7.32 (7.33).

4-Acetamido-cyclohexanol (**15**). The mixture of 0.78 g (0.024 mole) of $LiAlH_4$ and 0.404g (0.004mole) of 3-oximino-2-butanone in 50 m*l* of anhydrous ether (THF or diglyme) was refluxed for 3 hours. The rest of the procedures is the same as **18**. mp 106–106.5 °C. Ir: 3400 (ν_{O-H}), 2310–1850 ($\nu_{NH_3^+}$), 1647 ($\nu_{C=O}$). nmr; 5.89 (*d*, 1H), 6.95 (*m*, 1H), 7.70 (*s*, 3H), 7.92–8.53 (*m*, 1 OH).

Anal. Obs. (Calc. %) C, 59.60 (59.72),: H, 9.38(9.30); N, 8.49 (8.58).

2-Acetamido-1,2-diphenylethanol (**16**). The procedures is the same as **15**. mp 191–191.5 °C. Ir(KBr); 3300 (ν_{O-H}), 1650 ($\nu_{C=O}$), 1550 (ν_{N-H}). nmr: 2.29 (*m*, 1H), 2.70–2082 (*m*, 10H), 5.78–7.27 (*m*, 2H), 7.70 (*s*, 3H).

Anal. Obs (Calc. %) C, 74.10 (73.97); H, 6.61 (6.47); N, 5.40 (5.28).

3-Acetamide-2-Butanol (**17**). The procedure is the same as **15**. mp 77–78 °C. In(KBr); 3040 (ν_{O-H}), 2020–1960 ($\nu_{NH_3^+}$) 1650 ($\nu_{C=O}$) nmr; 2.67 (*m*, 1H), 6.10–6.40 (*m*, 1H), 7.03–7.47 (*m*, 8H), 7.75 (*s*, 3H).

Anal. Obs (Calc. %) C, 53.31 (53.21); H, 9.69 (9.74); N, 10.36 (10.09)

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Enzyme Kinetics of Multiple Inhibition in the Presence of Two Reversible Inhibitors

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In order to extend our understanding on the multiple inhibition enzyme kinetics, a general equation of an enzyme reaction in the presence of two different reversible inhibitors was derived by what we call "match-box mechanism" under the combined assumption of steady -state and quasi-equilibrium for inhibitor binding. Graphical methods were proposed to analyze the multiple inhibition of an enzyme by any given sets of different inhibitors, *i.e.*, competitive, noncompetitive, and uncompetitive inhibitors. This method not only gives an interaction factor (α) between two inhibitors, but also discerns α_1 and α_2 with and without substrate binding, respectively. The factors involved in the dissociation constants of inhibitors can also be evaluated by the present plot. It is also shown that the present kinetic approach can be extended to other forms of activators or hydrogen ions with some modification.

Introduction

Studies of the combined effect of two different inhibitors on enzyme systems are useful for understanding mechanisms of inhibition as well as active sites of an enzyme. This also provides useful information on the relationship of two inhibitors that interact with an enzyme. Triple relationship among inhibitor, hydrogen ion, and substrate interactions with an enzyme can also be analyzed by studying the kinetics of multiple inhibition.

Kinetic studies dealing with the combined effect of two inhibitors have been developed in various ways to analyze

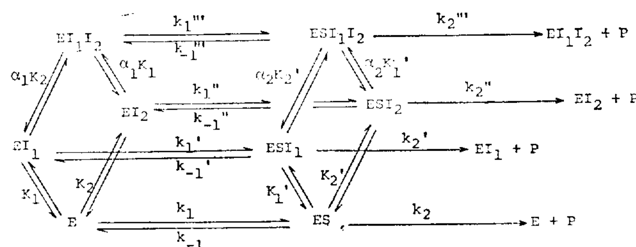
their own results by Yagi and Ozawa (1960), and Loewe (1957). Webb (1963) reviewed the multiple inhibition in general terms and formulated kinetic equations for enzyme inhibition by multiple inhibitors, while Yonetani and Theorell (1964) reported derivation and graphical analysis of steady-state kinetics involving two competitive inhibitors. This type of analysis of multiple inhibition have been applied to yeast alcohol dehydrogenase (Anderson & Reynolds, 1965a; 1965b; 1966a; 1966b; Fonda & Anderson, 1967; Heitz & Anderson, 1968). Special cases of multiple inhibition have been also reported. The cooperative (synergistic) pure competitive inhibition by two different nonexclusive inhibitors was reported for the case of glutamine: PRPP amido transferase (Caskey *et al.*, 1964; Nierlich & Magasanik, 1965), whereas the cooperative noncompetitive inhibition by two different nonexclusive inhibitors was reported for β-aspartylkinase of *Pseudomonas fluorescens* (Dungan & Datta, 1973). The partial noncompetitive inhibition by two inhibitors was also shown for glutamine synthetase of *Escherichia coli* (Stadman *et al.*, 1968). The fully competitive and noncompetitive inhibitions were reported for rabbit intestine sucrase (Semenza & Balthazar, 1974), and a simple kinetic test was given by them to ascertain the existence of a mutual competition between enzyme inhibitors. The use of multiple inhibition was extended to confirm the mechanism of pig heart triphosphopyridine nucleotide isocitrate dehydrogenase (Northrop & Cleland, 1974). Analytical methods were also reported for several cases of multiple inhibition (Segal, 1975).

It is felt, however, that a general approach is necessary to extend our understanding on the multiple effect of any two inhibitors instead of confining our interest to limited cases.

The present communication describes general rate equation for an enzyme reaction in the presence of two different inhibitors under the combined assumption of steady state and *quasi*-equilibrium for the inhibitor binding. Graphical analyses for the triple relationship between substrate and two inhibitors were discussed and compared with those previously known.

