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Relationships between Inhibition Constants and Fractional Inhibition in Enzyme-Catalyzed Reactions with Different Numbers of Reactants, Different Reaction Mechanisms, and Different Types and Mechanisms of Inhibition

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SUMMARY

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The relationship between the inhibition constant (K_i) and fractional inhibition (in particular I_{50}) in classical steady-state enzyme kinetics has been analyzed. The analysis covers enzyme reactions with different numbers of reactants, different reaction mechanisms, and different types and mechanisms of inhibition. The assumptions are made that the product concentrations are very low and that the products have much lower affinities for the enzyme than those of the substrates. The following generalized conclusions are drawn. (a) Fractional velocity (f_v) and fractional inhibition (f_i) in the presence of an inhibitor (I) can be expressed by $f_v = 1/[1 + (I/K_i)(E_x/E_t)]$ and $f_i = 1/[1 + (K_i/I)(E_t/E_x)]$, respectively, where E_t is the total amount of enzyme and E_x is the amount of the enzyme species with which the inhibitor may combine. (b) In a multisubstrate reaction, if all the substrates with which the inhibitor does not compete are at saturating concentrations, the relationship between K_i and I_{50} is the same as for a one-substrate reaction. (c) Inhibition of either the competitive, noncompetitive, or uncompetitive type produces a generalized relationship, $K_i/I_{50} = E_x/E_t$. This relationship indicates that K_i will never be greater than I_{50}, and that the ratio of K_i and I_{50} provides a simple experimental method for determination of the availability of the enzyme species for inhibitor binding or the distribution of enzyme forms in an enzyme reaction. (d) In partial noncompetitive inhibition, $K_{ii} > I_{50} > K_{is}$ or $K_{is} > I_{50} > K_{ii}$, depending upon whether the crossover point in the Lineweaver-Burk plot is above or below the horizontal axis (where K_{ii} and K_{is} are the K_i values obtained from the intercept and slope, respectively); in noncompetitive inhibition, K_i is always equal to I_{50}. Examples are cited of one-substrate reactions indicating that competitive, noncompetitive, or uncompetitive inhibition can be illustrated or detected by novel graphical methods different from those currently available.

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INTRODUCTION

In studying antimetabolites or other reversible inhibitors of enzyme-catalyzed reactions, the affinity of inhibitor for the enzyme is usually reported as the inhibition constant (K_i), and the relative inhibition potency is usually reported as fractional inhibition. The concentration of inhibitor required to reduce the reaction velocity by 50% (I_{50}) provides a practical and readily comprehensible potency index which permits the comparison of a series of inhibitors. However, the I_{50} value may be misleading in some cases, since its magnitude is profoundly influenced by the concentration of substrate(s), especially when the inhibitor is competitive or uncompetitive with respect to the substrate in question. On the other hand, K_i is the dissociation constant of an enzyme-inhibitor complex and is independent of the substrate concentration, which is varied. From a practical point of view, it is reasonable to ask how great the inhibition will be for a given enzyme reaction if the inhibitor concentration is at its K_i value, and how the inhibition will be influenced by substrate concentration(s). Since the Michaelis constant (K_m) has often been considered a counterpart of the inhibition constant, the relationship between K_i and fractional inhibition has led to confusion in the literature by the occasional error of illustrating or interpretiing K_i as I_{50}. Since little quantitative work has been done in this area, especially with regard to the relationship between K_i and I_{50} in different enzyme reaction mechanisms, certain basic facts pertaining to inhibition in cases of classical Michaelis-Menten kinetics appear not to have been examined. For a one-substrate enzyme reaction, the expression of inhibition in terms of fractional inhibition has been described by Webb (1). To my knowledge, no further information on this question is available for higher reactancies, especially with regard to the physical meaning of the ratio of K_i and I_{50}. As will be seen in the text, there are reasons to believe that I_{50} values should be accorded special attention in considering fractional inhibition. Although the measurement of I_{50} alone has little value for the characterization of an enzyme, it can be a powerful tool in providing valuable information on enzyme reaction mechanisms when considered in conjunction with K_i values.

The purpose of the present paper is to analyze systematically the relationship between K_i and I_{50} values in enzyme reactions with different numbers of reactants, with different reaction mechanisms, and with different types or mechanisms of inhibition. A study of the inhibition of a one-substrate reaction alone appears to be a special case which cannot be expected to lead to generalized conclusions, and the importance of a systematic analysis should not be overlooked. Furthermore, two- and three-substrate reactions are so common that their detailed quantitative analysis has been undertaken.

SYMBOLS AND DEFINITIONS

The notation of Cleland (2) is used. In addition, the following symbols and definitions are used:

v_i	Reaction velocity in the presence of inhibitor
f_v	Fractional velocity or the ratio of velocity in the presence and absence of inhibitor
f_i	Fractional inhibition (i.e., $1 - f_v$)
I_{50}	Inhibitor concentration required to reduce the reaction velocity one-half
A/K_a, $B/K_b \cdots$	Specific concentration of substrate A, B, $\cdots$
E_t	Amount or concentration of total enzyme
E_x	Amount or concentration of a specific enzyme species which is a fraction of E_t

It is assumed here that the binding of substrate or inhibitor to the enzyme is reversible and that the free concentration of substrate or inhibitor is the same as the total concentration of substrate or inhibitor added to the reaction system.

