[S]\left(1+\dfrac{[I]}{K_i}\right)}} \qquad \text{(III-21)}$$

The expression above differs from the usual Henri-Michaelis-Menten equation and from that derived earlier for competitive inhibition in that the K_S and [S] terms in the denominator are both multiplied by the factor $(1+[I]/K_i)$. We can better appreciate the effect of a noncompetitive inhibitor by dividing the denominators of both sides of the equation by $(1+[I]/K_i)$:

$$\boxed{\frac{\dfrac{v}{V_{max}}}{\left(1+\dfrac{[I]}{K_i}\right)} = \frac{[S]}{K_S+[S]}} \quad \text{or} \quad \frac{v}{V_{max_i}} = \frac{[S]}{K_S+[S]} \qquad \text{(III-22)}$$

where:

$$V_{max_i} = \frac{V_{max}}{\left(1+\dfrac{[I]}{K_i}\right)} = \text{the apparent } V_{max} \text{ at the given } [I]$$

As predicted, the only effect of a noncompetitive inhibitor is to decrease V_{max}. The K_S value remains unchanged. (The reader should not be confused by the fact that the K_S term in the original equation III-21 was multiplied by $(1+[I]/K_i)$. Before deciding whether K_S is affected, we must first modify the equation by removing any factor of the variable, [S].) The decrease in V_{max} does not mean that the inhibitor has decreased the rate constant for the breakdown of ES to E+P. This constant, k_p, is unchanged. It is the steady-state level of ES that is decreased. At any [S] and [I], the enzyme-substrate complex is present as a mixture of productive ES and nonproductive ESI forms ($k_p = 0$ for the ESI complex). The factor $(1+[I]/K_i)$ may be considered to be an [I]-dependent statistical factor describing the distribution of the enzyme-substrate complexes between the ES and ESI forms.

Systems are known in which the inhibitor does affect k_p (i.e., in which ESI forms product slower than ES). This type of system, called partial noncompetitive inhibition, is described in Chapter Four.

If k_p contributes significantly to the $K_m = (k_{-1} + k_p)/k_1$ relationship, then an inhibitor that affects the apparent k_p value will also affect the K_m value. In pure and partial noncompetitive systems, the apparent k_p changes without K_m changing. Consequently, k_p must be very small compared to k_{-1}, and K_m must be equivalent to K_S. In other words, classical noncompetitive inhibition in unireactant systems is obtained only under rapid equilibrium conditions. In fact, a steady-state treatment does not yield an equation of the Henri-Michaelis-Menten form, but rather a complex expression containing $[S]^2$ and $[I]^2$ terms. The reciprocal plots are theoretically nonlinear (although they may appear so because the nonlinear region may occur close to the $1/v$-axis). Figure III-15 shows a situation that could be at steady-state and still yield a simple velocity equation without $[S]^2$ terms (because there is only one reaction in which S adds).

An expression for the relative velocity in the presence of a noncompetitive inhibitor can be derived readily:

$$a = \frac{v_i}{v_0} = \frac{\dfrac{V_{max}[S]}{(K_m + [S])\left(1 + \dfrac{[I]}{K_i}\right)}}{\dfrac{V_{max}[S]}{K_m + [S]}} \quad \text{or} \quad \boxed{a = \frac{K_i}{K_i + [I]}} \qquad \text{(III-23)}$$

Thus a is independent of [S], or, in other words, a given concentration of I reduces the velocity by exactly the same factor at all substrate concentrations.

$$\therefore \quad \boxed{\frac{V_{max_i}}{V_{max}} = \frac{K_i}{K_i + [I]}} \qquad \text{(III-24)}$$

Although V_{max} is reduced to V_{max_i}, the specific substrate concentration required for any fraction of V_{max_i} is unchanged. For example, $[S]_{0.5} = K_m$, $[S]_{0.9} = 9K_m$, and $[S]_{0.1} = \frac{1}{9}K_m$. Thus the $[S]_{0.9}/[S]_{0.1}$ ratio is 81 at all inhibitor concentrations. As before, the constant ratio is expected, since the form of the equation is unchanged; only the absolute value of V_{max} is changed. The effect of a noncompetitive inhibitor is shown in Figure III-16.

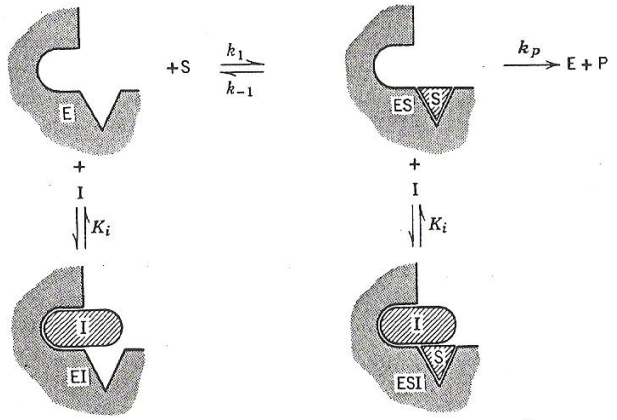


Fig. III-15. A third model for noncompetitive inhibition; I sterically hinders S binding. The velocity equation derived from steady-state assumptions would be the same as that derived from rapid equilibrium assumptions.

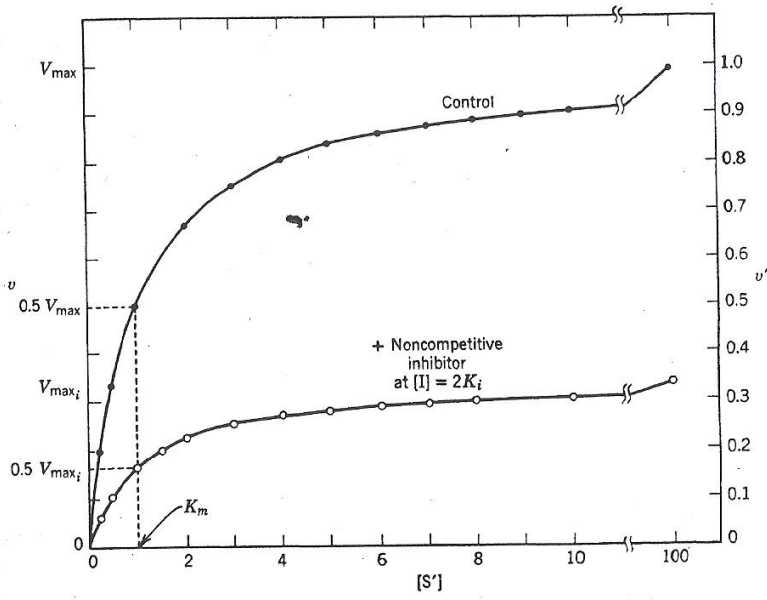


Fig. III-16. The v versus [S] plot in the presence of a noncompetitive inhibitor.

An expression for the fractional inhibition can be derived from equation III-23:

$$i = 1 - \frac{v_i}{v_0} = 1 - a = 1 - \frac{K_i}{K_i + [I]}$$

$$\boxed{i = \frac{[I]}{K_i + [I]}} \qquad \boxed{i_{\%} = 100\left(\frac{[I]}{K_i + [I]}\right)} \qquad \text{(III-25)}$$

At any [S], $[I]_{0.5} = K_i$, $[I]_{0.9} = 9K_i$, and $[I]_{0.1} = \frac{1}{9}K_i$. Thus the $[I]_{0.9}/[I]_{0.1}$ ratio is always 81.

General Principles

A classical noncompetitive inhibitor decreases V_{max}, but has no effect on the K_m value. The degree of inhibition in the presence of a noncompetitive inhibitor depends only upon [I] and K_i. The inhibited velocity (v_i) is always a constant fraction of v_0, regardless of the substrate concentration or the value of K_m. An increase in [S] causes both v_0 and v_i to increase by the same factor. The net effect of a noncompetitive inhibitor is to make it seem as if less enzyme were present. When $[I] = K_i$, we observe 50% inhibition at all substrate concentrations.

