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TIGHT-BINDING INHIBITORS—II

NON-STEADY STATE NATURE OF INHIBITION OF MILK XANTHINE OXIDASE BY ALLOPURINOL AND ALLOXANTHINE AND OF HUMAN ERYTHROCYTIC ADENOSINE DEAMINASE BY COFORMYCIN*

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Abstract—The non-steady state nature of the inhibition of milk xanthine oxidase by allopurinol and alloxanthine was demonstrated, and the kinetic data presented are consistent with the known mechanisms of inhibitions by these inhibitors. With the use of human erythrocytic adenosine deaminase and its tight-binding inhibitor, coformycin, it was demonstrated that the classical methods of enzyme kinetics based on the steady state assumptions are grossly inadequate for determining the inhibition mechanisms or inhibition constants for tight-binding inhibitors. The application of the Ackermann–Potter plot, I_{50}, the Easson–Stedman plot (or Henderson plot), and the rates of association and dissociation of enzyme–inhibititor complex were presented and their usefulness was evaluated. The molar equivalency and the catalytic number of human erythrocytic adenosine deaminase were estimated at about $1{\cdot}0 \times 10^{-10}$ mole/unit and $1{\cdot}0 \times 10^{4}$ min^{-1} respectively. It was also demonstrated that the K_i value of coformycin for this enzyme does not exceed $1{\cdot}2 \times 10^{-10}$ M, and that the second-order rate constant for the association of the enzyme with coformycin is approximately 2×10^{6} M^{-1} sec^{-1}. The biphasic nature of the dissociation of the deaminase–coformycin complex (*EI* complex) indicates that the *EI* complex undergoes a slow conformational change. The implications of these new kinetic approaches for the study of tight-binding inhibitors, including transition-state analogs, were discussed.

In the preceding article [1], the theoretical bases of the kinetic behavior of tight-binding inhibitors have been examined under both steady state and non-steady state conditions. It was emphasized that, in the presence of a potent tight-binding inhibitor, the transient or non-steady state phase may be so markedly prolonged that, under ordinary experimental conditions, the familiar methods based on steady state kinetics cannot be applied directly for the determination of the inhibition mechanisms or the inhibition constants.

Many examples of the difficulties encountered in the analyses of such cases may be found in the literature. For example, Hartman and Heidelberger [2] reported that thymidylate synthetase from Ehrlich ascites tumor cells is inhibited by 5-fluoro-2′-deoxyuridine 5′-monophosphate (FUdRP) competitively with deoxyuridylate (dUMP). However, Blakley [3] and Mathews and Cohen [4] later reported that the inhibition by FUdRP is non-competitive with dUMP, after a short preincubation of the enzyme with the inhibitor. Upon re-examination of the Ehrlich ascites tumor enzyme, Reyes and Heidelberger [5] found that both FUdRP and 5-trifluoromethyl-2′-deoxyuridine 5′-monophosphate (F_3TdRP) behaved as competitive inhibitors against dUMP when the enzyme was not preincubated, but when the enzyme was preincubated with the inhibitor, non-competitive patterns are obtained. Furthermore, K_i values calculated from the slopes of Lineweaver–Burk plots were 10- and 6-fold lower for FUdRP and F_3TdRP, respectively, when the enzyme was preincubated with the inhibitor. Among the numerous examples from the literature where problems of this type have been encountered, one might cite the studies of various dihydrofolate reductases and their interaction with the potent antifolate drugs such as methotrexate [6, 7] and the inhibition of liver alcohol dehydrogenase by compounds such as pyrazole [8, 9].

In the present publication, the non-steady state behavior of enzymic reactions in the presence of tight-binding inhibitors is examined with the xanthine oxidase reaction in the presence of allopurinol or alloxanthine and with human erythrocytic adenosine deaminase in the presence of coformycin. These two experimental systems were chosen because erythrocytic adenosine deaminase and its reaction mechanism represent a comparatively uncomplicated enzymic reaction; on the other hand, xanthine oxidase and the related enzyme, aldehyde oxidase, may be among the most complex enzymes yet identified [10], being capable of transporting electrons from a substrate such as hypoxanthine to atmospheric oxygen or other artificial electron acceptors through a complex series of electron carriers. Furthermore, the inhibition mechanism of xanthine oxidase by allopurinol is among the most complex studied. A preliminary dis-

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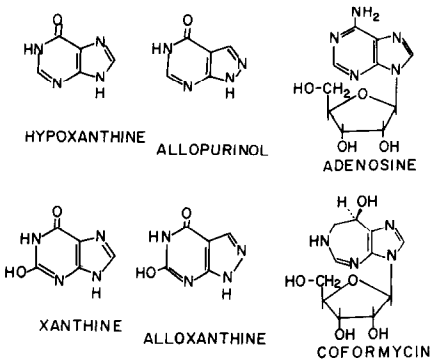

Fig. 1. Structures of substrates and inhibitors of xanthine oxidase and adenosine deaminase.

cussion of the marked time dependence of xanthine oxidase reaction velocity in the presence of allopurinol and alloxanthine has been presented [11]. Coformycin, an antibiotic discovered and recently characterized by Sawa *et al.* [12], has been found to be a potent inhibitor of adenosine deaminases from various sources [12–14]. The structures of coformycin, allopurinol and of alloxanthine are compared with the normal substrates in Fig. 1.

MATERIALS AND METHODS

Xanthine oxidase (EC 1.2.3.2) from buttermilk (0.4 unit/mg) was purchased from Sigma and had an AFR^{27} of 23·6 as defined by Avis *et al.* [15]. Since an AFR^{25} value of 160 has been indicated for the homogeneous enzyme [16], the preparation used in our studies was about 15 per cent pure in terms of the catalytically active flavin content. The concentration of enzyme used in each reaction was estimated at $3{\cdot}1 \times 10^{-8}$ M on the basis of total flavin content ($\epsilon_{450\,nm} = 11{\cdot}3 \times 10^3$) [16], and at $4{\cdot}2 \times 10^{-9}$ M in terms of the catalytically active form of flavin. Xanthine was obtained from Matheson, Collman & Bell. Allopurinol (4-hydroxypyrazolo[3,4-*d*]pyrimidine, $\epsilon_{261\,nm} = 7{\cdot}80 \times 10^3$ at pH 11) and alloxanthine (4,6-dihydroxypyrazolo[3,4-*d*]pyrimidine, $\epsilon_{242\,nm} = 8{\cdot}25 \times 10^3$ at pH 8·3) [16] were gifts from Dr. G. B. Elion of the Wellcome Research Laboratories. The assay mixture contained: potassium phosphate buffer, pH 7·8, 50 mM; Na_2EDTA, 0·01 mM; xanthine oxidase, $4{\cdot}2 \times 10^{-9}$ M; xanthine, 0·5 mM; and allopurinol or alloxanthine where indicated. The formation of uric acid was measured by determining the change in absorbancy at 295 nm.

