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TIGHT-BINDING INHIBITORS—VII

EXTENDED INTERPRETATION OF THE RATE EQUATION EXPERIMENTAL DESIGNS AND STATISTICAL METHODS*

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Abstract—The previously derived rate equation for tight-binding inhibitors has been reinterpreted to accommodate: (1) the case where the reaction is not started by the addition of enzyme, and (2) two-substrate reactions. Two major types of experimental design, i.e. equilibrium studies and time course studies, are developed. For each experimental design, graphical methods (if feasible) and statistical methods have been presented. Potential sources of errors are discussed.

In previous publications [1–5], the inadequacy of classical steady-state equations for the study of tight-binding and tight-binding inhibitors [4, 5]. To equation has been derived which accounts for the depletion of free inhibitor by binding and for the prolonged pre-steady-state phase of enzyme–inhibitor interactions [3]. Experimental procedures have been presented which are applicable to some special cases, e.g. where $I_t \gg E_t$, for the determination of kinetic parameters and inhibition mechanisms [3].

Recognizing the wide range of rates at which an inhibitor may interact with an enzyme, we have classified enzyme inhibitors, by simple criteria, into three classes, namely, readily reversible, semi-tight-binding and tight-binding inhibitors [4, 5]. To determine the class to which an inhibitor belongs, the enzyme is assayed in two ways, which differ only in whether or not the enzyme is preincubated with the inhibitor before addition of the substrate. The inhibitor is classified as readily reversible when the time courses of the two different assays are identical, as semi-tight-binding when they are different but attain the same steady state velocity within a reasonable time period, or as tight-binding when they are different and do not attain the same steady-state velocity over a long period of time. The terms "slow tight-binding" and "very slow tight-binding" inhibition have been introduced by others [6, 7] to describe the latter two classes.

In the present publication, the theory of tight-binding inhibitors is expanded. The rate equation is analyzed in detail to provide bases for more versatile and accurate experimental designs in the study of semi-tight and tight-binding inhibitors. Statistical methods for data analysis are also presented.

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SYMBOLS

Symbols are basically the same as before [1, 3] with a few changes and additions. Some of these symbols which may not be obvious are:

v, v_b	Enzyme velocity at any time and the background velocity in the absence of the enzyme;
v_0, v_i, v_z, v_s	Enzyme velocity in the absence of inhibitor, in the presence of inhibitor, at zero time and at steady state, respectively;
K_{is}	k_6/k_5, dissociation constant of EI complex;
K_{ii}	k_8/k_7, dissociation constant of ESI complex to ES and I;
K_m	Michaelis constant;
K'_m	Dissociation constant for $ESI \rightarrow EI + S$; k_{10}/k_9;
k_j	Rate constants; j is an odd number for the forward direction, and an even number for the reverse direction;
α	Apparent first-order rate constant (sec^{-1});
β	Apparent second-order rate constant ($M^{-1}\,sec^{-1}$);
F_i	Inhibition factor, or apparent K_i, formerly K'_i;
γ'	Depletion factor, general case;
γ	Standard depletion factor;
ψ	Fraction of E_t free of bound inhibitor at zero time;
λ	Decay factor (sec^{-1});
λ_0	Adjusted decay factor (sec^{-1});
ε	Molar equivalency of enzyme, molar concn (or equivalent) of enzyme = ε × (enzyme concn. in any arbitrary unit);
δ	Distribution fraction of inhibitor-free enzyme species;
δ'	Distribution fraction of inhibitor-bound enzyme species;

Q A square root term, $[(F_i + E_t + I_t)^2 - 4E_tI_t]^{\frac{1}{2}}$;

0 (superscript) Signifies the provisional value of a parameter, when used as a superscript.

BASIC MODEL AND RATE EQUATIONS

The basic model is the same as that used previously [1, 3]. In the following schematic representations of reaction mechanisms, double-headed single arrows (⇀↽) denote fast steps; double arrows (⇌), slow steps; and single-headed arrows (→), product-releasing steps.

Mechanism I: *Generalised non-competitive inhibition*

$$\begin{array}{ccc} E & \overset{K_m}{\rightleftharpoons} & ES \rightarrow \\ {\scriptstyle k_5 I}\downarrow\uparrow{\scriptstyle k_6} & & {\scriptstyle k_7 I}\downarrow\uparrow{\scriptstyle k_8} \\ EI & \underset{K'_m}{\rightleftharpoons} & ESI \end{array} \tag{1}$$

Previously, it was assumed that the reaction was started by the addition of the enzyme [3]. If this assumption is excluded (see Appendix for other assumptions), the rate equation should be modified to be:

$$v = \frac{v_s + [v_z(1 - \gamma') - v_s]\,e^{-\lambda t}}{1 - \gamma' e^{-\lambda t}} \tag{2}$$

or

$$\frac{v_z - v}{v_z - v_s} = \frac{1 - e^{-\lambda t}}{1 - \gamma' e^{-\lambda t}}. \tag{3}$$

If the reaction is started by the addition of the enzyme, the depletion factor for the general case (γ') becomes the standard depletion factor (γ), the initial velocity (v_z) equals the uninhibited velocity (v_0), and the rate equation becomes identical to that derived previously [3]

$$v = \frac{v_s + [v_0(1 - \gamma) - v_s]\,e^{-\lambda t}}{1 - \gamma e^{-\lambda t}}. \tag{4}$$

Various terms in these equations are defined as follows.

$$v_0 = VS/(K_m + S) \tag{5}$$

$$v_z = \phi v_0 \tag{6}$$

$$v_s = \frac{VS/K_m}{1 + I_t/K_{is} + (S/K_m)(1 + I_t/K_{ii})} \tag{7}$$

$$\gamma' = \frac{F_i + E_t(2\phi - 1) + I_t - Q}{F_i + E_t(2\phi - 1) + I_t + Q} \tag{8}$$

$$\gamma = \frac{F_i + E_t + I_t - Q}{F_i + E_t + I_t + Q} = \frac{(F_i + E_t + I_t - Q)^2}{4E_tI_t} \tag{9}$$

$$\lambda = \beta Q \tag{10}$$

$$\lambda_0 = \lambda(1 + \gamma)/(1 - \gamma) \tag{11}$$

$$= \lambda(F_i + E_t + I_t)/Q \tag{11a}$$

$$= \alpha + \beta(\varepsilon v_0 + I_t) \tag{11b}$$

$$\alpha = \Sigma k'_j\delta'_j = (k_6 + k_8S/K'_m)/(1 + S/K'_m) \tag{12}$$

$$\beta = \Sigma k_j\delta_j = (k_5 + k_7S/K_m)/(1 + S/K_m) \tag{13}$$

$$F_i = \alpha/\beta \tag{14}$$

$$= \frac{(1 + S/K_m)(k_6 + k_8S/K'_m)}{(1 + S/K'_m)(k_5 + k_7S/K_m)} \tag{14a}$$

