Deoxycytidylate Aminohydrolase. I. Preparation and Properties of the Homogeneous Enzyme*

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ABSTRACT: Deoxycytidylate aminohydrolase has been purified from donkey spleen; the specific activity of the purified enzyme is 4.8×10^4 times that of the enzyme in the centrifuged homogenate. The purification procedure involves ammonium sulfate fractionation, ethanol fractionation, and TEAE-cellulose column chromatography. The total enzyme recovery is about 5%. The purified enzyme is at least 95% homogeneous. It shows one protein band on starch gel electrophoresis at different pH values and one protein band on disc electrophoresis. The specific activity of the purified enzyme does not increase with TEAE-cellulose column chromatography. The enzyme is eluted in a symmetric peak of constant specific activity. The molecular weight of the enzyme, determined by sucrose gradient centrifugation and by Sephadex gel filtration, is $1.2 \times 10^5 \pm 1 \times 10^4$. Ultracentrifugal sedimentation analysis revealed only one component with $s_{20,w}$ 5.8 ± 0.1. The isoelectric point is 4.7. Certain kinetic parameters of the enzyme and the effect of deoxycytidine triphosphate (dCTP) and deoxythymidine triphosphate (dTTP) on the enzyme activity have been studied.

Knowledge of the enzymes of DNA biosynthesis is essential for understanding the mechanism of regulation of cell multiplication. Deoxycytidylate aminohydrolase (EC 3.5.4.5) (Scarano, 1958, 1960) appears to play an important role in the regulation of the pool of deoxynucleotides in higher organisms. dCMP[1]-aminohydrolase is an allosteric enzyme; it is activated by dCTP-Mg and is inhibited by dTTP-Mg (Scarano *et al.*, 1962a, 1963, 1964; Maley and Maley, 1962, 1964, 1965).

The study of the regulation of the activity of the enzyme and of the correlation between function and molecular structure requires a pure protein. The small amount of dCMP-aminohydrolase which can be obtained from the known sources (Scarano *et al.*, 1960; Talarico and Scarano, 1960; Scarano *et al.*, 1962b; Maley and Maley, 1964) is not sufficient to extend the purification up to homogeneity. To obtain an enzyme source easily available in large amounts, screening of organs from slaughterhouse animals was made and the spleen of the donkey was found to be the best source. The present paper reports (1) a preparation procedure which yields homogeneous dCMP-aminohydrolase and (2) the main properties of the enzyme.

Experimental Procedure

Materials and Methods. All reagents were analytical reagent grade. dCMP and 5-CH_3-dCMP were purchased from Calbiochem; Tris was obtained from Sigma Chemical Co.; DEAE- and TEAE-cellulose from Serva Entwicklungslabor; alumina gel C_γ from Schuchardt; starch hydrolyzed for gel electrophoresis from Connaught Medical Research Laboratories, Toronto.

From purification step 5 on, quartz-redistilled water was used to recrystallize the reagents and to prepare the solutions. Phosphate and potassium chloride were recrystallized once from 10 mM Versene and once from water. Tris was recrystallized from 85% ethanol; alumina gel C_γ was washed once with 10 mM Versene and once with water. The dialysis tubes were heated in 10 mM Versene at 95° for 20 min and then washed with water.

From purification step 4 on, glycerol was added to the buffers to protect the enzyme from inactivation. Two glycerol-containing buffers were used. Buffer A, Tris (10 mM)–Versene (1 mM)–50% glycerol–β-mercaptoethanol (7 mM), was adjusted to pH 8.0 with acetic acid. Buffer B had the same composition as buffer A except that the glycerol concentration which was 20%.

