Biochemical Pharmacology, Vol. 26, pp. 359–367. Pergamon Press, 1977. Printed in Great Britain.

TIGHT-BINDING INHIBITORS—IV. INHIBITION OF ADENOSINE DEAMINASES BY VARIOUS INHIBITORS*

RAM P. AGARWAL, THOMAS SPECTOR† and ROBERT E. PARKS, JR.

Section of Biochemical Pharmacology, Division of Biology and Medicine, Brown University, Providence, R.I. 02912, and Wellcome Research Laboratories, Research Triangle Park, NC 27709, U.S.A.

(*Received* 4 *June* 1976; *accepted* 30 *July* 1976)

Abstract—Three ADA (adenosine deaminase) inhibitors, DHMPR (1,6-dihydro-6-hydroxymethyl purine ribonucleoside); EHNA [erythro-9-(2-hydroxy-3 nonyl)adenine]; and deoxycoformycin [(R)-3-(2-deoxy-β-D-erythro-pento-furanosyl)-3,6,7,8-tetrahydroimidazo[4,5-*d*] [1,3-diazepin-8-ol] or Covidarabin, were compared with regard to their inhibitory behavior with ADAs from human erythrocytes and calf intestine. Marked differences in the times required for establishment of steady state between the enzyme and inhibitors were observed, e.g. DHMPR, virtually instantaneous; EHNA, 2–3 min; and deoxycoformycin, many hr. The parameters of the inhibition of human erythrocytic ADA by deoxycoformycin were as follows: the association rate constant (k_1) = 2.6×10^6 M^{-1} sec^{-1}; the dissociation rate constant of the enzyme–inhibitor complex (k_2) = 6.6×10^{-6} sec^{-1}; K_i (from k_2/k_1) = 2.5×10^{-12} M and K_i (from I_{50}) = 1.5×10^{-11} M. The K_i values for EHNA and DHMPR, as determined by classical methods after attainment of steady state, were 1.6×10^{-9} and 1.3×10^{-6} M, respectively, for human erythrocytic ADA. The kinetic parameters for EHNA and calf intestinal ADA were as follows: K_i = 6.5×10^{-9} M (by the method of I_{50}); k_1 = 0.7×10^6 M^{-1} sec^{-1} and k_2 = 4.6×10^{-3} sec^{-1}. On the basis of K_i values, the inhibitors, DHMPR, EHNA and deoxycoformycin (a transition state analog), were classified as readily reversible, semi-tight-binding and tight-binding inhibitors. The difficulties encountered in the kinetic analyses of different types of inhibitors and the methods for dealing with the problems of these inhibitors are discussed.

In a recent publication, Cha [1] described a new theoretical approach to the study of tight-binding ligands with enzymes or other macromolecules of biological interest. It was emphasized that under commonly used experimental conditions, in the presence of a tight-binding ligand, the non-steady state phase between the macromolecule and the ligand–macromolecular complex may be so prolonged that the familiar methods of analysis based on steady state kinetics cannot be applied directly, e.g. for the determination of the inhibition constants or mechanisms of tight-binding enzyme inhibitors. Studies designed to test this new theory that employed human erythrocytic adenosine deaminase (ADA)‡ and the tight-binding inhibitor, coformycin, clearly showed that the classical methods of kinetic analysis based on steady state assumptions were grossly inadequate for determination of either the inhibition constants or reaction mechanisms [2]. This new approach [1] has permitted the development of experimental procedures that enable ready measurement of such parameters as the K_i values, the individual velocity constants (k_1 and k_2) of the association and dissociation reactions between the enzyme and inhibitor, as well as the molar equivalency and catalytic numbers of the enzyme [2]. In a recent further theoretical advance, Cha [3] has devised methods for readily determining the inhibition mechanism, i.e. competitive, non-competitive, of the inhibitor–enzyme interaction.

In our initial studies with a partially purified preparation of human erythrocytic ADA, the kinetic parameters (K_m, relative V_{max} and K_i values) of the natural substrates, adenosine and 2′-deoxyadenosine and products inosine and 2′-deoxyinosine, as well as a number of adenosine analogs, were examined [4]. Also included were preliminary studies of coformycin [4] which attempted to employ the classical methods of inhibition analysis. These studies suggested that coformycin is a competitive inhibitor of the enzyme with a K_i value of about 1×10^{-8} M [2, 4].

More recent investigations of the interaction of coformycin and human erythrocytic ADA [2] that employed methodology derived from the theoretical approaches of Cha [1] demonstrated that the inhibition is much more potent than indicated by the earlier studies. For a reaction:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \qquad (1)$$

* This work was supported by USPHS Grant CA 07340.

† T. Spector is from the Wellcome Research Laboratories, Research Triangle Park, NC.

‡Abbreviations used: ADA, adenosine deaminase (adenosine aminohydrolase EC 3.5.4.4); DHMPR, 1,6-dihydro-6-hydroxymethyl purine ribonucleoside; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; and *d*-coformycin, (R)-3-(2-deoxy-β-D-erythro-pento-furanosyl)-3,6,7,8-tetrahydroimidazo[4,5-*d*][1,3]diazepin-8-ol, deoxycoformycin, Covidarabine.

the K_i value for coformycin was determined by two procedures.* It was possible to measure directly the second-order association rate constant (k_1) and the first-order dissociation rate constant of the EI complex (k_2). From these rate constants, the K_i value was calculated from the relation:

$$K_i = k_2/k_1 \quad (2)$$

A second method for determination of the inhibition constant was based on the equation derived by Cha [1]:

$$I_{50} = K_i + 1/2\, E_t \quad (3)$$

When the ADA was preincubated with coformycin for about 50 min, both methods yielded comparable K_i values, i.e. about 1×10^{-10} M. However, more prolonged incubation (10 hr) yielded K_i values that were about 10-fold lower and suggested the possibility that the EI complex undergoes an as yet undefined conformational change that increases the tightness with which the coformycin binds to the enzyme [2].

Since completion of these studies with coformycin, we have had the opportunity to test further these newer methods of inhibition analysis of ADA by the availability of several ADA inhibitors [5–8] that appear to vary significantly in potency (for structures, see Fig. 1). The reported K_i values for two of these compounds fell in the range of 1×10^{-6} to 10^{-8} M [5, 6], which suggested that the group included readily reversible, tight-binding and semi-tight-binding inhibitors. This novel group of ADA inhibitors has permitted us to compare the relative usefulness of several methods of analysis of enzymic inhibition, and the results of these studies are presented below. A preliminary report of some of these studies has been presented [9].

