BBA 65933

KINETICS OF THE REVERSIBLE INHIBITION OF ENZYME-CATALYSED REACTIONS BY TIGHT-BINDING INHIBITORS

J F MORRISON

Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, A C T (Australia)

(Received March 13th, 1969)

SUMMARY

A theoretical kinetic study has been undertaken to determine the kinetic characteristics of enzymic reactions that are reversibly inhibited by a substrate analogue at concentrations comparable to that of the enzyme A general rate equation, that allows for the combination of such a tight-binding inhibitor with one or more enzyme forms of any reaction mechanism, has been derived and shown to be quadratic with square and linear terms in v which represents the true initial steady-state velocity. It has been concluded that, from plots of v against total enzyme concentration at fixed total inhibitor concentration, the concentration of enzyme catalytic sites can be determined irrespective of the nature of the inhibition and the reaction mechanism. However, plots of v (or $1/v$) against total inhibitor concentration can be used for this purpose only if the dissociation constant associated with the inhibitor is virtually zero Particular initial rate equations have been obtained for the cases in which the tight-binding inhibitor gives rise to competitive, non-competitive and uncompetitive inhibition with respect to one of the substrates of a bireactant mechanism. From these equations it has been deduced that, in the presence of inhibitor, plots of $1/v$ against the reciprocals of the substrate concentrations yield families of concave-down, non-rectangular hyperbolas. The difficulties associated with the determination of inhibition constants for tight-binding inhibitors are discussed

INTRODUCTION

A number of enzymes involved in the metabolism of folate are strongly inhibited by compounds which are related structurally to the various substrates Indeed, it has been shown that these substrate analogues are effective at concentrations similar to that at which an enzyme is used for a study of its catalytic properties[1] Thus, for convenience, they can be classified loosely as tight-binding inhibitors and this generic name distinguishes them from the classical type of inhibitor which causes inhibition only at concentrations considerably in excess of the enzyme concentration. However, it cannot be considered that there is or will be a clear line of demarkation between

these two classes of inhibitor Undoubtedly, there will be developed compounds which exhibit a wide spectrum of ability to inhibit a particular enzyme reaction Nevertheless, it is convenient for the purposes of the present paper to define a tight-binding inhibitor as one that exerts its inhibitory effect at a sufficiently low concentration so that allowance must be made for a change in its concentration as a result of undergoing reaction with some form(s) of enzyme

While some tight-binding inhibitors cause irreversible inhibition of an enzyme as a consequence of the formation of a covalently bonded enzyme–inhibitor complex[2], it is not necessarily true that all tight-binding inhibitors must exert their effects in this manner Reversible inhibition remains a possibility and in this paper consideration is given to what kinetic effects might be expected with compounds that combine strongly with an enzyme in a reversible manner

The kinetics of the inhibition of enzymes by tight-binding inhibitors have been discussed by a number of authors including GOLDSTEIN[3], STRAUSS AND GOLDSTEIN[4], ACKERMANN AND POTTER[5] and REINER[6] However, in their respective treatments, these authors were primarily concerned with the qualitative aspects of the subject and thus considered only a unireactant mechanism Our interest in the action of this type of inhibitor arose from investigations carried out with highly purified preparations of dihydrofolate reductase from two strains of *Streptococcus faecium*[7] These enzymes were inhibited strongly by relatively low concentrations of a number of pteridine derivatives that are related structurally to the substrate, dihydrofolate Since the studies formed part of a general project which was directed towards an understanding of the action of anti-leukaemic drugs, it became apparent that there would be merit in determining the mechanism of the reactions, and the types of inhibition produced by the compounds, as well as values for the true inhibition constants The latter two aims necessitated the further development of the kinetic theory for a bireactant mechanism that took into account the order in which substrates add to and products dissociate from the enzyme

The present communication describes the general form of initial rate equation that is applicable to any reaction mechanism It allows for the combination of a tight-binding inhibitor with any number of enzyme forms and can be used to determine the characteristics of plots of initial velocity against the total concentration of enzyme or inhibitor In addition, there are given initial rate equations which were derived by assuming that the reaction under consideration has an ordered, sequential mechanism and that, by virtue of their structural similarity to the second reactant, the tight-binding inhibitors can give rise to competitive, non-competitive and uncompetitive inhibition The characteristics of these equations are illustrated by double reciprocal plots with the second reactant as the variable substrate and by plots of initial velocity as a function of the total concentrations of enzyme or inhibitor In all treatments, the further assumption has been made that the various equilibria between the enzyme, substrates and inhibitor are attained at a sufficiently rapid rate so as to enable true steady-state initial velocities to be determined

THEORY

The initial velocity equation for any enzyme-catalysed reaction, irrespective of the reaction mechanism, can be expressed in general form as

$$v = \frac{NE_t}{D} \tag{1}$$

where the term N contains the rate constants that determine the maximum velocity of the reaction, together with the concentrations of the reactants, E_t represents the total enzyme concentration and D represents the denominator of the rate equation. The distribution equation[8] for the various enzyme forms (E_i) that are produced can also be written in general form as

$$\frac{E_i}{E_t} = \frac{N_i}{D} \tag{2}$$

When an inhibitor (I) reacts with one of these various enzyme forms to give a complex (E_iI) which has a dissociation constant, K_i, the distribution equation for the complex becomes

$$\frac{E_iI}{E_t} = \frac{E_i}{E_t}\frac{I}{K_i} = \frac{N_i\left(\frac{I}{K_i}\right)}{D + N_i\left(\frac{I}{K_i}\right)} \tag{3}$$

If $K_i \gg E_t$, the initial velocity of the reaction in the presence of I is described by Eqn 4

$$v = \frac{NE_t}{D + N_i\left(\frac{I}{K_i}\right)} \tag{4}$$

If I reacts with multiple enzyme forms to give complexes with various dissociation constants (K_i) then

$$v = \frac{NE_t}{D + I\Sigma\left(\frac{N_i}{K_i}\right)} \tag{5}$$

When $K_i \approx E_t$, free I (I_f) is equal to total I (I_t) less that combined with various enzyme forms so

$$I_f = I_t - \Sigma(E_iI) \tag{6}$$

and

$$v = \frac{NE_t}{D + I_f\Sigma\left(\frac{N_i}{K_i}\right)} = \frac{NE_t}{D + [I_t - \Sigma(E_iI)]\Sigma\left(\frac{N_i}{K_i}\right)} \tag{7}$$

from which it may be determined that

$$\Sigma(E_iI) = I_t + \frac{\left(D - \frac{NE_t}{v}\right)}{\Sigma\left(\frac{N_i}{K_i}\right)} \tag{8}$$