Rationale

Since the steady-state approximation for a general rate equation of an enzyme reaction in the presence of two inhibitors appears too complicate to use in practice, it is assumed that the binding of inhibitors to an enzyme is in the state of *quasi*-equilibrium. This assumption for the inhibitor binding is reasonable for most of enzymes since bimolecular rate constant for the binding of a small molecule to an enzyme moiety reaches more than 10^6–10^8 M^{-1} sec^{-1} (Eigen, 1963). The transfer of a proton from a strong acid to the basic form of a weak acid is in general a diffusion controlled reaction with a bimolecular rate constant of the order of 10^{10} M^{-1} sec^{-1} in water at 25 °C (Eigen, 1960). The bimolecular rate constants for the binding of a small molecule or proton are so large that the assumption of *guasi*-equilibrium can be justified. The scheme of one intermediate



Scheme 1.

reaction in the presence of two reversible inhibitors is shown in Scheme 1. We should like to call it, a "Match-box mechanism".

In formulation of a general equation for the enzyme reaction in the presence of any two reversible inhibitors, interaction factors between the two inhibitors are assigned as α_1 and α_2 for the EI and ESI complexes, respectively. The factors for the equilibrium of other inhibitors with the free enzyme and with the ES complex are denoted as a and b, respectively.

K_1, K_2, α_1K_1 and α_1K_2 are the dissociation equilibrium constants for the respective enzyme–inhibitor complexes, and K_1', K_2', α_2K_1' and α_2K_2' are those for the respective enzyme - substrate - inhibitor complexes. Combining the above dissociation equilibrium constants, the Michaelis–Menten constants for E, EI_1, EI_2, and EI_1I_2 can be represented as eq. (1);

$$K_m=\frac{K_m'}{a}=\frac{K_m''}{b}=\frac{\alpha_1}{ab\alpha_2}K_m''' \tag{1}$$

where

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

The overall reaction velocity (ν) of an enzyme reaction sholn in Scheme 1 can be derived as eq. (2) or eq. (3) by applying the steady-state approximation, a conservation equation for the total enzyme, and a reaction velocity expression for the mode of a partial inhibitioon.

$$\nu=\frac{[E_t][s]}{\left(\frac{k_{-1}+k_2}{k_1k_2}\right)\left(\frac{\Phi_1}{\Phi_3}\right)+\frac{[s]}{k_2}\left(\frac{\Phi_2}{\Phi_3}\right)} \tag{2}$$

or

$$\nu=\frac{V_m[s]}{K_m\left(\frac{\Phi_1}{\Phi_3}\right)+[s]\left(\frac{\Phi_2}{\Phi_3}\right)} \tag{3}$$

where

$$V_m=k_2[E_t] \tag{4}$$

$$\Phi_1=1+\frac{[I_1]}{K_1}+\frac{[I_2]}{K_2}+\frac{[I_1][I_2]}{\alpha_1K_1K_2} \tag{5}$$

$$\Phi_2=1+\frac{[I_1]}{aK_1}+\frac{[I_2]}{bK_2}+\frac{[I_1][I_2]}{ab\alpha_2K_1K_2} \tag{6}$$

$$\Phi_3=1+\gamma\frac{[I_1]}{aK_1}+\gamma'\frac{[I_2]}{bK_2}+\gamma''\frac{[I_1][I_2]}{ab\alpha_2K_1K_2} \tag{7}$$

TABLE 1: The Values of Slope and Ordinate in the Secondary Plot of $\frac{V_m}{v}$ vs. $[I_1]$ or $[I_2]$

