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The Interaction between Full and Partial Inhibitors Acting on a Single Enzyme

A Theoretical Analysis

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SUMMARY

A theoretical analysis has been made of multiple inhibition systems involving a full and a partial inhibitor. This analysis applies to single- and multisubstrate enzyme systems obeying Michaelis-Menten kinetics. It has been shown that a plot of the reciprocal of the enzyme velocity versus the concentration of the full inhibitor, at constant substrate concentration, is linear in either the presence or the absence of a fixed level of the partial inhibitor. If the slope of the plot is increased or unaltered in the presence of a fixed concentration of the partial inhibitor, the two inhibitors are mutually nonexclusive. If the slope of the plot is decreased, the two inhibitors may be either mutually exclusive or nonexclusive. When a decrease in slope is observed, mutual exclusivity can be distinguished from nonexclusivity by the use of secondary plots based on the effect of the partial inhibitor on the slope or the abscissal intercept of the primary plot. The rules proposed for distinguishing mutually exclusive from nonexclusive inhibitors hold irrespective of the type of inhibition (competitive, noncompetitive, uncompetitive, mixed), so that a knowledge of the kinetic nature of the inhibitors is not required. The results of such an analysis are also discussed in terms of summation, antagonism, and synergism between inhibitors. It has been pointed out that independent inhibitor binding does not necessarily result in independent inhibitor effects, and the conditions necessary for observation of independent inhibitory effects have been defined.

INTRODUCTION

The case of more than one inhibitor acting on a single enzyme has been analyzed, experimentally or theoretically, by several authors (see, e.g., refs. 1–14). Among the graphical methods devised to analyze multiple inhibition, those proposed by Yagi and Ozawa (2, 3), Webb (4), and Chou and Talalay (14) allow a discrimination between mutually exclusive and nonexclusive inhibitors, based on the linearity or nonlinearity of the plots. The Dixon plot (5–9), which is linear in either case, appears to be a particularly versatile tool for analyzing multiple inhibition. Besides providing a simple diagnostic test for distinguishing mutual exclusivity from nonexclusivity, this graphical method allows in most cases a direct estimation of the relevant kinetic constants and the inhibitor interaction factor (9). It has also been used to determine enzyme kinetic mechanisms (7) and to analyze multiple inhibition in carrier-mediated transport phenomena (15, 16). A thorough Dixon analysis of multiple inhibition, based on the rapid equilibrium assumption, has been performed by Segel (9) for single-substrate Michaelian systems. This author has shown that, irrespective of the type of inhibition, the slope of the Dixon plot for a full (dead-end) inhibitor is increased in the presence of a constant concentration of a second, mutually nonexclusive, full inhibitor, whereas no slope effect is observed when two dead-end inhibitors are mutually exclusive. The potentialities of this method of analysis have not been fully explored for double-inhibition systems involving a full and a partial inhibitor. The term "partial" is used to designate an inhibitor which, by combining with the enzyme, does not give rise to a dead-end complex, as is the case of full inhibitors, but to an enzyme form which still retains some catalytic activity (17, 18). Besides serving as investigational instruments, partial enzyme inhibitors are often valuable pharmacological tools. For example, it has been shown that the cardiac effects of the spironolactone derivative, canrenone, are due to the partial inhibition of (Na^+-K^+)-ATPase at the digitalis receptor site (19). It has also been proposed that the partial inhibition of (Na^+-K^+)-ATPase contributes to the antitumor activity of the polypeptides cesalin and macromomycin (20). Very recently, certain local anesthetics have been proven to be partial inhibitors of mitochondrial ATPase (21). Attempts to characterize the interaction between full and partial inhibitors (19, 20) had to

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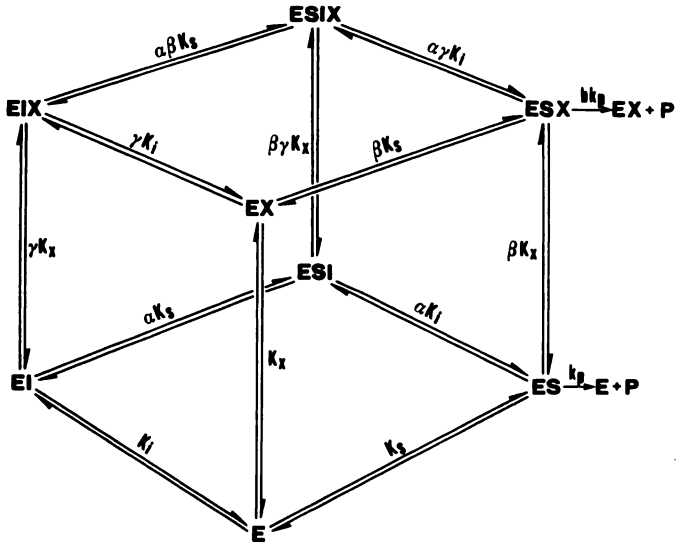
rely primarily on binding studies, since the results of inhibition experiments could not be interpreted unambiguously. The interaction between full and partial effectors is of particular pharmacological interest, since partial effectors are often used in pharmacological practice to modulate the activity of full effectors. Canrenone, for instance, has been employed to antagonize selectively the arrhythmic effects of cardiac glycosides (see ref. 19 and references therein).

The purpose of the present paper is to provide theoretical bases for studying the kinetic behavior of multiple-inhibition systems involving a full and a partial inhibitor. Accordingly, the Dixon equation is used to analyze the combined effects of a full and a partial inhibitor on the initial velocity of single-substrate enzymatic reactions obeying Michaelis-Menten kinetics. The assumptions are made that rapid equilibrium conditions apply and that each enzyme species cannot combine with more than one molecule of each inhibitor. Such an analysis shows that an unambiguous discrimination can be made between mutually exclusive and nonexclusive inhibitors only on the basis of kinetic evidence. It is also shown that the same type of analysis applies to multisubstrate enzyme systems. The applicability of the tests proposed for distinguishing mutually exclusive from nonexclusive inhibitors is demonstrated by analysis of the data previously obtained for the multiple inhibition of $(Na^+\text{-}K^+)$-ATPase by ouabain and canrenone (19).

PROPERTIES OF MULTIPLE INHIBITION SYSTEMS INVOLVING A FULL AND A PARTIAL INHIBITOR

A general mechanism for the reversible inhibition of a single-substrate enzyme by the combined action of a full and a partial inhibitor is shown in Scheme 1, where I is a full inhibitor and X is a partial inhibitor. K_s, K_i, and K_x are the dissociation constants for the ES, EI, and EX complexes, respectively. The interaction factors α and β represent the change in substrate affinity induced by I and X, respectively, or, vice versa, the alteration in the affinity for the inhibitors due to the bound substrate; k_p is the rate constant for the breakdown of the ES complex to the free enzyme plus the product, and b expresses the effect of X on the rate of product formation. The inhibitor interaction factor, γ, represents the mutual influence of the two inhibitors on the binding of each other. Inhibitor binding is independent when $\gamma = 1$, whereas values of γ lower or greater than unity denote mutual facilitation or hindering, respectively. When $\gamma = \infty$, the two inhibitors are mutually exclusive.



SCHEME 1

If rapid equilibrium conditions apply, the Dixon form of the rate equation for the above mechanism, assuming I as the varied inhibitor, is

$$\frac{1}{v} = \frac{1 + \dfrac{\alpha K_s}{[S]} + \dfrac{[X]}{\beta\gamma K_x} + \dfrac{\alpha K_s[X]}{\gamma K_x[S]}}{\alpha K_i V_m \left(1 + \dfrac{b[X]}{\beta K_x}\right)}[I] + \frac{1 + \dfrac{K_s}{[S]} + \dfrac{[X]}{\beta K_x} + \dfrac{K_s[X]}{K_x[S]}}{V_m \left(1 + \dfrac{b[X]}{\beta K_x}\right)} \quad (1)$$

where V_m is the maximal velocity of the uninhibited reaction and the other symbols are as defined above. At constant $[S]$ and $[X]$, a plot of $1/v$ versus $[I]$ will be linear. This would not be the case if X were the varied inhibitor. A plot of $1/v$ versus $[X]$, at constant $[S]$ and $[I]$, would be hyperbolic and hence not very informative. Equations describing the various special cases of multiple inhibition can be readily obtained from Eq. 1 by assigning appropriate values to the interaction constants (9, 17, 22).

