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KINETIC CONSEQUENCES OF A SLOW SUBSTRATE BINDING STEP IN GROUP TRANSFERASES

INTERPRETATION OF PRODUCT INHIBITION EXPERIMENTS.

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Summary

The steady state kinetic properties of a simple model for an enzyme catalyzed group transfer reaction between two substrates have been calculated. One substrate is assumed to bind slowly and the other rapidly to the enzyme. Apparent substrate inhibition or substrate activation by the rapidly binding substrate may result if the slowly binding substrate binds at unequal rates to the free enzyme and to the complex between the enzyme and the rapidly binding substrate. Competitive inhibition by each product with respect to its structurally analogous substrate is to be expected if both substrates are in rapid equilibrium with their enzyme-substrate complexes. This product inhibition pattern, however, may also be observed when one substrate binds slowly. Noncompetitive inhibition with respect to the rapidly binding substrate by its structurally analogous product may result if the slowly binding substrate binds more slowly to the enzyme-product complex than to the free enzyme. Inhibition by substrate analogs which are not products should follow the same rules as inhibition by products. Thus substrate analog inhibition experiments are not particularly informative. The form of inhibition by "transition state analog" inhibitors should reveal which substrate binds slowly.

There is no sharp conceptual distinction between ordered and random "kinetic mechanisms". I therefore suggest that the use of these concepts should be abandoned.

Introduction

Steady state kinetic and product inhibition experiments are widely used to obtain information concerning enzyme catalyzed reactions. It is now customary to interpret the results of such experiments in terms of the "kinetic mecha-

nisms" popularized by Cleland [1,2]. Although there has been considerable controversy concerning the interpretation of the kinetic properties of certain enzymes, hexokinase for example [3–6], it has been within the framework of Cleland's kinetic mechanisms. The adequacy of this framework has not been questioned.

In this paper, I will examine the steady state kinetic and product inhibition properties of a simple model for an enzyme catalyzed group transfer reaction between two substrates. In this model, I assume that one of the two substrates binds slowly to the enzyme and the other substrate equilibrates rapidly with its enzyme-substrate complexes. I will also consider the steady state kinetic consequences of a slow product dissociation step. The analysis presented here shows that the usefulness of the distinction customarily made between "ordered" and "random" kinetic mechanisms is questionable, and also that the interpretation of product inhibition experiments is more ambiguous than has been commonly assumed.

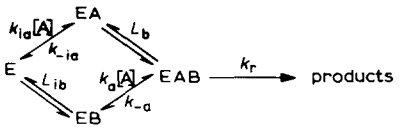
Theory

The slow substrate binding model

Consider an enzyme catalyzed group transfer reaction between two substrates with the following properties: (1) Both substrates must be bound to the enzyme in order for chemical reaction to occur, i.e. the "mechanism" is sequential, using the conventional terminology. (2) Product release is not rate-limiting. (3) Substrate B is in rapid equilibrium with its enzyme-substrate complexes, EB and EAB, at all times. (4) The interaction between substrate A and its enzyme-substrate complexes, EA and EAB, may be characterized by bimolecular, k_{on}, and unimolecular, k_{off}, rate constants. The k_{off} values may be similar to or much less than the rate constant for the conversion of EAB to products, therefore equilibrium will not, in general, be established between substrate A and its enzyme-substrate complexes at steady state.

These assumptions are summarized, in diagrammatic form, in Scheme I,

Scheme I: Slow Binding of Substrate A



where k_r, k_{-ia}, and k_{-a} are unimolecular rate constants, k_{ia} and k_a are bimolecular rate constants, and $L_{ib} = [E][B]/[EB]$ and $L_b = [EA][B]/[EAB]$ are dissociation constants. The letter, L, is used to represent dissociation constants in order to reserve the letter, K, to represent empirical kinetic parameters. When the Michaelis-Menten equation is obeyed, K_a and K_b will designate Michaelis constants for substrates A and B, that is the concentration of substrate necessary to produce half maximum velocity in the presence of a saturating concentration of the other substrate. K_{ia} and K_{ib} will designate the concentration of substrate necessary to produce half maximum velocity, extrapolated to zero concentration of the second substrate.

The rate equation corresponding to Scheme I was derived by the method of Cha [7]. If [E] + [EB] is designated E_T and [EA] + [EAB] is designated F_T, Scheme I may be represented in compact form as

$$E_T \underset{\Sigma k_{FE}F_{FE}/F_T}{\overset{\Sigma k_{EF}E_{EF}/E_T}{\rightleftharpoons}} F_T \xrightarrow{\Sigma k_r F_r/F_T} \text{products} \tag{1}$$

or

$$E_T \overset{\Sigma k_{EF}E_{EF}/E_T}{\underset{\Sigma k_{FE}F_{FE}/F_T + \Sigma k_r F_r/F_T}{\rightleftharpoons}} F_T \tag{2}$$

where $\Sigma k_{EF}E_{EF}$, $\Sigma k_{FE}F_{FE}$, and $\Sigma k_r F_r$ are sums of terms each of whcih is a product of a rate constant and a concentration. Each collection of terms represents the total rate of the process indicated by the subscript, thus $\Sigma k_{EF}E_{EF}$ is the rate of the E_T to F_T conversion, $\Sigma k_{FE}F_{FE}$ is the rate of the F_T to E_T conversion which does not lead to the formation of products, and $\Sigma k_r F_r$ is the rate of product formation. The rate equation corresponding to Scheme I in the form of Eqn. 1 is

$$v/E_o = \hat{v} = \frac{\Sigma k_r F_r/F_T \cdot \Sigma k_{EF}E_{EF}/E_T}{(\Sigma k_r F_r + \Sigma k_{FE}F_{FE})/F_T + \Sigma k_{EF}E_{EF}/E_T} \tag{3}$$

By multiplying the numerator and denominator of Eqn. 3 by $E_T F_T$, we obtain

$$v/E_o = \hat{v} = \frac{\Sigma k_r F_r \cdot \Sigma k_{EF}E_{EF}}{(\Sigma k_r F_r + \Sigma k_{FE}F_{FE}) \cdot E_T + \Sigma k_{EF}E_{EF} \cdot F_T} \tag{4}$$

The rate equation corresponding to Scheme I can now be written directly by substitution into Eqn. 4.

$$\hat{v} = \frac{[k_r[B]/L_b][k_{ia}[A] + k_a[A][B]/L_{ib}]}{[(k_r + k_{-a})[B]/L_b + k_{-ia}][1 + [B]/L_{ib}] + [k_{i\varepsilon}[A] + k_a[A][B]/L_{ib}][1 + [B]/L_b]} \tag{5}$$

Eqn. 5 shows that the velocity as a function of [A] for Scheme I obeys the Michaelis-Menten equation. The velocity as a function of [B] will in general be a 2/1 function of [B] (Eqn. 6).

$$v = \frac{d[B]^2 + e[B]}{a[B]^2 + b[B] + c} \tag{6}$$

If the reciprocal of Eqn. 6 is divided out, we obtain

$$1/v = c/e[1/[B]] + [b/e - cd/e^2] + \frac{[a/d - (b/e - cd/e^2)]}{[B] + e/d} \tag{7}$$

Eqn. 7 may be rewritten as the four parameter eqn. (Eqn. 8).

$$1/v = \frac{\bar{K}_b(\text{app})}{\bar{V}(\text{app})[B]} + \frac{1}{\bar{V}(\text{app})} + \frac{1/\bar{V}'(\text{app}) - 1/\bar{V}(\text{app})}{[B] + \bar{K}_b} \tag{8}$$

The parameters of Eqn. 8, $\overline{K}_b(app)$, $\overline{V}(app)$, and $\overline{V}'(app)$ are written as apparent constants to emphasize the fact that these parameters are functions of the concentration of substrate A. the overbar indicates they are associated with a four parameter equation. The dependence of the parameters of Eqn. 8 on [A] is:

$$\overline{V}(app) = \overline{V}[A]/[[A] + \overline{K}_a] \tag{9}$$

$$\overline{K}_b(app) = \overline{K}_{ib}[1 + [A]/\overline{K}_{ia}]/[1 + [A]/\overline{K}_a] \tag{10}$$

$$\overline{V}'(app) = \overline{V}'[A]/[[A] + \overline{K}'_a] \tag{11}$$

Values for the concentration-independent kinetic parameters of Eqns. 9 to 11, in terms of the constants of Scheme I, are listed in Table I.