$$= \frac{K_m + S}{\frac{K_m}{K_{is}} + \frac{S}{K_{ii}}} \tag{14b}$$

$$= K_{ii}(K_m + S)/(K'_m + S) \tag{14c}$$

$$= K_{is}(1 + S/K_m)/(1 + S/K'_m) \tag{14d}$$

$$Q = [(F_i + E_t + I_t)^2 - 4E_tI_t]^{\frac{1}{2}} \tag{15}$$

$$= [(F_i - E_t + I_t)^2 + 4F_iE_t]^{\frac{1}{2}} \tag{15a}$$

$$= [(F_i + E_t - I_t)^2 + 4F_iI_t]^{\frac{1}{2}}. \tag{15b}$$

The parameter ϕ represents the initial state of the enzyme, i.e. that fraction of total enzyme which is free of bound inhibitor. Thus, if the reaction is started by the addition of the enzyme:

$$\phi = 1. \tag{16}$$

Or, if the enzyme is preincubated with the inhibitor until equilibrium conditions have been reached, and the reaction is started by the addition of the substrate:

$$\phi = \{-(F_i - E_t + I_t) + [(F_i + E_t + I_t)^2 - 4E_tI_t]^{\frac{1}{2}}\}/(2E_t). \tag{16a}$$

New names have been assigned to three kinetic parameters: the inhibition factor (F_i), the depletion factor (γ) and the decay factor (λ). The significance of these are given below.

Inhibition factor, F_i

In the past, the inhibition factor F_i has been designated as the apparent K_i by various investigators [1, 7, 8] and denoted by various symbols such as K'_i, S, $D/\Sigma(N_i/K_i)$. F_i is an experimentally determinable parameter, from which K_{is} and K_{ii} can be estimated. The use of this factor simplifies the expressions of complicated rate equations. Since it is a function of the substrate, it would be improper to call F_i a constant. Therefore, this author prefers the term "inhibition factor" for F_i and also prefers to reserve the term "inhibition constants" for dissociation constants of various enzyme–inhibitor complexes.

F_i is the ratio of the apparent first-order rate constant (α) and the apparent second-order rate constant (β) as shown in equation 14. The expressions of α and β in constants and concentration terms can be easily obtained from equations 12 and 13, which are based on the concept of steady-state segment [9]. The term δ_j is the distribution fraction of an inhibitor-free enzyme species, i.e. the ratio of the concentration of the enzyme species (to which the inhibitor binds with the second-order rate constant, k_j) to the sum of the concentrations of all inhibitor-free enzyme species. For example, δ_j for E in Mechanism IV (equation 22) corresponds to $E/(E + EA + EAB)$ at the steady state, and k_j is k_5. Similarly δ'_j denotes the ratio of the concentration of inhibitor-bound jth enzyme species (from which the inhibitor dissociates with the first-order rate constant, k'_j) to the sum of the concentrations of all inhibitor-bound enzyme species.

Depletion factors, γ' and γ

The general depletion factor (γ') accounts for the effect of depletion of the free inhibitor by binding to the enzyme. This factor may have any numerical value between 0 and 1. It is a function of F_i, E_t and I_t as well as ϕ. If the enzyme is entirely free of bound inhibitor at zero time, as may be the case when the reaction is started by the addition of the enzyme, the value of ϕ is 1, and the value of γ' is that of the standard depletion factor (γ). The values of γ as a function of E_t, I_t and F_i are presented in Fig. 1. A truly stoichiometric inhibitor may be considered as the extreme case where $F_i = 0$, and the value of γ takes the upper limit, i.e. I_t/E_t if $I_t < E_t$; E_t/I_t if $I_t > E_t$; or 1 if $I_t = E_t$. In practical terms, the depletion factor is the product of the fraction of total enzyme bound to inhibitor and the fraction of total inhibitor bound to the enzyme at equilibrium, or

$$\gamma = \left(\frac{\Sigma E_b}{E_t}\right)\left(\frac{\Sigma I_b}{I_t}\right) = \frac{(\text{sum of } EI \text{ complexes})^2}{E_t I_t} = \frac{(E_t - \Sigma E_f)^2}{E_t I_t}. \quad (17)$$

From this, the following relationship can be deduced:

$$\gamma/\varepsilon = (v_0 - v_s)^2/(I_t v_0). \quad (18)$$

However, as will be discussed below, some inherent errors exist within this relationship. Nevertheless, it can be used for gross estimations of γ or ε when one of these factors is known.

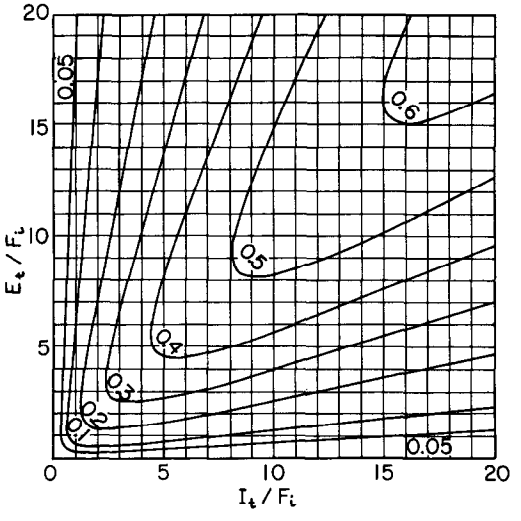


Fig. 1. Values of the standard depletion factor (γ) as a function of E_t, I_t and F_i.

Decay factor, γ

The exponential constant λ, which is also a function of E_t, I_t, S and the rate constants, is named the "decay factor" because of its similarity to radioactivity decay constants. The time taken for the velocity to reach halfway between v_0 and v_s is easily found from equation 3:

$$t_{\frac{1}{2}} = \frac{\ln(2 - \gamma')}{\lambda}. \quad (19)$$

Note that when $\gamma' = 0$, this expression is identical to that for first-order decay. This experimentally determinable decay factor accounts for the slow interactions of the enzyme and inhibitor.