Donkey spleens were obtained from the slaughterhouse of Corato (Bari, Italy). The spleens were collected

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[1] Abbreviations used: dCMP, deoxycytidine monophosphate; dUMP, deoxyuridine monophosphate; dTTP, deoxythymidine triphosphate; dCTP, deoxycytidine triphosphate; PPO, 2,5-diphenyloxazole; dimethyl-POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]; dAMP, deoxyadenosine monophosphate; dCR, deoxycytidine; dGMP, deoxyguanosine monophosphate; LDH, lactic dehydrogenase; G-6-PDH, glucose 6-phosphate dehydrogenase; ADH, alcohol dehydrogenase; AR-deaminase, adenosine deaminase.

as soon as the animals were killed and immediately frozen at −30°. To standardize conditions the spleens were used only after 5 days of storage at −30°. The enzyme is labile if the spleens are stored at 4°; at this temperature no dCMP-aminohydrolase activity is detectable after 24 hr.

The electric band saw and the electric meat mincer were obtained from Bizerba, Milan, Italy. Proteins were determined by the method of Lowry *et al.* (1951) except for a few cases reported in the text, in which protein concentration was determined spectrophotometrically (Warburg and Christian, 1942).

Ammonium sulfate was determined by the following turbidimetric method. $BaCl_2$ (0.1 ml of 0.05 M) in 0.1 M HCl was added to 0.5 ml of a solution containing from 0.1 to 0.5 μmole of ammonium sulfate in 0.01 M HCl. The sample was mixed and the optical density at 540 mμ was measured after 3 min. A standard curve was obtained using appropriate amounts of a solution of Na_2SO_4 in 0.01 M HCl. Ammonia was determined colorimetrically with the Nessler reagent, prepared according to the formula of Bock and Benedict (Hawk *et al.*, 1954).

Enzyme Assay. The purification procedure was followed to step 3 by assaying the enzyme activity at 38° with the spectrophotometric method I already described (Scarano *et al.*, 1962b) using dCMP as substrate. From step 4 on the activity was determined by the spectrophotometric method II (Scarano *et al.*, 1962b) using dCMP as substrate at 38°. The absorbancy measurements were made with a Beckman Model DU spectrophotometer equipped with a Pyrocell microattachment and a thermostatic chamber set at 38°. One unit of enzyme is defined as the amount of enzyme which deaminates 1 μmole of dCMP per min per ml of incubation mixture at 38°.

The following assay method was employed to determine the pH optimum of the enzyme. dCMP-2-^{14}C (20 mM, 4.7 × 10^4 cpm/μmole) was incubated at 38° in a buffer containing 0.075 M phosphate–0.075 M Tris–0.075 M glycine, adjusted at different pH values by addition of HCl or NaOH in a final volume of 0.25 ml. At 0 and 5 min, 25 μl of 15% $HClO_4$ was added to aliquots of 50 μl. No precipitate appears when the purified enzyme is used. The clear solution (50 μl) was neutralized by addition of 20 μl of 0.6 M K_2CO_3 in 0.1 M Tris buffer, pH 7.5. The suspension was kept at 4° for 30 min and the $KClO_4$ precipitate was eliminated by centrifugation at 4°. Carrier dUMP (0.1 μmole) was added to 5 μl of the supernatant and spotted on a thin layer of DEAE-cellulose. The chromatogram was developed with 0.02 M HCl (Grippo *et al.*, 1965). The dCMP and dUMP spots were scraped off the cellulose layer and suspended in 15 ml of a liquid scintillator (4 g/l. of PPO and 100 mg/l. of dimethyl-POPOP in toluene). The samples were counted in a Packard Tri-Carb Model 314EX.

Purification Procedure. Table I lists the initial specific activity of dCMP-aminohydrolase from the different sources that were found to have high activity, and the specific activity of the purified preparations obtained so far. The purification procedure and the results of an average preparation are summarized in Table II. The specific activity of the purified enzyme was 4.8 × 10^4 times that of the centrifuged homogenate. All operations except the ethanol fractionation were done at 4°.

