Cytidine Deaminase from *Escherichia coli* B. Purification and Enzymatic and Molecular Properties†

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ABSTRACT: Cytidine deaminase (cytidine aminohydrolase, EC 3.5.4.5) from *Escherichia coli* has been purified to homogeneity through a rapid and efficient two-step procedure consisting of anion-exchange chromatography followed by preparative electrophoresis. The final preparation is homogeneous, as judged by a single band obtained by disc gel electrophoresis performed in the absence and presence of denaturing agents. The native protein molecular weight determined by gel filtration is 56000. Sodium dodecyl sulfate disc gel electrophoresis experiments conducted upon previous incubation of the enzyme with dimethyl suberimidate suggest an oligomeric structure of two identical subunits of 33000 molecular weight. The absorption spectrum of the protein reveals a maximum at 277 nm and a minimum at 255 nm. The isoelectric point is at pH 4.35. Amino acid analysis indicates an excess of acidic amino acid residues as well as six half-cystine residues. No interchain disulfide groups have been evidenced. According to Cleland's nomenclature, kinetic analysis shows a rapid-equilibrium random Uni-Bi mechanism. Cytidine deaminase is competitively inhibited by various nucleosides. K_m values for cytidine, deoxycytidine, and 5-methylcytidine are 1.8×10^{-4}, 0.9×10^{-4}, and 12.5×10^{-4} M, respectively.

Pyrimidine nucleosides are readily taken up and metabolized by *Escherichia coli* cells. Their utilization requires the concerted action of pyrimidine metabolizing enzymes (Munch-Petersen et al., 1979). The main feature in the catabolism of nucleosides is the phosphorolytic cleavage of the N-glycosidic bond between the base and the pentose moieties of the nucleosides. In the case of cytidine and deoxycytidine, deamination must occur before phosphorolytic cleavage takes place. The deamination is catalyzed by cytidine deaminase which has been detected and purified from a variety of microbial and animal cells (Ipata et al., 1970; Wisdom & Orsi, 1969; Tomchick et al., 1968; Wang et al., 1950; Trimble & Moley, 1971; Hosono & Kuno, 1973), but never to homogeneity.

In *Escherichia coli*, the enzyme is induced if cytidine, adenosine, or CMP is added to growth media (Vita et al., 1983). In the case of cytidine, the enzyme specific activity increases up to 8 times its original value. Addition of glucose to *Escherichia coli* growing cells in the presence of cytidine causes a drop of cytidine deaminase specific activity, which after 2 h approaches the activity value obtained by growing cells on inorganic salts–glucose media (Vita et al., 1983). In order to assess whether this phenomenon is due to an inactivation mechanism, i.e., an alteration of the molecular structure of the protein, or to cessation of enzyme synthesis, we have developed a rapid and efficient purification procedure, allowing characterization of cytidine deaminase obtained from cells grown in the above-described conditions. A preliminary note, describing the purification procedure and the major properties of the enzyme, has been recently reported (Vita et al., 1984).

Experimental Procedures

Materials. Diaflo membranes were purchased from Amicon Corp.; DE-52 cellulose was Whatman; bases, nucleosides, nucleotides, tris(hydroxymethyl)aminomethane (Tris)[1] (Trizma base), 2-mercaptoethanol, sodium dodecyl sulfate, protein markers, and other fine chemicals were from Sigma Chemical Co. Other chemicals, reagent grade, were obtained from J. T. Baker Chemicals B.V. Ampholine carrier ampholytes were from LKB. Solutions of amino acid standards (2.5 μmol/mL), constant-boiling HCl (6 N), fluoraldehyde OPA reagent solution, and sodium acetate (pH 9, buffer grade) were obtained from Pierce.

Cell Growth and Preparation of Crude Extract. Cells of *Escherichia coli* were grown aerobically at 37 °C, up to 0.9 ODU at 660 nm, in 1.5 L of inorganic salts medium (19 mM glutamate, 1.7 mM $MgSO_4$, 14.3 mM K_2SO_4, 43 mM NaCl, and 100 mM phosphate buffer, pH 7.0) in the presence of 11 mM glucose. In preparations of induced cytidine deaminase, glucose was replaced by 5 mM cytidine. the cells were harvested by centrifugation, washed twice with 100 mL of 20 mM Tris-HCl, pH 7.5, resuspended with 25 mL of the same buffer containing 5 mM 2-mercaptoethanol (buffer A), and disrupted by passage through an Aminco French pressure cell at 20000 lb/in^2. The homogenate was centrifuged at 40000*g* for 30 min, and the supernatant was dialyzed overnight against buffer A and taken as the crude extract. All operations subsequent to the harvesting procedure were performed at 0–4 °C.