From Eqn 3, it follows that

$$\Sigma(E_1I) = \frac{I_f E_t \Sigma\left(\frac{N_1}{K_i}\right)}{D + I_f \Sigma\left(\frac{N_1}{K_i}\right)} \tag{9}$$

Substitution of the relationships for I_f (Eqn 6) and $\Sigma(E_1I)$ (Eqn 8) into Eqn 9 gives

$$\left[I_t + \frac{D - \frac{NE_t}{v}}{\Sigma\left(\frac{N_1}{K_i}\right)}\right]\left(\frac{NE_t}{v}\right) = E_t\left(\frac{NE_t}{v} - D\right) \tag{10}$$

which on multiplication by $v^2/N^2E_t^2D$ and re-arrangement gives

$$v^2 + N\left[\frac{1}{\Sigma\left(\frac{N_1}{K_i}\right)} + \frac{I_t - E_t}{D}\right]v - \frac{N^2E_t}{D\Sigma\left(\frac{N_1}{K_i}\right)} = 0 \tag{11}$$

or

$$v = \frac{N}{2}\left[\sqrt{\left(\frac{1}{\Sigma\left(\frac{N_1}{K_i}\right)} + \frac{I_t - E_t}{D}\right)^2 + \frac{4E_t}{D\Sigma\left(\frac{N_1}{K_i}\right)}} - \left(\frac{1}{\Sigma\left(\frac{N_1}{K_i}\right)} + \frac{I_t - E_t}{D}\right)\right] \tag{12}$$

Either Eqn 11 or Eqn 12 represents the initial steady-state rate equation for any enzyme-catalysed reaction in the presence of a tight-binding, reversible inhibitor Eqn 12 may also be written as

$$v = \sqrt{\left(\frac{1}{\Sigma\left(\frac{N_1}{K_i}\right)} + \frac{E_t}{D}\right)^2 + 2\left(\frac{1}{\Sigma\left(\frac{N_1}{K_i}\right)} - \frac{E_t}{D}\right)\frac{I_t}{D} + \left(\frac{I_t}{D}\right)^2} - \left(\frac{1}{\Sigma\left(\frac{N_1}{K_i}\right)} + \frac{I_t - E_t}{D}\right) \tag{12a}$$

or

$$v = \sqrt{\left(\frac{1}{\Sigma\left(\frac{N_1}{K_i}\right)} + \frac{I_t}{D}\right)^2 + 2\left(\frac{1}{\Sigma\left(\frac{N_1}{K_i}\right)} - \frac{I_t}{D}\right)\frac{E_t}{D} + \left(\frac{E_t}{D}\right)^2} - \left(\frac{1}{\Sigma\left(\frac{N_1}{K_i}\right)} + \frac{I_t - E_t}{D}\right) \tag{12b}$$

It may be shown readily that if $I_t = 0$, Eqn 12a reduces to Eqn 1 Further, if

$$E_t \ll K_i \quad \left(E_t \ll \frac{D}{\Sigma\left(\frac{N_1}{K_i}\right)}\right),$$

Eqn 12b can be simplified to Eqn 5 (The proof depends on the fact that if $b \ll a$

$$\sqrt{a + b} = \sqrt{a} + \frac{b}{2\sqrt{a}}\Big)$$

Characteristics of the initial velocity equation in the presence of a reversible, tight-binding inhibitor

Initial velocity as a function of total enzyme concentration

For a reaction conforming to Eqn 11, plots of initial velocity (v) against total enzyme concentration (E_t) will pass through the origin and have an asymptote (*cf* Fig. 1), the equation of which may be derived by putting $F = v^2 - NE_tv/D$ and using the relationship

$$\frac{\delta F}{\delta v} \cdot v + \frac{\delta F}{\delta E_t} E_t + \text{linear terms in } v \text{ and } E_t \text{ of Eqn 11} = 0 \tag{13}$$

This gives Eqn 14 which reduces to Eqn 15 by substitution of the relationship $v/E_t = N/D$ as obtained by setting $F = 0$

$$\left(\frac{2v}{E_t} - \frac{N}{D}\right)v - \left(\frac{N}{D} - \frac{v}{E_t}\right)E_t + N\left(\frac{1}{\Sigma\left(\frac{N_i}{K_i}\right)} + \frac{I_t}{D}\right)\frac{v}{E_t} - \frac{N^2}{D\Sigma\left(\frac{N_i}{K_i}\right)} = 0 \tag{14}$$

$$v = \frac{N}{D}(E_t - I_t) \tag{15}$$

When $v = 0$, the asymptote will cut the abscissa at the point where the concentration of the enzyme catalytic sites is equivalent to that of the inhibitor This conclusion is, of course, based on the premise that only one molecule of inhibitor reacts at each catalytic site.

The tangent to the curve at the point where $E_t = 0$ may be determined using the relationship

$$\text{Initial slope of curve} = \frac{dv}{dE_t} = -\frac{\frac{\delta F}{\delta E_t}}{\frac{\delta F}{\delta v}} \tag{16}$$

where F is now represented by Eqn 11 This gives the expression

$$\frac{\frac{Nv}{D} + \frac{N^2}{D\Sigma\left(\frac{N_i}{K_i}\right)}}{2v + \frac{N}{\Sigma\left(\frac{N_i}{K_i}\right)} + \frac{NI_t}{D}} \tag{17}$$

From Eqn. 11 it may be determined that when $E_t = 0$, $v = 0$ which on substitution into Eqn 17 gives

$$\text{Initial slope of curve} = \frac{N}{D + \Sigma\left(\frac{N_i}{K_i}\right)I_t} \tag{18}$$

Initial velocity as a function of total inhibitor concentration

The general shape of the curves that are obtained when initial velocity (v) is plotted as a function of total inhibitor concentration (I_t) is illustrated in Fig 2. The slope of the tangent to the curve at the point where $I_t = 0$ may be obtained using the

relationship given in Eqn 16 and the fact that when $I_t = 0$, the initial velocity equation is $v = NE_t/D$ The resulting expression for the tangent to the curve is

$$v = -\frac{NE_t}{D\left[E_t + \frac{D}{\Sigma\left(\frac{N_i}{K_i}\right)}\right]} I_t + \frac{NE_t}{D} \tag{19}$$