Preincubation of the xanthine oxidase with an inhibitor under anaerobic conditions was carried out by gently blowing nitrogen gas into the enzyme solution for 1 min or more before addition of allopurinol or alloxanthine; after the inhibitor was mixed, a gentle flow of nitrogen gas over the mixture was maintained until the mixture in a small volume was added to the buffer solution containing xanthine. Aerobic incubations were carried out in atmospheric oxygen without any special treatment.

The adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) used in these studies was a partially purified preparation (0·33 unit/mg) from human erythrocytes as described earlier [14]. The activity of adenosine deaminase was measured spectrophotometrically as described previously [14] by following the decrease in absorbancy at 265 nm in 50 mM phosphate buffer, pH 7·5, at room temperature. Coformycin (3-β-D-ribofuranosyl-6,7,8-trihydroimidazo [3,4-*d*][1,3]diazepin-8(R)-ol)[17] was a gift from Dr. Umezawa of the Institute of Microbial Chemistry, Tokyo. The hemoglobin-coated charcoal used in the study of dissociation of the adenosine deaminase–coformycin complex was prepared as described by Waxman *et al.* [18].

RESULTS AND DISCUSSION

Studies of xanthine oxidase

As an example of the marked time-dependency of the inhibition of enzymes by tight-binding inhibitors, milk xanthine oxidase was chosen because of the ready availability of both the enzyme and two tight-binding inhibitors, allopurinol (a hypoxanthine analog, Fig. 1) and alloxanthine (a xanthine analog, Fig. 1) [19, 20]. These inhibitors have been thoroughly examined and their mechanisms of action, although very complex, are reasonably well understood [21, 22]. The K_i values for allopurinol and alloxanthine have been estimated at $6{\cdot}3 \times 10^{-10}$ M and $5{\cdot}4 \times 10^{-10}$ M, respectively [21], and the mechanism of inhibition, as now generally accepted, is as follows [21, 22]. The enzyme contains FAD, non-heme iron, labile sulfur and molybdenum, and these electron carriers are rapidly reduced by a substrate such as hypoxanthine or xanthine, or by the oxidizable inhibitor, allopurinol. In the presence of oxygen, these electron carriers are reoxidized. However, in the presence of alloxanthine or allopurinol, the enzyme is trapped in a partially reduced form in which both FAD and

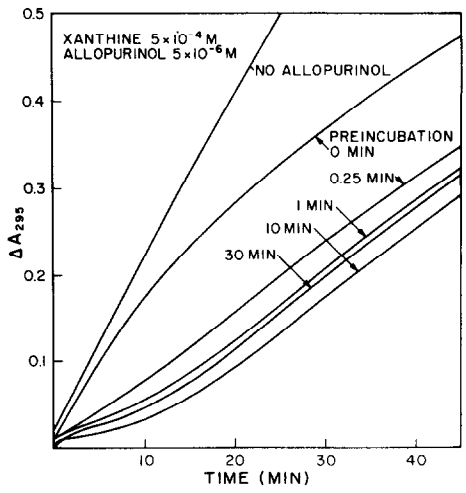

Fig. 2. Effect of preincubation with allopurinol on the xanthine oxidase reaction. The enzyme was preincubated at room temperature with allopurinol in 2·9 ml reaction mixture for various lengths of time. The reactions were started by adding 0·1 ml xanthine solution. The buffer and the enzyme concentration were as described in Materials and Methods. The concentrations indicated in the graph were those in the final reaction mixture. In order to present several tracings on one graph without undue overcrowding, the absorbancies at zero time were chosen arbitrarily.

iron-sulfur are reoxidized but molybdenum remains at the reduced state, Mo (IV) level. It is believed that this complex between the enzyme and alloxanthine (or allopurinol) is responsible for the strong inhibition by the inhibitors. However, in the presence of an artificial electron acceptor such as potassium ferricyanide, allopurinol serves as a good substrate.

Time-dependency of inhibition by allopurinol. As shown in Fig. 2, preincubation of the enzyme with allopurinol caused markedly greater inhibition than when the reaction was initiated by addition of the enzyme. Figure 2 also shows that inhibition of the preincubated enzyme was slowly but progressively reversed upon addition of a high concentration of the substrate, xanthine. For such phenomena, a term, hysteretic, or relaxable inhibiton has been proposed [11]. The fact that the inhibition is gradually and partially overcome is particularly noteworthy because inhibitors such as allopurinol are often referred to as "titrating" or "stoichiometric" inhibitors. It should also be noted that, when the enzyme was not preincubated with inhibitor, initially the degree of inhibition was negligible, but the enzymic reaction velocity gradually decreased with time. Furthermore, a final steady state rate was reached that was essentially identical whether or not the enzyme was preincubated with inhibitor. These findings indicate that the formation and dissociation of *EI* complex are markedly slower than the reactions between the enzyme and the substrate. Thus, the results of Fig. 2 illustrated one of the major theoretical points developed in the previous paper [1].

During these studies, an unexpected and as yet unexplained phenomenon was observed, i.e. in experiments where the enzyme was preincubated under aerobic conditions with allopurinol for 10 min or longer, initial rapid velocities were observed, as shown in Fig. 3. It can be seen that when the preincubation period was 10 min or longer, triphasic reaction tracings were obtained, i.e. initially fast, then slow, followed by a faster steady state rate. The significance of these triphasic reaction curves deserves further study by technics other than simple spectrophotometry.

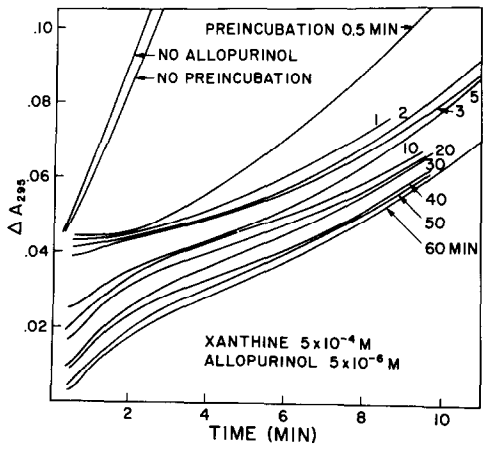

Fig. 3. Effect of long-term preincubation with allopurinol on the xanthine oxidase reaction. The experimental conditions are similar to those of Fig. 2. The zero time absorbancies were chosen arbitrarily.