Assay. Cytidine deaminase activity was determined spectrophotometrically as described by Cohen & Wolfenden (1971). The method is based on the differential absorption existing between cytidine and uridine at 282 nm (ΔE_{mM} at 282 nm = 3.60). The reaction mixture consisted of 0.167 mM cytidine and 100 mM phosphate buffer, pH 7.5, in a final volume of 1.0 mL. The reaction was initiated by the addition of 0.02–0.04 enzyme unit, and the reaction rates was followed

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[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; ODS, octadecylsilyl (bonded phase); HPLC, high-performance liquid chromatography.

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by using a Varian Cary 118C spectrophotometer. One enzyme unit is defined as the amount of enzyme which catalyzes the deamination of 1 μmol of cytidine per minute at 30 °C. Assay conditions of inhibition experiments are reported in the figure legends.

Protein Determination. Protein was measured by the dye-binding procedure of Bradford (1976) for routine determinations and by the method of Schacterle & Pollack (1973) for more accurate determinations. In both methods, bovine serum albumin was used as the standard.

Electrophoresis. Discontinuous polyacrylamide gel electrophoresis was carried out as described by Ornstein (1964) and Davis (1964). $NaDodSO_4$–polyacrylamide gel electrophoresis was performed according to Laemmli (1970). Protein was stained with Coomassie brilliant blue R-250. To correlate the stained band with enzyme activity, triplicate nondenaturing gels (Ornstein, 1964; Davis, 1964) loaded with 2 units of cytidine deaminase activity were run simultaneously at 4 °C. One gel was fixed and stained for proteins. The second gel was sliced in 1-mm segments and placed in 200 μL of 100 mM phosphate buffer, pH 7.5, for 30 min at 4 °C. The enzyme activity was assayed on 50 μL of supernatant. The third gel was used to detect enzyme activity in situ following a modified procedure of Teng et al. (1975). Three milliliters of 2% ion–agar solution containing 15 μg of cytidine, 10 mg of dithiothreitol, and 3 mg of methylthiazolyltetrazolium was poured in a tube containing the gel and left at room temperature until a reddish purple band appeared on the agar. The staining reaction was stopped by addition of 5 mL of 3% acetic acid. Preparative polyacrylamide gel electrophoresis was carried out in a "Uniphor 7900" apparatus from LKB. The gels and the reservoir buffer were prepared according to Ornstein (1964) and Davis (1964). Polymerization of the polyacrylamide gel (10%) was carried out on a glass column (2.5 × 30 cm). The sample containing 5% glycerol and bromophenol blue was applied on top of the gel. Electrophoresis was conducted at 17 W (constant power), and the temperature was kept at 4 °C. Elution using the reservoir buffer was performed at a flow rate of 10 mL/h, collecting 1.8-mL fractions.

Isoelectrofocusing. Isoelectrofocusing experiments were conducted at 4 °C using LKB 8100 ampholine electrofocusing equipment. Runs were carried out in a 110-mL column filled with a linear gradient from 560 to 0 g/L sucrose containing 2% (w/v) pH 3.5–6 carrier ampholytes. The lower electrode solution (anode) contained 600 g/L sucrose and 1% H_2SO_4, while the upper electrode solution contained 1% NaOH (cathode). Approximately 30 units of cytidine deaminase were added to the dense sucrose solution before the gradient was poured. The power supply was maintained at 15 W for 1 h. At the end of the run, the column was eluted at a flow rate of 60 mL/h, collecting 1.0-mL fractions. The fractions were then assayed for cytidine deaminase activity and pH values.

Molecular Weight Determination. The molecular weight of the purified native enzyme was determined by gel filtration chromatography as described by Andrews (1969), using a Sephacryl S-300 superfine column (1.5 × 70 cm) equilibrated with 100 mM Tris-HCl, pH 7.5, and 100 mM KCl. One-milliliter aliquots of samples containing cytidine deaminase and/or protein markers were applied to the column, eluted at a flow rate of 18 mL/h, and collected in 1.0-mL fractions. For estimation of subunit molecular weights, protein markers, cross-linked aldolase and cross-linked pure cytidine deaminase, were incubated at 100 °C for 5 min in a denaturing mixture containing 2% $NaDodSO_4$, 5% 2-mercaptoethanol, and 0.125 M Tris-HCl, pH 6.8. Samples were loaded on 8-cm $NaDodSO_4$–polyacrylamide gels (Laemmli, 1970) and run at 5 mA per gel. Cross-linking reactions were performed as described by Davies & Stark (1970).

