On the Interaction of 3,4,5,6-Tetrahydrouridine with Human Liver Cytidine Deaminase†

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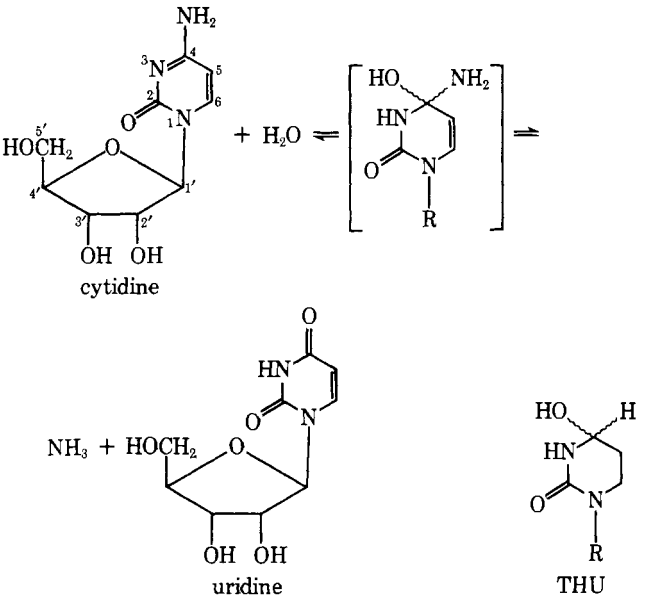
ABSTRACT: In contrast to the rapid inhibition of bacterial cytidine deaminase by 3,4,5,6-tetrahydrouridine, the onset of inhibition of the enzyme from human liver was found to be relatively slow. Inhibition was found to be reversible, and the corrected rate constants for binding (k^{on} = 2.4 × 10^4 M^{-1} sec^{-1}) and release (k^{off} = 5.6 × 10^{-4} sec^{-1}) were in reasonable agreement with a K_i value (2.9 × 10^{-8} M) measured separately under steady-state conditions, which was several orders of magnitude lower than estimates previously reported in the literature. Rates of binding and release of this potential transition state analogue were not appreciably affected by the substitution of deuterium oxide for solvent water. The slow onset of inhibition, which was also observed for cytidine deaminase from HeLa cells, suggests that structural reorganization precedes the formation of a stable enzyme–inhibitor complex. 6-Azacytidine, which favors a "high-anti" configuration at the glycosidic bond, was found to be active as a substrate for cytidine deaminase, with a turnover number exceeding that of cytidine. 2,2′-Anhydro-1-β-D-arabinofuranosylcytosine, which is restricted to the "syn" configuration, was found to be without activity as a substrate or an inhibitor.

3,4,5,6-Tetrahydrouridine inhibits cytidine deaminase from bacterial and mammalian sources at concentrations much lower than the K_m values of substrates (Camiener, 1968; Cohen and Wolfenden, 1971; Chabner et al., 1973), and the corresponding deoxynucleoside 5′-monophosphate is similarly effective as an inhibitor of avian deoxycytidylate deaminase (Maley and Maley, 1971).

The very high affinity of THU[1] for cytidine deaminase may be due to its resemblance to a tetrahedral intermediate formed by addition of water across the 3,4-double bond of cytidine as shown in Scheme I. In the inhibitor, a carbon-bound hydrogen replaces the variable leaving group (ammonia, in the case of the substrate cytidine) at position 4 of the hypothetical intermediate. According to this hypothesis, 5,6-dihydrocytidine might also be expected to serve as a substrate for cytidine deaminase since THU resembles the corresponding intermediate in deamination of this compound even more closely than it resembles the hypothetical intermediate in cytidine deamination. Cytidine deaminase has recently been found to be an excellent catalyst for this nonphysiological reaction (Evans et al., 1975).

Equally compatible with these observations is the alternative hypothesis that the actual inhibitor is not THU itself, but a dehydrated form of THU such as I or II, which might be present as a minor species in solutions of THU. All samples of THU prepared in this laboratory have been similarly effective as inhibitors of cytidine deaminase, before and after exhaustive purification, and their solutions are stable to prolonged storage under refrigeration as judged by their inhibitory properties. Dehydrated species might always be present, however, in rapid equilibrium with THU. While there is no evidence that such species are present in samples of THU (Hanze, 1967; and unpublished experiments of G. N. Mitchell in this laboratory), their presence would pass undetected in nuclear magnetic resonance (NMR) spectra of the parent compound at levels of 2% or less. If such a compound, present as a very minor species, were the true inhibitor, its affinity would presumably be very much greater than the apparent affinity measured for THU itself. Inhibition might then be attributed, for example, to addition by enzyme at the position of unsaturation, yielding an adduct stabilized by interactions similar to those of an intermediate in double displacement (Scheme II).