It is apparent that the intercept term of Eq. 1 does not depend on the type of interaction between the two inhibitors, since it does not contain γ. On the other hand, this was expected, since the intercept term of the Dixon equation represents the reciprocal of the enzyme velocity in the absence of the varied inhibitor. In this case, the intercept reflects the effect of X alone on the enzyme velocity. The addition of X results in either an increase or a decrease in the intercept of the Dixon plot for I depending on the relative values of β and b. If $\beta \geq b$, the intercept can only be increased, but it may be decreased if $\beta < b$. Since for inhibitors $b \leq 1$, the latter condition implies that the affinity of the enzyme for the substrate is increased in the presence of X. This ligand will therefore act as an activator or an inhibitor, depending on which effect prevails: the increase in the affinity for the substrate or the decrease in the rate constant of product formation. Partial uncompetitive inhibition can be considered a limiting case of this type of inhibition where $\beta = 0$ (17). It can be shown that, when $\beta < b$, the intercept of the Dixon plot for I is increased, unaffected, or decreased by X, depending on whether the substrate concentration is greater than, equal to, or lower than $K_s(b - \beta)/(1 - b)$. Unlike the intercept term, the slope term of Eq. 1 is a function of γ. An analysis of this term will therefore provide information on the type of interaction between the inhibitors. As γ increases from 0 (infinite

cooperativity between the two inhibitors) to infinity (mutual exclusivity), the slope of the Dixon plot for I, in the presence of a finite concentration of X, decreases from infinity to a finite value (Fig. 1). Thus, for $\gamma = 0$, the Dixon plot coincides with the vertical axis. As γ increases, the Dixon plot pivots clockwise about the point of intersection with the vertical axis (which is independent of γ) until, for $\gamma = \infty$, a limiting positive slope is reached. When $\gamma = \infty$, Eq. 1 reduces to

$$\frac{1}{v} = \frac{1 + \dfrac{\alpha K_s}{[S]}}{\alpha K_i V_m \left(1 + \dfrac{b[X]}{\beta K_x}\right)} [I] + \frac{1 + \dfrac{K_s}{[S]} + \dfrac{[X]}{\beta K_x} + \dfrac{K_s[X]}{K_x[S]}}{V_m \left(1 + \dfrac{b[X]}{\beta K_x}\right)} \quad (2)$$

The slope term of this equation differs from that of the Dixon equation for I alone in that the denominator is multiplied by the factor $(1 + b[X]/\phi\beta K_x)$. Thus, for $\gamma = \infty$, the slope in the presence of X is always lower than the slope in its absence. It can therefore be concluded that, in case of mutual exclusivity, the effect of a fixed concentration of a partial inhibitor will be to reduce the slope of the Dixon plot for a full inhibitor. Since a decrease in slope cannot be observed when the fixed inhibitor inhibits totally (9), this graphical method may be useful in distinguishing partial from total inhibitors (19). From Fig. 1 it is also apparent that, in case of nonexclusivity ($0 < \gamma < \infty$), a partial inhibitor may have either a positive or a negative slope effect, depending on whether the value of the inhibitor interaction factor, γ, is lower or greater than a particular value which depends on the nature of the two inhibitors (competitive, noncompetitive, uncompetitive, mixed). This value is shown in Table 1 for various possible types of double inhibition. The double-inhibition system involving a fully competitive and a partially uncompetitive inhibitor is not shown, since in this case the two inhibitors are mutually exclusive and only a decrease in slope can be observed (the fully competitive inhibitor, by totally preventing substrate binding, prevents also the binding of the partially uncompetitive inhibitor, and vice versa).

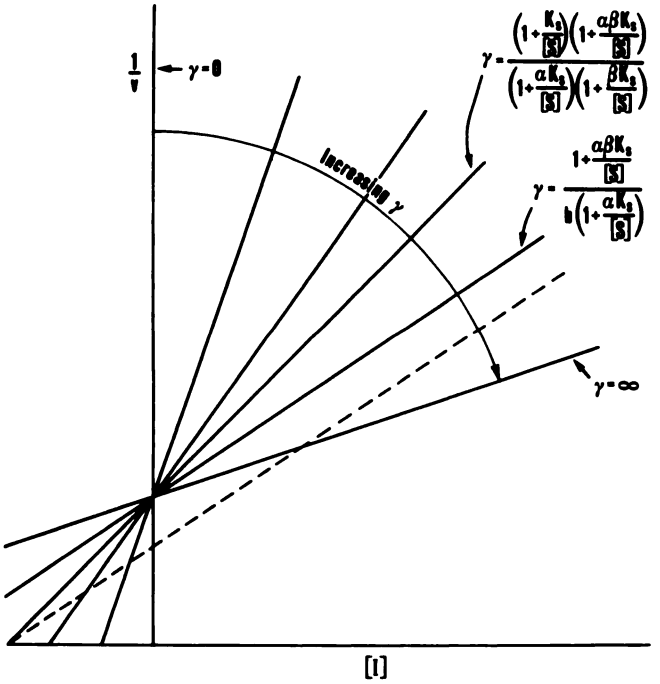


FIG. 1. *Dixon plot for a full inhibitor, I, in the presence of a fixed level of a partial inhibitor, X, and at constant substrate concentration, showing how the slope of the plot varies with varying the value of the inhibitor interaction factor, γ*

The continuous lines represent the Dixon plot for I in the presence of X, whereas the *broken line* represents the plot in the absence of X. Kinetic constants were chosen arbitrarily. The values of γ are shown at which the plots in the presence and in the absence of the partial inhibitor cross on the horizontal axis, or have the same slope. These values refer to the general case (Scheme 1) in which I is a full (linear) mixed-type inhibitor and X is a partial (hyperbolic) mixed-type inhibitor. In particular cases (see text), the intercept of the plot in the presence of X may be equal to, or lower than, that of the plot for I alone, but the dependence of the slope on γ is the same.

In Table 1, the value of γ is also shown at which the plots in the absence and the presence of X cross on the horizontal axis. This value is of interest since, as shown in Appendix I, an intersecting pattern that crosses on the horizontal axis is indicative of summation of inhibitions, i.e., independent inhibitory effects (4). Values of γ lower or greater than this value give rise to synergistic or antagonistic inhibitory actions, respectively, the intersection point being in the upper left-hand quadrant in the former case and in the lower left-hand quadrant or the upper right-hand quadrant in the latter case. In Table 1, it can be seen that summation of inhibitions coincides with independent binding ($\gamma = 1$) only when at least one of the two inhibitors is noncompetitive. An identical limitation applies to multiple-inhibition systems involving two full inhibitors (13). If neither inhibitor is noncompetitive, independent binding does not result in independent inhibitory effects, but in synergistic (Cases 1 and 5) or antagonistic (Case 7) actions. For independent binding, γ (which is equal to 1) is in fact lower than the value at which summation of inhibitions occurs in Cases 1 and 5, whereas it is greater in Case 7. The fact that two inhibitors that bind at independent sites may not produce independent inhibitory effects can be easily understood if one considers that two inhibitors also can influence indirectly the binding of each other through altering the substrate affinity. For example, two independently bound inhibitors that compete (fully or partially) with the substrate will act synergistically, since, by decreasing the affinity of the substrate, they facilitate the binding of each other. Conversely, two inhibitors that bind independently of one another but exert opposite effects on substrate affinity (i.e., one competitive and the other uncompetitive) will act antagonistically. It already has been observed, in this connection, that a fully competitive inhibitor is necessarily mutually exclusive with respect to an uncompetitive inhibitor. Such an indirect, substrate-mediated, mutual influence cannot be exerted only when one (or both) of the two independently bound

TABLE 1

Values of the inhibitor interaction constant, γ, in various types of double inhibition

The values of γ are shown at which the slope or the abscissal intercept of the Dixon plot for the full inhibitor, *I*, is independent of the concentration of the partial inhibitor, *X*. All theoretically possible combinations of pure inhibitors are shown, except the case of a fully competitive and a partially uncompetitive inhibitor. In this case, a parallel pattern cannot be observed, since the two inhibitors are mutually exclusive (see text), and the plots in the absence and presence of *X* intersect above the horizontal axis at a height of $1/bV_m$.