Thus when the tangent is extrapolated to cut the abscissa, it will do so at the point where

$$I_t = E_t + \frac{D}{\Sigma\left(\frac{N_i}{K_i}\right)} \tag{20}$$

Reciprocal of initial velocity as a function of total inhibitor concentration

Eqn 11 may be re-arranged as

$$y^2 - \frac{D\Sigma\left(\frac{N_i}{K_i}\right)}{NE_t}\left[\frac{1}{\Sigma\left(\frac{N_i}{K_i}\right)} + \frac{I_t - E_t}{D}\right] y - \frac{D\Sigma\left(\frac{N_i}{K_i}\right)}{N^2E_t} = 0 \tag{21}$$

where $y = 1/v$, and used in conjunction with the relationships given in Eqns 13 and 16 to show that plots of $1/v$ against I_t will be non-rectangular hyperbolas whose characteristics are defined by Eqns 22–26

$$\text{Initial slope of curve} = \frac{\Sigma\left(\frac{N_i}{K_i}\right)}{NE_t\left[1 + \frac{\Sigma\left(\frac{N_i}{K_i}\right)E_t}{D}\right]} \tag{22}$$

$$\text{Vertical intercept of curve} = \frac{D}{NE_t} \tag{23}$$

$$\text{Slope of asymptote} = \frac{\Sigma\left(\frac{N_i}{K_i}\right)}{NE_t} \tag{24}$$

$$\text{Extrapolated vertical intercept of asymptote} = \frac{D - \Sigma\left(\frac{N_i}{K_i}\right)E_t}{NE_t} \tag{25}$$

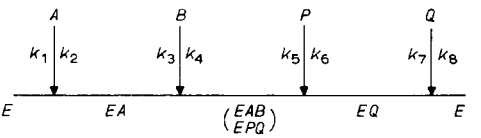
$$\text{Extrapolated horizontal intercept of asymptote} = E_t - \frac{D}{\Sigma\left(\frac{N_i}{K_i}\right)} \tag{26}$$

Since the initial slope of the curve (Eqn 22) is less than the slope of the asymptote (Eqn 24), the non-rectangular hyperbolas will always be concave-up

Double reciprocal plots of initial velocity as a function of substrate concentration

While the equations for the initial velocity of an enzyme-catalysed reaction, with either E_t or I_t as the independent variable, can be written in general form, irrespective of the number or type of enzyme species with which the tight-binding

inhibitor undergoes reaction, this is not so with substrate as the independent variable Thus, to obtain the initial velocity equation for any mechanism, it becomes necessary to substitute the appropriate expressions for N, N_1, D and $\Sigma (N_1/K_t)$ For the purposes of illustration, the particular mechanism chosen is one for which there is an obligatory order of addition of two substrates (A and B) so that A adds to the enzyme before B Similarly, it is considered that there is a compulsory order of release of the two products (P and Q) so that P, the product arising from B, dissociates before Q which arises from A Such an ordered sequential mechanism can be illustrated, using the shorthand method of CLELAND[8], as



Scheme I

The initial steady-state velocity equation may be expressed as

$$v = \frac{V_1 AB}{K_{1a}K_b + K_a B + K_b A + AB} \qquad (27)$$

where K_a and K_b are Michaelis constants equal to $k_5k_7/k_3(k_5+k_7)$ and $k_7(k_4+k_5)/k_3(k_5+k_7)$, respectively, K_{1a} is the dissociation constant for the EA complex equal to k_2/k_1, V_1 is the maximum velocity of the reaction in the forward direction equal to $k_5k_7E_t/k_5+k_7$ or kE_t

When a classical inhibitor combines with one or more enzyme–substrate complexes, such as those of Scheme I, plots of the reciprocals of the initial velocities against the reciprocals of the variable substrate concentrations are linear both in the absence and presence of the inhibitor. Further, the inhibition can be competitive, non-competitive or uncompetitive according to whether the slopes, the slopes and vertical intercepts or the vertical intercepts of linear double reciprocal plots vary as a function of the inhibitor concentration As will be shown below, in the presence of a tight-binding inhibitor, similar plots are non-linear and have shapes that are determined by (a) the initial slope of the curve, (b) the vertical intercept of the curve, (c) the slope of the asymptote and (d) the extrapolated vertical intercept of the asymptote Since each of these four characteristics can vary with the total concentration of a tight-binding inhibitor, it might be argued that new terms should be introduced to describe the inhibition caused by such inhibitors. However, there would appear to be merit in retaining the same three classes of inhibition and this has been done by modifying the aforementioned definitions Thus, for plots of $1/v$ against 1/substrate, inhibition by a tight-binding inhibitor will be called 'competitive' when all the curves at different total concentrations of inhibitor have the same vertical intercepts, 'non-competitive' when the vertical intercepts and slopes of the asymptotes vary with the total concentration of inhibitor, 'uncompetitive' when the vertical intercepts vary with, and the slopes of the asymptotes are independent of, the total concentration of inhibitor On the basis of these definitions, the combination of either a classical or tight-binding inhibitor with the same enzyme form(s) will give rise to the same type of inhibition

Non-competitive inhibition by tight-binding inhibitors with B as the variable sub-

strate Under conditions where the usual steady-state assumptions are applicable, it is likely that an analogue of B (Scheme 1) can cause non-competitive inhibition with respect to B by virtue of its ability to combine with both the EA and EQ forms of enzyme The same type of reaction mechanism could be invoked to account for the non-competitive inhibition in relation to B which can be produced by a tight-binding analogue of B The reactions involving the inhibitor can be expressed as

$$EA + I \overset{K_i}{\rightleftharpoons} EAI, \; EQ + I \overset{K_I}{\rightleftharpoons} EQI \tag{28}$$

and the distribution equations for EA and EQ (ref 8) as

$$\frac{EA}{E_t} = \frac{K_b A}{D}, \; \frac{EQ}{E_t} = \frac{V_1 K_q A B}{V_2 K_{iq} D} \tag{29}$$

where, in addition to the terms previously defined, D represents the denominator of Eqn 27, V_2 is the maximum velocity of the reaction in the reverse direction equal to $k_2 k_4 E_t/(k_2+k_4)$ or $k'E_t$, K_q is the Michaelis constant for Q equal to $k_2 k_4/k_8(k_2 + k_4)$, K_{iq} is the dissociation constant for the EQ complex equal to k_7/k_8. From the distribution equations, it may be determined that

$$\Sigma\left(\frac{N_1}{K_1}\right) = \frac{K_b A + \gamma A B}{K_i} \tag{30}$$

where $\gamma = V_1 K_q K_i / V_2 K_{iq} K_I$ When the relationship given in Eqn 30 is substituted into Eqn 11, together with the relationships $N = kAB$, $D = K_{ra}K_b + K_a B + K_b A + AB$, $y = 1/v$ and $x = 1/B$, the reciprocal form of the initial velocity equation becomes

$$\begin{aligned} k^2 K_i E_t A y^2 - kK_i A\left(1 + \frac{K_a}{A} + \frac{\gamma I_t - \gamma E_t}{K_i}\right)y - kK_b A\left(\frac{K_i K_{ia}}{A} + K_i + I_t - E_t\right)xy \\ - K_b(K_a + A + K_{ia}\gamma + A\gamma)x - K_b^2(K_{ia} + A)x^2 - \gamma(K_a + A) = 0 \end{aligned} \tag{31}$$