Inhibition mode		Cases	Slope		Ordinate	
			$[I_1]$	$[I_2]$	$[I_1]$	$[I_2]$
I_1 I_2	NC NC	a=b=1	$\frac{K_m}{[S]}(\frac{1}{K_1}+\frac{[I_2]}{\alpha_1K_1K_2})+(\frac{1}{K_1'}+\frac{[I_2]}{\alpha_2K_1K_2})$	$\frac{K_m}{[S]}(\frac{1}{K_2}+\frac{[I_1]}{\alpha_1K_1K_2})+(\frac{1}{K_2}+\frac{[I_1]}{\alpha_2K_1K_2})$	$1+\frac{K_m}{[S]}+\frac{[I_2]}{K_2}+\frac{K_m[I_2]}{K_2[S]}$	$1+\frac{K_m}{[S]}+\frac{[I_1]}{K_1}+\frac{K_m[I_1]}{[S]K_1}$
I_1 I_2	C C	a=b=∞	$\frac{K_m}{[S]}(\frac{1}{K_1}+\frac{[I_2]}{\alpha_1K_1K_2})$	$\frac{K_m}{[S]}(\frac{1}{K_2}+\frac{[I_1]}{\alpha_1K_1K_2})$	$1+\frac{K_m}{[S]}+\frac{K_m[I_2]}{K_2[S]}$	$1+\frac{K_m}{[S]}+\frac{K_m[I_1]}{[S]K_1}$
I_1 I_2	C NC	a=∞ b=1	$\frac{K_m}{[S]}(\frac{1}{K_2}+\frac{[I_2]}{\alpha_1K_1K_2})$	$\frac{K_m}{[S]}(\frac{1}{K_2}+\frac{[I_1]}{\alpha_1K_1K_2})$	$1+\frac{K_m}{[S]}+\frac{K_m[I_2]}{[S]K_2}+\frac{[I_2]}{K_2}$	$1+\frac{K_m}{[S]}+\frac{K_m[I_1]}{[S]K_1}$
I_1 I_2	NC C	a=1 b=∞	$\frac{K_m}{[S]}(\frac{1}{K_1}+\frac{[I_2]}{\alpha_1K_1K_2})+\frac{1}{K_1}$	$\frac{K_m}{[S]}(\frac{1}{K_2}+\frac{[I_1]}{\alpha_1K_1K_2})$	$1+\frac{K_m}{[S]}+\frac{K_m}{[S]}\frac{[I_2]}{K_2}$	$1+\frac{K_m}{[S]}+\frac{K_m}{[S]}\frac{[I_1]}{K_1}+\frac{[I_1]}{K_1}$
I_1 I_2	UC C	a→0 b=∞	$\frac{1}{K_1'}$	$\frac{K_m}{[S]}\frac{1}{K_2}$	$1+\frac{K_m}{[S]}+\frac{K_m}{[S]}\frac{[I_2]}{K_2}$	$1+\frac{K_m}{[S]}+\frac{K_m}{[S]}\frac{[I_1]}{K_1}+\frac{[I_1]}{K_1'}$
I_1 I_2	UC NC	a→0 b→∞	$\frac{1}{K_1'}+\frac{[I_2]}{\alpha_2K_1'K_2}$	$\frac{1}{K_2'}+\frac{[I_1]}{\alpha_2K_1'K_2}$	$1+\frac{K_m}{[S]}+\frac{K_m}{[S]}\frac{[I_2]}{K_2}+\frac{[I_2]}{K_2}$	$1+\frac{K_m}{[S]}+\frac{[I_1]}{K_1'}$
I_1 I_2	UC UC	a→0 b→0	$\frac{1}{K_1'}+\frac{[I_2]}{\alpha_2K_1'K_2'}$	$\frac{1}{K_2'}+\frac{[I_1]}{\alpha_2K_1'K_2'}$	$1+\frac{K_m}{[S]}+\frac{[I_2]}{K_2'}$	$1+\frac{K_m}{[S]}+\frac{[I_1]}{K_1'}$
I_1 I_2	NC UC	a=1 b→0	$\frac{K_m}{[S]}+\frac{1}{K_1}+\frac{[I_2]}{\alpha_2K_1K_2'}$	$\frac{1}{K_2'}+\frac{[I_1]}{\alpha_2K_1K_2'}$	$1+\frac{K_m}{[S]}+\frac{[I_2]}{K_2'}$	$1+\frac{K_m}{[S]}+\frac{K_m}{[S]}\frac{[I_1]}{K_1}+\frac{[I_1]}{K_1}$
I_1 I_2	C UC	a→∞ b→0	$\frac{K_m}{[S]}\frac{1}{K_1}+\frac{[I_2]}{\alpha_2K_1'K_2'}$	$\frac{1}{K_2'}$	$1+\frac{K_m}{[S]}+\frac{[I_2]}{K_2'}$	$1+\frac{K_m}{[S]}+\frac{K_m}{[S]}\frac{[I_1]}{K_1}$

Different inhibition modes are obtained from eq.(9) where $\gamma'=\gamma''=\gamma'''=0$. From eq.(9),

$$\frac{V_m}{v}=\frac{K_m}{S}(1+\frac{[I_1]}{K_1}+\frac{[I_2]}{K_2}+\frac{[I_1][I_2]}{\alpha_1K_1K_2})+(1+\frac{[I_1]}{K_1'}+\frac{[I_2]}{K_2'}+\frac{[I_1][I_2]}{\alpha_2K_1'K_2'})=[I_1]\ \{\frac{K_m}{[S]}(\frac{1}{K_1}+\frac{[I_2]}{\alpha_1K_1K_2})+\frac{1}{K_1'}+\frac{[I_2]}{\alpha_2K_1'K_2'}\}+(1+\frac{K_m}{[S]}+\frac{K_m}{[S]}\frac{[I_2]}{K_2}+\frac{[I_2]}{K_2'})$$

$$=[I_2]\ \{\frac{K_m}{[S]}(\frac{1}{K_2}+\frac{[I_1]}{\alpha_1K_1K_2})+\frac{1}{K_2'}\quad\frac{[I_1]}{\alpha_2K_1'K_2'}\}+(1+\frac{K_m}{[S]}+\frac{K_m}{[S]}\frac{[I_1]}{K_1}+\frac{[I_1]}{K_1'})$$

where $K_1'=aK_1$ and $K_2'=bK_2$. In case of $0<a<\infty$, $a\neq 1$ and $0<b<\infty$, $b\neq 1$, it will be a mixed type inhibition.

and

$$\gamma=\frac{k_2'}{k_2},\quad \gamma'=\frac{k_2''}{k_2},\quad \gamma''=\frac{k_2'''}{k_2} \tag{8}$$

By double reciprocal plot, eq. (3) becomes;

$$\frac{V_m}{v}=\frac{K_m}{[s]}\left(\frac{\Phi_1}{\Phi_3}\right)+\frac{\Phi_2}{\Phi_3} \tag{9}$$

Thus,

$$\frac{V_m}{v}=\frac{1}{k_a}\ \frac{1}{[s]}+\frac{1}{k_b} \tag{10}$$

where

$$\frac{1}{k_a}=K_m\left(\frac{\Phi_1}{\Phi_3}\right),\quad \frac{1}{k_b}=\frac{\Phi_2}{\Phi_3} \tag{11}$$