Inhibitor	Case and type of inhibition							
	1	2	3	4	5	6	7	8
I *X*	*C*,[a] $\alpha = \infty$ *C*, $b = 1$ $1 < \beta < \infty$	*NC*, $\alpha = 1$ *NC*, $0 < b < 1$ $\beta = 1$	*C*, $\alpha = \infty$ *NC*, $0 < b < 1$ $\beta = 1$	*NC*, $\alpha = 1$ *C*, $b = 1$ $1 < \beta < \infty$	*UC*, $\alpha = 0$ *UC*, $0 < b < 1$ $\beta = 0$	*UC*, $\alpha = 0$ *NC*, $0 < b < 1$ $\beta = 1$	*UC*, $\alpha = 0$ *C*, $b = 1$ $1 < \beta < \infty$	*NC*, $\alpha = 1$ *UC*, $0 < b < 1$ $\beta = 0$
Value of γ at which the slope is independent of [*X*]	β	$\frac{1}{b}$	$\frac{1}{b}$	$\frac{1+\frac{\beta K_s}{[S]}}{1+\frac{K_s}{[S]}}$	$\frac{1}{b}$	$\frac{1}{b}$	1	$\frac{1}{b\left(1+\frac{K_s}{[S]}\right)}$
Value of γ at which the abscissal intercept is independent of [*X*]	$\frac{\beta\left(1+\frac{K_s}{[S]}\right)}{1+\frac{\beta K_s}{[S]}}$	1	1	1	$1+\frac{K_s}{[S]}$	1	$\frac{1+\frac{K_s}{[S]}}{1+\frac{\beta K_s}{[S]}}$	1

[a] *C*, competitive; *NC*, noncompetitive; *UC*, uncompetitive.

inhibitors is noncompetitive. The noncompetitive inhibitor, having no effect on substrate affinity, does not affect the binding of the other inhibitor. Conversely, the other inhibitor, whatever its mode of inhibition, does not affect the binding of the noncompetitive inhibitor, since the affinity of a noncompetitive inhibitor is independent of the substrate affinity. From the foregoing considerations it follows that independent inhibitor binding, if inferred only from binding studies in the absence of the substrate, does not exclude synergistic or antagonistic inhibitory effects. In certain limiting conditions, however, summation of inhibitions can be observed even when neither of two independently bound inhibitors is noncompetitive. In Case 1 (Table 1), the value of γ at which summation of inhibitions occurs approaches 1 as [*S*] approaches 0. Conversely, it approaches 1 as [*S*] approaches infinity, in Cases 5 and 7. Thus, summation of inhibitions is predicted at very low substrate concentration in case of a fully competitive and a partially competitive inhibitor that bind independently. Conversely, summation of inhibitions is predicted at saturating substrate concentration when two independently bound inhibitors are either both uncompetitive, or one is fully uncompetitive and the other is partially competitive.

GRAPHICAL PROCEDURE FOR DISTINGUISHING MUTUALLY EXCLUSIVE FROM MUTUALLY NONEXCLUSIVE INHIBITORS

The procedure for analyzing the mutual interaction of two inhibitors consists of plotting the reciprocal of the enzyme velocity versus the concentration of one inhibitor at a fixed substrate concentration and at different constant levels of the other inhibitor. The types of plots to be expected when $1/v$ is plotted versus [*I*] at various constant concentrations of *X* are illustrated below for the simple case where *I* is a fully noncompetitive inhibitor and *X* is a partially noncompetitive inhibitor ($\alpha = \beta = 1$; $0 < b < 1$). Considering a simple case will render the interpretation of the equations more straightforward, without affecting the general validity of the conclusions. As outlined above, the effect of *X* on the slope of the Dixon plot for *I* is qualitatively identical for all double-inhibition systems, the only difference being the value of γ, which determines whether *X* has a positive or a negative slope effect. Plots analogous to those shown below therefore would be obtained in other cases. The equation describing a double-inhibition system involving a total noncompetitive inhibitor and a partial noncompetitive inhibitor, mutually exclusive with respect to each other ($\gamma = \infty$), is

$$\frac{1}{v} = \frac{1+\frac{K_s}{[S]}}{K_i V_m\left(1+\frac{b[X]}{K_x}\right)}[I] + \frac{\left(1+\frac{K_s}{[S]}\right)\left(1+\frac{[X]}{K_x}\right)}{V_m\left(1+\frac{b[X]}{K_x}\right)} \quad (3)$$

Since *X* appears only in the denominator of the slope factor, it follows that the slope of the Dixon plot for *I* decreases as [*X*] increases, the limiting slope being 0 at $[X] = \infty$. On the other hand, the intercept increases with increasing [*X*]. Consequently, the family of plots obtained at different fixed concentrations of *X* intersect to the right of the vertical axis at a point corresponding to $(1 + K_s/[S])/bV_m$ on this axis and to $K_i(1 - b)/b$ on the abscissal axis (Fig. 2). It is thus evident that *X* has an additive inhibitory effect at low concentrations of *I*, has no effect on the enzyme velocity at any concentration when $[I] = K_i(1 - b)/b$, whereas it acts as a "deinhibitor" (the total inhibition is less than the inhibition in the absence of *X*) at higher concentrations of *I*. This "deinhibitory" effect is due to the fact that, at the higher

concentrations of I, the decrease in k_p caused by X is more than compensated for by the decrease in the apparent affinity for the full inhibitor.

Besides providing information on the nature of the interaction between the inhibitors, this type of plot may provide an alternative method of determining the inhibition constants for the partial inhibitor. It is known that such constants cannot be determined directly from a Dixon plot, which is nonlinear, but require the use of special types of replots (18, 23). In the case under consideration (Fig. 2), b can be directly obtained from the coordinates of the crossover point, since K_i and $(1 + K_s/[S])/V_m$ are known from the abscissal and ordinal intercepts, respectively, of the plot in the absence of X. Analogously, K_x can be estimated from the abscissal intercepts of the plots in the presence of X. Obviously, such determinations require previous knowledge of the type of inhibition produced by each of the two inhibitors, since a Dixon plot at a single substrate concentration does not provide information on the mode of inhibition. Moreover, it must be known whether the two inhibitors are mutually exclusive or nonexclusive, since a similar plot may be obtained in case of nonexclusivity (see below).

In case of nonexclusivity ($0 < \gamma < \infty$), Eq. 3 becomes

$$\frac{1}{v} = \frac{\left(1 + \frac{K_s}{[S]}\right)\left(1 + \frac{[X]}{\gamma K_x}\right)}{K_i V_m \left(1 + \frac{b[X]}{K_x}\right)}[I] + \frac{\left(1 + \frac{K_s}{[S]}\right)\left(1 + \frac{[X]}{K_x}\right)}{V_m \left(1 + \frac{b[X]}{K_x}\right)} \quad (4)$$

Since the slope of the Dixon plot for I alone is $(1 + K_s/[S])/K_i v_m$, it is evident that the addition of X will increase, leave unaltered, or decrease the slope, depending on whether γ is less than, equal to, or greater than $1/b$. Such plots are shown in Fig. 3. When $\gamma < 1/b$ (increased slope), the family of plots may intersect above, on, or below the horizontal axis. If $\gamma < 1$ (mutually facilitated inhibitor binding; Fig. 3A), the lines intersect above the horizontal axis. If $\gamma = 1$ (independent binding; Fig. 3B), the crossover point lies on the horizontal axis. If γ is between 1 and $1/b$ (mutual hindering; Fig. 3C), the lines cross below the horizontal axis. For this double-inhibition system, the inhibitor interaction factor, γ, as well as the inhibition constants for the partial inhibitor cannot be determined separately from a Dixon plot. It will be shown, however, that a determination is possible from suitable secondary plots. Plots for other special cases of multiple inhibition may differ in that the crossover point for $\gamma = 1$ is either above or below the horizontal axis (Table 1). In addition, if the partial inhibitor is uncompetitive, the vertical intercept of the Dixon plot in the presence of X may be decreased. However, partially uncompetitive inhibition is a purely theoretical possibility which has not yet been reported for any single-substrate enzyme (18).

