Biochemical Pharmacology, Vol. 25, pp. 2695–2702. Pergamon Press, 1976. Printed in Great Britain.

TIGHT-BINDING INHIBITORS—III

A NEW APPROACH FOR THE DETERMINATION OF COMPETITION BETWEEN TIGHT-BINDING INHIBITORS AND SUBSTRATES—INHIBITION OF ADENOSINE DEAMINASE BY COFORMYCIN*

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(*Received* 26 *March* 1976; *accepted* 12 *May* 1976)

Abstract—Equations have been developed which provide the basis for the elucidation of the inhibition mechanism of a tight-binding inhibitor by studying the manner in which the substrate interferes with the binding of the inhibitor to the enzyme. The procedure involves the determination of the pseudo-first-order rate constants at various concentrations of the substrate and the inhibitor. This method is applicable to very tightly binding inhibitors, including irreversible inhibitors. Methods for the graphical as well as statistical analyses of the data are presented. By the application of these methods, it is demonstrated that coformycin competes with adenosine for adenosine deaminase from calf intestinal mucosa. The kinetic parameters (±S. E.) for the binding of coformycin with adenosine deaminase were determined at 22° and pH 7.4; the second-order rate constant, 1.01 (±0.06) × 10^6 M^{-1} sec^{-1}; the first-order rate constant, 2.2 (±1.0) × 10^{-4} sec^{-1}; and the dissociation constant of the *EI* complex, 2.2 (±0.76) × 10^{-10} M.

In previous publications [1, 2], it has been demonstrated that under usual experimental conditions both the association and the dissociation of tight-binding inhibitors with enzymes are slow processes, that the classical steady state equations are inadequate for determining inhibition mechanisms or inhibition constants, and that I_{50} values can be used effectively to determine the dissociation or inhibition constant (K_i) of the enzyme–inhibitor complex. In order to describe the time course of the inhibited enzyme reaction, the following equation was derived under the assumption that the depletion of free inhibitor by binding to the enzyme is negligible.

$$v = v_s + [v_z - v_s]e^{-\lambda t} \quad (1)$$

It was also pointed out that, depending on the inhibition mechanism, λ is a different function of various rate constants and the concentrations of the substrate and the inhibitor, and that λ can be determined experimentally in certain cases from the values of v, v_z and v_s, the velocities at time t, 0 and ∞ respectively. Thus, it was suggested that the inhibition mechanisms may be elucidated from the analyses of the λ values determined at various concentrations of the substrate and the inhibitor. In these publications, however, no practical method was presented.

In the present publication, a theory is developed which provides the basis for the elucidation of the inhibition mechanism of a tight-binding inhibitor by studying the manner in which the substrate interferes with the binding of the inhibitor to the enzyme. A practical application of the theory is demonstrated by the competition between coformycin and adenosine for calf intestinal adenosine deaminase.

* This work was supported by USPHS Grants CA 12531 and CA 13943. The mathematical derivations of equations and the computer program are available upon request.

MATERIALS AND METHODS

Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) from calf intestinal mucosa was purchased from Sigma as a suspension in 3.2 M ammonium sulfate (255 units/mg). An aliquot of the suspension was centrifuged, the pellet was dissolved in 50 mM potassium phosphate buffer, pH 7.4, and the solution was passed through a Sephadex G-25 column (1 × 20 cm) equilibrated with 25% glycerol in the same buffer to remove ammonium sulfate. The enzyme was kept in a refrigerator as a stock solution in 25% glycerol in 50 mM phosphate buffer. Enzyme assays were carried out by adding a small volume (10–15 μl) of the stock enzyme solution to a mixture of adenosine and coformycin in 50 mM potassium phosphate buffer, pH 7.4, at 22°. The absorbance decrease at 265 nm due to the hydrolysis of adenosine to inosine was recorded by the use of a Gilford spectrophotometer [3, 4].

Coformycin (3-β-D-ribofuranosyl-6,7,8-trihydroimidazo [3,4-*d*][1,3] diazepin-8(R)-ol) was a gift from Dr. H. Umezawa of the Institute of Microbial Chemistry, Tokyo [5, 6].

Statistical analyses were performed on a Wang model 2200 computer with various programs written in BASIC language.

THEORY

Symbols

In addition to the symbols defined in the previous publication [1], the following new symbols are used.

α: apparent first-order rate constant, sec^{-1}.
β: apparent second-order rate constant, M^{-1} sec^{-1}.
α_0: apparent first-order rate constant in the absence of the substrate, sec^{-1}.

β_0: apparent second-order rate constant in the absence of the substrate, $M^{-1}\,sec^{-1}$.
$\Delta\alpha$: $\alpha_0 - \alpha$.
$\Delta\beta$: $\beta_0 - \beta$.
$\Delta\alpha_{max}$: $\Delta\alpha$ extrapolated to that for $S = \infty$.
$\Delta\beta_{max}$: $\Delta\beta$ extrapolated to that for $S = \infty$.
γ: factor that accounts for depletion of the free inhibitor.
λ: exponential constant; apparent pseudo-first-order rate constant, sec^{-1}; k' in the previous publication [1].
v_z, v_s: velocity at zero time and at steady state respectively.