Analysis of Eqn 31, using the relationships of Eqns 13 and 16, indicates that plots of $1/v$ against $1/B$ at different concentrations of I_t can yield a series of non-rectangular hyperbolas (*cf* Fig 6) for which the initial slopes and vertical intercepts of the curves, as well as the slopes and extrapolated vertical intercepts of the asymptotes, will vary as a function of the I_t concentration The relationship for the vertical intercept of the curves is shown in Eqn 32, but the others have not been given because of their complexity

$$\begin{aligned} \text{Vertical intercept of curve} = \frac{1}{2V_1}\Bigg[\left(1 + \frac{K_a}{A} + \frac{\gamma I_t - \gamma E_t}{K_i}\right) \\ + \left\{\left(1 + \frac{K_a}{A} + \frac{\gamma I_t - \gamma E_t}{K_i}\right)^2 + \frac{4\gamma E_t(K_a + A)}{K_i A}\right\}^{\frac{1}{2}}\Bigg] \end{aligned} \tag{32}$$

Competitive inhibition by tight-binding inhibitors with B as the variable substrate If a tight-binding inhibitor reacts only with the EA complex (Scheme I), competitive inhibition would be expected as the inhibitor and the variable substrate, B, combine with the same form of enzyme The initial velocity equation for this reaction mechanism may be derived from Eqn 31 by setting $K_I = \infty$ This results in the elimination of all terms containing γ which, as indicated previously, is equal to $V_1 K_q K_i / V_2 K_{iq} K_I$ The equation so obtained is

$$k^2K_iE_tAy^2 - [kK_i(K_a + A)]y - \left[kK_bA\left(\frac{K_{ia}K_i}{A} + K_i + I_t - E_t\right)\right]xy - [K_b(K_a + A)]x - [K_b^2(K_{ia} + A)]x^2 = 0 \quad (33)$$

Using the relationships given in Eqns. 13 and 16, it may be shown that plots of $1/v$ against $1/B$ at different I_t concentrations will yield a family of non-rectangular hyperbolas that have the same vertical intercepts (*cf* Fig 5) The characteristics of these curves are given by Eqns. 34–37.

$$\text{Initial slope of curve} = \frac{K_b}{V_1K_i}I_t + \frac{K_b}{V_1}\left(1 + \frac{K_{ia}}{A}\right) \quad (34)$$

$$\text{Vertical intercept of curve} = \frac{K_a + A}{V_1A} \quad (35)$$

$$\text{Slope of asymptote} = \frac{2K_b\left(K_{ia} + A + \frac{\alpha\beta A}{4K_iE_t}\right)}{kA(\beta - \alpha)} \quad (36)$$

$$\text{Extrapolated vertical intercept of asymptote} = \frac{(K_a + A)\left(1 + \frac{\beta}{2E_t}\right)}{kA(\beta - \alpha)} \quad (37)$$

where $\alpha = \frac{K_{ia}K_i}{A} + K_i + I_t - E_t$

and $\beta = \alpha + \left[\alpha^2 + \frac{4K_iE_t(K_{ia} + A)}{A}\right]^{\frac{1}{2}}$

It is apparent that while there is a linear relationship between the initial slopes of the curves and the total inhibitor concentration (Eqn 34), no such simple relationship is obtained with respect to the slopes and extrapolated vertical intercepts of the asymptotes (Eqns. 36 and 37)

Uncompetitive inhibition by tight-binding inhibitors with B as the variable substrate

When B is the variable substrate, the combination of a tight-binding inhibitor with only the EQ complex of Scheme I will give rise to what has been defined as uncompetitive inhibition The rate equation for this type of inhibition may be derived from Eqn. 31 by setting $K_i = \infty$ This yields Eqn 38

$$y^2 - \frac{K_q}{V_2K_{iq}}\left(\frac{V_2K_{iq}K_a}{V_1K_qA} + \frac{V_2K_{iq}}{V_1K_q} + \frac{I_t - E_t}{K_I}\right)y - \frac{K_b}{V_1}\left(\frac{K_{ia}}{A} + 1\right)xy - \frac{K_bK_q}{kV_2K_{iq}K_I}\left(\frac{K_{ia}}{A} + 1\right)x - \frac{K_q}{kV_2K_{iq}K_I}\left(\frac{K_a}{A} + 1\right) = 0 \quad (38)$$

from which it may be determined that plots of $1/v$ against $1/B$ at different concentrations of I_t will have the shape of non-rectangular hyperbolas By procedures similar to those outlined above, it may be shown that

$$\text{Slope of asymptote} = \frac{K_b}{V_1}\left(\frac{K_{ia}}{A} + 1\right) \quad (39)$$

$$\text{Extrapolated vertical intercept of asymptote} = \frac{K_q}{V_2K_{iq}K_I}I_t + \frac{1}{V_1}\left(\frac{K_a}{A} + 1\right) \quad (40)$$

It is apparent from Eqn 39 that the slope of the asymptote is independent of the I_t concentration and that the expression is the same as that for the slope of the line in the absence of inhibitor Thus, at lower concentrations of B, all the lines of a double reciprocal plot will be parallel In this respect, there is a similarity in the results expected with tight-binding and classical uncompetitive inhibitors Eqn 40 indicates that the extrapolated intercept of the asymptote will vary as a linear function of the I_t concentration No simple relationships were found between the concentration of I_t, on one hand, and the vertical intercepts and initial slopes of the curves, on the other Therefore, these equations have been omitted

Graphical representation of plots

For the purpose of illustrating the type of curves to be expected for plots of v against E_t, v against I_t and $1/v$ against I_t, it was assumed that the reaction occurs by means of an ordered, sequential mechanism (Scheme I) Further, it was supposed that the tight-binding inhibitor reacts with the EA form of enzyme and thus causes competitive inhibition with respect to substrate, B The velocity equation that describes the mechanism was derived from Eqn 11 by substituting the appropriate relationships for N, D and $\Sigma (N_i/K_i)$ and is given in the legend to Fig 1