Reciprocal Plot for Noncompetitive Inhibition Systems

In the reciprocal form, the velocity equation for noncompetitive inhibition is:

$$\boxed{\frac{1}{v} = \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)\frac{1}{[S]} + \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)} \qquad \text{(III-26)}$$

The equation indicates that both the slope and the $1/v$-axis intercept of the reciprocal plot are increased by the factor $(1 + [I]/K_i)$ compared to the "control" plot. If the slope *and* the $1/v$-axis intercept increase by the same factor, then the $1/[S]$-axis intercept will remain the same (equal to $-1/K_m$). The K_i can be calculated from the slope or the $1/v$-axis intercept. When $1/[S] = 0$, the $1/v$ intercept gives $1/V_{max_i}$, where $1/V_{max_i} = 1/V_{max}(1 + [I]/K_i)$. For each fixed inhibitor concentration, a new reciprocal

plot can be drawn (Fig. III-17). As [I] increases, the "plus inhibitor" curves increase in slope and $1/v$-axis intercept, pivoting counterclockwise about the point of intersection with the control curve (at $-1/K_m$ on the $1/[S]$-axis). Because the initial velocity can be driven to zero at a saturating inhibitor concentration, the limiting slope will be a vertical line through $-1/K_m$ and parallel to the $1/v$-axis.

Replots of $Slope_{1/S}$ and $1/V_{max_i}$ Versus [I]

The slope of the reciprocal plot in the presence of a pure noncompetitive inhibitor is a linear function of [I] (Fig. III-18*a*) as shown earlier for pure competitive inhibition (Fig. III-5). The $1/v$-axis intercept ($1/V_{max_i}$) is also a linear function of [I] (Fig. III-18*b*):

$$\boxed{\frac{1}{V_{max_i}} = \frac{1}{V_{max}K_i}[I] + \frac{1}{V_{max}}} \qquad \text{(III-27)}$$

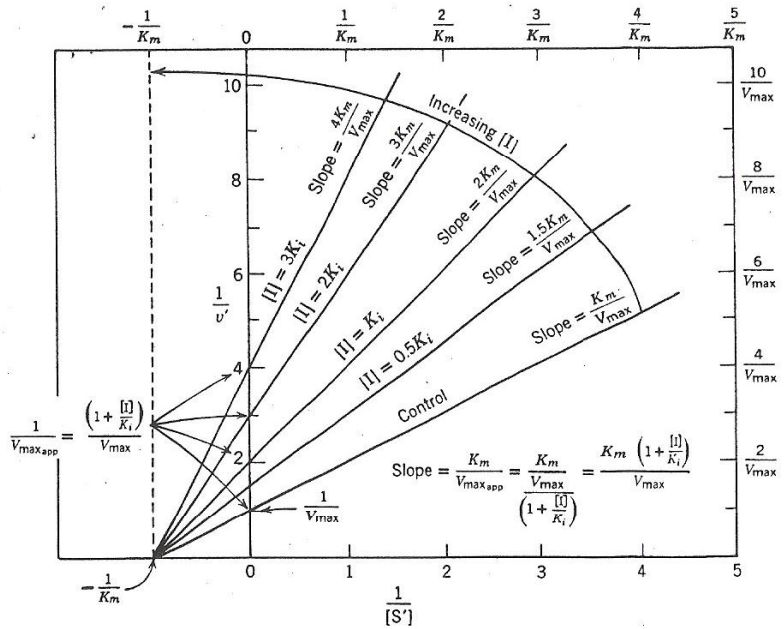


Fig. III-17. The $1/v$ versus $1/[S]$ plot in the presence of different fixed concentrations of a noncompetitive inhibitor.

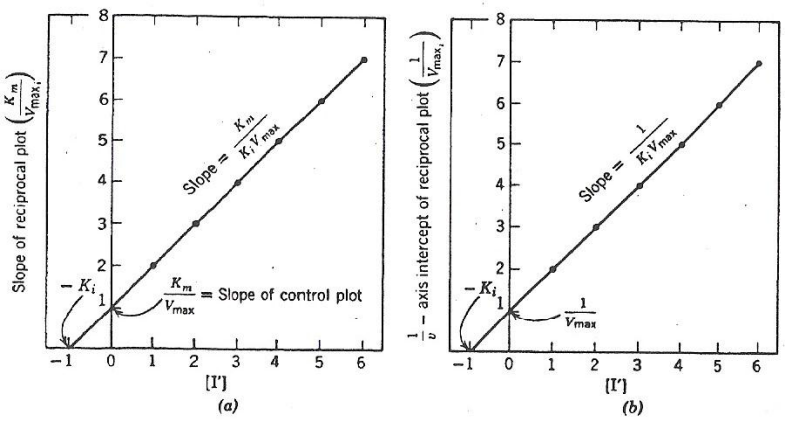


Fig. III-18. Replots of data taken from the reciprocal plot. (*a*) $Slope_{1/S}$ versus [I]. (*b*) $1/v$-axis intercept (i.e., $1/V_{max_i}$) versus [I].

Linear replots of $slope_{1/S}$ versus [I] and $1/V_{max_i}$ versus [I] distinguish pure noncompetitive inhibition from partial noncompetitive inhibition. The latter yields hyperbolic replots (Chapter Four).

Dixon Plot for Noncompetitive Inhibition: $1/v$ Versus [I]

The reciprocal equation for noncompetitive inhibition may be rearranged to:

$$\frac{1}{v} = \frac{\left(1+\frac{K_m}{[S]}\right)}{V_{max}K_i}[I] + \frac{1}{V_{max}}\left(1+\frac{K_m}{[S]}\right) \qquad \text{(III-28)}$$

A plot of $1/v$ versus [I] will be a straight line (Fig. III-19*a*). The slope of the Dixon plot is given by:

$$slope = \frac{K_m}{V_{max}K_i}\frac{1}{[S]} + \frac{1}{V_{max}K_i} \qquad \text{(III-29)}$$

A replot of *slope* versus the corresponding $1/[S]$ (Fig. III-19*b*) is a straight line with a slope of $K_m/V_{max}K_i$ and an intercept of $1/V_{max}K_i$ on the *slope*-axis. When $slope = 0$, the intercept on the $1/[S]$-axis gives $-1/K_m$. (In contrast, the slope replot for competitive inhibition passes through the origin.) The replot of $1/v$-axis intercept versus the corresponding $1/[S]$ is

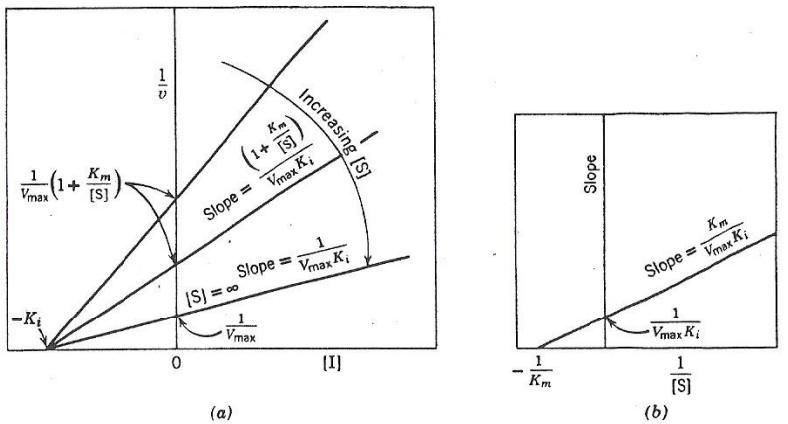


Fig. III-19. (*a*) Dixon plot for a noncompetitive inhibitor: $1/v$ versus [I] in the presence of different fixed concentrations of substrate. (*b*) *Slope* replot.

identical to the usual reciprocal plot in the absence of I. Other plots for noncompetitive inhibition are described in Chapter Four.

Integrated Rate Equation in the Presence of a Noncompetitive Inhibitor

If the reaction has a large K_{eq} and none of the products have an appreciable affinity for the enzyme, then the time course of the reaction in the presence of a noncompetitive inhibitor can be expressed as:

$$\frac{2.3}{t}\log\frac{[S]_0}{[S]} = -\frac{1}{K_m}\frac{[P]}{t} + \frac{V_{max}}{K_m\left(1+\frac{[I]}{K_i}\right)} \qquad \text{(III-30)}$$

where $[P] = [S]_0 - [S]$.

The equation assumes a constant inhibitor concentration during the time course of the reaction. A determination of [P] at various times during the course of the reaction will allow K_m and $V_{max_{app}}$ to be determined. A family of plots can be obtained for different inhibitor concentrations (Fig. III-20). The lines will be parallel since the slope is independent of [I]. The values of V_{max} and K_i can be obtained from appropriate replots of vertical or horizontal axes intercepts.

