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ANALYSIS OF CYTIDINE DEAMINASE AND TETRAHYDROURIDINE INTERACTION BY USE OF LIGAND TECHNIQUES

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Abstract—Ligand techniques, as employed in radio-immunossay and radioreceptor assay, offer a sensitive and precise method for characterizing the interaction of enzymes and tight-binding inhibitors. Tetrahydrouridine (H_4U) inhibition of human liver cytidine deaminase (EC 3.5.4.5) has been examined by direct measurement of enzyme–inhibitor (*EI*) binding and release. With partially purified enzyme from human liver, the *EI* complex was found to have a dissociation constant (K_D) of 4.5×10^{-8} M at 37°, in close agreement with estimates based on inhibition of enzyme activity by H_4U. The presence of steady state conditions during competitive binding analysis was confirmed by direct measurement of the rate constants for *EI* binding at 25° and 37° (k^{on} 1.7×10^4 and 5.6×10^4 M^{-1} sec^{-1} respectively). The rate constant for *EI* release at 37° was also determined experimentally ($k^{off} = 4.0 \times 10^{-3}$ sec^{-1}), and was in close agreement with the k^{off} value calculated from the experimentally determined K_D and k^{on} values ($K_D = k^{off}/k^{on}$). Scatchard analysis of H_4U–enzyme binding, both in the presence and in the absence of 10^{-3} M cytidine, showed no variation in total enzyme concentration (E_T) but a decrease in apparent inhibitor affinity for enzyme, suggesting that cytidine and H_4U compete for the same binding sites on cytidine deaminase, and confirming the competitive inhibition suggested by Lineweaver–Burk analysis. The turnover number for cytidine deaminase based on per H_4U binding sites was 3.9×10^3 min^{-1}. Thermodynamic constants for cytidine deaminase–tetrahydrouridine binding were derived from data on the temperature dependence of binding and included an enthalpy change (ΔH) = −12.7 kcal/mole, entropy change (ΔS) = −8.68 cal/deg/mole and Gibbs free energy change (ΔG) = −9.96 kcal/mole at 37.1°. This study indicates that ligand techniques can be applied to the difficult problem of characterizing the interaction of enzymes and tight-binding inhibitors.

3,4,5,6-Tetrahydrouridine (H_4U) is a potent inhibitor of human cytidine deaminase (EC 2.5.4.5) binding to the enzyme three orders of magnitude more tightly than does the physiologic substrate, cytidine. Studies by Evans *et al.* [1] suggest that the effectiveness of H_4U as a tight-binding inhibitor is due to its structural resemblance to a hypothetical transition state intermediate formed in the deamination of cytidine [2]. While initial work suggested that H_4U exhibited a complex, non-linear pattern of cytidine deaminase inhibition by a Lineweaver–Burk plot [3], more recent studies, based on Michaelis–Menten kinetic analysis, have provided support for a purely competitive mechanism of inhibition [4]. However, due to both the depletion of free inhibitor by binding [5], and the prolonged non-steady state between enzyme and tight-binding inhibitor, Morrison [6], Henderson [7], Cha [8] and Goldstein [9] have pointed out the possible inaccuracy of using steady state equations to describe the kinetics of such tight-binding inhibitors.

The present work was undertaken to study the kinetics of tetrahydrouridine inhibition of human liver cytidine deaminase using both classical Michaelis–Menten kinetic analysis ("enzyme activity inhibition" studies) and a new method of direct measurement of H_4U–deaminase binding by ligand techniques. The ligand method allowed for direct quantitation of enzyme–inhibitor (EI) binding (k^{on}), and release (k^{off}) and calculation of the thermodynamic constants of the binding reaction. The results of these experiments demonstrate the advantages of ligand techniques in characterizing the interaction of tight-binding inhibitors and target enzymes.

MATERIALS AND METHODS

Materials

Activated charcoal (acid washed with phosphoric and sulfuric acid), bovine serum albumin (fraction V), high molecular weight dextran, and all non-radiolabeled nucleosides were obtained from Sigma Chemical Co., St. Louis, MO, except where otherwise noted. Ara-C, H_4U and [2-^{14}C]H_4U [56 Ci/mole, radiochemical purity >97 per cent, thin-layer chromatography (TLC) Silica gel chloroform–methanol (1:1)] were kindly supplied by Dr. Harry Wood of the Drug Research and Development Branch, National Cancer Institute (NCI), Bethesda, MD. [2-^{14}C]cytidine and [2-^{3}H]ara-C were obtained from New England Nuclear Corp., Boston, MA. All other chemicals were reagent grade and were supplied by Sigma Chemical Co., St. Louis, MO.