Scheme I



The work described in this paper, undertaken in an effort to resolve this dilemma, was prompted by apparent discrepancies between the behavior of cytidine deaminase from bacteria and from human liver. THU was found to exhibit rapid and reversible inhibition of the enzyme from *Escherichia coli*, and the inhibition was competitive in character (Cohen and Wolfenden, 1971). Earlier experiments on the

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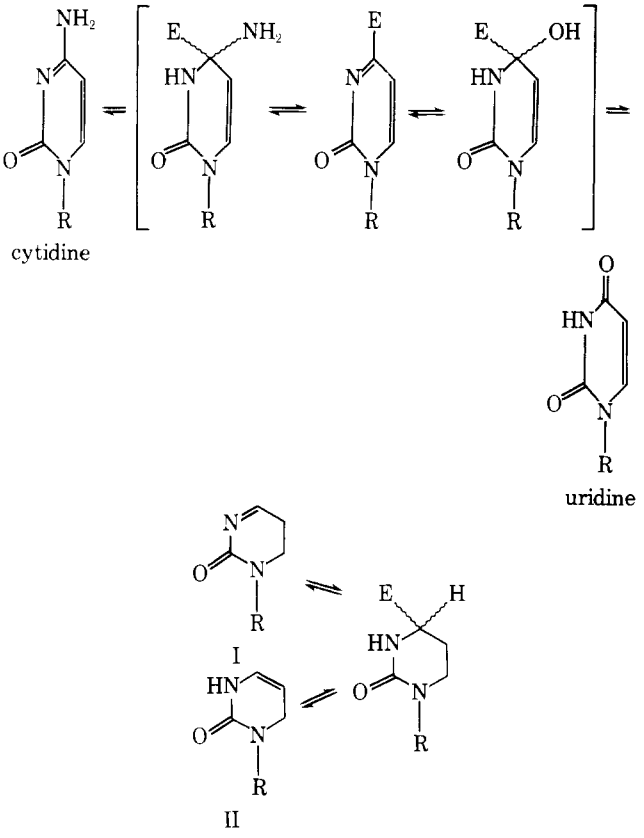
[1] Abbreviations used are: THU, 3,4,5,6-tetrahydrouridine; cyclocytidine, 2,2′-anhydro-1-β-D-arabinofuranosylcytosine.

Scheme II



enzyme from human liver (Camiener, 1968) revealed a more complex pattern of inhibition, and there were indications that the course of inhibition might be time dependent.

In view of these findings, it was of interest to determine whether inhibition of the enzyme from human liver by THU is reversible and competitive as expected for a transition state analog, and whether it might differ in some fundamental respect from the inhibition of the bacterial enzyme. If the onset of inhibition were time dependent, its characteristics might provide further information about interactions in the enzyme–inhibitor complex.

Experimental Section

Cytidine, uridine, 5-azacytidine, and 6-azauridine were obtained from Sigma Chemical Co.; 6-azacytidine was obtained from Calbiochem Corp.; cyclocytidine was obtained from Aldrich Chemical Co., Inc.; and deuterium oxide (99.8%) was purchased from Stohler Isotope Chemicals Co. THU was synthesized by the method of Hanze (1967); the proton magnetic resonance spectrum of the product was identical with the published spectrum.

The deamination of cytidine was followed by measuring the decrease in $A_{290\ nm}$ with a Perkin-Elmer recording spectrophotometer equipped with a recorder giving full scale deflection for an absorbance change of 0.10, and with a thermally regulated cell block. Assays were conducted at 25° in solutions which contained, unless otherwise specified, 2×10^{-4} *M* cytidine and Tris-HCl buffer (0.01 *M*, pH 8.0). One unit of activity was defined as that amount of enzyme required to deaminate 1 μmol of cytidine per min under these conditions. Cuvettes of 1-cm light path were used except in the case of experiments with 6-azacytidine, in which cuvettes of 0.5-cm light path were used.

Changes in extinction coefficient, corresponding to complete deamination at pH 8.0, were determined from the change in spectrum after prolonged incubation with enzyme, and also from the observed extinction of authentic samples of reactant and product in the case of cytidine and 6-azacytidine, and were as follows: cytidine, −2100 (290 nm); 6-azacytidine, −800 (305 nm); 5-azacytidine, −1300 (270 nm). The extinction change for 5-azacytidine could be determined only by the enzymatic method, since 5-azauridine appears to be of limited stability even under mild conditions, readily isomerizing to a cyclic species (Piskala and Sorm, 1964), and a pure sample of the authentic product, prepared by a nonenzymatic method, was accordingly not available. The action of human liver cytidine deaminase on all three substrates was inhibited effectively by low concentrations of THU.