Model and assumptions

A general non-competitive inhibition mechanism is represented by:

$$\begin{array}{ccccc} E & \underset{k_2}{\overset{k_1S}{\rightleftharpoons}} & ES & \underset{k_4P}{\overset{k_3}{\rightleftharpoons}} & E+P \\ {\scriptstyle k_5I}\downarrow\uparrow{\scriptstyle k_6} & & & & {\scriptstyle k_7I}\downarrow\uparrow{\scriptstyle k_8} \\ EI & \underset{k_{10}}{\overset{k_9S}{\rightleftharpoons}} & ESI & & \end{array} \qquad (2)$$

The kinetic constants are defined as

$$K_m = (k_2 + k_3)/k_1 \qquad (3)$$

$$K'_m = k_{10}/k_9 \qquad (4)$$

$$V = k_3E_t \qquad (5)$$

$$K_{is} = k_6/k_5 \qquad (6)$$

$$K_{ii} = k_8/k_7 \qquad (7)$$

The following assumptions are made, which are reasonable for the reactions inhibited by a tight-binder.

(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) on the reaction velocity is negligible.

(5) The reaction is started by the addition of the enzyme.

(6) The concentration of product is negligibly small.

Rate equation

Under these assumptions, the following rate equation was derived (see footnote).

$$v = \frac{v_s + [v_0(1-\gamma) - v_s]e^{-\lambda' t}}{1 - \gamma e^{-\lambda' t}} \qquad (8)$$

where

$$\gamma = \frac{K'_i + E_t + I_t - Q}{K'_i + E_t + I_t + Q} \qquad (9)$$

$$v_0 = v_z = \frac{VS}{K_m + S} \qquad (10)$$

$$v_s = v_0\left(\frac{-(K'_i - E_t + I_t) + Q}{2E_t}\right) \qquad (11)$$

Table 1. Parameters of Equation 8

	Non-competitive inhibition		Competitive inhibition	
	General case	$E_t \ll I_t$	General case	$E_t \ll I_t$
K'_i	$\frac{K_{is}K_{ii}(K_m+S)}{K_{ii}K_m + K_{is}S}$	$\frac{K_{is}K_{ii}(K_m+S)}{K_{ii}K_m + K_{is}S}$	$K_i\left(1+\frac{S}{K_m}\right)$	$K_i\left(1+\frac{S}{K_m}\right)$
Q	$\sqrt{(K'_i + E_t + I_t)^2 - 4E_tI_t}$	$K'_i + I_t$	$\sqrt{(K'_i + E_t + I_t)^2 - 4E_tI_t}$	$K_i\left(1+\frac{I}{K_i}+\frac{S}{K_m}\right)$
γ	$\frac{K'_i + E_t + I_t - Q}{K'_i + E_t + I_t + Q}$	0	$\frac{K'_i + E_t + I_t - Q}{K'_i + E_t + I_t + Q}$	0
v_s	$\frac{v_0(-K'_i + E_t - I_t + Q)}{2E_t}$	$\frac{V\frac{S}{K_m}}{1+\frac{I_t}{K_{is}}+\frac{S}{K_m}\left(1+\frac{I_t}{K_{ii}}\right)}$	$\frac{v_0(-K'_i + E_t - I_t + Q)}{2E_t}$	$\frac{V\frac{S}{K_m}}{1+\frac{I_t}{K_i}+\frac{S}{K_m}}$
λ	βQ	βQ	βQ	βQ
β	$\frac{k_5 + \frac{k_7S}{K_m}}{1+\frac{S}{K_m}}$	$\frac{k_5 + \frac{k_7S}{K_m}}{1+\frac{S}{K_m}}$	$\frac{k_5}{1+\frac{S}{K_m}}$	$\frac{k_5}{1+\frac{S}{K_m}}$
$\Delta\beta$	$\frac{(k_5 - k_7)S}{K_m + S}$	$\frac{(k_5 - k_7)S}{K_m + S}$	$\frac{k_5S}{K_m + S}$	$\frac{k_5S}{K_m + S}$

$$\lambda = \frac{k_5 + \frac{k_7 S}{K_m}}{1 + \frac{S}{K_m}} Q \tag{12}$$

$$K_i' = \frac{\left(1 + \frac{S}{K_m}\right)\left(k_6 + \frac{k_8 S}{K_m'}\right)}{\left(1 + \frac{S}{K_m'}\right)\left(k_5 + \frac{k_7 S}{K_m}\right)} = \frac{1 + \frac{S}{K_m}}{\frac{1}{K_{is}} + \frac{S}{K_{ii} K_m}} \tag{13}$$

$$Q = \sqrt{(K_i' - E_t + I_t)^2 + 4K_i' E_t} = \sqrt{(K_i' + E_t + I_t)^2 - 4E_t I_t} \tag{14}$$

It can be shown that $\gamma \cong 0$ when $E_t \ll I_t$. Thus, the factor, γ, accounts for the depletion of free inhibitor due to binding, and when it is set equal to zero, Equation 8 reduced to Equation 1 as expected. It must be emphasized, however, that according to Assumptions 3 and 4, Equation 8 does not account for the depletion of the substrate by conversion to the product or by binding to the enzyme.