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Enzyme purification

Post-mortem human liver samples were obtained through the cooperation of the Laboratory of Pathology, NCI.

In a typical experiment, 96 g tissue was homogenized in a Waring blender in 150 ml of 0.05 M Tris–Cl buffer, pH 7.5 (buffer A). The homogenate was centrifuged for 30 min at 12,000 *g* (Sorvall RC-2 centrifuge), and the supernatant heated to 70° for 6 min with constant agitation. The denatured protein was removed by centrifugation, and the supernatant was brought to 60% saturation by the addition of ammonium sulfate, 36.1 g/100 ml. The precipitate was collected by centrifugation at 12,000 *g* for 20 min, resuspended in buffer A containing 2.5 mM dithiothreitol (DTT) to a total volume of 12 ml, and applied to an upward-flow Sephadex G-200 column (90 × 3 cm) equilibrated in buffer A containing 2.5 mM DTT. Four-ml fractions were collected. Peak enzyme activity eluted between 200 and 228 ml; these fractions were pooled and saved for use in the H_4U ligand assay. This enzyme was purified 30-fold as compared to the liver supernatant enzyme, and had a specific activity of 1.9×10^3 units/mg of protein (Table 1). In experiments examining kinetic features of H_4U inhibition of deaminase, it was desirable to further purify the enzyme. To the pooled G-200 fractions (volume 28 ml) was added 7.5 g of aged calcium phosphate gel (Sigma Chemical Co., St. Louis, MO). The supernatant was discarded after centrifugation at 5000 *g* for 10 min, and the enzyme was then eluted by resuspending the gel in 30 ml of 0.05 M sodium phosphate buffer, pH 7.0. The calcium phosphate gel was removed by centrifugation, and the supernatant brought to 60% ammonium sulfate saturation. The resulting precipitate was dissolved in 1.3 ml of buffer A containing 2.5 mM DTT and applied to a downward-flow Sephadex G-150 column (60 × 1 cm) equilibrated in buffer A with DTT. The enzyme peak eluted between 12 and 16 ml. Potassium chloride was added to the pooled peak fractions, bringing the enzyme solution to a final concentration of 0.05 M KCl. This solution was then added to a slurry of 1.0 ml of DEAE–cellulose resin which had been previously equilibrated with buffer A with 0.05 M KCl. The resin was collected by centrifugation at 1000 *g* for 10 min, washed three times with 2.0 ml of starting buffer, and the enzyme was then eluted with four 2.0-ml washes of buffer A with 0.2 M KCl. The four washes containing peak enzyme activity were pooled and stored at 4.0 . The final purification of these fractions was 1300-fold, as shown in Table 1.

At 4 , highly purified enzyme was stable for only 2–4 days whereas crude extracts were stable for several months without loss of activity.

Protein determination. Protein concentrations were determined according to the method of Lowry *et al.* [10], using bovine serum albumin fraction V as the standard.

Enzyme units. One unit of enzyme activity was defined as the hydrolysis of 1 nmole substrate/hr at 37 .

Assay of enzyme activity

Catalytic activity of cytidine deaminase was determined by a previously described method [4]. Enzyme (150–450 units) and substrate were incubated with 15 μmoles Tris–Cl, pH 7.5, in a total volume of 0.3 ml for a period of 15 60 min. The reaction was terminated by the addition of 0.2 ml of 0.1 N HCl, and substrate and product were separated by passage over Dowex 50 H^+ resin, as initially described by Maley and Maley [11], and modified by Steuart and Burke [12].

Assay of enzyme–inhibitor binding

This assay was based on the high affinity binding of H_4U to cytidine deaminase with subsequent removal of unbound inhibitor by charcoal adsorption. Preliminary experiments demonstrated that charcoal was capable of quantitatively removing unbound H_4U from the assay solution at H_4U concentrations as high as 1 mM, while enzyme and enzyme–inhibitor complex remained in solution. When enzyme was added to an incubation mixture containing an excess of unlabeled H_4U (0.30 μmole), the binding of [^{14}C]H_4U (0.11 nmole) to enzyme was prevented, as indicated by quantitative removal of labeled drug from solution with activated charcoal (Table 2).