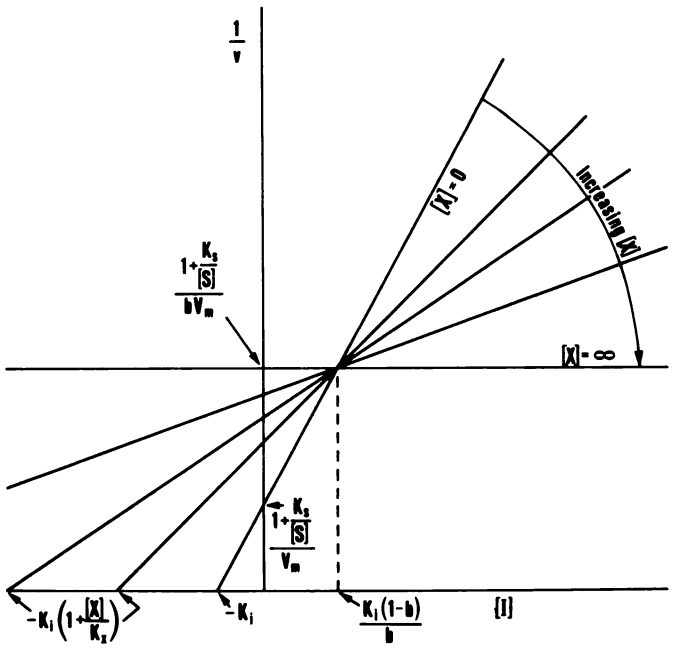


FIG. 2. *Dixon plot for I at different fixed concentrations of X, in case of mutually exclusive inhibitor binding*

I is a fully noncompetitive inhibitor and *X* is a partially noncompetitive inhibitor. In this and the following plots kinetic constants in the absence of X were chosen arbitrarily. $b = 0.4$.

Since only a decrease in slope can be observed in case of mutually exclusive inhibitor binding (Fig. 2), it can be concluded that, when the slope of the Dixon plot for I is increased or unaffected in the presence of X, the two inhibitors are mutually nonexclusive. When, on the contrary, a reduction in slope is observed, I and X may be either mutually exclusive or nonexclusive. Thus, unlike the case of two full inhibitors, this graphical method may or may not be able to discriminate between mutually exclusive and nonexclusive inhibitor binding when one of the inhibitors inhibits partially. However, it can be noted that in case of mutual exclusivity (Fig. 2), the limiting plot at $[X] = \infty$ is a horizontal line, whereas it has a finite positive slope when the two inhibitors are nonexclusive (Fig. 3). By the same token, the absolute value of the abscissal intercept increases indefinitely with increasing $[X]$ if the two inhibitors are mutually exclusive. It reaches instead a finite value at $[X] = \infty$ in case of nonexclusivity. These differential effects on slope and abscissal intercept allow mutual exclusivity to be distinguished from nonexclusivity by the use of suitable secondary plots. Two replots regarding the effect of X on the abscissal intercept are illustrated below. The equations refer to the double-inhibition system considered above (a fully noncompetitive and a partially noncompetitive inhibitor).

Replot of $A_0/\Delta A$ versus $1/[X]$, where A_0 is the absolute value of the abscissal intercept of the Dixon plot for I in the absence of X, and $\Delta A = A - A_0$ is the difference between the absolute values of the abscissal intercept in the presence and the absence of X. In case of nonexclusive inhibitor binding ($0 < \gamma < \infty$), the relevant equation is

$$\frac{A_0}{\Delta A} = \frac{1}{\gamma - 1} + \frac{\gamma K_x}{\gamma - 1}\frac{1}{[X]} \quad (5)$$

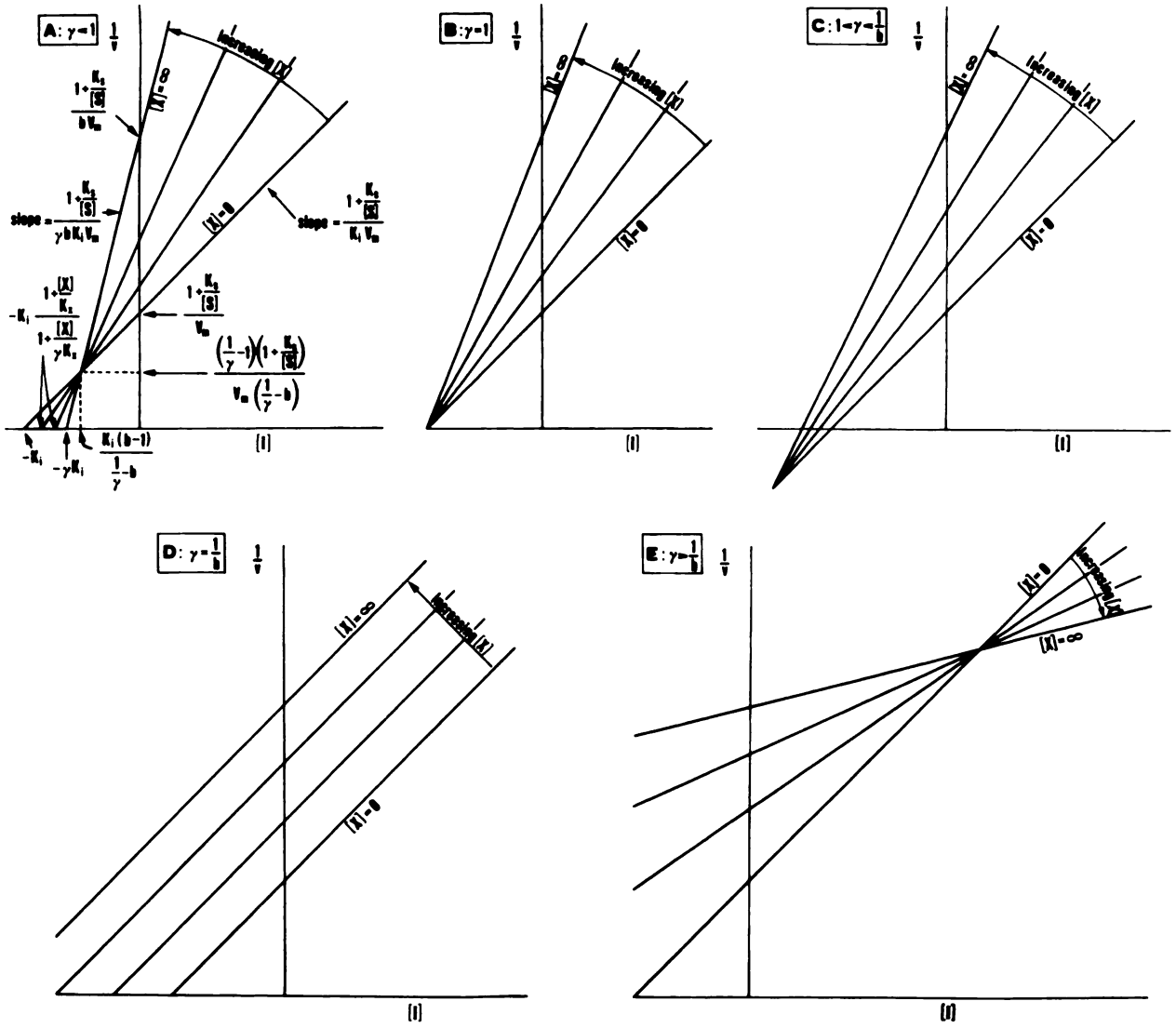


FIG. 3. *Dixon plots for I at various constant levels of X, in case of mutually nonexclusive inhibitor binding*

I is a fully noncompetitive inhibitor and *X* is a partially noncompetitive inhibitor. $b = 0.4$. Values for γ are as follows: A, 0.625; B, 1; C, 1.25; D, 2.5; E, 10. The limiting plots at $[X] = \infty$ have a finite slope which, apart from Case D, depends on γ and b, and a finite vertical intercept which depends on b. The type of information on kinetic parameters that can be obtained from such plots is summarized in A.

This equation represents a straight line which has a finite vertical intercept. Both slope and intercept are positive or negative depending on whether γ is greater or lower than unity (mutually hindered or facilitated inhibitor binding, respectively). Obviously, for $\gamma = 1$ (no effect of *X* on the abscissal intercept of the Dixon plot; see Table 1, Case 2) Eq. 5 makes no sense (both slope and intercept would be infinite). When $\gamma = \infty$ (mutually exclusive inhibitor binding), Eq. 5 reduces to

$$\frac{A_0}{\Delta A} = \frac{K_x}{[X]} \tag{6}$$

It is evident that in case of mutual exclusivity a replot of $A_0/\Delta A$ versus $1/[X]$ will be a straight line which goes through the origin instead of having a finite vertical intercept. Thus, when *X* causes a decrease in the slope of the Dixon plot for *I*, nonexclusivity can be distinguished from exclusivity by replotting $A_0/\Delta A$ versus $1/[X]$. A line with a finite vertical intercept is obtained in the former case, whereas the replot goes through the origin in the latter case. Figure 4 shows how the replot varies with varying γ. This type of replot may also be used when nonexclusivity has been already determined from the primary plot, since it can be of help in determining the kinetic constants involved. In this instance, the values of γ and K_x can be obtained from the vertical and horizontal intercepts, respectively.