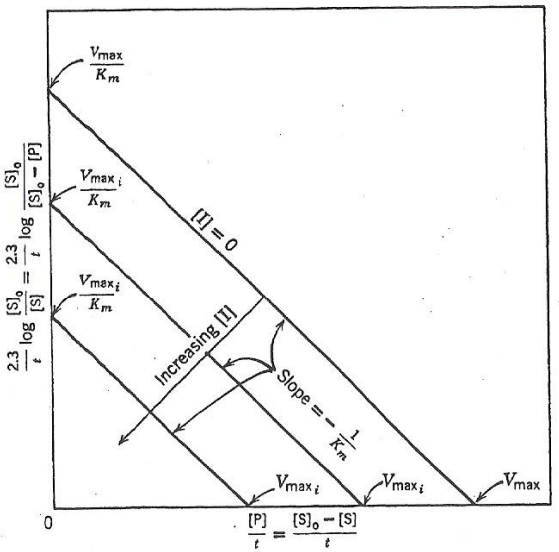


Fig. III-20. Plot of the integrated velocity equation in the presence of a noncompetitive inhibitor.

C. UNCOMPETITIVE INHIBITION (SIMPLE LINEAR UNCOMPETITIVE INHIBITION)

A classical uncompetitive inhibitor is a compound that binds reversibly to the enzyme-substrate complex yielding an inactive ESI complex (Fig. III-21). The inhibitor does not bind to the free enzyme. Pure uncompetitive inhibition (also called anticompetitive inhibition and coupling inhibition) may be rare in unireactant systems. Nevertheless, it is worth considering because it is a simple example of the sequential addition of two enzyme ligands in an obligate order. Uncompetitive inhibition is common in steady-state multireactant systems (Chapter Nine) for reasons similar to those described here. That is, I will be uncompetitive with respect to a given substrate if I binds to the enzyme only after the substrate binds (although I rarely binds to a central complex where all the substrate binding sites are filled). Classical uncompetitive inhibition is described by the following

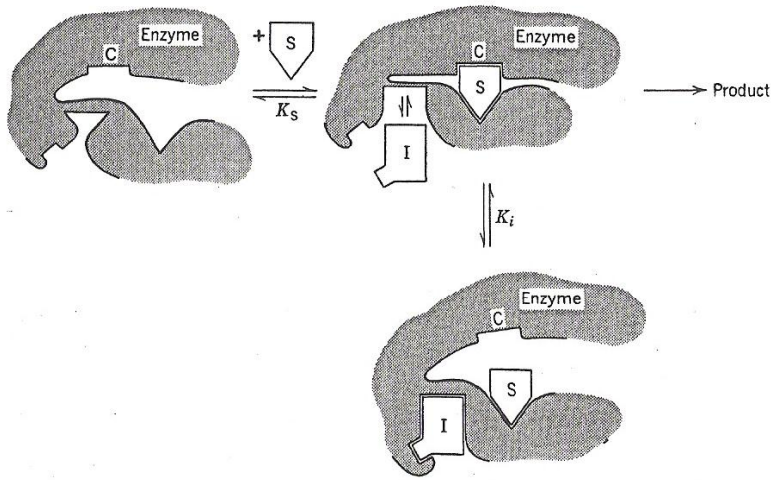


Fig. III-21. Uncompetitive inhibition; I binds only to the ES complex. When S binds, a conformational change occurs in the enzyme which forms or unmasks the I site. The resulting ESI complex is catalytically inactive; C represents the catalytic center of the enzyme.

equilibria:

$$E + S \underset{}{\overset{K_S}{\rightleftharpoons}} ES \xrightarrow{k_p} E + P$$
$$+$$
$$I$$
$$K_i \updownarrow$$
$$ESI$$

The equilibria show that at any [I] an infinitely high substrate concentration will not drive all of the enzyme to the ES form; some nonproductive ESI complex will always be present. Consequently, we can predict that V_{max} in the presence of an uncompetitive inhibitor (V_{max_i}) will be lower than the V_{max} in the absence of inhibitor. Unlike noncompetitive inhibition, however, the apparent K_m value will decrease. The decrease occurs because the reaction ES+I→ESI removes some ES causing the reaction E+S→ES to proceed to the right. Under certain conditions, a mixed-type inhibitor can produce the same effects as an uncompetitive inhibitor. The specific conditions are discussed in Chapter Four.

An expression relating v, V_{max}, [S], K_S, [I], and K_i in the presence of an uncompetitive inhibitor is derived below:

$$v = k_p[ES]$$

$$\frac{v}{[E]_t} = \frac{k_p[ES]}{[E]+[ES]+[ESI]}$$

$$\boxed{\frac{v}{V_{max}} = \frac{\frac{[S]}{K_S}}{1+\frac{[S]}{K_S}+\frac{[S][I]}{K_S K_i}}} \qquad \text{(III-31)}$$

The equation does not contain an $[I]/K_i$ term because no EI complex forms. Multiplying numerator and denominator of equation III-31 by K_S and factoring:

$$\boxed{\frac{v}{V_{max}} = \frac{[S]}{K_S+[S]\left(1+\frac{[I]}{K_i}\right)}} \qquad \text{(III-32)}$$

A steady-state treatment yields the same equation with K_m replacing K_S.

The velocity equation differs from the usual Henri-Michaelis-Menten expression in that the [S] term in the denominator is multiplied by the factor $(1+[I]/K_i)$. We can better appreciate the effect of an uncompetitive inhibitor on the kinetic constants by dividing the denominators of both sides of the equation by $(1+[I]/K_i)$.

$$\boxed{\frac{\frac{v}{V_{max}}}{\left(1+\frac{[I]}{K_i}\right)} = \frac{[S]}{\frac{K_m}{\left(1+\frac{[I]}{K_i}\right)}+[S]}} \qquad \text{(III-33)}$$

or

$$\boxed{\frac{v}{V_{max_i}} = \frac{[S]}{K_{m_{app}}+[S]}} \qquad \text{(III-34)}$$

where

$$V_{max_i} = \frac{V_{max}}{\left(1+\frac{[I]}{K_i}\right)} \quad \text{and} \quad K_{m_{app}} = \frac{K_m}{\left(1+\frac{[I]}{K_i}\right)}$$

In other words, an uncompetitive inhibitor decreases V_{max} and K_m to the same extent (Fig. III-22).

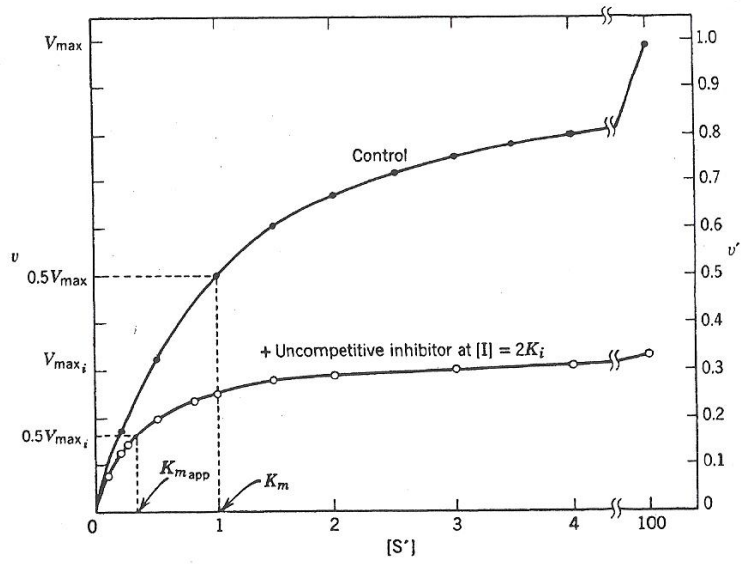


Fig. III-22. The v versus [S] plot in the presence of an uncompetitive inhibitor.