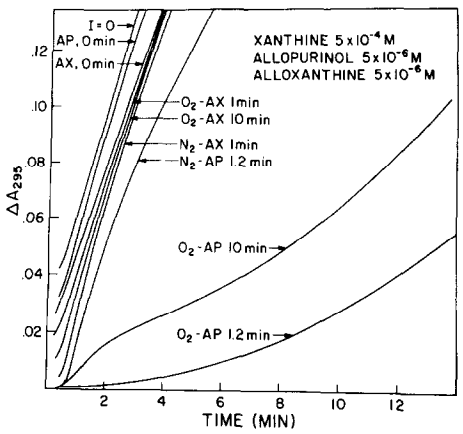

Fig. 4. Requirement for oxygen in the strong inhibition by allopurinol and the lack of inhibition by alloxanthine in early periods of the xanthine oxidase reaction. The enzyme was incubated at room temperature for various lengths of time, with allopurinol (AP) or with alloxanthine (AX) under aerobic (atmospheric oxygen, O_2) or anaerobic (nitrogen gas, N_2, see Materials and Methods for the procedure) conditions. A volume of 0·1 ml preincubation mixture then was added to 2·9 ml xanthine solution in buffer which had been maintained under aerobic conditions. The concentrations presented in the graph were those of the final reaction mixtures. The zero time absorbancies were chosen arbitrarily.

Requirement for reducing agent and oxygen for inhibition. The data in Fig. 4 demonstrate the effect of oxygen on the inhibition by allopurinol. If the reaction is started by addition of enzyme, the presence of allopurinol (AP, 0 min) or alloxanthine (AX, 0 min) does not cause an impressive difference in the initial enzymic velocity. Preincubation of the enzyme with allopurinol under aerobic conditions (O_2–AP, 1·2 min; O_2–AP, 10 min) causes a marked inhibition of the reaction which is progressively reversed upon addition of the substrate, xanthine, while the initial velocity obtained after anaerobic preincubation with allopurinol for the same length of time (N_2–AP, 1·2 min) was essentially the same as the uninhibited reaction ($I = 0$). Unlike allopurinol, alloxanthine (which is incapable of donating electrons to, and reducing the enzyme) did not initially inhibit the enzyme after preincubation under aerobic conditions (O_2–AX, 1 min and O_2–AX, 10 min) or anaerobic conditions (N_2–AX, 1 min). These data are consistent with the view that the partially reduced form of the enzyme is the inhibited species [21].

Figure 5 illustrates the time-course of the xanthine oxidase reaction in the presence of alloxanthine monitored for longer periods of time than in the experiments presented in Fig. 4. In the presence of alloxanthine, the initial velocity was not very different from that of the uninhibited reaction (X, 0·5 mM; AX, 0) whether or not the enzyme was preincubated under aerobic conditions for 5 min. However, the reaction became progressively inhibited after the addition of the substrate (third and fourth curves from the top). At a 10-fold lower concentration of the substrate and in the absence of inhibitor (X, 0·05 mM; AX, 0), due to the lack of substrate inhibition [23, 24], the reaction proceeded more rapidly than at high substrate

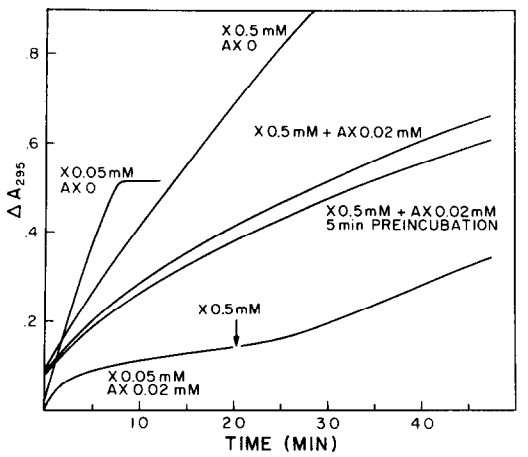

Fig. 5. Requirement for a reducing agent in the inhibition of xanthine oxidase by alloxanthine. Whether preincubated (the second curve from bottom) or not (the third curve from bottom), alloxanthine (AX) has little effect on the reaction velocity compared to the control (X, 0·5 mM; AX, 0) in the initial period of time, whereas marked inhibitory effects become apparent later. In the presence of low concentrations of the oxidizable substrate, xanthine (X, 0·05 mM; AX, 0·02 mM), the reaction almost ceased long before the substrate was exhausted, as evidenced by comparison with the control reaction (X, 0·05 mM; AX, 0). This marked inhibition was partially and gradually reversed after addition of a larger amount of the substrate (at the arrow on the bottom curve).

concentration until the substrate was depleted. When the enzyme was added to a reaction mixture containing alloxanthine and the same small amount of xanthine (X, 0·05 mM; AX, 0·02 mM), the full inhibitory effect was expressed within 10 min, which was partially reversed by the addition of a large amount of xanthine (0·5 mM at the arrow on the bottom curve in Fig. 5).

These phenomena, which are consistent with prior reports by other investigators [21, 22], further support the thesis that the enzyme must be in the reduced state before it can be inhibited by substrate analogs, such as alloxanthine. Reduction of the enzyme can be achieved by a substrate such as xanthine or hypoxanthine, by a substrate analog such as allopurinol, or by reducing agents such as dithionite [16, 21, 22].

Studies of human erythrocytic adenosine deaminase

Ackermann–Potter analysis. In a preliminary kinetic study [14] with human erythrocytic adenosine deaminase, the inhibition constant (K_i) of coformycin was estimated by the classical methods of analysis (based on steady state assumptions) as approximately 1×10^{-8} M. In order to determine whether coformycin behaves as a tight-binding inhibitor, varying amounts of the enzyme were incubated with several concentrations of coformycin for 50 min at room temperature, after which the residual enzymic activity was assayed at an adenosine concentration of 0·1 mM. The results are presented in Fig. 6A. This is a classical Ackermann–Potter plot [25] which clearly indicates that coformycin is a "stoichiometric inhibitor." However, as emphasized in the previous paper [1], apparent stoichiometric or titrating inhibition does not necessarily indicate the formation of a covalent bond between the enzyme and inhibitor, but may be explained by a very much slower equilibration between the inhibitor and the enzyme than the rate of equilibration between the enzyme and substrate.