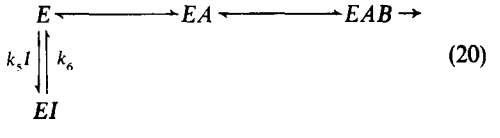
Adjusted decay factor, λ_0

As shown in equation 11, the decay factor (λ) adjusted for the standard depletion factor (γ) has a rather simple expression. In equation 11b, v_0 represents the enzyme concentration expressed as uninhibited velocity in any arbitrary unit (e.g. ΔA/min). This equation allows the estimation of the values of α, β and ε from the values of λ_0 determined for three or more different concentrations each of enzyme and inhibitor. Then, the set of values of α and β determined from at least four (three if K_m is known) different concentrations of the substrate allows computation of k_5, k_6, k_7, k_8, K_m and K'_m, from which, in turn, the values of K_{is} and K_{ii} can be calculated.

EXTENSION OF THE RATE EQUATION TO TWO-SUBSTRATE REACTIONS

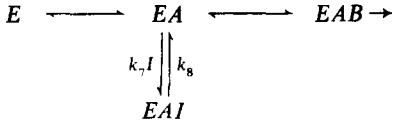
The following are some representative inhibition mechanisms involving two-substrate enzyme reactions.

Mechanism II: *Ordered bi–bi, competition with A*



(20)

Mechanism III: *Ordered bi–bi, competition with B*



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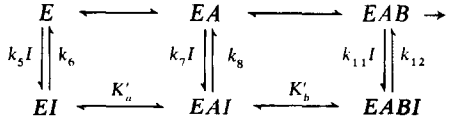
Mechanism IV: *Ordered bi–bi, non-competitive*



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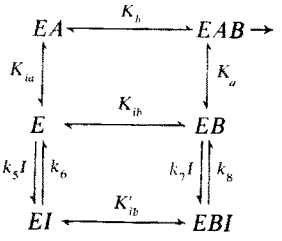
Mechanism V: *Rapid equilibrium random bi–bi, competitive with one substrate*



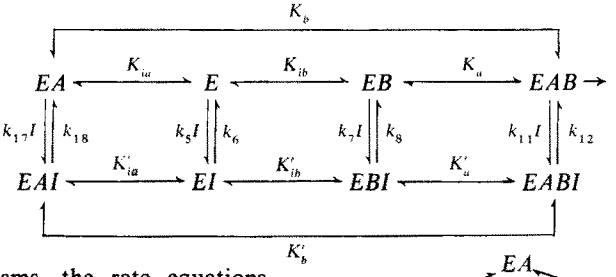
(23)

expressions of α and β, and hence of F_i (i.e. α/β), are readily derived according to equations 12, 13 and 14, as listed in Table 1. These modifications are important important for the analysis of the data to determine the inhibition mechanisms and rate constants.

EQUILIBRIUM STUDIES

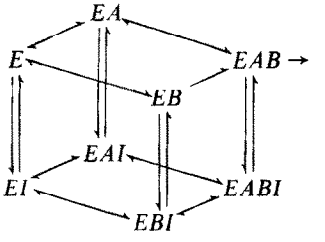
The use of the Ackerman–Potter plot [1, 2] and the plot of I_{50} versus E_t, as well as the subsequent determination of various kinetic parameters and inhibition

Mechanism VI: *Rapid equilibrium random bi–bi, non-competitive*



(24)

For all these mechanisms, the rate equations derived for the single substrate (equations 2 and 3) hold when the definitions of some terms are modified. Definitions of γ', γ, λ and Q remain unchanged. Those of v_z, v_0 and v_s should be changed according to the reaction and inhibition mechanisms; however, they are inconsequential for the further analysis of the data concerning tight-binding inhibitors. The



(24a)

Table 1. Apparent rate constants for association and dissociation of tight-binding inhibitor

Mechanism	First-order (α)*	Second-order (β)
I (non-comp)	$\dfrac{k_6K'_m + k_8S}{K'_m + S}$	$\dfrac{k_5K_m + k_7S}{K_m + S}$
I (comp)	k_6	$k_5K_m/(K_m + S)$
I (uncomp)	k_8	$k_7S/(K_m + S)$
II	k_6	$\dfrac{k_5(K_{ia}K_b + K_aB)}{K_{ia}K_b + K_aB + K_bA + AB}$
III	k_8	$\dfrac{k_7K_bA}{K_{ia}K_b + K_aB + K_bA + AB}$
IV	$\dfrac{k_6K'_{ia}K'_b + k_8K'_bA + k_{12}AB}{K'_{ia}K'_b + K'_bA + AB}$	$\dfrac{k_5(K_{ia}K_b + K_aB) + k_7K_bA + k_{11}AB}{K_{ia}K_b + K_bA + K_aB + AB}$
V	$\dfrac{k_6K'_{ib} + k_8B}{K'_{ib} + B}$	$\dfrac{k_5K_{ia}K_b + k_7K_aB}{K_{ia}K_b + K_bA + K_aB + AB}$
VI	$\dfrac{k_6K'_{ia}K'_b + k_8K'_aB + k_{18}K'_bA + k_{12}AB}{K'_{ia}K'_b + K'_bA + K'_aB + AB}$	$\dfrac{k_5K_{ia}K_b + k_7K_aB + k_{17}K_bA + k_{11}AB}{K_{ia}K_b + K_bA + K_aB + AB}$
VII	k_6	$\dfrac{k_5K_aB}{K_bA + K_aB + AB}$
VIII†	$\dfrac{k_6K'_aB + k_{16}K'_bA + [k_8(1-\rho') + k_{18}\rho']AB}{K'_ak'_b + K'_bA + K'_aB + AB}$	$\dfrac{k_5K_aB + k_{15}K_bA + [k_7(1-\rho) + k_{17}\rho]AB}{K_aK_b + K_bA + K_aB + AB}$

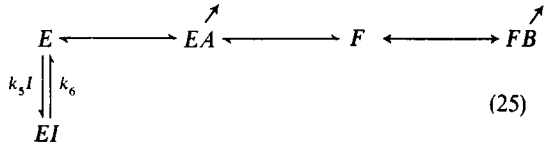
* Primed inhibition constants and Michaelis constants e.g. K'_m, K'_{ia}, K'_a, etc., denote those of the steady-state segment consisting of inhibitor-bound enzyme species.

