Biochemical Pharmacology, Vol. 24, pp. 2177–2185. Pergamon Press, 1975. Printed in Great Britain.

TIGHT-BINDING INHIBITORS—I

KINETIC BEHAVIOR*

SUNGMAN CHA

Divison of Biological and Medical Sciences, Brown University, Providence, R.I. 02912, U.S.A.

(*Received* 30 *December* 1974; *accepted* 21 *February* 1975)

Abstract—Various aspects of the kinetic behavior of the interaction between tight-binding ligands and macromolecules have been examined. The relationships between 50 per cent inhibitory concentration and dissociation constants of the complex are shown for various mechanisms of inhibition, and several methods for the estimation of very low K_i values are presented. The properties of the Ackermann–Potter plot are examined, and precautions are offered for the usage of the term stoichiometric or titrating inhibition. Both association and dissociation processes between a tight binder and a macromolecule are slow under ordinary laboratory conditions, and steady state rate equations are not applicable. In the presence of a tight-binding inhibitor, the initial velocity of an enzymic reaction depends on the order of addition of the components, and the extent of lag period can be used for the calculation of the rate constant for the slowest step and for diagnosis of the inhibition mechanism. It is also pointed out that the Lineweaver–Burk analysis of competition between two ligands may lead to erroneous conclusions unless the slope of the plot is carefully examined.

Compounds that bind tightly to macromolecules, such as enzymes, drug or hormone receptors or carrier proteins, are often valuable as drugs or as research tools. Since Straus and Goldstein [1] and Goldstein [2] dealt with the effect of binding on the depletion of free inhibitors, many authors have considered the kinetics of tight-binders from various points of view [1–11]. Morrison [9] derived the steady state rate equations for multi-substrate reactions in the presence of tight-binding inhibitors, and showed that the usual double reciprocal plot by the method of Lineweaver and Burk is non-linear. Because of this non-linearity, the analysis of kinetic data by conventional methods is complicated. Henderson [11] presented a linear equation that describes the steady state kinetics of enzymes with tight-binding inhibitors, and more recently Cheng and Prusoff [12] and Chou [13] analyzed independently the relationship between I_{50} (50 per cent inhibitory concentration of an inhibitor) and the inhibition constants for various enzymic reaction mechanisms.

The significance of I_{50}, the time-course of inhibited reactions, the theoretical bases for determining low dissociation constants and some kinetic problems that may be encountered in the study of high affinity substances that bind tightly to non-enzymic proteins, such as receptors, are described in this paper.

THEORY

Symbols and definitions

E: enzyme or other macromolecule, or the molar concentration (or molar equivalent in the case of multivalent enzyme) of the free form (i.e. unbound to substrate or inhibitor).

E_t: total enzyme (or macromolecule) concentration (M or molar equivalent).

E_{50}: total enzyme concentration at which 50 per cent of the total inhibitor is in the bound form.

I: inhibitor (or ligand), or its concentration.

I_t: total inhibitor concentration, i.e. sum of free and bound inhibitor concentration.

I_{50}: total inhibitor concentration at which the enzyme reaction velocity is 50 per cent of the uninhibited reaction.

( ): the parenthesis is used to denote the molar (or equivalent) concentration only when ambiguities may arise.

v, K_m, V: enzyme reaction velocity, Michaelis constant, and maximal velocity respectively.

k_x: k_1, k_2, etc. are the first- or second-order rate constants. Odd and even number subscripts are assigned for the forward and backward rate constants respectively.

k': an exponential decay constant defined for each individual case.

v_0, v_i, v_z, v_s: enzyme reaction velocity in the absence of inhibitor, in the presence of inhibitor, at zero time, and at steady state respectively. These symbols are used only when distinction is necessary.

K_i: inhibition constant or the dissociation constant of EI complex (even when

* This work was supported by USPHS Grants CA 12531 and CA 13943. Mathematical proofs of some statements and derivations of various equations will be made available upon request.

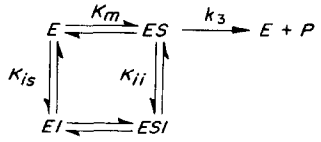
E is not an enzyme). For complicated inhibition mechanisms, the definitions are presented individually.

K_{is}, K_{ii}: inhibition constants estimated from the slope and the intercept of a Lineweaver–Burk plot as defined by Cleland [14]; dissociation constants of EI and ESI (or EIS) complexes respectively. Replaced by K_i when the distinction between K_{is} and K_{ii} is unnecessary.

The terminology of Cleland [14] was employed for inhibition mechanisms. Competitive and uncompetitive inhibitions were treated as limiting cases of non-competitive inhibition where $K_{ii} = \infty$ and $K_{is} = \infty$ respectively. Thus, 'non-competitive inhibition' and 'mixed inhibition' as defined by Dixon and Webb [15] correspond to non-competitive inhibition where $K_{is} = K_{ii}$ and $K_{is} < K_{ii}$ respectively.

Steady state rate equation and I_{50}

For simple mechanisms of inhibition, Henderson [11] has derived a linear steady state rate equation, based on steady state assumptions, which accounts for the depletion of both free enzyme and free inhibitor by binding. A general non-competitive inhibition mechanism may be represented as:



(1)

The symbol K_i is used for inhibition constant where distinction between K_{is} and K_{ii} is unnecessary, e.g. when $K_{is} = K_{ii}$, $K_{ii} = \infty$, or $K_{is} = \infty$.

The generalized inhibition equation presented by Henderson, which accounts for the depletion of free inhibitor by binding, may be written as:

$$\frac{I_t}{\left(1 - \frac{v_i}{v_0}\right)} = E_t + \left(\frac{\frac{S + K_m}{K_m}}{\frac{K_{is}}{S} + \frac{S}{K_{ii}}}\right)\frac{v_0}{v_i} \qquad (2)$$

A plot of $I_t/(1 - v_i/v_0)$ vs v_0/v_i (Henderson plot) yields a straight line.