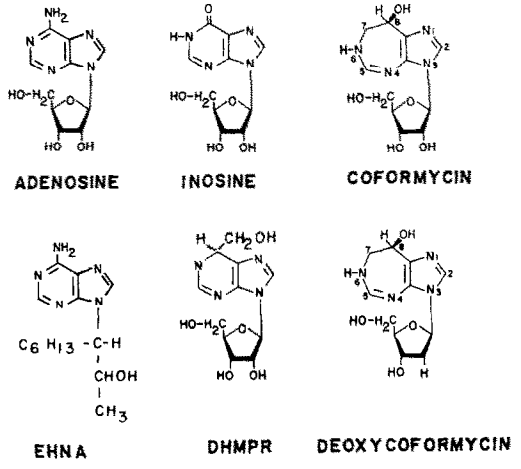

Fig. 1. Structures of substrates and inhibitors of adenosine deaminase.

*Symbols are defined as follows: k_1 = rate constant for forward reaction (association); k_2 = rate constant for backward reaction (dissociation); v_0, v_i = enzyme reaction velocity in the absence of inhibitor and in the presence of inhibitor, respectively; K_i = inhibition constant or the dissociation constant of the EI complex; E = enzyme (or macromolecule); E_t = total enzyme; I = inhibitor (or ligand); EI = enzyme–inhibitor complex; and I_{50} = total inhibitor concentration at which the enzyme reaction velocity is 50 per cent of the uninhibited reaction.

MATERIALS AND METHODS

Adenosine was purchased from P. L. Biochemicals, Inc., Milwaukee, Wisc. *d*-Coformycin [(R)-3-(2-deoxy-β-erythro-pento-furanosyl)-3,6,7,8-tetrahydroimidazo [4,5-*d*][1,3]diazepin-8-ol] was a gift from Dr. H. W. Dion of Parke, Davis & Co., Detroit, Mich. [8]. DHMPR (1,6-dihydro-6-hydroxymethyl purine ribonucleoside) was a gift from Dr. R. V. Wolfenden of the University of North Carolina, Chapel Hill, N.C. [5]. EHNA [erythro-9-(2-hydroxy-3-nonyl) adenine] was prepared by Schaeffer and Schwender [6] and was obtained through Dr. G. B. Elion of the Wellcome Research Laboratories, Research Triangle Park, NC. The concentrations of solution of *d*-coformycin ($\epsilon_{282\ nm}$ in $H_2O = 8.0 \times 10^3$) [7], DHMPR ($\epsilon_{293\ nm}$ in $H_2O = 4.17 \times 10^3$) [6], and EHNA ($\epsilon_{261\ nm}$ in $H_2O = 14.2 \times 10^3$) were determined by spectrophotometry at the wavelengths of the absorption maxima.

The human erythrocytic adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4, ADA) was a preparation (0.36 units/mg of protein), partially purified as described earlier [4]. The activity of the erythrocytic adenosine deaminase was measured spectrophotometrically as described previously [4], by following the decrease in absorbancy at 265 nm in 50 mM phosphate buffer, pH 7.5, at room temperature. Calf intestinal adenosine deaminase, purchased from Boehringer Mannheim (200 units/mg), was assayed in 20 mM morpholilopropane sulfonic acid (MOPS) buffer, pH 6.8, at 25°. The concentration of adenosine for these studies was 0.1 mM. The K_m for adenosine with the calf intestinal enzyme was 0.037 mM (data not shown), whereas the K_m for the human erythrocytic enzyme is 0.025 mM [4]. The hemoglobin-coated charcoal used in the study of dissociation of the adenosine deaminase–*d*-coformycin complex was prepared as described by Waxman *et al.* [10].

RESULTS

Interaction between various inhibitors and human erythrocytic adenosine deaminase. Figure 2 shows spectrophotometric tracings of the adenosine deaminase reaction using adenosine as the substrate in the presence of various inhibitors. Two methods were used to study the reaction.

In method 1, the substrate and inhibitor were added to the assay reaction mixture minus the enzyme, and the reaction was started by adding ADA. In method 2, the enzyme and the inhibitor were preincubated for a specific time period in the assay reaction mixture minus the substrate, and the reaction was started by addition of the substrate.

As is evident from frame A, DHMPR inhibited the enzymic reaction but no difference was observed between methods 1 and 2, suggesting that steady state conditions between the enzyme and inhibitor were established quickly. Frame B presents the reaction with EHNA and ADA. When the reaction was started by addition of the enzyme (method 1), initially (during min 1) significant inhibition was not observed. How-