In typical experiments, enzyme (5–25 pmoles of H_4U binding activity) was incubated with 15 μmoles Tris–Cl, pH 7.5, and between 7.6 and 535 pmoles [^{14}C]H_4U (56 Ci/mole), in a total reaction volume of 0.3 ml. After a 30-min incubation, 300 μl of a charcoal slurry, kept at 4 , was added (acid-washed activated charcoal, 10 g/100 ml; bovine serum albumin,

Table 1. Purification of human liver cytidine deaminase

Step	Total activity (units × 10^{-5})	Specific activity (units/mg protein)	Recovery (%)	Purification (fold)
Starting extract (95.5 g liver)	2.59	58.0		
Heat precipitation	1.51	85.0	58.3	1.47
First ammonium sulfate precipitate	1.00	254	38.6	4.38
Sephadex G-200	1.10	1850	42.6	31.9
Calcium phosphate gel elution at 50 mM K_2HPO_4	0.92	4020	35.6	69.3
Second ammonium-sulfate precipitate (0–60%)	0.74	6200	28.5	107
Sephadex G-150	0.60	15,400	23.1	266
DEAE–cellulose	0.22	78,800	8.5	1360

Table 2. Separation of protein-bound from free tetrahydrouridine (H_4U)

[^{14}C]H_4U (0.11 nmole)	H_4U (0.30 μmole)	Binding protein*	Activity remaining in supernatant after charcoal (dis./min)
+	−	−	25
+	−	+	382
+	+	+	54

* H_4U binding activity is 0.40 nmole.

fraction V, 2.5 g/100 ml; and high molecular weight dextran, 0.1 g/100 ml), and samples were mixed by Vortex agitation and placed immediately in an ice-water bath. After cooling, samples were centrifuged at 700 *g* for 45 min at 4°. A 200-μl aliquot of the supernatant was then placed in 18 ml Aquasol (New England Nuclear Corp.) and the enzyme-bound [^{14}C]H_4U determined by counting in a liquid scintillation spectrophotometer (Searle Mark III model 6880). In order to determine the degree of non-specific binding, competed reactions containing 0.30 μmole of unlabeled H_4U were run at each concentration of [^{14}C]H_4U studied. Binding studies were done at various temperatures between 26.7 and 41.2°. A thermocouple laboratory thermometer with an accuracy of +/−0.05° was used to monitor water-bath temperature (model BAT-4, Bailey Instrument Co., Inc., Saddle Brook, NJ).

Calculations

Scatchard analysis of deaminase–H_4U binding [13] provided data for the calculation of the following parameters: (1) specifically bound (B) H_4U concentration (pmoles/ml of reaction mixture) and the ratio of bound/free (B/F) H_4U, and (2) the K_D, the E_T value (concentration of total enzyme binding sites) and the regression coefficient.

The rate constants for binding (k^{on}) were calculated using the MLAB differential equation solving program [14], running on a PDP-10 digital computer. The data were fit to a non-linear regression under the following conditions:

assuming: $E + I \underset{k^{off}}{\overset{k^{on}}{\rightleftharpoons}} EI$

then: $$\frac{d[EI]}{dt} = k^{on}[E]_t[I]_t - k^{off}[EI]_t$$

where: $$[E]_t = [E]_0 - [EI]_t$$
$$[I]_t = [I]_0 - [EI]_t$$
$$[EI]_0 = 0$$

$[E]$, $[I]$ and $[EI]$ refer to the concentrations of enzyme, H_4U, and enzyme–H_4U complex, respectively, and the subscripts 0 and t refer to time zero and time t.

