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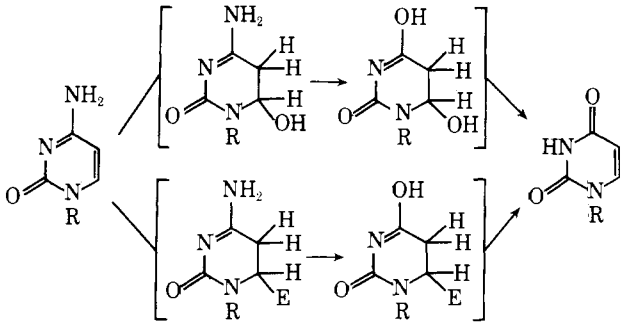
The Action of Bacterial Cytidine Deaminase on 5,6-Dihydrocytidine†

Ben E. Evans, Gordon N. Mitchell, and Richard Wolfenden*

ABSTRACT: Cytidine deaminase from *Escherichia coli* was found to catalyze the hydrolytic deamination of 5,6-dihydrocytidine, at a rate slightly lower than its rate of action on the normal substrate. The results suggest that nucleophilic addition by the enzyme at the 5,6 position of the substrate is not an essential part of catalysis, unless the active site is so flexible that deamination can occur with addition in one case (cytidine) and without addition in another case (5,6-dihydrocytidine). 3,4,5,6-Tetrahydrouridine bears a close structural resemblance to a hypothetical "tetrahedral" intermediate formed by direct water addition to 5,6-dihydrocytidine. The hydrolytic activity of the enzyme toward 5,6-dihydrocytidine and its potent inhibition by 3,4,5,6-tetrahydrouridine are presumably related by the ability of the active site to stabilize structures of this kind by tight binding. Cytidine deaminase shows no detectable activity as a catalyst for the dehydration of 6-hydroxy-5,6-dihydrouridine.

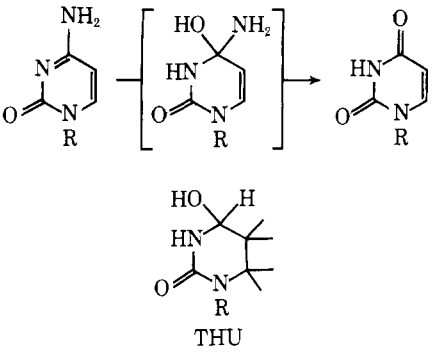
Recent studies have shown that a variety of nucleophiles catalyze the deamination of cytidine by adding to the 5,6-double bond (Shapiro and Klein, 1966, 1967; Notari, 1967; Wechter, 1970; Wechter and Kelly, 1970). These models suggest that the action of cytidine deaminase (cytidine aminohydrolase, EC 2.5.4.5) might similarly involve addition of the enzyme (or of enzyme-bound water) at the 5,6-double bond of cytidine as part of the catalytic mechanism (Scheme I).

SCHEME I



An alternative mechanism would involve direct addition of water at the 3,4-double bond, followed by elimination of ammonia as in the enzymatic deamination of adenosine (Evans and Wolfenden, 1970, 1972, 1973). This might explain the potent inhibition of cytidine deaminase by 3,4,5,6-tetrahydrouridine (THU), which resembles a possible intermediate which might be formed by addition of water to cytidine (Scheme II) (Cohen and Wolfenden, 1971).

SCHEME II



A distinction between THU and the intermediate enclosed in brackets in Scheme II is in the saturation of the 5,6 bond. If this interpretation of the inhibition by THU is correct, THU might actually be considered an analog of an intermediate in the deamination of 5,6-dihydrocytidine rather than cytidine itself, and cytidine deaminase might catalyze deamination of 5,6-dihydrocytidine. If, on the other hand, the reaction proceeds through addition of enzyme or water at the 5,6 position (Scheme I), catalysis should be effectively *blocked* in the case of 5,6-dihydrocytidine. In this communication, we describe experiments undertaken in an attempt to decide between these alternatives.