In the special case of competitive inhibition, K_{ii} may be regarded as infinite, and K_i may be replaced for K_{is}. Therefore, the Henderson plot yields:

$$\text{Slope} = K_i\left(1 + \frac{S}{K_m}\right) \quad \text{and} \quad \text{intercept} = E_t \qquad (3)$$

For the classical non-competitive inhibition, where K_{is} equals K_{ii}, and both may be replaced by K_i, the plot yields:

$$\text{Slope} = K_i \quad \text{and} \quad \text{intercept} = E_t \qquad (4)$$

It should be pointed out that the Henderson equation for the classical non-competitive inhibition (Eq. 4) is identical to that of Easson and Stedman [16]. For uncompetitive inhibition, where $K_{is} = \infty$, and $K_i = K_{ii}$, the plot yields:

$$\text{Slope} = K_i\left(1 + \frac{K_m}{S}\right) \quad \text{and} \quad \text{intercept} = E_t \qquad (5)$$

Henderson has proposed the use of equation 2 for estimating E_t and the inhibition constants, as well as for distinguishing the type of inhibition. In the field of pharmacology, however, the parameter I_{50} has been extensively used for the analyses of structure–activity relationships and for comparative studies of enzymes, e.g. measurement of the relative potency of folate analogs as inhibitors of dihydrofolate reductases from various species [17, 18]. Detailed analyses of I_{50} for the cases where the depletion of free inhibitor due to binding to enzyme is negligible were published recently by Cheng and Prusoff [12] and by Chou [13]. The equations derived by those authors have now been extended to accommodate tight-binding inhibitors.

From equations 2–5 it follows that

$$I_{50} = \tfrac{1}{2}E_t + \frac{S + K_m}{\frac{K_m}{K_{is}} + \frac{S}{K_{ii}}} \qquad \text{Non-competitive} \qquad (6)$$

$$I_{50} = (\tfrac{1}{2}E_t + K_i) + \frac{K_i S}{K_m} \qquad \text{Competitive} \qquad (7)$$

$$I_{50} = (\tfrac{1}{2}E_t + K_i) + \frac{K_i K_m}{S} \qquad \text{Uncompetitive} \qquad (8)$$

$$I_{50} = \tfrac{1}{2}E_t + K_i \qquad \text{Non-competitive where } K_{is} = K_{ii} \qquad (9)$$

Note that these equations can be readily derived from the Henderson equation (equation 2) and that they are identical with those derived by others [12–13] except for the additional term $(\frac{1}{2}E_t)$, which accounts for the depletion of free inhibitor by binding. By the use of these equations, one may distinguish among different types of inhibition and estimate K_i and E_t by determining I_{50} values at several levels of S and E_t. Although they do not offer any intrinsic advantages over the equation of Henderson, the above equations can be helpful in the evaluation of data published in terms of I_{50}. Graphical analyses of equations 6–9 are simple. For competitive inhibition, the plot of I_{50} against S gives a straight line with the I_{50}-intercept of $(\frac{1}{2}E_t + K_i)$ and a slope equal to K_i/K_m according to equation 7. Determination of such intercepts at two or more levels of E_t will provide K_i and E_t. For uncompetitive inhibition (equation 8), a similar analysis can be performed with plots of I_{50} against $1/S$. For the classical non-competitive inhibition, I_{50} is independent of the substrate concentration (equation 9). The plot of I_{50} against S for non-competitive inhibition (equation 6) is non-linear. I_{50} may be determined easily by either a plot of v_i/v_0 against I_t or a plot of v_0/v_i against I_t.

As will be presented in detail below, the usage of equations derived on the basis of steady state assumptions (e.g. equations 2, 6–9) can be grossly inadequate in the study of tight-binders, because steady state conditions may not be reached between the enzyme, the fast-reacting substrate and the slow-reacting inhibitor. In fact, if the enzyme is preincubated with a tight-binding inhibitor, the observed I_{50} values are independent of the substrate concentration regardless of the inhibition mechanism. Thus, equation 9 becomes applicable for all inhibition mechanisms.

Determination of low dissociation constants from E_{50}

The determination of enzymic activity of I_{50} in the presence of a tight-binding inhibitor is essentially a measurement of the concentration of unbound free enzyme. However, occasionally it is more convenient to determine the bound or unbound ligand rather than the enzymic activity. This is especially true when the molecules under study are not enzymes, e.g. binding of a hormone to a receptor protein. When a ligand binds tightly to a macromolecule which may or may not be an enzyme, it may be possible, through methods such as gel filtration or adsorption, to separate the complex from the free ligand without significant loss by dissociation during the isolation procedure. Sometimes, the concentration of free ligand or complex may be determined by differences in light absorption or fluorescence, e.g. the determination of the concentration of the complex formed between glutamate aspartate transaminase and erythro-β-hydroxy-L-aspartate as described by Jenkins [19]. When one measures changes in the free ligand concentrations, the dissociation constant can be estimated by determining E_{50} at different concentrations of I, according to the following equation.

$$E_{50} = K_i + I_t/2 \tag{10}$$

where K_i is the dissociation constant of EI complex. Equation 10 is formally identical to that of Jenkins [19]. A plot of E_{50} against I_t gives K_i as the intercept on the E_{50}-axis and the extension of the straight line intersects the I_t-axis at $-2K_i$. When the molar equivalent concentration of E is not known, it may be determined from the I_t-intercept. It is obvious that this method will be useful and accurate only when both E_t and I_t are of the same order of magnitude as K_i.