Plots of v against E_t at different concentrations of I_t

Fig 1 illustrates the type of plot that is obtained when a tight-binding inhibitor causes competitive inhibition with respect to B and when both substrates are present at fixed concentrations Similar plots would be obtained if the inhibitor gave rise to

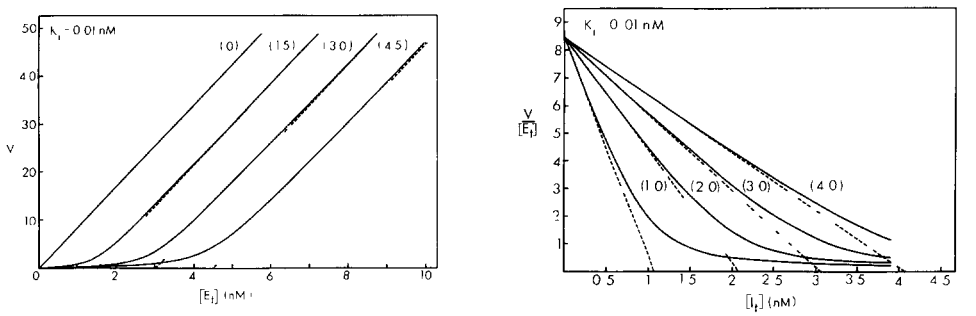


Fig 1 Theoretical plots of initial velocity (v) as a function of total enzyme concentration (E_t) in the absence and presence of different concentrations of a tight-binding inhibitor The initial velocity equation

$$v^2 + kAB\left[\frac{K_i}{K_b A} + \frac{I_t - E_t}{D}\right]v - \frac{k^2 K_i E_t A B^2}{K_b D} = 0$$

where $D = K_{ia}K_b + K_a B + K_b A + AB$, was obtained by substitution into Eqn 11 of the appropriate relationships for N and $\Sigma(N_i/K_i)$ and used to calculate the velocity data together with the following values for the kinetic parameters K_a, 1 5 μM, K_{ia}, 0 5 μM, K_b, 5 0 μM, k, 10 μmoles per min per nmole of enzyme, A, 100 μM, B, 30 μM, K_i, 0 01 nM The figures in parentheses above each curve represent the total concentration (nM) of the inhibitor The broken lines represent the calculated asymptotes to the curves in the presence of inhibitor

Fig 2 Theoretical curves for the variation of the velocity as a function of the total concentration of tight-binding inhibitor at different concentrations of total enzyme The conditions were as described in Fig 1 The figures in parentheses to the right of each curve represent the total enzyme concentration (nM) while the broken lines represent the calculated tangents to the curves at the point where $I_t = 0$

either non-competitive or uncompetitive inhibition (Eqn 11) Since the equation for the asymptote of the curve is the same, irrespective of the nature of the inhibition (Eqn. 15), plots of this type offer a potential means of determining the concentration of enzyme catalytic sites under conditions where one molecule of inhibitor combines at each active centre However, it is apparent from Fig 1 that when inhibitor is present, the slope of the curve at higher concentrations of E_t does not always represent the slope of the asymptote Thus, depending on the range of E_t concentrations that are used and the accuracy of the experimental data, significant error can be introduced into the determination as a result of graphical extrapolation to zero velocity of what appears to be an asymptote This error, which will be a function of the dissociation constant for the enzyme–inhibitor complex and increase as the value for this constant increases, would undoubtedly be reduced as a result of statistical analysis of the data

Plots of v against I_t at different concentrations of E_t

The theoretical curves illustrated in Figs 2 and 3 were obtained by using the equation which describes the competitive inhibition by a tight-binding inhibitor with respect to B (see legend to Fig 1) But the shape of the curves would be similar if the inhibition were non-competitive or uncompetitive (Eqn 11) This type of plot has been used to determine the concentration of enzyme catalytic sites by assuming that the initial slope represents the tangent to the curve at the point where $I_t = 0$ which is then extrapolated to cut the abscissa[9] However, extrapolation of the true tangent does not give an estimate of the concentration of catalytic sites since, as shown in Eqn 20, its intersection point on the abscissa is not only a function of the amount of enzyme added, but also of the values for the kinetic constants, including the dissociation constant(s) for the enzyme–inhibitor complex(es), and the substrate concentration(s) This same equation also indicates that the difference between the estimated and true concentration of the enzyme catalytic sites will reduce as the value(s) for the inhibition

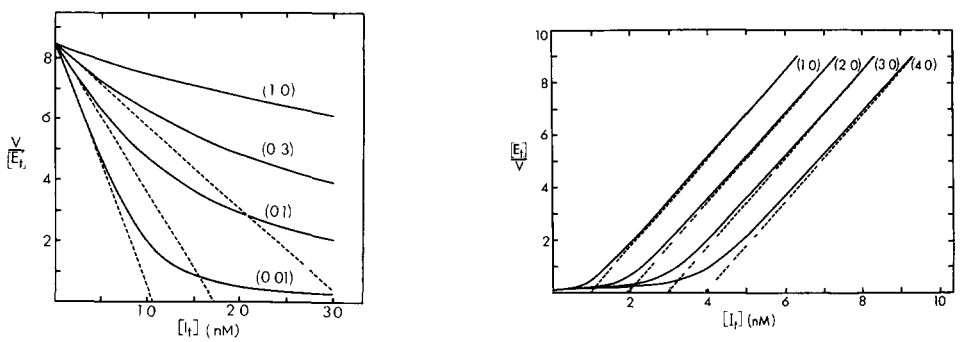


Fig 3 The effect of the magnitude of the K_i value for a tight-binding inhibitor on theoretical plots of velocity as a function of total inhibitor concentration The total enzyme concentration was 1 0 nM while the other conditions were the same as those given in the legend to Fig 1 The figures in parentheses above the curves are K_i values expressed as nM The broken lines are the calculated tangents to the curves at the point where $I_t = 0$

Fig 4 Theoretical plots of the reciprocal of the velocity ($1/v$) as a function of the total concentration of tight-binding inhibitor (I_t) at different concentrations of total enzyme The conditions were as described in Fig 1 The figures in parentheses to the right of each curve represent the total enzyme concentration (nM) while the broken lines represent the calculated asymptotes of the curves

constant(s) decreases A further difficulty is associated with the drawing, by graphical methods, of the tangent to the curve Fig 2 demonstrates that, over a particular range of inhibitor concentrations and with a relatively low K_i value, a more accurate determination of the tangent can be made at higher total enzyme concentrations Fig 3 shows that the nature of the plot changes as a function of the value for K_i so that for relatively high K_i values, it will be virtually impossible to draw a tangent to the curve