Gibbs free energy change (ΔG) for the binding of H_4U to deaminase was calculated from the experimentally determined association constant (K_a) according to the relationship:

$$\Delta G = -2.3\,RT \log K_a$$

Enthalpy change (ΔH) was calculated from the slope of the plot of K_a as a function of temperature, as defined by the van't Hoff equation:

$$\Delta H = -2.3R \frac{d \log K_a}{d(T^{-1})}$$

Entropy (ΔS) was calculated from the equation:

$$\Delta S = \frac{(\Delta H - \Delta G)}{T}$$

RESULTS

Enzyme activity inhibition

Inhibition of enzyme calalytic activity by H_4U appeared to be competitive, as suggested by both Lineweaver–Burk and Dixon plots (Fig. 1) [15, 16]. No change in V_{max} or deviation from linearity was observed in Lineweaver–Burk plots of H_4U inhibition

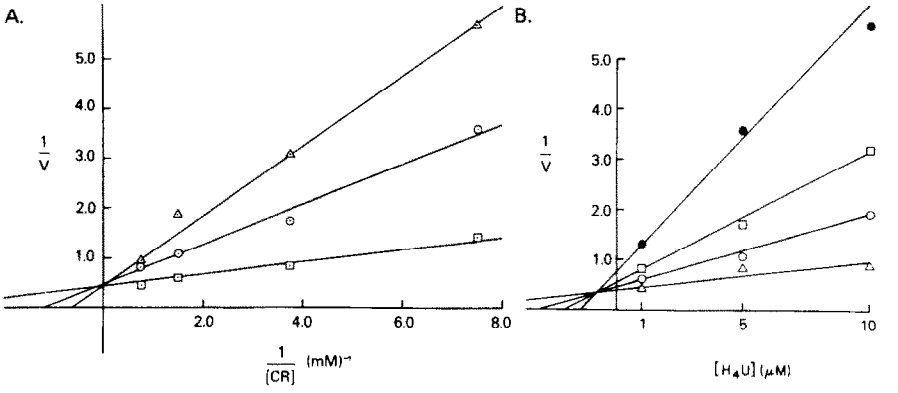

Fig. 1(A). Lineweaver–Burk plot of cytidine deamination in the presence of 10^{-5} M H_4U (△——△), 5×10^{-6} M H_4U (○——○) and 10^{-6} M H_4U (□——□). Cytidine deaminase (263 units), sp. act. 10,500 units/mg protein, was used in each assay. Reaction were initiated by the addition of enzyme, and were run for 15 min at 37°. (B) Dixon plot of inhibitor concentration versus the reciprocal of reaction velocity for the inhibitor H_4U in the presence of 0.14 mM cytidine (●——●), 0.27 mM cytidine (□——□), 0.68 mM cytidine (○——○) and 1.37 mM cytidine (△——△). Reaction conditions were as described for Fig. 1A.

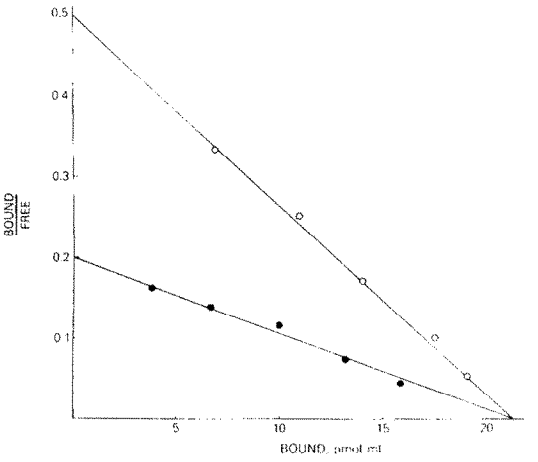

Fig. 2. Scatchard plot of H_4U binding to cytidine deaminase in the absence of competitive substrate (O—O) and in the presence of 1 mM cytidine (●—●). Enzyme (6.4 pmoles of H_4U binding capacity) was incubated with 15 μmoles Tris–Cl, pH 7.5, and 7.6 to 107 pmoles [^{14}C]-H_4U (56 Ci/mole), and indicated substrate concentration in a total volume of 0.3 ml. The reaction was initiated by the addition of enzyme. After a 30-min incubation at 37°, the concentration of enzyme-bound H_4U was determined as described in Materials and Methods.

or partially purified cytidine deaminase, using either cytidine (Fig. 1A) or ara-C as substrate. Furthermore, H_4U was a competitive inhibitor using either crude supernatant (K_i 3.3×10^{-8} M) or partially purified enzyme (K_i 2.8×10^{-8} M) from human liver.

Product formation using either cytidine or ara-C as substrate was linear for up to 60 min. Lineweaver Burk plots were used to determine substrate affinity for both cytidine (K_m 1.8×10^{-5} M) and cytosine arabinoside (K_m 2.5×10^{-4} M) at 37°.