Estimation of K_i *from the Lineweaver–Burk plot*

Occasionally the concentration of the EI complex can be measured in the absence of substrate, e.g. by physical isolation of isotopically labeled inhibitors bound to the macromolecule, or by measuring the difference in the light absorption spectrum or fluorescence. Under such circumstances, the concentrations of the EI complex at equilibrium at various values of I_t may be used for the determination of K_i. The saturation equation is:

$$(EI) = \tfrac{1}{2}(K_i + I_t + E_t - \sqrt{[(K_i + I_t + E_t)^2 - 4I_tE_t]}) \tag{11}$$

The double reciprocal plot ($1/EI$ vs $1/I_t$) of this equation is a concave upward hyperbola (equations 5, 9), which has an asymptote:

$$\frac{1}{(EI)} = \frac{1}{E_t\left(1 + \dfrac{E_t}{K_i}\right)} + \frac{K_i}{I_t}\left(\frac{1}{E_t} + \frac{1}{K_i}\right) \tag{12}$$

This asymptote intersects the $1/(EI)$-axis and $1/I_t$-axis at $1/E_t(1 + E_t/K_i)$ and $-1/K_i(1 + E_t/K_i)^2$ respectively [5]. If we define $K_i(1 + E_t/K_i)^2$ as appK_i:

$$\text{app}K_i = \sqrt{K_i} + E_t/\sqrt{K_i} \tag{13}$$

Therefore, a plot of $\sqrt{\text{app}K_i}$ vs E_t yields $\sqrt{K_i}$ from the intercept on the $\sqrt{\text{app}K_i}$-axis.

Ackermann–Potter plot and stoichiometric binding

The steady state rate equation for simple competitive inhibition which accounts for depletion of free inhibitor [9, 20] may be written as:

$$v = \frac{k_3S}{2(K_m + S)}\left\{-\left[K_i\left(1 + \frac{S}{K_m}\right) + I_t - E_t\right] + \sqrt{\left(\left[K_i\left(1 + \frac{S}{K_m}\right) + I_t + E_t\right]^2 - 4I_tE_t\right)}\right\} \tag{14}$$

where k_3 is the first-order rate constant for the reaction, $ES \rightarrow E + P$. If the value of S is much greater than K_m, this equation becomes identical to that derived by Ackermann and Potter [20].

$$v = \frac{k_3}{2}\left[-\left(\frac{K_iS}{K_m} + I_t - E_t\right) + \sqrt{\left\{\left(\frac{K_iS}{K_m} + I_t + E_t\right)^2 - 4I_tE_t\right\}}\right] \tag{15}$$

The plot of v against E_t at various levels of I_t, known as the Ackermann–Potter plot [20], is often used to demonstrate the so-called pseudo-irreversible, titrating or stoichiometric inhibition. The usual experimental procedure is to preincubate various amounts of enzyme with the inhibitor for a sufficiently long period of time, then to assay an aliquot of the mixture for enzymic activity at a given concentration of the substrate. Under these experimental conditions, the enzyme in the form of EI complex would not contribute to the observed enzymic reaction velocity because of the slow dissociation of the EI complex. Therefore, the above steady state equations (equations 14 and 15) do not hold. Instead, a rate equation more suitable for the experimental conditions is:

$$v = \frac{k_3S}{2(K_m + S)}\left[-(K_i + I_t - E_t) + \sqrt{\{(K_i + I_t + E_t)^2 - 4I_tE_t\}}\right] \tag{16}$$

As illustrated in Fig. 1, the plot of v against E_t at various levels of I_t according to equation 16 is a curve having an asymptote (a linear portion) described by the following equation:

$$v = \left(\frac{k_3S}{K_m + S}\right)E_t - \frac{k_3I_tS}{K_m + S} \tag{17}$$

This asymptote intersects the E_t-axis at $E_t = I_t$ and the v-axis at $v = -k_3I_tS/(K_m + S)$. As noted by Ackermann and Potter [20], since I_t, S and K_m are usually known values, the slope or intercept permits ready evaluation of the catalytic number, k_3 and the molar equivalency of E_t. If the assay is performed at a substrate concentration much greater than K_m, the slope and intercept of the plot of v vs E_t reduce to k_3 and k_3I_t, respectively, thus simplifying the evaluation of the molar equivalency of E_t and k_3.

Equation 16 indicates that the velocity is not zero in the presence of a stoichiometric amount of inhibitor; therefore, the term 'titrating inhibitor' must be used with caution. Figure 2 illustrates that a tight-binding inhibitor resembles a titrating inhibitor only when the concentration of E_t is very high compared

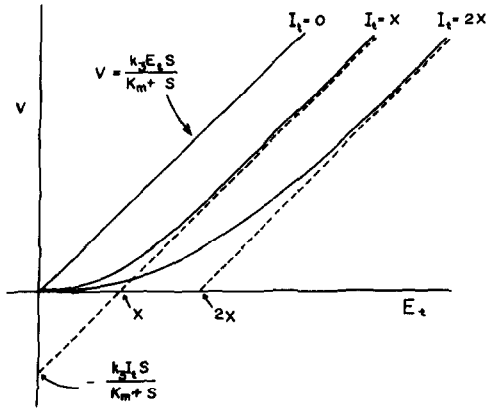


Fig. 1. Schematic illustration of Ackermann–Potter plot according to equation 16.

to K_i or when E_t is much greater than I_t. In fact, the fraction of inhibitor bound to the enzyme cannot exceed $E_t/(K_i + E_t)$. This means that if the enzyme concentration is at the K_i value, the enzyme cannot bind more than 50 per cent of inhibitor, and that for an inhibitor to be regarded as 'stoichiometric', the K_i value must be much lower than the enzyme concentration. Therefore, in interpreting reactions of tight-binding inhibittors *in vivo*, one should not assume stoichiometric titration of the enzyme unless the local concentration of the enzyme is known to be much greater than K_i and the inhibitor concentration, or that the binding is truly irreversible as occurs with covalent binding, e.g. the inactivation of acetylcholinesterase by an alkylphosphate inhibitor.