The first two terms of Eqn. 8 represent the conventional double reciprocal form of the Michaelis-Menten equation. Therefore, Michaelis-Menten linearity will be observed whenever the third term of Eqn. 8 drops out, that is whenever $\overline{V}(app) = \overline{V}'(app)$ or K'_b becomes infinite. We see from Eqns. 9 and 11 that $\overline{V}(app) = \overline{V}'(app)$ when $\overline{K}_a = \overline{K}'_a$. $\overline{K}_a$ will equal $\overline{K}'_a$ if and only if $k_{ia} = k_a$ (Table I). The condition for Michaelis-Menten linearity with respect to [B] in Scheme I is therefore simply $k_{ia} = k_a$, the rate constants for binding of substrate A to E and to EB are equal. In general, apparent substrate inhibition by B will be observed when $k_{ia} > k_a$, and apparent substrate activation by B will be observed when $k_{ia} < k_a$.

If $k_{ia} = k_a$, $\overline{K}_{ia}$, $\overline{K}_a$, $\overline{K}_{ib}$, $\overline{K}_b$, and $\overline{V}$ become identical to the conventional Michaelis-Menten parameters, K_{ia}, K_a, K_{ib}, K_b and V. We see from Table I that for Scheme I the dissociation constant $k_{-ia}/k_{ia} = L_{ia} = \overline{K}_{ia}$ and $L_b = \overline{K}_b$. However, $\overline{K}_{ib} < L_{ib}$ and $k_{-a}/k_a = L_a < \overline{K}_a$. These results are a direct consequence of the fact that the concentration of EA at steady state is less than it would be if equilibrium were achieved between the two substrates and their enzyme-substrate complexes. Similarly, the concentration of EB at steady state is greater

TABLE I

KINETIC PARAMETERS ASSOCIATED WITH SLOW BINDING OF SUBSTRATE A

Kinetic parameters are defined by Eqns. 8 to 11. Kinetic constants are defined by Scheme I ($L_{ia} = k_{-ia}/k_{ia}$, $L_a = k_{-a}/k_a$).

Kinetic parameter	Value of parameter in terms of Scheme I constants
$\overline{V}$	k_r
$\overline{V}'$	k_r
$\overline{K}_{ia}$	L_{ia}
$\overline{K}_a$	$L_a[1 + k_r L_{ib}/k_{-ia}L_b] = L_a + k_r/k_{ia}$
$\overline{K}'_a$	$L_a[1 + k_r/k_{-a}] = L_a + k_r/k_a$
$\overline{K}_{ib}$	$L_{ib}/[1 + k_r L_{ib}/k_{-ia}L_b]$
$\overline{K}_b$	L_b
$\overline{K}'_b$	$L_{ib}k_{ia}/k_a = L_b k_{-ia}/k_{-a}$
$\overline{K}_a/\overline{K}_{ia} = \overline{K}_b/\overline{K}_{ib}$	$L_b/L_{ib} + k_r/k_{-ia}$

than the equilibrium concentration of EB. The ratio, $L_{ib}/\bar{K}_{ib} = \bar{K}_a/L_a = 1 + (k_r L_{ib})/(k_{-ia} L_b)$, is a measure of the degree to which equilibration of the enzyme with its enzyme-substrate complexes is not achieved at steady state, thus it can be considered to represent a kind of "disequilibrium index" for the enzyme catalyzed reaction.

The qualitative effects of changes in the kinetic constants of Scheme I on the expected steady state kinetic properties of an enzyme catalyzed reaction following Scheme I can be appreciated by reference to Table II. The first five columns of Table II contain sets of assumed rate and equilibrium constants. The last two columns list quantities which can in principle be obtained by experimental measurement. L_{ib}/K_{ib} is the ratio between the kinetic parameter K_{ib} and the enzyme-substrate dissociation constant, L_{ib}. K_b/K_{ib} is a convenient measure of the steady state kinetic pattern. Low values of this ratio ($K_b/K_{ib} \ll 1$) indicate an apparent synergistic substrate-substrate binding interaction. Set I represents what would usually be called a "rapid equilibrium random mechanism." As the binding step becomes progressively more irreversible (sets I through VI) the apparent substrate-substrate binding interaction, represented by quantity, K_b/K_{ib}, becomes more anti-synergistic. If the binding step is completely irreversible, as in set VI, the "parallel" initial rate pattern sometimes considered to be characteristic of a "ping-pong mechanism" will be observed. Other things being equal, bad substrates, defined either by a low V or high K_m, will tend to be more equilibrated with their enzyme-substrate complexes at steady state, and thus will tend to show a less "parallel" and more "intersecting" kinetic pattern. This is a likely explanation for the more "intersecting" initial

TABLE II

SLOW BINDING OF SUBSTRATE A (SCHEME I): CALCULATED KINETIC PARAMETERS FROM SELECTED SETS OF RATE AND EQUILIBRIUM CONSTANTS

Kinetic constants are defined by Scheme I. Kinetic parameters were calculated from these constants by reference to Table I.

Set number	Kinetic constants					Kinetic parameters	
	L_{ib}/L_b	k_{-ia}/k_r	k_{ia}/k_r	k_{-a}/k_r	k_a/k_r	L_{ib}/K_{ib}	K_b/K_{ib}
I	1	∞	1	∞	1	1	1
II	1	10	1	10	1	1.1	1.1
III	1	1	1	1	1	2	2
IV	1	0.1	1	0.1	1	11	11
V	1	0.01	1	0.01	1	101	101
VI	1	0	1	0	1	∞	∞
VII	10	0.1	1	0.01	1	101	10.1
VIII	100	0.1	1	0.001	1	1001	10.01
IX	∞	0.1	1	immaterial		∞	10
X	1	0.1	1	0.01	0.1	substrate inhibition	
XI	1	0.1	1	0	0	substrate inhibition	
XII	1	0.01	0.1	0.1	1	substrate activation	
XIII	1	0	0	0.1	1	1,11 [a]	1
XIV	1	∞	1	0	0	1	1
XV	1	0	0	∞	1	1	1

[a] $L_{ib}/K_{ib} = 1$; $K_a/L_a = 11$.

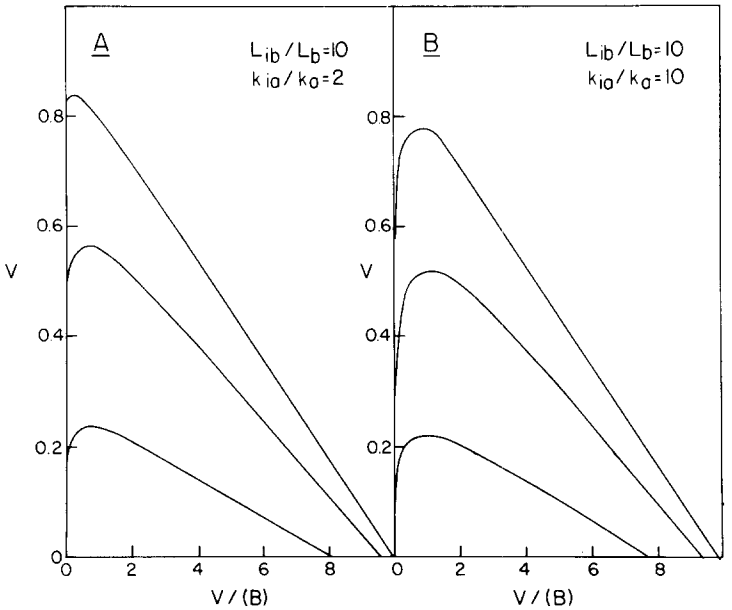


Fig. 1. Eadie plots demonstrating apparent substrate inhibition by substrate B when substrate A binds slowly. Velocities were calculated from Eqns. Assumed values of constants are: $L_{ib} = 1$, $L_b = 0.1$, $k_r = 1$, $k_{ia} = 1$, $k_{-ia} = 0.1$; (A) $k_a = 0.5$, $k_{-a} = 0.005$; (B) $k_a = 0.1$, $k_{-a} = 0.001$. Lines are drawn at $[A]/L_{ia} = 0.4$, 2, 10.

steady state kinetic pattern of fructose as compared to glucose with rat brain hexokinase [8].