As discussed earlier [1], an extension of the linear portion of the curve to the E_t-axis and v-axis enables one to estimate both the molar equivalency of the enzyme concentrations and the value of k_3, the catalytic number (see equation 17 and Fig. 1 of Ref. 1). From the slope of the plot of the E_t-axis intercepts vs I_t as shown in Fig. 6B, it was estimated that 1 unit of adenosine deaminase is equal to $1{\cdot}0 \times 10^{-10}$ molar equivalent. The catalytic number, k_3, i.e. the moles of substrate converted to product per mole of active center per min, can be calculated in two ways, i.e. from the slopes or from the v-intercepts of the asymptotes (linear portions) of the Ackermann–Potter plots according to the following equation (equation 17 and Fig. 1 of Ref. 1):

$$v = \left(\frac{k_3 S}{K_m + S}\right) E_t - \frac{k_3 I_t S}{K_m + S} \qquad (1)$$

The slope of the Ackermann–Potter plot is $k_3S/(K_m + S)$ when E_t is expressed in terms of molar equivalents. In Fig. 6A, the slope is $8{\cdot}9 \times 10^{-6}$ (M/min)/(unit/liter), and since 1 unit/liter corresponds to about $1{\cdot}0 \times 10^{-10}$ M, the slope is 8900 (M/min)/M, or 8900 min^{-1}. Since the values of S (0·1 mM) and K_m (0·025 mM) [14] are known, k_3 can be calculated from

$$\text{Slope} = \frac{k_3 S}{K_m + S} = 8900\ \text{min}^{-1} \qquad (2)$$

as $1{\cdot}11 \times 10^4\ min^{-1}$. Alternatively, k_3 can be estimated from the plot of v-axis intercepts vs I_t, as shown in Fig. 6C. The value obtained in this manner is $0{\cdot}995 \times 10^4\ min^{-1}$, which is in good agreement with the value of $1{\cdot}11 \times 10^4\ min^{-1}$.

Thus, it has been demonstrated that the Ackermann–Potter plot may be used for diagnostic purposes as well as for the estimation of molar equivalencies and catalytic numbers of enzymes. However, it should be noted that the value of K_i cannot be estimated readily from this plot.

Non-steady state nature of inhibited reaction and Lineweaver–Burk analyses. Figure 7 shows several spectrophotometric tracings of adenosine deaminase reactions with 0·05 mM adenosine in the presence of various concentrations of coformycin. In these cases, the substrate and inhibitor were added to the reaction mixtures and the reactions were started by the addition of enzyme. This type of experimental procedure is usually satisfactory for initial velocity determinations in the presence of relatively "weak" inhibitors. In the present case, however, as may be seen in Fig. 7, there is little difference in the very early reaction velocities, regardless of the inhibitor concentration, despite the fact that profound differences in the velocities become obvious after a few min of reaction. In order to illustrate some of the errors that are possible in the analyses of such data by the usual methods of evaluating the mechanisms of inhibition, the velocities that occurred during the first 2 min of the reactions in experiments similar to that presented in Fig.

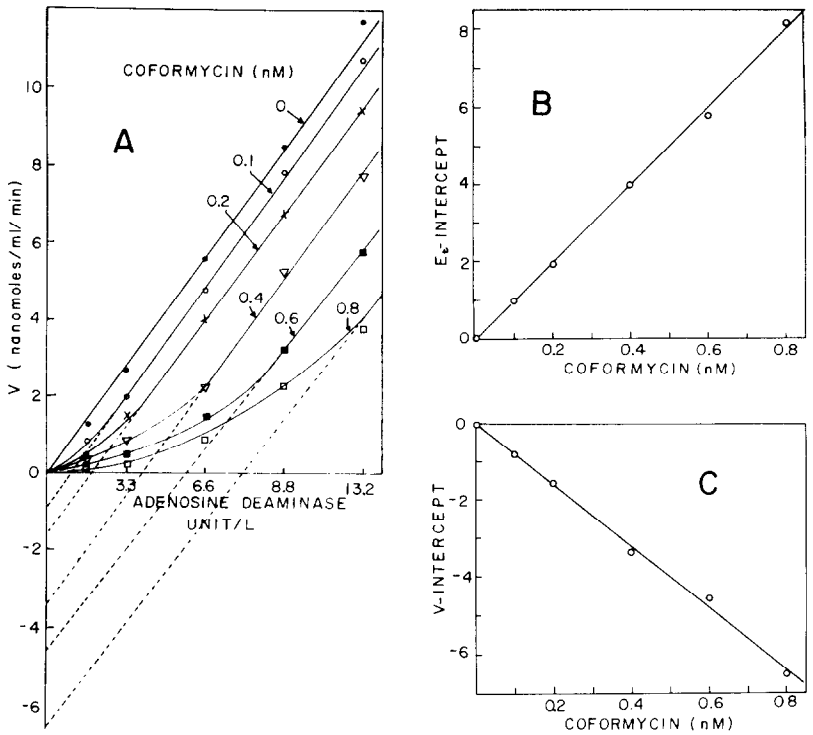

Fig. 6. Ackermann–Potter plot of adenosine deaminase preincubated with coformycin. Various amounts of adenosine deaminase were incubated in a total volume of 0·99 ml containing 50 mM potassium phosphate buffer, pH 7·5, and varying concentrations of coformycin. After incubation for 50 min at room temperature, the enzymic reaction was started by the addition of 10 μl of 10 mM adenosine (final concentration, 0·1 mM). The reaction was followed by measuring the decrease in absorbancy at 265 nm at room temperature. Frame A: plot of the enzymic velocities (nmoles/ml/min) vs the enzymic concentration (units/liter). Frame B: plot of E_t-intercept (from frame A) vs coformycin concentration. From this plot it was calculated that 1 unit/liter corresponds to about $1{\cdot}0 \times 10^{-10}$ M. Frame C: plot of v-intercept (from frame A) vs coformycin concentration. From the slope of the line, the k_3 value was calculated to be $0{\cdot}995 \times 10^4$ min^{-1}.

7 were assumed to represent "initial velocities," and from these values a Lineweaver–Burk plot was constructed, as shown in Fig. 8. Although considerable scatter occurs among the data points, this Lineweaver–Burk plot might readily be considered diagnostic of simple competitive inhibition, and an attempt might be made to estimate the value of K_i as done previously [14]. However, it is obvious from further examination of Fig. 7, e.g. the velocities that occur beyond 5 min of incubation, that the data points used for the Lineweaver–Burk plot of Fig. 8 did not represent steady state velocities; therefore, conclusion about the mechanism of inhibition or the estimation of K_i might be grossly in error. It would appear that the use of data such as these has resulted in considerable confusion in previous studies of other tight-binding inhibitors.

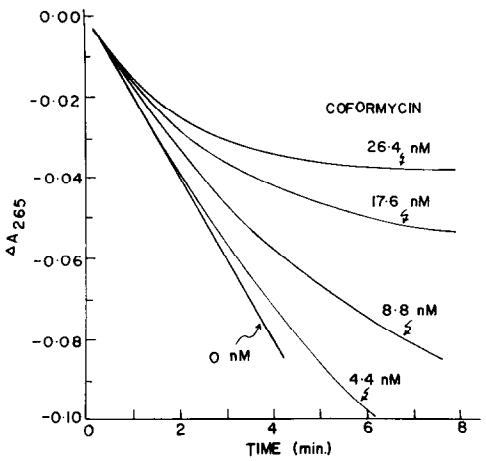

Fig. 7. Spectrophotometric tracings of adenosine deaminase reactions in the presence of coformycin. Reaction mixtures contained: phosphate buffer, 50 mM, pH 7·5; adenosine, 0·10 mM; and coformycin (as indicated) at 30°. The reaction was started by addition of 0·0033 unit of adenosine deaminase in 10 μl.