Replot of A versus [X], where *A* is the absolute value of the abscissal intercept. In case of nonexclusive inhibitor binding ($0 < \gamma < \infty$), the equation is

$$A = K_i \frac{1 + \dfrac{[X]}{K_x}}{1 + \dfrac{[X]}{\gamma K_x}} \tag{7}$$

Thus, in case of nonexclusivity, this plot will be a monotonically increasing or decreasing hyperbola depending

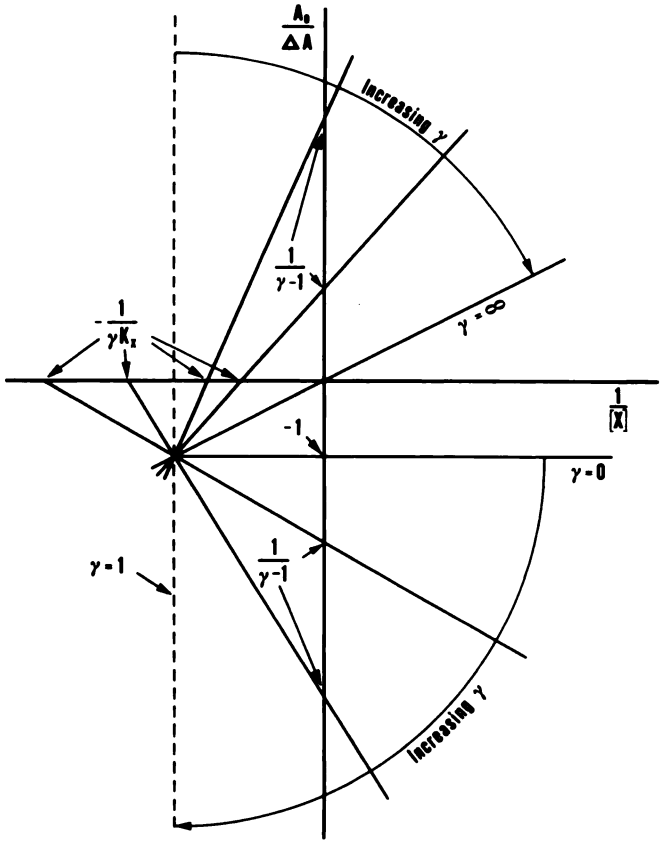


FIG. 4. *Replot of $A_0/\Delta A$ versus $1/[X]$*

For $\gamma = 1$ (independent inhibitor binding), the slope of the plot is infinite. For values of γ lower than unity, the slope is negative, whereas for greater values of γ, the slope is positive. The replot goes through the origin only when $\gamma = \infty$. Identical replots would be obtained in other special cases of multiple inhibition, the only difference being the value of γ, which determines whether the slope of the replot is positive or negative. This is the value of γ at which the abscissal intercept of the primary plot is independent of $[X]$ (see Table 1).

on whether γ is greater or lower than 1. For $\gamma = 1$ (independent inhibitor binding), a horizontal line will be obtained. When $\gamma = \infty$ (mutually exclusive inhibitor binding), Eq. 7 reduces to

$$A = K_i + \frac{K_i}{K_x}[X] \qquad (8)$$

which is the equation of a straight line with positive slope. A linear replot with positive slope is therefore indicative of mutually exclusive inhibitor binding, whereas a hyperbolic replot or a horizontal line indicates nonexclusive binding (Fig. 5). Equations 5–8 for the general mechanism (Scheme 1) as well as analogous equations regarding the effect of X on the slope of the Dixon plot for I are given in Appendix II.

Although derived for single-substrate enzymes, the above rules for distinguishing mutual exclusivity from nonexclusivity hold also for multireactant systems. Equations describing the multiple inhibition of multisubstrate enzyme reactions, although apparently more complex, have in fact the same algebraic form as those for single-substrate reactions. This similarity can be readily appreciated from Appendix III, where various examples are given of equations describing the combined inhibition by a full and a partial inhibitor of rapid equilibrium random and ordered multireactant systems.

In certain conditions, partial inhibition also can be observed with alternate substrates (24) or products (25). It might be shown that, if the present analysis is applied to multiple-inhibition systems involving such partial inhibitors, equations and plots analogous to those described above are obtained. Thus, if equilibrium conditions prevail, any Michaelian system involving a full inhibitor and a partial inhibitor may be represented by a Dixon equation of the following general form:

$$\frac{1}{v} = \frac{l + \dfrac{m[X]}{\gamma}}{1 + n[X]}[I] + \frac{p + q[X]}{1 + n[X]} \qquad (9)$$

where l, m, n, p, and q are constants, at constant substrate concentration, and γ is the inhibitor interaction factor defined above. The slope of this equation increases or decreases with increasing $[X]$, depending on whether γ is less than or greater than m/ln. For $\gamma = m/ln$ the slope is independent of $[X]$. In case of exclusive binding ($\gamma = \infty$), the term containing $[X]$ in the numerator of the slope expression reduces to zero and, consequently, the slope decreases as $[X]$ increases. Equations for $A_0/\Delta A$ and A

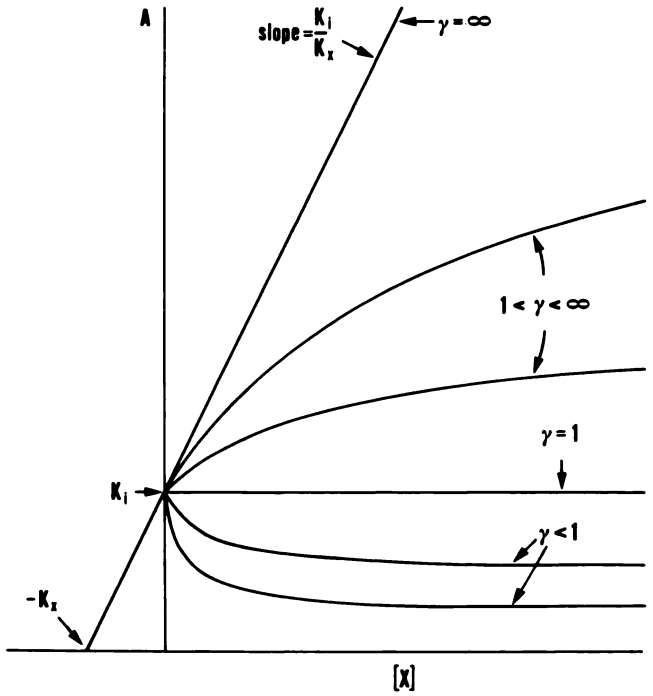


FIG. 5. *Replot of A versus $[X]$*

In case of mutually exclusive inhibitor binding ($\gamma = \infty$), the replot is a straight line with positive slope. In case of nonexclusive inhibitor binding ($\gamma < \infty$), the plot is hyperbolic, or a horizontal line when $\gamma = 1$. The γ values for hyperbolic curves are, from top to bottom: 4; 2; 0.5; 0.25. The same types of replots would be obtained in other special cases of multiple inhibition, the only difference being the value of γ at which the replot is a horizontal line (the value at which the abscissal intercept of the primary plot is independent of $[X]$; see Table 1).

have the following form:

$$A_0/\Delta A = \frac{1}{\gamma\frac{lq}{mp} - 1} + \frac{\gamma\frac{l}{m}}{\gamma\frac{lq}{mp} - 1}\frac{1}{[X]} \quad (10)$$

(for nonexclusive binding)

$$A_0/\Delta A = \frac{p}{q}\frac{1}{[X]} \quad \text{(for exclusive binding)} \quad (11)$$

$$A = \frac{p + q[X]}{l + \frac{m}{\gamma}[X]} \quad \text{(for nonexclusive binding)} \quad (12)$$

$$A = \frac{p}{l} + \frac{q}{l}[X] \quad \text{(for exclusive binding)} \quad (13)$$

Equations for $A_0/\Delta A$ and A can be obtained readily for any special case of multiple inhibition of either single- or multisubstrate enzymes by writing the Dixon equation for the system in question in the form of Eq. 9 and then inserting the appropriate expression for $l \ldots q$ in the above equations. Equations 10–13 are identical in form with Eqs. 5–8. This proves the general validity of the tests proposed for discriminating between exclusive and nonexclusive inhibitors.