It had been previously reported that preincubation of enzyme and inhibitor was required to demonstrate inhibition of cytidine deaminase by H_4U [3]. The enzyme activity inhibition assay was used to assess this reported preincubation requirement. The degree of enzyme inhibition seen after 5-min preincubation with inhibitor at 37°, as determined by incubation with substrate for 15–60 min, did not significantly differ from the deaminase inhibition seen without preincubation. This lack of enhancement of inhibition by preincubation was noted over a wide substrate concentration range with either ara-C (0.2 to 5.0 mM) or cytidine (0.067 to 1.34 mM) as substrate and using either crude liver homogenate or partially purified liver cytidine deaminase.

Enzyme–inhibitor binding

A primary objective of this study was to directly measure the rate of formation of the *EI* complex and to compare these results with the analysis of inhibition based on enzyme activity. Using the enzyme-inhibitor binding assay, both in the presence and in the absence of substrate, such direct measurements were possible.

EI *dissociation constant* (K_D)

The linear Scatchard plot of H_4U–deaminase binding (Fig. 2) indicated a single homogeneous class of

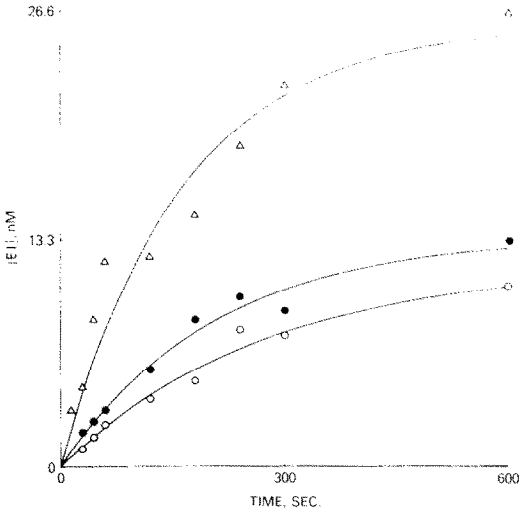

Fig. 3. Rate of cytidine deaminase-H_4U (*EI*) association at 25°. Reactants were preequilibrated at 25°. The binding reaction was begun by the addition of cytidine deaminase (10.3 pmoles of H_4U binding capacity) to a reaction mixture containing 15 μmoles Tris–Cl, pH 7.5, and either 15.2 pmoles [^{14}C]H_4U (O—O), 26.7 pmoles [^{14}C]H_4U (●—●) or 107 pmoles [^{14}C]H_4U (△—△) (total reaction volume 0.3 ml). The reaction was terminated at timed intervals, from 15 sec to 10 min after the addition of enzyme, by adding 0.1 μmole of unlabeled H_4U. The reactants were mixed by Vortex agitation and placed immediately in a 4° ice bath. The concentration of enzyme-bound [^{14}C]H_4U (*EI*) was determined as described in Materials and Methods.

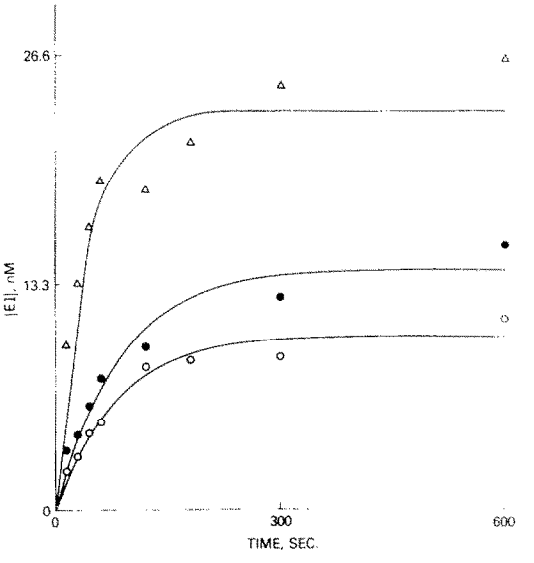

Fig. 4. Rate of cytidine deaminase-H_4U (*EI*) association at 37°. Reactants were individually preequilibrated to 37°. Cytidine deaminase (10.3 pmoles of H_4U binding capacity) was then incubated with varying amounts of [^{14}C]H_4U: 15.2 pmoles (O—O), 26.7 pmoles (●—●) and 107 pmoles (△—△) under experimental conditions described in Fig. 3.