When $k_{ia} > k_a$, as in sets X and XI, there will be an apparent substrate inhibition by substrate B (Fig. 1). An apparent substrate activation by substrate B will result when $k_a > k_{ia}$, as in set XII (Fig. 2). When $k_{ia} = 0$ (set XIII) Michaelis-Menten linearity returns as the first two terms of Eqn. 8 drop out. The series represented by sets IV, VII, VIII, and IX is of particular interest. Set IV is random in the sense that both EA and EB complexes are formed. Moreover, the dissociation constants of the enzyme-substrate complexes are the same whether or not the other substrate is also bound to the enzyme, implying the absence of a net substrate-substrate binding interaction. Sets VII and VIII differ from set IV by the existence of a positive, or synergistic substrate-substrate binding interaction. This is carried to the limit in set IX, in which an infinitely synergistic substrate-substrate binding interaction is assumed. Set IX clearly represents the situation normally characterized as a "compulsory ordered mechanism." Note, however, that the steady state kinetic pattern of set IV ($K_b/K_{ib} = 11$) differs only slighly from that of set IX ($K_b/K_{ib} = 10$). Sets IV, VII, VIII, and IX are points along a smooth continuum. Surely in the real world the substrate-substrate binding interaction, although it may be highly synergistic, will be finite. If the constants of set IV are characterized as a "steady state random mechanism" and the constants of set IX are characterized as a "compulsory ordered mechanism," where is the line to be drawn between them? More fundamentally, does it make sense to draw such a line? In a recent paper,

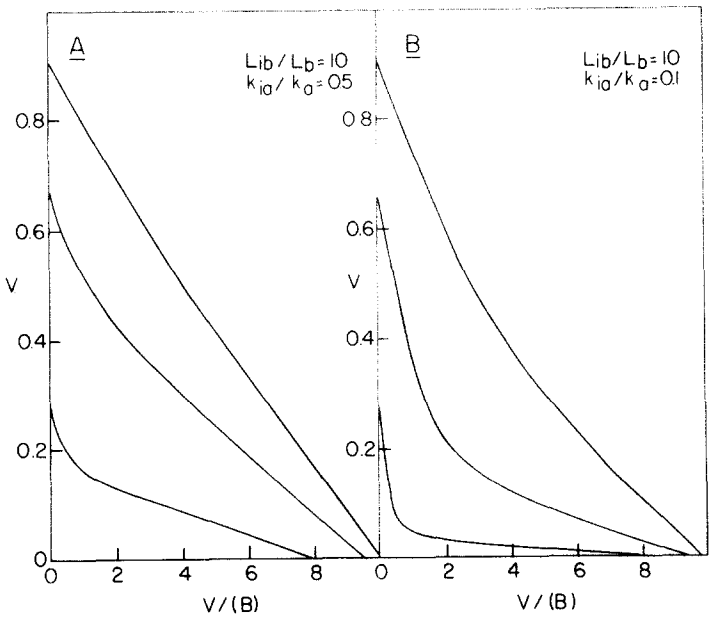


Fig. 2. Eadie plots demonstrating apparent substrate activation by substrate B when substrate A binds slowly. Velocities were calculated from Eqn. 5. Assumed values of constants are: $L_{ib} = 1$, $L_b = 0.1$, $k_r = 1$, $k_a = 1$, $k_{-a} = 0.01$; (A) $k_{ia} = 0.5$, $k_{-ia} = 0.05$; (B) $k_{ia} = 0.1$, $k_{-ia} = 0.01$. Lines are drawn at $[A]/L_{ia} = 0.4, 2, 10$.

Danenberg and Cleland [6] postulated a set of constants with $L_{ib}/L_b = 50$, i.e. very close to my set VIII, to explain the kinetic properties of yeast hexokinase, and yet characterized it as representing a "random mechanism." Conceptually, the distinction between and "ordered" and "random" mechanism is fuzzy. In practice, this distinction is usually made on the basis of product or substrate analog inhibition experiments. There is no doubt that different product inhibition patterns do exist for enzyme catalyzed reactions. What do the observed product inhibition patterns mean? This question will be discussed in the following section.

Product inhibition

In an enzyme catalyzed group transfer reaction between two substrates, which we are considering here, each substrate is transformed to a structurally analogous product either by donating a group to or accepting a group from the other substrate. There are thus two analogous substrate-product pairs which would be expected to compete for a common binding site, and two non-analogous substrate-product pairs. The smaller non-analogous substrate-product pair, or the two "acceptors," certainly would be expected to form a dead end enzyme-substrate-product complex. The two "donors" may or may not form a dead end complex depending on whether the enzyme binding surface can accomodate two of the transferred groups. If the transferred group is small, as in the dehydrogenases, donor-donor dead end complexes are to be expected.

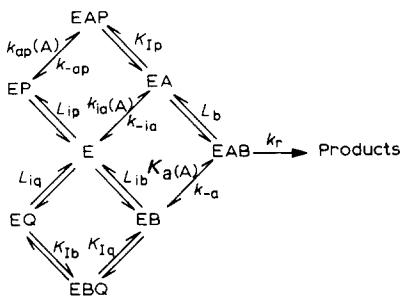
If the Michaelis-Menten equation is obeyed, competitive and non-competitive

inhibition are defined by reference to Eqn. 12 [1].

$$v_{obs} = \frac{V[A]/[1 + [I]/K_{i\,uncomp}]}{[A] + \{K_a[1 + [I]/K_{i\,comp}]\}\{[1 + [I]/K_{i\,uncomp}]\}^{-1}} \tag{12}$$

If $[I]/K_{i\,comp}$ is measurable and $[I]/K_{i\,uncomp}$ is not, inhibition by I is competitive with respect to A. If both $[I]/K_{i\,comp}$ and $[I]/K_{i\,uncomp}$ are measurable, inhibition by I is noncompetitive with respect to A. Finally, if $[I]/K_{i\,uncomp}$ is measurable and $[I]/K_{i\,comp}$ is not, inhibition by I is uncompetitive with respect to A. Somewhat arbitrarily, but in accord with customary practice [1,2], I have designated the product structurally analogous to substrate A by the letter Q and the product structurally analogous to substrate B by the letter P. The complexes which can be formed by these two classes of inhibitor, when substrate A binds slowly are illustrated in Scheme II. The rate equations corresponding to Scheme II, written in the form of Eqn. 4, are given below as Eqns. 13 and 14. Dissociation constants associated with products P and Q are designated by the letter L in order to reserve the letter K to represent kinetic parameters for the reaction in the reverse direction.