Human liver cytidine deaminase was prepared from frozen tissue which had been removed at autopsy from a normal juvenile female. A chilled suspension of tissue (100 g) in Tris-HCl buffer (300 ml, 0.013 *M*, pH 8.0) was homogenized for 2 min in a Waring Blendor. The homogenate was rapidly frozen and thawed, and then cleared of cell debris by centrifugation at 0° for 20 min at 20000 *g* in a Sorvall centrifuge. In view of the observed heat stability of this enzyme (Camiener, 1967), the supernatant was incubated in 10-ml portions at 75° for 10 min, rapidly cooled, and then cleared by centrifugation at 0° for 60 min at 20000*g*. This heat treatment procedure was repeated once. The supernatant was then adjusted to 40% saturation by addition of solid ammonium sulfate (24 g/100 ml of supernatant) with stirring at room temperature, and stirring was continued for 30 min. After removal of the precipitate, the active supernatant was adjusted to 70% saturation by further addition of ammonium sulfate (22.9 g/100 ml of supernatant). The resulting precipitate, recovered by centrifugation, was dissolved in 5 ml of Tris-HCl buffer (0.01 *M*, pH 8.0) and dialyzed three times against 250 ml of the same buffer. This procedure was found to result in a 21-fold increase in the specific activity of the enzyme as compared with the crude extract so that the stock enzyme solution contained 0.2 unit of activity and 8 mg of protein/ml.

Cytidine deaminase was also prepared from HeLa cells, kindly provided by Dr. Hans Zweerink. Wet cells (8 g) were suspended in 24 ml of Tris-HCl buffer (0.01 *M*, pH 8.0). After 20 strokes with a chilled Potter-Elvehjem homogenizer, cell debris was removed by centrifugation at 10000*g* at 0° for 10 min, followed by centrifugation at 90000*g* at 0° for 4.5 hr in a Spinco preparative ultracentrifuge. The supernatant in 3-ml portions was heated at 70° for 5 min, and the denatured protein was removed by centrifugation. The heat step was found to result in a sixfold increase in specific activity as compared with the crude extract. After fivefold reduction in volume, the enzyme solution contained 0.07 unit of activity and 4 mg of protein/ml.

Bacterial cytidine deaminase was prepared from *E. coli* as described previously (Cohen and Wolfenden, 1971).

The rate of change of activity of the enzyme, during the onset or release of inhibition, was determined by published procedures (Fridovich, 1968; Wentworth and Wolfenden, 1974). Figure 1 shows the increase in the rate of enzymatic deamination of cytidine which occurred after the enzyme was equilibrated with THU and the mixture was then diluted into the assay solution. The final steady-state rate of cytidine deamination was extrapolated to zero time (broken line), and the solid line traces the appearance of enzyme activity. If the enzyme is converted from an inhibited to an active state, then the vertical distance between the broken line

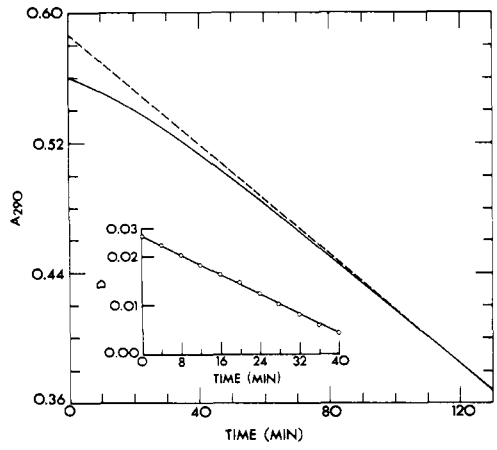


FIGURE 1: Slow release from THU inhibition. Enzyme from human liver, 0.01 unit, was preincubated with 4×10^{-7} M THU in 0.01 M Tris-HCl (pH 8.0), 100 μl total volume, for at least 60 min, and 10 μl of this mixture was then diluted 100-fold into assay solution containing 2×10^{-4} M cytidine and 0.01 M Tris-HCl (pH 8.0). The lag phase is emphasized here. The dashed line is an extrapolation of the final linear rate of cytidine deamination, which was found to be linear beyond 180 min. The vertical distance (D) between the solid line (the course of enzyme activation) and the dashed line is proportional to the amount of enzyme remaining in the inhibited state. k^{off} was obtained from a semilogarithmic plot (insert) of D vs. time.