H_4U binding sites, with an *EI* dissociation constant (K_D) of 4.5×10^{-8} M. Due to the requirement for substantial quantities of enzyme for these studies, enzyme purified only through the Sephadex G-200 step was used in the binding studies. The linearity of the Scatchard plot and the close agreement of the K_D value with kinetically determined K_i values provided substantial evidence that cytidine deaminase was the major protein responsible for binding of H_4U in these experiments. When this same experiment measuring H_4U–deaminase binding was done in the presence of 1 mM cytidine, Scatchard analysis showed an increase in the apparent *EI* dissociation constant, but no change in the total number of binding sites (E_T) as would be expected for a competitive binding ligand (Fig. 2). It therefore appears that H_4U and cytidine compete for the same binding sites, a finding consistent with the competitive inhibition by H_4U of cytidine deaminase (K_i 3.3×10^{-8} M) (Fig. 1A). The cytidine deaminase turnover number (k_p), calculated per H_4U binding site, was 3.9×10^3 min^{-1}.

Furthermore, when cytidine deaminase from granulocytes of a patient with chronic myelogenous leukemia (CML) was purified through the Sephadex G-200 step and was used in the enzyme–inhibitor binding assay, the Scatchard plot was again linear ($r = 0.99$) with a similar K_D value of 4.3×10^{-8} M.

Rate constant for EI *binding* (k^{on})

As Scatchard analysis assumes equilibrium binding data, the rate constant for *EI* binding (k^{on}) at both 25 and 37° was determined experimentally in order to confirm that, under the conditions used to determine K_D, the *EI* complex had reached a steady state concentration. As k^{on} is a second-order rate constant, experimental data of $[EI]$ vs time were used to solve the differential equation:

$$\frac{d[EI]}{dt} = k^{on}[E]_t[I]_t - k^{off}[EI]_t$$

using the MLAB program as outlined in Methods. The computer program fit the data to a non-linear regression, generating rate constants for enzyme–inhibitor binding (k^{on}) and release (k^{off}) at both temperatures (25 and 37°) studied. At 25°, the $k^{on} = 1.7 \times 10^4$ M^{-1} sec^{-1} and $k^{off} = 2.1 \times 10^{-3}$ sec^{-1} (Fig. 3), whereas at 37°, k^{on} increased to 5.6×10^4 M^{-1} sec^{-1} and k^{off} increased to 8.1×10^{-3} sec^{-1} (Fig. 4). At 25°, with an enzyme concentration of 10.3 pmoles of H_4U binding capacity/0.3 ml, the formation of the *EI* complex approached equilibrium in 10 min (Fig. 3), even at the lowest concentration of [^{14}C]-H_4U (15.2 pmoles/0.3 ml). At higher concentrations of [^{14}C]H_4U and at higher temperatures, equilibrium was approached more rapidly (Figs. 3 and 4). For the Scatchard plots (Figs. 2 and 6), deaminase (6–18 pmoles of H_4U binding capacity) and [^{14}C]-H_4U (7.6 to 107 pmoles) were incubated together in a total volume of 0.3 ml for a period of 30 min, at temperatures ranging from 26.7 to 41.2°. From the experimental determination of the rate constants for binding (Figs. 3 and 4), it can be concluded that measurements of the *EI* dissociation constant (K_D) (Figs. 2 and 6) were performed under equilibrium conditions.

Rate constant for EI *release* (k^{off})

The calculation of k^{off} by the MLAB program ($k^{off} = 8.1 \times 10^{-3}$ sec^{-1} at 37°) was confirmed both experimentally and mathematically. Having determined the K_D and k^{on} experimentally, and knowing that $K_D = k^{off}/k^{on}$, the rate constant for release (k^{off}) at 37° was calculated to be 2.5×10^{-3} sec^{-1}. Experimentally, the k^{off} was determined by incubating [^{14}C]H_4U and enzyme for a variable period of time, then adding a 3 log excess of unlabeled H_4U and measuring the rate of deaminase–[^{14}C]H_4U dissociation. As the rate constant for release is a first-order function, the slope of a plot of $\ln[EI]$ against time is a measure of the off-rate. Using this technique, the rate constant for release of deaminase–H_4U binding was measured after both a 15- and 60-min incubation of enzyme and inhibitor at 37°. The k^{off} was identical ($k^{off} = 4.0 \times 10^{-3}$ sec^{-1}) under both these experimental conditions (Fig. 5). The $T_{1/2}$ for *EI* dissociation at 37° was 2.9 min. Furthermore, at 0° the k^{off} was negligible and the $T_{1/2}$ was therefore infinite.