In the case of competitive inhibition, the same rate equation (Equation 8) holds, except the expressions of various terms can be simplified to those listed in Table 1 by setting $k_7 = 0$ (or $K_{ii} = \infty$), and $k_9 = 0$ (or $K_m' = \infty$).

Determination of rate constants

The slope of the semilog plot of ln v vs t according to Equation 8 is:

$$\frac{d(\ln v)}{dt} = \frac{-(1 - \gamma)(v_0 - v_s)\lambda e^{-\lambda t}}{(1 - \gamma e^{-\lambda t})\{v_s + [v_0(1 - \gamma) - v_s]e^{-\lambda t}\}} \tag{15}$$

As may be seen from this equation, the slope of the semilog plot is not a constant. In general, the plot of ln v vs t is a concave curve. Under special conditions, however, the semilog plot is a straight line. If the concentration of the inhibitor is much higher than both that of the enzyme and that of the apparent inhibition constant (K_i'), i.e. if $I_t \gg E_t$ and $I_t \gg K_i'$, the factor, γ, becomes negligible compared to 1, and v_s becomes negligibly small compared to v_0. Thus, Equations 8 and 12 reduce to:

$$v = v_0 e^{-\lambda t} \tag{16}$$

$$\lambda = \frac{k_6 + \frac{k_8 S}{K_m'}}{1 + \frac{S}{K_m'}} + \left(\frac{k_5 + \frac{k_7 S}{K_m}}{1 + \frac{S}{K_m}}\right) I_t \tag{17}$$

From Equation 16

$$\ln v = \ln v_0 - \lambda t \tag{18}$$

Therefore, under these conditions the semilog plot is a straight line and the value of λ can be determined from the slope. Let

$$\alpha = \frac{k_6 + \frac{k_8 S}{K_m'}}{1 + \frac{S}{K_m'}} \tag{19}$$

and

$$\beta = \frac{k_5 + \frac{k_7 S}{K_m}}{1 + \frac{S}{K_m}} \tag{20}$$

then Equation 17 becomes:

$$\lambda = \alpha + \beta(I_t) \tag{21}$$

The plots of α vs S, β vs S, $1/\alpha$ vs S, and $1/\beta$ vs S are all hyperbolas. However, if the inhibition mechanism is competitive, $K_m' = \infty$ and $k_7 = 0$.

$$\alpha = k_6 \tag{22}$$

$$\frac{1}{\beta} = \frac{1}{k_5} + \frac{S}{k_5 K_m}. \tag{23}$$

Let α_0 and β_0 be α and β at $S = 0$, then from Equations 19 and 20:

$$\alpha_0 = k_6 \tag{24}$$

$$\beta_0 = k_5 \tag{25}$$

Let

$$\Delta\alpha = \alpha_0 - \alpha \tag{26}$$

$$\Delta\beta = \beta_0 - \beta \tag{27}$$

$$\Delta\alpha_{max} = k_6 - k_8 \tag{28}$$

$$\Delta\beta_{max} = k_5 - k_7 \tag{29}$$

From Equations 19, 20 and 24–29:

$$\Delta\alpha = \frac{\Delta\alpha_{max} S}{K_m' + S} \tag{30}$$

$$\Delta\beta = \frac{\Delta\beta_{max} S}{K_m + S} \tag{31}$$

The double reciprocal forms of these equations are:

$$\frac{1}{\Delta\alpha} = \frac{K_m'}{\Delta\alpha_{max}}\left(\frac{1}{S}\right) + \frac{1}{\Delta\alpha_{max}} \tag{32}$$

$$\frac{1}{\Delta\beta} = \frac{K_m}{\Delta\beta_{max}}\left(\frac{1}{S}\right) + \frac{1}{\Delta\beta_{max}}. \tag{33}$$

The above equations (Eqns. 19, 20 and 30–33) provide the basis for a new method for the determination of the rate constants, k_5, k_6, k_7 and k_8, and the Michaelis constants K_m and K_m', which in turn permit the estimation of K_{is} and K_{ii}.

EXPERIMENTAL PROCEDURE

The experimental procedure to determine the values of k_5, k_6, k_7, k_8, K_m and K_m' and the methods of statistical analysis are considered below.

Determination of the constant, λ

Two different experimental procedures may have to be employed. First, in the absence of substrate or in the presence of an incomplete set of substrates (e.g. only one substrate in the case of a multi-substrate reaction), the enzyme is incubated with the inhibitor, and aliquots are assayed at various time intervals.

If the inhibitor is a tight-binder, the observed velocities are proportional to the remaining free enzyme, because the dissociation of the *EI* complex is so slow that the enzyme in the *EI* form does not contribute significantly to the enzyme reaction velocity.