Figure 9 presents a Lineweaver–Burk plot constructed from data obtained by a different design from that used in the experiment of Fig. 8. In this case, the enzyme was preincubated in reaction mixtures containing coformycin for 50 min. The assay reactions were then started by adding the substrate. From the theoretical considerations developed in the previous paper [1], one should expect to see three phenomena. First, as occurred with allopurinol and xanthine oxidase (Fig. 1), the inhibition of adenosine deaminase by coformycin might have been gradually overcome after addition of the substrate. This phenomenon, however, was not observed, suggesting that dissociation of the adenosine deaminase–coformycin complex

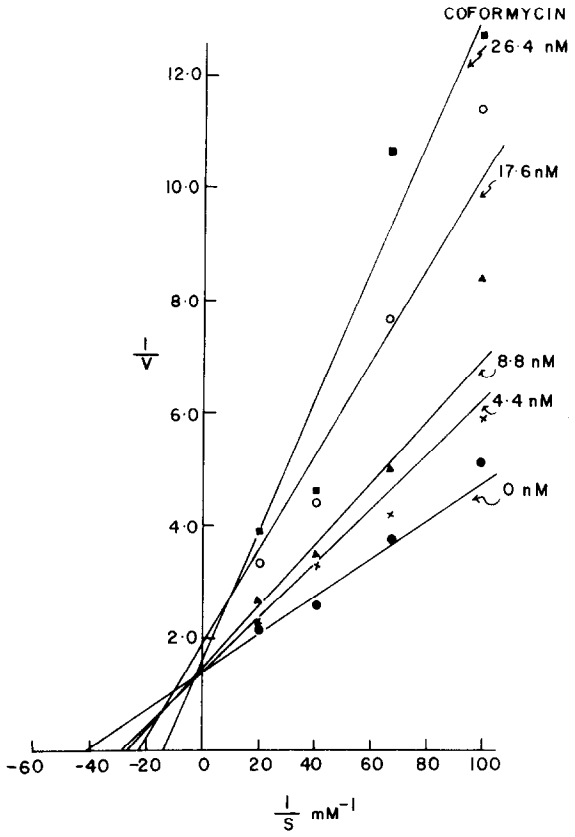

Fig. 8. Lineweaver–Burk plots of reactions started by addition of adenosine deaminase. The experimental conditions were similar to those of Fig. 7, except that various concentrations of adenosine were used. The decreases in absorbancy at 265 nm during the first 2 min were taken as 'initial velocities.'

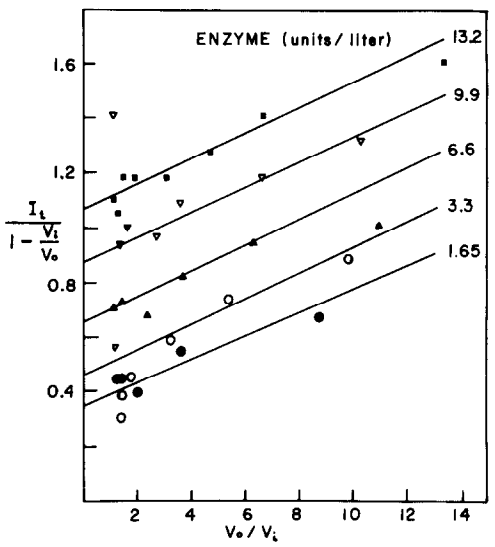

Fig. 10. Henderson (or Easson–Stedman) plot of the data of Fig. 6A.

was too slow to contribute a significant quantity of free enzyme during the brief assay period (less than 10 min). The second predictable consequence of this experimental design that was found was a Lineweaver–Burk plot resembling that of classical non-competitive inhibition (Fig. 9). However, one may not conclude on the basis of this plot alone that true non-competitive inhibition occurred, as pointed out much earlier by Easson and Stedman [26]. Here again, a very slow dissociation of the *EI* complex can lead to a misinterpretation of the true inhibition mechanism. The third phenomenon predicted for this experimental design is the appearance of a non-linear replot of the $1/v$ intercept vs the inhibitor concentration as seen in the inset of Fig. 9. The dotted line illustrates how an erroneous estimation of K_i could have resulted if the data point for the highest inhibitor concentration had been ignored in order to draw a straight line. The above data in Figs. 8 and 9 clearly demonstrate the inadequacy of the classic Lineweaver–Burk analytic methods based on steady state assumptions for the determination of either the inhibition mechanism or the K_i values in the study of tight-binding inhibitors.

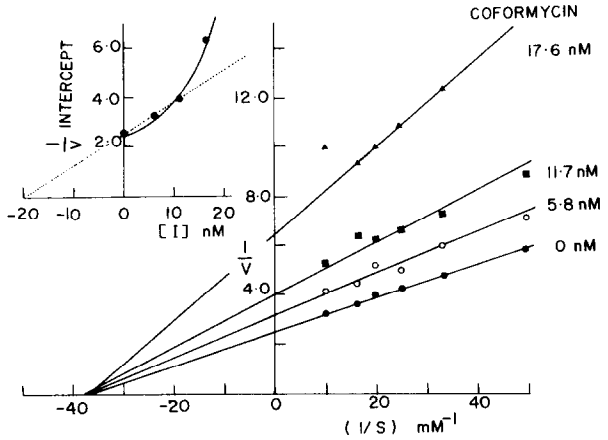

Fig. 9. Lineweaver–Burk plots of reactions started by addition of substrate. Adenosine deaminase (0·033 unit) was preincubated in 0·12 ml with coformycin (concentrations as indicated) for 50 min or more at room temperature. Ten μl of this pretreated enzyme was used for determination of activity at varying concentrations of adenosine. The concentrations of coformycin indicated are those of the preincubated mixture. The inset is a replot of $1/v$-intercept vs coformycin concentration.