Thermodynamics of EI *binding*

The enthalpy change for the binding of H_4U to cytidine deaminase was calculated from the slope of the van't Hoff plot (Fig. 6). The binding reaction is exothermic with $\Delta H = -12.7$ kcal/mole. At 37.1°, the negative entropy ($\Delta S = -8.68$ cal/deg/mole) indicates that the binding of H_4U to deaminase, to form the *EI* complex, represents the formation of a more ordered structure, a finding not inconsistent with the binding of a transition-state analog to an enzyme [2, 17, 18]. The Gibbs free energy (ΔG) at 37.1° was calculated to be −9.96 kcal/mole.

DISCUSSION

H_4U has been characterized in this and previous studies [4, 19] as a potent inhibitor of cytidine deaminase, the enzyme which catalyzes the degradation of the antineoplastic agents, cytosine arabinoside and 5-azacytidine. In the present investigation, we have used deaminase–H_4U interaction as a model for examining the value of ligand techniques in characterizing the formation of enzyme–inhibitor complexes. Using the ligand assay, the rate constants for enzyme–inhibitor binding and release (k^{on} and k^{off}) could be measured directly. The k^{on} value of 1.7×10^4 M^{-1} sec^{-1} at 25° was in close agreement with the value reported previously by Wentworth and Wolfenden [19] (2.4×10^4 M^{-1} sec^{-1}), who analyzed the binding reaction by determining the lag in achievement of steady state catalytic activity. Analysis of the rate of *EI* complex formation defined the time required for achievement of steady state conditions and validated the determination of the dissociation constant by Scatchard analysis, as shown in Fig. 6. In particular, it was shown that the rate of deaminase–H_4U complex formation was temperature dependent, with a 3-fold rate increase at 37° as compared to 25°.

Previous workers noting the dissociation of several tight-binding inhibitor–enzyme complexes to be

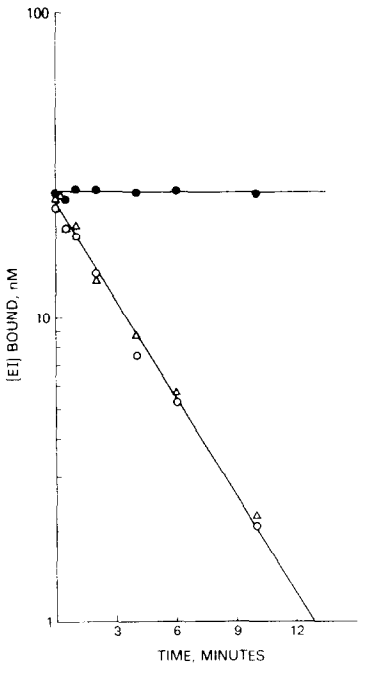

Fig. 5. Rate of cytidine deaminase–H_4U (*EI*) dissociation at 37° (○——○, △——△) and 4° (●——●). Cytidine deaminase (24.1 pmoles of H_4U binding capacity) was incubated with 15 μmoles Tris–Cl, pH 7.5, and 534 pmoles [^{14}C]H_4U (56 Ci/mole) in a total volume of 0.3 ml. After either a 15-min (△——△) or 60-min (○——○) preincubation of enzyme and inhibitor at 37°, an excess of unlabeled H_4U (0.1 μmole) was added to the reaction mixture (time 0), and the reaction stopped at time intervals, from 15 sec to 10 min, by the addition of 300 μl of the charcoal slurry. The reaction mixture was then placed immediately in a 4° ice bath, and the concentration of enzyme-bound [^{14}C]H_4U determined as described in Materials and Methods. To confirm that *EI* complex dissociation was inhibited in the 4° ice bath, enzyme and [^{14}C]H_4U were incubated together for 10 min at 37° as noted above. The reaction mixture was then placed in a 4° ice bath, and after 5 min an excess of unlabeled H_4U (0.1 μmole) was added to the iced incubation mixture (time 0). Again the reaction was stopped at time intervals by the addition of 300 μl of the charcoal slurry and the concentration of enzyme-bound [^{14}C]H_4U determined (●——●). The data were plotted by linear regression ($r = 0.99$) on semi-log paper. The slope of this line is the first-order rate constant for release (k^{off}).