The above plots make clear that the concentration of a tight-binding inhibitor which is required to obtain 50% inhibition of a reaction depends on the concentration of the enzyme Considering the data of Fig 2, it may be deduced that the concentrations of I_t required to achieve this degree of inhibition at enzyme concentrations of 1, 2, 3 and 4 nM are 0 575, 1 075, 1 575 and 2 075 nM, respectively Therefore, the practice of reporting this type of result without stating the concentration of enzyme should be avoided as it does not give any meaningful information about the effectiveness of the inhibitor

Plots of 1/v against I_t at different concentrations of E_t

The family of concave-up, non-rectangular hyperbolas (Fig 4), which were obtained by considering that the tight-binding inhibitor acts competitively in relation to substrate, B, are illustrative of the type of curves that would be obtained irrespective of the nature of the inhibition (Eqn 11) The use of this type of plot to determine the concentration of enzyme catalytic sites suffers from the same weaknesses as those described in connection with plots of v against I_t However, it should be noted that the equivalent concentration of the enzyme can be determined by averaging the values, at a particular E_t concentration, for the true horizontal intercept of the tangent to the curve for a plot of v against I_t (Eqn 20) and the true horizontal intercept of the asymptote of the curve for a plot of $1/v$ against I_t (Eqn 26)

Double reciprocal plots of velocity as a function of the concentration of B at different I_t concentrations

Although the reaction under consideration involves two substrates (A and B), either of which could be varied, only the results to be expected with B as the variable substrate will be discussed in the following section From the theoretical curves illus-

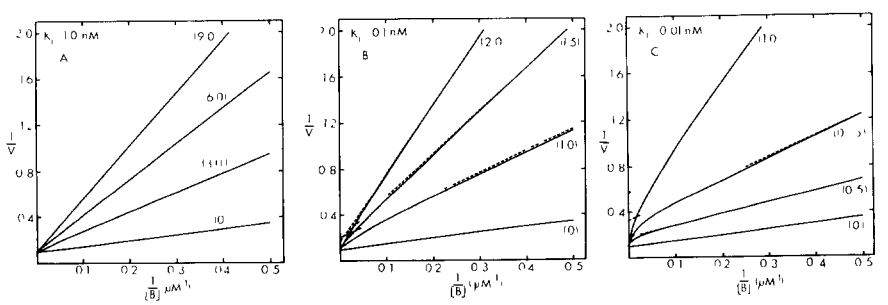


Fig 5 Theoretical double reciprocal plots for the competitive inhibition of a reaction by tight-binding inhibitors which react with the same form of enzyme (EA) as the variable substrate and which have different inhibition constants The conditions were the same as those described in the legend to Fig 1 except that the enzyme concentration was 1 0 nM, the concentration of B was varied and the velocity data were calculated from Eqn 33 The figures in parentheses represent the total concentrations of inhibitor, expressed as nM ,while the broken lines represent the calculated asymptotes to the curves

trated in Fig 5, it is apparent that at an infinite concentration of B, the same maximum velocity is obtained, irrespective of the inhibitor concentration Thus a tight-binding inhibitor that reacts with the same form of enzyme as the variable substrate will give rise to competitive inhibition as judged by the fact that all lines have a common point of intersection on the vertical ordinate It is also apparent that the shape of the curves is dependent on the value for the inhibition constant At relatively high values (Fig 5A), the plot approximates to that obtained under conditions where the usual steady-state assumptions are applicable That is, the initial slope and vertical intercept of the curve are virtually equal to the slope and extrapolated intercept of the asymptote, respectively (Eqns 34–37) Under these circumstances, the slope of the lines is almost a linear function of the inhibitor concentration (Eqn 34) However, the type of result obtained is dependent on the concentration of enzyme It has been shown that a 10-fold increase in enzyme concentration not only reduces the degree of inhibition, but also makes apparent the non-linearity of the plots in the presence of inhibitor as well as the non-linear relationship between the slopes of the asymptotes to the curves and the inhibitor concentration

As the value for K_i is decreased, with the enzyme at the same concentration as in Fig 5A, the curves are no longer linear (Figs 5B and 5C) They become concave-down, non-rectangular hyperbolas whose initial slopes are greater than those of the asymptotes which, on extrapolation, cut the vertical ordinate at points higher than the intercept of the curve The same plots illustrate the non-linear relation between the slope of the asymptotes and the concentration of inhibitor and in addition, draw attention to the fact that the non-linearity becomes more apparent at lower values of K_i. Two further points of practical interest are the difficulties that can be associated with the graphical determination of the true slope and true vertical intercept of the asymptotes and the fact that an inhibitor, which combines with the same enzyme form as the variable substrate, can appear to act in a non-competitive manner if initial velocities are determined only at lower concentrations of a substrate

When a tight-binding inhibitor gives rise to non-competitive inhibition, double reciprocal plots can have the shape of a non-rectangular hyperbola and thus have

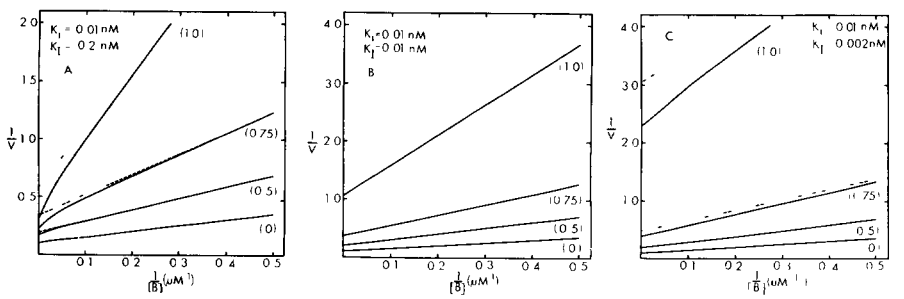


Fig 6 Theoretical double reciprocal plots for the non-competitive inhibition of a reaction by tight-binding inhibitors which react with two enzyme forms (EA and EQ) and which have either the same or different inhibition constants for reaction with the two forms In addition to the values for the kinetic parameters given in the legend to Fig 1, the following values were used K_Q, 10 0 μM, K_{IQ}, 20 0 μM, k', 5 μmoles per min per nmole of enzyme, E_t, 1 0 nM, A, 100 μM Velocity data were calculated from Eqn 31 The figures in parentheses represent the total concentrations of inhibitor, expressed as nM The broken lines represent the calculated asymptotes to the curves

features in common with those for competitive inhibition However, the former plots differ from the latter in that the intersection points of the curves with the vertical ordinate vary as a function of the inhibitor concentration (Fig 6A, Eqns 31 and 32) It is of interest to note that in the special case where $K_i = K_I$, the plots are linear although the slopes and intercepts of the curves are not a linear function of the inhibitor concentration (Fig 6B) Fig 6C shows that when the value for K_I is less than that for K_i, the non-linearity of the double reciprocal plots is less apparent, although the non-linear relationship between vertical intercepts of the curves (and asymptotes) and the inhibitor concentration is emphasized