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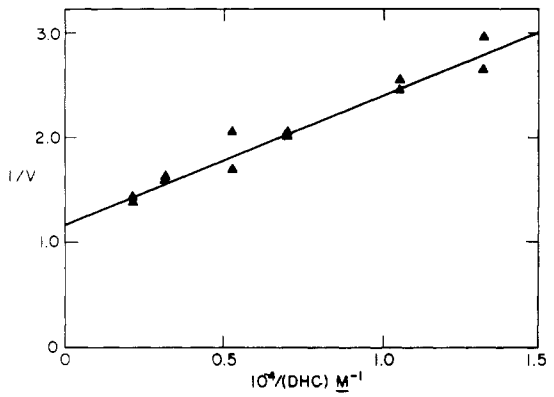

FIGURE 1: Double reciprocal plot of the initial rate of deamination of 5,6-dihydrocytidine catalyzed by cytidine deaminase in potassium phosphate buffer (0.2 M, pH 7.5) at 25°, as a function of substrate concentration. Reaction velocity is expressed as μmol min^{-1} mg of protein^{-1}.

Materials and Methods

5,6-Dihydrouridine was prepared by hydrogenation of uridine as described by Green and Cohen (1957). Uridine (4 g) was dissolved in water (100 ml) and shaken at ambient temperature in a Parr hydrogenation apparatus with 1.05 g of 5% Rhodium/alumina catalyst under 50 psig of hydrogen. Hydrogen uptake ceased abruptly after 8 min, with a total absorption of 1.0 molar equiv of hydrogen. Filtered free of catalyst, the mixture was shown by thin-layer chromatography (Brinkmann Silica Gel GF-254, 35% methanol in chloroform, visualization by background fluorescence shadow and by phosphomolybdic acid stain) to contain but one detectable component (R_F 0.4) and to be free of starting material (uridine R_F 0.75). The product, obtained by lyophilization, exhibited an ultraviolet absorption spectrum in agreement with that reported (Hanze, 1967) for 5,6-dihydrouridine.

5,6-Dihydrocytidine was prepared by a procedure similar to that previously applied to the synthesis of related compounds (Janion and Shugar, 1960; Green and Cohen, 1957). Cytidine (0.5 g) was shaken over a period of 3 days in a Parr apparatus at ambient temperature with 5% Rhodium/alumina catalyst (0.2 g) in anhydrous methanol (150 ml, dried with 3 Å molecular sieves) under 50 psig of hydrogen. Additional catalyst (0.07 g) was introduced after 12 hr, and again after 24 hr, when samples were removed for analysis by thin-layer chromatography (Brinkmann Silica Gel GF-254, 35% methanol in chloroform: cytidine R_F 0.15, dihydrocytidine R_F 0.10). At the end of the 3-day period, the level of unreacted cytidine was less than 1% of the level present at the outset, and the final product was closely comparable in ultraviolet spectrum with that reported for 5,6-dihydrocytidine 2′,3′-phosphate (Janion and Shugar, 1960), for which firm structural proof was not given. Identification of this material was confirmed by comparison of its ultraviolet spectrum (λ_{max} 242.5 nm and 212.5 nm (log ϵ 3.87, 3.92); λ_{min} 230 (log ϵ 3.83) in potassium phosphate buffer (0.2 M, pH 7.5)) and proton magnetic resonance spectrum (characteristic peaks at δ 5.8 and 2.8 (t) in deuterium oxide) with those very recently published for 5,6-dihydrocytidine (Skaric *et al.*, 1974); and by its enzymatic and nonenzymatic hydrolysis to 5,6-dihydrouridine (see Results).