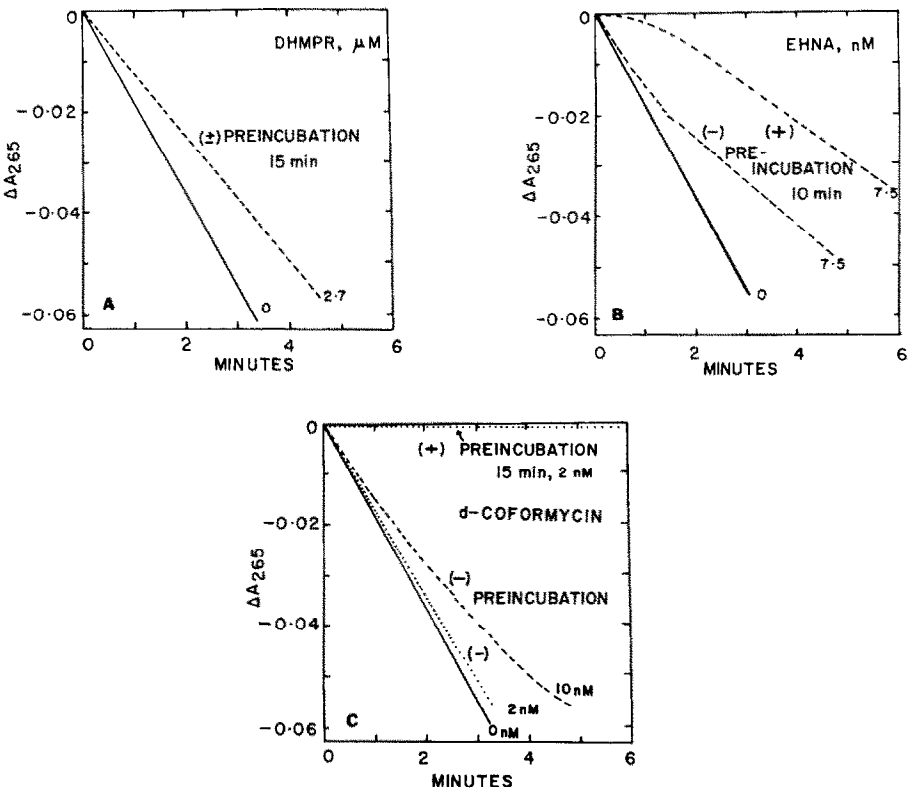

Fig. 2. Spectrophotometric tracings of the reaction of human erythrocytic adenosine deaminase in the presence of DHMPR (frame A), EHNA (frame B) and *d*-coformycin (frame C). Reaction mixtures in a total volume of 1.0 ml contained: phosphate buffer, 50 mM, pH 7.5; adenosine 0.1 mM; human erythrocytic ADA, 0.0033 units and inhibitors (concentration as indicated). Where inhibitors were used, two methods were employed: preincubation of enzyme and inhibitor (+), where the enzyme and inhibitor were preincubated for the time indicated in 0.99 ml phosphate buffer at room temperature and the reaction was started by addition of substrate, adenosine (10 μl); without preincubation (−), where the reaction was started by addition of ADA (10 μl) to a mixture of adenosine and inhibitor in phosphate buffer. The reactions were followed at room temperature by measuring decrease in absorbance at 265 nm in a Beckman spectrophotometer equipped with a Gilford recorder.

ever, inhibition of the reaction became apparent after 1 min and reached steady state at 2–3 min. On the other hand, when the enzyme and inhibitor were preincubated for 10 min prior to addition of the substrate (method 2), the reaction was completely inhibited, initially. This inhibition was slowly released, and after 2–3 min, the reaction rate was essentially similar to that of method 1, suggesting that steady state conditions were also established after 2–3 min.

In contrast to the above examples where the order of addition of the reactants or preincubation of ADA with an inhibitor had either no effect (DHMPR) or a relatively transient effect (EHNA), the results with *d*-coformycin (Fig. 2, frame C) yielded markedly different results. Here, when the reaction mixture contained a very small but completely inhibitory amount of *d*-coformycin (2.0 nM) and the assay was started by addition of the enzyme, little or no inhibition was observed over the first 2–3 min and only slight inhibition was seen after 5–6 min. Also, at the higher concentration of *d*-coformycin (10 nM), initially there was only a little inhibition, but this inhibition became progressively more pronounced over a 5-min period. However, if ADA and *d*-coformycin (2.0 nM) were preincubated for 15 min, and the reaction was started by addition of adenosine, almost total inhibition of the enzyme was observed throughout the 6 min of the experiment. Results comparable to these were described earlier with the tight-binding inhibitor, coformycin [2]. Thus, in this example, the order of addition of reactants and the period of preincubation of enzyme and inhibitor had a profound effect on the experimental results obtained.

Analyses of the inhibitions of human erythrocytic adenosine deaminase by DHMPR and EHNA. As observed above, steady state conditions were achieved very rapidly between DHMPR and ADA and after a period of 2–3 min with the combination of EHNA and ADA. On the other hand, no evidence of the attainment of steady state conditions was seen with the combination of *d*-coformycin and ADA, even after 5 min of incubation. These observations indicated that the classical methods of determination of inhibition constants (K_i) could be utilized directly for the study of DHMPR and after a period of 3 or more min of preincubation of ADA with EHNA. On the other hand, these methods would be of negligible value in the study of the tight-binding inhibitor, *d*-coformycin.

Figure 3 presents the results of a kinetic analysis of DHMPR and the determination of the K_i values through the use of the standard double reciprocal

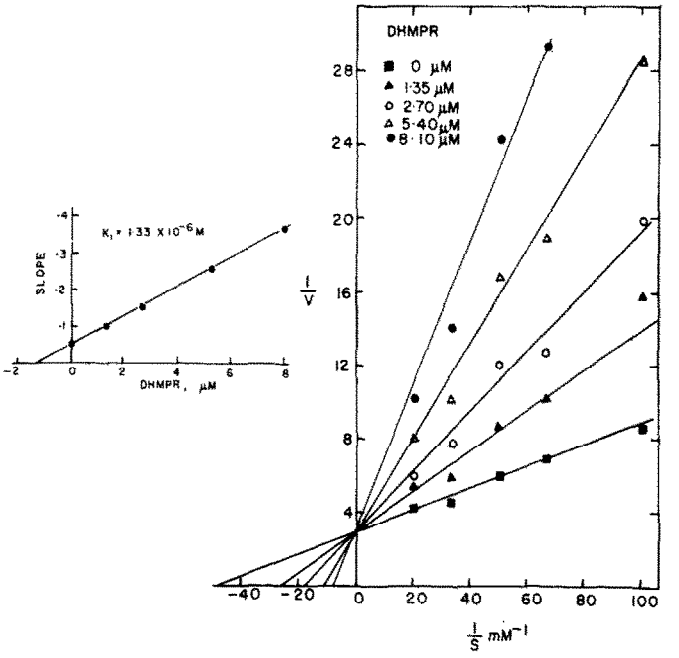

Fig. 3. Double reciprocal plots of the adenosine deaminase reaction in the presence of DHMPR. In all cases, the reactions were started by addition of 0.0033 units of human erythrocytic ADA. The inset is a replot of slope vs DHMPR concentrations. From the replot the K_i value of DHMPR was estimated to be 1.3×10^{-6} M.

plots with subsequent replotting of the slopes vs the inhibitor concentration. A classical pattern of competitive inhibition was obtained and the K_i value of DHMPR was estimated to be 1.3×10^{-6} M, which is somewhat lower than the value of 7.6×10^{-6} M reported for this compound with calf intestinal ADA [5].

Figure 4 shows the results of a kinetic analysis performed with EHNA, which employed velocity measurements determined after the attainment of steady state conditions between the inhibitor and enzyme (after about 3 min). The double reciprocal plots yielded patterns consistent with classical competitive inhibition. The replot of the slope vs EHNA concentration was linear and yielded a K_i value of 1.6×10^{-9} M.