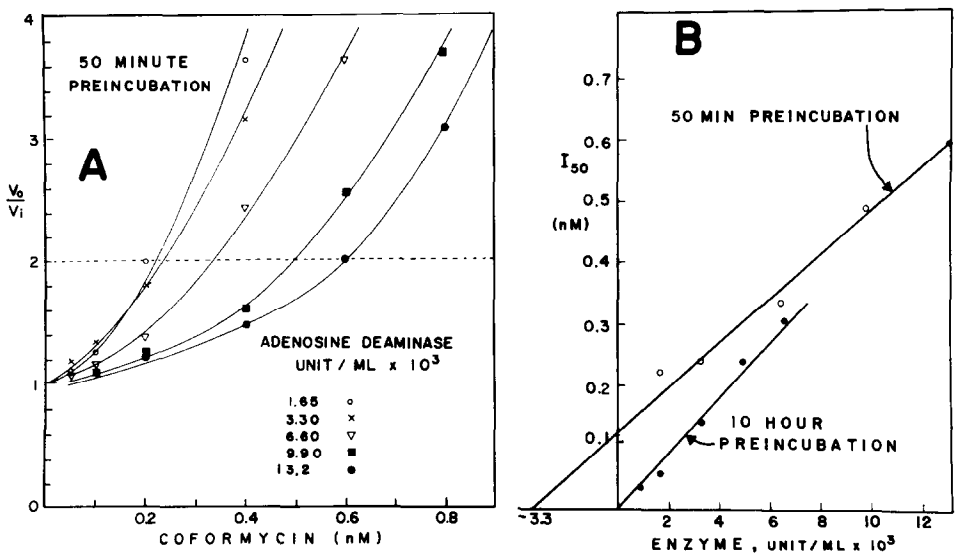

Fig. 11. Determination of I_{50}. Frame A: a plot of v_0/v_i vs coformycin concentration. The same data as in Fig. 6 were used; Frame B: I_{50} values were estimated from frame A and are plotted against the enzyme concentration (○——○). The I_{50} values from a similar experiment in which the enzyme was preincubated for 10 hr are also presented (●——●).

Use of I_{50} for estimation of K_i *and molar equivalency of enzyme.* While the classical Lineweaver–Burk plot is inadequate for the determination of very low K_i values as demonstrated above, several other methods such as the Henderson plot [1, 27] (or the Easson–Stedman plot [26]), or a plot of I_{50} vs I_t may be applied. Shown in Fig. 10 is the Henderson plot (see equation 2, Ref. 1), i.e. $I_t/(1 - v_i/v_0)$ vs v_0/v_i, of the same experimental data presented in Fig. 6A. Unfortunately the complex mathematical transformations inherent in the Henderson equation can so greatly amplify small errors that considerable scatter of the data points can occur, as seen in Fig. 10. Obviously little or no meaningful information can be obtained from such a plot unless one applies an elaborate statistical analysis such as that described by Henderson [28]. On the other hand, Fig. 11A shows a plot of v_0/v_i vs I_t (coformycin) also using the data of Fig. 6A, and from this plot, I_{50} values were estimated. The plot of I_{50} values against the enzyme concentration is shown in Fig. 11B (50-min incubation). Equation 9 in the previous paper [1], $I_{50} = \frac{1}{2}E_t + K_i$, indicates that the straight line in Fig. 11B should intersect the I_{50}-axis at K_i and the E_t-axis at $-2K_i$. The K_i value estimated from the I_{50}-intercept is $1{\cdot}2 \times 10^{-10}$ M. The E_t-intercept, which should be $-2K_i$, was at $-3{\cdot}3$ units/liter. Therefore, 1 unit of enzyme corresponds to $0{\cdot}8 \times 10^{-10}$ molar equivalents, which is in reasonably good agreement with the value of $1{\cdot}0 \times 10^{-10}$ molar equivalents estimated from the Ackermann–Potter plot above (Fig. 6). This demonstrates the usefulness of I_{50} measurements in the determination of both K_i values and molar equivalencies of an enzyme. However, Fig. 11B also shows the plot based on an experiment in which the enzyme was preincubated with coformycin for 10 hr. In this case, the straight line extrapolates to a point near the origin. This fact indicates either that the reaction between the enzyme and inhibitor was not at equilibrium at the end of the 50-min preincubation, or that some unrecognized events, such as enzymic denaturation or conformational change, took place during the long preincubation time of 10 hr at room temperature. In any case, this experiment suggests that the true K_i value may be much lower than the estimated value, $1{\cdot}2 \times 10^{-10}$ M. This question will be subjected to further study.

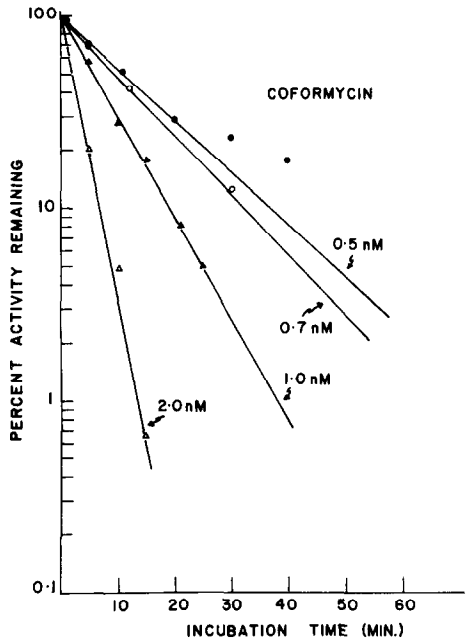

Fig. 12. Rate of association of adenosine deaminase and coformycin. The enzyme was incubated with several concentrations of coformycin in 0·99 ml phosphate buffer, 50 mM, pH 7·5. After incubation at 30° for various time intervals, the reactions were started by addition of adenosine (0·1 mM).

Measurement of velocity constants of association and dissociation of EI complex. It was suggested in the accompanying paper [1] that, under favorable experimental conditions, the rate of association of an enzyme and a potent inhibitor and the rate of dissociation of the *EI* complex may be studied without the need of special instrumentation, and that it may be possible to determine the first- and second-order rate constants. Figure 12 shows that the association reaction between coformycin and the enzyme to form the *EI* complex is a pseudo-first-order reaction at high concentrations of the inhibitor. Note that the plots of logarithms of the per cent of the enzymic activity remaining vs incubation time are apparently linear during the initial time period. The concentration of enzyme used in this experiment was approximately $1{\cdot}2 \times 10^{-10}$ molar equivalents. From the slope of these plots of Fig. 12, the value of k_1 was estimated at $2 \times 10^6\ M^{-1}\ sec^{-1}$ as presented in Table 1.