ANALYSIS

One-Substrate Reactions

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \begin{pmatrix} EA \\ EP \end{pmatrix} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + P$$

SCHEME 1

For the reaction mechanism shown in Scheme 1, the initial rate of the reaction, v, is described by the Michaelis-Menten equation (3) as shown in Eq. 1. In the presence of an inhibitor, the initial rate equation is modified in various ways, depending on the type of inhibition, as indicated in Eqs. 2–4. The derivation of these equations for one-substrate reactions and their interpretation for each type of inhibition can be readily obtained elsewhere (4–6).

No inhibition:

$$v = \frac{VA}{K_a + A} \tag{1}$$

Competitive inhibition:

$$v_i = \frac{VA}{K_a (1 + I/K_i) + A} \tag{2}$$

Partial noncompetitive inhibition:[2]

$$v_i = \frac{VA}{K_a (1 + I/K_{is}) + A (1 + I/K_{ii})} \tag{3}$$

Uncompetitive inhibition:

$$v_i = \frac{VA}{K_a + A (1 + I/K_i)} \tag{4}$$

Case 1: competitive inhibition. The fractional velocity (f_v) observed after the addition of a competitive inhibitor can be expressed by the ratio Eq. 2/Eq. 1, which is equal to

$$f_v = \frac{1}{1 + (I/K_i)\,[K_a/(K_a + A)]} \tag{5}$$

where $K_a/(K_a + A) = E/E_t$, which represents the distribution of the free enzyme form in the absence of the product (see Eq. 24).

When the inhibitor inhibits the enzyme activity 50%, the concentration of that inhibitor is called I_{50}. Thus, at I_{50}, $f_v = 0.5$ and Eq. 5 becomes

$$\frac{I_{50}}{K_i} = 1 + \frac{A}{K_a} \tag{6}$$

Therefore, in competitive inhibition, I_{50} is always greater than K_i. When the substrate concentration is numerically equal to its Michaelis constant (i.e., $A = K_a$), we get $I_{50} = 2K_i$. It is generally believed that many enzymes *in vivo* operate at substrate concentrations not far removed from their Michaelis constants, although some auxiliary reactants (such as cofactors) may be saturating. Otherwise the catalytic potential of the enzymes is wasted. Therefore *in vivo* I_{50} is approximately equal to twice the K_i value.

From Eq. 5 we get

$$f_i = 1 - f_v = \frac{1}{1 + (K_i/I)\,[(K_a + A)/K_a]} \tag{7}$$

Equation 6 can also be obtained from Eq. 7 by setting $f_i = 0.5$ and $I = I_{50}$.

The theoretical plots for Eq. 6 are given in Figs. 1–3. I_{50} will approach K_i only when

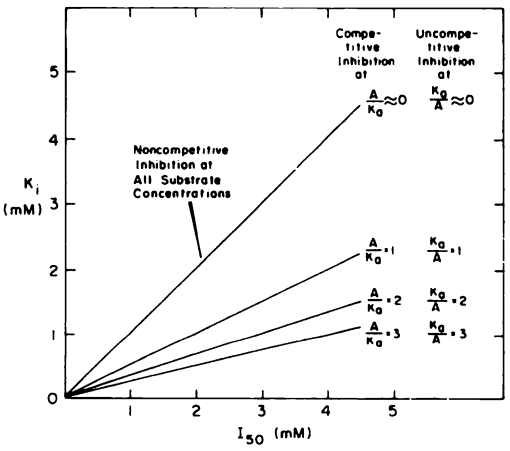

FIG. 1. *Relationship of values of K_i and I_{50} in one-substrate reactions at different levels of substrate*

The plots are theoretical curves of Eq. 6 (competitive inhibition), $I_{50} = K_i$ (noncompetitive inhibition), and Eq. 15 (uncompetitive inhibition). The slopes of the lines (from top) are 1, ½, ⅓, and ¼, respectively.

[2] In pure noncompetitive inhibition $K_{is} = K_{ii} = K_i$; in partial noncompetitive inhibition, $K_{is} \neq K_{ii}$.

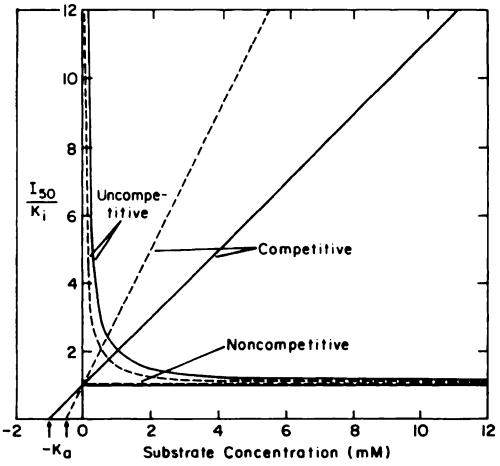

FIG. 2. *Ratio of I_{50} and K_i as a function of substrate concentration in one-substrate reactions, assuming Michaelis constant (K_a) is 1 mM(——) or 0.5 mM (- - -)*

The plots are theoretical curves of Eq. 6 (competitive inhibition), $I_{50} = K_i$ (noncompetitive inhibition), and Eq. 15 (uncompetitive inhibition). Notice that K_i is a constant; therefore the plots represent I_{50} as a function of substrate concentration. The value of I_{50}/K_i is linearly increased by increasing substrate concentration in competitive inhibition, nonlinearly decreased by increasing substrate concentration in uncompetitive inhibition, and unaffected by changing substrate concentration in noncompetitive inhibition.

the substrate specific concentration approaches zero; when the substrate concentration is increased, I_{50} will also increase.