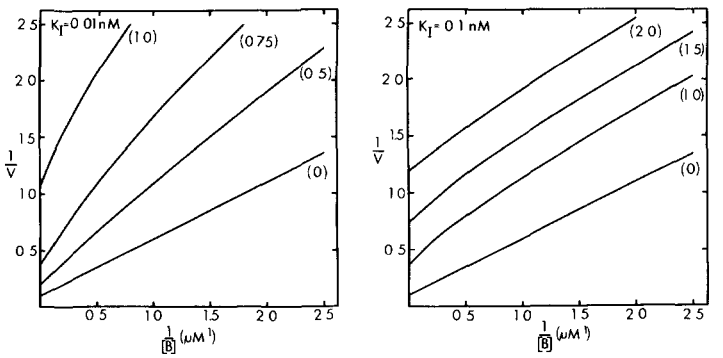


Fig 7 Theoretical double reciprocal plots for the uncompetitive inhibition of a reaction by tight-binding inhibitors which react with one enzyme form *(EQ)* and which have different inhibition constants The conditions were the same as those given in the legend to Fig 6, except that the velocity data were calculated from Eqn 38 The figures in parentheses represent the total concentrations of inhibitor

The plots illustrated in Fig 7 show the poorly defined non-rectangular hyperbolas that are obtained, under the chosen conditions, for the uncompetitive inhibition by a tight-binding inhibitor which is assumed to react only with the *EQ* (Scheme I) form of enzyme (Eqn 38) While the variation of the vertical intercepts of the curves with the inhibitor concentration is clear, the independence of the slopes of the asymptotes on the inhibitor concentration is not, even though the variable substrate has been reduced to concentrations well below its K_m value Thus it is likely that there will be practical difficulties associated with the detection of parallel asymptotes which is necessary in order to distinguish uncompetitive from non-competitive inhibition

DISCUSSION

The work reported in the present paper was undertaken with the aim of gaining a better understanding of the kinetic theory relating to the action of reversible, tight-binding inhibitors and of clarifying some of the misconceptions that have arisen in connection with the interpretation of results obtained with such inhibitors It was carried out as a prelude to detailed kinetic investigations of the inhibition of dihydrofolate reductase by analogues of dihydrofolate Steady-state kinetic studies in this laboratory have shown that each of the reactions catalysed by this enzyme from two

strains of *S faecium* has an ordered, sequential mechanism for which NADPH (*A*) must combine with the enzyme before the addition of dihydrofolate (*B*) Further, the results obtained with folate, which is a classical inhibitor, indicate that there is a corresponding ordered release of the products so that tetrahydrofolate (*P*) dissociates before $NADP^+$ (*Q*), and that inhibition can occur as a consequence of the reaction of folate with the *EA* and *EQ* complexes Therefore, the same overall reaction mechanism has been considered in connection with the kinetic effects of tight-binding inhibitors, and the theoretical curves have been drawn using values for the kinetic constants which, for the most part, were of a similar magnitude to those obtained for the reactions catalysed by dihydrofolate reductase In addition, it has been assumed that inhibitions which are competitive, non-competitive or uncompetitive with respect to B can arise as a result of the combination of the inhibitor with the *EA*, *EA* and *EQ*, or *EQ* forms of enzyme, respectively Of course, it is possible that the non-competitive inhibition could occur because of the reaction of an inhibitor with both *EA* and free enzyme and that another mechanism could apply to a particular enzyme reaction However, the elaboration, in the THEORY section, of the procedures used to derive the initial rate equations and to determine the characteristics of the various plots, should facilitate further extension of the kinetic theory for other cases.

In deriving initial velocity equations to account for the kinetic effects of tight-binding inhibitors, it cannot be assumed that the concentration of inhibitor remains constant as is done when the usual steady-state assumptions are made As a direct consequence of the low dissociation constant for this type of inhibitor, inhibition will occur when it is added at concentrations comparable to that of the enzyme. Thus allowance must be made for the reduction in the concentration of free inhibitor as a result of its reaction with various enzyme forms The equations so obtained have forms different from those which describe the various types of inhibition under the usual steady-state conditions Irrespective of whether allowance is made for competitive, non-competitive or uncompetitive inhibition, they are quadratic with squared and linear terms in v which represents the true steady-state initial velocity In this connection, it should be emphasized that the equations given are valid only when the establishment of the equilibria for the reactions of a particular enzyme form with the variable substrate and inhibitor are fast compared with the time taken to determine the velocity The inhibition constant, being a ratio of the unimolecular and bimolecular rate constants for the reaction of an enzyme form with inhibitor, gives no indication of the absolute magnitude of the rate constants. These values could be such as to preclude the rapid establishment of steady-state equilibria between inhibitor, substrate and the enzyme forms with which they react and hence invalidate the interpretation of velocity data on the basis of the postulates outlined above If true steady-state initial velocities are not measured then for the situation in which an inhibitor combines with the same form of enzyme as does the variable substrate, the data would suggest non-competitive, rather than competitive inhibition Similar results would be obtained if, for instance, an *EAI* complex formed from *EA* and *I* were to undergo an isomerization reaction which for all practical purposes was irreversible These conclusions draw attention to the necessity of carrying out preliminary experiments in order to establish that one of the fundamental premises on which the theory is based is, in fact, correct

The initial rate equations have also been derived on the assumptions that only one molecule of inhibitor reacts at each catalytic site and that the complex so formed

can be regarded as a dead-end one that can only dissociate back to the reactants from which it was formed But it should be borne in mind that the reaction of an inhibitor with an EQ complex may form an EQI complex from which Q is released at a slower rate than from an EQ complex (Scheme I) Such a reaction would introduce additional terms into the initial velocity equations given here