General consideration of reaction rate with tight-binders

Consider the binding of a compound, I, to a macromolecule, E,

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \qquad (18)$$

When the binding is very tight, in other words when K_i, i.e. k_2/k_1, is very small (e.g. less than 10^{-9} M), it follows that both the dissociation and association reactions are very slow under the conditions commonly used for enzymic assays for the following

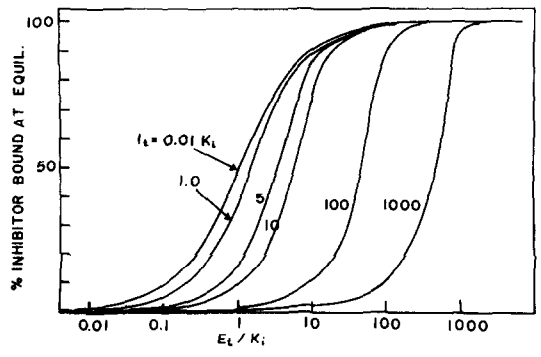


Fig. 2. Calculated values of per cent inhibitor bound at equilibrium at various concentrations of the enzyme and inhibitor. The values of $(EI)/I_t$ were calculated as a function of E_t/K_i according to equation 11.

reasons. K_i will be very small when k_2 is very small or k_1 is very large; but k_1, the second-order rate constant, has its inherent upper limit set by the diffusion rates of the interacting molecules. Mahler and Cordes [21] listed k_1 values for 31 pairs of enzymes and substrates, which range from $1{\cdot}2 \times 10^4$ to $10^9\ M^{-1}\ sec^{-1}$, whereas the values of k_2 have the much wider range of 10^{-4} to $4{\cdot}5 \times 10^4\ sec^{-1}$. To illustrate the significance of these parameters, let us consider a hypothetical enzyme with a tight-binding inhibitor having the kinetic parameters:

$$k_1 = 10^7\ M^{-1}\ sec^{-1}, \text{ and } K_i = 10^{-10}\ M.$$

Then

$$k_2 = (K_i)k_1 = 10^{-10}(M) \times 10^7(M^{-1}\ sec^{-1}) = 10^{-3}\ sec^{-1} \qquad (19)$$

Therefore, the half-time ($T_{1/2}$) for dissociation is $0{\cdot}693/k_2$ or 11·6 min.

On the other hand, the rate of association reaction is $k_1(I)(E)$, a second-order reaction with regard to (E). However, if (I_t) is much larger than (E_t) throughout the reaction, (I) does not differ very much from (I_t); therefore, $k_1(I) \cong k_1(I_t)$. Thus, the association reaction may be considered as pseudo-first-order with regard to E with a rate constant of $k_1(I_t)$. In the above example, assume (I_t) is 10^{-9} M, ten times greater than K_i, and that (E_t) is in the order of K_i or less. Then, the pseudo-first-order rate constant may be calculated as:

$$k_1(I_t) = 10^7(M^{-1}\ sec^{-1}) \times 10^{-9}(M) = 10^{-2}\ sec^{-1} \qquad (20)$$

Therefore, the half-time for association is 1·2 min. Since $k_1(I_t) \gg k_2$, initially the reaction resembles irreversible binding, and the concentration of E changes according to

$$E = E_t \cdot e^{-k_1(I_t)t} \quad \text{or} \quad \log(E) = \log(E_t) - \frac{k_1(I_t)}{2{\cdot}3} \cdot t \qquad (21)$$

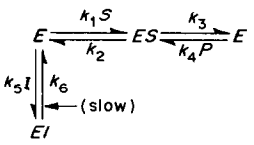
Therefore, in a plot of log(E) vs time, the pseudo-first-order velocity constant $k_1(I_t)$ is readily determined from the slope, i.e. $-k_1(I_t)/2{\cdot}3$. On the other hand, k_2 may be estimated by calculation from known values of K_i and k_1 as in equation 16 or experimentally by isolating highly concentrated EI complex free of I followed by sufficient dilution to permit determination of free E as it is liberated from EI. If sufficient dilution to minimize the re-formation of EI by the forward reaction is not practical, it may be possible to "trap" or to remove free I as it is released from EI, e.g. by adsorption of I on charcoal, or to "trap" E in the form of an ES complex by the addition of excess substrate. When a tight-binding inhibitor is studied, its concentration is of necessity kept low so that the ratios of I/K_i and S/K_m are of the same order of magnitude. Otherwise the degree of inhibition is too little or too much for study. Under such circumstances, as the above hypothetical example illustrates, both the association and dissociation processes between E and I are very slow in comparison to ordinary enzyme–substrate interactions which usually take place within a fraction of a second. Therefore, it becomes apparent that in the presence

of tight-binding inhibitors at a very low concentration, the reaction between the substrate and enzyme may be at the steady state, but that between the enzyme and inhibitor may be still at a pre-steady state (or transient phase) even after the substrate is almost completely depleted. Therefore, the ordinary steady state rate equations become grossly inadequate to describe these reactions. On the basis of these arguments, the time-course of enzymic reactions in the presence of a tight-binding inhibitor will be examined in the following section.

Time-course of enzymic reaction in the presence of tight-binding competitive or non-competitive inhibitor

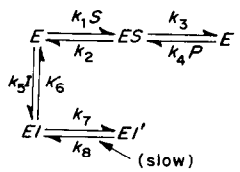
Frieden [22] pointed out that a lag period should be observed in an enzymic reaction if the free enzyme undergoes a slow conformational change and if the equilibrium favors the less active form. He termed this phenomenon the hysteretic property. A similar phenomenon can take place with tight-binding inhibitors as will be shown below. Although such phenomena should occur regardless of the inhibition mechanism, only a few simple types of inhibition will be dealt with for the sake of mathematical simplicity. In addition to classical competitive inhibition, two other competitive mechanisms involving conformational changes of either the free enzyme or the *EI* complex as well as the non-competitive mechanism will be considered.

Mechanism A, competitive inhibition



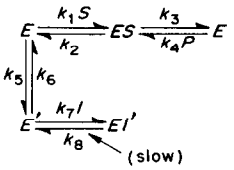
(22)

Mechanism B, competitive inhibition with conformational change of *EI* complex



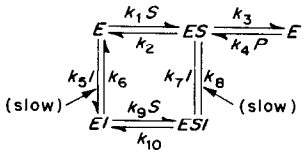
(23)

Mechanism C, Monod–Wyman–Changeux-type competitive inhibition



(24)

Mechanism D, non-competitive inhibition



(25)

In all cases, I is assumed to be a tight-binding inhibitor. In mechanism A, according to the argument developed above, k_5I and k_6 are much smaller than k_1S and k_2. Therefore, a near approach to steady state is achieved much more quickly between E and ES than between E and EI. In mechanism B, k_5I and k_6 may not be very different from k_1S and k_2, but I is a tight binder by virtue of EI undergoing to a slow conformational change to EI' with the equilibrium constant strongly favoring the formation of EI'. In mechanism C, it is assumed that the enzyme exists in two forms, an active form E and an inactive form E', that I is accessible only to E' and that the equilibrium between EI and EI' lies far toward EI'. This mechanism is the simplest example of the Monod *et al.* [23] inhibition model. In mechanism D, which is a general non-competitive inhibition, both the association and dissociation of the inhibitor are assumed to be very slow compared to the reactions of the substrate.