Second, in the presence of the substrate in a single-substrate reaction, the reaction is started by addition of the enzyme to the reaction mixture containing the substrate and the inhibitor. The concentration of the substrate or product is continuously recorded. The reaction velocities at various time periods after the addition of the enzyme are determined from the slope of the tangent to the concentration vs time spectrophotometric tracings.

With either procedure, the natural logarithm of the velocity is plotted against time. There are three possible outcomes of the experiment. First, if the velocity remains constant for a significant period of time, the inhibitor binding is probably rapidly reversible. In this case, classical steady state kinetic methods should be employed. Second, if the semilog plot is a curve leveling off to a horizontal line, i.e. the velocity approaches a finite non-zero value as time progresses, the present method is not applicable. In this case, one can employ a more complex method to estimate the value of λ, as will be the subject of a future publication. If practical, one can repeat the experiment employing higher inhibitor concentrations, a lower enzyme concentration, or both. Third, if the semilog plot is a straight line, the value of λ is estimated from the graph or preferably by a linear regression.

Determination of the apparent first- and second-order rate constants, α and β

The values of λ determined at various levels of S are plotted against I_t according to Equation 21. The values of α and β are obtained as the intercepts and the slopes respectively.

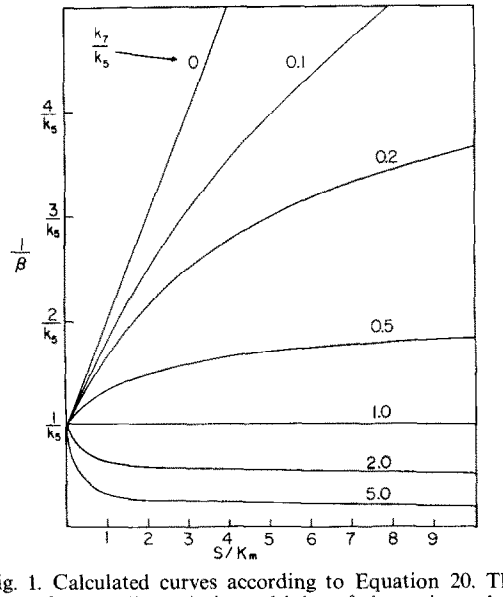

Fig. 1. Calculated curves according to Equation 20. The scale of the ordinate is in multiples of the reciprocal of k_5, and that of the abscissa in multiples of K_m. The number on each curve represents the ratio of k_7 to k_5. If the inhibition is competitive, $k_7 = 0$.

If the values of α at various levels of S are constant within experimental error, probably the inhibition mechanism is competitive, and the value of α is an estimate of the rate constant, k_6. On the other hand, if α shows an obvious dependency on S, Equation 30 or Equation 32 should be employed to estimate K'_m and $\Delta\alpha_{max}$. If the value of α_0 is available, $k_6 = \alpha_0$ and $k_8 = \alpha_0 - \Delta\alpha_{max}$.

The value of β, obtained as the slope of λ vs I_t plot, will be a function of S except when $k_5 = k_7$. A convenient diagnostic plot would be that of $1/\beta$ vs S as illustrated in Fig. 1. If the inhibition is competitive, the plot would be a straight line. In the case of non-competitive inhibition, the plot would be a hyperbola, except when $k_5 = k_7$, in which case a horizontal line is produced. Thus, this plot provides information on the inhibition mechanism, but an analysis of the data according to Equation 30 would provide more quantitative information. A similar diagnostic plot of $1/\alpha$ vs S may also be employed.

Evaluation of rate constants, K_m, and inhibition mechanism

Equations 30–33 are similar to the Michaelis–Menten equation and its double reciprocal form respectively. Therefore, Wilkinson's method for the statistical analysis of the Michaelis–Menten equation [7, 8] is directly applicable. The variance of $\Delta\beta$ is reasonably homogeneous, as may be seen in the real example given in Table 2 below. Therefore, $(n_i - 2)(\Delta\beta)^4$ is an adequate weight for the first step of Wilkinson's procedure, in which a linear regression line is fitted to the double reciprocal form of the equation to obtain provisional values of K_m and V_{max} (K_m and $k_5 - k_7$ in the present case). The degree of freedom for each value of $\Delta\beta$, i.e. $(n_i - 2)$, rather than the number of original assays (n_i), should be used in the weighting factor. For the second part of Wilkinson's analysis in which a hyperbola is directly fitted to the Michaelis–Menten equation (Equation 22 in the present case), $(n_i - 2)$ is the proper weight. Thus $k_5 - k_7$ and its standard error of estimation, S. E. $(k_5 - k_7)$, may be obtained with a degree of freedom, $\Sigma(n_i - 2)$, where $\Sigma(n_i - 2)$ is the sum of all the degrees of freedom for the individual determination of β except that at $S = 0$. Finally, the inhibition mechanism, i.e. whether or not the inhibition is competitive, can be ascertained by a statistical test of the null hypothesis that there is no difference between β_0 (an estimate of k_5) and $\Delta\beta_{max}$ (an estimate of $k_5 - k_7$). The affirmative test for this null hypothesis indicates that $k_7 = 0$, i.e. the inhibition is competitive. The test statistics t is given by:

$$t = d/S_{\bar{d}} \tag{34}$$

where d and $S_{\bar{d}}$ are the difference between β_0 and $\Delta\beta_{max}$, and its standard error respectively. The value of $S_{\bar{d}}$ can be calculated by:

$$S_{\bar{d}} = \sqrt{\left(\frac{df_1 S_1^2 + df_2 S_2^2}{df_1 + df_2}\right)\left(\frac{1}{n_1} + \frac{1}{n_2}\right)} \tag{35}$$

where df and n represent the degrees of freedom and the number of samples respectively. The P value may

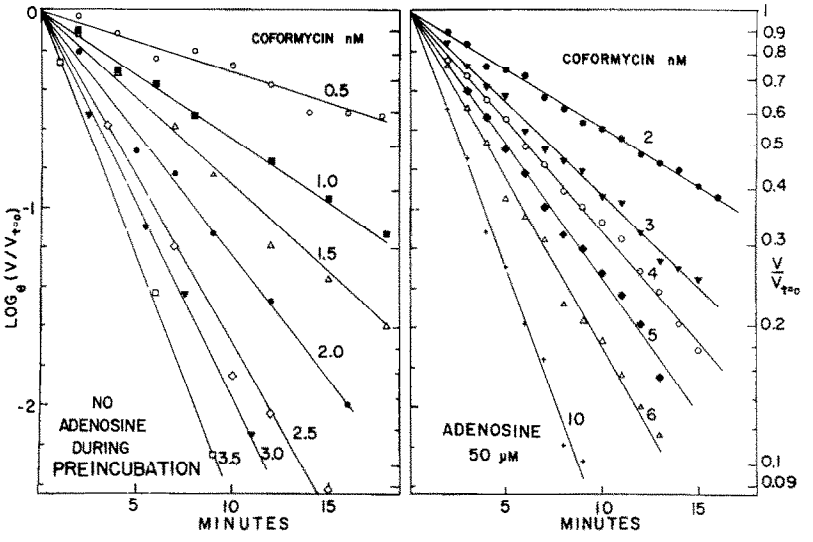

Fig. 2. Determination of the pseudo-first-order rate constants (λ) by the semilog plot of $\ln v$ vs t according to Equation 18. The velocity at zero time was taken as 1. The concentration of the enzyme was approximately 0.001 unit/ml of the reaction mixture. Left panel: data obtained in the absence of adenosine in the preincubation mixture. Aliquots of the preincubation mixture were assayed for the enzymic activity by adding the substrate at various time periods. Right panel: results of the experiments in the presence of 50 μM adenosine (see Fig. 3). The straight lines were drawn by linear regression analysis.

be calculated or obtained from a table of Student's t-distribution.

RESULTS

The results of the experiments which demonstrated the competition between the substrate, adenosine, and the inhibitor, coformycin, for binding to the adenosine deaminase from calf intestinal mucosa are as follows.

Determination of K_m *by the conventional method*

The average and the standard error of estimation of the K_m value determined six times by the conventional method at pH 7.4 and 22° were 3.88 ($\pm$1.35) $\times 10^{-5}$ M. The variations among the determinations were disappointingly large with a coefficient of variation of as much as 37 per cent. Nevertheless, the observed value was in good agreement with the reported values of 3.53×10^{-5} M at pH 7.4 and 20° [9] and 2.4×10^{-5} M at pH 7.0 and 20.8° [10].

Determination of the pseudo-first-order rate constant, λ

To measure the constant, λ, in the absence of the substrate, the enzyme (0.001 unit/ml of the final volume) was mixed with the buffer solution (50 mM potassium phosphate, pH 7.4) containing various amounts of the inhibitor, coformycin. Aliquots of the preincubation mixture were assayed for the enzyme activity at various time periods after addition of the substrate. The logarithms of the velocities were plotted against time as shown in the left panel of Fig. 2.

To determine the values of λ in the presence of the substrate, the enzyme was added to the reaction mixture containing both the substrate and the inhibitor. Absorbance at 265 nm was continuously recorded. Velocities at various time periods were determined by the slope of tangents to the tracing as illustrated in Fig. 3. The natural logarithms of the velocities were plotted against time as shown in the right panel of Fig. 2. Note that the slopes of the straight lines for the same concentrations of the inhibitor are much steeper in the absence of the substrate (left panel of Fig. 2) than in the presence of the substrate (right panel), indicating that the substrate indeed impedes the binding of the inhibitor to the enzyme. The slopes of the straight lines in Fig. 2 are the pseudo-first-order rate constants, λ.