6-Hydroxy-5,6-dihydrouridine was prepared by irradiation of a solution of uridine (5×10^{-3} M) in water (Wang, 1962) in a rotating quartz tube, cooled to 4° by a continuous stream of ice-water, in the presence of a semicircular array of germicidal lamps (Sylvania G15T8) over a period of several hours. The hydration reaction under these conditions proceeded to approximately 90% completion, as determined from the disappearance of ultraviolet absorbance at 262 nm; and the product mixture, concentrated by evaporation and dissolved in D_2O, was virtually identical in nmr spectrum with that characterized by Wechter and Smith (1968). The aqueous solution resulting from irradiation was assayed without purification (see Results) for susceptibility to enzyme attack.

Cytidine deaminase was obtained from *Escherichia coli* as described by Cohen and Wolfenden (1971), and its activity on cytidine was measured spectrophotometrically at 25° according to the procedure reported earlier, at 282 nm. Deamination of 5,6-dihydrocytidine was followed, both nonenzymatically and enzymatically, by the decrease in absorption at 247.5 nm, where the observed extinction coefficients for dihydrocytidine and dihydrouridine were 7100 and 100, respectively, in reasonable agreement with values reported in the literature for 5,6-dihydrocytidine 2′,3′-phosphate and 1-methyl-5,6-dihydrouracil, respectively (Janion and Shugar, 1960), in neutral solution. Kinetic measurements were routinely carried out in potassium phosphate buffer (0.2 M, pH 7.5), at 25°.

Results

Nonenzymatic Deamination of 5,6-Dihydrocytidine. When allowed to stand in potassium phosphate buffer (0.2 M, pH 7.5) for 3 days, a sample of dihydrocytidine (0.05 M) was transformed completely to dihydrouridine as indicated by thin-layer chromatography and by comparison of ultraviolet spectra with that of an authentic sample. When the course of this reaction was followed as a function of time (see Materials and Methods) at lower concentrations convenient for spectrophotometric observation, product formation was observed to occur with satisfactory first-order kinetics, exhibiting a half-time of approximately 105 min at 25°. The corresponding first-order rate constant, 1.1×10^{-4} sec^{-1}, may be compared with a value, 4×10^{-5} sec^{-1}, very recently reported for the rate of deamination of 1-methyl-5,6-dihydrocytosine in dilute Tris buffer at 30° (Shapiro *et al.*, 1974). Skaric *et al.* (1974) report 66% hydrolysis of 5,6-dihydrocytidine after 8 days in water at room temperature.

Enzymatic Deamination of 5,6-Dihydrocytidine. In the presence of bacterial cytidine deaminase, 5,6-dihydrocytidine was very much more rapidly deaminated than in the absence of this enzyme, and the product, as before, was characterized as 5,6-dihydrouridine by thin-layer chromatography and comparison of ultraviolet spectra. For kinetic measurements, a stock solution of 5,6-dihydrocytidine (2.2×10^{-3} M) was prepared in anhydrous methanol. Measured samples were diluted in potassium phosphate buffer (0.2 M, pH 7.5, total volume 3.0 ml), and the rate of nonenzymatic deamination was determined from the rate of decrease of ultraviolet absorbance at 247.5 nm. A similar sample was incubated with added enzyme, and the difference between the two rates observed was taken as the rate of the enzymatic process alone. In no case was more than 5% of the initial substrate consumed before the completion of the run. The observed rates, obtained in duplicate over a range of substrate concentrations from 3.8×10^{-5} to 2.4×10^{-4} M, are shown as a double reciprocal plot in Figure 1. Similar

Table I: Kinetic Parameters for Substrates and Inhibitors of Cytidine Deaminase, in Potassium Phosphate Buffer (0.2 M, pH 7.5) at 25°.