Ackermann–Potter analysis with human erythrocytic adenosine deaminase. Figure 5A presents an Ackermann–Potter plot [11], using the ADA–*d*-coformycin combination, which clearly indicates that *d*-coformycin is a tight-binding inhibitor. On the other hand, EHNA did not produce a classical Ackermann–Potter

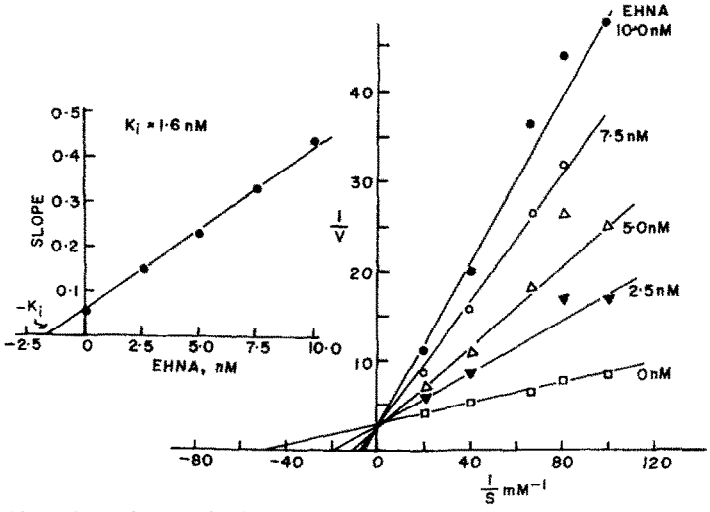

Fig. 4. Double reciprocal plot of adenosine deaminase reaction in the presence of EHNA. Human erythrocytic ADA (0.0033 units) and EHNA (concentrations as indicated) were incubated at room temperature for 10 min in a total volume of 0.99 ml containing 50 mM phosphate buffer, pH 7.5. The steady state velocities were taken 3 min after the start of the reaction. From a replot of slope vs EHNA concentrations (inset), the K_i value of EHNA was estimated to be 1.6×10^{-9} M.

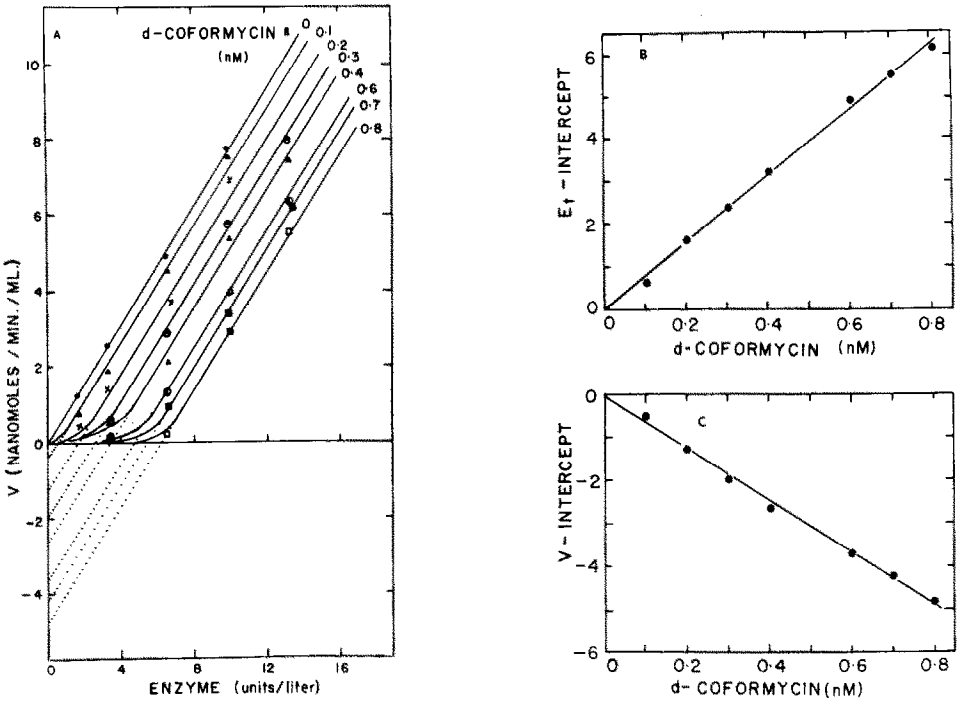

Fig. 5. Ackermann–Potter plot of the reaction of human erythrocytic adenosine deaminase with *d*-coformycin. Various amounts of ADA were incubated in a total volume of 0.99 ml containing 50 mM phosphate buffer, pH 7.5, and varying concentrations of *d*-coformycin. After incubation for 1 hr at room temperature, the enzymic reaction was started by addition of 10 μl of 10 mM adenosine (final concn 0.1 mM). The reaction was followed by measuring the decrease in absorbancy at 265 nm at room temperature. Frame A: plot of enzymic velocity, V (nmoles/min/ml) vs the enzymic concentration (units/liter). Frame B: plot of E_t (units/liter) intercept (from frame A) vs *d*-coformycin concentration. From this plot it was observed that 1 unit ADA/liter corresponds to a concentration of about 1.3×10^{-10} M. Frame C: plot of V-intercept (from frame A) vs *d*-coformycin concentration. From the slope, the value of the catalytic number was calculated to be 0.8×10^4 min^{-1}.

plot with the experimental conditions used (data not shown). As reported earlier [1, 2], the finding of an apparent stoichiometric inhibitory pattern does not necessarily indicate the formation of a covalent bond between the enzyme and inhibitor, but may be explained by a much slower equilibration between the inhibitor and the enzyme than between the enzyme and substrate. Although the Ackermann–Potter plot cannot be used to determine the K_i value of *d*-coformycin, the E_t- and V-intercepts may be utilized to calculate the molar equivalency and the catalytic number* of the enzyme. From a plot of E_t vs *d*-coformycin concentration (Fig. 5B), the molar equivalency of the enzyme was calculated to be about 1 unit/liter, corresponding to 1.3×10^{-10} M, a value close to that obtained earlier with coformycin (1.0×10^{-10} M) [2]. From the slope of the graph of the V-intercept vs the *d*-coformycin concentration (Fig. 5C), the value of the catalytic number was calculated to be about 0.8×10^4 min^{-1}, which approximates the value of about 1.0×10^4 min^{-1} reported previously with coformycin [2].