Results and Discussion

Special Cases and the Analytical Method for α_1, α_2, a, and b. The rate equation (10) is the form that is converted to a linear function of the reciprocals of $[S]$ and ν, whose ordinate and slope are dependent upon the variables of the two inhibitors. Obviously, if all variables for the two inhibitors are valid, the equation of $1/\nu$ as a function of inhibitor concentration will not represent a linear relationship. The complete rate equation is not useful in practice, in evaluating kinetic and equilibrium constants. However this equation can be simplified by taking several reasonable assumptions which can represent special cases for certain characteristics of inhibitors. If both inhibitors block the enzyme reaction completely, *i.e.*, $\gamma=\gamma'=\gamma''=0$, then,

$$\frac{1}{k_a}=K_m\Phi_1 \text{ and } \frac{1}{k_b}=\Phi_2.$$

Table 1 summarizes the inhibitor dependent values of ordinate ($1/k_b$) and slope ($1/k_a$) in the rate equation (10). Secondary plots of $[I]$ vs. $1/k_a$ and $1/k_b$ will also give linear lines if the experimental data fit in one of those specific cases listed in Table 1. The interaction factors, α_1 and α_2 between the two inhibitors can be evaluated from these secondary plots as seen in the following equations;

$$\frac{1}{k_a}=K_m\left(1+\frac{[I_1]}{K_1}+\frac{[I_2]}{K_2}+\frac{[I_1][I_2]}{\alpha_1K_1K_2}\right) \tag{12}$$

$$\frac{1}{k_b}=1+\frac{[I_1]}{aK_1}+\frac{[I_2]}{bK_2}+\frac{[I_1][I_2]}{ab\alpha_2K_1K_2} \tag{13}$$

Figure 1 shows the secondary plot of slope ($1/k_a$) against inhibitor concentration. From this plot one can obtain the interaction factor α_1 between the two inhibitors, regardless of the species of inhibitor, I_1 or I_2. If $\alpha=\infty$, the group of lines will be linear and parallel whose slope is equal to $1/K_1$ or $1/K_2$, respectively. This slope increases gradually as the α value decreases without affecting the value of ordinate.

This plot is similar to that described by Yonetani and Theorell (1964); the plot of $1/\nu$ vs. $[I_1]$ in the presence of I_2 in various concentration. In this case, differences are found in the values of ordinate and slope as seen in Table 1 for the various combinations of two different inhibitors.

The secondary plot of ordinate ($1/k_b$) against inhibitor concentration is shown in Figure 2. In this plot, more precise information on the multiple inhibition can be obtained. Besides interaction factor α_2, the factors on the dissociation equilibrium constant of the enzyme-substrate complex

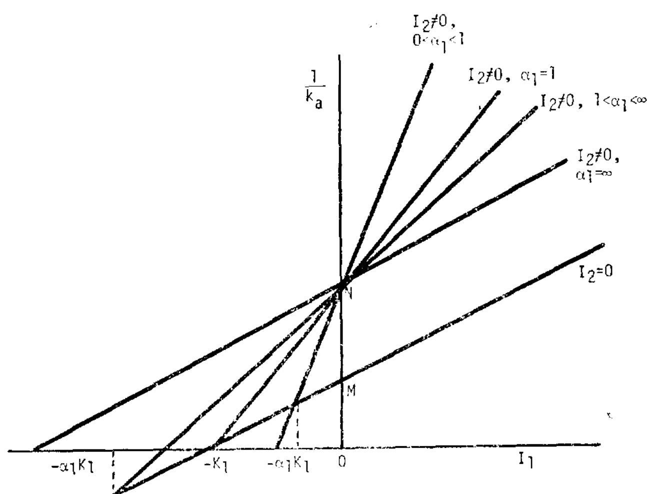


Figure 1. Secondary plot of $\frac{1}{k_a}$ *vs.* I_1. The values of M and N are K_m and $K_m\left(1+\frac{[I_2]}{K_2}\right)$, respectively. $\frac{1}{k_a}$ (slope) value can be obtained from the plot of $\frac{V_m}{v}$ *vs.* $\frac{1}{[S]}$ in the presence of two inhibitors. Same information can be drawn by plot of $\frac{1}{k_a}$ *vs.* I_2.

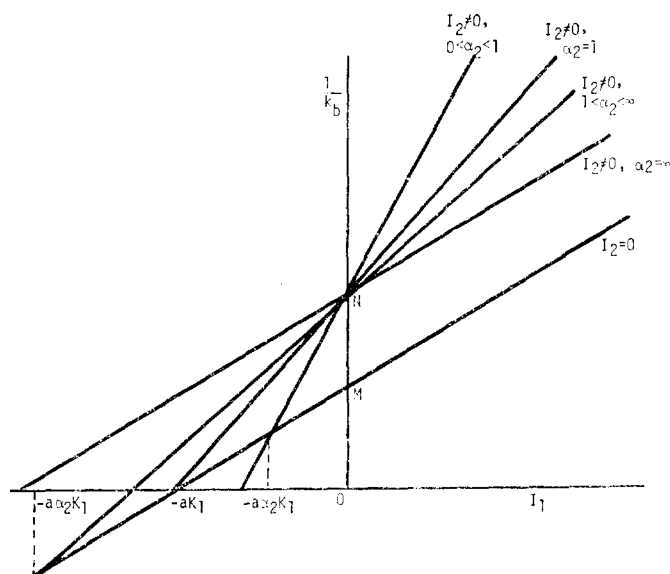


Figure 2. Secondary plot of $\frac{1}{k_b}$ *vs.* I_1. The values of M and N are 1 and $\left(1+\frac{[I_2]}{bK_2}\right)$, respectively $\frac{1}{k_b}$ (ordinate) value can be obtained from the plot of $\frac{V_m}{v}$ *vs.* $\frac{1}{[S]}$ in the presence of two inhibitors.

can also be obtained. The factor a can be calculated from the intersection point between the two lines where $I_2=0$ and $I_2\neq 0$, and b, from the ordinate value of this plot in a given I_2 value. By the combined procedure of the plot of $1/k_a$ *vs.* $[I_1]$ and $1/k_b$ *vs.* $[I_1]$, precise information are given on the values of α_1, α_2, a, and b.