In a series of papers, Hijazi and Laidler [24–27] derived non-steady state (or transient phase) rate equations for enzymic reactions in the presence or absence of an activator or an inhibitor. Vassent [28] also examined extensively the time dependence of ligand binding. However, the transient phase rate equations for enzymic reactions in the presence of a tight-binding inhibitor become much simpler than those derived by these authors, if an assumption is made that a steady state between E and S and a non-steady state between E and I exist according to the arguments developed above. For the sake of mathematical simplicity, a further assumption will be made that depletion of the free inhibitor by binding is negligible, which may or may not be true depending on the relative magnitudes of (I_t), (E_t) and K_i. Under these assumptions, a time-dependent rate equation for mechanism A may be derived as follows:

$$E + ES + EI = E_t \tag{26}$$

$$(S)(E) - K_m(ES) = 0 \tag{27}$$

$$-k_5(I)(E) + k_6(EI) = \frac{d(E)}{dt} = \frac{K_m}{S}\frac{d(ES)}{dt} \tag{28}$$

Solution of these simultaneous equations yields:

$$v = v_s + [v_z - v_s]e^{-k't} \tag{29}$$

where $k' = k_6(1 + I/K_i + S/K_m)$, $K_i = k_6/k_5$, $K_m = (k_2 + k_3)/k_1$, v_s = velocity at overall steady state and v_z = initial velocity. This equation is identical to that derived by Frieden [22] for hysteretic enzymic reactions except for the expression of k'. The same equation may also be derived for other mechanisms except that the significance of k' is different, as shown in Table 1.

In all four mechanisms, if an overall steady state is ever reached before significant depletion of the substrate takes place, the steady state rate (v_s) is the same regardless of the order of addition of the reaction components, and double reciprocal plots should give the classical competitive inhibition pattern for mechanisms A, B, and C, and the non-competitive pattern for mechanism D.

For mechanisms A, B and C,

$$v_s = \frac{V\dfrac{S}{K_m}}{1 + \dfrac{I}{K_i} + \dfrac{S}{K_m}} \tag{30}$$

Table 1. Comparison of different competitive and non-competitive inhibition mechanisms

	Mechanism A	Mechanism B	Mechanism C	Mechanism D
Assumptions	$K_m \gg K_i$ $k_2 \gg k_6$ $k_1S \gg k_5I$	$K_m \gg K_i$ $k_2 \gg k_7$ $k_2 \gg k_8$	$K_m \gg K_i$ $k_2 \gg k_6 \gg k_5$	$K_m \gg K_i$ $k_2 \gg k_6$, $k_2 \gg k_8$ $k_1S \gg k_5I$, $k_1S \gg k_7I$
Definitions				
K_m	$(k_2 + k_3)/k_1$	$(k_2 + k_3)/k_1$	$(k_2 + k_3)(k_5 + k_6)/k_1k_6$	$K_m = (k_2 + k_3)/k_1$, $K'_m = k_{10}/k_9$
K_i	k_6/k_5	$k_6k_8/k_5(k_7 + k_8)$	$(k_5 + k_6)k_8/k_5k_7$	$K_{is} = k_6/k_5$, $K_{ii} = k_8/k_7$
k'	$k_6\left(1 + \frac{I}{K_i} + \frac{S}{K_m}\right)$	$\frac{k_8\left(1 + \frac{I}{K_i} + \frac{S}{K_m}\right)}{\frac{k_5I}{k_6}}$	$\frac{k_8(k_5+k_6)\left(1+\frac{I}{K_i}+\frac{S}{K_m}\right)}{k_5}$	$\frac{\left(k_5+\frac{k_7S}{K_m}\right)I}{1+\frac{S}{K'_m}} + \frac{k_6+k_8\frac{S}{K'_m}}{1+\frac{S}{K'_m}}$
Diagnostic plots				
k' vs S	Linear	Linear	Linear	Hyperbola
k' vs I	Linear	Hyperbola	Linear	Linear

and for mechanism D

$$v_s = \frac{V\frac{S}{K_m}}{1 + \frac{I}{K_{is}} + \left(1 + \frac{I}{K_{ii}}\right)\frac{S}{K_m}} \tag{31}$$

In contrast to this, however, the initial velocity, v_z, would be the same as the uninhibited reaction if the reaction is started with the enzyme, because no significant amount of EI would have been formed initially. Therefore:

$$v_z = \frac{V\frac{S}{K_m}}{1 + \frac{S}{K_m}} \tag{32}$$

However, if the enzyme is preincubated with the inhibitor before the addition of the substrate, the reaction velocity observed will be due to the unbound free enzyme alone and any contribution by enzymes in the EI complex to the reaction velocity would be negligible initially, because the rate of dissociation of the EI complex is too slow. Therefore, the inhibitor would appear to have completely inactivated a certain fraction of enzyme. Thus, the double reciprocal plot would yield a family of straight lines that intersect at one point on the $1/S$-axis. This pattern would resemble that of classical non-competitive inhibition as defined by Dixon and Webb [15]. However, close examination of these double reciprocal plots will reveal a fundamental difference. The initial velocity when the enzyme and inhibitor are equilibrated before the addition of substrate, with the depletion of free inhibitor taken into account, can be shown as follows:

$$v_z = \frac{V\frac{S}{K_m}}{\left(\frac{K_i + I_t - E_t + \sqrt{[(K_i + I_t + E_t)^2 - 4I_tE_t]}}{2K_i}\right) \times \left(1 + \frac{S}{K_m}\right)} \tag{33}$$