As will be seen from the general treatment given under DISCUSSION, the K_i/I_{50} ratio represents the availability of the enzyme species with which the inhibitor may combine. In the case of competitive inhibition, the availability of the free enzyme form for inhibitor binding (i.e., E/E_t) decreases hyperbolically when the specific concentration of the substrate (i.e., A/K_a) is increased (Fig. 3).

Equation 7 can be plotted in a double-reciprocal form (Fig. 4A), which was first presented by Webb (8).

Case 2: partial noncompetitive inhibition. Equation 3 can be rearranged to a double-reciprocal form suitable for a Lineweaver-Burk plot (9), as shown in Eq. 8.

$$\frac{1}{v_i} = \frac{K_a}{V}\left(1 + \frac{I}{K_{is}}\right)\frac{1}{A} + \frac{1}{V}\left(1 + \frac{I}{K_{ii}}\right) \quad (8)$$

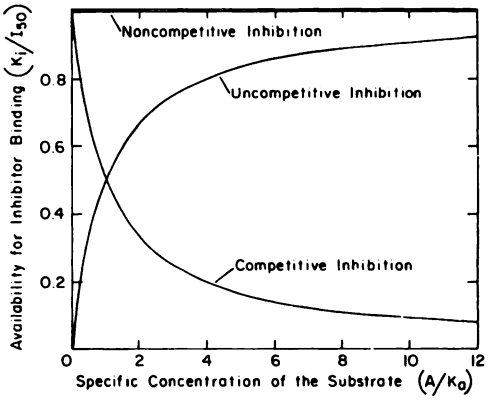

FIG. 3. *Fractional availability of enzyme for inhibitor binding as a function of specific concentration of substrate (A/K_a)*

The K_i/I_{50} ratio is defined as the availability of the enzyme species for inhibitor binding because it is equal to E_x/E_t, where E_x is the amount (or the concentration) of enzyme species that may combine with the inhibitor, and E_t is the amount (or the concentration) of total enzyme (see DISCUSSION for details). The plots are theoretical curves of Eq. 6 (competitive inhibition), $I_{50} = K_i$ (noncompetitive inhibition), and Eq. 15 (uncompetitive inhibition). Notice that the fraction of the enzyme that combines with the inhibitor is hyperbolically decreased by the linear increase in specific concentration of the substrate in competitive inhibition, is hyperbolically increased by the linear increase in specific concentration of the substrate in uncompetitive inhibition, and is unaffected by changing substrate concentration in noncompetitive inhibition. It is clear that, in noncompetitive inhibition, the inhibitor interacts with the total enzyme as a whole, regardless of the constituent enzyme species involved in the reaction mechanism. Therefore noncompetitive inhibition is mechanistically nonspecific.

A series of reciprocal plots represented by this equation always intersect at one point to the left of the vertical axis, and the lines cross above, below, or on the horizontal axis when K_{is} is smaller than, greater than, or equal to K_{ii}, respectively.

The fractional velocity in the presence of a noncompetitive inhibitor with $K_{is} \neq K_{ii}$, as obtained from Eq. 3/Eq. 1, is

$$f_v = \left[1 + \frac{I(K_a/K_{is} + A/K_{ii})}{K_a + A}\right]^{-1} \quad (9)$$

When the concentration of inhibitor is

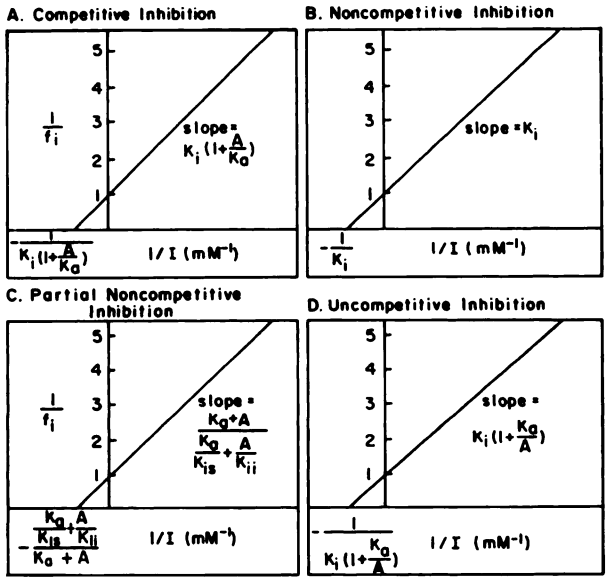

FIG. 4. *Relationship between reciprocal of inhibitor concentration and reciprocal of fractional inhibition in one-substrate reactions*

The plots are theoretical curves of Eq. 7 (A, competitive inhibition), Eq. 13 (B, noncompetitive inhibition, Eq. 12 (C, partial noncompetitive inhibition), and Eq. 16 (D, uncompetitive inhibition). The slopes and horizontal intercepts are given as shown. Notice that the vertical intercepts are all equal to unity since, when inhibitor concentration is infinity, the enzyme will be completely inhibited.