† In the inhibitor-free steady-state segment of the ping–pong bi–bi reaction:

$$E \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} (EA \rightleftharpoons FP) \underset{k_4P}{\overset{k_3}{\rightleftharpoons}} F \underset{k'_2}{\overset{k'_1B}{\rightleftharpoons}} (FB \rightleftharpoons EQ) \underset{k'_4Q}{\overset{k'_3}{\rightleftharpoons}} E$$

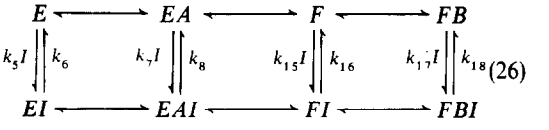
$\rho = k'_3/(k_3 + k'_3)$; $1 - \rho = k_3/(k_3 + k'_3)$; and ρ' is defined similarly for the inhibitor-bound steady-state segment.

Mechanism VII: *Ping–pong bi–bi, competitive with one substrate*



(25)

Mechanism VIII: *Ping–pong bi–bi, non-competitive*



(26)

mechanisms [2, 4] have been demonstrated previously. Improved experimental designs, various graphical methods and statistical methods of data analysis are presented below.

Experimental procedures

In the absence of substrate, or in the presence of an incomplete set of substrates in the case of multi-substrate reactions, the enzyme and inhibitor are incubated at three or more concentration levels of each for a period of time sufficient to establish virtual equilibrium conditions. Then the missing substrate(s) is added to start the reaction at the standard conditions (in terms of the final concentrations of substrates), and the initial velocity is determined. It is important to measure the initial velocity before any significant portion of the enzyme in *EI* complex(es) has dissociated and contributed to catalysis.

In the case of a single substrate reaction such as in Mechanism I, this procedure, carried out in the absence of the substrate during the preincubation, provides information on only K_{is} (as F_i) and the molar equivalency of the enzyme ε. If the substrate is present from the beginning, accurate determination of K_{ii} from equilibrium or steady-state data may not be feasible because steady-state conditions are not established simultaneously among all enzyme species before the concentrations of the substrate and the product are changed significantly. Therefore, the determination of the type of inhibition of single substrate reactions (i.e. competitive or non-competitive) is difficult from equilibrium studies or steady-state kinetics, but can be done by the time-course studies as shown before [3] and below.

The above procedure, however, may be applied readily to multi-substrate reactions for the determination of the inhibition mechanism. For example, in a two-substrate reaction inhibited by a tight-binding inhibitor (e.g. dihydrofolate reductase inhibited by methotrexate), if one substrate (e.g. NADPH) is included in the preincubation mixture, the value of F_i changes with the concentration of this substrate, either linearly or hyperbolically, depending on whether the inhibitor and the substrate are competitive (e.g. A and I in Mechanism V) or non-competitive. Thus, equilibrium studies, which are not applicable to the study of readily reversible inhibitors, can be a versatile tool in studies of tight-binding inhibitors.

Graphical method for the estimation of F_i *and* ε

Data obtained from experiments of the inhibition of dihydrofolate reductase (*Lactobacillus casei*) by methotrexate are shown in Fig. 2 as an example of how the graphical method of analysis can be used. Here, the observed velocities at various levels of the enzyme are plotted against the inhibitor concentrations. Uninhibited velocities are taken as the enzyme concentrations in arbitrary units. For each level of the enzyme, the location on the experimental line where $v = \frac{1}{2}v_0$ is marked. This point corresponds to

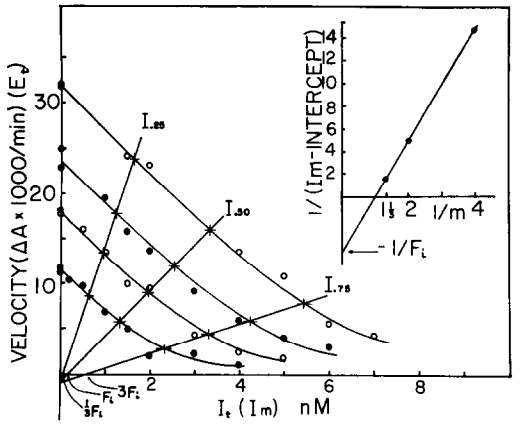


Fig. 2. Example of an equilibrium study. Velocity ($\Delta A \times 100$/min) of dihydrofolate reductase (*L. casei*) reaction after a 75-min preincubation of the enzyme with methotrexate (I_t) in the presence of 70 μM NADPH. The curves represent the values calculated on the basis of parameters estimated by the computer program.

the I_{50} value for that enzyme concentration. The best fitting straight line is drawn through these points. The intercept on the abscissa is the value of F_i and that on the ordinate equals $-2F_i/\varepsilon$ from which ε may be computed.

This graphical method is essentially the same as that for the I_{50} method presented obviously [1, 2], except that the determination of I_{50} values and the analysis of I_{50} versus E_t plot are carried out on one graph instead of two. Disadvantages of these graphical methods include the likelihood of making substantial errors in the determination of I_{50} values, and the fact that a large body of information from data points not located in the vicinity of 50 per cent of v_0 is wasted. It also must be pointed out that this method is most accurate when the ranges of both E_t and I_t studied are closest to the F_i value.

Recently Greco and Hakala [10] evaluated the magnitudes of errors of various methods for estimating F_i values of tight-binding inhibitors. It was their conclusion that the graphical method of I_{50} vs E_t may lead to moderate errors. They also noted that the term E_t can be eliminated if two equations for the expression of I_m (I_t to cause m% inhibition) are solved simultaneously. As shown below, a slight extension of their approach leads to an analytical method which allows the use of more available information and which hopefully would lead to reduced errors for the estimation of F_i and ε.

The Henderson steady-state rate equation [11] for Mechanism I in the form of an Easson–Stedman plot [12] is:

$$\frac{I_t}{1 - v_i/v_0} = E_t + \left(\frac{S + K_m}{(K_m/K_{is}) + S/(K_{ii})}\right)\left(\frac{v_o}{v_i}\right). \quad (27)$$

Let

$$m = 1 - v_i/v_0 \quad (28)$$

$$v_0/v_i = 1/(1 - m) \quad (28a)$$

and express m as a fraction rather than a percentage. Then, substituting F_i for the coefficient of v_0/v_i according to equation 14b, we have:

$$I_m = mE_t + [m/(1 - m)]F_i. \quad (29)$$

On graphs of v against I_t, such as Fig. 2, a straight line can be drawn for a given value of m (e.g. 0.25, 0.50 or 0.75) through points corresponding to I_m values at each level of enzyme. Then the intercepts on the abscissa and ordinate are $F_i[m/(1 - m)]$ and $-F_i/(1 - m)$, respectively. Therefore:

$$\frac{1}{I_t - \text{intercept}} = \left(\frac{1}{m}\right)\frac{1}{F_i} - \frac{1}{F_i}. \quad (30)$$

As illustrated in the inset of Fig. 2, the plot of $1/(I_t$-intercept) against $1/m$ has a slope of $1/F_i$ and the intercept on the abscissa equals $-1/F_i$, which allows the estimation of F_i. Once the F_i value is known, the value of the molar equivalency conversion factor ε may be estimated from a v-intercept:

$$\varepsilon(v\text{-intercept}) = -F_i/(1 - m). \quad (31)$$

Among many methods examined by Greco and Hakala [10], the most well-known are the methods of Dixon [13, 14] and of Henderson [11, 15]. The method of Dixon is the best in its simplicity, but its precision for normally obtainable data is no better than the I_{50} method [10]. The method of Henderson [11, 15] according to equation 27 is based on steady-state kinetics in the presence of all components; therefore, it may be suitable for the study of certain semi-tight binding inhibitors but would be inadequate for those more tightly binding inhibitors.