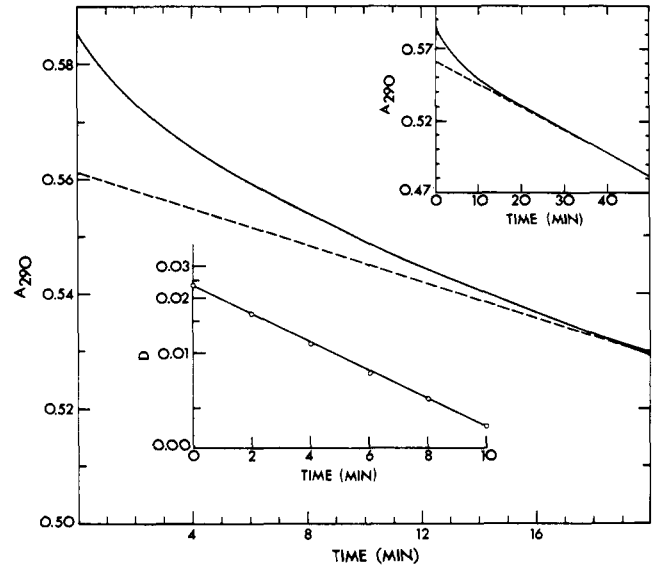


FIGURE 2: Slow onset of THU inhibition. Enzyme from human liver, 0.002 unit, was added at zero time to 1 ml of assay solution containing 2.4×10^{-6} M THU, 2×10^{-4} M cytidine, and 0.01 M Tris-HCl (pH 8.0). The burst phase is emphasized here. The dashed line is an extrapolation of the steady-state rate of cytidine deamination finally achieved, which was found to be linear beyond 50 min. The vertical distance (D) between the solid line (course of enzyme inhibition) and the dashed line is proportional to the difference in activity remaining at time t and the amount of activity remaining after the steady state was attained. k^{app}, the rate constant for the approach to the steady state, was obtained from a semilogarithmic plot of D vs. time (insert), at the corresponding THU concentration.

and the solid line is proportional to the amount of inhibited enzyme remaining. If this occurs with first-order kinetics, a plot of log vertical distance as a function of time will be linear (inset to Figure 1), and the apparent first-order rate constant can be obtained from the observed half-time. A similar procedure was used to determine the pseudo-first-order rate constant for combination of the enzyme with inhibitor (Figure 2), and the second-order rate constant for combination of enzyme with inhibitor was determined from the dependence of these values on the concentration of inhibitor present, after appropriate correction for substrate competition (see Results).

Protein concentrations were determined by the method of Lowry et al. (1951). Measurements of pH were made with the glass electrode of a Corning Model 7 pH meter, and pD values were assigned by adding 0.40 to the pH meter reading in D_2O solutions (Glasoe and Long, 1960).

Results

When THU was preincubated with human liver cytidine deaminase and the mixture was diluted into an assay solution containing substrate, the deamination of cytidine was found to approach a constant rate after a lag period. Typical behavior is shown in Figure 1, in which a sample of the preincubation mixture containing 4×10^{-7} M THU (approximately 10 K_i) was diluted 100-fold into an assay solution containing 2×10^{-4} M cytidine (approximately 20 K_m), so that the eventual breakdown of the enzyme–inhibitor complex was virtually complete. The decay of inhibition appeared to be a first-order process (inset to Figure 1), with an apparent rate constant (k^{off}) of 5.6×10^{-4} sec^{-1}. The onset of inhibition was observed by a similar procedure. When enzyme was added to an assay solution containing THU, the approach to a steady rate of cytidine deamination was preceded by a burst of activity as shown in Figure 2. Semilogarithmic plots of the approach to constant activity were linear (inset to Figure 2), and yielded apparent first-order rate constants which varied with the concentration of THU present in the assay mixture (Figure 3).

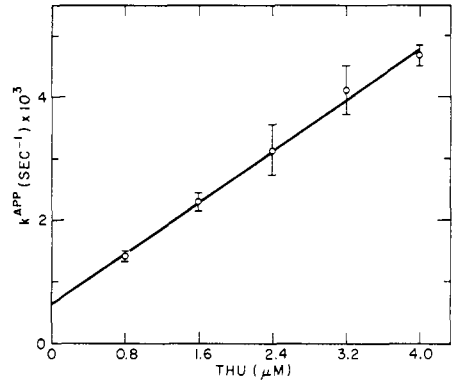


FIGURE 3: Determination of k^{on}, the THU on-rate constant. The data shown were obtained with the human liver enzyme; the points represent the average of four determinations, and the error bars are standard deviations. k^{app}, the pseudo-first-order rate constant for the approach to the steady state, is plotted as a function of THU concentration. The slope and intercept were obtained by a least-squares fit of the data to eq 2. The apparent second-order rate constant for THU binding, obtained from the slope of this plot, was corrected for substrate competition by multiplying by the factor $(1 + S/K_m)$.

Since the binding of THU is reversible (eq 1), the pseudo-first-order rate constant k^{app} for approach to equilibrium of the inhibited and uninhibited enzyme includes terms (eq 2) for binding and release of the inhibitor (Gutfreund, 1955). According to eq 2, k^{off} should be equivalent to the intercept of Figure 3, which was found to be 6.3×10^{-4} sec^{-1}. This value was in reasonable agreement with the value, 5.6×10^{-4} sec^{-1}, obtained from the rate of decay of inhibition as indicated by lag curves such as Figure 1.

$$E + I \underset{k^{off}}{\overset{k^{on}}{\rightleftharpoons}} EI \qquad (1)$$

$$k^{app} = k^{on}(I) + k^{off} \quad (2)$$

The apparent second-order rate constant for binding of THU, obtained from the slope in Figure 3, required correction for competitive binding of substrate, which was present at a level of approximately 20 K_m. Assuming that the equilibrium of substrate binding was established rapidly in comparison with the binding of THU, the apparent second-order rate constant was multiplied by the factor $(1 + S/K_m)$ in order to obtain a corrected second-order rate constant, k^{on}, for binding of THU in the absence of substrate. Accordingly, a value of $2.4 \times 10^4\ M^{-1}\ sec^{-1}$ was calculated for k^{on}.