An expression for the relative velocity in the presence of an uncompetitive inhibitor can be derived:

$$a = \frac{v_i}{v_0} = \frac{\dfrac{V_{max}[S]}{K_m + [S]\left(1 + \dfrac{[I]}{K_i}\right)}}{\dfrac{V_{max}[S]}{K_m + [S]}}$$

$$\boxed{a = \frac{K_m + [S]}{K_m + [S]\left(1 + \dfrac{[I]}{K_i}\right)}} \qquad \text{(III-35)}$$

The fractional inhibition caused by an uncompetitive inhibitor is given by:

$$i = 1 - a \qquad \text{or} \qquad \boxed{i = \frac{[I]}{K_i\left(1 + \dfrac{K_m}{[S]}\right) + [I]}} \qquad \text{(III-36)}$$

When $i = 0.5$:

$$\boxed{[I]_{0.5} = \left(1 + \frac{K_m}{[S]}\right)K_i} \qquad \text{(III-37)}$$

Note that the relationship between K_m and [S] is opposite to that for competitive inhibition. Similarly, we can show that:

$$[I]_{0.9} = 9\left(1 + \frac{K_m}{[S]}\right)K_i, \qquad [I]_{0.1} = \frac{1}{9}\left(1 + \frac{K_m}{[S]}\right)K_i$$

Thus the $[I]_{0.9}/[I]_{0.1}$ ratio is 81 for all substrate concentrations regardless of the absolute values of K_m and K_i. The degree of inhibition depends on the substrate concentration, but, unlike competitive inhibition, the degree of inhibition *increases* as [S] increases. This is to be expected, because an uncompetitive inhibitor combines only with the ES complex, and the concentration of ES increases as [S] increases. An uncompetitive inhibitor inhibits because of its effect on V_{max}. The inhibitor is actually an activator with respect to K_m ($K_{m_{app}} < K_m$). If the substrate concentration is low enough so that the reaction is essentially first-order, the effect of an uncompetitive inhibitor on V_{max} will be almost completely canceled by its opposite effect on K_m and little or no inhibition will be observed.

Reciprocal Plot for Uncompetitive Inhibition

The reciprocal form of the velocity equation for uncompetitive inhibition is:

$$\boxed{\frac{1}{v} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)} \qquad \text{(III-38)}$$

The slope of the plot is still K_m/V_{max}, but the $1/v$-axis intercept is increased by the factor $(1 + [I]/K_i)$ which multiplied the [S] term in the original equation. Consequently, the "plus inhibitor" and control curves will be parallel. As [I] increases, the $1/v$-axis intercepts increase, yielding a series of parallel plots (Fig. III-23). A saturating inhibitor concentration will drive the velocity to zero. Consequently, the displacement of the "plus inhibitor" plots from the control plot increases without limit.

Replots of $1/V_{max_i}$ and $1/K_{m_{app}}$ Versus [I]

A replot of $1/V_{max_i}$ versus [I] will be linear (Fig. III-24*a*) with intercepts of $1/V_{max}$ and $-K_i$ as shown for pure noncompetitive inhibition. The $K_{m_{app}}$ varies inversely with [I] (Fig. III-24*b*):

$$\boxed{\frac{1}{K_{m_{app}}} = \frac{1}{K_i K_m}[I] + \frac{1}{K_m}} \qquad \text{(III-39)}$$

The linear replots will distinguish pure uncompetitive inhibition from a mixed-type system in which K_m and V_{max} change by the same factor (see mixed-type inhibition System C4 where $\alpha = \beta$, in Chapter Four).

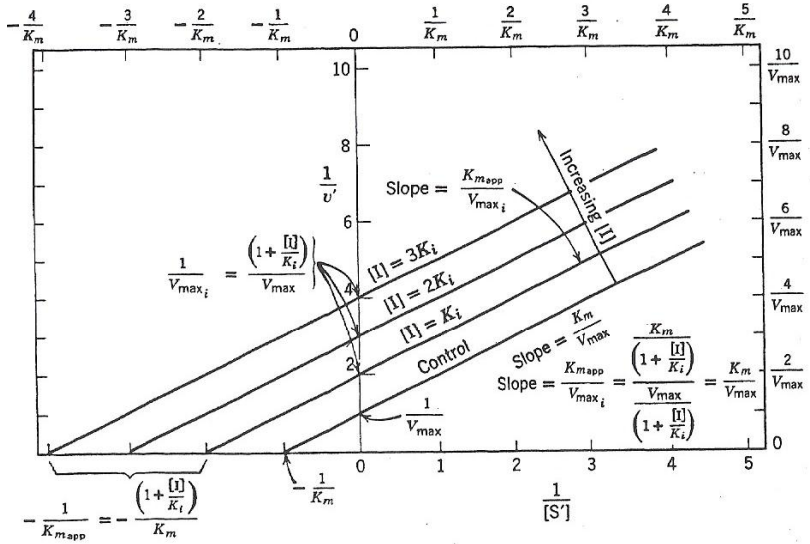


Fig. III-23. The $1/v$ versus $1/[S]$ plot in the presence of different fixed concentrations of an uncompetitive inhibitor.

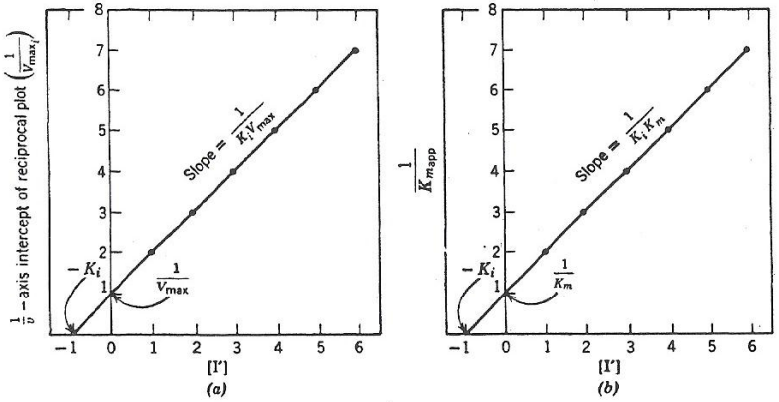


Fig. III-24. Replots of data taken from the reciprocal plot. (*a*) $1/v$-axis intercept (i.e., $1/V_{max_i}$) versus [I]. (*b*) $1/K_{m_{app}}$ versus [I].

Dixon Plot for Uncompetitive Inhibition: $1/v$ Versus [I]

The equation for the Dixon plot is:

$$\frac{1}{v} = \frac{1}{V_{max}K_i}[I] + \frac{1}{V_{max}}\left(1+\frac{K_m}{[S]}\right) \qquad \text{(III-40)}$$

The slope expression does not contain an [S] term. Hence we can expect the plots to be parallel at all substrate concentrations (Fig. III-25).

Integrated Rate Equation in the Presence of an Uncompetitive Inhibitor

The integrated rate equation in the presence of an uncompetitive inhibitor is:

$$\frac{2.3}{t}\log\frac{[S]_0}{[S]} = -\frac{\left(1+\frac{[I]}{K_i}\right)}{K_m}\frac{[P]}{t} + \frac{V_{max}}{K_m} \qquad \text{(III-41)}$$

where $[P] = [S]_0 - [S]$. Figure III-26 shows the time course of the reaction at various fixed inhibitor concentrations plotted according to equation III-41.

D. EFFECTS OF CONTAMINATING INHIBITORS ON THE INITIAL VELOCITY VERSUS ENZYME CONCENTRATION PLOT

Enzyme preparations frequently contain inhibitors. These may be endogenous metabolites or substances introduced during the cell breakage or fractionation procedures. If the presence of inhibitors is not recognized, errors may be introduced into the determination of K_m or K_i values, or total enzyme units. The problems caused by contaminating endogenous inhibitors can be avoided usually if the enzyme is partially purified. Indeed, recoveries greater than 100% of the original activity after a preliminary purification step are not uncommon. However, many times a large number of cell free extracts must be assayed under conditions where a preliminary purification of each one is not feasible. Under such circumstances, it would be desirable

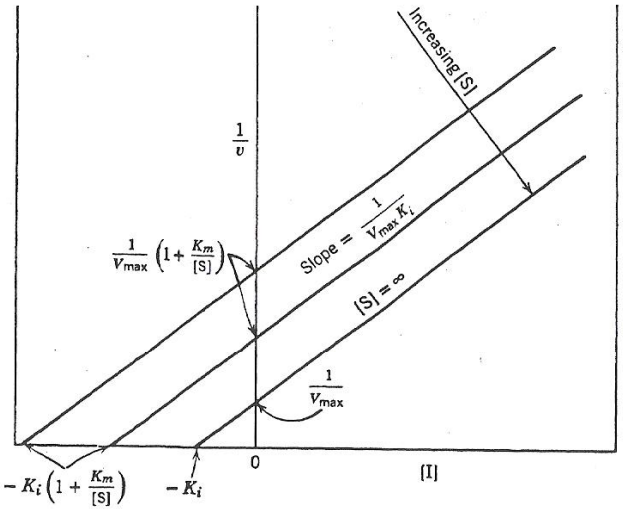


Fig. III-25. Dixon plot for an uncompetitive inhibitor: $1/v$ versus [I] at different fixed concentrations of S.