Statistical method for F_i *and* ε

The method of least squares can be applied to fit a family of curves to data points by employing iterative Newton–Raphson approximations as described by Wilkinson [16] and Cleland [17].

Since the uninhibited velocity may be considered an arbitrary expression of the enzyme concentration, E_t may be replaced by εv_0. Then, from equations 6 and 16a we have:

$$v_z/v_0 = \{-(F_i - \varepsilon v_0 + I_t) + [(F_i + \varepsilon v_0 + I_t)^2 - 4\varepsilon v_0 I_t]^{\frac{1}{2}}\}/(2\varepsilon v_0), \quad (32)$$

where v_z is the initial velocity after the preincubation.

In the first step of computation, the values of v_0, although subject to experimental error, are taken as known parameters, and a bivariate (F_i and ε) linear regression procedure is carried out on the approximation of equation 32.

$$\frac{v_z}{v_0} - \left(\frac{v_z}{v_0}\right)_0 = (F_i - F_i^0)X_1 + (\varepsilon - \varepsilon^0)X_2, \quad (33)$$

where

$$X_1 = \partial(v_z/v_0)/\partial F_i = \{-1 + (F_i + \varepsilon v_0 + I_t)/Q\}/ \times (2\varepsilon v_0) \quad (33a)$$

$$X_2 = \partial(v_z/v_0)/\partial\varepsilon = -\{(F_i + I_t)(F_i + I_t - Q) + \varepsilon v_0(F_i - I_t)\}/(2\varepsilon^2 v_0 Q). \quad (33b)$$

The subscript 0 outside of the parentheses denotes that the functions inside are evaluated at F_i^0 and ε^0. Since the proper weighting factor is generally n/σ^2 [17], under the assumption that the variance of v is constant throughout the experimental range, equation 33 may be weighted with:

$$w = v_0^4/(v_0^2 + v_i^2). \quad (34)$$

The provisional values, F_i and ε, must be provided before the regression procedure is performed. If mathematical impossibilities such as the square root of a negative number are encountered during the computation, or if the iterative procedure does not converge, a new set of provisional values must be provided. In a non-linear regression procedure such as this, the iterative approximation of parameters may converge to a false set of values. However, this has not been a serious problem in the case of data for methotrexate-inhibited dihydrofolate reductase. On rare occasions when this has occurred, the parameter values were so unrealistic that they were readily recognizable, and the correct values were obtained by repeating the procedure with a new set of provisional values.

In the second step, the best fitting value of v_0 is determined for each level of enzyme as the average of v_0 values computed for all experimental points from the equation below (equation 35) which can be derived by rearranging equation 32:

$$v_0 = v_z[1 + I_t/(\varepsilon v_z + F_i)]. \quad (35)$$

The weighting factor for this procedure is:

$$w = (\varepsilon v_z + F_i)^4/[(\varepsilon v_z + F_i)^2 + F_i I_t]. \quad (36)$$

Finally, with the newly calculated set of v_0 values, the first step (evaluation of F_i and ε) and the second step (calculation of v_0) are repeated until no further significant refinement of all values can be attained. In the process, I_{50} values and the standard errors of F_i and ε are also computed according to the procedures presented by Cleland [17].

Determination of K_{is}, K_{ii} *and inhibition mechanism*

The value of F_i determined in the absence of a substrate during the preincubation period equals K_{is} regardless of whether the inhibition is competitive or non-competitive. Therefore, in this case, no information on the inhibition mechanism can be attained.

However, if one of the substrates (but not a complete set) is present, in a multi-substrate reaction, during the preincubation, F_i becomes a function of the substrate concentration unless $K_{is} = K_{ii}$. From this relationship the values of K_m, K'_m, K_{is} and K_{ii} may be estimated by another iterative procedure, according to the linear approximation of equation 14d.

$$F_i = K_1\left(\frac{1}{1 + K_3^0 S}\right) + K_2\left(\frac{S}{1 + K_3^0 S}\right) + K_1(K_3 - K_3^0)\left(\frac{-(1 + K_2^0 S)S}{(1 + K_3^0 S)^2}\right), \quad (37)$$

where

$$K_1 = K_{is}, K_2 = 1/K_m \text{ and } K_3 = 1/K'_m.$$

K_{ii} now can be computed as $K_{ii} = K_{is}K'_m/K_m$. If the inhibitor and the preincubated substrate compete with each other, $K'_m = \infty$, and F_i will increase linearly with S according to:

$$F_i = K_{is}(1 + S/K_m). \quad (38)$$

If S and I are non-competitive, the plot of F_i versus S becomes a hyperbola with either a positive slope when $K_{is} < K_{ii}$, or a negative slope when $K_{is} > K_{ii}$. When $K_{is} = K_{ii}$, the value of F_i is independent of S. A statistical test (e.g. Student's t-test) for the null hypothesis (H_0: $1/K'_m = 0$) determines the probability of the inhibition being competitive.

TIME COURSE STUDIES

The prolonged transient phase makes the study of tight-binding inhibitors by steady-state kinetics difficult. The slow rates of enzyme–inhibition interactions, however, make possible the determination of the rate constants without sophisticated instruments. One can simply determine the time course of the reaction and estimate the decay factor and the depletion factor, from which all the other parameters may be computed.

Integration of equation 2, taking into account the background velocity which may be due to instability of substrate or slow non-enzymic side reactions, yields:

$$P = P_0 + (v_s + v_b)t - \left(\frac{v_s - v_z}{\lambda}\right)\left(\frac{1 - \gamma'}{\gamma'}\right) \times \ln\left(\frac{1 - \gamma' e^{-\lambda t}}{1 - \gamma'}\right), \quad (39)$$

where P_0 and P are product (or substrate) concentrations at zero time and time t, respectively. If the disappearance of the substrate is measured, the velocities take negative values.