K_i for THU was determined from linear rates of deamination which were observed in the presence of THU ($1 \times 10^{-6}\ M$) after the transient burst phase. The final steady-state rates indicated competitive inhibition with $K_i = 2.9 \times 10^{-8}\ M$. This value was in reasonable agreement with the value ($2.3 \times 10^{-8}\ M$) calculated from the ratio of rate constants (k^{off}/k^{on}).

Following the report of Meyers et al. (1973), we found that HeLa cells contained substantial quantitites of cytidine deaminase activity. Employing methods and conditions similar to those used with the human liver enzyme, inhibition by THU was found to be time dependent for the enzyme from HeLa cells, with $k^{off} = 1.1 \times 10^{-3}\ sec^{-1}$ and $k^{on} = 2.3 \times 10^4\ M^{-1}\ sec^{-1}$. A competitive pattern of inhibition was observed in double reciprocal plots of final steady-state rates in the presence and absence of THU, which yielded $K_i = 4.0 \times 10^{-8}\ M$, in good agreement with the ratio of rate constants for binding and release.

A possible basis for the observed lag in inhibition by THU (burst in deaminase activity) might be that THU is converted to a more potent inhibitor by an extraneous enzyme present in the cytidine deaminase preparations. This possibility was tested as follows. Human liver enzyme, 0.002 unit/ml, was incubated with $2.2 \times 10^{-6}\ M$ THU in 0.01 M Tris-HCl (pH 8.0) for 60 min, then heated at 95° for 30 min, after which denatured protein was removed by centrifugation. To 0.9 ml of this solution, now devoid of cytidine deaminase activity, was added 0.1 ml of cytidine (final concentration $2 \times 10^{-4}\ M$) and 10 μl of cytidine deaminase, and the rate of onset of THU inhibition was found to be 78% of that observed with THU which had not been preincubated with enzyme but under otherwise identical conditions. The small decrease in the on-rate of THU may be accounted for by the degradation of THU by heat, since in a control experiment a solution of $2.2 \times 10^{-6}\ M$ THU in 0.01 M Tris-HCl (pH 8.0) containing no enzyme was held at 95° for 30 min, and the on-rate observed with this THU sample was 109% of that obtained with the above preincubated sample. If a potent inhibitor were generated from THU during this preincubation period, one would have expected to observe little or no lag in the onset of inhibition but no increase in the on-rate was observed. This result, as well as the good agreement between the K_i value obtained from the ratio (k^{off}/k^{on}) and the K_i value obtained from conventional double reciprocal plots, tend to rule out the possibility that the transient kinetics observed with THU arise from the interaction of THU with enzymes other than cytidine deaminase.

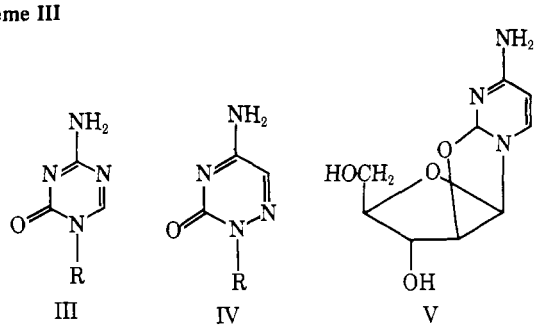
It seemed possible that the low second-order rate constants observed for combination of THU with these enzymes might be due to the obligatory participation of a rare species of THU (perhaps an anhydrous form). If inhibition were due to a rare species such as I or II which combined with cytidine deaminases from different species at similar rates approaching the diffusion control limit, then the expected rates of THU release might be predictable on the basis of the relative K_i values for cytidine deaminases from different species, and it was therefore of interest to re-examine the behavior of the enzyme from *E. coli*. K_i for THU inhibition of this enzyme was found to be $2 \times 10^{-7}\ M$ (Cohen and Wolfenden, 1971), and on the basis of the present kinetic constants observed for the enzyme from human liver, one would predict that k^{off} for the bacterial enzyme might be roughly $3.9 \times 10^{-3}\ sec^{-1}$, corresponding to a half-time of 3 min. To test this argument, enzyme from *E. coli* was preincubated with $2 \times 10^{-3}\ M$ THU (approximately $10K_i$), and the mixture was then diluted 100-fold into an assay solution containing $1 \times 10^{-3}\ M$ cytidine (approximately $5K_m$), so that the eventual release of THU was practically complete. It was estimated that a lag phase with a half-time of 0.3 min could be detected without difficulty in the experimental system. No lag phase was in fact detected, confirming previous results (Cohen and Wolfenden, 1971). It was concluded that the slow binding of THU by the enzymes from human liver and HeLa cells was unlikely to be simply due to the obligatory participation of a rare species of the inhibitor, since this effect would be expected to be independent of the source of the enzyme.