biphasic (e.g. coformycin–adenosine deaminase, oxalate–lactate oxidase) [20, 21] have suggested that these complexes may undergo secondary rearrangements after the initial binding reaction. In the present study, there were no indications of such secondary conformational changes, as H_4U–deaminase dissociation followed simple first-order decay kinetics with a k^{off} of 4.3×10^{-3} sec^{-1} at 37°. The $T_{1/2}$ of 2.9 min at 37° contrasts with a $T_{1/2}$ of 9.5 hr for the dissociation of coformycin–adenosine deaminase [20]. Thus, the difference in affinity of these two inhibitors for their respective enzymes (4×10^{-8} M for H_4U versus $< 10^{-10}$ M for coformycin) can be principally accounted for by differences in the relative off-rates (k^{off}) of the *EI* complexes.

These results can be used to explain the apparent discrepancies between the earlier work of Caminier [3] and more recent studies of H_4U inhibition kinetics [4]. Caminier found maximal inhibition of deaminase activity required preincubation of enzyme and inhibitor in the absence of substrate, and characterized this inhibition as partially non-competitive. These experiments were performed with preincubation times of 5 min, followed by incubation with substrate for 1–2 min. As judged by our current study, the short duration of incubation was not sufficient to allow establishment of an equilibrium between the bound and free forms of inhibitor and substrate during the 1–2 min assay period. The present experiments utilized longer periods of assay of 15–60 min, and equilibrium was achieved for most of the assay period, as verified by analysis of the enzyme–inhibitor complex. Under these conditions, the reaction kinetics were consistent with a competitive inhibition mechanism.

This work has shown that ligand techniques can be applied to the problem of quantifying and characterizing the interaction of an enzyme and inhibitor of intermediate potency. The same techniques have also been used to compare the relative binding affinities of a more potent class of inhibitors, the antifolates [22, 23], and thus provide a useful alternative to the often difficult and indirect analysis of enzyme–inhibitor interactions by kinetic means.

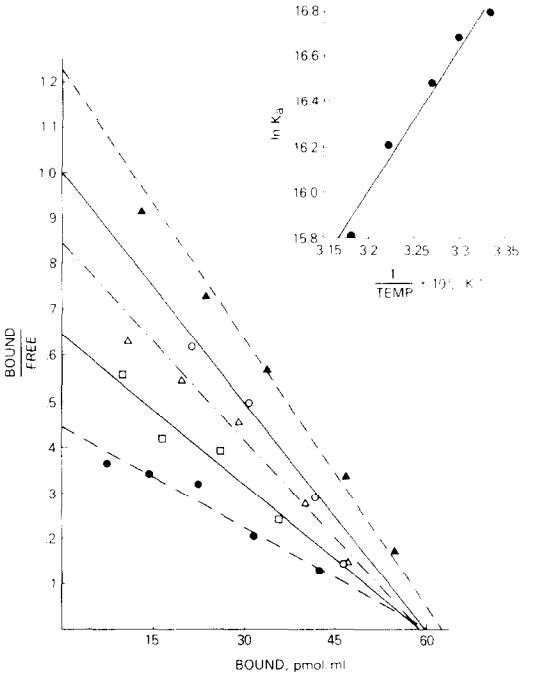

Fig. 6. Scatchard plots of H_4U binding to cytidine deaminase at 26.7° (▲——▲), 30.0° (○——○), 33.7° (△——△), 37.1° (□——□), and 41.2° (●——●). Cytidine deaminase (18.1 *p*moles of H_4U binding capacity) was incubated for 30 min with 15 μmoles Tris–Cl, pH 7.5, and from 7.6 to 107 *p*moles [^{14}C]H_4U (56 Ci/mole) in a total volume of 0.3 ml. Inset: van't Hoff plot of the reciprocals of absolute temperature versus natural logarithm of the association constant. Data were plotted by linear regression ($r = 0.98$). The slope of this line equals $-\Delta H/R$, where R is the gas constant and ΔH is the enthalpy change for H_4U–cytidine deaminase binding.

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