Unlike the classical non-competitive inhibition, the intercept of the double reciprocal plot of equation 33 is not a linear function of I_t. A plot of the intercept vs I_t is a hyperbola with an asymptote which fits the equation, intercept $= I_t/K_iV + (K_i - E_t)/K_iV$, and intersects the I_t-axis at $E_t - K_i$. Therefore, unless $E_t \ll K_i$, at least two such I_t-intercepts representing different levels of E_t are required for the estimation of K_i. In order to obtain a meaningful I_t-intercept of the asymptote of the hyperbola on the replot, a large number of experimental points covering a sufficiently wide range of values of I_t is also required. A plot of the I_t-intercepts against I_t is a straight line intersecting the I_t-intercept-axis at $-K_i$ and E_t-axis at K_i, enabling the estimation of both K_i and the molar equivalency of E_t.

Determination of k′ *and differentiation of mechanisms*

Integration of equation 29 permits evaluation of formation of the reaction product, P, as a function of time.

$$P = v_st - \frac{1}{k'}(v_s - v_z)(1 - e^{-k't}) \tag{34}$$

As illustrated in Fig. 3, a concave upward curve will be obtained if the enzyme is preincubated with the inhibitor and the reaction is started by adding the substrate (curve A). Conversely a concave downward curve is seen when the reaction is started by adding the enzyme (curve B). If one extends the linear portion of curve A in Fig. 4 to the t-axis, and that of curve B to the P-axis, the intercepts τ_0 and π_0 have the following expressions:

$$\tau_0 = \frac{v_s - v_z}{v_sk'} \quad \text{therefore} \quad k' = \frac{1}{\tau_0}\frac{v_s - v_z}{v_s} \tag{35}$$

$$\pi_0 = \frac{v_z - v_s}{k'} \quad \text{therefore} \quad k' = \frac{1}{\pi_0}(v_z - v_s) \tag{36}$$

From these equations, k' can be determined experimentally. Note that v_z in curve B is equal to v_0, the uninhibited velocity.

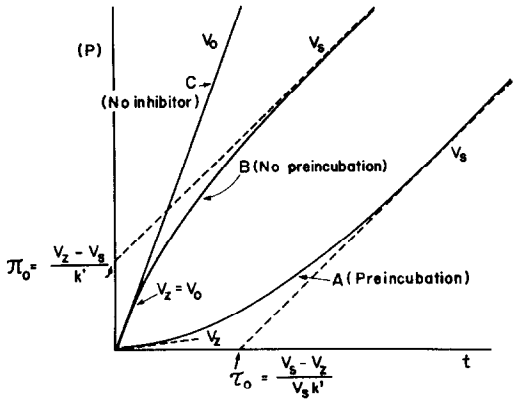


Fig. 3. Schematic illustration of lag periods in the rate of formation of product in an enzymatic reaction in the presence of a tight-binding inhibitor according to equation 34. Curve A, reaction curve for the case where the enzyme is preincubated with the inhibitor and the reaction is started with addition of the substrate; curve B, the reaction is started by adding the enzyme in the mixture containing both the substrate and inhibitor; and curve C, the control in the absence of the inhibitor.

Theoretically, the value of k' can also be determined from a single dynamic assay of the reaction velocity by the use of a plot of the logarithmic form of equation 29.

$$\log \frac{v - v_s}{v_z - v_s} = \frac{-k't}{2{\cdot}30} \tag{37}$$

As can be seen from the expression of k' in Table 1, some of the different mechanisms can be distinguished from the plot of k' vs S and that of k' vs I. It must be pointed out that the significance of k' listed in Table 1 holds true only when the depletion of free inhibitor by binding is negligible. Therefore, for this approach to be valid, the enzyme concentration must be an order of magnitude or more lower than the inhibitor concentration, which in turn must be kept sufficiently low with regard to K_i so that the enzyme is not completely inhibited (see Fig. 2). Furthermore, the value of v_s is required for estimating the value of k', but if K_i is very low, a steady state between the inhibitor and enzyme may not be attained before a significant amount of the substrate is converted to the product, so that one may not be able to measure meaningful v_s. Because of these limitations, this non-steady state approach measuring k'

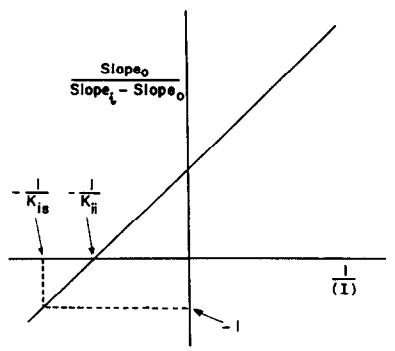
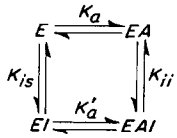


Fig. 4. Graphical method for estimating K_{is} and K_{ii} for the hyperbolic compeitive inhibition (equation 40).

diminishes in usefulness if K_i is much lower than the optimal concentration of the enzyme for assay.

Competition between two ligands

When two ligands A and I (not necessarily tight-binders) bind to a macromolecule E (not necessarily an enzyme), and if the same EAI complex is formed regardless of the order of addition of A and I, the general reaction scheme may be written as follows:



(38)

where K_a, K'_a, K_{is} and K_{ii} are the dissociation constants of the individual steps. In the classical type of competition, K'_a and K_{ii} are considered as infinite, so that EAI is never formed. If the assay method employed permits the determination of the sum of EA and EAI but not the individual species, and if A and I are in the great excess of E_t so that depletions by binding can be neglected, a generalized equation may be derived for scheme 38 by a previously published procedure [14].

$$\frac{A_{\text{bound}}}{E_t} = \frac{EA + EAI}{E + EA + EI + EAI} = \frac{\frac{A}{K_a}\left(1 + \frac{I}{K_{ii}}\right)}{1 + \frac{I}{K_{is}} + \frac{A}{K_a}\left(1 + \frac{I}{K_{ii}}\right)} \tag{39}$$