Special cases, graphical methods

Under the special circumstances where $I_t \gg E_t$ and $I_t \gg F_i$, (therefore, $\gamma' = 0$ and $v_s \simeq 0$), equation 3 reduces to:

$$v = v_0 e^{-\lambda t}. \quad (40)$$

The pseudo first-order rate constant λ can be estimated from the slope of a straight line on a plot of log v versus t. The values of α, β and all other kinetic constants can be evaluated from the values of λ at various substrate concentrations, and subsequently the inhibition mechanisms can be determined. Detailed procedures have been documented previously, for studies with adenosine deaminase inhibited by coformycin [3].

In general, the time course according to equation 39 is a concave downward or upward curve depending on whether $v_z > v_s$ or $v_z < v_s$ as shown in Fig. 3. The equation for the asymptote of this curve is:

$$P = P_0 + (v_s + v_b)t + \left(\frac{v_s - v_z}{\lambda}\right)\left(\frac{1 - \gamma'}{\gamma'}\right)\ln(1 - \gamma'). \quad (41)$$

The intercepts of the asymptote on the t-axis (τ_0), and on the y-axis (π_0) are:

$$\tau_0 = -\left[P_0 + \left(\frac{v_s - v_z}{\lambda}\right)\left(\frac{1 - \gamma'}{\gamma'}\right)\ln(1 - \gamma')\right] \Big/ \times (v_s + v_b) \quad (42)$$

$$\pi_0 = P_0 + \left(\frac{v_s - v_z}{\lambda}\right)\left(\frac{1 - \gamma'}{\gamma'}\right)\ln(1 - \gamma'). \quad (43)$$

If $\gamma' = 0$, the expressions become simpler:

$$\tau_0 = \left(\frac{v_s - v_z}{\lambda} - P_0\right) \Big/ (v_s + v_b) \quad (44)$$

$$\pi_0 = P_0 - \frac{v_s - v_z}{\lambda}. \quad (45)$$

Therefore

$$\lambda = (v_s - v_z)/[\tau_0(v_s + v_b) + P_0] \quad (46)$$

$$= (v_s - v_z)/(P_0 - \pi_0). \quad (46a)$$

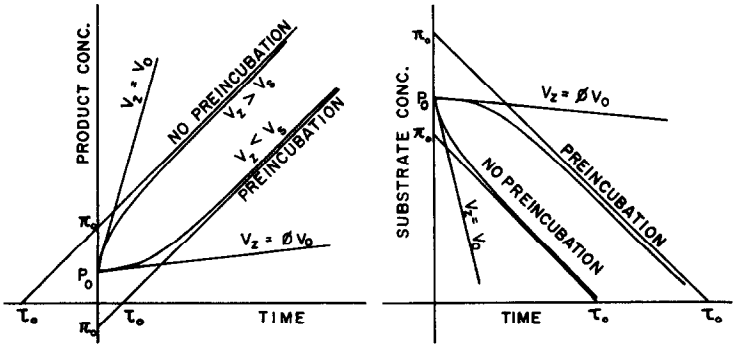


Fig. 3. Schematic illustration of the time course of enzymic reaction in the presence of semi-tight-binding inhibitor.

According to this equation, λ may be calculated in principle, from graphically estimated values of τ_0 or π_0, even when $v_s \neq 0$, provided $\gamma' \simeq 0$. However, the errors of estimation of τ_0 or π_0 could be considerable.

General case, statistical method

In general cases, the value of the depletion factor (γ') is not only a function of E_t, I_t and F_t, but also of the initial extent of the binding of the inhibitor with enzyme as shown in equation 8. When the reaction is started by the addition of enzyme, the value of ϕ is 1 by definition, and the general depletion factor (γ') may be replaced by the standard depletion factor (γ). Therefore, this mode of enzyme assay is the method of choice for time course studies, and the equation for the time course would be identical with equation 39 except for the substitution of γ for γ', and v_0 for v_z.

$$P = P_0 + (v_s + v_b)t - \left(\frac{v_s - v_0}{\lambda}\right)\left(\frac{1-\gamma}{\gamma}\right) \times \ln\left(\frac{1-\gamma\,e^{-\lambda t}}{1-\gamma}\right), \quad (47)$$

where v_b is non-enzymic background velocity.

This laboratory has been unsuccessful in developing an algorithm to estimate the five parameters (P_0, v_0, v_s, γ and λ with known v_b) of this integrated equation from a set of data comprising various P values at various times. Several versions of iterative approximation procedures have been tested. They all either converged to incorrect sets of parameter values, or did not converge at all. Therefore, at the risk of slightly greater errors, the following approach has been adopted, and has proven to be satisfactory with both simulated error-free data and real data for coformycin-inhibited adenosine deaminase and for methotrexate-inhibited dihydrofolate reductase.

The multi-dimensional Newton–Raphson method was applied for the least squares fitting of data to equation 4 which has two linear (v_0 and v_s) and two non-linear (γ and λ) parameters.

Step 1: *Data input.* The data consisting of the concentration of product or substrate (P) in arbitrary units (e.g. absorbance or reading on the recording chart) and the corresponding time (t) may be input in an increasing time sequence by any convenient means (e.g. keyboard, digitizer, etc.). It has been found that thirty or more such data points are required for a reasonably accurate estimation of the parameters. The velocity at each time point (except the first and last) is calculated as:

$$v_j = [(P_{j+1} - P_j)/(t_{j+1} - t_j) + (P_j - P_{j-1})/(t_j - t_{j-1})]/2 - v_b. \quad (48)$$

For the first and the last time points:

$$v_1 = (P_2 - P_1)/(t_2 - t_1) - v_b \quad (49)$$

$$v_n = (P_n - P_{n-1})/(t_n - t_{n-1}) - v_b. \quad (50)$$

Note that the non-enzymic background rate, if any, is subtracted from each velocity.

Step 2: *Provisional value input.* The provisional values of v_0 and v_s are input. Usually the absolute value of v_0 is greater than that of v_1, and that of v_s is smaller than that of v_n.

Step 3: *Provisional values of* γ *and* λ. The value of λ is computed for each assumed value of γ ranging from 0.05 to 0.95 at 0.05 intervals. The decay factor λ can be computed for each data point from equation 4, provided that v_0, v_s and γ are known. The average of such values serves as a satisfactory preliminary guess. Thus, for a given assumed value of γ:

$$\lambda = -\frac{1}{n}\sum\frac{1}{t_j}\ln\left(\frac{v_j - v_s}{v_0 - v_s - \gamma(v_0 - v_j)}\right). \quad (51)$$

If the time is 0 or the term after the logarithmic sign is not greater than zero, that particular data point is omitted from the computation at the present step. The set of γ and λ values that gives the smallest sum of squares is taken as the provisional values.