Scheme II: Slow binding of substrate A; inhibition by products or substrate analog



Slow substrate A binding, inhibition by product Q or substrate A analog

$$\hat{v} = \{[k_r[B]/L_b] \cdot [k_{ia}[A] + k_a[A][B]/L_{ib}]\}\{[(k_r + k_{-a})[B]/L_b + k_{-ia}] \cdot [1 + [B]/L_{ib} + [Q]/L_{ib}K_{Iq}] + [k_{ia}[A] + k_a[A][B]/L_{ib}] \cdot [1 + [B]/L_b]\}^{-1} \tag{13}$$

Slow substrate A binding, inhibition by product P or substrate B analog

$$\hat{v} = \{[k_r[B]/L_b] \cdot [k_{ia}[A] + k_a[A][B]/L_{ib} + k_{ap}[A][P]/L_{ip}]\}\{[(k_r + k_{-a})[B]/L_b + k_{-ia} + k_{-ap}[P]/K_{Ip}] \cdot [1 + [B]/L_{ib} + [P]/L_{ip}] + [k_{ia}[A] + k_a[A][B]/L_{ib} + k_{ap}[A][P]/L_{ip}] \cdot [1 + [B]/L_b + [P]/K_{Ip}]\}^{-1} \tag{14}$$

Eqn. 13 is of course in the Michaelis-Menten form when substrate A is the variable substrate, and can easily be rearranged to yield inhibition constants

TABLE III

SUMMARY OF INHIBITION CONSTANTS

Rate and equilibrium constants appearing in this table are defined by Scheme II. Kinetic parameters $\bar{K}_{ia}$, $\bar{K}_a$, $\bar{K}'_a$, $\bar{K}_{ib}$, and $\bar{K}_b$ associated with the slow A binding model are defined by Eqns. 8 to 11.

Type of constant	Kinetic property of reaction	Inhibition constants for analogs of substrate A	Inhibition constants for analogs of substrate B, $k_a = k_{ia} = k_{ap}$	Inhibition constants for "transition state analog" inhibitors (no dead-end complexes)
K_i comp (app) vs. A	Slow A binding	$\frac{L_{iq}[1 + [B]/L_{ib}]}{[1 + [B]/K_{Ib}]}$	$L_{ip}[1 + [B]/K_{ib}]$	$L_{ip}[1 + [B]/L_{ib}]$
	Rapid equilibrium	$\frac{L_{iq}[1 + [B]/K_{ib}]}{[1 + [B]/K_{Ib}]}$	$L_{ip}[1 + [B]/K_{ib}]$	$L_{ip}[1 + [B]/K_{ib}]$
K_i uncomp (app) vs. A	Slow A binding	∞	$K_{Ip}[1 + [B]/K_b]$	∞
	Rapid equilibrium	∞	$K_{Ip}[1 + [B]/K_b]$	∞
K_i comp (app) vs. B	Slow A binding	$L_{iq}[1 + [A]/\bar{K}_{ia}]$	$\frac{L_{ip}[1 + [A]/K_{ia}]}{[1 + [A]/K_{Ia}]}$	$L_{ip}[1 + [A]/\bar{K}_{ia}]$
	Rapid equilibrium	$L_{iq}[1 + [A]/K_{ia}]$	$\frac{L_{ip}[1 + [A]/K_{ia}]}{[1 + [A]/K_{Ia}]}$	$L_{ip}[1 + [A]/K_{ia}]$
K_i uncomp (app) vs. B	Slow A binding	$\frac{1}{K_{iq(app)}} = \left[\frac{1}{K_{Iq}L_{ib}/\bar{K}_{ib}} + \frac{11}{L_{iq}L_{ib}/(L_{ib} - \bar{K}_{ib})}\right] \cdot \left[\frac{1}{1 + [A]/\bar{K}_a}\right]$	∞	$L_{ip}[1 + [A]/\bar{K}_a]L_{ib}/(L_{ib} - \bar{K}_{ib})$
$\bar{K}'_q$(app) vs. B	Slow A binding	$K_{Iq}[1 + [A]/\bar{K}'_a]$		

which are listed in Table III. Although when substrate B is the variable substrate, Michaelis-Menten kinetics are not in general obeyed, it can be rearranged to the form of Eqn. 8 in which the first two terms represent a Michaelis-Menten equation. The inhibition constants listed in Table III refer to the effect of inhibitors on these first two terms. The constant K'_q is defined by the effect of Q in the reducing V' to $V'/[1 + (Q)/K'_q]$ (see Eqn. 8).

Eqn. 14, which describes inhibition by P, an analog of B, the rapidly binding substrate, is complicated by the possibility that the rate constant for binding of A to the EP complex will not be the same as the rate constants for binding of A to E and to EB. Inhibition constants for two special cases of Eqn. 14, one in which the EAP complex does not exist, and the other in which $k_{ap} = k_{ia} = k_a$, are given in Table III. In the first special case, the enzyme-inhibitor complex can bind neither substrate. It is anticipated that "transition state analog" inhibitors [9] will have this property. "Donor" product inhibitors may have this property if the transferred group is large, but "acceptor" product inhibitors will almost certainly not have this property.

The calculated inhibition constants associated with substrate analog inhibition are summarized in Table III for the case in which substrate A binds slowly, and the rapid equilibrium case. In the rapid equilibrium case, inhibition by a substrate analog or product inhibitor will be competitive with respect to substrates with which an enzyme-substrate-inhibitor dead end complex is not formed, and noncompetitive (or uncompetitive) with respect to substrates with which dead end complexes are formed.

Table III also shows that the presence of a slow substrate binding step is not necessarily revealed by the form of product inhibition. For example, if both EBQ and EAP dead end complexes exist, and if $k_{ia} = k_{ap}$, inhibition by products will be competitive vs. analogous substrates, and noncompetitive (or uncompetitive) vs. nonanalogous substrates, just as in the rapid equilibrium case. There are quantitative differences, however, by which the two cases can sometimes be distinguished. The competitive Q vs. A inhibition constant has limiting values of K_{iq} at low [B] and K_{Iq} at high [B] whether binding of substrate A is slow or rapid. If $K_{iq} = K_{Iq}$, product inhibition experiments will not distinguish the two cases. If Q binds preferentially to the EB complex, i.e. $K_{Iq} \ll K_{iq}$, inhibition by Q will be uncompetitive with respect to B in all cases. However, in the slow binding case, K_{iq}(app) will be approximately $K_{iq}/2$ at [B] = K_{ib} and decrease to a much lower value, K_{Iq}, as [B] is increased, whereas in the rapid equilibrium case, K_{iq}(app) will be approximately $2K_{Iq}$ at [B] = K_{ib} and increase to a much higher value, K_{iq}, as [B] is decreased. Another phenomenon which may be observed in the slow A binding case if Q binds preferentially to the EB complex is an apparent substrate inhibition by substrate B in the presence of Q, an anlog of A. This occurs because the presence of Q will decrease $\overline{V}'$(app) to a greater extent than $\overline{V}$(app) because $\overline{K}'_q$(app) is smaller than $K_{q\ uncomp}$(app) [see Table III]. A phenomenon of this type seems to be responsible for the observed apparent substrate inhibition by ATP in the presence of lyxose in yeast hexokinase [10]. A similar explanation for this observation has in fact been given by Danenberg and Cleland [6].