I_{50}, Eq. 9 becomes

$$\frac{A}{K_a} = \frac{1 - I_{50}/K_{is}}{I_{50}/K_{ii} - 1} \qquad (10)$$

Since A/K_a has a finite positive value, the conditions which satisfy Eq. 10 are either $I_{50}/K_{is} < 1$ and $I_{50}/K_{ii} > 1$, or $I_{50}/K_{is} > 1$ and $I_{50}/K_{ii} < 1$. Therefore $K_{is} > I_{50} > K_{ii}$ or $K_{ii} > I_{50} > K_{is}$, depending on the relative values of K_{is} and K_{ii}, or depending on whether the crossover point in the Lineweaver-Burk plot is below (more like uncompetitive inhibition) or above (more like competitive inhibition) the horizonal axis.

In Eq. 10, when $A = K_a$, we obtain

$$I_{50} = \frac{2K_{is}K_{ii}}{K_{is} + K_{ii}} \qquad (11)$$

which indicates that I_{50} is the harmonic mean of K_{is} and K_{ii}.

From Eq. 9 we obtain

$$f_i = \left[1 + \frac{K_a + A}{I\,(K_a/K_{is} + A/K_{ii})}\right]^{-1} \qquad (12)$$

A theoretical plot of Eq. 12 is shown in Fig. 4C.

Case 3: noncompetitive inhibition. In Eq. 12, when $K_{is} = K_{ii}$ (i.e., for pure noncompetitive inhibition), we obtain

$$f_i = 1/(1 + K_i/I) \qquad (13)$$

At $I = I_{50}$, we get $I_{50} = K_i$.

Therefore, in pure noncompetitive inhibition, I_{50} is always equal to K_i regardless of the substrate concentration. Since K_i/I_{50} represents the availability of the enzyme species with which the inhibitor may combine (see DISCUSSION), the fact that this ratio is unity indicates that the inhibitor interacts with the enzyme as a whole, irrespective of the constituent enzyme species in a given reaction mechanism, and thus the inhibition is nonspecific mechanistically. In partial noncompetitive inhibition, either K_{is} or K_{ii} is greater than I_{50}, suggesting that the inhibitor combines nonspecifically with the enzyme as a whole and yet has additional influences, such as steric hindrance, on some species of the enzyme.

Case 4: uncompetitive inhibition. For this case the following equations are obtained.

$$f_v = \frac{1}{I + (I/K_i)[A/(K_a + A)]} \quad (14)$$

where $A/(K_a + A) = (EA-EP)/E_t$, which represents the distribution of the central complex enzyme species in the absence of the product (see Eq. 23).

At $I = I_{50}$, Eq. 14 becomes

$$\frac{I_{50}}{K_i} = 1 + \frac{K_a}{A} \quad (15)$$

The theoretical plots for Eq. 15 are given in Figs. 1–3. It can be seen in Fig. 3 that with uncompetitive inhibition the availability of the enzyme species which may combine with the inhibitor increases hyperbolically with the increase of the specific concentration of the substrate.

From Eq. 14 we get

$$f_i = \frac{1}{1 + (K_i/I)[(K_a + A)/A]} \quad (16)$$

A plot of Eq. 16 is given in Fig. 4D.

In cases when more than one enzyme-substrate intermediate is formed, or more than one product is released, the initial rate equations have the same coefficient form (2, 10), and the present analysis of one-substrate reactions remains valid.

Notice that Figs. 1–3 illustrate the three types of inhibition in three different ways. When noncompetitive and uncompetitive inhibition is difficult to distinguish by Lineweaver-Burk plots in which subjective biases may occur because of weighting factors, the graphical methods developed here may be used for alternative diagnostic purposes, since the shapes, slopes, and intercepts are distinct in the three types of inhibition, although other alternatives are available (11). The physical meanings of competitive, noncompetitive, and uncompetitive inhibition are most explicitly illustrated in Fig. 3.

Fractional velocity and fractional inhibition and the relationship between I_{50} and K_i in one-substrate reactions with different types of inhibition are summarized in Table 1.

Multiple-Substrate Reactions

The over-all rate equations for multiple-substrate reactions can be obtained by solving simultaneous equations for the steady state or, more conveniently, by using the procedure of King and Altman (12). The resulting over-all rate equations may be transformed to equations involving kinetic constants and concentration factors by using the procedure of Cleland (2). The initial rate equation is obtained by setting product concentration(s) in the over-all rate equation equal to zero. The initial velocity of the reaction in the presence of an inhibitor is obtained by multiplying certain terms in the denominator of the initial rate equation by the factor $(1 + I/K_i)$. The terms multiplied by this factor are those appearing in the numerator of the distribution equation (2). Therefore the fractional velocity (and fractional inhibition) in the presence of an inhibitor can be obtained, and enzyme reactions with different mechanisms and different types and mechanisms of inhibition can be analyzed systematically.

The relationships between I_{50} and K_i in two-substrate reactions with Ordered Bi and Ping Pong Bi mechanisms are summarized in Table 2.[3] Some of these relationships in Ping Pong Bi mechanisms are given in Figs. 5 and 6.

Three-substrate reactions with one stable enzyme form (Ordered Ter-x mechanisms), two stable enzyme forms (Uni-x Bi-y Ping Pong or Bi-x Uni-y Ping Pong mechanisms) and three stable enzyme forms (Uni-x Uni-y Uni-z Ping Pong mechanisms) are similarly analyzed. The results are summarized in Table 3.[3]

DISCUSSION

In all cases that have been analyzed in this paper, regardless of the differences in the number of substrates, reaction mechanisms, and types and mechanisms of inhibition (see Eqs. 5, 7, 13, 14, and 16, and Tables 1 and 3), except for mixed inhibition, where K_{is} and K_{ii} are involved (see Eqs. 9 and 12), the following generalized conclusions can be deduced.