The reciprocal form is

$$\frac{1}{A_{\text{bound}}} = \frac{K_a}{E_t}\left(\frac{1 + \frac{I}{K_{is}}}{1 + \frac{I}{K_{ii}}}\right)\frac{1}{A} + \frac{1}{E_t} \tag{40}$$

This equation indicates that even when A and I are non-competitive in the classical sense, the double reciprocal plot ($1/A_{\text{bound}}$ vs $1/A$) will give an apparent competitive pattern, i.e. lines intersecting on the y-axis. However, in such a case a plot of slope vs (I) gives a hyperbola rather than a straight line. In the case of true competitive inhibition of the binding, $K_{ii} = \infty$, therefore, a plot of slope (of the Lineweaver–Burk plot) vs (I) will yield a straight line with y- and x-intercepts at $1/E_t$ and $-K_i$ respectively.

Both values of K_{is} and K_{ii} can be estimated according to the procedure suggested by Cleland [14]. From equation 40:

$$\text{Slope} = \frac{K_a\left(1 + \frac{(I)}{K_{is}}\right)}{E_t\left(1 + \frac{(I)}{K_{ii}}\right)} \tag{41}$$

Therefore,

$$\frac{\text{Slope}_0}{\text{Slope}_i - \text{Slope}_0} = \frac{K_{is}}{K_{ii} - K_{is}}\left(1 + \frac{K_{ii}}{(I)}\right) \tag{42}$$

where Slope_0 is the slope of the straight line on the

Lineweaver–Burk plot for $I = 0$, and Slope$_i$ is that for $I \neq 0$. K_{is} and K_{ii} can be estimated as shown in Fig. 4. Then the value of K'_a can be calculated from known value of K_a and the thermodynamic relationship, $K_a K_{ii} = K'_a K_{is}$.

DISCUSSION

Elucidation of the mechanisms of inhibitions and determination of the dissociation constants of various macromolecule–ligand complexes are subjects of major interest to many investigators in the field of enzymology and biochemical pharmacology. Frequently the inhibitors (or ligands) that bind tightly to enzymes (or other macromolecules) are important as drugs or as research tools. Recognizing the need for theoretical bases for understanding the behavior of tight-binding ligands, investigators such as Morrison [9] and Henderson [11] have attempted to describe the kinetic behavior of tight-binding inhibitors in terms of steady state equations. In the present paper, arguments are presented that the time-honored steady state approach often is not applicable to inhibitors that bind to enzymes very tightly. One might consider use of the non-steady state rate equations such as those derived by Hijazi and Laidler [24–27] or by Vassent [28]. Unfortunately, these rate equations are so complicated that investigators who do not specialize in enzyme kinetics may find the usage of those equations difficult. One of the themes developed in the present work is that a steady state can be assumed for the reactions between the enzyme and the substrate, but not between the enzyme and a tight-binding inhibitor. Rate equations derived for such situations may be of greater practical value than the complete non-steady state equations because they are sufficiently accurate to describe most systems and are simpler to apply than the latter. This partial steady state and partial non-steady state approach to the problem of understanding the kinetic behavior of tight-binding ligands may be considered as a logical extension of the partial equilibrium and partial steady state approach which has been successfully applied to the derivation of enzyme rate equations for complicated reaction mechanisms by this author [29].

When a tight-binder is being studied, two facts must be considered. First, the depletion of free ligand by binding cannot be ignored. Second, both the association and dissociation reactions may be so slow that classical steady state enzyme kinetics may not be applicable. If the dissociation constant is not too low, i.e. roughly 10^{-7} M or higher, the reaction system may be studied under near steady state conditions. If the enzyme concentration is in the same order of magnitude as the inhibitor concentration, however, even when the system is under quasi-steady state conditions, the classical rate equations for steady state cannot be used. In such cases, the depletion of both free enzyme and free inhibitor must be taken into account through use of one of the several equations presented above, equations 2–13.

If a ligand is a tight-binder with a very low dissociation constant (10^{-9} M or less), the steady state kinetics may not be applicable. For instance, suppose a reaction is started by adding the enzyme to the reaction mixture that contains both the substrate and a low concentration of a potent inhibitor. A significant amount of substrate may be converted to the product long before steady state conditions are reached between the enzyme and inhibitor. Even if depletion of the substrate is not significant, the degree of inhibition is notably time dependent, i.e. the reaction velocity decreases gradually as the concentration of the enzyme–inhibitor complex increases. In such cases, the term 'initial velocity' becomes meaningless. If one adheres to the classical concept of the initial velocity of inhibited reactions, and measures 'initial velocity' in some arbitrary way, e.g. the amount of substrate which disappears in the first 2 min and then tries to analyze the data by a double reciprocal plot, not only do many data appear uninterpretable, but also any conclusions drawn from these data may be erroneous. In any case, it will be difficult, if not impossible, to evaluate correctly the inhibition mechanism.

On the other hand, if the enzyme is preincubated for a sufficient time with the inhibitor, and the reaction is started by addition of the substrate, one, in fact, measures the amount of free enzyme that remains at the end of the preincubation period. Therefore, a plot of reciprocal of the initial velocity against reciprocal of the substrate concentration would yield a pattern in which all lines intersect at one point on the $1/S$-axis, a result which could be erroneously interpreted as classical 'non-competitive' inhibition. Furthermore, the replot of intercept vs I_t would be a hyperbola instead of linear, and a different replot would be obtained when different amounts of the enzymes were used. Therefore, the mode of inhibition or value of K_i cannot be determined by this method.