Inhibition by "transition state analog" inhibitors, which compete for the binding sites of both substrates, will be noncompetitive with respect to B, the

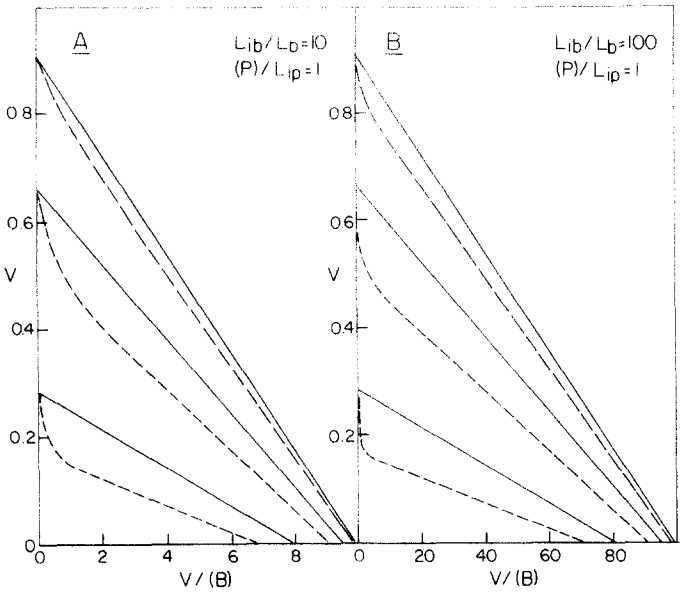


Fig. 3. Eadie plots demonstrating apparent noncompetitive inhibition by "transition state analog" inhibitors with respect to the rapidly binding substrate. Velocities were calculated from Eqn. 14. Assumed values of constants are: $L_{ib} = 1$; (A) $L_b = 0.1$; (B) $L_b = 0.01$; $k_r = 1$, $k_{ia} = 1$, $k_{-ia} = 0.1$, $k_a = 1$, $K_{ip}/K_{Ip} = 0$, ———, $[P]/L_{ip} = 0$; - - - - -, $[P]/L_{ip} = 1$. Lines are drawn at $[A]/L_{ia} = 0.4, 2, 10$.

rapidly binding substrate, if one substrate binds slowly. This is in contrast to the competitive inhibition expected in the rapid equilibrium case. The calculated form of inhibition by "transition state analog" inhibitors is illustrated in Fig. 3. The apparent nonlinearity of the V vs. $V/[B]$ plots in the presence of the inhibitor is due to the fact that V(app) but not V'(app) is depressed by the presence of the inhibitor, leading to the condition $\overline{V}'(\text{app}) > \overline{V}(\text{app})$ and apparent substrate activation by B. Fig. 3 also illustrates the fact that if the substrate-substrate binding interaction is highly synergistic, deviation from Michaelis-Menten linearity by B in the presence of inhibitor will only be observable at very high concentrations of B, and in practice will probably not be observed.

If an inhibitor P, an analog of substrate B, forms an EAP complex which is formed more slowly from EP than is the EA complex from E, i.e. $k_{ap} < k_{ia}$, we have a situation intermediate between that represented by the second and third columns of Table III. In this situation inhibition by P may appear to be noncompetitive with respect to both substrates. Apparent noncompetitive P vs. B inhibition when $k_{ap}/k_{ia} = 0.5$ is illustrated in Fig. 4.

The form of product and substrate analog inhibition is summarized in Table IV. Of note is the fact that when substrate A binds slowly to the enzyme, inhibition by P may look competitive vs. A and noncompetitive vs. B, or competitive vs. B and noncompetitive vs. A, or noncompetitive vs. both A and B, depending on the relative rate of formation of EA from E and EAP (if it exists) from EP. This fact alone should give pause to those who attempt to assign "kinetic mechanisms" on the basis of the results of product inhibition experiments.

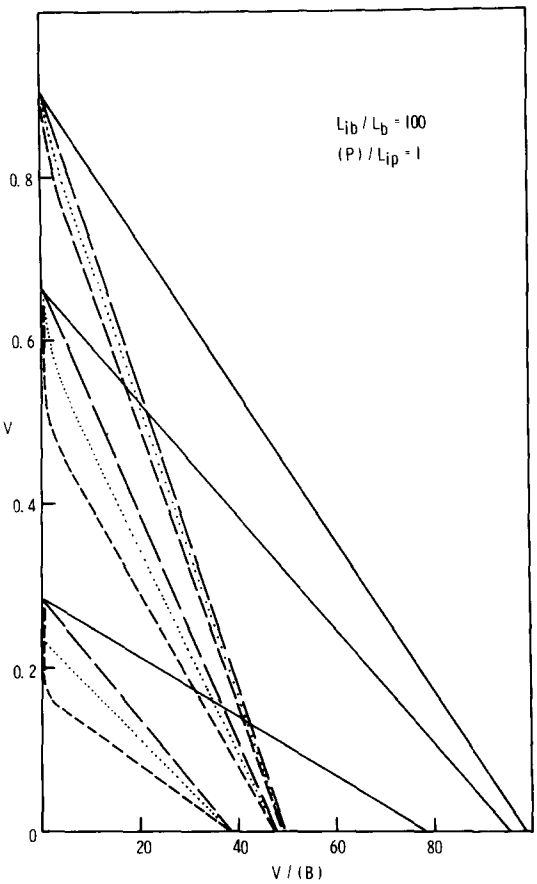


Fig. 4. Eadie plots demonstrating the effect of the rate of substrate binding to the enzyme-inhibitor complex on the form of inhibition by analogs of the rapidly binding substrate with respect to the rapidly binding substrate. Velocities were calculated from Eqn. 14. Assumed values of constants are: $L_{ib} = 1$, $L_b = 0.01$, $L_{ip}/K_{Ip} = 1$, $k_{ia} = 1$, $k_{-ia} = 0.1$, $k_r = 1$; ———, $[P]/L_{ip} = 0$; — — —, $[P]/L_{ip} = 1$, $k_{ap} = 1$, $k_{-ap} = 0.1$; . . . . . , $[P]/L_{ip} = 1$, $k_{ap} = 0.5$, $k_{-ap} = 0.05$; - - - - - -, $[P]/L_{ip} = 1$, $k_{ap} = 0$. Lines are drawn at $[A]/L_{ia} = 0.4, 2, 10$.

TABLE IV

PRODUCT OR SUBSTRATE ANALOG INHIBITION PATTERNS FOR THE SLOW SUBSTRATE A BINDING CASE AND THE RAPID EQUILIBRIUM SUBSTRATE BINDING CASE

Inhibitor class	Kinetic property of reaction	Form of inhibition vs. substrate A	Form of inhibition vs. substrate B
Substrate A analog	Slow A binding	competitive	noncompetitive *
	Rapid equilibrium	competitive	noncompetitive *
Substrate B analog	Slow A binding	noncompetitive *	competitive
$k_{ap} = k_{ia}$	Rapid equilibrium	noncompetitive *	competitive
Substrate B analog	Slow A binding	noncompetitive *	noncompetitive
$k_{ap} < k_{ia}$	Rapid equilibrium	noncompetitive *	competitive
"Transition state"	Slow A binding	competitive	noncompetitive
analog	Rapid equilibrium	competitive	competitive

* The form of this inhibition may appear to be uncompetitive.

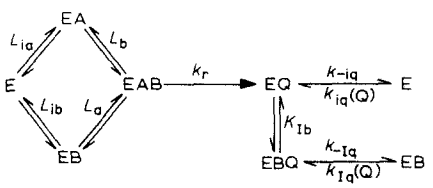
The slow substrate binding model for which I have calculated kinetic parameters and inhibition constants is perhaps oversimplified. The interaction of the slowly binding substrate with the enzyme is probably better approximated for most enzymes by assuming that a weak enzyme-substrate complex forms rapidly, followed by a slow "isomerization" to a more stable enzyme-substrate complex. There are a number of examples of enzyme-substrate complexes which seem to show this behavior [11]. I have worked out the properties of such a model, and found that they differ only in very minor respects from the slow binding model presented here. One difference is that, if a slow isomerization of an EA complex is assumed, apparent substrate inhibition by B is not necessarily overcome by saturation by substrate A, as it is if A is assumed to bind slowly in a bimolecular step (Fig. 1). Expressions for inhibition constants for the slow EA complex isomerization model are identical to the slow substrate A binding model.