	Cytidine	5,6-Dihydrocytidine	Uridine	5,6-Dihydrouridine	Tetrahydrouridine
K_m(M)	1.29×10^{-4}	1.13×10^{-4}			
K_i(M)[a]			2.5×10^{-3}	3.4×10^{-3}	2.4×10^{-7}
V_{max} (μmol per min per mg of protein	9.0	1.1			
Wavelength (nm)	282	247.5			
$\Delta\epsilon$	−3500	−7000			

[a] Values from Cohen and Wolfenden (1971).

data were obtained for deamination of the normal substrate, cytidine. Kinetic parameters calculated from these data are shown in Table I.

Like the deamination of cytidine, the deamination of 5,6-dihydrocytidine by bacterial cytidine deaminase was found to be very strongly inhibited by 3,4,5,6-tetrahydrouridine. This inhibitor, which exhibits $K_i = 2.4 \times 10^{-7}$ M for cytidine under similar conditions (Cohen and Wolfenden, 1971), virtually eliminated any observable enzymatic contribution to the rate of deamination of 5,6-dihydrocytidine, when the inhibitor was present at a concentration comparable to that of the substrate.

Failure of the Enzyme to Catalyze Dehydration of Uridine Photohydrate. When uridine was converted photochemically to a steady-state mixture consisting mainly of 6-hydroxy-5,6-dihydrouridine (see Materials and Methods), the resulting mixture was found to revert slowly to uridine, with approximately 19% reversion after 4 days in Tris-HCl buffer (pH 7.5, 0.05 M) at 25°. Gattner and Fahr (1964) have reported half-times for spontaneous dehydration at elevated temperature as follows: 400 hr at 50° in water (unbuffered); 9 hr at 50° in phosphate buffer (pH 7.0, 0.15 M).

In the presence of cytidine deaminase (0.05 unit/ml) the rate of spontaneous dehydration in Tris-HCl buffer (pH 7.5, 0.05 M) at 25° was not detectably enhanced above that in the absence of enzyme. In these experiments the reversion of the photohydrate (5×10^{-4} M) to uridine was followed by the appearance of ultraviolet absorption at 262 nm.

Discussion

The kinetic results presented in Table I show that cytidine and 5,6-dihydrocytidine are similarly effective as substrates for cytidine deaminase from *E. coli*. The enzyme addition mechanism shown in Scheme I cannot function with 5,6-dihydrocytidine as substrate, and therefore appears to be unlikely for cytidine, the natural substrate.

The present findings also appear to render improbable the alternative mechanism in Scheme I, involving formation of a 5,6-hydrate intermediate. Catalysis would presumably be required for hydrate formation, and the failure of the enzyme to affect the rate of dehydration of uridine hydrate suggests that the enzyme is incapable of providing catalysis for this kind of reaction, which proceeds spontaneously at a very slow rate.

The observed activity of the enzyme on 5,6-dihydrocytidine appears to be consistent with the mechanism of Scheme II, involving addition of water to form a tetrahedral intermediate from which ammonia is subsequently eliminated. This mechanism was proposed earlier as an explanation of the unusual effectiveness of 3,4,5,6-tetrahydrouridine as an inhibitor, in view of the inhibitor's resemblance to the proposed intermediate (Cohen and Wolfenden, 1971). In comparing structures, it seems clear that the saturation of the 5,6 bond is *not* the principal source of inhibitory potency, since conversion of either cytidine or uridine to the 5,6-dihydro form (Table I) has very little effect on the observed Michaelis constant for cytidine or the observed K_i for uridine.

The spontaneous rate of hydrolysis of cytidine in neutral solution at room temperature is not accurately known, but almost certainly corresponds to a half-time of some years (Sanchez and Orgel, 1970), vastly longer than the half-time for hydrolysis of 5,6-dihydrocytidine, approximately 105 min under the conditions used in the present experiments. It therefore appears somewhat surprising that the rates of enzymatic deamination are so similar for the two compounds. It seems most reasonable to suppose that the rates of the enzyme reactions are limited, at least in part, by a step or steps which do not involve chemical transformation of the substrate. This would be consistent with the absence of a deuterium solvent isotope effect on the rate of enzymatic hydrolysis of cytidine, as previously reported (Cohen and Wolfenden, 1971). In this respect, as well as in its general mechanism of action, cytidine deaminase appears to resemble adenosine deaminase (Evans and Wolfenden, 1973).