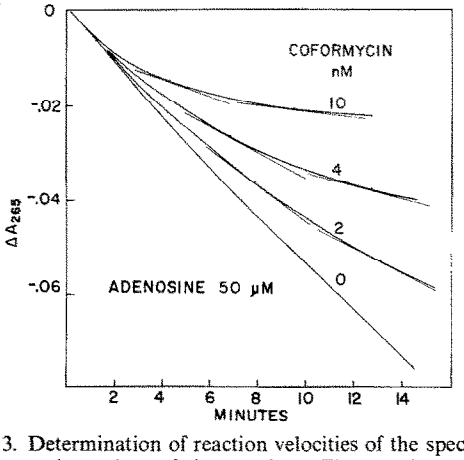

Fig. 3. Determination of reaction velocities of the spectrophotometric tracings of the reactions. The reactions were started by the addition of the enzyme to the reaction mixture containing 50 μM adenosine and various concentrations of coformycin. The reaction velocities at various time periods were measured as the slopes of tangents to the tracings.

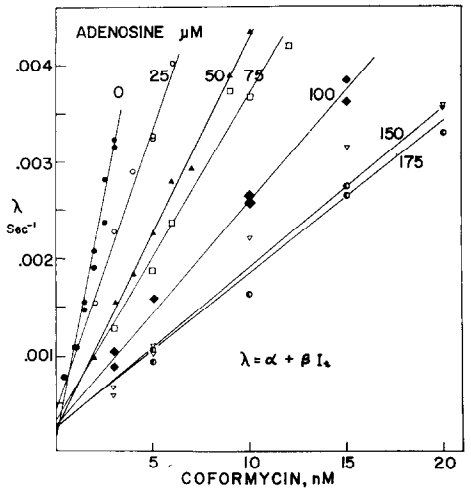

Fig. 4. Estimation of the apparent rate constants, α and β, according to Equation 21. The values of λ obtained from Fig. 1 are plotted against coformycin concentration. The linear regression lines were computed by the least squares method. The numerical values are presented in Table 2.

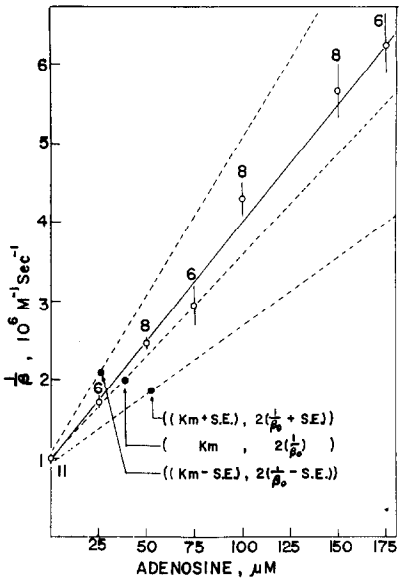

Fig. 5. Plot of the reciprocal of the apparent second-order rate constant vs adenosine concentration according to Equations 20 and 23. The circles and the perpendicular bars indicate the reciprocal of apparent second-order rate constants and their standard errors of estimation (see Table 2). The number on each data point indicates the number of replicates. The middle dashed line indicates the theoretical expectation on the basis of competitive inhibition (i.e. $k_7 = 0$), and the values of k_5 and K_m being $1.009 \times 10^6\ M^{-1}\,sec^{-1}$ and $38.8\ \mu M$ respectively. The upper and lower dashed lines represent the cases where the true values of $1/k_5$ and K_m are $(1/k_5) \pm$ S. E. $(1/k_5)$ and $K_m \pm$ S. E. (K_m) respectively. The coordinates of one point on each of these three theoretical lines are given in the figure.

First-order rate constant (k_6) *and inhibition constant*

The pseudo-first-order rate constants (λ) determined from data such as that illustrated in Fig. 2 were plotted against the inhibitor concentration (I_t) as shown in Fig. 4. Linear regression lines were computed by the least squares method. The intercepts (α), the slopes (β), and their respective standard errors at various levels of adenosine are given in Table 2. The data obtained at the adenosine concentration of 125 μM were obviously in gross error; therefore, they were omitted from further analyses.

Note that there is no apparent correlation between the values of α and the adenosine concentrations. This fact suggests that coformycin may compete with adenosine; therefore, $K'_m = \infty$ in Equation 19 and the observed values of α are indeed estimates of k_6. The weighted average of α is $0.219 \times 10^{-3}\ sec^{-1}$ with a standard error of $0.104 \times 10^{-3}\ sec^{-1}$.

From these values it can be calculated that:

$$K_i = \frac{k_6}{k_5} = \frac{\alpha}{\beta_0} = 2.17 \times 10^{-10}\ M$$

with the observed range of 0.8×10^{-10} M to 4.4×10^{-10} M, and the standard error of estimation:

$$\text{S. E. } (K_i) = K_i[(0.104/0.219) + (0.0569/1.009)] = 0.76 \times 10^{-10}\ M.$$

The 95 per cent confidence limits of K_i value are approximately 0.68×10^{-10} M and 3.66×10^{-10} M.