Amino Acid Analysis. Amino acid analysis was performed essentially as described by Umagat et al. (1982). The HPLC system consisted of two Beckman Model 112 pumps, Beckman Model 420 microprocessor for generation of elution gradients, and a Gilson Model 121 filter fluorometer (excitation filter 305–395 nm, emission filter 475–650 nm). Chromatographic peaks were recorded and integrated by a Hewlett Packard Model 3390A integrator. Separations were carried out on a 150 × 4.6 mm i.d. Ultrasphere column packed with 5-μm ODS particles connected to a precolumn (50 × 4.6 mm i.d.) packed with the same material. The samples were introduced with a Beckman Model 210 injection valve, equipped with a 20-μL external loop. Gradients were formed between two degassed solvents at a flow rate of 0.7 mL/min. Solvent A was tetrahydrofuran–0.05 M sodium acetate, pH 6.6 (1:99), and solvent B was methanol. The gradient program was linear steps from 15% to 23% solvent B for 15 min, linear steps to 60% solvent B for 30 min, and linear steps to 80% solvent B for 5 min. The column was thermostated at 21 °C. Hydrolysis of 30 μg of pure enzyme, using 6 M redistilled HCl at 110 °C in a hydrolysis tube sealed under vacuum, was carried out for 24, 48, and 72 h in order to allow extrapolation to zero (Ser, Thr) or infinite (Val, Ile, Leu) time of hydrolysis. After hydrolysis, HCl was removed by lyophilization and the resulting residue dissolved in 50 μL of water. The derivatization with *o*-phthaldialdehyde reagent was performed as described by Umagat et al. (1982). Cysteine plus cystine was determined as cysteic acid after performic acid oxidation. In this case, to shorten the time course, an isocratic elution step at 70% solvent B was carried out.

RESULTS

Purification of Cytidine Deaminase. A 46-mL volume of induced crude extract, prepared as described under Experimental Procedures, was applied to a DE-52 cellulose column (2.5 × 24 cm) equilibrated with dialysis buffer A at a flow rate of 16 mL/h. The column was then washed with the same buffer, containing 0.1 M KCl, until protein elution ceased. A linear salt gradient, 0.1–0.4 M KCl in buffer A, was then applied to the column and eluted at a flow rate of 32 mL/h. The fractions (3.6 mL) containing activity were pooled and dialyzed against 20 mM Tris-HCl, pH 7.5. The dialyzed sample was concentrated by ultrafiltration up to 2 mL using an Amicon PM-10 membrane and submitted to preparative electrophoresis, performed as described under Experimental Procedures. The activity-containing fractions were pooled, concentrated by ultrafiltration, and stored at −20 °C.

The final enzyme preparation was homogeneous as judged by the single band observed by running 10% polyacrylamide gel electrophoresis under both nondenaturing (Ornstein, 1964; Davis, 1964) and denaturing (Laemmli, 1970) conditions. A single band of cytidine deaminase activity showing the same relative mobility (0.53) as the Coomassie blue stained band was detected on separate gels, as described under Experimental Procedures.

After 2 weeks at 4 °C, the pure enzyme retained 63% of its original activity when stored in 50 mM Tris-HCl, pH 7.5, and only 13% in 50 mM phosphate buffer, pH 7.5. After 1 month at −20 °C, the activity was 80% in Tris-HCl and 70% in phosphate buffer (Table I).

Molecular Weight Determination. The molecular weight of the native enzyme estimted by gel filtration as described

Table I: Purification of Cytidine Deaminase from *E. coli* B

step	total protein (mg)	total act. (units)	sp act. (units/mg)	x-fold purification	% yield
crude extract	348	591	1.7		100
DEAE-cellulose	13	227	17	10	38
electrophoresis	0.95	144	152	90	24