Fractional velocity in the presence of the inhibitor is

$$f_v = \frac{1}{1 + (I/K_i)(E_x/E_t)} \quad (17)$$

[3] Details of this analysis are available upon request from the author.

TABLE 1

Summary of relationships between I_{50} and K_i in one-substrate reactions with different types of inhibition

Type of inhibition	Fractional velocity, f_v[a]	Relationship between I_{50} and K_i			
		For over-all reaction, A = finite value	At specific substrate concentration, $A = K_a$	At extreme substrate concentration	
				$\lim_{A\to\infty}$	$\lim_{A\to 0}$
Competitive	$\dfrac{1}{1+(I/K_i)[K_a/(K_a+A)]}$	$I_{50} = \left(1+\dfrac{A}{K_a}\right)K_i$	$I_{50} = 2K_i$	$\dfrac{I_{50}}{K_i} = \infty$	$I_{50} = K_i$
Noncompetitive[b]	$\dfrac{1}{1+I/K_i}$	$I_{50} = K_i$	$I_{50} = K_i$	$I_{50} = K_i$	$I_{50} = K_i$
Partial noncompetitive[b]	$\dfrac{1}{1+[I(K_a/K_{ia})+(A/K_{ii})]/(K_a+A)}$	$K_{ii} > I_{50} > K_{ia}$ $K_{ia} > I_{50} > K_{ii}$	$I_{50} = \dfrac{2K_{ia}K_{ii}}{K_{ia}+K_{ii}}$	$I_{50} < \dfrac{2K_{ia}K_{ii}}{K_{ia}+K_{ii}}$	$I_{50} > \dfrac{2K_{ia}K_{ii}}{K_{ia}+K_{ii}}$
Uncompetitive	$\dfrac{1}{1+(I/K_i)[A/(K_a+A)]}$	$I_{50} = \left(1+\dfrac{K_a}{A}\right)K_i$	$I_{50} = 2K_i$	$I_{50} = K_i$	$\dfrac{I_{50}}{K_i} = \infty$

[a] Fractional inhibition (f_i) can be obtained by $1 - f_v$.

[b] In the Lineweaver-Burk plot, when the crossover point is on the horizontal axis (i.e., $K_{ia} = K_{ii}$), inhibition is called noncompetitive; when the crossover point is above the horizontal axis (i.e., $K_{ii} > K_{ia}$) or below the horizontal axis (i.e., $K_{ia} > K_{ii}$), it is partial noncompetitive. Notice that $2K_{ia}K_{ii}/(K_{ia}+K_{ii})$ is the harmonic mean of K_{ia} and K_{ii}.

TABLE 2

Summary of relationships between I_{50} and K_i in two-substrate reactions with different mechanisms of inhibition

Reaction mechanism	Inhibition mechanism	Resulting inhibition	I_{50}/K_i							
			For over-all reaction, A = finite value	At specific substrate concentration			At extreme substrate concentration			
				$A = K_a$ $(=K_{ia})$	$B = K_b$	$A = K_a$ $(=K_{ia})$, $B = K_b$	$\lim_{A\to\infty}$	$\lim_{B\to\infty}$	$\lim_{A\to 0}$	$\lim_{B\to 0}$
Ordered Bi	*I* Combines with *E*	Competitive with *A* (non-competitive with *B*)	$1 + \frac{AB + K_bA}{K_aB + K_{ia}K_b}$	2	$1 + \frac{2A}{K_a + K_{ia}}$	2	∞	$1 + \frac{A}{K_a}$	1	$1 + \frac{A}{K_{ia}}$
	I Combines with *EA*	Competitive with *B* (uncompetitive with *A*)	$1 + \frac{AB + K_aB + K_{ia}K_b}{K_bA}$	$2 + \frac{2B}{K_b}$	$2 + \frac{K_a + K_{ia}}{A}$	4	$1 + \frac{B}{K_b}$	∞	∞	$1 + \frac{K_{ia}}{A}$
Ping Pong Bi	*I* Combines with *E*	Competitive with *A* (uncompetitive with *B*)	$1 + \frac{AB + K_bA}{K_aB}$	$2 + \frac{K_b}{B}$	$1 + \frac{2A}{K_a}$	3	∞	$1 + \frac{A}{K_a}$	1	∞
	I Combines with *F*	Competitive with *B* (uncompetitive with *A*	$1 + \frac{AB + K_aB}{K_bA}$	$1 + \frac{2B}{K_b}$	$2 + \frac{K_a}{A}$	3	$1 + \frac{B}{K_b}$	∞	∞	1

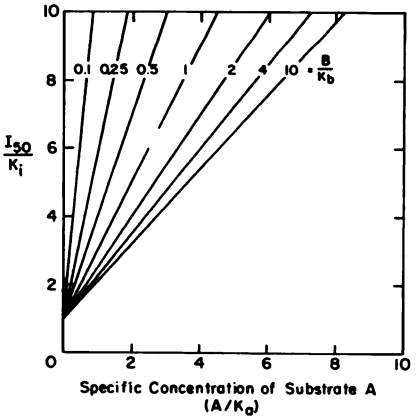

FIG. 5. *I_{50}/K_i ratio as a function of specific concentration of substrate A at different specific concentrations of substrate B in Ping Pong Bi mechanisms, where inhibitor is competitive with respect to A and uncompetitive with respect to B*

The plots are theoretical curves of $I_{50}/K_i = 1 + [(AB + K_bA)/K_aB]$ in Table 2. I_{50}/K_i is linearly increased by the linear increase in A/K_a. This increase is particularly prominent at low levels of B.