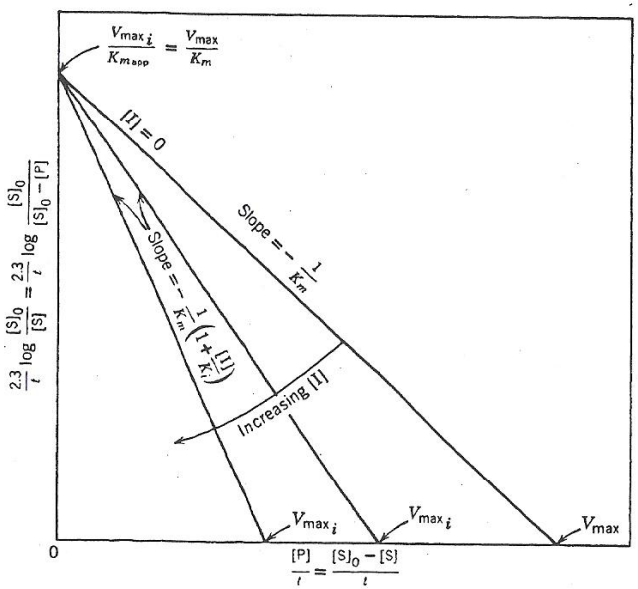


Fig. III-26. Plot of the integrated velocity equation in the presence of an uncompetitive inhibitor.

to know whether the enzyme preparation contains inhibitors. This can be checked by determining the response of the assay to increasing amounts of enzyme preparation. A departure of the v versus $[E]_t$ plot from linearity indicates the presence of inhibitors in the enzyme preparation. Suppose an extract contains a competitive inhibitor such that 10 μl gives a final concentration of $3K_i$ in the assay mixture. The assay is conducted at $[S]=10K_m$. The 10 μl of extract contains an amount of enzyme sufficient to yield a V_{max} under the assay conditions of 100 arbitrary units in the absence of any inhibitors. At the assay substrate concentration ($10K_m$), the theoretical (uninhibited) v is 91 units. (Of course, the amount of enzyme present is not known—the object of the assay is to determine how much enzyme is present.) The observed activity in the presence of $[I]=3K_i$ will be:

$$v_{10\,\mu l}=\frac{k_p[E]_t[S]}{K_{m_{app}}+[S]}=\frac{V_{max}[S]}{K_m\left(1+\frac{[I]}{K_i}\right)+[S]}$$

$$=\frac{(100)(10K_m)}{K(1+3)+10K_m}=\frac{1000K_m}{14K_m}$$

$$v_{10\,\mu l}=71.4 \text{ units}$$

If the presence of the inhibitor is unrecognized, the value of 71.4 units would be taken as a measure of the amount of enzyme present. The true velocity should have been 91 units; thus the assay will give a value 20% lower than the true value. Now, let us double the amount of enzyme preparation used. If 10 μl produced a v of 71.4 units and this represents an uninhibited velocity, then 20 μl should yield a v of 142.8 units (doubling $[E]_t$ doubles V_{max}, since $V_{max}=k_p[E]_t$). If the preparation contains an inhibitor, then doubling $[E]_t$ will also double [I].

$$v_{20\,\mu l}=\frac{2V_{max}[S]}{K_m\left(1+\frac{2[I]}{K_i}\right)+[S]}=\frac{(2)(100)(10K_m)}{K_m(1+6)+10K_m}$$

$$v_{20\,\mu l}=\frac{2000K_m}{17K_m}=118 \text{ units}$$

Similarly, if only 5 μl of preparation are used and the v obtained at 10 μl

represents an uninhibited v, then $v_{5\ \mu l}$ should be 35.7 units.

$$v_{5\ \mu l} = \frac{0.5\,V_{max}[S]}{K_m\left(1+\frac{0.5[I]}{K_i}\right)+[S]} = \frac{(0.5)(100)(10K_m)}{K_m(1+1.5)+10K_m}$$

$$= \frac{500K_m}{12.5K_m} = 40 \text{ units}$$

We see that doubling the enzyme concentration produced less than a doubling of the velocity, and halving the enzyme concentration produced more than half the velocity. It is obvious that v is not directly proportional to $[E]_t$.

Figure III-27 shows the effect of a contaminating competitive inhibitor and a contaminating noncompetitive inhibitor on the v versus $[E]_t$ curve at $[S]=10K_m$ and $[I]=3K_i$ in the 10 μl assay. The curve for a contaminating uncompetitive inhibitor would be slightly higher than that shown for the noncompetitive inhibitor.

If the enzyme preparation contains an activator then the v versus $[E]_t$ plot

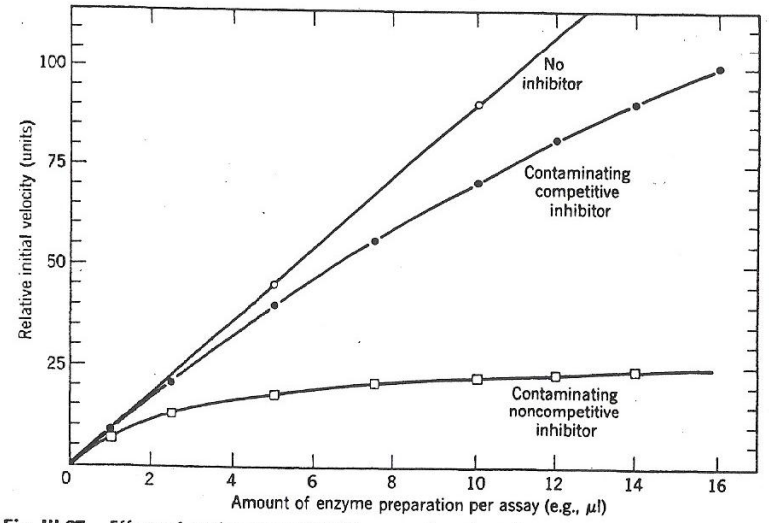


Fig. III-27. Effect of endogenous inhibitors on the plot of v versus amount of enzyme preparation added. Each 10 μl enzyme preparation contains 100 units of activity at saturating [S], and $[I]=3K_i$; $[S]=10K_m$ in all assays.

will curve upward, giving a greater than proportionate increase in v for a given increment of $[E]_t$.

Other Factors Producing Nonlinear v Versus $[E]_t$ Plots

Nonlinear v versus $[E]_t$ plots can result from a number of factors besides the presence of endogenous inhibitors in the enzyme preparation. Some of these are as follows. (*a*) The enzyme preparation may be stored at a pH or ionic strength significantly different from the optimum of the reaction. As the amount of enzyme preparation in the assay is increased, the "carry-over" will cause the assay pH or ionic strength to depart more and more from the optimum. Similarly, the enzyme may be stored in the presence of certain stabilizing agents (e.g., EDTA, thiols, and specific cations) which may inhibit the reaction. (*b*) The measured velocity may not be a true initial velocity. This could occur if the substrate concentration decreased significantly (i.e., out of the zero-order range) before the first product measurement. Similarly, if the pH of the assay mixture changes or a product inhibitor accumulates to a significant level before the first measurement, the calculated velocity will be lower than the true initial velocity. (*c*) The enzyme preparation may contain enzymes that convert the product to another compound that escapes detection by the assay method. (*d*) The enzyme may be unstable at the assay temperature, pH, ionic strength, and so on. If a significant amount of denaturation occurs before the first product measurement, the calculated velocity will be lower than the true initial velocity. (*e*) The enzyme preparation may contain proteolytic enzymes that are inactive under the storage conditions but degrade the enzyme rapidly in the assay mixture. (*f*) The assay method may be inaccurate at high product concentrations. For example, in spectrophotometric assays, high optical densities may be impossible to read accurately with the spectrophotometer used. In assays employing radioactive substrates, the radioactivity in the product may exceed the resolution time of the detector. These problems can be avoided by diluting the product before final measurement. For radioactive assays, a "coincidence" correction can be applied. (*g*) The reagents used to convert the product into a measureable form may be limiting. Similarly, "coupling" enzymes included in the original assay mixture may be the rate limiting factor, rather than the enzyme being assayed. (See Chapter Two, Section H.)