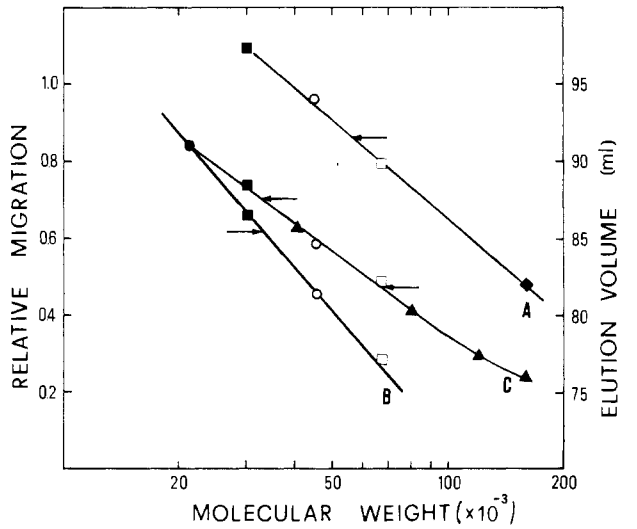

FIGURE 1: Determination of molecular weight and number of subunits of purified cytidine deaminase on (A) a Sephacryl S-300 superfine column, (B) a $NaDodSO_4$–10% polyacrylamide disc gel, and (C) a $NaDodSO_4$–5% polyacrylamide disc gel. The arrows indicate the interpolation values for the enzyme. Protein markers used were the following: (♦) aldolase (M_r 160 000); (□) bovine serum albumin (M_r 67 000); (○) ovalbumin (M_r 45 000); (■) carbonic anhydrase (M_r 31 000); (●) soybean trypsin inhibitor (M_r 21 500) (▲) cross-linked aldolase (M_r 40 000, 80 000, 120 000, and 160 000). See Experimental Procedures for details.

under Experimental Procedures was about 56 000. When run on denaturing acrylamide gels (see Experimental Procedures), the purified enzyme consisted of a single protein band of molecular weight 33 000. A bifunctional cross-linking reagent, dimethyl suberimidate, was employed to resolve this discrepancy. When active enzyme was cross-linked and analyzed by $NaDodSO_4$–5% polyacrylamide gel electrophoresis, two protein bands of molecular weight 66 000 and 33 000 were observed. These results, shown in Figure 1, suggest that cytidine deaminase is a dimer composed of two apparently identical subunits. A similar $NaDodSO_4$–polyacrylamide gel electrophoresis pattern, obtained upon incubation of the enzyme either in the presence or in the absence of 2-mercaptoethanol, revealed the absence of disulfide linkages between the two subunits.

Isoelectric Point. The activity and pH profile of an eluate from an isoelectric focusing column (see Experimental Procedures) shows that one catalytically active component with an isoelectric pH value of 4.35 was present.

Amino Acid Composition. Amino acid analyses were performed as described under Experimental Procedures. The results of five independent amino acid hydrolyses are present in Table II.

Ultraviolet Absorption Spectrum. The absorption spectrum of cytidine deaminase shows, in the ultraviolet range, a maximum at 277 nm and a minimum at 255 nm with a small shoulder at 290 nm. The percent absorption coefficient was determined to be 17 by using the Folin–Ciocalteus protein determination method modified according to Schacterle & Pollack (1973).

Table II: Amino Acid Composition of Cytidine Deaminase from *E. coli* B

amino acid	residues per subunit[a]	amino acid	residues per subunit[a]
aspartic acid and asparagine	35	alanine	40
		tyrosine	8
glutamic acid and glutamine	31	methionine	4
		valine	10[c]
serine	15[b]	phenylalanine	12
histidine	7	isoleucine	12[c]
glycine	18	leucine	33[c]
threonine	11[b]	lysine	8
arginine	12	half-cystine	6[d]

[a] All calculations based on a molecular weight of 33 000. [b] Values were extrapolated to zero time of hydrolysis. [c] Values were extrapolated to infinite time of hydrolysis. [d] Determined as cysteic acid after performic acid oxidation.