Predictive coordinate values of the two different intersecting points, the one between lines plotted for various $[I]$ at the constant $[S]$ (point I) and the other between lines plotted for various $[S]$ at constant $[I]$ (point II) are listed in Table 2

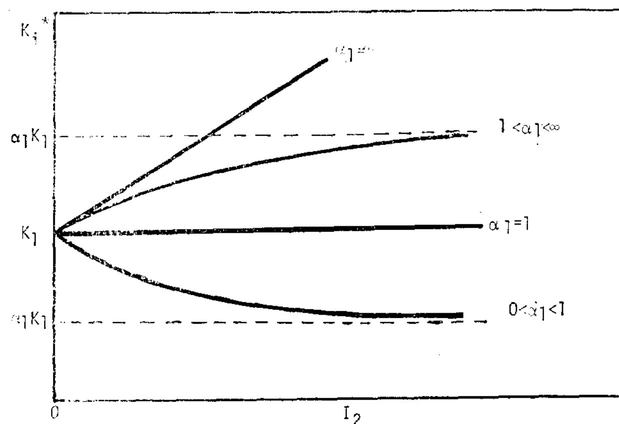


Figure 3. Plot of K_i^* *vs.* inhibitor concentration. K_i^* is the abscissa value of intersecting point II, between lines plotted for various $[S]$ at constant $[I_2]$ as listed in Table 2.

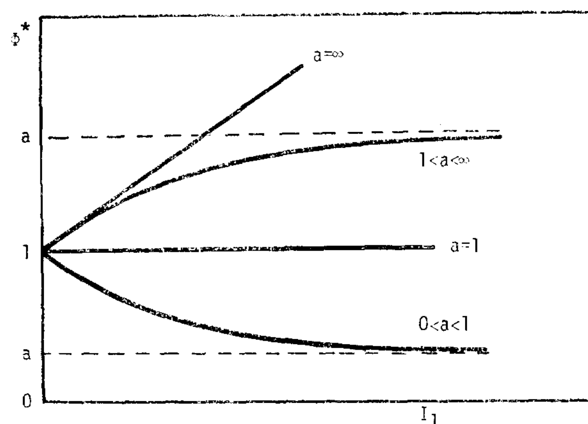


Figure 4. Plot of Φ^* *vs.* I_1 Φ^* is the ratio of slope and intercept $\left(\frac{1}{k_a}/\frac{1}{k_b}\right)$. Respective $\frac{1}{k_a}$ and $\frac{1}{k_b}$ values can be obtained from the plot of $\frac{V_m}{v}$ *vs.* $\frac{1}{[S]}$ in the presence of two inhibitors.

for all possible combinations of three different kinds of inhibitors. The intersecting point I has been discussed by Yonetani and Theorell (1964), and we thus try to avoid duplication. In any event, evaluation of this point is valuable for determining the value of interaction factor α.

The intersecting point II is of interest as well, since the interaction factor and the dissociation equilibrium constants of I_1 and I_2 can be determined, respectively. In the case of multiple inhibition of two noncompetitive inhibitors, two competitive inhibitors, and combination of a competitive and a noncompetitive inhibitor, the abscissa of this point II (K_i^*) is expressed as $K_i^*=-K_i(1+I_j/K_j)$ if $\alpha=\infty$ and $K_i^*=-\alpha_j K_i\left(\frac{K_j+I_j}{\alpha_j K_j+I_j}\right)$ or $-\alpha_i K_j\left(\frac{K_i+I_i}{\alpha_i K_i+I_i}\right)$ if $0<\alpha<\infty$ where i, j are either 1 or 2.

Thus, these equations lead to a secondary function between K_i^* and inhibitor concentration, whose plot will be linear if $\alpha=\infty$, and a hyperbola with a horizontal asymtote if $0<\alpha<\infty$ (Figure 3). A downward concave curve is obtained

TABLE 2: Intersecting Points between Lines in the Plot of $\frac{V_m}{V}$ vs. Inhibitor Concentration