Use of I_{50} *for the estimation of the* K_i *of* d-*coformycin with human erythrocytic adenosine deaminase.* Since the data of Fig. 2C clearly demonstrate the inapplicability of the classical methods for the determination of the K_i value of *d*-coformycin with human erythrocytic ADA, procedures proposed in recent publications from this group were employed, therefore, for the estimation of this parameter [1, 2]. One of these procedures involves the determination of I_{50} values at various concentrations of the enzyme [1] and analysis of the data in accord with equation 3. As pointed out earlier, it may be practical to employ the same data used for the Ackermann–Potter plot (Fig. 5A) for the I_{50} analysis; in this case, one plots v_0/v_i vs the concentration of the inhibitor *d*-coformycin. The I_{50} values correspond to inhibitor concentrations at the points where the ratio of v_0/v_i equals 2 (Fig. 6A).

The replot of the I_{50} vs E_t according to equation 3 yields a straight line that intersects the I_{50} axis at a point corresponding to the K_i value, and the E_t axis at $-2K_i$. From this plot of I_{50} vs E_t, the K_i value for *d*-coformycin was estimated to be about 1.5×10^{-11} M, a value about 6-fold greater than that determined by the measurement of the velocity constants of the association and dissociation reactions as described below. It should be noted that in Fig. 6B, the line derived from the plot of I_{50} vs E_t intersects both axes at points very close to the origin. Therefore, as noted in the discussion below, the estimation of K_i from this experiment may be less accu-

* The catalytic number is defined as the number of moles of substrate converted to product/mole of active center of the enzyme/min under the defined conditions.

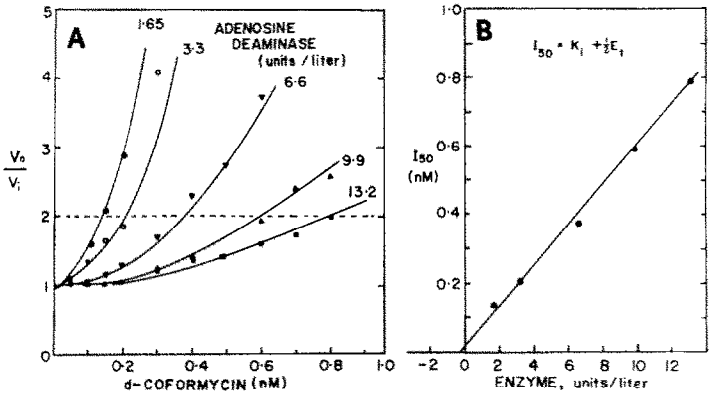

Fig. 6. Determination of I_{50} and the K_i values of *d*-coformycin. Frame A: the data of Fig. 5, frame A, were replotted as v_0/v_i vs *d*-coformycin concentration. Frame B: a plot of I_{50} vs enzyme concentration. From this plot the K_i value of *d*-coformycin was calculated to be 1.5×10^{-11} M.

rate than that calculated from the velocity constants k_1 and k_2.

*Determination of velocity constants of the human erythrocytic adenosine deaminase–*d*-coformycin interaction.* It was shown previously that under favorable experimental conditions the velocity constants of the association and dissociation reactions between a potent, tight-binding inhibitor and an enzyme may be determined directly. As shown in Fig. 7, it has been possible to establish conditions whereby the second-order association velocity constant of the ADA–*d*-coformycin reaction could be measured. The technique employed was to use *d*-coformycin concentrations sufficiently high that significant depletion of the inhibitor did not occur as the result of interaction with the enzyme. Thus, a family of pseudo-first-order velocity constants was determined at different concentrations of *d*-coformycin according to the following relationship:

$$\text{Pseudo-first-order velocity constant} = k_1 \times (I) \quad (4)$$

From the pseudo-first-order velocity constants, calculated from Fig. 7, the value of k_1 was estimated at $2.6 \times 10^6\ M^{-1}\ sec^{-1}$ as described previously [1, 2]. This value is close to the k_1 of the ADA—coformycin reaction ($2.1 \times 10^6\ M^{-1}\ sec^{-1}$) [2]. The similarity of these values is not surprising, since we are dealing with the same enzyme and two inhibitors that are similar both in structure and physical properties. Therefore, the velocity of the interaction should be strongly influenced by rates of diffusion and collision frequencies which should be similar with both inhibitors.

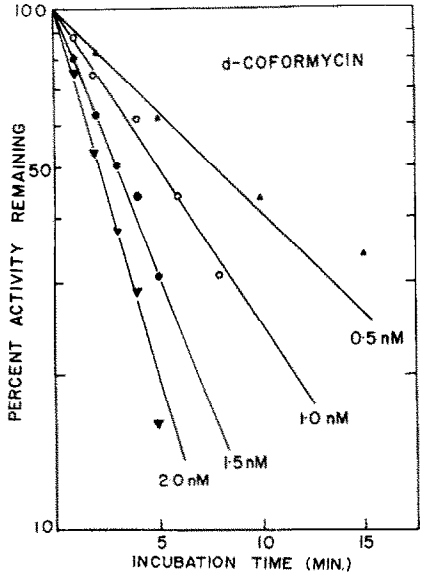

Fig. 7. Rate of association of human erythrocytic adenosine deaminase and *d*-coformycin. ADA (0.0033 units) was incubated with various concentrations of *d*-coformycin in 0.99 ml phosphate buffer. After incubation at room temperature for various time intervals, the reactions were started by addition of 10 μl of 10 mM adenosine (final concentration 0.1 mM).