Contaminating Inhibitors in the Substrate

Inhibitors may be present as contaminants in substrates. These inhibitors may be structural or stereoisomers of the true substrate, or unrelated compounds (e.g., the buffer used to prepare the substrate stock solution, or a preservative). A contaminant may go undetected if it acts as a competitive

inhibitor. For example, suppose [I], the concentration of the contaminant in the stock solution of substrate, is x[S]. As [S] is varied, [I] will also vary. However, [I] in the assay mixture will always be a constant fraction or multiple of [S]. In other words, [S] and [I] will be varied together at a constant ratio. The velocity equation is:

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

or

$$\boxed{\frac{\frac{v}{V_{max}}}{\left(1+\frac{xK_m}{K_i}\right)} = \frac{[S]}{\frac{K_m}{\left(1+\frac{xK_m}{K_i}\right)}+[S]}} \qquad \text{(III-42)}$$

The velocity curve and reciprocal plot will appear normal, but the kinetic constants are only apparent constants. The observed reciprocal plot will be parallel to and above the true plot.

The situation is different if I acts noncompetitively. In this case the velocity equation is:

$$\frac{v}{V_{max}} = \frac{\frac{[S]}{K_m}}{1+\frac{[S]}{K_m}+\frac{[I]}{K_i}+\frac{[S][I]}{K_mK_i}} = \frac{\frac{[S]}{K_m}}{1+\frac{[S]}{K_m}+\frac{x[S]}{K_i}+\frac{x[S]^2}{K_mK_i}}$$

$$\boxed{\frac{v}{V_{max}} = \frac{[S]}{K_m+[S]\left(1+\frac{xK_m}{K_i}+\frac{x[S]}{K_i}\right)}} \qquad \text{(III-43)}$$

The equation does not have the usual form of the Henri-Michaelis-Menten equation. In effect, the denominator contains an $[S]^2$ term resulting in

apparent substrate inhibition. The equation for the reciprocal plot can be written as:

$$\frac{1}{v} = \frac{K_m}{V_{max}}\left(\frac{1}{[S]}\right)+\frac{1}{V_{max}}\left(1+\frac{xK_m}{K_i}\right)+\frac{x}{V_{max}K_i}\left(\frac{1}{[S]}\right)^{-1} \qquad \text{(III-44)}$$

If we take the first derivative of equation III-44 and set it equal to zero, we find that the plot has a minimum.

$$\frac{d(1/v)}{d(1/[S])} = \frac{K_m}{V_{max}} - \frac{x}{V_{max}K_i}\left(\frac{1}{[S]}\right)^{-2} = 0$$

$$\therefore (1/[S])^{-2} = \frac{K_mK_i}{x}, \qquad (1/[S])^2 = \frac{x}{K_mK_i}$$

The minimum occurs at:

$$\frac{1}{[S]} = \sqrt{\frac{x}{K_mK_i}} \qquad \text{or} \qquad [S] = \sqrt{\frac{K_mK_i}{x}}$$

The plot of v versus [S] will increase, pass through a maximum, and then decrease to zero. The plot of $1/v$ versus $1/[S]$ will bend upward as it approaches the $1/v$-axis (Fig. III-28). At very low [S] (i.e., high $1/[S]$), the

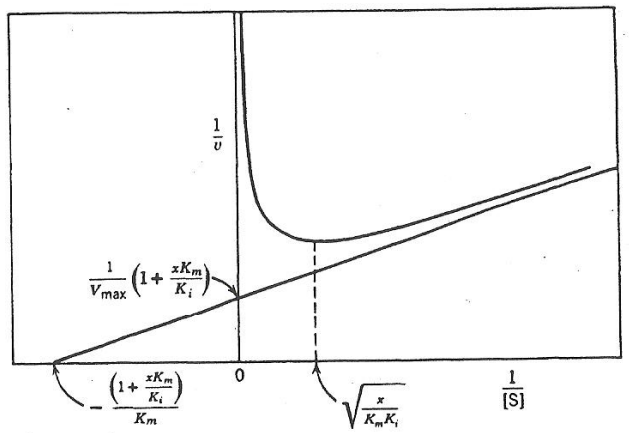


Fig. III-28. Effect of a contaminating noncompetitive inhibitor in the substrate on the $1/v$ versus $1/[S]$ plot.

reciprocal plot approaches a straight line asymptotically. In this region of $1/[S]$, the effect of I is minimal ($[I]$ is very low compared to K_i). Extrapolation of the asymptote yields apparent values for K_m and V_{max}. An uncompetitive inhibitor will behave similarly since the velocity equation will contain the $x[S]^2/K_mK_i$ term. However, the extrapolated K_m and V_{max} values will be the real values, since the velocity equation will not contain $x[S]/K_i$ (i.e., an $[I]/K_i$) term.

The treatment above assumes that I represents a very small fraction of the added substrate so that $[S] \cong [S]_{added}$. If, however, x is not $\ll 1$, then the true substrate concentration will be significantly less than $[S]_{added}$ and the $1/[S]$ coordinate of a given velocity point will be in error. Suppose that I is a competitive inhibitor. Let $[I] = x[S]_{added}$, then $[S] = (1-x)[S]_{added}$, where $[S]$ is the true substrate concentration. The velocity is given by:

$$\frac{v}{V_{max}} = \frac{\dfrac{(1-x)[S]_{added}}{K_m}}{1 + \dfrac{(1-x)[S]_{added}}{K_m} + \dfrac{x[S]_{added}}{K_i}} = \frac{[S]_{added}}{\dfrac{K_m}{(1-x)} + [S]_{added} + \dfrac{xK_m[S]_{added}}{(1-x)K_i}}$$

or

$$\boxed{\frac{v}{\dfrac{V_{max}}{\left(1 + \dfrac{xK_m}{(1-x)K_i}\right)}} = \frac{[S]_{added}}{\left[\dfrac{K_m}{(1-x) + \dfrac{xK_m}{K_i}}\right] + [S]_{added}}} \qquad \text{(III-45)}$$

The reciprocal plot is still linear but now the observed K_m and V_{max} are altered by different factors. When $x \ll 1$, equation III-45 reduces to equation III-42, and the effects of the incorrect assumed values of [S] disappear (only the effects of the competitive inhibition by I remain). When $K_i \gg K_m$ (i.e., the impurity is not inhibitory), equation III-45 reduces to equation II-35 [with $(1-x)$ representing the fractional purity of S, called y in deriving equation II-35].

E. TIGHTLY BOUND INHIBITORS

The equations derived in the previous sections assume that there is no depletion of the inhibitor by the enzyme. That is, that the formation of EI and ESI does not significantly change the concentration of free inhibitor.

Thus the [I] of the equations (which represents the concentration of free inhibitor) is assumed to be identical to $[I]_t$, the concentration of added I. If, however, the enzyme has a very high affinity for the inhibitor, it will be necessary to use very low inhibitor concentrations in initial velocity studies. Consequently, a significant proportion of the total inhibitor present may be enzyme-bound and the usual graphical methods of determining K_i will not be valid. Dixon has devised a simple graphical method that permits K_i to be determined and at the same time tells us whether a significant fraction of $[I]_t$ is enzyme-bound. The method is based on the same procedures outlined in Chapter Two for systems involving tightly bound substrates (or systems in which $[E]_t \cong [S]_t$). In the derivations given below, it is assumed that only the inhibitor is depleted by the enzyme and $[S] = [S]_t$.