Inhibitors	Modes	α	Variables of abscissa	Point I (between various[I] at constant[S])		Point II (between various[S] at constant[I])	
				absicissa	ordinate	abscissa	ordinate
I_1 I_2	NC NC	∞ $0<\alpha<\infty$	$[I_1]$	∞ $-\alpha K_1$	 $(1+K_m/[S])(1-\alpha)$	$-K_1(1+[I_2]/K_2)$ $-\alpha K_1(K_2+[I_2])/(\alpha K_2+[I_2])$	0 0
I_1 I_2	C C	∞ $0<\alpha<\infty$	$[I_1]$	∞ $-\alpha K_1$	 $1+K_m(1-\alpha)/[S]$	$-K_1(1+[I_2]/K_2)$ $-\alpha K_1(K_2+[I_2])/(\alpha K_2+[I_2])$	1 1
I_1 I_2	UC UC	∞ $0<\alpha<\infty$	$[I_1]$	∞ $-\alpha K_1'$	 $1-\alpha$	∞	
I_1	C	∞ $0<\alpha<\infty$	$[I_1]$	∞ $-\alpha K_1(1+[S]/K_m)$	 $(1+K_m/[S])(1-\alpha)$	$-K_1(1+[I_2]/K_2)$ $-\alpha K_1(K_2+[I_2])/(\alpha K_2+[I_2])$	$1+[I_2]/K_2$ $1+[I_2]/K_2$
I_2	NC	∞ $0<\alpha<\infty$	$[I_2]$	∞ $-\alpha K_2$	 $(1+K_m/[S])(1-\alpha)$	$-K_2(1+[I_1]/K_1)$ $-\alpha K_2(K_1+[I_1])/(\alpha K_1+[I_1])$	$-[I_1]/K_1$ $[I_1](1-\alpha)/(\alpha K_1+[I_1])$
I_1	NC	∞ $0<\alpha<\infty$	$[I_1]$	∞ $-\alpha K_1$	 $(1+K_m/[S])(1-\alpha)$	$-K_1(1+[I_2]/K_2)$ $-\alpha K_1(K_2+[I_2])/(\alpha K_2+[I_2])$	$1+[I_1]/K_1$ $1+[I_1]/K_1$
I_2	C	∞ $0<\alpha<\infty$	$[I_2]$	∞ $-\alpha K_2(1+[S]/K_m)$	 $(1+K_m/[S])(1-\alpha)$	$-K_2(1+[I_1]/K_1)$ $-\alpha K_2(K_1+[I_1])/(\alpha K_1+[I_1])$	$1+[I_2]/K_2$ $1+[I_2]/K_2$
I_1	UC	∞ $0<\alpha<\infty$	$[I_1]$	∞ $-\alpha K_1'(1+K_m/[S])$	 $(1+K_m/[S])(1-\alpha)$	∞	
I_2	NC	∞ $0<\alpha<\infty$	$[I_2]$	∞ $-\alpha K_2$	 $1+K_m(1-\alpha)/[S]$	$-K_2$ $-K_2$	$[I_1]/K_1'$ $[I_1](1-\alpha)/K_1'$
I_1	NC	∞ $0<\alpha<\infty$	$[I_1]$	∞ $-\alpha K_1$	 $(1+K_m/[S])(1-\alpha)$	$-K_1$ $-K_1$	$[I_2]/K_2'$ $[I_2](1-\alpha)/K_2'$
I_2	UC	∞ $0<\alpha<\infty$	$[I_2]$	∞ $-K_2'(1+K_m/[S])$	 $(1+K_m/[S])(1-\alpha)$	∞	
I_1 I_2	UC C	∞ $0<\alpha<\infty$	$[I_2]$	∞		$-K_2$ $-K_2$	$1+[I_1]/K_1'$ $1+[I_1]/K_1'$
I_1 I_2	C UC	∞ $0<\alpha<\infty$	$[I_1]$	∞		$-K_1$ $-K_1$	$1+[I_2]/K_2'$ $1+[I_2]/K_2'$

Abscissa value of point I is given as $I_1=-\frac{\alpha_1\alpha_2K_1K_1'(K_mK_2'+[S]K_2)}{[S]K_1K_2\alpha_1+K_mK_1'K_2'\alpha_2}$, $I_2=-\frac{\alpha_1\alpha_2K_2K_2'(K_mK_1'+[S]K_1)}{[S]K_1K_2\alpha_1+K_mK_1'K_2'\alpha_2}$. The value of point I is calculated from this in case of $\alpha_1=\alpha_2=\alpha$. Abscissa value of point II is given as $I_1=-\frac{\alpha K_1(K_2+[I_2])}{\alpha K_2+[I_2]}$, $I_2=-\frac{\alpha K_2(K_1+[I_1])}{\alpha K_1+[I_1]}$. ∞ represents parallel lines with no intercept values.

for the case of $0<\alpha<1$, and an upward convex for $1<\alpha<\infty$. If $\alpha=1$, $K_1{}^*$ is simply equal to $-K$, which is independent of $[I_2]$.

A simple test of the factors on the dissociation equilibrium constant of the enzyme-substrate complex is depicted in Figure 4. It is essentially based on the plot of $[I]$ and the ratio of slope and intercept $\left(\frac{1}{k_a}/\frac{1}{k_b}\right)$ obtained by double reciprocal plots of substrate concentration and initial velocity in the presence of any two inhibitors. If any effect is not exerted on the dissociation of ESI complex by substrate binding, the change of Φ^* will not be observed. Instead, an upward concave curve in case of facilitated dissociation of ESI complex and a downward convex curve in case of retardation of it will be observed. The curve will approach a horizontal asymptotic value a (or b).

When a straight line with positive slope is observed, the value of a (or b) is thought to be infinite. In this case, the inhibition mode reduces to pure competitive. The physical significance of this plot will be substantiated by the following calculation;

$$\Phi^*=\frac{1}{k_a}/\frac{1}{k_b}=K_m\left(\frac{\Phi_1}{\Phi_2}\right)$$

$$=\frac{1+\frac{[I_1]}{K_1}+\frac{[I_2]}{K_2}+\frac{[I_1][I_2]}{\alpha_1K_1K_2}}{1+\frac{[I_1]}{aK_1}+\frac{[I_2]}{bK_2}+\frac{[I_1][I_2]}{ab\alpha_2K_1K_2}} \quad (14)$$

$$\text{if}\quad [I_2]=0,\quad \Phi^*=\frac{a(K_1+[I_1])}{aK_1+[I_1]} \quad (15)$$

$$\text{if}\quad [I_1]=0,\quad \Phi^*=\frac{b(K_2+[I_2])}{bK_2+[I_2]} \quad (16)$$

Eq. (15) and (16) are not dependent of α values, but are functions of a and b. Ey examining the line behavior of the plot of Φ^* vs. $[I_1]$ or $[I_2]$, the variation of a and b values can be observed, respectively, in the whole range of zero to infinite.

Significance of the Interaction Factor α. The specific physical significance of the interaction factor α is of interest in a broad sense. However, kinetically the value indicates the

degree of interrelationship between interaction sites for two inhibitors. It can be either a 'steric factor' or 'allosteric factor' depending on the characteristics of the interaction. α can be classified into four different groups; (1) when $\alpha=\infty$, $[I_1]$ and $[I_2]$ are antagonistic inhibitors that may interact at the same site on an enzyme or that may be exclusive each other due to indirect influence to the affinity of the other inhibitor; (2) when $1<\alpha<\infty$, these two inhibitors are negatively interactive, *i.e.*, one inhibitor causes decrease in the affinity of the other; (3) when $\alpha=1$, two inhibitors are non-interactive in such a way that each inhibitor acts on an enzyme independently without affecting the affinity of the other; (4) when $0<\alpha<1$, synergistic inhibitory effect will be noted in which the affinity of an inhibitor increases in the presence of the other.