Step 4: *Tetravariate non-linear regression.* Equation 4 is non-linear with respect to two (γ and λ) of the four parameters. The Newton approximation of equation 4 may be written as:

$$v = v_0X_1 + v_sX_2 + (\gamma - \gamma^0)X_3 + (\lambda - \lambda^0)X_4, \quad (52)$$

where

$$X_1 = \left(\frac{\partial v}{\partial v_0}\right)_0 = (1-\gamma^0)\,e^{-\lambda^0 t}/(1-\gamma^0\,e^{-\lambda^0 t}) \quad (52a)$$

$$X_2 = \left(\frac{\partial v}{\partial v_s}\right)_0 = (1-e^{-\lambda^0 t})/(1-\gamma^0\,e^{-\lambda^0 t}) \quad (52b)$$

$$X_3 = \left(\frac{\partial v}{\partial \gamma}\right)_0 = -(v_0^0 - v_s^0)(1-e^{-\lambda^0 t})\,e^{-\lambda^0 t}/(1-\gamma^0\,e^{-\lambda^0 t})^2 \quad (52c)$$

$$X_4 = \left(\frac{\partial v}{\partial \lambda}\right)_0 = -(v_0^0 - v_s)(1-\gamma^0)t\,e^{-\lambda^0 t}. \quad (52d)$$

By employing the linear regression procedures, a new set of values of v_0, v_s, γ and λ is obtained. If the sum of squares calculated with these new parameter values is close enough (six or more significant digits) to the previous one, Step 5 is carried out. If not, Step 3 is repeated with the current values of the parameters as the new provisional values. If the values do not converge within several (e.g. seven) iterations, the entire procedure is repeated from Step 2 with newly input provisional values. If none of the reasonable sets of provisional values leads to convergence, then the data must be re-evaluated. Input of more data points often leads to a satisfactory solution of the problems.

Step 5: *Computation of variances and standard errors.* Variances, covariances and standard errors of estimation of all parameters are computed by the method described by Cleland [17].

Step 6: *Additional calculations.* The adjusted decay factor (λ_0), which is useful for further analysis of the data, can be calculated according to equation 11, and its standard error from the variances and the covariance of γ and λ. If desired, the theoretical values of P

at any time can also be calculated by using equation 47 and the estimated parameters.

Further analyses of data

Once the adjusted decay factors are estimated at a given concentration (including zero) of substrate(s) at several levels of E_t and I_t, the values of α, β and ε are computed by a simple linear regression on equation 11b, and then F_i is calculated by equation 14. The values of α, β and F_i estimated at various levels of the substrate(s) then enable one to compute individual rate constants and to determine the inhibition mechanism according to equation 14 and the expressions of α and β listed in Table 1.

DISCUSSION

Classical steady-state kinetics can be applied to studies of readily reversible and semi-tight-binding inhibitors, whereas the methods described in this paper are suitable for those of semi-tight-binding and tight-binding inhibitors. The interpretation of the rate equation (equations 2–4) has been expanded to accommodate a wide variety of reaction and inhibition mechanisms. It must be emphasized, however, that the above rate equations have been derived under certain assumptions. Therefore, under real conditions where one or more assumptions do not hold strictly, there may be some discrepancy between the actual time course of the reaction and that predicted by the rate equation.

Among six assumptions made previously [3] for the derivation of equation 3, four are reasonable for reactions involving tight-binding inhibitors and need no further discussion. The fact that the depletion factor takes different values, depending on whether the reaction is started by the addition of the enzyme or the substrate, has been dealt with in detail above.

The assumption that the concentration of product is negligibly low may not hold under certain experimental conditions, and may become a source of significant error. During time course studies, the overall steady-state velocity (v_s) may not be reached until a significant portion of the substrate has been converted to product. F_i, v_s and all other parameters (α, β, γ, λ, etc.) are functions of S (the concentration of substrate, if present) which changes with time. For example, the definitions of v_0 and v_s for Mechanism I are as in equation 5 and equation 7. When the inhibition is readily reversible, these two equations hold simultaneously. But if I is a tight-binding inhibitor, the S term in v_s may be significantly smaller than in v_0, because v_s might be reached only after significant depletion of S has taken place. Furthermore, the expression of v_s may have to be corrected for the non-negligible product concentration. Under such circumstances, the determination of various parameters based on the above equations will certainly include some inherent errors, somewhat similar to those in steady-state approaches. Ideally, one could employ complete rate equations based on no such restrictive assumptions. Those of Vassent [18] and those of Hijazi and Laidler [19] are examples. Unfortunately, these equations are so complicated and have so many parameters that no simple methods are available for the evaluation of all the necessary parameters. Even if a method of numerical analysis is developed in the future, it is doubtful whether or not a sufficiently large number of accurate data can be obtained to allow the estimation of so many parameters. Therefore, given the present state of the art, it would be best to design experiments so that the steady-state velocity is reached before no more than a small fraction (e.g. 10 per cent of the total) of the substrate is depleted before the velocity reaches near v_s. One way to accomplish this would be to work at relatively high substrate concentrations with respect to the Michaelis constant and a relatively high concentration of I with respect to F_i.

A second source of error is the inaccuracy of assigning zero time. Initial velocity and time are among the important parameters of the equation describing the time course of the reaction (equation 47). Unless some kind of rapid mixing device is employed, assignment of zero time can be considerably inaccurate. In order to minimize this type of error, one could choose concentrations of various components (especially that of enzyme) so that the initial velocity is reasonably slow.

A large number of therapeutically important compounds belong to the class of tight-binding inhibitors, e.g. methotrexate, 5′-deoxyfluorouridylic acid, allopurinol and deoxycoformycin. Yet, a property as basic as the inhibition constant cannot be estimated accurately by classical steady-state kinetics. On the other hand, the methods of studying tight-binding inhibitors presented above include some inherent sources of error and obviously require a far greater amount of work than studies of readily reversible inhibitors. These methods, however, enable not only reasonably accurate estimations of inhibition constants but also of the reaction and inhibition mechanisms. Particularly, the time course study provides the first- and second-order rate constants which cannot be obtained by equilibrium or steady-state studies alone. Measurement of these parameters may illuminate the structure–activity relationships of certain compounds better than the relative activities alone, thus contributing to better understanding and more rational designs of drugs.