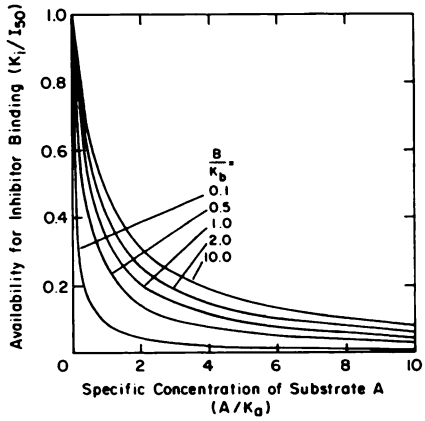

FIG. 6. *Fractional availability of enzyme for inhibitor binding as a function of specific concentration of A, and at different specific concentrations of substrate B, in Ping Pong Bi mechanisms, with inhibitor competitive with respect to A and uncompetitive with respect to B*

The plots are theoretical curves of $I_{50}/K_i = 1 + [(AB + K_bA)/K_aB]$ in Table 2. The K_i/I_{50} ratio is defined as the availability of the enzyme species for inhibitor binding, because it is equal to E_x/E_t, where E_x is the amount (or concentration) of the enzyme species that may combine with the inhibitor, and E_t is the amount (or concentration of total enzyme (see DISCUSSION for details). Notice that the fraction of the enzyme that combines with the inhibitor is hyperbolically decreased by the linear increase in specific concentration of substrate A. This decrease is particularly prominent when substrate B concentration is low. When the specific concentration of substrate B approaches infinity, the curve obtained approaches the one-substrate competitive case in Fig. 3.

where E_x/E_t is expressed by the distribution equation which in turn can be expressed by K_i/I_{50} (see Eq. 19). Therefore, f_v is equal to $1/(1 + I/I_{50})$ and f_i is equal to $1/(1 + I_{50}/I)$.

Fractional inhibition in the presence of the inhibitor is

$$f_i = \frac{1}{1 + (K_i/I)(E_t/E_x)} \qquad (18)$$

When the concentration of the inhibitor is I_{50} (i.e., $f_v = f_i = 0.5$), Eq. 17 or Eq. 18 becomes

$$\frac{K_i}{I_{50}} = \frac{E_x}{E_t} \qquad (19)$$

Therefore determination of the K_i/I_{50} ratio in effect determines the availability of the fraction of the total enzyme with which the inhibitor may combine, and thus provides a simple experimental procedure for measuring the distribution of enzyme forms for a reaction.

Notice that E_x will never exceed E_t; therefore K_i will never exceed I_{50}.

Since the product concentrations are usually much lower than those of substrate, the terms containing product concentration factors in the distribution equations are ignored. Low product concentrations alone are necessary but not sufficient to justify neglecting product-containing terms. For example, the over-all rate equation for Scheme 1 is (see ref. 2)

$$v = \frac{V_1 (A - P/K_{eq})}{K_a + A + (V_1P/V_2K_{eq})} \qquad (20)$$

where $V_1 = k_3E_t$, $V_2 = k_2E_t$, $K_a = (k_2 + k_3)/k_1$, and $K_{eq} = k_1k_3/k_2k_4$. Applying the Haldane relationship (2),

$$K_{eq} = \frac{V_1}{V_2}\left(\frac{K_p}{K_a}\right) \qquad (21)$$

TABLE 3

Inhibition of different three-substrate reactions with inhibitors of different inhibition mechanisms

Symbols are defined under SYMBOLS AND DEFINITIONS. x, y or z refers to unspecified number of product(s) in a reaction sequence.