A number of possible causes that may influence on the interaction factor of two inhibitors have been discussed by Yonetani and Theorell (1964). For any interaction forces that exist between small molecules, inhibitors, and macromolecular unit, it would be attributed to the degree of interaction of two inhibitors in an enzyme system. Characteristic interaction forces of electrostatic, van der Waals, dipole-dipole, hydrophobic, and hydrophilic, can be important factors that determine the binding affinity as well as simple steric hindrance. Other important factors include complex of changes protein conformation that can bring about structural modification of the binding site for the other inhibitor, and that can alter any molecular interaction forces indirectly.

Table 3 summarizes the values of α for various enzyme systems reported from several laboratories. The pairs of multiple inhibitor we cited herein exclude those for infinite value of $\alpha\,(\alpha=\infty)$ since these cases confer no great meaning for the present analytical purposes. It is of interest that even simple competitive inhibitors on an enzyme demonstrate different behavior in multiple inhibition by different pairs of inhibitors, which appears in the value of α.

Although exact physical meaning of α values is difficult to evaluate, it shows at least how a pair of inhibitors are interrelated in their inhibitory action on an enzyme. Anderson and his coworkers attempted to elucidate various portions of the binding site of coenzyme molecule on yeast alcohol dehydrogenase with evaluation of the parameter α obtained by studies of competitive inhibitor pairs of the coenzyme analogs with different side chains or the analogs to only a portion of the coenzyme structure (Anderson & Reynolds, 1965a, 1965b, 1966a, 1966b; Fonda & Anderson, 1967). The evaluation of interaction range of structural analogs or side chains of inhibitors can be achieved by determining the value of α only if those inhibitors can bind with enzymes without causing distortion of enzyme conformation. If $\alpha=\infty$, it is interpreted as that there is no possible EI_1I_2 ternary complex formation due to competition for the same portion of the binding site, whereas $0<\alpha<\infty$ suggests the existence of the complex formation and the interacting portion of the effective radius of an inhibitor binding site roughly correlated with the value of α. The present analytical method can be applied for the clarification of the conflicting mechanism of penicillin amidase reaction proposed by two independent workers (Ryu *et al.*, 1972: Warburton *et al.*, 1973). Since the main difference between these two proposed rate equation is the existence of the EI_1I_2 complex where I_1 is 6-APA and I_2 is phenylacetic acid, the difference can be easily visualized by the plot of $\frac{1}{k_a}$ *vs.* $[I_1]$ in the presence of I_2. Furthermore, this plot can provide the degree of formation of the EI_1I_2 complex denoted by the value of α, and a simple test of which is the plot of K^* *vs.* $[I_1]$ or $[I_2]$ (Figure 3). While an inhibitor has either a steric hindrance or a repulsive effect on the other's binding in case of $1<\alpha<\infty$, the α value would appear in the range of $0<\alpha<1$ when the effect between two inhibitors is synergistic or attractive. In the usual cases, however, it is very difficult to differentiate such a simple primary effect by inhibitors the-

TABLE 3: Reported values of α for Various Enzyme Systems

Enzymes	Inhibitors I_1	Inhibitors I_2	α	References
Succinic dehydrogenase	Flouride	Phosphate	0.0034	Slater & Bonner, 1952
D-Amino acid oxidase	Riboflavin-5'-sulfate	Adenosine-5'-sulfate	0.2	Yagi & Ozawa, 1960
Horse liver alcohol dehydrogenase	*O*-Phenanthroline	ADPR	1	Yonetani & Theorell, 1964
	O-Phenanthroline	ADP	0.5	
	O-Phenanthroline	AMP	0.3	
Yeast alcohol dehydrogenase	N'-methylnicotinamide -HCl	ADPR	1.8	Anderson & Reynolds, 1965
	N'-Alkylammonium chloride	AMP	0.74-0.76	Fonda & Anderson, 1967
	N'-Alkylammonium	ADPR	0.66-1.15	
	N-Alkylnicotinamide	ADPR	0.25-0.55	
	N-Alkylnicotinamide	AMP	0.36-0.51	
	N-Alkylnicotinamide	NADH	0.33-1.03	
	N'-Methylnicotinamide -adenylic acid	N'-Benylnicotinamide	1	Heitz & Anderson, 1968
NADPH-isocitrate dehydrogenase	NADPH	Oxylylglycine	2.0±0.5	Northrop & Cleland, 1974

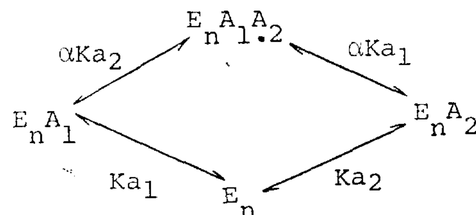
mselves from a secondary effect due to the conformational change of an enzyme protein induced by the binding of inhibitors.

Significance of the Factors a and b. The factors involved in dissociation constants of inhibitors were denoted in such a way that $K_1'=aK_1$ and $K_2'=aK_2$, where K_1 and K_2 are the dissociation equilibrium constants of I_1 and I_2 from the inhibitor complex of free enzyme, and K_1' and K_2' from that of the enzyme-substrate complex, respectively. The larger the value of the factors a and b, the more the dissociation of the complex is favorable. The difference between K_1 and K_1' (or K_1 and K_2') appeared as the factor a (or b) can be induced by the binding of substrate to the free enzyme. The values of factor a and b can be evaluated by secondary plot of $\frac{1}{k_b}$ *vs.* $[I]$ (Figure 2) after finding K_1 and α_1 values from the secondary plot of $\frac{1}{k_a}$ *vs.* $[I]$ (Figure 1).