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APPENDIX
DERIVATION OF EQUATIONS FOR MECHANISM I

Equation numbers preceded by T identify the equations in the text.

Assumptions

(1) Steady-state conditions are reached instantaneously between E and ES, and between EI and ESI.

(2) Prolonged non-steady-state conditions exist between E and EI, and between ES and ESI.

(3) The substrate concentration is much greater than the enzyme concentration, so that the depletion of free substrate by binding to the enzyme is negligible.

(4) Experimental observations are made only while the effect of substrate depletion (by conversion to the product) and the effect of production inhibition on the reaction velocity are negligible.

(5) The reaction is started by the addition of the enzyme, *or* the substrate.

Conservation equations

$$E_t = E + ES + EI + ESI \quad (1)$$

$$I_t = I + EI + ESI \quad (2)$$

$$S_t = S + ES + ESI \cong S \text{ (from Assumption No. 3)} \quad (3)$$

Steady-state expressions (Assumption No. 1)

$$E = (K/S)(ES) \quad (K \text{ is the Michaelis constant}) \quad (4)$$

$$EI = (K'/S)(ESI) \quad (K' \text{ is the } K_d \text{ for } ESI \rightarrow EI + S) \quad (5)$$

Non-steady-state expression (Assumption No. 2)

$$d(E + ES)/dt = -k_5(E)(I) - k_7(ES)(I) + k_6(EI) + k_8(ESI) \quad (6)$$

From equation 4:

$$d(E + ES) = d[(K/S)(ES) + (ES)] = (K/S + 1)d(ES). \quad (7)$$

From equations 1, 2 and 5:

$$I = I_t - E_t + (K/S + 1)(ES). \quad (8)$$

From equations 2, 5 and 8:

$$EI = [E_t - (ES)(K/S + 1)]/(1 + S/K'). \quad (9)$$

From equations 5 and 9:

$$ESI = [E_t - (ES)(K/S + 1)](S/K')/(1 + S/K'). \quad (10)$$

Substituting equations 7–10 into equation 6 and rearranging, we have:

$$d(ES)/dt = \beta[-E_tF_i(S/K)/(1 + S/K) + (ES)(F_i + I_t - E_t) + (ES)^2(1 + S/K)/(S/K)], \quad (11)$$

where

$$F_i = \alpha/\beta \quad (12)$$

$$\alpha = (k_6 + k_8S/K')/(1 + S/K') \quad (13, T12)$$

$$\beta = (k_5 + k_7S/K)/(1 + S/K). \quad (14, T13)$$

F_i defined in equation 12 can be expressed in several different ways by the use of the identity, $KK_{ii} = K'K_{is}$.

$$F_i = \alpha/\beta = [(1 + S/K)/(1 + S/K')][(k_6 + k_8S/K')/(k_5 + k_7S/K)] = [(1 + S/K)/(1 + S/K')](k_6/k_5)\left(\frac{1 + k_8S/(k_6K')}{1 + k_7S/(k_5K)}\right) \quad (15)$$

Compare the coefficients of S in the numerator and the denominator of the last term, and use $k_6/k_5 = K_{is}$, $k_8/k_7 = K_{ii}$ and $KK_{ii} = K'K_{is}$.

$$\left(\frac{k_8}{k_6K'}\right)\Big/\left(\frac{k_7}{k_5K}\right) = \left(\frac{k_8}{k_7}\right)\left(\frac{k_5}{k_6}\right)\left(\frac{K}{K'}\right) = \frac{K_{ii}K}{K_{is}K'} = 1. \quad (16)$$

Therefore, the last two terms of equation 15 equal 1, thus equation 15 is identical to equation T14d, from which equation T14a, T14b and T14c can be derived.

Now let ϕ be the fraction of enzyme free of bound inhibitor at $t = 0$. From Assumption No. 1, ES at zero time (ES_0) is:

$$ES_0 = \phi E_tS/(K + S). \quad (17)$$

Substitute this into equation 11 and let:

$$a = -(ES_0/\phi)F_i \quad (18)$$

$$b = F_i + I_t - E_t \quad (19)$$

$$c = \phi E_t/ES_0 \quad (20)$$

$$Q = (b^2 - 4ac)^{\frac{1}{2}} \quad (21)$$

$$X = ES \quad (22)$$

$$X_0 = ES_0. \quad (23)$$

Integrating equation 11, we have:

$$\int_{x_0}^{x} dX/dt = -\beta\int_0^t (a + bX + cX^2). \quad (24)$$

The solution of this equation is:

$$\ln \gamma'(2cX + b - Q)/(2cX + b + Q) = -\lambda t, \quad (25)$$

where

$$\gamma' = (2cX_0 + b - Q)/(2cX_0 + b + Q) \quad (26)$$

$$\lambda = \beta Q. \quad (27, T10)$$

Substitution of equations 17 and 19–21 into equation 26 yields equation T8. If $\phi = 1$, as may be the case where the reaction is started by the addition of the enzyme (Assumption No. 5), equation T9 holds.

Solving equation 25 for X, we have:

$$X = \frac{(-b + Q)/(2c) + [\gamma'(b + Q)/(2c)]e^{-\lambda t}}{1 - \gamma' e^{-\lambda t}}. \quad (28)$$

Let $X = X_0$ at $t = 0$; and $X = X_\infty$ at $t = \infty$. Then from equation 28, we have:

$$X_\infty = (-b + Q)/(2c) \quad (29)$$

$$X_0 = [-b + Q + \gamma'(b + Q)]/[2c(1 - \gamma')]. \quad (30)$$

Substitution of equation 29 into equation 30 yields:

$$\gamma'(b + Q)/(2c) = X_0(1 - \gamma') - X_\infty. \quad (31)$$

Substituting equation 29 and equation 31 into equation 28, we have:

$$X = \frac{X_\infty + [X_0(1 - \gamma') - X_\infty]e^{-\lambda t}}{1 - \gamma' e^{-\lambda t}}. \quad (32)$$

Let the first-order rate constant for the release of the product from ES complex be k. Multiply both sides of equation 32 by k. Replace $kX_0 = k(ES_0) = v_0$ and $kX_\infty = k(ES_\infty) = v_s$. Then equation 32 becomes equation T2 which, in turn, can be rearranged to equation T3.

Derivation of equation for asymptote (equation T41)

The parameters of the equation of asymptote, $y = ax + b$, for a curve, $y = f(x)$ are:

$$a = \lim_{x\to\infty} y/x \quad (33)$$

$$b = \lim_{x\to\infty} (y - ax). \quad (34)$$

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