Competitive Inhibitors

In the presence of a competitive inhibitor, the enzyme is distributed among three forms:

$$[E]_t = [E] + [ES] + [EI] \qquad \text{(III-46)}$$

From the velocity-dependence equation, $v = k_p[ES]$, we obtain:

$$[ES] = \frac{v}{k_p} \qquad \text{(III-47)}$$

From the definition of $K_m = [E][S]/[ES]$, we obtain, after substituting [ES] as given by equation III-47:

$$K_m = \frac{[E][S]k_p}{v} \qquad \therefore \quad [E] = \frac{K_m v}{k_p[S]} \qquad \text{(III-48)}$$

From the definition of $V_{max} = k_p[E]_t$, we obtain:

$$[E]_t = \frac{V_{max}}{k_p} \qquad \text{(III-49)}$$

Now substituting for [E], [ES], and $[E]_t$ in the mass balance equation III-46, we obtain:

$$\frac{V_{max}}{k_p} = \frac{K_m v}{k_p[S]} + \frac{v}{k_p} + [EI] \qquad \text{(III-50)}$$

$$V_{max} - \frac{K_m}{[S]}v - v = k_p[EI]$$

$$V_{max} - v\left(1 + \frac{K_m}{[S]}\right) = k_p[EI] \qquad \text{(III-51)}$$

The K_i is given by:

$$K_i = \frac{[E][I]}{[EI]} = \frac{[E]([I]_t - [EI])}{[EI]} = [E]\left(\frac{[I]_t}{[EI]} - 1\right) \qquad \text{(III-52)}$$

Substituting for [E] from equation III-48:

$$K_i = \frac{K_m v}{k_p[S]}\left(\frac{[I]_t}{[EI]} - 1\right) = \frac{K_m v[I]_t}{k_p[S][EI]} - \frac{K_m v}{k_p[S]} \qquad \text{(III-53)}$$

Rearranging:

$$K_i + \frac{K_m v}{k_p[S]} = \frac{K_m v[I]_t}{k_p[S][EI]}$$

$$\frac{[I]_t}{k_p[EI]} = \frac{K_i[S]}{K_m v} + \frac{1}{k_p} \qquad \text{(III-54)}$$

From equation III-53:

$$[EI] = \frac{V_{max}}{k_p} - \frac{K_m v}{k_p[S]} - \frac{v}{k_p} = \frac{1}{k_p}\left[V_{max} - v\left(1 + \frac{K_m}{[S]}\right)\right] \qquad \text{(III-55)}$$

Substituting the solution above for [EI] into equation III-54:

$$\frac{[I]_t}{V_{max} - v\left(1 + \frac{K_m}{[S]}\right)} = \frac{K_i[S]}{K_m v} + \frac{1}{k_p} \qquad \text{(III-56)}$$

At any substrate concentration in the absence of I, the velocity, v_0, is given by:

$$v_0 = \frac{[S]V_{max}}{K_m + [S]} = \frac{V_{max}}{\left(1 + \frac{K_m}{[S]}\right)} \qquad \text{(III-57)}$$

Now consider some point on the v versus [I] plot where the velocity, v_i, is some fraction of v_0; that is,

$$v_i = \frac{v_0}{n} = \frac{V_{max}}{n\left(1 + \frac{K_m}{[S]}\right)} \qquad \text{(III-58)}$$

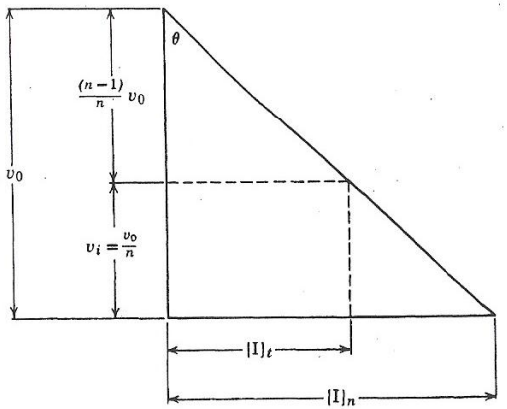
Substituting v_i given by equation III-58 for v in the left-hand part of equation III-56, we obtain:

$$\frac{[I]_t}{V_{max} - \frac{V_{max}}{n}} = \frac{[I]_t}{V_{max}\left(1 - \frac{1}{n}\right)} = \frac{n[I]_t}{(n-1)V_{max}} \qquad \text{(III-59)}$$

And substituting v_0/n for v in the right-hand part of equation III-56, we obtain:

$$\frac{n[I]_t}{(n-1)V_{max}} = \frac{nK_i[S]}{K_m v_0} + \frac{1}{k_p} \qquad \text{(III-60)}$$

A line drawn from v_0 through any v_i intersects the $[I]_t$-axis at some value, $[I]_n$ (e.g., $[I]_2$ for $v_i = v_0/2$, $[I]_3$ for $v_i = v_0/3$, etc.). The geometry of the situation is shown below.



$$\tan\theta = \frac{[I]_n}{v_0} = \frac{n[I]_t}{(n-1)v_0}$$

or

$$[I]_n = \frac{n}{(n-1)}[I]_t \quad \text{and} \quad [I]_t = \frac{(n-1)}{n}[I]_n \qquad \text{(III-61)}$$

Substituting for $[I]_t$ in equation III-60:

$$\frac{[I]_n}{V_{max}} = \frac{nK_i[S]}{K_m v_0} + \frac{1}{k_p}$$

$$[I]_n = nK_i \frac{[S]V_{max}}{K_m v_0} + \frac{V_{max}}{k_p} \qquad \text{(III-62)}$$

but

$$\frac{V_{max}}{v_0} = \left(1 + \frac{K_m}{[S]}\right) \quad \text{and} \quad \frac{V_{max}}{k_p} = [E]_t$$

$$\therefore \quad \boxed{[I]_n = nK_i\left(1 + \frac{[S]}{K_m}\right) + [E]_t} \qquad \text{(III-63)}$$

The plot is shown in Figure III-29. The distance between intercepts for successive values of n is $K_{i_{app}}$, where:

$$K_{i_{app}} = K_i\left(1 + \frac{[S]}{K_m}\right)$$

If several plots are made at different fixed substrate concentrations, $K_{i_{app}}$ can be replotted against [S] (Fig. III-29*b*). The intercepts of the replot give K_m and K_i.

A line drawn from v_0 to the $[I]_t$-axis that intercepts the $[I]_t$-axis one $K_{i_{app}}$ distance to the left of $[I]_2$, represents the line for $n=1$. This is a line tangent to the v versus $[I]_t$ curve at v_0. Another line drawn from v_0 that intercepts the $[I]_t$-axis one $K_{i_{app}}$ distance to the left of $[I]_1$ gives $[E]_t$. If only a very small fraction of the inhibitor is tied up as EI, this $n=0$ line will coincide with the vertical axis. Figure III-29*c*, which represents the left hand portion of the v versus $[I]_t$ plot, shows how the distribution of enzyme forms can be calculated.

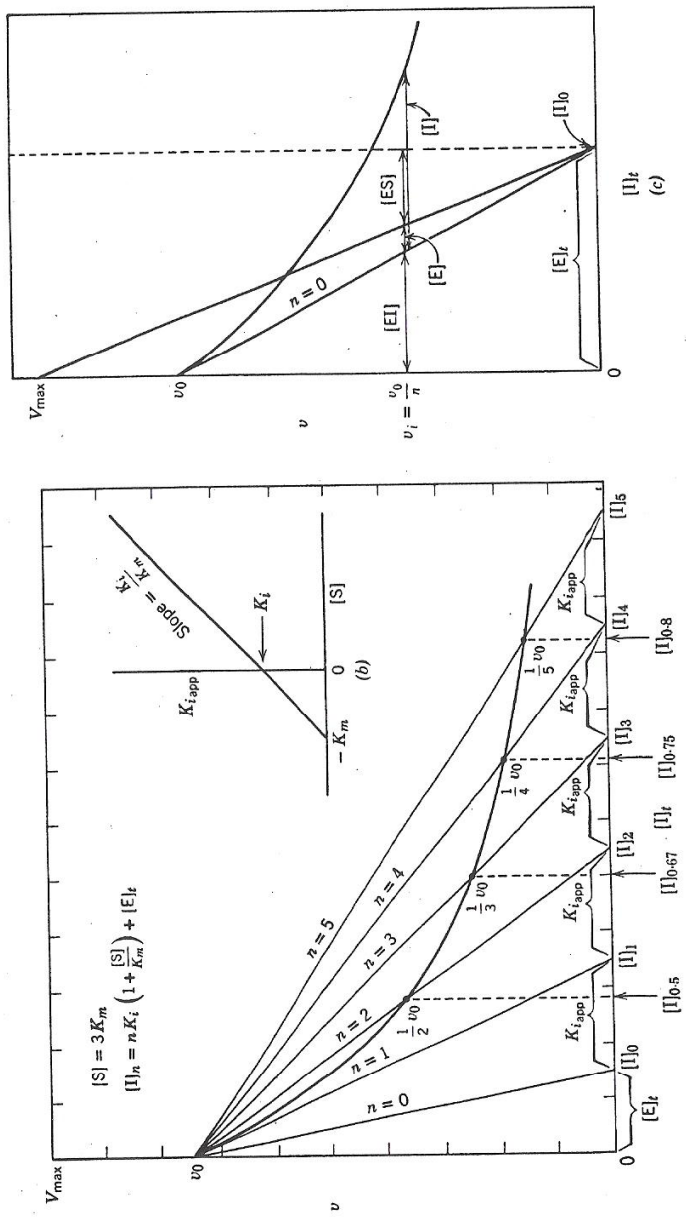


Fig. III-29. (*a*) Plot suggested by Dixon to determine $[E]_t$ when a significant fraction of competitive inhibitor is bound by the enzyme; [S] is assumed to be equal to $3K_m$ ($\therefore v_0 = 0.75V_{max}$). (*b*) Replot of $K_{i_{app}}$ versus [S]. (*c*) Left-hand portion of Fig. III-29*a* showing distribution of enzyme species at a given v_0/n.