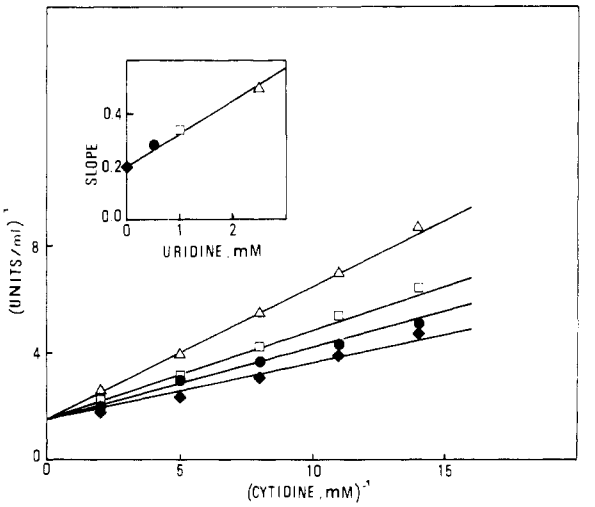

FIGURE 2: Product inhibition by uridine at various fixed concentrations: (♦) none; (●) 0.5 mM; (□) 1.0 mM; (△) 2.5 mM. The reaction mixture contained 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.04 unit of enzyme, and cytidine and uridine at the concentrations indicated. The assay was performed at 30 °C and the reaction rate followed at 290 nm (ΔA_{mM} = 2.1). Inset: Secondary plot of slope vs. uridine concentration for the determination of K_i (intercept on the abscissa).

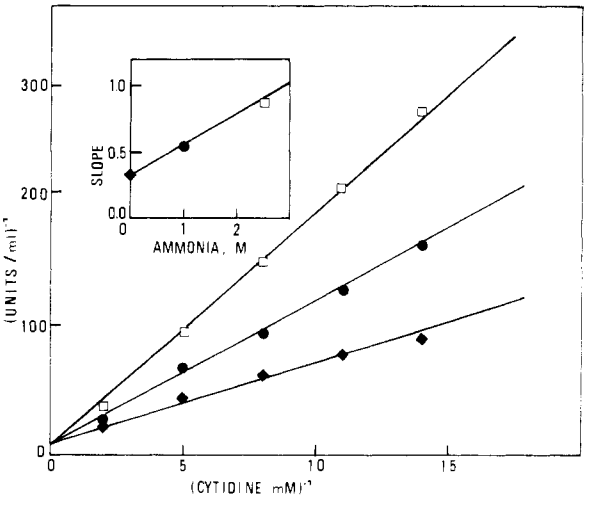

FIGURE 3: Product inhibition by ammonia at various fixed concentrations: (♦) none; (●) 1.0 M; (□) 2.5 M. The reaction mixture contained 50 mM Tris-HCl, pH 10, 100 mM KCl, 0.04 unit of enzyme, and cytidine and ammonia at the concentrations indicated. Experimental conditions as in Figure 2. Inset: Secondary plot of slope vs. ammonia concentration.

Kinetic Analysis. To establish the reaction mechanism catalyzed by cytidine deaminase, product inhibition experi-

Table III: Inhibition Kinetics of Cytidine Deaminase[a]

compd	app K_i (μM)	type of inhibn
5,6-dihydrouridine	0.03	competitive
dihydrothymidine	0.35	competitive
deoxyuridine	0.40	competitive
1-methyladenosine	0.80	competitive
thymidine	0.83	competitive
thymine riboside	1.35	competitive
deoxyadenosine	2.30	competitive

[a] Experimental conditions as described in the legend of Figure 6.

ments have been performed by using a homogeneous preparation of the enzyme. Double-reciprocal plots of initial velocities at various concentrations of cytidine and at different fixed concentrations of uridine (Figure 2) and ammonia (Figure 3) both resulted in a family of lines intersection on the ordinate axis. The competitive nature of both product inhibition patterns clearly indicated that the catalytic mechanism was rapid-equilibrium random Uni-Bi according to Cleland's nomenclature (Cleland, 1963). The secondary plot of slope vs. product concentration (insets of Figures 2 and 3) shows that both inhibitions are linear. The inhibition constants (K_i) calculated from the insets of Figures 2 and 3 were 1.6 mM for uridine and 1.4 M for ammonia. K_m and V_{max} values calculated from Figure 2 were 0.18 mM and 317 units/mg, respectively. In addition, cytidine deaminase catalyzes the deamination of deoxycytidine and 5-methylcytidine, being inactive toward a variety of pyrimidine and purine nucleosides. No halogenated pyrimidine nucleosides have been tested. The K_m values for deoxycytidine and 5-methylcytidine are 0.9×10^{-4} and 12.5×10^{-4} M, respectively. A variety of compounds including pyrimidine and purine bases, oxy- and deoxynucleosides, oxy- and deoxynucleotides, cyclic AMP, cyclic CMP, cyclic GMP, dihydrothymine, several halogenated and methylated nucleobases, oxy- and deoxyribose, oxy- and deoxyribose 1-phosphate, oxy- and deoxyribose 5-phosphate, glucose, glucose 1-p hosphate, glucose 6-phosphte, glucose 1,6-diphosphate, fructose, fructose 1,6-diphosphate, 5-phosphorylribose 1-pyrophosphate, inorganic phosphate, and pyrophosphate were examined for their ability to cause variations of enzyme activity. Among them, only the compounds listed in Table III inhibit cytidine deaminase. All inhibitors displayed linear competitive inhibition (plots not shown).