Figure 8 demonstrates an experiment in which the rate of dissociation of the ADA–*d*-coformycin complex was measured by a method similar to that described earlier [2]. In this case, the ADA was incubated for 1 hr with an excess of *d*-coformycin, conditions under which the enzyme is completely inhibited, after which the unreacted inhibitor was removed by treatment of the reaction mixture with charcoal. The enzyme–inhibitor complex was then diluted about 50-fold and stirred at room temperature in the presence of charcoal. Periodically aliquots were removed and centrifuged and the supernatant fluids were assayed for the presence of released, non-inhibited ADA. As shown in Fig. 8B, when the logarithm of the remaining *EI* complex was plotted vs time, a straight line was obtained over a 25- to 30-hr period, that made possible the extrapolation of a $T_{1/2}$ value of about 29 hr as well as the k_2 value from the relation:

$$k_2 = \frac{0.693}{T_{\frac{1}{2}}} \quad (5)$$

The value of k_2 was determined to be about $6.6 \times 10^{-6}\ sec^{-1}$. It should be noted that several differences were seen between this experiment and that reported earlier for the dissociation of the ADA coformycin complex [2]. In the earlier experiments, a diphasic, rather than linear semilogarithmic plot was obtained and the $T_{1/2}$ values were 75 min and

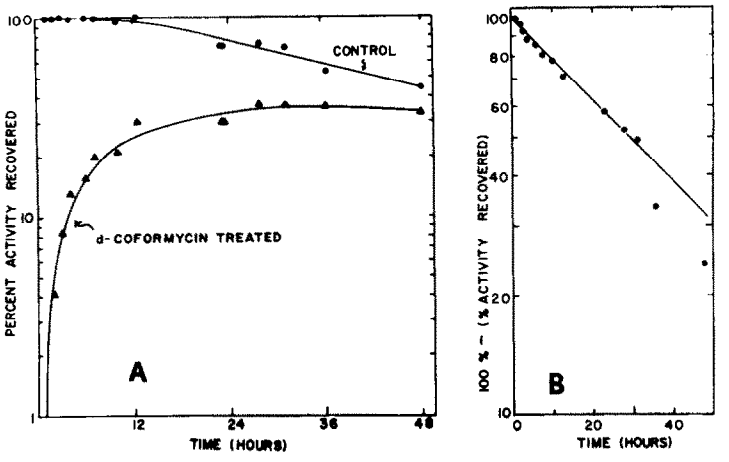

Fig. 8. Dissociation of adenosine deaminase–*d*-coformycin complex. In a reaction mixture of 0.5 ml, human erythrocytic ADA (0.13 units), *d*-coformycin (4×10^{-11} moles) and phosphate buffer (25 μmoles, pH 7.5) were incubated at room temperature for 1 hr. *d*-Coformycin was replaced by an equal volume of water in control experiments. After incubation, 0.4 ml of hemoglobin-coated charcoal in 50 mM phosphate buffer, pH 7.5, was added. After stirring occasionally for 5 min the charcoal was removed by centrifugation. Immediately, the supernatant fluids were diluted 50-fold with 50 mM phosphate buffer, pH 7.5, containing charcoal (0.5 ml), Na_2 EDTA (0.5 mM), and bovine plasma albumin (0.03%, w/v). The mixtures were stirred at room temperature using a magnetic stirrer. Aliquots (1.2 ml) of these suspensions were withdrawn at different time intervals (time immediately after dilution was recorded as zero time) and centrifuged for 5 min, and 1.0 ml of the supernatants was assayed at room temperature for enzymic activity in the presence of 0.1 mM adenosine. Frame A: recovery of activity from enzyme–*d*-coformycin (*EI*) complex with time. The 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 (per cent of enzyme remaining as *EI* complex) vs time. The per cent of activity recovered was calculated on the basis of the activity of the control sample at each particular time taken as 100 per cent. From this plot the $T_{\frac{1}{2}}$ value was estimated at about 29 hr.

7.9 hr, in contrast to the 29 hr observed here. By substitution of the values of k_1 and k_2 in equation 2, the inhibition constant, K_i, of *d*-coformycin with human erythrocytic ADA was calculated to be about 2.5×10^{-12} M, a value significantly lower than that reported for coformycin [2].

Interactions of EHNA and calf intestinal adenosine deaminase. Since EHNA binds to erythrocytic ADA with an affinity that lies between that of a tight-binding and a readily reversible inhibitor, it was of interest to compare the behavior of EHNA with an ADA from another source. Therefore, the interactions of EHNA and calf intestinal ADA were investigated. The spectral tracings of Fig. 9 indicate that, while there was a quantitative difference in the binding of EHNA to human erythrocytic and bovine ADAs, qualitatively they were similar (cf. Fig. 2B). When the reaction was initiated with ADA (method 1), the inhibition progressively developed during the first few min. Conversely, initiation of the reaction by addition of adenosine after a 10-min preincubation of EHNA and ADA (method 2) resulted in the progressive reversal of the inhibition. In both reactions, as with the human erythrocytic enzyme, the final steady state levels of inhibition were approximately equal. In order to avoid the problems of substrate depletion that occurs prior to the establishment of the steady state, the K_i for this enzyme was determined by the I_{50} method that was used in the above study with *d*-coformycin. Replots of I_{50} values vs E_t over a 6.5-fold range of ADA (using method 2) resulted in a horizontal line with a y-axis value of 6.5 nM (data not shown). This indicates that the K_i is considerably greater than 1/2 E_t and the value of 6.5 nM approximates the K_i^*. This

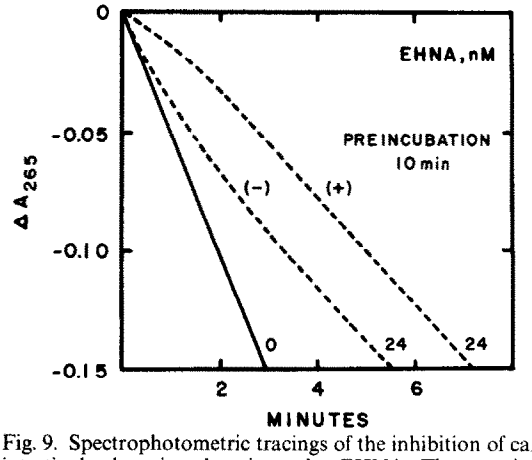

Fig. 9. Spectrophotometric tracings of the inhibition of calf intestinal adenosine deaminase by EHNA. The reaction conditions described in Materials and Methods for calf intestinal ADA were applied to the protocol described in the legend of Fig. 2B. Reactions were initiated either with 10 μl of 20 mM adenosine after 10 min of preincubation (+) of 0.0137 units of calf intestinal ADA and EHNA, or with ADA without preincubation (−). The final volume was 2.0 ml.

* The I_{50} concentration is equal to the K_i for non-competitive inhibitors. Since the velocity measurements for this experiment were determined from tangents drawn to the initial portions of reactions with pre-equilibrated *EI* complexes (i.e. prior to the displacement of *I* by substrate), this inhibition was operationally non-competitive.

value is about 4-fold higher than the K_i of 1.6 nM determined above with human ADA.