Step 1. Ammonium Sulfate Fractionation I. Frozen spleens were cut in small cubes with an electric band saw and immediately minced in an electric meat mincer. Minced spleen (10 kg) was suspended in 30 l. of 0.15 M phosphate–1 mM β-mercaptoethanol, pH 7.3, and stirred for 30 min. The suspension was centrifuged at 4000*g* for 15 min and the supernatant was collected. Finely powdered ammonium sulfate (up to 30% saturation) was added slowly under mechanical stirring to the supernatant. After 30 min of additional stirring the mixture was centrifuged at 4000*g* for 30 min, and the

TABLE I: Deoxycytidylate Aminohydrolase from Higher Organisms.[a]

Enzyme Source	Centrifuged Homogenate	Purified Prepn	References
Sphaerechinus granularis eggs	0.02	16	Scarano *et al.* (1960)
Monkey liver	0.007	70	Scarano *et al.* (1962)
Rabbit liver	0.014	4	Scarano *et al.* (1962)
Chick embryos			
5 days old	0.07	...	Talarico and Scarano
7 days old	0.04	...	(1960)
Chick embryos			
6 days old	0.06	6	Maley and Maley (1964)
Donkey spleen	0.015	720	Present paper

[a] Specific activity in micromoles of deoxycytidylate deaminated per minute and per milligram of protein at 38°.

TABLE II: Purification of Donkey Spleen Deoxycytidylate Aminohydrolase.

Fraction No. and Step	Vol. (l.)	Protein (mg/ml)	Enzyme Act. (units/ml)	Sp Act. (units/mg of protein)	Total Units	Recov (%)
(1) Centrifuged homogenate	31.0	25.0	0.363	0.015	11,160	100
(2) Ammonium sulfate fraction I	12.2	25.0	0.760	0.030	9,292	83
(3) Ethanol fraction	3.04	4.0	2.432	0.608	8,035	72
(4) Ammonium sulfate fraction II	2.83	2.08	2.270	1.09	6,473	58
(5) Ammonium sulfate fraction pH 5.1	0.312	8.35	18.20	2.18	5,691	51
(6) Dialysis and protamine pptn	0.212	6.7	22.00	3.29	4,687	42
(7) TEAE-cellulose I	0.061	1.17	38.42	32.9	2,344	21
(8) TEAE-cellulose II	0.006	0.87	186.0	277.5	1,116	10
(9) TEAE-cellulose III	0.002	0.42	300.0	720.0	600	5.3

precipitate was discarded. Solid ammonium sulfate to 60% saturation was added slowly under mechanical stirring to the supernatant. After 30 min of additional stirring the suspension was centrifuged at 4000*g* for 1 hr and the supernatant was discarded.

Step 2. Ethanol Fractionation. The ammonium sulfate paste from step 1 was dissolved in 0.02 M phosphate–1 mM β-mercaptoethanol, pH 7.3. The protein concentration was adjusted to 25 mg/ml and the ammonium sulfate to 0.8 M. Absolute ethanol, chilled to about −20°, was added slowly under continuous stirring to achieve a final concentration of 40% (v/v). During the addition of the ethanol which takes 12 min the temperature is increased to 10 ± 1°. After 15 min of additional stirring at 10 ± 1°, the suspension was centrifuged at 9000*g* at 0° for 15 min. The supernatant was collected and brought to 0°. Ethanol at −30° was added to a final concentration of 55% (v/v). During the addition of the ethanol, 12 min, the temperature was kept at 0 ± 1°. After 15 min of stirring at the same temperature the suspension was centrifuged as above. The precipitate was collected, suspended in 0.02 M phosphate–1 mM β-mercaptoethanol, pH 7.3, and homogenized with a glass homogenizer. After 30 min of mechanical stirring the suspension was centrifuged at 30,000*g* for 30 min. The precipitate was discarded and the clear supernatant was saved for the next step.

Step 3. Ammonium Sulfate Fractionation II. The enzyme solution was diluted to 4 mg of protein/ml with 0.02 M phosphate–1 mM β-mercaptoethanol, pH 7.3. The ammonium sulfate concentration of the solution was brought to 43% saturation by addition of solid ammonium sulfate as described in the first step. After 30 min of mechanical stirring the suspension was centrifuged at 9000*g* at 0° for 30 min. The precipitate was discarded and the supernatant was brought to 53% saturation and stirred for 30 min. The precipitate was collected by centrifugation as above and dissolved to a concentration of 2.5 mg of proteins/ml in 0.05 M phosphate–1 mM β-mercaptoethanol–1 mM Versene, pH 7.3.