Apparent second-order rate constants and diagnostic plot

The reciprocals of the values of β were plotted against S as shown in Fig. 5. The linear regression line computed with weights of $(n_i - 2)/(\text{S. E.})^2$ was represented by the solid line in the figure. The correlation coefficient for this line was 0.998. It can be seen that the regression line is in good agreement with the theoretical line (the middle dashed line). The latter was drawn under the assumptions that coformycin and adenosine are competitive, and that the values of K_m and k_5 are 3.88×10^{-5} M and $1.009 \times 10^6\ M^{-1}\,sec^{-1}$ (see Table 2) respectively. If

Table 2. Estimated parameters and standard errors

AdR (μM)	N	α ($10^{-3}\ sec^{-1}$)	β ($10^6\ M^{-1}\,sec^{-1}$)	$1/\beta$ (10^{-6} M sec)	$\Delta\beta$ ($10^6\ M^{-1}\,sec^{-1}$)	$1/\Delta\beta$ (10^{-6} M sec)
0	11	0.080 ± 0.115	1.009 ± 0.057	0.991 ± 0.056	0	∞
25	6	0.447 ± 0.156	0.584 ± 0.036	1.714 ± 0.104	0.426 ± 0.051	2.350 ± 0.283
50	8	0.230 ± 0.080	0.408 ± 0.013	2.451 ± 0.076	0.601 ± 0.045	1.644 ± 0.124
75	6	0.288 ± 0.236	0.342 ± 0.029	2.924 ± 0.249	0.667 ± 0.050	1.499 ± 0.112
100	8	0.249 ± 0.113	0.233 ± 0.012	4.283 ± 0.220	0.776 ± 0.045	1.289 ± 0.074
125	8	0.813 ± 0.162	0.167 ± 0.012	5.974 ± 0.422	0.842 ± 0.045	1.188 ± 0.063
150	8	0.200 ± 0.129	0.177 ± 0.011	5.646 ± 0.336	0.832 ± 0.045	1.202 ± 0.064
175	6	0.203 ± 0.160	0.160 ± 0.010	6.244 ± 0.386	0.849 ± 0.048	1.178 ± 0.066

one standard deviation unit of error in each of the estimates of K_m and k_5 (β_0) is allowed, the theoretical line will lie between the upper and the lower dashed lines shown in the figure. It can be seen that the experimental points and their standard errors lie well within the theoretically predicted limits. Therefore, Fig. 5 suggests that coformycin and adenosine compete with each other for binding to the enzyme.

Statistical tests

In order to apply more rigorous statistical tests, the values of β were analyzed according to Equation 31 by the method of Wilkinson. The results are:

$$\Delta\beta_{max} = 1.031 \pm 1.234\ (10^6\ M^{-1}\ sec^{-1})$$

$$K_m = 3.61 \pm 0.120\ (10^{-5}\ M)$$

with 28 degrees of freedom.

The estimate of the common variance of $\Delta\beta_{max}$ and β_0 (i.e. the estimate of k_5 or β at $S = 0$) is:

$$S_d^2 = \frac{(11-2)(0.057)^2 + (28-2)(1.234)^2}{11+28-4} = 1.132025.$$

The standard error of the difference is:

$$S_{\bar{d}} = \sqrt{1.132}\sqrt{\frac{1}{11} - \frac{1}{28}} = 0.378604$$

The t-value is then:

$$t = \frac{d}{S_{\bar{d}}} = \frac{1.031 - 1.009}{0.3786} = 0.5811$$

with $df = (11-2) + (28-2) = 35$ and $P = 0.9528$.

Therefore, the probability of there being no difference between $\Delta\beta_{max}$ and β_0, i.e. the probability of $k_7 = 0$, hence the probability of coformycin being a competitive inhibitor of the adenosine deaminase, is greater than 95 per cent on the basis of the data.

Similarly, the two K_m values, one measured by the conventional method, 3.88 ($\pm$1.35) $\times$ 10^{-5} M, and the other estimated from Fig. 6, 3.61 ($\pm$0.120) $\times$ 10^{-5} M, are in excellent agreement.

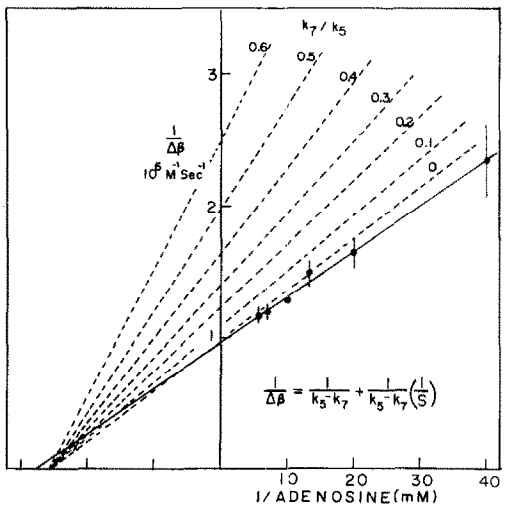

Fig. 6. Double reciprocal plot according to Equation 33. The solid line was drawn on the bases of parameters computed by the statistical method of Wilkinson. The dashed lines are those expected from a competitive inhibition ($k_7 = 0$) and non-competitive inhibitions with various magnitudes of k_7 values relative to k_5. The intercepts on x- and y-axes are the estimates of $(k_5 - k_7)$ and $-1/K_m$ respectively.