Additional studies with the calf intestinal enzyme were performed to determine the rate of association of EHNA and ADA (k_1). When the method that was used for *d*-coformycin was applied to this study, the k_1 value of $7 \times 10^5\ M^{-1}\ sec^{-1}$ was obtained (data not shown). The rate of dissociation (k_2) was calculated by substituting k_1 and K_i into equation 2. This value was $4.6 \times 10^{-3}\ sec^{-1}$. This corresponds to a $T_{1/2}$ of about 150 sec or 2.5 min.

DISCUSSION

The studies presented in this paper offer an unusual opportunity to compare and evaluate some of the problems encountered in studying various enzyme inhibitors, including readily reversible, semi-tight-binding and tight-binding inhibitors, with K_i values ranging from about 1×10^{-6} to 2.5×10^{-12} M. Since each type of inhibitor requires its own experimental approach, it is essential, initially, to examine the inhibition of the enzymic reaction by different procedures, i.e. first, preincubation of the inhibitor and enzyme, with initiation of the enzymic reaction by addition of the substrate, and second, incubation of the substrate and inhibitor in the reaction mixture, with initiation of the reaction by addition of the enzyme. If essentially identical inhibited initial velocity reactions are observed by both procedures, one may assume that the inhibitor is readily reversible and the conventional methods of inhibitor analysis may be employed, e.g. as in the case of DHMPR above. If, on the other hand, one is dealing with an enzyme-inhibitor complex that is slow to equilibrate, the conditions of preincubation and the order of addition of reactants can produce dramatically different results, as shown with the experiments with EHNA and *d*-coformycin (Figs. 2 and 9). Although when such an inhibitor is encountered it is necessary to employ special experimental conditions and methods, in such cases one is also presented with a unique opportunity to derive significantly more information about the enzyme and its reaction than is ordinarily obtained by the standard methods of kinetic analysis. For example, as shown above and elsewhere [1–3], even with relatively impure enzymes, it may be possible to readily determine such important parameters as the catalytic number, the molar equivalency of the enzyme, and the individual velocity constants of the association and dissociation reactions, in addition to estimation of the K_i value.

Several unique problems may be encountered in the study of semi-tight-binding inhibitors, where the attainment of equilibrium between the enzyme, inhibitor and substrate may be prolonged for several min. For example, excessive amounts of substrate may be consumed before the steady state is attained, thus rendering inaccurate the evaluation of the kinetic parameters. Therefore, in such instances it is essential to employ a rapid and highly sensitive assay and the least practical amount of enzyme that does not consume significant quantities of substrate. Furthermore, it is preferable to perform such experiments by preincubating the enzyme with the inhibitor, with initiation of the reaction by addition of substrate, since, under these conditions, less substrate would be consumed before the attainment of steady state conditions than occurs if the reaction is initiated by the addition of enzyme (Figs. 2B and 9).

In another approach that might be employed with certain multi-substrate reactions, or those that require special cofactors, one could omit the cofactor or second substrate until the appropriate equilibrium is established, with the enzymic reaction then initiated by addition of the missing substrate or cofactor. Of course, this approach would not be satisfactory for certain enzymic reactions with ordered reaction sequences, or where the cofactor is required for substrate or inhibitor binding.

An especially intriguing point revealed by the present study is that the K_i value of *d*-coformycin (2.5×10^{-12} M) is about 4-fold lower than the value determined for coformycin after prolonged incubation with ADA (about 1×10^{-11} M). The chemical difference between these two tight-binding inhibitors is in the pentose moiety, i.e. coformycin has a ribose and *d*-coformycin a 2′-deoxyribose moiety. These relative values are in good agreement with our earlier reports of the K_m and K_i values of several substrates and inhibitors of human erythrocytic ADA [4]. For example, the K_m of adenosine is 25 μM and that of 2′-deoxyadenosine, 7 μM; the K_i of 2-fluoroadenosine is 60 μM, and that of 2′-deoxyribosyl-2-fluoroadenine, 19 μM. The K_i values of the reaction products inosine and 2′-deoxyinosine are 116 and 60 μM respectively. Thus, in each case the 2′-deoxyribose-containing compound binds to the enzyme two to four times more tightly than does the corresponding ribose-containing compound. A likely explanation for these differences is offered in the present paper. The apparent 4-fold difference in the K_i values of coformycin and *d*-coformycin with erythrocytic ADA appears to reside in the 3- to 4-fold difference in the velocity constant, k_2 [$2.4 \times 10^{-5}\ sec^{-1}$ for coformycin [2] and $6.6 \times 10^{-6}\ sec^{-1}$ for *d*-coformycin (see above)]. In both cases, the asssociation velocity constants (k_1) were similar: i.e. $2.1 \times 10^6\ M^{-1}\ sec^{-1}$ for coformycin [2] and $2.6 \times 10^6\ M^{-1}\ sec^{-1}$ for *d*-coformycin. The very slow dissociation of the ADA–*d*-coformycin complex ($T_{1/2}$ about 29 hr) could have a profound impact on its future usefulness as a biochemical and pharmacological tool, as well as a potential therapeutic agent.

Another finding worthy of discussion is that the rates of association (k_1) of inhibitors and ADA appear to be 2- to 3-fold faster with the human erythrocytic enzyme than with the calf intestinal enzyme. For example, coformycin [2] and *d*-coformycin have k_1 values with human erythrocytic ADA of 2.1 to $2.6 \times 10^6\ M^{-1}\ sec^{-1}$, while coformycin [3] and EHNA have k_1 values of 0.7 to $1.0 \times 10^6\ M^{-1}\ sec^{-1}$ with calf intestinal ADA. Therefore, it is interesting to speculate that the lower K_m [e.g. adenosine and 2′-deoxy-adenosine (see Ref. 4 for review)] and K_i values (e.g. DHMPR [5], coformycin [2, 3] and EHNA) observed with the human enzyme as compared to the calf enzyme may be the result of these faster rates of association.

The above studies with *d*-coformycin and EHNA are interesting in that they point to the limitations of the I_{50} method. When the ratio of E_t/K_i is greater

than 10, it becomes difficult to distinguish empirically the I_{50} value from the concentration of $1/2\,E_t$. In this range a plot of I_{50} vs E_t will intersect the y-axis very close to the origin. Therefore, the K_i value of *d*-coformycin (2.5×10^{-12}) calculated from the individual rate constants k_1 and k_2 is a more valid estimation of this parameter. If the concentration of ADA were lowered 50- to 100-fold, the I_{50} method would then be valid in this case. However, a more sensitive assay would be required, e.g. an assay employing radiolabeled adenosine of high specific activity. A third approach to this determination would involve equilibrium dialysis. This method would depend upon the availability of suitably labeled *d*-coformycin.