Step 4. Ammonium Sulfate Fractionation at pH 5.1. The ammonium sulfate concentration of the solution from step 3 was adjusted to 53 mg/ml. The solution was then brought to pH 5.1 by slow addition, under mechanical stirring, of 1 M acetic acid. After 10 min of stirring the suspension was centrifuged at 9000*g* for 15 min and the precipitate was discarded. To the clear supernatant, solid ammonium sulfate was added to a concentration of 230 mg/ml and stirred for 15 min. The suspension was centrifuged as above, and the precipitate was collected and immediately dissolved in buffer A. The amount of buffer A used was such that 20% glycerol (v/v) was present in the final solution. The pH of the solution was adjusted to pH 8.0 by addition of a few drops of a saturated Tris solution to obtain complete solution of the proteins.

Step 5. Protamine Sulfate Fractionation. To the enzyme solution from the preceding step 100 mg of protamine sulfate was added for each 170 mg of protein. The protamine was added as 1% solution in buffer B. The solution, which remains clear, was dialyzed against 50 volumes of buffer B. Every 7 hr the external solution was changed until no more ammonium sulfate was detected in the dialysate. The heavy precipitate which forms during the dialysis was discarded by centrifugation at 9000*g* for 15 min. The clear supernatant was used for step 6.

Step 6. TEAE-Cellulose Chromatography I. TEAE-cellulose, freed of light particles and degassed under vacuum, was washed with 0.5 N NaOH until the NaOH solution had no absorbancy at 280 mμ. The cellulose was washed with water until the washings were neutral. The same procedure was repeated using 0.5 N HCl. The washed TEAE-cellulose was suspended in buffer B and packed into a column 4.4 × 40 cm. The column was equilibrated with the above buffer and the rate of effluent was adjusted to 5 ml/min by means of a constant-flow pump. The same rate was maintained throughout the whole column chromatography. The enzyme solution was adsorbed on the column and the column was washed with 100 ml of buffer B. The elution of the proteins was carried out with a linear KCl

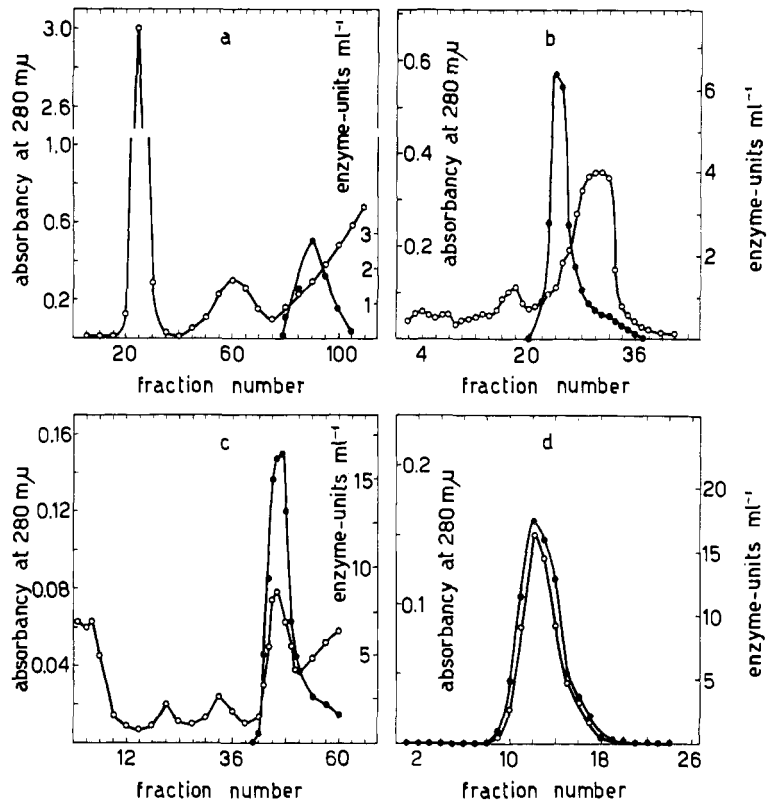

FIGURE 1: Elution patterns of TEAE-cellulose column chromatographies. (○—○), optical density at 280 mμ; (●—●) enzyme activity. For details see the text.