Thus, it is difficult to determine whether a tight-binding inhibitor is competitive or non-competitive. If quasi-steady state conditions can be established between the inhibitor and the enzyme, the Henderson equation (equation 2) or equations 6–9 may be used effectively, and analysis of the transient phase (or non-steady state phase) (equation 29 and Table 1) may provide additional information with regard to the rate constants of the slow reaction steps. However, if the duration of the transient phase between the inhibitor and the enzyme is too long, none of the steady state rate equations becomes applicable. A practical test for the applicability of the transient phase analysis according to equation 29 and Table 1 is to carry out three assays: the first without inhibitor; the second in the presence of the inhibitor, with the reaction started by addition of the enzyme; and the third in which the reaction is started by addition of the substrate to the preincubated mixture of enzyme and inhibitor. If the steady state velocity (v_s in Fig. 3) is reached before the depletion of a significant amount of the substrate, the transient phase analysis may be used to evaluate the value of k' (equation 26) and to determine the inhibition mechanism from k' values.

If the transient phase analysis is not applicable, the dissociation constant can be best estimated under equilibrium conditions by incubating the enzyme with the inhibitor in the presence of substrate for a sufficiently long period of time. Then I_{50} (equation 9), E_{50} (equation 10) or apparent K_i (equation 13) can be used for estimation of the dissociation constant

of the *EI* complex. Although determination of inhibition mechanisms and inhibition constants is more difficult for a tight-binding inhibitor than for a readily equilibrating inhibitor, because of a favorable time factor, the first- and second-order rate constants can be estimated directly for a tight-binder without the need of sophisticated instrumentation such as a stopped-flow apparatus.

Throughout the present paper, it was assumed that the enzymic reaction was a one-substrate–one-product reaction, and that the dissociation of *ES* complex to *E* and *P* was irreversible. These assumptions are, of course, incorrect for most enzymic reactions. However, just as the Michaelis–Menten theory is applicable to so many more complex enzyme mechanisms, the above theoretical considerations will hold as long as the reaction velocity as a function of substrate concentration follows the Michaelis–Menten kinetics. For instance, in the case of a multisubstrate–multiproduct reaction, the above theories will be adequate if concentrations of substrate other than the varied one are held constant and the product concentrations are kept zero, and if the maximal velocity, Michaelis constant and catalytic number are understood as the apparent parameters. In the case of multivalent enzymes with significant allosteric interactions among protomers, however, interpretation of some of the above equations would require some caution. Complete mathematical description of such allosteric systems in the presence of tightly binding inhibitor would be a formidable task at the present time.

Since tight-binding ligands, such as enzyme inhibitors and hormone or carrier protein competitors, often serve as excellent investigational tools and occasionally are potent therapeutic agents, knowledge of the kinetic behavior of tight-binders should play an important role. Therefore, in this article the kinetic behavior of tight-binders has been presented from theoretical bases. Several actual examples selected from the literature and from our own studies are presented in the accompanying paper [30].

Acknowledgements—The author is indebted to Dr. R. E. Parks, Jr., Dr. G. W. Crabtree and Dr. David P. Baccanari for their constructive criticisms during the preparation of this manuscript.

REFERENCES

1. O. H. Straus and A. Goldstein, *J. gen. Physiol.* **26**, 559 (1943).
2. A. Goldstein, *J. gen. Physiol.* **27**, 529 (1944).
3. J. L. Webb, *Enzyme and Metabolic Inhibitors*, Vol. 1, p. 66. Academic Press, New York (1963).
4. J. M. Reiner, *Behavior of Enzyme Systems*, p. 82. Van Nostrand Reinhold, New York (1969).
5. S. Cha, *J. biol. Chem.* **245**, 4814 (1970).
6. C.-J. M. Cha and S. Cha, *Molec. Pharmac.* **1**, 178 (1965).
7. H. C. Hemker, P. W. Hemker and E. A. Loeliger, *Thromb. Diath. haemorrh.* **13**, 155 (1965).
8. G. Vassent and S. Jard, *C.r. hebd. Séanc. Acad. Sci. Paris* **272**, 880 (1971).
9. J. F. Morrison, *Biochim. biophys. Acta* **185**, 269 (1969).
10. D. G. Rhoads and D. Garfinkel, *Fedn Proc.* **30**, 1131 (1971).
11. P. J. F. Henderson, *Biochem. J.* **127**, 321 (1972).
12. Y. C. Cheng and W. H. Prusoff, *Biochem. Pharmac.* **22**, 3099 (1973).
13. T. C. Chou, *Molec. Pharmac.* **10**, 235 (1974).
14. W. W. Cleland, *Biochim. biophys. Acta* **67**, 173 (1963).
15. M. Dixon and E. C. Webb, *Enzymes*, 2nd Edn p. 316. Academic Press, New York (1964).
16. L. H. Easson and E. Stedman, *Proc. R. Soc. B* **112**, 142 (1936).
17. J. J. Burchall and G. H. Hitchings, *Molec. Pharmac.* **1**, 126 (1965).
18. R. Ferone, J. J. Burchall and G. H. Hitchings, *Molec. Pharmac.* **5**, 49 (1969).
19. W. T. Jenkins, *Analyt. Biochem.* **38**, 409 (1970).
20. W. W. Ackermann and V. R. Potter, *Proc. Soc. exp. Biol. Med.* **72**, 1 (1949).
21. H. R. Mahler and E. H. Cordes, *Biological Chemistry*, 2nd Edn, p. 322. Harper & Row, New York (1971).
22. C. Frieden, *J. biol. Chem.* **245**, 5788 (1970).
23. J. Monod, J. Wyman and J. P. Changeux, *J. molec. Biol.* **12**, 88 (1965).
24. N. H. Hijazi and K. J. Laidler, *Can. J. Biochem. Physiol.* **51**, 806 (1973).
25. N. H. Hijazi and K. J. Laidler, *Can. J. Biochem. Physiol.* **51**, 815 (1973).
26. N. H. Hijazi and K. J. Laidler, *Can. J. Biochem. Physiol.* **51**, 822 (1973).
27. N. H. Hijazi and K. J. Laidler, *Can. J. Biochem. Physiol.* **51**, 832 (1973).
28. G. Vassent, *J. theoret. Biol.* **44**, 241 (1974).
29. S. Cha, *J. biol. Chem.* **243**, 820 (1968).
30. S. Cha, R. P. Agarwal and R. E. Parks, Jr., *Biochem. Pharmac.*, **24**, 2187 (1975).