An application to a real system of the replotting techniques illustrated above is shown in Fig. 6. The data refer to the multiple inhibition of (Na^+-K^+)-ATPase by the full inhibitor ouabain and the partial inhibitor canrenone

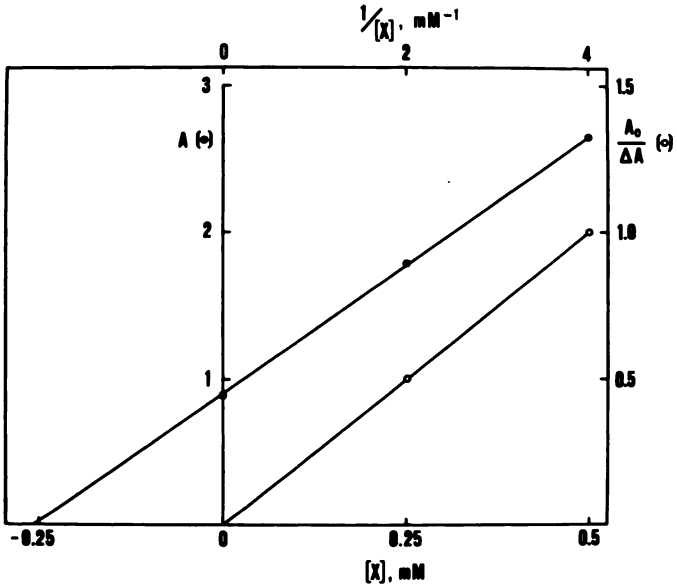


FIG. 6. *Replots of A versus [X] and $A_0/\Delta A$ versus $1/[X]$ for the multiple inhibition of (Na^+-K^+)ATPase by ouabain and canrenone*

The data are taken from the Dixon plot shown in Figure 5 of ref. 19. *X* is the partial inhibitor canrenone. The line for the *A* versus [*X*] plot was obtained by linear regression analysis (r = 0.9998). Since both inhibitors were shown to be noncompetitive with respect to the substrate, the abscissal intercept of this plot gives the dissociation constant for the partial inhibitor (see Fig. 5).

(19). In that study, Dixon plots for ouabain were obtained in the absence and presence of two different concentrations of canrenone. The slope of the Dixon plot for ouabain was found to decrease as the concentration of canrenone was increased, leaving unresolved the question of whether the two inhibitors were mutually exclusive or nonexclusive. Figure 6 shows that a replot of A (the abscissal intercept of the Dixon plot for ouabain) versus $[X]$ (the concentration of canrenone) is a straight line with positive slope, and a replot of $A_0/\Delta A$ versus $1/[X]$ goes through the origin. Both replots thus indicate that ouabain and canrenone are mutually exclusive inhibitors, in accordance with the conclusion previously reached with the aid of binding experiments and further inhibition studies (19).

CONCLUSIONS

In the present study, the Dixon equation has been used to analyze the combined inhibition by a full and a partial inhibitor of single- and multisubstrate enzyme systems obeying Michaelis-Menten kinetics. This analysis has shown that a Dixon plot versus the concentration of the full inhibitor, at constant substrate concentration, is linear in either the absence or the presence of a fixed concentration of the partial inhibitor.

It should be noted that such predictions of linearity are based on the rapid equilibrium assumption. Steady-state treatment of kinetic systems involving a partial inhibitor would give rise to very complex equations containing higher-degree terms both in substrate and inhibitor concentrations (18, 25). Therefore, if an enzymatic reaction obeys steady-state kinetics, it is theoretically possible that a Lineweaver-Burk or a Dixon plot is found to be linear in the absence, but curved in the presence, of a partial inhibitor. However, in the cases reported (19, 26), such predictions of nonlinearity have not been verified. It may be noted that steady-state analysis predicts nonlinear plots also for classical noncompetitive inhibition of single-substrate enzymes (18). As a matter of fact, linear noncompetitive inhibition is currently recorded. This indicates that either in most instances equilibrium conditions are indeed approached or the curvature is too small to be detected; either way, the equilibrium assumption is a reasonable approximation.

The proposed procedure for discriminating between exclusive and nonexclusive inhibitors involves plotting the reciprocal of the initial velocity versus the concentration of the full inhibitor in the absence and in the presence of a constant concentration of the partial inhibitor. When the slope of the Dixon plot for the full inhibitor is increased or unaltered in the presence of the partial inhibitor, the two inhibitors are mutually nonexclusive. When a decrease in slope is observed, the two inhibitors may be either mutually exclusive or nonexclusive. In case of decreased slope, a Dixon plot in the presence of various fixed levels of the partial inhibitor must be obtained first. Mutual exclusivity can then be distinguished from nonexclusivity by the use of each of the following secondary plots:

1. Replot of $A_0/\Delta A$ versus the reciprocal of the concentration of the partial inhibitor, where A_0 is the abso-

lute value of the abscissal intercept of the Dixon plot in the absence of the partial inhibitor, and ΔA is the difference between the absolute values of the abscissal intercept in the presence and absence of the partial inhibitor.

2. Replot of slope/Δslope versus the reciprocal of the concentration of the partial inhibitor, where Δslope is the difference between the slope in the absence and the presence of the partial inhibitor.

The two replots have a finite vertical intercept in case of nonexclusive inhibitor binding, whereas they go through the origin when the two inhibitors are mutually exclusive.

3. Replot of A versus the concentration of the partial inhibitor, where A is the absolute value of the abscissal intercept of the Dixon plot.

4. Replot of slope_0/slope versus the concentration of the partial inhibitor, where slope_0 is the slope in the absence of the partial inhibitor.

In case of nonexclusivity, Replots 3 and 4 are generally hyperbolic, but, under particular conditions, a line parallel to the abscissal axis may be obtained. In case of mutual exclusivity, linear replots with positive slope will be obtained.

Previous approaches to the study of the interaction between full and partial inhibitors had to be based mainly on binding experiments (19, 20). Alternatively, the kinetic analysis had to be restricted to low concentrations of the partial inhibitor, where the inhibition was approximately linear and the system could be studied as though composed of two full inhibitors (27). Such a method has, however, a very narrow range of applicability, since it constitutes a reasonable approximation only when the partial inhibitor is almost linear; i.e., it produces nearly complete inhibition. The method presented in this paper permits a rigorous analysis of the interaction between full and partial inhibitors and enables a clear distinction to be made between exclusive and nonexclusive inhibitors on the basis of kinetic evidence alone. It permits, therefore, an accurate analysis of such double-inhibition systems also when neither inhibitor is available in labeled form. This method has a wide range of applications, since (a) the rules proposed for discriminating between mutually exclusive and nonexclusive inhibitors are general, as they are valid for either single- or multisubstrate enzyme reactions regardless of the mechanism (random or ordered) of the reaction. They also hold irrespective of the kinetic nature of the inhibitors (competitive, noncompetitive, uncompetitive, mixed), so that a knowledge of the type of inhibition is not required. (b) Linear primary and secondary plots can be obtained in case of either mutually exclusive or nonexclusive inhibitor binding, which makes easier the determination of the kinetic constants involved. (c) It provides a criterion for the summation of inhibitory effects. (d) It offers a simple test of whether a partial inhibitor is potentially useful *in vivo* to counteract the effects of a full inhibitor acting on the same enzyme. The effect of a full inhibitor is reduced in the presence of a partial inhibitor only when the partial inhibitor causes a reduction in the slope of the Dixon plot for the full inhibitor (Figs. 2 and 3E) and the concentration of this inhibitor exceeds a certain critical concentration (the concentration given by the abscissa of the crossover point). Thus, when a reduction in slope is observed, the partial inhibitor may be tested for possible antagonistic effects *in vivo*.