Slow product release

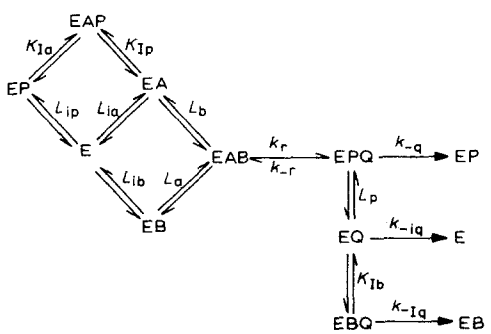
The assumption that product release is not rate limiting may not always be valid. The next section will therefore explore some kinetic consequences of a rate-limiting product dissociation step.

If we assume that product Q dissociates slowly from the enzyme, Scheme III illustrates the complexes which may exist in the presence of added Q, and Scheme IV illustrates the complexes which may exist in the presence of added P.

Scheme III: Slow Dissociation by Product Q; Inhibition by Product Q



Scheme IV: Slow dissociation by product Q; inhibition by product P



Scheme IV, in the same manner as Scheme I, can be represented schematically by Eqn. 1. Scheme III can be represented schematically by Eqn. 15 which can be made identical to Eqn. 2

$$E_T \underset{\Sigma k_{FE} F_{FE}/F_T}{\overset{-k_{EF} E_{EF}/E_T + \Sigma k_r E_r/E_T}{\rightleftharpoons}} F_T \tag{15}$$

by reversing E and F. The rate equations for Schemes III and IV, written in the form of Eqn. 4 are:

$$\hat{v}\,(\text{Scheme III}) = \{[k_r[A][B]/L_{ia}L_b] \cdot [k_{-iq} + k_{-Iq}[B]/K_{Ib}]\}\{[k_r[A][B]/L_{ia}L_b + k_{iq}[Q] + k_{Iq}[B][Q]/L_{ib}] \cdot [1 + [B]/K_{Ib}] + [k_{-iq} + k_{-Iq}[B]/K_{Ib}] \cdot [1 + [A]/L_{ia} + [B]/L_{ib} + [A][B]/L_{ia}L_b]\}^{-1} \tag{16}$$

$$\hat{v}\,(\text{Scheme IV}) = \{[k_r[A][B]/L_{ia}L_b] \cdot [k_{-iq} + k_{-q}[P]/L_p + k_{-Iq}[B]/K_{Ib}]\}\{[k_{-iq} + (k_{-q} + k_{-r})[B]/L_p + k_{-Iq}[B]/K_{Ib}] \cdot [1 + [A]/L_{ia} + [B]/L_{ib} + [A][B]/L_{ia}L_b + [P]/L_{ip} + [A][P]/L_{ia}K_{Ip}] + [k_r[A][B]/L_{ia}L_b] \cdot [1 + [B]/K_{Ib} + [P]/L_p]\}^{-1} \tag{17}$$

In the absence of added inhibitors, the empirical steady state kinetic parameters associated with Scheme IV (assuming $k_{-Iq} = k_{-iq}$ or that the EBQ complex does not exist) are: $V/E_0 = k_r k_{-iq}/(k_r + k_{-iq})$, $K_{ia} = L_{ia}$, $K_{ib} = L_{ib}$, $K_a = L_a k_{-iq}/(k_r + k_{-iq})$, $K_b = L_b k_{-iq}/(k_r + k_{-iq})$. Therefore in the absence of added inhibitor, the effect of a slow product dissociation step is to decrease the apparent Michaelis constants, K_a and K_b, by the factor $k_{-iq}/(k_r + k_{-iq})$, thus the apparent substrate-substrate binding interaction as measured by K_{ia}/K_a will appear to be more synergistic than it is. This effect is strikingly illustrated by the properties of horse liver alcohol dehydrogenase, in which there is evidence that the release of NADH is rate limiting in the direction of alcohol oxidation [12]. In the direction of ethanol oxidation, $K_{ia}/K_a = 30$, whereas in the direction of acetaldehyde reduction, NADH does not have time to equilibrate with complexes with the enzyme and $K_{ia}/K_a = 0.05$ [13].

Apparent substrate inhibition by B can result if the rate of product dissociation from the EBQ complex (Scheme III) is less than from the EQ complex. This effect is illustrated in Fig. 5. If the slowly dissociating product is added initially to the reaction mixture, the inhibition will be competitive with respect to the analogous substrate, and noncompetitive with respect to the nonanalogous substrate. If the product P, which dissociates rapidly, is added to the reaction mixture, some of the EQ complex will be converted to the EPQ complex. If, as is likely because of synergistic binding interaction between P and Q, Q dissociates more slowly from the EPQ complex than from the EQ complex, P will inhibit the reaction noncompetitively with respect to both substrates (see Fig. 6). The product inhibition effects possible when one product dissociates slowly are summarized in Table V. We see that a slow product dissociation step makes possible noncompetitive inhibition between one analogous substrate-product pair but not both. Inhibition by the slowly dissociating product will be competitive with respect to its analogous substrate. We have previously seen that inhibition by the product analogous to the slowly binding substrate will be competitive with respect to the slowly binding substrate. In general we would expect that if an enzyme is so constructed that one substrate binds slowly, the analogous product will tend to dissociate slowly. We would therefore expect that this substrate-product pair would be competitive inhibitors of each other

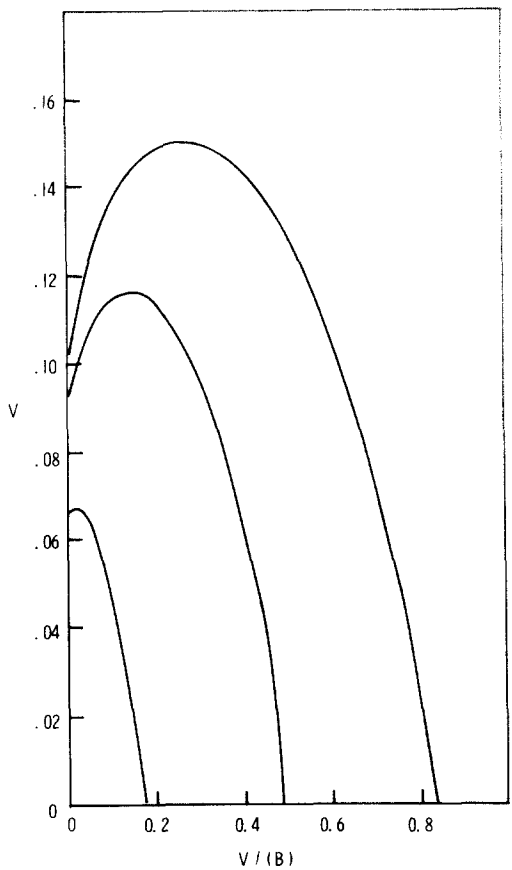


Fig. 5. Eadie plots demonstrating apparent substrate inhibition by substrate B when product Q dissociates slowly. Velocities were calculated from Eqn. 16. Assumed values of constants are: $L_{ib} = 1$, $L_b = 10$, $k_r = 10$, $k_{-iq} = 1$, $k_{-Iq} = 0.1$, $K_{Ib} = 0.1$. Lines are drawn at $[A]/L_{ia} = 0.2, 1, 5$.

even if both substrate binding and product dissociation steps were partially rate-limiting, and we would not expect to find enzymes in which inhibition by both products is noncompetitive with respect to their analogous substrates.