In order to examine the effect of substitution of deuterium oxide for solvent water on the rates of binding and release of THU by human liver cytidine deaminase, the kinetics of cytidine deamination in this solvent were first examined. The substitution of D_2O (final concentration 98%) for solvent water resulted in no appreciable effect on the initial rate of deamination of $10^{-4}\ M$ cytidine ($k^{H_2O}/k^{D_2O} = 0.98$) in 0.01 M Tris-HCl buffer (pH = pD = 8.0) at 25°, and similar observations were made for $10^{-3}\ M$ 6-azacytidine ($k^{H_2O}/k^{D_2O} = 1.03$). Like the enzyme from *E. coli* (Cohen and Wolfenden, 1971), the enzyme from human liver was kinetically insensitive to changing pH in this range, exhibiting rates of deamination of $10^{-4}\ M$ cytidine which were identical within experimental error in 0.01 M potassium acetate buffer (pH 5.0); 0.01 M Tris-HCl buffer (pH 8.0); and 0.005 M glycine-KOH buffer (pH 10.0).

To test for an isotope effect on the rate of inhibitor binding, it was first established that the K_m for cytidine did not change significantly in D_2O. The rate constant for approach to the steady state was then determined in H_2O and in D_2O (Table I) and no appreciable isotope effect was found ($k^{H_2O}/k^{D_2O} = 1.03$). Similarly negative results were obtained when k^{off} was examined for a possible isotope effect, by incubating enzyme from human liver with $4 \times 10^{-6}\ M$ THU (approximately 10 K_i) for 1 hr in either H_2O or D_2O (pD 8.0), and then diluting aliquots 100-fold into assay solutions (pH or pD = 8.0) containing $2 \times 10^{-4}\ M$ cytidine (approximately 20 K_m), so that virtually no enzyme-inhibitor complex remained after the steady state had been established (see above).

K_m and V_{max} values observed for cytidine, 5-azacytidine (III), and 6-azacytidine (IV) (Scheme III), determined for the enzyme from human liver under parallel conditions, are compared in Table II. The deamination of all three substrates was strongly inhibited by THU. It was found necessary to correct deamination rates observed with 5-azacytidine for a nonenzymatic reaction which took place in Tris-HCl buffer or triethanolamine hydrochloride buffer alone, and which was not further characterized. Without correc-

Scheme III



III IV V

tion, misleadingly high values were obtained for the K_m of this substrates. Such a correction was apparently not made in the observations of Chabner et al. (1973) on the enzyme from human granulocytes.

Cyclocytidine, or 2,2′-anhydro-1-β-D-arabinofuranosylcytosine (V), appears to be fixed in the "syn" configuration, whereas 6-azacytidine adopts the "high-anti" configuration in the solid state (Singh and Hodgson, 1974a,c). In view of the relatively high V_{max} and K_m values exhibited by 6-azacytidine, it was of interest to test cyclocytidine as a substrate. When cyclocytidine (10^{-4} M) was incubated with the usual amount of enzyme at pH 8.0 for 24 hr at 25°, no change in ultraviolet spectrum was observed. Cyclocytidine also showed no activity as an inhibitor, producing no detectable inhibition of the deamination of cytidine when present in assay mixtures at a concentration (10^{-4} M) equal to that of the substrate cytidine.

Discussion

The slow onset of inhibition by THU of human liver cytidine deaminase obscures, in initial rate studies, the very high affinity of this inhibitor for the enzyme, which exceeds its remarkable affinity for the enzyme from *E. coli*. Both enzymes appear to bind THU approximately four orders of magnitude more tightly than they bind uridine, the substrate for the reverse reaction, and the 5′-monophosphate of tetrahydrodeoxyuridine is bound with comparable avidity by deoxycytidylate deaminase (Maley and Maley, 1971). Numerically, THU can thus be considered a potential transition state analog, and the mechanism of action of all three of these enzymes is presumably similar.

A fundamental question which may be asked about the mechanism is whether cytidine deaminases catalyze direct attack by water on the substrate like adenosine deaminase (Evans and Wolfenden, 1973), or whether a covalent derivative of the enzyme is formed in a double displacement mechanism (see introduction). It appeared that the slow onset of inhibition of the enzyme from human liver might reflect a chemical process consistent with the latter possibility, as in the binding of D-galactal by bacterial β-galactosidase (Wentworth and Wolfenden, 1974).

Addition of a nucleophilic group of the enzyme, along with a solvent proton (or deuteron), across the double bond of an anhydrous species of THU, might have been expected to show an appreciable solvent deuterium isotope effect on the rate of onset of inhibition, if this were the rate-determining step. Alternatively, a nucleophilic group of the enzyme might simply replace the hydroxyl residue at the 4 position of THU. A solvent deuterium isotope effect might then have been anticipated if, in the transition state, a bond to hydrogen (or to exchangeable deuterium) were partly broken from the attacking nucleophile or formed to the departing hydroxyl group. The absence of a solvent deuterium isotope effect on the rate of onset of inhibition by THU appears to argue against these interpretations. These results do not, however, allow the unequivocal conclusion that THU is bound without chemical alteration. It is quite possible, for example, that an isotope effect on a partial reaction in THU binding may pass undetected because the overall rate is limited by a conformational change.