A ratio of E_t/K_i of less than 0.2 represents the other extreme limit for the I_{50} method (e.g. EHNA). Below this value, the I_{50} concentration approaches the K_i value. Therefore, the possible errors here would be much greater for the measurement of E_t, the molar equivalency of the enzyme, than for the K_i value. The I_{50} method could still be used with semi-tight-binding inhibitors as EHNA to determine E_t and the catalytic number if the concentration of ADA were raised 50- to 100-fold, and the reactions were analyzed by a rapid method such as stopped-flow spectrophotometry.

In an earlier report of this series, it was noted that the tight-binding ADA inhibitor, coformycin, binds to the enzyme one to ten million times more tightly than does inosine, the structurally related reaction product [2, 4]. It was pointed out that coformycin possesses many of the structural features that one might expect to find in a "transition state" analog [2]. Since the heterocyclic ring structures of coformycin and *d*-coformycin are identical, similar speculation may be applied to *d*-coformycin, i.e. it may be considered as a transition state analog. On the other hand, the semi-tight-binding inhibitor, EHNA, has a natural purine ring structure, but differs from natural nucleosides in the attachment of a large lipophilic group to N-9 of the purine ring. In this case, evidence has been presented [6] that the tightness of binding is due to the attraction of this lipophilic moiety to a hydrophobic center in the enzymic protein region that is in close proximity to the active site. Of interest is the relative binding of the substrate, adenosine ($K_m = 2.5 \times 10^{-5}$ M) and the inhibitor, EHNA ($K_i = 1.6 \times 10^{-9}$ M). Thus, replacement of the ribose moiety by the erythrononyl group greatly increases the tightness of binding. An intriguing point and a challenge to the ingenuity of the medicinal chemist is the possibility of preparing a potential ADA inhibitor that contains both the heterocyclic ring structure of coformycin or *d*-coformycin and the erythrononyl group. It is conceivable that such a compound might combine the tight-binding inhibitory features of the transition state analog with those of lipophilic attraction, thus producing an inhibitor with a K_i value perhaps as low as 10^{-15} to 10^{-16} M, e.g. an inhibition with potency approaching that of covalent bond formation.

A question not dealt with in the present manuscript is the nature of the inhibition by *d*-coformycin, i.e. is it competitive, non-competitive or uncompetitive? It should be noted, however, that Cha [3] has recently made further advances in the theoretical treatment of tight-binding enzyme inhibitors that now make it possible to determine the nature of the inhibition. In a recent study he has offered evidence that coformycin competes with adenosine for calf intestinal ADA [3]. On the basis of these observations, it seems highly likely that *d*-coformycin will also prove to be competitive with adenosine. As shown above, the kinetic analyses of DHMPR and EHNA yielded double reciprocal plots that are typical of competitive inhibition (Figs. 3 and 4).

Recently, widespread and increasing interest has developed in the possible use of ADA inhibitors (alone or in combination with other analogs) as anti-tumor, anti-viral or immunosuppressive agents [4, 12–19]. Thus, the availability of new ADA inhibitors of widely different potencies offers numerous interesting and promising therapeutic opportunities.

Acknowledgement—We wish to thank Dr. Sungman Cha for many helpful discussions during the preparation of this manuscript.

REFERENCES

1. S. Cha, *Biochem. Pharmac.* **24,** 2177 (1975).
2. S. Cha, R. P. Agarwal and R. E. Parks, Jr., *Biochem. Pharmac.* **24,** 2187 (1975).
3. S. Cha, *Biochem. Pharmac.* **25,** 2695 (1976).
4. R. P. Agarwal, S. M. Sagar and R. E. Parks, Jr., *Biochem. Pharmac.* **24,** 693 (1975).
5. B. Evans and R. Wolfenden, *J. Am. chem. Soc.* **92,** 4751 (1970).
6. H. J. Schaeffer and C. F. Schwender, *J. med. Chem.* **17,** 6 (1974).
7. P. W. K. Woo, H. W. Dion, S. M. Lange, L. F. Dahl and L. J. Durham, *J. heterocyclic Chem.* **11,** 641 (1974).
8. H. W. Dion, P. W. K. Woo and A. Ryder, *Ann. N.Y. Acad. Sci.*, in press.
9. R. P. Agarwal and R. E. Parks, Jr., *The New England Pharmacologists Annual Meetings*, University of Connecticut Health Center, Farmington, Conn. (Jan. 30–31, 1976).
10. S. Waxman, C. Schrieber and V. Herbert, *Blood* **38,** 219 (1971).
11. W. W. Ackermann and V. R. Potter, *Proc. Soc. exp. Biol. Med.* **72,** 1 (1949).
12. R. E. Parks, Jr., G. W. Crabtree, C. M. Kong, R. P. Agarwal, K. C. Agarwal and E. M. Scholar, *Ann. N.Y. Acad. Sci.* **255,** 412 (1975).
13. R. P. Agarwal, G. W. Crabtree, R. E. Parks, Jr., J. A. Nelson, R. Keightley, R. Parkman, F. S. Rosen, R. C. Stern and S. H. Polmar, *J. clin. Invest.* **57,** 1025 (1976).
14. J. J. Ballet, R. Insel, E. Merler and F. S. Rosen, *J. exp. Med.* **143,** 1271 (1976).
15. R. Wolberg, T. P. Zimmerman, K. Hiemstra, M. Winston and L. C. Chu, *Science, N.Y.* **187,** 957 (1975).
16. W. Plunckett and S. S. Cohen, *Cancer Res.* **35,** 1547 (1975).
17. F. M. Schabel, Jr., M. W. Trader and W. R. Laster, *Proc. Am. Ass. Cancer Res.* **17,** 46 (1976).
18. A. P. Kimball, G. A. LePage, L. S. Worth and S. H. Lee, *Proc. Am. Ass. Cancer Res.* **17,** 168 (1976).
19. R. W. Brockman, S. C. Shaddix, L. M. Rose and J. Carpenter, *Proc. Am. Ass. Cancer Res.* **17,** 52 (1976).