In summary, we may conclude that for a two substrate group transferase, if inhibition by both products is competitive with respect to their analogous substrates, the result is compatible with rapid equilibrium substrate binding, but is

TABLE V

PRODUCT INHIBITION PATTERNS, ONE PRODUCT DISSOCIATES SLOWLY

Inhibitor	Form of inhibition vs. analogous substrate	Form of inhibition vs. nonanalogous substrate
Slowly dissociating product	Competitive	Noncompetitive
Rapidly dissociating product	Noncompetitive or competitive	Noncompetitive

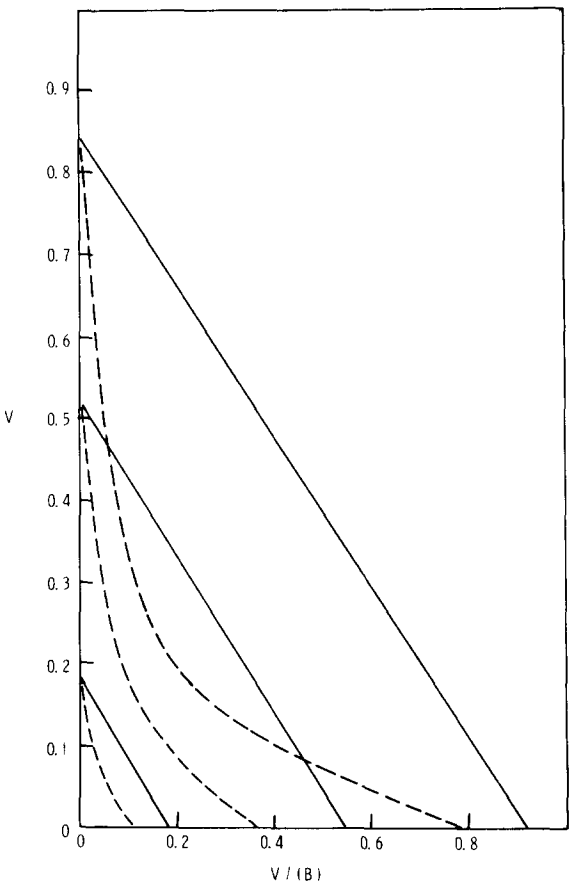


Fig. 6. Eadie plots demonstrating inhibition by product P when product Q dissociates slowly. Velocities were calculated from Eqn. 17. Assumed values of constants are: $L_{ib} = 1$, $L_b = 10$, $k_r = 10$, $k_{-iq} = 1$, $k_{-r} = 0$, $k_{-q} = 0.1$, $L_{ip}/L_p = 10$, $L_{ib}/K_{Ib} = 10$; ———, $[P]/L_{ip} = 0$; - - - - - -, $[P]/L_{ip} = 1$. Lines are drawn at $[A]/L_{ia} = 0.2, 1, 5$.

also compatible with slow binding of one substrate under certain conditions. This result is therefore not definitive. If inhibition by one product is noncompetitive with respect to its analogous substrate, and inhibition by the other product is competitive with respect to its analogous substrate, the result is not compatible with rapid equilibrium substrate binding, and the competitive substrate-product pair includes the slowly binding substrate. If inhibition by both products are noncompetitive with respect to their analogous substrates, the result is incompatible with the simple model considered here. The principles which apply to inhibition by substrate analogs which are not products are the same as which apply to inhibition by products, a fact which suggests that substrate analog inhibition experiments are unlikely to contribute important additional information if product inhibition experiments have already been carried out. "Transition state analog" inhibitors, or inhibitors with a structure which precludes the binding of either substrate to its enzyme-inhibitor complex, may provide additional information, however. If inhibition by such inhibitors is

noncompetitive with respect to a substrate, this is an indication that the other substrate binds slowly.

Discussion

Comparison with Cleland's treatment of kinetic mechanisms

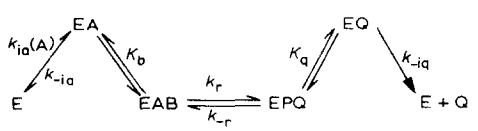
The theory presented in this paper covers much the same ground as that presented by Cleland [1,2,14,15]. In this section I will summarize the points of similarity and difference between my treatment and that of Cleland with emphasis on those respects in which Cleland's treatment is inadequate. First of all, Cleland assumes implicitly that a sharp conceptual distinction can be made between a "random mechanism" and an "ordered mechanism." I have argued that this is not so (see Table II and accompanying discussion). Also, even in those cases which closely approximate the "ordered mechanism" situation such as set VIII of Table II, the actual "order" of substrate addition will be dependent on the concentrations of the two substrates and is in no sense "compulsory." Conversely, one could imagaine (somewhat implausibly to be sure) that one of four binding steps (B to E, B to EA, A to E, A to EB) did not occur while the other three were in rapid equilibrium (sets XIV and XV of Table II). Since this latter condition would ensure equilibrium of the substrates with all enzyme-substrate complexes, the steady state kinetic properties would be indistinguishable from the rapid equilibrium case (set I of Table II) although the binding sequence would be absolutely compulsory.

The use of the word "mechanism" to describe situations which differ only with respect to relative values of some rate constants also seems inappropriate to me. For these reasons, the phrases "compulsory ordered mechanism" and "random mechanism" are highly misleading. The use of these terms appears to be an example of what has been called coercive language [16], in that they have helped to obscure rather than elucidate the meaning of enzyme kinetic experiments.

As generally used, the product inhibition criteria of Cleland [1,2] boil down to two simple rules. (1) If inhibition by each product is competitive with respect to its analogous substrate, a rapid equilibrium random mechanism is postulated. (2) If inhibition by one product is noncompetitive with respect to its analogous substrate, an ordered mechanism is postulated with the competitive substrate-product pair being the first to bind and last to dissociate.

There is roughly identification which can be made between my slow substrate binding case, and Cleland's ordered mechanism in which my slowly binding substrate is identified with Cleland's "first on" substrate. This correspondence is not entirely accidental, as can be seen by writing Cleland's "ordered bi bi mechanism" in the form below.

Scheme V: A Representation of Cleland's Ordered Bi-Bi Mechanism



Noncompetitive P vs. B inhibition in Cleland's "ordered bi bi" model arises entirely from the diversion of EQ to EPQ which is assumed not to release Q at all. My analysis suggests this may indeed occur, but suggests in addition that noncompetitive P vs. B inhibition can also sometimes occur if substrate A binds slowly even though product release is not rate limiting. Of course, in my model, competitive P vs. B inhibition is not incompatible with slow A binding and suggests the observation cannot be safely used as evidence for rapid equilibrium substrate binding.

The shorthand stick diagrams introduced by Cleland [1] to represent "kinetic mechanisms" focus one's attention on supposed compulsory sequences of substrate binding and product release, and thus have provided a neat, but, as we have seen, false picture of enzyme behavior. I suggest their use should be abandoned. Several treatments of more complex enzyme catalyzed reactions than have been considered here, e.g. those with three substrates, three products, have been advanced [15,17—21]. These treatments are supposed to tell how to distinguish "kinetic mechanisms" based principally on dubious distinctions between "ordered" and "random" sequences of substrate binding and product release. Since these treatments rest largely on false premises, it is unlikely that conclusions derived from them are correct.

Experimental approaches to the discovery of slow substrate binding steps

Although, as we have seen, observation of competitive inhibition by both products with respect to their analogous substrates is insufficient to establish rapid equilibrium substrate binding, there are a number of ways to demonstrate the existence of a slow substrate binding step. One is to measure the relative rates of isotopic exchange at equilibrium between the two analogous substrate-product pairs. If both exchange rates are not equal, it is an indication of a slow substrate binding or product release step. This method, which has been applied to a number of enzymes, has been discussed in detail elsewhere [22]. Galactokinase [23] and arginine kinase [24] are examples of enzymes with apparently "rapid equilibrium random" mechanisms by product inhibition criteria but which have unequal equilibrium isotopic exchange rates.

A slow substrate binding step will cause the kinetic parameter, K_{ib}, to be less than the dissociation constant for the EB complex. Therefore, if this dissociation constant can be measured independently and found to be much greater than K_{ib}, it is an indication that the other substrate binds slowly to the enzyme. This criterion has in fact been applied in much this way for a number of enzymes, including ornithine transcarbamylase [25], hexokinase [26,27] and citrate synthase [28]. A fairly direct method of demonstrating the lack of equilibrium between enzyme and substrate at steady state is the isotope trapping method in which labeled substrate is preincubated with enzyme, and then a mixture of unlabeled substrate and the other substrate added. If product molecules are found to be more heavily labeled than would be expected if equilibration of labeled and unlabeled enzyme-substrate complexes was achieved prior to chemical reaction, this suggests that the rate of dissociation of substrate from its enzyme-substrate complex is lower than the rate of product formation. This method has been used to show that glucose dissociates slowly from yeast hexokinase [29].