Noncompetitive Inhibitors

In the presence of a noncompetitive inhibitor, the enzyme is distributed among four forms:

$$[E]_t = [E] + [ES] + [EI] + [ESI]$$

$$[EI] + [ESI] = [E]_t - [E] - [ES]$$

$$= \frac{V_{max}}{k_p} - \frac{K_m v}{k_p [S]} - \frac{v}{k_p}$$

$$= \frac{V_{max} - v\left(1 + \frac{K_m}{[S]}\right)}{k_p} = \frac{U}{k_p} \qquad \text{(III-64)}$$

where, for convenience, the numerator of equation III-64 is written as U. The mass balance equation for I is:

$$[I]_t = [I] + [EI] + [ESI]$$

$$[I] = [I]_t - ([EI] + [ESI]) = [I]_t - \frac{U}{k_p} \qquad \text{(III-65)}$$

The K_i is defined as:

$$K_i = \frac{[E][I]}{[EI]} = \frac{[ES][I]}{[ESI]}$$

$$\therefore \quad [EI] = \frac{[E][I]}{K_i} \qquad \text{and} \qquad [ESI] = \frac{[ES][I]}{K_i}$$

Since $[ES] = v/k_p$,

$$[ESI] = \frac{v[I]}{k_p K_i} = \frac{v}{k_p K_i}\left([I]_t - \frac{U}{k_p}\right) \qquad \text{(III-66)}$$

From equation III-64:

$$[EI] + [ESI] = \frac{U}{k_p}$$

$$[EI] = \frac{U}{k_p} - [ESI] = \frac{U}{k_p} - \frac{v}{k_p K_i}\left([I]_t - \frac{U}{k_p}\right) \qquad \text{(III-67)}$$

Now, substituting into the expression for K_i where:

$$K_i = \frac{[E][I]}{[EI]} \qquad \text{and} \qquad [E] = \frac{K_m v}{k_p [S]}$$

$$K_i = \frac{K_m v}{k_p [S]}\left[\frac{[I]_t - \frac{U}{k_p}}{\frac{U}{k_p} - \frac{v}{k_p K_i}\left([I]_t - \frac{U}{k_p}\right)}\right] \qquad \text{(III-68)}$$

which simplifies to:

$$\frac{[I]_t}{V_{max} - v\left(1 + \frac{K_m}{[S]}\right)} = \frac{K_i}{v}\left(\frac{[S]}{K_m + [S]}\right) + \frac{1}{k_p} \qquad \text{(III-69)}$$

Now considering some point on the v versus $[I]_t$ curve where $v_i = v_0/n$ and proceeding as shown earlier for a competitive inhibitor, we obtain:

$$[I]_n = nK_i\left(\frac{[S]}{K_m + [S]}\right)\frac{V_{max}}{v_0} + [E]_t \qquad \text{(III-70)}$$

But

$$\frac{V_{max}}{v_0} = \left(1 + \frac{K_m}{[S]}\right) = \frac{[S] + K_m}{[S]}$$

so that

$$\boxed{[I]_n = nK_i + [E]_t} \qquad \text{(III-71)}$$

The plot is identical to that shown in Figure III-29 except now the distance between successive intercepts on the $[I]_t$-axis gives K_i directly. Figure III-30 shows how the concentrations of the four enzyme forms can be determined.

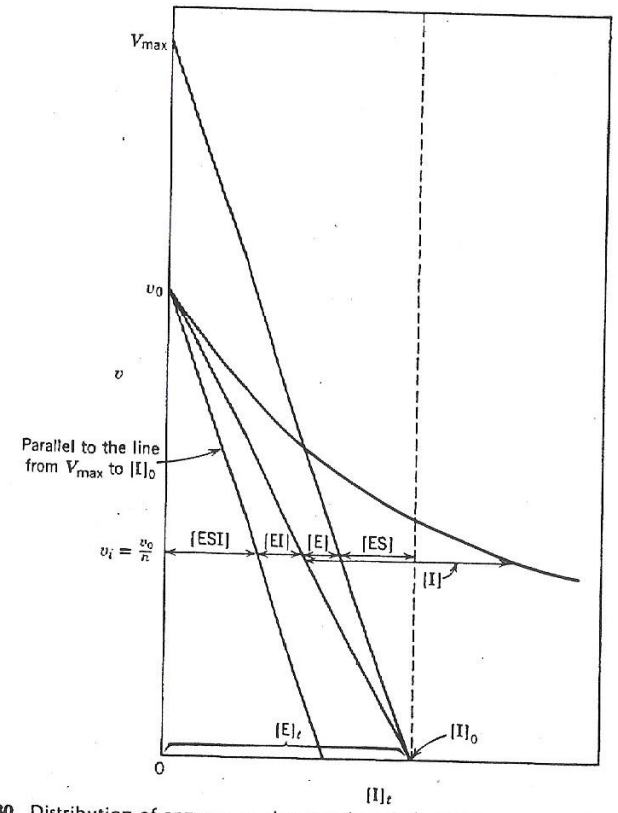


Fig. III-30. Distribution of enzyme species at a given v_0/n in the presence of a noncompetitive inhibitor.

Henderson (1972) has suggested a linear plot of $[I]_t/(1-v_i/v_0)$ versus v_0/v_i to determine K_i and $[E]_t$ in systems where a substantial fraction of the added inhibitor is bound. The equations for the plots are shown below.

$$\text{Competitive} \quad \boxed{\frac{[I]_t}{\left(1-\frac{v_i}{v_0}\right)} = K_i\left(1+\frac{[S]}{K_m}\right)\frac{v_0}{v_i}+[E]_t} \qquad \text{(III-72)}$$

$$\text{Noncompetitive} \quad \boxed{\frac{[I]_t}{\left(1-\frac{v_i}{v_0}\right)} = K_i\frac{v_0}{v_i}+[E]_t} \qquad \text{(III-73)}$$

$$\text{Uncompetitive} \quad \boxed{\frac{[I]_t}{\left(1-\frac{v_i}{v_0}\right)} = K_i\left(1+\frac{K_m}{[S]}\right)\frac{v_0}{v_i}+[E]_t} \qquad \text{(III-74)}$$

The concentration of inhibitor is varied at different fixed substrate concentrations and a constant enzyme concentration. For each fixed [S] and $[E]_t$, the values of v_i/v_0 and v_0/v_i are calculated and plotted as described above. The family of plots for different fixed [S] intersect on the vertical-axis at $[E]_t$. The value of K_i can be calculated from the slopes of the plots or from a *slope* versus [S] (competitive) or *slope* versus $1/[S]$ (uncompetitive) replot. The value of [S] in equations III-72 to III-74 can be taken as $[S]_t$ as long as there is no depletion because of tight substrate binding. Otherwise, $[S]_t - [ES]$ can be substituted for [S]. The concentration of ES can be calculated from equation II-53*b*. The plot remains linear with a vertical-axis intercept of $[E]_t$, but slope replots will be nonlinear. (See equations IV-76 to IV-80 for a plot useful when both S and I are tightly bound to the enzyme [Best-Belpomme and Dessen, 1973]).

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See General References at the end of Chapter Two and also references on plotting inhibition data at the end of Chapter Four.

Tightly Bound Inhibitors

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