Reaction mechanism	Inhibition mechanism	K_i/I_{50}	Fractional velocity, f_v	Fractional inhibition, f_i	Distribution equation[b]
Ordered Ter-x (one stable enzyme form)	I combines with E	$\frac{E}{E_t}$	$\frac{1}{1+(I/K_i)(E/E_t)}$	$\frac{1}{1+(K_i/I)(E_t/E)}$	$\frac{E}{E_t}=\frac{K_{ia}K_{ib}K_c+K_aBC+K_{ia}K_bC}{K_{ia}K_{ib}K_c+K_{ib}K_cA+K_{ia}K_bC+K_cAB+K_bAC+K_aBC+ABC}$
	I combines with EA	$\frac{EA}{E_t}$	$\frac{1}{1+(I/K_i)(EA/E_t)}$	$\frac{1}{1+(K_i/I)(E_t/EA)}$	$\frac{EA}{E_t}=\frac{K_{ib}K_cA+K_bAC}{\sum}$
	I combines with EAB	$\frac{EAB}{E_t}$	$\frac{1}{1+(I/K_i)(EAB/E_t)}$	$\frac{1}{1+(K_i/I)(E_t/EAB)}$	$\frac{EAB}{E_t}=\frac{K_cAB}{\sum}$
Uni-x Uni-y Uni-z Ping Pong (three stable enzyme forms)	I combines with E	$\frac{E}{E_t}$	$\frac{1}{1+(I/K_i)(E/E_t)}$	$\frac{1}{1+(K_i/I)(E_t/E)}$	$\frac{E}{E_t}=\frac{K_aBC}{K_cAB+K_bAC+K_aBC+ABC}$
	I combines with F	$\frac{F}{E_t}$	$\frac{1}{1+(I/K_i)(F/E_t)}$	$\frac{1}{1+(K_i/I)(E_t/F)}$	$\frac{F}{E_t}=\frac{K_bAC}{\sum}$
	I combines with G	$\frac{G}{E_t}$	$\frac{1}{1+(I/K_i)(G/E_t)}$	$\frac{1}{1+(K_i/I)(E_t/G)}$	$\frac{G}{E_t}=\frac{K_cAB}{\sum}$
Bi-x Uni-y or Uni-x Bi-y[a] Ping Pong (two stable enzyme forms)	I combines with E	$\frac{E}{E_t}$	$\frac{1}{1+(I/K_i)(E/E_t)}$	$\frac{1}{1+(K_i/I)(E_t/E)}$	$\frac{E}{E_t}=\frac{K_{ia}K_bC+K_aBC}{K_{ia}K_bC+K_cAB+K_bAC+K_aBC+ABC}$
	I combines with EA	$\frac{EA}{E_t}$	$\frac{1}{1+(I/K_i)(EA/E_t)}$	$\frac{1}{1+(K_i/I)(E_t/EA)}$	$\frac{EA}{E_t}=\frac{K_bAC}{\sum}$
	I combines with F	$\frac{F}{E_t}$	$\frac{1}{1+(I/K_i)(F/E_t)}$	$\frac{1}{1+(K_i/I)(E_t/F)}$	$\frac{F}{E_t}=\frac{K_cAB}{\sum}$

[a] All the relationships are the same as in the Bi-x Uni-y Ping Pong mechanism except that the symbols are changed as follows: E becomes F, B becomes A, C becomes B, and A becomes C.

[b] Notice that distribution equation can be expressed by K_i/I_{50} (see Eq. 19), therefore f_v and f_i are $1/(1+I/I_{50})$ and $1/(1+I_{50}/I)$, respectively.

where $K_p = (k_2 + k_3)/k_4$, we obtain

$$v = \frac{V_1 (A - P/K_{eq})}{K_a (1 + P/K_p) + A} \quad (22)$$

Notice that when P approaches zero, we obtain Eq. 1. From the distribution equations (see refs. 2 and 10) we obtain

$$\frac{\left(\frac{EA}{EP}\right)}{E_t} = \frac{k_1A + k_4P}{(k_2 + k_3) + k_1A + k_4P} = \frac{A + PK_a/K_p}{K_a (1 + P/K_p) + A} \quad (23)$$

$$\frac{E}{E_t} = \frac{k_2 + k_3}{(k_2 + k_3) + k_1A + k_4P} = \frac{K_a}{K_a (1 + P/K_p) + A} \quad (24)$$

Notice that $E + (EA\text{-}EP) = E_t$, and the conditions which permit neglect of product-containing terms in Eq. 23 or Eq. 24 to allow approximation of these equations to $A/(K_a + A)$ or $K_a/(K_a + A)$, respectively, is $P \ll K_p$ and/or $K_p \gg K_a$. If the inhibitor combines with the free enzyme form, E, the over-all rate equation in the presence of the product becomes

$$v_i = \frac{V_1(A - P/K_{eq})}{K_a[1 + (P/K_p) + (I/K_i)] + A} \quad (25)$$

The fractional velocity is Eq. 25/Eq. 22, which gives

$$f_v = \frac{K_a(1 + P/K_p) + A}{K_a[1 + (P/K_p) + (I/K_i)] + A} \quad (26)$$

When $I = I_{50}$, $f_v = 0.5$, and we obtain

$$\left[\frac{I_{50}}{K_i}\right]_{real} = 1 + \frac{A}{K_a} + \frac{P}{K_p} \quad (27)$$

Comparing Eq. 27 (with a real I_{50}/K_i ratio) and Eq. 6 (with an approximated I_{50}/K_i ratio), it is clear that

$$\left[\frac{I_{50}}{K_i}\right]_{approx} < \left[\frac{I_{50}}{K_i}\right]_{real} \quad (28)$$

and the two sides of this expression will be equal only when P approaches zero or K_p approaches infinity. A generalized restriction can be formulated for the present analyses by stating that the value of $(P \cdot Q \cdot R \cdots V_1)/(K_{eq}V_2)$ should be small, where P, Q, $R \cdots$ are product concentrations, K_{eq} is thermodynamic equilibrium constant, and V_1 and V_2 are forward and backward maximal velocities.

The Haldane relationship (2) can be represented by

$$K_{eq} = \left(\frac{V_1}{V_2}\right)^n \frac{K_{(p)}K_{(q)}K_{(r)} \cdots}{K_{(a)}K_{(b)}K_{(c)} \cdots} \quad (29)$$

where $K_{(a)}$ may be K_a or K_{ia}, and so forth. There exists $n = 1$, at least, but there may also be Haldane relationships when n has some other values. Applying the Haldane relationship with $n = 1$ to the above restriction, we can state that the $P \cdot Q \cdot R \cdots (K_{(a)} \cdot K_{(b)}K_{(c)} \cdots / K_{(p)}K_{(q)}K_{(r)} \cdots)$ value should be small in order to neglect the product-containing terms. Since it is known that products $P \cdot Q \cdot R \cdots$ have very low values, an additional condition to keep the whole term low will be $K_{(a)}K_{(b)}K_{(c)} \ll K_{(p)}K_{(q)}K_{(r)}$. Therefore, in general, a higher affinity of the enzyme for substrates and lower affinity for the products will favor this condition.