"Transition state analog" inhibitors, or at least inhibitors whose structure is such that neither substrate can bind to its enzyme-inhibitor complex, can be used to demonstrate slow substrate binding. Inhibition by such inhibitors will be competitive only with respect to the slowly binding substrate, and will be noncompetitive with respect to the other substrate (Table III). The noncompetitive inhibition by *N*-(phosphonacetyl)-L-aspartate with respect to aspartate has been used to demonstrate the non-random binding of substrates to the catalytic subunit of *Escherichia coli* aspartate transcarbamylase [30]. Interestingly, inhibition by carbamyl aspartate is noncompetitive with respect to carbamyl phosphate which suggests that an enzyme-carbamyl aspartate-carbamyl phosphate complex exists [31]. Inhibition by carbamyl aspartate with respect to aspartate is noncompetitive at pH 7 [32], competitive at pH 8 [31]. The present analysis suggests that carbamyl phosphate binds more slowly to the enzyme-carbamyl aspartate complex than to the free enzyme at pH 7, but that these two binding rates are the same at the same at pH 8.

Experimental evidence relevant to the proposed model

In this section I will review the kinetic behavior of real enzymes in order to indicate the utility of the proposed analysis as compared to the methods which are currently employed to analyze steady state kinetic and product inhibition experiments. Table III leads us to expect to find two classes of enzymes, those in which inhibition by both products is competitive with respect to their analogous substrates, and those in which inhibition by one product is competitive,

TABLE VI

PRODUCT INHIBITION PROPERTIES OF ENZYMES

This table represents a survey of the major biochemical journals for the years 1971—1974. Those two substrate group transferases, for which the form of inhibition by both products was determined, are listed below.

Inhibition by both products is competitive vs. analogous substrates	Inhibition by one of two products is noncompetitive vs. analogous substrates	Inhibition by both products is noncompetitive vs. analogous substrates
D-Mannonate oxidoreductase [33]	Phosphofructokinase [41,42]	Hexokinase [5,58]
Phosphofructokinase [34,35]	Aspartate transcarbamylase [43,44]	Phosphoribosyl pyrophosphate synthetase [59]
Arginine kinase [36]	D-Altronate-NAD-oxidoreductase [45]	Adenine phosphoribosyltransferase [60]
UDP glucuronyl transferase [37]	diacetyl reductase [46]	
Aspartate transcarbamylase [38]	β-Hydroxybutyrate dehydrogenase [47]	
Diacetyl reductase [39]	Shikimate dehydrogenase [48]	
Protein kinase [40]	Gluconokinase [49]	
	5-Aminolevulinic acid synthetase [50]	
	Uridine phosphorylase [51]	
	Thymidine phosphorylase [52]	
	1-Phosphofructokinase [53]	
	IMP dehydrogenase [54]	
	ATP-sulfate adenylyltransferase [55]	
	Sucrose synthetase [56]	
	Phosphoribosyl pyrophosphate synthetase [57]	

and the other noncompetitive with respect to their analogous substrates. Noncompetitive inhibition by both products is not expected. The result of a survey of the major biochemical journals for the four year period 1971—1974 is given in Table VI.

The only enzymes in Table VI for which noncompetitive inhibition by both products with respect to their analogous substrates has been observed are hexokinase, phosphoribosyl pyrophosphate synthetase, and adenine phosphoribosyltransferase. The "mechanism" of yeast hexokinase has been the subject of a great deal of controvery, principally between the groups of Fromm [4,5,10] who favors a "steady state random mechanism," and Ricard[3,26] who favors an "ordered mechanism." A number of observations, including the slower rate of isotopic exchange at equilibrium for the sugars than for the nucleotides [61], the weak binding of ATP to the free enzyme [26,27], and the slow dissociation of glucose from the enzyme as revealed by isotopic trapping experiments [29], suggest that glucose binds slowly to the enzyme. By current standards these observations point to an ordered mechanism with glucose binding first. We definitely would expect competitive glucose 6-phosphate vs. glucose inhibition which has indeed been observed by some workers [3]. Of the other two "deviant" enzymes, we may perhaps explain away the properties of phosphoribosyl pyrophosphate synthetase [59] as arising somehow from the fact one substrate, ribose 5-phosphate, is also an analog of part of ATP, the other substrate. The model presented in this paper cannot easily explain the published properties of adenine phosphoribosyl-transferase [60].

The largest group of enzymes in Table VI are those in which inhibition by only one of the two products is competitive with respect to its analogous substrate. This result is incompatible with rapid equilibrium binding of both substrates to the enzyme. It is likely that rapid equilibrium binding of substrates also does not occur in many of the enzymes in which competitive inhibition by both products with respect to their analogous substrates is bound. We have seen that some enzymes with this property have nonidentical isotopic exchange rates at equilibrium for the two substrate-product pairs [23,24]. Another indication of this is the fact that K_b/K_{ib} ratios of near or greater than unity are often observed. Highly synergistic binding interactions between substrates and substrate analogs have been regularly observed in enzymes. Some examples are lyxose-ATP in yeast hexokinase [27], succinate-carbamyl phosphate in aspartate transcarbamylase [62], norvaline-carbamyl phosphate in ornithine transcarbamylase [25], as well as NADPH-glutamate [63] and NADPH-α-ketoglutarate [64,65] in glutamate dehydrogenase and adenosine-phenylalanine and ATP-phenylalanine in phenylalanyl-tRNA synthetase [66]. Reasoning by analogy, we might expect that the substrate-substrate binding interaction is highly synergistic for most enzymes, i.e. L_b/L_{ib} is low. (See, however, ref. 66). If K_b/K_{ib} is very high, it probably largely reflects the ratio, k_r/k_{-ia}. Recall that if substrate A binds slowly, $K_b/K_{ib} = L_b/L_{ib} + k_r/k_{-ia}$ (Table I).

Experimental tests of proposed model

The existence of enzymes whose properties cannot be simply explained by the theory proposed in this paper suggests that it would be valuable to have experimental tests of its validity in cases where the steady state kinetic and pro-

duct inhibition properties can be interpreted in terms of the proposed theory. A key feature of the proposed theory is the postulation of slow steps associated with the binding interaction of substrates and inhibitors with enzymes. A good experimental test of the ideas set forth in this paper would therefore be direct measurements of the rate of formation of enzyme-substrate complexes. If we assume one substrate binds slowly and product release is not rate-limiting, then the following relationship should hold (Table I).

$$k_{-ia} = \frac{k_r}{(K_b/K_{ib})[1 - K_{ib}/L_{ib}]} \tag{18}$$

The constants K_b and K_{ib} can be obtained from steady state kinetics, k_r can be obtained from a combination of steady state kinetics and the determination of the molecular weight per active site, L_{ib} can be obtained from an equilibrium binding experiment, and k_{-ia} can be obtained by measuring the rate of enzyme-substrate complex formation. Although all of these measurements have been carried out on various enzymes, rate of binding experiments have normally been carried out in different laboratories, often under different experimental conditions than steady state kinetic experiments. I was unable to locate a single example in the literature of data of sufficient quality and self-consistency to test by the use of Eqn. 18. A related test of the theory proposed in this paper would be to measure the relative rates of formation of the EA complex from E and the EAP complex from EP for enzymes in which substrate A binds slowly. I would expect competitive P vs. B inhibition to be associated with equal rates of binding of A to E and to EP, and noncompetitive P vs. B inhibition to be associated with a faster rate of binding of A to E than A to EP.

Acknowledgement

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