Table I: Effect of D_2O on k^{app}.[a]

k^{app} (sec^{-1})	
H_2O[b]	D_2O[c,d]
0.0028	0.0026
0.0026	0.0026
0.0031	0.0029
0.0029	0.0030

[a] Assay solution contained 2.4 μM THU and 0.2 mM cytidine. [b] pH 8.0. [c] pD 8.0. [d] Enzyme had been dialyzed against D_2O, pD 8.0.

Table II: Kinetic Parameters for Aza Analogs of Cytidine.

	K_m (M)	Rel V_{max}[a]
Cytidine	9.2×10^{-6}	1[b]
5-Azacytidine	5.8×10^{-5}	0.17[c]
6-Azacytidine	4.2×10^{-3}	6.4[d]

[a] Calculated from double reciprocal plot using $\Delta\epsilon$ for designated wavelength. [b] 290 nm. [c] 270 nm. [d] 305 nm.

It is difficult to avoid the conclusion, indeed, that a reorganization of the structure of the enzyme–inhibitor complex must be largely responsible for the slow binding of THU. The slow binding of inhibitors has now been observed for a number of enzymes, including aspartate aminotransferase (Czerlinski and Malkewitz, 1965; Hammes and Haslam, 1968, 1969), acetoacetate decarboxylase (Colman, 1962; Tagaki et al., 1968; Fridovich, 1968), enolase (Spring and Wold, 1971), ribulose diphosphate carboxylase (Siegel and Lane, 1972), β-galactosidase (Wentworth and Wolfenden, 1974), and lactate oxidase (Ghisla and Massey, 1975). In some cases (e.g., lactate oxidase), the kinetics of onset of inhibition indicate that initial complexes are formed rapidly, and that these undergo slow conversion to more stable complexes. In other cases, rare species of the enzyme or inhibitor may be required for successful encounter.

It is unclear whether the slow onset of THU inhibition of human liver cytidine deaminase is due to events occurring before or after encounter, but there does appear to be a substantial kinetic barrier to the achievement of structural complementarity. This may be related to other unusual properties of the enzyme, such as its kinetic insensitivity to the substitution of deuterium oxide for solvent water (Cohen and Wolfenden, 1971, and present results). Since water is a substrate, this suggests that, as in the case of adenosine deaminase (Evans and Wolfenden, 1973; Wolfenden, 1969), the rate of enzymatic hydrolysis of substrates may be limited by an isomerization process which does not involve bond making or breaking. Binding of an analog of an intermediate in substrate transformation might be subject to similar kinetic restraints.

6-Azacytidine serves as a fairly effective substrate for cytidine deaminase. Together with the observed activity of bacterial cytidine deaminase on 5,6-dihydrocytidine (Evans

et al., 1975), this suggests that covalent addition of the enzyme at the 6 position of cytidine is unlikely to be an essential element of the catalytic process. Singh and Hodgson (1974a,c) have shown that the glycosyl bond of this compound is in the "high-anti" configuration in the crystalline state, in contrast to the more conventional "anti" configuration of cytidine (Furberg et al., 1965) and 6-azacytidine also exhibits an unusual negative Cotton effect in solution (Rogers and Ulbricht, 1971). The high K_m and high V_{max} observed for 6-azacytidine (Table II) may be compared with the high values also observed for calf adenosine deaminase acting on 8-azaadenosine (Simon et al., 1970) and for human erythrocyte adenosine deaminase acting on formycin A (Agarwal et al., 1975). Like 6-azacytidine, 8-azaadenosine (Singh and Hodgson, 1974b) and formycin A (Prusiner et al., 1973) adopt the "high-anti" configuration in the crystalline state. Cyclocytidine, which is fixed instead in the "syn" configuration, does not serve as a substrate or an inhibitor for cytidine deaminase.

The action of cytidine deaminase on the antineoplastic agent cytosine arabinoside (Camiener and Smith, 1965) to yield an apparently inert product (Chu and Fischer, 1962; Wilkoff et al., 1972) presumably limits this agent's effectiveness in the treatment of human leukemias (Body et al., 1969). The observation that THU is a potent inhibitor of the enzyme from human liver and from HeLa cells, as well as the enzyme from human granulocytes as previously reported by Chabner et al. (1974), suggests the chemotherapeutic use of THU with cytosine arabinoside. 5-Azacytidine (Piskala and Sorm, 1964) is also an antineoplastic agent (Sorm and Vesely, 1964), and has shown promising results in the treatment of acute leukemia (McCredie et al., 1972). 5-Azacytidine is deaminated by the enzyme from human liver, as well as by the enzyme from human granulocytes (Chabner et al., 1973), and it has been suggested in several reports that the product of deamination of 5-azacytidine is an inactive metabolite. It should be noted, however, that Doskocil and Sorm (1970) found that significant inhibition of protein synthesis by 5-azacytidine in *E. coli* required the presence of cytidine deaminase, even though 5-azacytidine was incorporated into RNA, and that inhibition of protein synthesis occurred when 5-azauridine was introduced into a strain deficient in cytidine deaminase.