In Eq. 6, since K_a and K_i are constants, it is possible in theory to use a reversible competitive inhibitor as a tool to assay the unknown substrate concentration. This may be of particular value for studies *in vivo*, since the potency of the competitive inhibition is not critical and because only the ratio I_{50}/K_i is involved. Therefore a nonmetabolized, stable inhibitor can be used. From Eq. 6 it is clear that I_{50} will be a function of the substrate concentration at the locus or the compartment of cells or tissues where the enzyme reaction actually takes place. Permeability or transport and metabolism of the inhibitor may affect the concentration of inhibitor at the site where inhibition is manifested; however, this problem can be circumvented in part by selecting a metabolically inert analogue of the substrate as an inhibitor. In addition, the concentration of the inhibitor at the cellular level may be measured directly by using isotopic tracer techniques, for example. If the reaction under consideration is a multiple-substrate reaction, the substrate with which the inhibitor does not compete must be at high

concentration with respect to its corresponding K_m value (Table 2) in order for this study to be valid. It is concluded that the relationship between K_i and I_{50} in a multiple-substrate reaction can be analyzed as if it were a single-substrate reaction, provided that the substrate(s) with which the inhibitor does not compete is kept at saturation concentration. For the general case, based on the same assumptions as in the present analyses, it has been shown (13) that in reactions with S number of substrates, P products, and n enzyme forms, the number of possible mechanism patterns (i.e., the number of possible over-all rate equations) can be calculated.

The generalized conclusions indicating that, in competitive and uncompetitive inhibition, K_i will never exceed I_{50} at any substrate concentration and that, in noncompetitive inhibition, K_i will always be equal to I_{50}, suggest a potential practical application. Investigators have usually characterized the kinetic properties of an enzyme at artificially contrived optimal conditions *in vitro*, without knowledge of whether the kinetic parameters determined under such conditions bear any relation to the conditions prevailing *in vivo*. In any case, when K_i (*in vitro*) is greater than I_{50} (*in vivo*), it would indicate a contradiction with the central dogma. For instance, studies of the inhibition of DNA polymerase in mouse L-cells by 1-β-D-arabinofuranosylcytosine 5′-triphosphate (a competitive structural analogue of deoxycytosine 5′-triphosphate) gave a K_i value *in vitro* of 8.7 μM and an I_{50} value *in vivo* of less than 50 nM, with an extrapolated value of 35 nM (14). The exceedingly high value for K_i in comparison with I_{50} may indicate that the conventional assay conditions for DNA polymerase bear little relation to the conditions that obtain *in vivo*, unless we assume an unusually uneven intracellular distribution of the inhibitor for the I_{50} measurement. The suggestion of the infidelity of DNA polymerase assay system can be reconciled with the current observations that DNA replication *in vivo* may require an enzyme complex containing nuclease, polymerase, and ligase activities (15) as well as DNA-unwinding protein (16), and that some of the DNA polymerases isolated from *Escherichia coli* may not be responsible for the major synthesis of DNA, but may only act as repair enzymes (17, 18). On the other hand, studies in this laboratory on the metabolism of 1-β-D-arabinofuranosylcytosine in L1210-bearing mice indicated that 1 hr after subcutaneous injection of the drug the ratio of distribution of 1-β-D-arabinofuranosylcytosine in ascites cells as opposed to ascites fluid is 1.6, whereas the ratio of distribution of 1-β-D-arabinofuranosylcytosine 5′-triphosphate is 2260, strongly suggesting an unusually efficient trapping mechanism for the active metabolite in the L1210 cells.[4]

Usually variation of the slope or intercept with inhibitor concentration in Lineweaver-Burk plots is a linear function. However, in more complex cases, the slope or intercept can be a hyperbolic or parabolic function with respect to inhibitor concentration (6). The physical meanings of these more complex cases remain to be explored.

The present studies assume that binding of substrate or inhibitor to the enzyme is reversible and that the concentration of free substrate or inhibitor is the same as the total concentration of substrate or inhibitor added to the reaction system. This assumption will not be true if the enzyme has such a high affinity for the inhibitor (or substrate) that a considerable portion may be bound by the enzyme. The kinetics of tight-binding inhibitors has been analyzed by Henderson (19) and Dixon (20).

The procedure used in the present studies may prove useful for analysis of biological systems other than enzymes, such as pharmacological or physiological receptor systems, transport carrier systems, or other processes involving input-output sequences that may follow Michaelis-Menten kinetics. Attempts have been made to extrapolate the kinetics of enzyme systems to drug-receptor systems (21, 22). For purposes of formal theory, the following terms may be used interchangeably: enzyme and receptor, substrate and drug, alternative substrate and agonist, inhibitor and antagonist, velocity and effect, maximal velocity and maximal efficacy, Michaelis constant and

[4] Unpublished observations.

affinity (reciprocal). Therefore, by using a competitive antagonist as a tool, it may be possible to measure the distribution of receptor forms or the occupancy of the receptor by a drug when a given effect (or a response) is produced.

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ADDENDUM

Following the acceptance for publication of the present paper, a work similar in title but different in extent of analysis was published by Y-C. Cheng and W. H. Prusoff in *Biochemical Pharmacology*, **22**, 3099–3108 (1973).

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