If one assumes that the reaction between adenosine deaminase and coformycin is a simple one represented by

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \qquad (3)$$

and that the value of K_i estimated above is correct, then the dissociation of the *EI* complex should follow simple logarithmic decay kinetics with the first-order rate constant, $k_2 = K_i \times k_1 = (1{\cdot}2 \times 10^{-10}) \times (2 \times 10^6) = 2{\cdot}4 \times 10^{-4}\ sec^{-1}$. Therefore, in the absence of free coformycin, 50 per cent of the *EI* complex should dissociate in about $T_{1/2} = 0{\cdot}693/(2{\cdot}4 \times 10^{-4}\ sec^{-1}) = 2900$ sec = 48 min. The result of this type of dissociation experiment is shown in Fig. 13, (A and B). Two facts are immediately apparent. First, the dissociation (50 per cent in 9·5 hr) is much slower than expected (48 min); and second, the dissociation follows biphasic exponential decay kinetics. In fact, the data in Fig. 13B can be resolved into two exponential decay components illustrated by the two straight lines in the semilog plot. Thus, the experimental points may be fitted to the following empirical formula by a method similar to the procedure commonly used in studying the decay of mixtures of radioisotopes.

$$y = C_1 e^{-\lambda_1 t} + C_2 e^{-\lambda_2 t} \qquad (4)$$

where $C_1 = 1{\cdot}18$, $C_2 = -0{\cdot}18$, $\lambda_1 = 2{\cdot}44 \times 10^{-5}\ sec^{-1}$, and $\lambda_2 = 1{\cdot}54 \times 10^{-4}\ sec^{-1}$. Therefore, these data strongly suggest that the reaction between

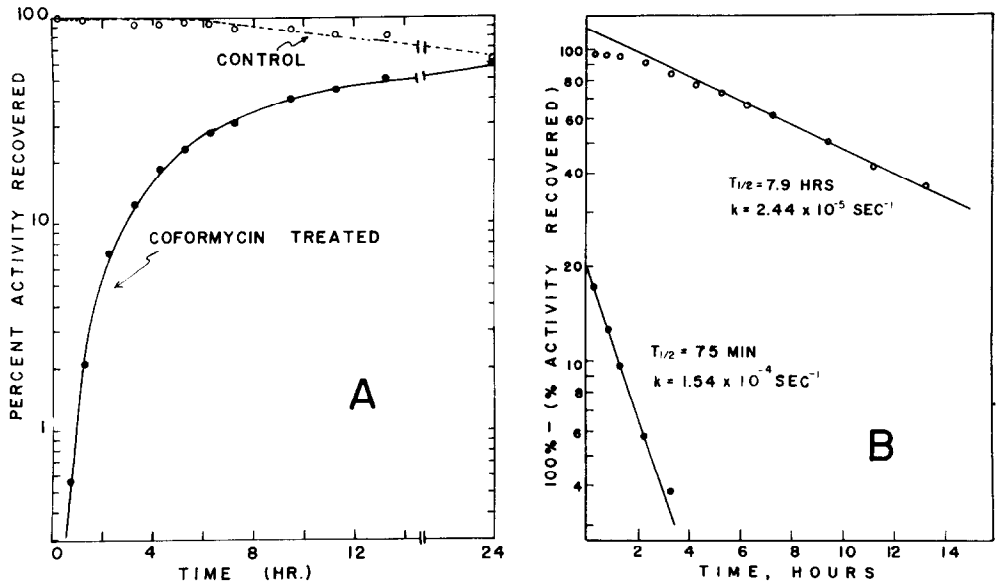

Fig. 13. Dissociation of adenosine deaminase–coformycin complex. Adenosine deaminase (0·26 unit/ml) and coformycin (40 nM) in 50 mM phosphate buffer, pH 7·5, were incubated at room temperature. Coformycin was omitted in the control reaction mixture. After 50 min, an equal volume of a suspension of hemoglobin-coated charcoal (5%, w/v) in 50 mM phosphate buffer, pH 7·5, was added. After stirring occasionally for 5 min, the charcoal was removed by centrifugation in a clinical centrifuge. The supernatant fluid was diluted 50-fold with 50 mM phosphate buffer, pH 7·5, containing 0·03% bovine serum albumin and 0·07% charcoal. The mixture was stirred at room temperature using a magnetic stirrer. Aliquots of this suspension were withdrawn at different time intervals, centrifuged for 5 min in a clinical centrifuge, and the enzymic activity in the supernatant fluids was assayed. Frame A: recovery of activity from inhibited enzyme–coformycin complex with time. Per cent of activity recovered was calculated on the basis of the activity of control at zero time. Frame B: Semilog plot of 100 per cent minus the per cent of activity recovered, i.e. per cent of enzyme remaining as *EI* complex vs time. The activity recovered was calculated with the activity of the control sample at each particular time taken as 100 per cent. The semilog plot of the actual data can be resolved into two exponential components (straight lines on a semilog plot). A straight line having a decay constant of $2{\cdot}44 \times 10^{-5}\ sec^{-1}$ is drawn through the linear portion of the semilog plot of the actual data (O——O). The logarithms of the differences between the values represented by this straight line and the actual values are plotted against time (●——●). This plot is also linear having a decay constant of $1{\cdot}54 \times 10^{-4}\ sec^{-1}$.

Table 1. Association constant (k_1) of adenosine deaminase and coformycin

Coformycin (I_t) (nM)	$T^*_{1/2}$ (sec)	k† (sec^{-1})	$k_1 = \frac{k}{(I_t)}$ ($M^{-1}\ sec^{-1}$)
0·5	660	$1·05 \times 10^{-3}$	$2·10 \times 10^6$
0·7	570	$1·22 \times 10^{-3}$	$1·74 \times 10^6$
1·0	360	$1·93 \times 10^{-3}$	$1·93 \times 10^6$
2·0	150	$4·62 \times 10^{-3}$	$2·31 \times 10^6$
			Mean $k_1 = 2·06 \times 10^6$

* $T_{1/2}$, the time for 50 per cent activity remaining, was taken from the straight line portions of Fig. 12.
† Pseudo-first-order rate constant = $k_1(I_t)$.

coformycin and adenosine deaminase takes place in more than one step which may be represented by:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^* \qquad (5)$$

where EI* represents a different conformational state of the enzyme–inhibitor complex. Then y in equation 4 represents

$$y = \frac{(EI) + (EI^*)}{(EI)_0 + (EI^*)_0}$$

where $(EI)_0$ and $(EI^*)_0$ are (EI) and (EI^*) at zero time respectively, i.e. at the end of preincubation. It is conceivable that the change from EI to EI^* can take place in more than one step. Even so reaction scheme 5 is still adequate to describe such a reaction if k_3 and k_4 are understood to be composite rate constants. The significance of the parameters in equation 4 will be analyzed thoroughly when more extensive experimental data are obtained. The intriguing hypothesis that the interaction between coformycin and adenosine deaminase occurs in multiple steps, probably involving slow changes in the conformation state of the enzyme–inhibitor complex, obviously is worthy of intensive study.