DISCUSSION

As demonstrated in previous publications [1, 2], the prolonged non-steady state period of the binding of a potent inhibitor to an enzyme makes classical steady state kinetic theories inapplicable. Therefore, it was thought until recently that the elucidation of inhibition mechanisms for these tight-binding inhibitors would be extremely difficult. The prolonged non-steady state exhibited by such inhibitors permits, however, the measurement of the rate constants for the binding of the inhibitor to the enzyme without special equipment such as stopped-flow or temperature-jump apparatus [11]. Furthermore, the manner in which these rate constants are affected by the presence of the substrate can be used to elucidate the inhibition mechanism. This was demonstrated above by the inhibition of adenosine deaminase by coformycin.

While the mathematical derivation of the equations is somewhat complicated, the basic principle of the method can be readily understood on an intuitive basis. If the inhibition is competitive, in the presence of the substrate only the free enzyme (E) of the uninhibited enzyme species (E and ES) is available for the binding of the inhibitor. Therefore, the effect of the presence of the substrate on the rate of formation of the EI complex is that the concentration of the enzyme appears to be reduced by a factor of $1/(1 + S/K_m)$, which is equivalent to the ratio $(E)/[(E) + (ES)]$, and the apparent second-order rate constant becomes $k_5/(1 + S/K_m)$. Thus, in the presence of a saturating concentration of the substrate, the binding of the inhibitor could be completely blocked and the apparent second-order rate constant would become zero. In contrast, in non-competitive inhibition, even if the system is saturated with the substrate, the binding of the inhibitor to the ES complex would proceed with a rate constant of k_7. In the presence of non-saturating concentrations of the substrate, the rates of association of the inhibitor with E and ES would be $k_5/(1 + S/K_m)$ and $k_7(S/K_m)/(1 + S/K_m)$, respectively, and the sum of these rates would be given by β as in Equation 20. Note that the factor $(S/K_m)/(1 + S/K_m)$ is equivalent to $(ES)/[(E) + (ES)]$ when the steady state conditions are reached between E and ES. Thus, the apparent rate constant will change from k_5 to k_7 as the concentration of S changes from 0 to ∞. The value of β at any finite concentration of the substrate is determined by Equation 20.

It should be emphasized that the present method is applicable only when the inhibitor concentration is sufficiently higher than both K_i' and E_t. In general, the condition $I_t \gg E_t$ can be readily met. However, if K_i' is relatively high, and if a high concentration of the inhibitor is used to meet the condition $I_t \gg K_i'$, the steady state conditions may be reached in a relatively short period of time, as was the case with xan-

thine oxidase inhibition by allopurinol [2]. In such cases, λ can be estimated in principle, as suggested previously, either directly from the time course tracing of the reaction (Fig. 3 of Ref. 1) or indirectly from a plot of log $[(v - v_s)/(v_z - v_s)]$ vs t (Equation 3 of Ref. 1). In practice, however, the accuracy of any determination of λ will be greatly affected by the accuracy of the values of the initial velocity (v_z) and the steady state velocity (v_s). These values may be difficult to obtain with the desired degree of accuracy. Therefore, a method for the determination of λ' which is less sensitive to the accuracy of v_z and v_s values is highly desirable. Currently this subject is under investigation.

For the human erythrocytic adenosine deaminase, the K_i value of coformycin was estimated at 1.2×10^{-10} M from the values of I_{50} determined after 50 min of incubation of the enzyme with the inhibitor. It was also suggested that the EI complex probably undergoes a slow conformational change to the $E'I$ form. Whether or not a similar phenomenon exists with the enzyme from calf intestinal mucosa has not been examined. Nevertheless it is noteworthy that the K_i values of 2.2×10^{-10} M obtained in the present study is very similar to that of the human erythrocytic enzyme. If the EI complex indeed undergoes a conformational change, these K_i values probably represent the dissociation constant of the initial complex, EI, rather than the overall dissociation constant of $(EI) + (E'I)$.

Given the close structural similarity between adenosine and coformycin, the competition between the two compounds for adenosine deaminase is not surprising. However, in the case of other potent inhibitors in which the structural analogy between the substrate and inhibitor may not be so apparent, information on whether or not the inhibitor competes with the substrate would be of invaluable aid for the understanding of the inhibition mechanism. For instance, some inhibitors which alkylate SH groups react slowly with the enzyme. Unequivocal determination of the competition between the thiol reagent and the substrate might provide valuable insights as to whether or not the reactive thiol group is located at or close to the substrate-binding site of the enzyme.

Acknowledgement—The author is indebted to Dr. Hamao Umezawa for many stimulating discussions and for the generous gift of coformycin.

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