Under conditions where the aforementioned requirements are satisfied, the initial velocity equations are valid irrespective of the magnitude of the dissociation constant Indeed, it has been shown in the case of competitive inhibition that when the dissociation constant for the inhibitor is high relative to the enzyme concentration, regular linear double reciprocal plots can be obtained (*cf* Fig 5A) Thus there would appear to be little merit in endeavouring to define zones of behaviour, according to the affinity of a reactant for an enzyme form, as proposed by GOLDSTEIN[3] and STRAUSS AND GOLDSTEIN[4] The initial velocity equations given (Eqns 11, 31, 33, 38), allow for the full range of results that can be obtained with inhibitors exhibiting a wide spectrum of dissociation constants It is for this same reason that it is not possible to give a precise definition for a tight-binding inhibitor In this connection, it should be noted that studies with this type of inhibitor differ from those with classical inhibitors in that the results obtained are dependent on the concentration of enzyme

The theoretical analyses presented herein indicate that irrespective of the form(s) of enzyme with which one molecule of inhibitor reacts, plots of initial velocity as a function of increasing amounts of the enzyme solution at different concentrations of a tight-binding inhibitor offers a means of determining the concentration of the enzyme (or catalytic sites), provided that the asymptote of the curve is clearly defined (Eqn 15, Fig 1) This is not true for plots of velocity against the concentration of tight-binding inhibitor with varying amounts of enzyme unless the dissociation constant for the inhibitor is virtually zero The higher is the dissociation constant for any fixed enzyme concentration, the greater will be the interval between the intersection point on the abscissa and that which corresponds to the true concentration of catalytic sites (Eqn 20, Figs 2 and 3) Difficulties are likely to be encountered in regard to the drawing of the tangent to the curve at the point where $I_t = 0$ (Figs 2 and 3) But if this can be done, the reaction mechanism is known and values are available for certain kinetic parameters, then both the slope and extrapolated intercept of the tangent (Eqns 19 and 20) may be used for the calculation of the dissociation constant and concentration of catalytic sites for what has been referred to as competitive and uncompetitive inhibitions If the inhibitor acts in a non-competitive manner, data from at least two curves must be obtained for the determination of the two K_t values An alternative method for determining directly the equivalent concentration of an enzyme, with I_t as the independent variable, is to use the same set of experimental data for plots of v against I_t and $1/v$ against I_t and to average the values for the horizontal intercepts of the curves (Eqns 20 and 26) It is of importance to note that plots of velocity against inhibitor concentration do not enable any conclusions to be reached about the type of inhibition Thus the study of the inhibition as a function of substrate concentration becomes mandatory

The theoretical curves illustrated in the form of double reciprocal plots indicate that it should be possible to distinguish competitive from non-competitive and uncompetitive inhibition (Figs 5–7) But to do so, it is necessary to use relatively high substrate concentrations and problems could arise because of substrate inhibition,

limited solubility of the substrate or non-specific effects due to an increase in ionic strength However, it is not true, as claimed by WERKHEISER[10], that under steady-state conditions a competitive tight-binding inhibitor will give rise to a double reciprocal plot which is characteristic of non-competitive inhibition This conclusion was reached because of a misreading of the report by GOLDSTEIN[3] It is clear (Figs 5B and 5C) that erroneous conclusions could be reached if the inhibition were to be studied only over a limited range of lower substrate concentrations which would not permit detection of the downward curvature of the plot There would appear to be no easy means of distinguishing between non-competitive and uncompetitive inhibition (Figs 6 and 7) unless it were possible to determine velocities at substrate concentrations, well below and above its K_m value It appears that it will be necessary to make a least squares fit of the data to the appropriate velocity equations and to deduce the type of inhibition from the analysis that gives the lower variance The use of such a technique would require accurate experimental data

The equations describing the three types of inhibition by a tight-binding inhibitor with respect to B are too complex for direct analysis because of the number of kinetic parameters that each contains (Eqns 31, 33 and 38) However, from studies in the absence of inhibitor, of the initial velocity of the reaction in each direction as a function of the concentration of substrates, it is possible to determine values for the kinetic constants associated with the substrates[8] These data would also yield values for the rate constants, k and k', associated with the maximum velocities of the reaction in the forward and reverse directions (*cf* Eqns 27 and 29), provided that a well characterised, pure enzyme was used For investigations with an impure enzyme preparation, the concentration of enzyme catalytic sites would have to be determined, as previously indicated (Eqn 15, Fig 1), and used to calculate values for k and k' Substitution of the values for the kinetic and rate constants into the appropriate rate equation would bring about a marked simplification. It should then be possible, by the use of a least squares fitting procedure, to determine if the experimental data are consistent with a particular hypothesis and to obtain true values for one or both inhibition constants It is proposed that values for the inhibition constants be determined by statistical analysis of the velocity data because accurate values are unlikely to be obtained by graphical methods[11] Indeed, such analysis should be performed with all data that give non-linear plots In connection with the determination of true values for the inhibition constants from non-competitive and uncompetitive inhibitions, attention should be drawn to the fact that these calculations require a knowledge of the values for K_{iq}, K_q and V_2 (Eqns 31 and 38) The latter two values can be obtained only if the equilibrium of a particular reaction does not markedly favour the formation of products[8]

It is apparent from the above discussion that many difficulties are associated with studies of the effects of tight-binding inhibitors on enzyme catalysed reactions which are carried out with the aims of elucidating the mechanism of the inhibition and determining true inhibition constants These difficulties have not always been appreciated and it is to be hoped that an awareness of them will prove to be of value with respect to future developments in this area of enzyme inhibition

ACKNOWLEDGEMENT

Thanks are due to Dr W W Cleland for his comments on the manuscript which included the suggestion that certain equations be given in general form

REFERENCES

1 R L BLAKLEY, *The Biochemistry of Folic Acid and Related Pteridines*, North Holland, Amsterdam, 1968
2 B R BAKER, *Design of Active-Site-Directed Irreversible Enzyme Inhibitors*, Wiley, New York, 1967
3 A GOLDSTEIN, *J Gen Physiol*, 27 (1944) 529
4 O H STRAUSS AND A GOLDSTEIN, *J Gen Physiol*, 26 (1943) 559
5 W W ACKERMANN AND V R POTTER, *Proc Soc Exptl Biol Med*, 72 (1949) 1
6 J M REINER, *Behaviour of Enzyme Systems*, Burgess Publishing Co, Minneapolis, 1959, p 148
7 P F NIXON AND R L BLAKLEY, *J Biol Chem*, 243 (1968) 4722
8 W W CLELAND, *Biochim Biophys Acta*, 67 (1963) 104
9 A W SCHRECKER AND F M HUENNEKENS, *Biochem Pharmacol*, 13 (1964) 731
10 W C WERKHEISER, *J Biol Chem*, 236 (1961) 888
11 J KOWALIK AND J F MORRISON, *Math Biosci*, 2 (1968) 57