COMMENTS

The above studies clearly demonstrated the validity of some of the theoretical concepts and approaches developed in the accompanying paper [1]. Perhaps the most important point illustrated is the markedly prolonged non-steady state phase of the interaction between tight-binding inhibitors and enzymes under ordinary experimental conditions. Because of this prolonged non-steady state phase, the classical kinetic theory based on steady state assumptions cannot be applied. In fact, the interaction between the inhibitor and enzyme may be so slow that it is possible to measure directly the rate constants for association and dissociation of the enzyme–inhibitor complex, as demonstrated in the studies with coformycin (Figs. 12 and 13). It has been our experience that the most useful type of experiment in the study of tight-binding inhibitors is that illustrated by Figs. 6 and 11. Here, different quantities of the enzyme were preincubated with various concentrations of the inhibitor for a time sufficient to reach an equilibrium state. From these data one may estimate I_{50} values which, in turn, may be employed to evaluate both E_t (the molar equivalence of enzyme) and K_i (Fig. 11). If the same data are plotted according to the method of Ackermann and Potter, (Fig. 6), one may also determine E_t and k_3, the catalytic number. Thus, from one type of experiment it is possible to determine several important parameters, i.e. K_i, k_3 (the catalytic number) and E_t. Hopefully, these concepts and experimental approaches will aid in the clarification of many confusing results and contradictory interpretations that have been encountered in the study of other tight-binding inhibitors.

In comparison with the relatively complex reaction system of xanthine oxidase and allopurinol, the interaction between adenosine deaminase and coformycin appears to offer certain advantages as a model system for the study of tight-binding inhibitors. First of all, the enzyme is easily and sensitively assayed by a variety of possible methods, both with isolated enzymes and intact cells [14, 29, 30]. The enzymic reaction is a basically simple hydrolysis involving only one substrate (in addition to water) and no cofactors. Although the human erythrocytic enzyme is easily prepared in a state of purity adequate for kinetic studies [14], it may also be obtained from other sources including the commercially available calf intestinal enzyme. Another significant advantage of human erythrocytic adenosine deaminase is its relative stability, which makes possible prolonged incubations at room temperature (more than 12 hr) without unacceptable losses in activity, e.g. see Fig. 13A.

As noted above in the experiments of Figs. 11 and 13, it is probable that conformational changes are a component of the inhibitory mechanism of coformycin with adenosine deaminase. In view of the several advantages of this experimental system discussed above, further study of the inhibitory mechanisms, including the effects of temperature and additional kinetic studies, may reveal such factors as a large entropy change, which would be consistent with a marked alteration in conformation during the binding of the inhibitor to the enzyme. Unfortunately, preparations of adenosine deaminase are not available in either sufficient quantity or purity to permit application of more powerful physical techniques, such as X-ray crystallography, in the presence and absence of inhibitor.

It should be noted that the inhibitor, coformycin (an analog of inosine), has many of the structural features of a transition-state analog, as originally postulated by Pauling [31] in 1948 and discussed recently in reviews by Wolfenden [32] and Lienhard [33]. As reported earlier [14], the K_m of adenosine is $2·5 \times 10^{-5}$ M and the K_i value of inosine, a product of the adenosine deaminase reaction, is about $1·2 \times 10^{-4}$ M for human erythrocytic adenosine deaminase. In striking contrast, the K_i value of coformycin measured above is in the range of 1×10^{-10} to 1×10^{-11} M, i.e. in the order of a million times more potent. As seen in Fig. 1, the ring system of coformycin resembles that of a purine with a methylene group interposed between N-1 and C-6 of the purine ring. As noted by Nakamura *et al.* [17], the 7-membered ring of coformycin is not planar but rather is puckered, and its conjugated system is interrupted by the methylene group. As a consequence, the keto-enol

tautomerism normally seen with inosine is not possible and the hydroxyl group is, in fact, a secondary alcohol.

Mention should be made of certain structural similarities between coformycin and the "transition-state" inhibitors of adenosine deaminase and deoxycytidine deaminase used in the poineering studies of Wolfenden and Evans [32, 34, 35]. Especially interesting is the fact that the potent inhibitor of adenosine deaminase described by Evans and Wolfenden [35], 1,6-dihydro-6-hydroxymethyl purine ribonucleoside, was actually employed as an intermediate in the chemical synthesis of coformycin recently reported by Ohno *et al.* [36]. Since the K_i values of coformycin presented above are 100- to 1000-fold lower than the value estimated previously by the use of classical methods for the study of enzymic inhibitors [14], one wonders whether significantly lower K_i values might not be found with 1,6-dihydro-6-hydroxymethyl purine ribonucleoside [35] if certain of the technics described above were applied. A similar question might be asked about the K_i values reported for 3,4,5,6-tetrahydrouridine, a potent inhibitor of several cytidine deaminases, which has been reported to bind to the enzyme about 3000 times more tightly than the substrate [34]. It should also be noted that interesting similarities can be seen between the structures of coformycin and 3,4,5,6-tetrahydrouridine.

Another significant point is that the addition of the methylene group in coformycin significantly increases the size of the ring structure in comparison with inosine. An interesting analogy might be made with recent findings on a class of potent inhibitors of adenylate kinase, a group of di(adenosine)-polyphosphate compounds [37]. Among those studies P^1,P^5-di(adenosine-5′) pentaphosphate was over 1000 times more potent as an inhibitor than the tetraphosphate analog. Since the proposed "transition-state" complex between this enzyme and its substrates, AMP and ATP, would include only four phosphates, this observation suggests that significantly more powerful inhibition may be achieved by having an analog somewhat greater in size than the "transition-state" complex. These observations suggest a hypothesis that during the catalytic event the active site becomes somewhat enlarged and that the "transition-state" analog "locks" the enzyme into this stretched position. Of course, physical studies, such as X-ray crystallography, will probably be necessary to verify this hypothesis. Also, as noted in the present study, it seems probable that further slow conformational changes occur after the initial formation of the *EI* complex that significantly enhance the binding.

From the equation $\Delta G^{0\prime} = -RT\ln K_{eq}$, a 6–7 logarithmic difference between the binding of inosine and corformycin to adenosine deaminase indicates a difference in the free energy of binding of -8 to -10 kcal/mole. Since the free energy of binding of single hydrogen bonds or electrostatic bonds is of the order of -3 to -5 kcal/mole, it appears that, compared to inosine, more than one additional interatomic attraction is present in the binding of coformycin to the enzyme. Consideration should also be given to the possible steric entrapment of the inhibitor in a cage-like structure formed slowly by the enzyme. It will be very interesting to compare the behavior of coformycin with that of another class of potent adenosine deaminase inhibitors (recently prepared by Schaeffer and Schwender [38]) that do not appear to be transition-state analogs. These compounds consist of normal purine ring structures with hydrophobic groups attached to N-9. It seems most likely that the inhibitor potency of these compounds results from the formation of hydrophobic bonds with the enzyme rather than from their resemblance to the transition-state complex.

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