Properties of the Enzyme from Uninduced Cells. The major properties shown by the enzyme purified from *E. coli* cells grown in the presence of cytidine, including molecular weight, kinetic behavior, and susceptibility to various inhibitors, are identical with those possessed by cytidine deaminase, purified with the same procedure, from cells grown in the presence of glucose (see Experimental Procedures).

Discussion

In *Escherichia coli*, cytidine deaminase plays an important role in the transport and utilization of pyrimidine nucleosides (Von Dippe et al., 1975; Hochstadt, 1979; Vita et al., 1983). The enzyme has a molecular weight, estimated by gel filtration, of 56 000. It is composed of two identical subunits of 33 000 molecular weight. $NaDodSO_4$–polyacrylamide gel electrophoresis conducted in the absence of reducing agents reveals the absence of interchain disulfide group(s). The discrepancy existing between the molecular weight values estimated by gel filtration and $NaDodSO_4$–polyacrylamide electrophoresis experiments might be explained by abnormal behavior of the macromolecule during gel filtration as described for other proteins (Andrews, 1969). The molecular weight of the native protein agrees with that estimated by Hosono & Kuno (1973) but is lower than the value reported by Cohen & Wolfenden (1971). Hosono & Kuno (1973) do not observe any indication of quaternary structure when they subject the enzyme to gel filtration in the presence of 6 M urea. Molecular weight values for the enzyme from other sources are in the range 51 000–74 000 (Ipata et al., 1970; Chabuer et al., 1974; Malathi & Silber, 1971). Cytidine deaminase exhibits an absorption spectrum typical of that expected for a protein with no visible absorbing cofactor. Amino acid analysis indicates the presence of at least 262 residues per subunit, with an excess of aspartic and glutamic acids. The acidic residues predominance agrees with the isoelectric pH value of 4.35, observed by submitting cytidine deaminase to isoelectrofocusing. The presence of six half-cystine residues in the protein molecule deserves some attention. It has been observed that the enzyme from *Escherichia coli* is insensitive to the usually employed sulfhydryl group reagents (Hosono & Kuno, 1973) whereas that from other sources shows a different degree of reactivity (Ipata et al., 1970; Malathi & Silber, 1971). Analysis of the nature of cytidine deaminase half-cystine groups needs, therefore, a more detailed study. Cytidine deaminase from *Escherichia coli* displays linear kinetics. Among the substrates tested, it shows the highest affinity for deoxycytidine, in agreement with the results obtained by others (Hosono & Kuno, 1973; Wang et al., 1950). The enzyme from yeast (Ipata et al., 1970) possesses the same preferential affinity for deoxycytidine whereas cytidine deaminase from some animal cells exhibits higher affinity for cytidine (Malathi & Silber, 1971). *Escherichia coli* enzyme is inhibited by the reaction products uridine and ammonia. The data show competitive inhibition by both compounds, suggesting a kinetic mechanism of the rapid-equilibrium radom Uni-Bi type according to the nomenclature adapted by Cleland (1963). The high K_i value for ammonia, exceeding 1 M concentrations, has also been postulated by Cohen & Wolfenden (1971). Furthermore, the enzyme is also inhibited in a competitive fashion by various nucleosides. Similar observations have been reported by Hosono & Kuno (1973).

The enzyme from other sources is inhibited by nucleosides as well as by nucleotides, suggesting that it is allosterically regulated and can be involved in the "salvage pathways" of pyrimidine nucleotide metabolism (Ipata et al., 1970; Wisdom & Orsi, 1969).

Acknowledgments

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Registry No. Cytidine deaminase, 9025-06-3; cytidine, 65-46-3; deoxycytidine, 951-77-9; 5-methylcytidine, 2140-61-6; 5,6-dihydrouridine, 5627-05-4; dihydrothymidine, 5627-00-9; deoxyuridine, 951-78-0; 1-methyladenosine, 15763-06-1; thymidine, 50-89-5; thymidine riboside, 1463-10-1; deoxyadenosine, 958-09-8.

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