Physical significance of the factor a and b can also he discussed to some extent in terms of extent of competition between an inhibitor and a substrate. If $1<a<\infty$, the inhibition is competitive, if $a=1$, noncompetitive. In the present section we should like to extend our discussion to the relationship between this factor and steady-state rate constants appeared in the "match-box" mechanism. In the derivation of steady-state concentration of the enzyme-substrate intermediate (E_1) in the two step reaction, it is seen that the relationship between the factors and the steady-state rate constants as;

$$\left(\frac{k_{-1}+k_2}{k_1}\right)=\frac{1}{a}\left(\frac{k_{-1}'+k_2'}{k_1'}\right)=\frac{1}{b}\left(\frac{k_{-1}''+k_2''}{k_2''}\right) \quad (17)$$

This relationship indicates that the values of the factor a and b also represent the changes in the steady-state rate constants that are involved in different pathways for the formation of steady-state concentration of E_1 complex. In other words, the steady-state concentration of E_1 complex is same irrespective of the reaction pathways; *i.e.*, through a direct pathway, $E_0 \cdots E_1$; indirect pathways *via* enzyme-inhibitor complex, $E_0 \cdots E_{01} \cdots E_{11} \cdots E_1$; $E_0 \cdots E_{02} \cdots E_{12} \cdots E_1$, $E_0 \cdots E_{01} \cdots E_{012} \cdots E_{112} \cdots E_{11} \cdots E_1$. Thus, any changes in the rate constants by inhibitors would appear in the factor a and b and also in the dissociation equilibrium constants of inhibitors. Consequently, if there is any changes in the rate constants by the presence of inhibitors, a strict non-competitive inhibition (in a classical point of view) will not be observed because $K_1 \neq K_1'$ unless $k_2=k_1=0$ and $a=1$. Instead, it will be a mixed type inhibition. In the other way around, if kinetic experiments demonstrate a noncompetitive inhibition, it means that $\frac{k_{-1}+k_2}{k_1}=\frac{k_{-1}'+k_2'}{k_1'}$ and thus $K_1=K_1'$. The inhibition must be a 'dead-end' as to $k_2'=k_2''=k_2'''=0$ and thus $\frac{k_{-1}+k_2}{k_1}=\frac{k_{-1}'}{k_1'}$. If $k_2 \ll k_{-1}$, then $k_s=k_s'$. In this case, a noncompetitive inhibition can be obtained experimentally, and the system must be in *quasi*-equilibrium in the case of two step mechanism (Moreales, 1965). In the case of *multi*-step enzyme reaction, however, the inhibitory patterns are entirely dependent on the rate determining steps in reaction sequences (Kaplan & Liaidler, 1967a; 1967b).



Scheme 2.

Alternative use of Funcation Φ. Φ denotes the equilibrium function for two inhibitors. This function can be modified to other forms for activators or hydrogen ions, or for combination of an inhibitor, an activator, and hydrogen ion if these small molecules are in *quasi*-equilibrium with an enzyme. Thus, the bimolecular rate constant for the formation of the enzyme-small molecule complex ought to be larger than any other unimolecular steady-state rate constants of an enzyme system.

The equilibrium system between nth enzyme intermediate (E_n) and small molecules (A) can generally be written as the following Scheme 2.

In this scheme, if A is an inhibitor, active species among different intermediate complexes will be the free form of enzyme intermediates (E_n) for the inhibitor binding. If A is an activator, the enzyme-activator complex (E_nA) will be an active form instead of the free enzyme intermediates. Depending upon the active form of intermediate complex, the following three different expressions for Φ function derived from the mass law can be obtained.

1. $1+\frac{[A_1]}{K_{a_1}}+\frac{[A_2]}{K_{a_2}}+\frac{[A_1][A_2]}{\alpha K_{a_1}K_{a_2}}$ for active E_n
2. $1+\frac{K_{a_1}}{[A_1]}+\frac{K_{a2}}{[A_2]}+\frac{\alpha K_{a_1}K_{a_2}}{[A_1][A_2]}$ for active $E_nA_1A_2$
3. $1+\frac{K_{a_1}}{[A_1]}+\frac{[A_2]}{K_{a_2}}+\frac{K_{a_1}[A_2]}{K_{a_2}[A_1]}$ for active E_nA_1

or

$(1+\frac{[A_1]}{K_{a_1}}+\frac{K_{a_2}}{[A_2]}+\frac{K_{a_2}[A_1]}{K_{a_1}[A_2]}$ for active $E_nA_2)$

Generally, the first expression is for the inhibitor reaction, and the second one for the reaction involving activator or cofactors. In the case of hydrogen ion equilibrium involving two protons, however, there will be no E_nA_2 complex, thus the reaction with hydrogen ion reaches an equilibrium system, $E_n \overset{K_1}{\rightleftharpoons} E_nA_1 \overset{K_2}{\rightleftharpoons} E_nA_1A_2$ where $A_1=A_2$ and A represents hydrogen ion concentration. The Φ functions can be simplified to simple pH functions envolving two ionizable groups depending upon the active species of the intermediate complexes. General derivation and discussion for the pH dependent steady-state rate expression of multi-step enzyme reaction system can be consulted with other references (Laidler, 1955; Alberty & Bloomfield, 1963; Kaplan & Laidler, 1967; Ottolenghi, 1971).

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