In constructing models for the chemotherapy of leukemia, the kinetic parameters of 5-azacytidine may be useful, and it would be of obvious interest to know whether in mammals the deamination of 5-azacytidine is a mechanism of resistance or the first step in the process which results in the inhibition of cell proliferation. It has been suggested that therapy with cytosine arabinoside may result in elevated levels of cytidine deaminase (Stewart and Burke, 1971; Meyers et al., 1973). If the deamination of 5-azacytidine leads to an active antimetabolite, it might then be rational to attempt therapy with 5-azacytidine after resistance to cytosine arabinoside had developed.

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Human Brain and Placental Choline Acetyltransferase: Purification and Properties†

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ABSTRACT: Choline acetyltransferase (EC 2.3.1.6) catalyzes the biosynthesis of acetylcholine according to the following chemical equation: acetyl-CoA + choline ⇌ acetylcholine + CoA. In addition to nervous tissue, primate placenta is the only other animal source which contains appreciable acetylcholine and its biosynthetic enzyme. Human brain caudate nucleus and human placental choline acetyltransferase were purified to electrophoretic homogeneity using ion-exchange and blue dextran-Sepharose affinity chromatography. The molecular weights determined by Sephadex G-150 gel filtration and sodium dodecyl sulfate gel electrophoresis are 67000 ± 3000. *N*-Ethylmaleimide, *p*-chloromercuribenzoate, and dithiobis(2-nitrobenzoic acid) inhibit the enzyme. Dithiothreitol reverses the inhibition produced by the latter two reagents. The pK_a of the group associated with *N*-ethylmaleimide inhibition is 8.6 ± 0.3. A chemically competent acetyl-thioenzyme is isolable by Sephadex gel filtration. The enzymes from the brain and placenta are thus far physically and biochemically indistinguishable.

Acetylcholine is the neurotransmitter at the vertebrate neuromuscular junction and at specific synapses in the autonomic nervous system. It is also an alleged neurotransmitter in the vertebrate central nervous system. The primate placenta is the only firmly established nonneuronal source of acetylcholine in animals; its function in placenta is unknown (cf. Potter, 1970). Choline acetyltransferase (EC 2.3.1.6) catalyzes the bioformation of acetylcholine with the stoichiometry given in the following chemical equation: acetyl-CoA + choline ⇌ acetylcholine + CoA. Thiol reagents inhibit the transferase prepared from rat (Potter et al., 1968) and bovine brain (Choa and Wolfgram, 1973; Roskoski, 1974a), human placenta (Schuberth, 1966), torpedo (Morris, 1967), and squid head ganglia (Reisberg, 1954). Previous studies with the bovine brain enzyme support the notion that an active site -SH group mediates the transfer of the acetyl group from donor to acceptor substrate (Roskoski, 1974b). Currier and Mautner (1974) were unable to isolate the postulated acetyl-enzyme intermediate from a purified preparation from squid head ganglia. These results may be related to species differences.

Choa and Wolfgram (1973) and Singh and McGeer (1974) reported methods for purifying the bovine and human brain transferase, respectively, to electrophoretic homogeneity. Using modifications of these procedures, and blue dextran-Sepharose affinity chromatography (Ryan and Vestling, 1974; Thompson and coworkers, 1975), we have purified the human brain caudate nucleus and human placenta choline acetyltransferase to electrophoretic homogeneity. The variable yield, however, is only 1–10%; this seems to be related, inter alia, to enzyme instability in the latter stages of the purification.

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Experimental Section

Materials. Normal caudate nuclei were obtained postmortem. The placentas were obtained after normal term deliveries. The tissues were stored at −20° for periods up to 3 months without loss of enzyme activity.

Buffers, protein standards, Sepharose 4B, and blue dextran were purchased from Sigma Chemical Co. The blue dextran-Sepharose resin was prepared by the procedure of Ryan and Vestling (1974). C.I. (Color Index) Reactive blue 2 (Cibachron Blue 3-GA), the chromophore of blue dextran, was a generous gift of Ciba-Geigy (Basel, Switzerland). The sources of other materials and the methods for radioactivity determination and Sephadex G-50 gel filtration have been documented previously (Roskoski, 1973, 1974c).

Choline acetyltransferase activity was measured as previously described (Roskoski, 1973) except that 100 μM [^{14}C]acetyl-CoA (40 Ci/mol) and 7.5 mM choline (final concentrations) were used and incubations were 10 min at 30°.

Preparation of Human Placental Choline Acetyltransferase. Frozen placenta (500 g) was cut into 3-cm cubes. All subsequent steps were carried out at 0–4°. The placenta was disrupted batchwise in 5 volumes of buffer A (5 mM potassium phosphate–0.1 mM EDTA (pH 7.4)) in a blender (2 min, high speed). The pH was adjusted to 7.4, if necessary, with 1 M K_2HPO_4 prior to centrifugation at 11000g