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APPENDIX I

Condition for summation of inhibitions as deduced from a Dixon plot

Summation of inhibitory effects has been defined by Webb (4) as the condition for which the following relationship holds:

$$i_{1,2} = i_1 + i_2 - i_1 i_2 \qquad \text{(A-1)}$$

where i_1 and i_2 are the fractional inhibitions in the presence of inhibitors I_1 and I_2, respectively, and $i_{1,2}$ is the fractional inhibition in the presence of both inhibitors at the same concentrations as those giving i_1 and i_2. This may be expressed alternatively as follows (13):

$$v_{1,2} = v_1 v_2 / v_0 \qquad \text{(A-2)}$$

where $v_{1,2}$ is the enzyme velocity in the simultaneous presence of the two inhibitors, v_1 and v_2 are the velocities in the presence of each inhibitor separately, and v_0 is the velocity of the uninhibited reaction. These equations represent the mathematical equivalent to the statement that the degree of inhibition produced by each inhibitor is independent of the presence of the other inhibitor. If, for example, each inhibitor separately reduces the enzyme velocity by 50%, a 75% inhibition will be observed in the simultaneous presence of the two inhibitors. A total inhibition of less than 75% is called antagonism, whereas a greater effect is called synergism. The condition expressed by Eqs. A-1 and A-2 can be also deduced from a Dixon plot. The slopes and the ordinal intercepts of two straight lines that intersect on the abscissal axis differ by the same factor (see any textbook of analytic geometry). It follows that the ratio between the ordinal coordinates of the points of the two lines that have the same abscissal coordinate is constant. Thus, when in a Dixon plot at constant substrate concentration (Fig. A-1), the lines in the absence and the presence of a fixed level of a second inhibitor cross on the base-line, the following equality holds:

$$\frac{1/v_2}{1/v_0} = \frac{1/v_{1,2}}{1/v_1} \qquad \text{(A-3)}$$

whence

$$v_{1,2} = \frac{v_1 v_2}{v_0} \qquad \text{(A-4)}$$

Equation A-4 is identical with Eq. A-2, which expresses the condition for summation of inhibitions.

APPENDIX II

Equations for secondary plots when both inhibitors are mixed (mechanism shown in Scheme 1)

Symbols are defined in the text.

Replot of $A_0/\Delta A$ *versus* $1/[X]$. Mutually nonexclusive

inhibitor binding ($0 < \gamma < \infty$):

$$\frac{A_0}{\Delta A} = \frac{1}{\dfrac{\left(1+\dfrac{\alpha K_s}{[S]}\right)\left(1+\dfrac{\beta K_s}{[S]}\right)}{\gamma\left(1+\dfrac{K_s}{[S]}\right)\left(1+\dfrac{\alpha\beta K_s}{[S]}\right)} - 1} + \frac{\beta\gamma K_x}{1+\dfrac{\beta K_s}{[S]}} \frac{1}{1+\dfrac{\alpha\beta K_s}{[S]}} \frac{1}{[X]} \qquad \text{(A-5)}$$

$$\gamma\frac{1}{1+\dfrac{K_s}{[S]}} - \frac{1}{1+\dfrac{\alpha K_s}{[S]}}$$

Mutually exclusive inhibitor binding ($\gamma = \infty$):

$$\frac{A_0}{\Delta A} = \frac{\beta K_x\left(1+\dfrac{K_s}{[S]}\right)}{1+\dfrac{\beta K_s}{[S]}} \frac{1}{[X]} \qquad \text{(A-6)}$$

Replot of A versus $[X]$. Mutually nonexclusive inhibitor binding ($0 < \gamma < \infty$):

$$A = \alpha K_i \frac{1+\dfrac{K_s}{[S]} + \left(1+\dfrac{\beta K_s}{[S]}\right)\dfrac{[X]}{\beta K_x}}{1+\dfrac{\alpha K_s}{[S]} + \left(1+\dfrac{\alpha\beta K_s}{[S]}\right)\dfrac{[X]}{\beta\gamma K_x}} \qquad \text{(A-7)}$$

Mutually exclusive inhibitor binding ($\gamma = \infty$):

$$A = \alpha K_i \frac{1+\dfrac{K_s}{[S]}}{1+\dfrac{\alpha K_s}{[S]}} + \frac{\alpha K_i\left(1+\dfrac{\beta K_s}{[S]}\right)}{\beta K_x\left(1+\dfrac{\alpha K_s}{[S]}\right)}[X] \qquad \text{(A-8)}$$

Replot of slope/Δslope versus $1/[X]$, where Δslope is defined as the difference between the slope of the Dixon plot for I in the absence and the presence of X. Mutually nonexclusive inhibitor binding ($0 < \gamma < \infty$):

$$\frac{\text{Slope}}{\Delta\text{Slope}} = \frac{1}{\gamma b\dfrac{1+\dfrac{\alpha K_s}{[S]}}{1+\dfrac{\alpha\beta K_s}{[S]}} - 1} + \frac{\beta\gamma K_x}{\gamma b - \dfrac{1+\dfrac{\alpha\beta K_s}{[S]}}{1+\dfrac{\alpha K_s}{[S]}}} \frac{1}{[X]} \qquad \text{(A-9)}$$

Mutually exclusive inhibitor binding ($\gamma = \infty$):

$$\frac{\text{Slope}}{\Delta\text{Slope}} = \frac{\beta K_x}{b}\frac{1}{[X]} \qquad \text{(A-10)}$$

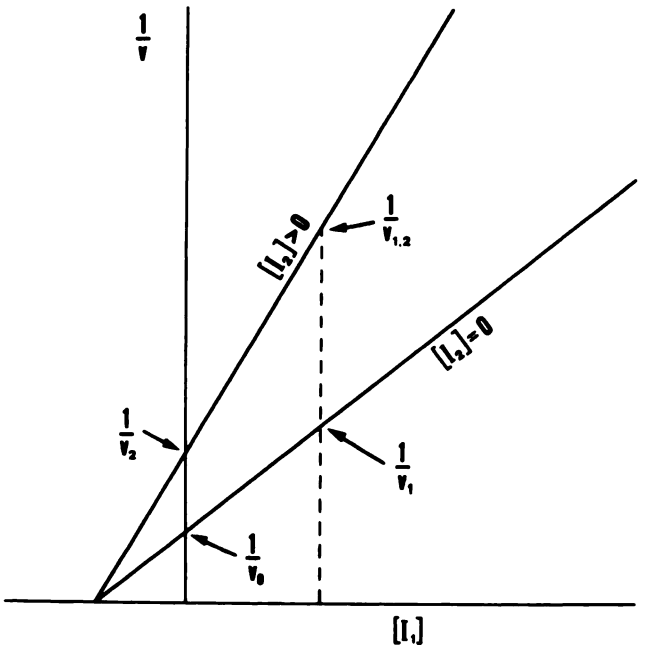


FIG. A-1. *Dixon plot for a full inhibitor, I_1, in the absence and in the presence of a constant concentration of a second (full or partial) inhibitor, I_2, illustrating the condition for summation of inhibitions according to the definition of Webb (4)*

Equations A-9 and A-10 have the same form as Eqs. A-5 and A-6. Replots equivalent to those shown in Fig. 4 are therefore obtained when slope/Δslope is plotted versus $1/[X]$.

Replot of slope₀/slope versus $[X]$, where slope₀ is the slope in the absence of X. Mutually nonexclusive inhibitor binding ($0 < \gamma < \infty$):

$$\frac{\text{Slope}_0}{\text{Slope}} = \frac{1+\dfrac{b[X]}{\beta K_x}}{1+\dfrac{1+\dfrac{\alpha\beta K_s}{[S]}}{1+\dfrac{\alpha K_s}{[S]}}\dfrac{[X]}{\beta\gamma K_x}} \qquad \text{(A-11)}$$

Mutually exclusive inhibitor binding ($\gamma = \infty$):

$$\frac{\text{Slope}_0}{\text{Slope}} = 1 + \frac{b}{\beta K_x}[X] \qquad \text{(A-12)}$$

Equations A-11 and A-12 have the same form as Eqs. A-7 and A-8. Replots similar to those shown in Fig. 5 are therefore obtained when slope₀/slope is plotted versus $[X]$.

APPENDIX III

Some cases of multiple inhibition by a full inhibitor and a partial inhibitor of rapid equilibrium multireactant systems

The notation and nomenclature of Segel (ref. 9, Chap. 6) are used.