The present experiments, showing that cytidine deaminase catalyzes the deamination of 5,6-dihydrocytidine, were suggested by the original observation that 3,4,5,6-tetrahydrouridine was a potent inhibitor of deaminase action on the normal physiological substrate cytidine. This newly demonstrated activity, against a nonphysiological substrate, is presumably the reaction for which 3,4,5,6-tetrahydrouridine is most closely analogous in structure to an activated intermediate. According to this hypothesis 3,4-dihydrouridine (an analog of the corresponding intermediate in hydrolysis of cytidine, for which the enzyme brings about a larger rate enhancement than for 5,6-dihydrocytidine) might be expected to be an even more effective inhibitor than 3,4,5,6-tetrahydrouridine. If this prediction proves correct, it may provide a basis for enhancing the clinical effectiveness of aracytidine by protecting this antileukemic agent from deaminase attack.

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Resonance Raman Investigation of an Enzyme–Inhibitor Complex†

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ABSTRACT: The resonance Raman spectrum has been recorded for two different binary complexes formed between 2-carboxy-2′-hydroxy-5′-sulfoformazylbenzene (zincon) and liver alcohol dehydrogenase. The shifts in the zincon spectrum upon complexation with enzyme in one complex are similar to those in model compounds containing azo or formazyl linkages upon complexation of these with zinc. The results are interpreted in terms of complexation of zincon to a zinc atom at the enzyme active site. Since zincon is a coenzyme competitive inhibitor, it is probably bound at or near the coenzyme binding site; the results of this study, therefore, are useful in understanding the chemistry of zinc at the enzyme active site.

The method of resonance Raman spectroscopy has recently received attention as a means of obtaining the vibrational spectra of biochemical systems with the attendent wealth of bonding and structural information inherent to vibrational spectroscopy. The potential of the resonance Raman method for answering important questions concerning chemical bonding in a molecule derives from the fact that resonance Raman spectra can be obtained in solution at concentrations of 10^{-5} M or less for samples as small as 50–100 μl and that water, as solvent, interferes minimally in the spectrum. The resonance Raman spectrum results from enhancement of Raman band intensities, often by several orders of magnitude, due to coupling of Raman active vibrations with electronic transitions of the molecule being studied. Such a spectrum can thus be obtained when the Raman excitation frequency is near an electronic absorption band for the molecule. Several macromolecules which contain chromophores in the visible region have already been studied by the resonance Raman method (Dunn *et al.,* 1973; Salmeen *et al.,* 1973; Spiro and Strekas, 1974). Noncovalent interactions of the chromophore Methyl Orange with bovine serum albumin and of a hapten–antibody complex have also been reported (Carey *et al.,* 1972; Carey *et al.,* 1973). However, there has been no report to date of the use of this technique to study the interaction between an enzyme and a chromophore known to bind reversibly at the active site of the enzyme.[1] The bound chromophore might serve as an excellent probe of the active site *via* changes in its Raman spectrum upon complexation, thus providing specific information about geometry and binding sites of the enzyme. For this study, we have chosen as our resonance Raman probe the molecule, 2-carboxy-2′-hydroxy-5′-sulfoformazylbenzene (zincon) (Figure 1a). This dye molecule is known to form a 1:1 complex with zinc (Rush and Yoe, 1954) by coordination through the azo bond and N-4 of the formazyl system and through carboxylate and phenol oxygens (Figure 1b). We report here a resonance Raman study of the binding of zincon to liver alcohol dehydrogenase.

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[1] Since submission of this manuscript, it has been brought to our attention that recent Raman experiments have been performed on acylchymotrypsin (Carey and Schneider, 1974), an irreversibly bound chromophore.