gradient. The mixing chamber was filled with 750 ml of buffer B and the reservoir was filled with 750 ml of 0.15 M KCl in buffer B. Fractions of 20 ml were collected and assayed for proteins and for enzyme activity. Protein concentration was determined spectrophotometrically. The pattern of the chromatography is shown in Figure 1a. The fractions with specific activity higher than 18 were pooled and the enzyme was precipitated by addition of 2.5 volumes of absolute ethanol previously chilled to −20°. During the ethanol addition the temperature was lowered to −15°. When the addition of ethanol was completed the suspension was kept under magnetic stirring for 5 min at −15° and then centrifuged at −15° at 9000*g* for 10 min. The precipitate was collected and suspended in about 50 ml of buffer B. The suspension was centrifuged at 20,000*g* for 10 min and the precipitate was discarded.

Step 7. TEAE-Cellulose Chromatography II. The enzyme solution from step 6 was dialyzed for 4 hr against 500 volumes of buffer B. The dialyzed enzyme was adsorbed onto a TEAE-cellulose column 1.7 × 50 cm, prepared as previously described. The chromatography was developed with a linear KCl concentration gradient prepared with 400 ml of buffer B in the mixing chamber and with 400 ml of 0.12 M KCl in buffer B in the reservoir. Fractions of 12 ml were collected. The elution pattern is shown in Figure 1b. The fractions with specific activity higher than 200 were combined. A suspension (2 ml) of 2% alumina gel C_γ for 100 ml of column eluate was added to adsorb the enzyme. The suspension was kept under magnetic stirring for 30 min and then centrifuged. The enzyme was eluted by stirring the gel for 15 min in 2–3 ml of buffer A, containing 10% ammonium sulfate. The gel was collected by centrifugation and eluted again as above.

Step 8. TEAE-Cellulose Chromatography III. The enzyme from step 7 was dialyzed against buffer B until no ammonium sulfate was detected in the dialysate. The enzyme solution was adsorbed on a TEAE-cellulose column 2 × 19 cm prepared as previously described. The column was washed with 15 ml of buffer B and the enzyme was eluted by means of a linear KCl concentration gradient (mixing chamber 200 ml of 0.006 M KCl in buffer B and 200 ml of 0.1 M KCl in buffer B in the reservoir). Fractions of 5 ml were collected (Figure 1c). The fractions with the highest specific activity were pooled and the enzyme was adsorbed on alumina gel C_γ and eluted as described in step 7.

Properties of the Enzyme

Stability. The enzyme, up to step 5, is unstable and cannot be stored in solution; the daily loss of activity is about 40%. The ammonium sulfate fractions

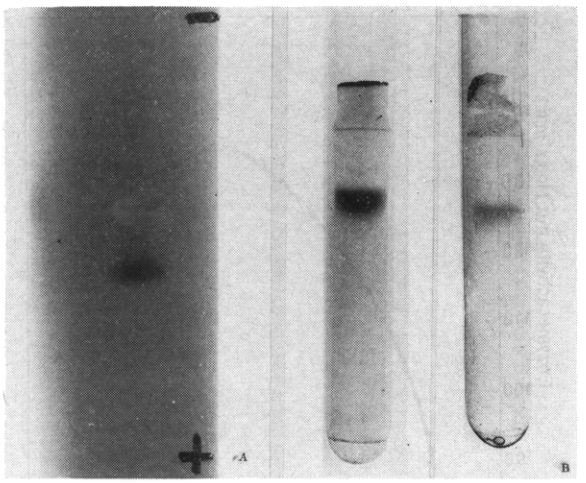

FIGURE 2: dCMP-aminohydrolase electrophoresis at pH 8 of the purified enzyme. (A) Starch gel electropherogram (70 μg of protein). (B) Polyacrylamide gel electropherogram (40 and 5 μg of protein).