Random Bireactant Mechanism

Case 1. I is a full inhibitor, competitive with respect to substrate A and mixed-type with respect to substrate

B. *X* is a partial, mixed-type inhibitor with respect to both substrates, *A* and *B* (Scheme A-1). The relevant Dixon equation is:

$$\frac{1}{v} = \frac{\alpha K_A\left(1 + \frac{K_B}{[B]} + \frac{[X]}{\beta\gamma K_x} + \frac{K_B[X]}{\gamma K_x[B]}\right)}{[A]K_iV_m\left(1 + \frac{b[X]}{\beta K_x}\right)}[I]$$

$$+ \frac{1 + \frac{\alpha K_A}{[A]} + \frac{\alpha K_B}{[B]} + \frac{\alpha K_AK_B}{[A][B]} + \frac{[X]}{\beta K_x}\left(1 + \frac{\alpha K_A}{[A]} + \frac{\alpha\beta K_B}{[B]} + \frac{\alpha\beta K_AK_B}{[A][B]}\right)}{V_m\left(1 + \frac{b[X]}{\beta K_x}\right)} \quad \text{(A-13)}$$

The effect of *X* is to increase, leave unaltered, or decrease the slope of the Dixon plot for *I* depending on whether γ is less than, equal to, or greater than $(1 + \beta K_B/[B])/b(1 + K_B/[B])$. For $\gamma < \infty$ (mutually nonexclusive inhibitor binding), the slope approaches a finite positive value as $[X]$ approaches infinity. For $\gamma = \infty$ (mutually exclusive inhibitor binding), the terms containing $[X]$ in the numerator of the slope factor reduce to zero, and the slope approaches zero as $[X]$ approaches infinity. Consequently, for $[X] = \infty$, the absolute value of the abscissal intercept is finite or infinite depending on whether γ is less than or equal to infinity. Exclusivity and nonexclusivity can therefore be distinguished by means of the same rules obtained for single-substrate reactions. It can be easily verified that the same holds for the systems illustrated below.

Case 2. *I* is fully competitive with respect to *A* and noncompetitive with respect to *B*. *X* is partially noncompetitive with respect to either substrate (Scheme A-1, $\alpha = \beta = 1$). The equation is:

$$\frac{1}{v} = \frac{K_A\left(1 + \frac{K_B}{[B]}\right)\left(1 + \frac{[X]}{\gamma K_x}\right)}{[A]K_iV_m\left(1 + \frac{b[X]}{K_x}\right)}[I]$$

$$+ \frac{\left(1 + \frac{[X]}{K_x}\right)\left(1 + \frac{K_A}{[A]} + \frac{K_B}{[B]} + \frac{K_AK_B}{[A][B]}\right)}{V_m\left(1 + \frac{b[X]}{K_x}\right)} \quad \text{(A-14)}$$

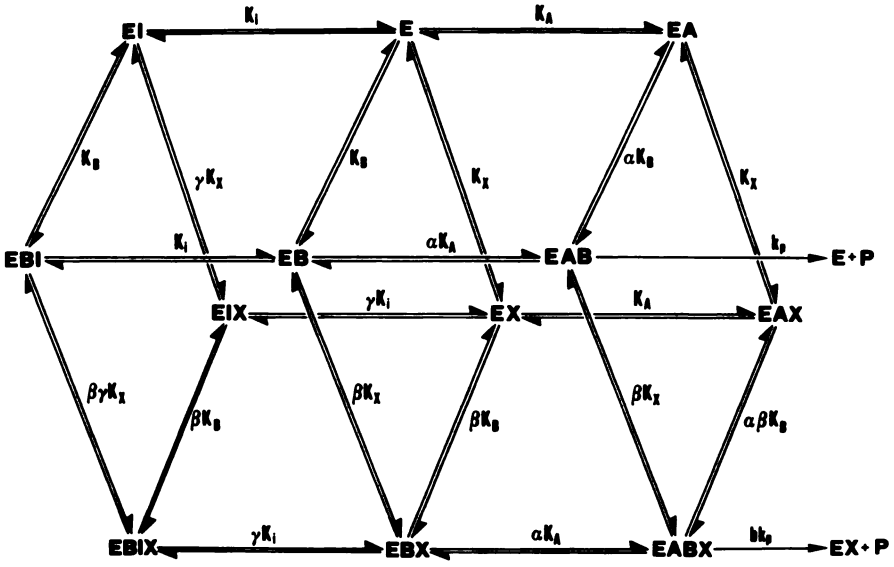
In the presence of *X*, the slope is increased, unaffected, or decreased depending on whether γ is less than, equal to, or greater than $1/b$.

Ordered Bireactant Mechanism

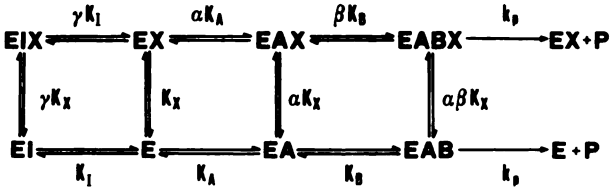
Case 3. *I* and *X* bind to *E*. *I* is a full inhibitor competitive with respect to *A* and *B*. *X* is a partial inhibitor, mixed-type with respect to *A* and competitive with respect to *B* (Scheme A-2). The equation is:

$$\frac{1}{v} = \frac{K_AK_B\left(1 + \frac{[X]}{\gamma K_x}\right)}{[A][B]K_iV_m\left(1 + \frac{[X]}{\alpha\beta K_x}\right)}[I]$$

$$+ \frac{1 + \frac{K_B}{[B]} + \frac{K_AK_B}{[A][B]} + \frac{[X]}{\alpha\beta K_x}\left(1 + \frac{\beta K_B}{[B]} + \frac{\alpha\beta K_AK_B}{[A][B]}\right)}{V_m\left(1 + \frac{[X]}{\alpha\beta K_x}\right)} \quad \text{(A-15)}$$



SCHEME A-1



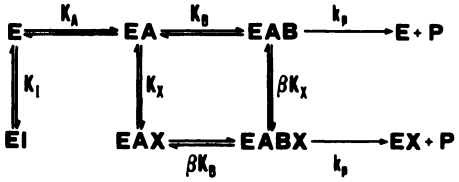
SCHEME A-2

In the presence of X, the slope is increased, unaffected, or decreased depending on whether γ is less than, equal to, or greater than $\alpha\beta$.

Case 4. I binds to E and X binds to EA. I is fully competitive with respect to A and B. X is partially competitive with respect to B and partially uncompetitive with respect to A (Scheme A-3). The Dixon equation is:

$$\frac{1}{v} = \frac{K_A K_B}{[A][B]K_i V_m\left(1+\frac{[X]}{\beta K_x}\right)}[I] + \frac{1+\frac{K_B}{[B]}+\frac{K_A K_B}{[A][B]}+\frac{[X]}{\beta K_x}+\frac{K_B[X]}{K_x[B]}}{V_m\left(1+\frac{[X]}{\beta K_x}\right)} \quad \text{(A-16)}$$

In this case, the addition of X will cause a decrease in slope, in accordance with the fact that the two inhibitors are mutually exclusive.



SCHEME A-3

Ordered Terreactant Mechanism

Case 5. I and X bind to E. I is a full inhibitor competitive with respect to A, B, and C. X is a partial inhibitor, mixed-type with respect to A and B, and competitive with respect to C. The Dixon equation is:

$$\frac{1}{v} = \frac{K_A K_B K_C\left(1+\frac{[X]}{\gamma K_x}\right)}{[A][B][C]K_i V_m\left(1+\frac{[X]}{\alpha\beta\delta K_x}\right)}[I] + \frac{1+\frac{K_C}{[C]}+\frac{K_B K_C}{[B][C]}+\frac{K_A K_B K_C}{[A][B][C]}+\frac{[X]}{\alpha\beta\delta K_x}+\frac{\left(1+\frac{\delta K_C}{[C]}+\frac{\beta\delta K_B K_C}{[B][C]}+\frac{\alpha\beta\delta K_A K_B K_C}{[A][B][C]}\right)}{}}{V_m\left(1+\frac{[X]}{\alpha\beta\delta K_x}\right)} \quad \text{(A-17)}$$

where δ represents the change in the affinity for substrate C, induced by X. For the other symbols see Scheme A-2. A comparison with Eq. A-15 shows that, for a given mode of inhibition, increasing the number of substrates has only the effect of increasing the number of terms in the numerator of the intercept expression, but the form of the equation remains unchanged.

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