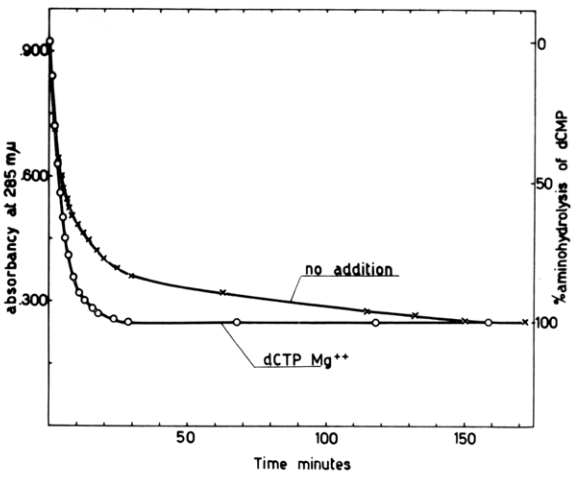

FIGURE 3: Time dependence of the enzymatic aminohydrolysis of dCMP and activation by dCTP-Mg. (x—x), 2 mM dCMP; (○—○), 2 mM dCMP + 20 μM dCTP; enzyme (0.2 unit) in 0.3 ml of incubation mixture, containing 2 mM $MgCl_2$ and 0.1 M phosphate buffer, pH 7.5, temperature, 38°. Optical density was measured at 285 mμ; 0.1-cm light path.

can be kept as a paste at −20° for 1 week without any appreciable loss of activity. From step 7 on the enzyme can be stored at −20° in buffer A. In buffer B at −20° the enzyme undergoes a slow loss of activity of about 5%/week.

Homogeneity. Tests of homogeneity were performed by chromatography on TEAE-cellulose and by electrophoresis on starch gel and on polyacrylamide gel. Purified enzyme (1.2 mg) was rechromatographed on a TEAE-cellulose column 1 × 9 cm, prepared as already described. The elution was performed by a linear KCl concentration gradient. The mixing chamber contained 30 ml of buffer B, and the reservoir 30 ml of 0.2 M KCl in the same buffer. Fractions of 2 ml were collected. The elution pattern is shown in Figure 1d. The specific activity of the enzyme does not increase and is constant in all the fractions within experimental error.

Starch gel electrophoresis of the enzyme was performed at 4°, 5 ma/cm²; 6 v/cm for 14 hr in 20 mM phosphate–7 mM β-mercaptoethanol–1 mM Versene, pH 7.5. Disc polyacrylamide gel electrophoresis was performed according to Reisfeld *et al.* (1962). One protein band was obtained with both methods staining with Amido-Schwarz. The band obtained with starch gel electrophoresis was tested also for enzyme activity. At 38° a mixture of 2% agar in 2 mM dCMP containing 7 mM β-mercaptoethanol, 5% of a saturated solution of phenol red adjusted to pH 7.0, was layered on the half of the electropherogram obtained by cutting the gel to half-thickness. By incubation at 38° the position of the enzyme is revealed, since the ammonia produced by the enzymatic activity changes the color of the indicator to a brownish-red that shows up well on the yellow background. The results are shown in Figure 2.

By starch gel electrophoresis it is possible to detect as much as 4 μg of protein. Since up to 70 μg of enzyme were used in the starch gel electrophoresis of Figure 2, and one band was observed in every instance, the preparation should be not less than 95% pure. One band was obtained also in starch gel electropherograms in citrate buffer from pH 4.5 to 6.5 (see isoelectric point determination).

Identification of the Product and Equilibrium of the Reaction. The time dependence of the enzymatic aminohydrolysis of dCMP and the influence of dCTP-Mg on the reaction are shown in Figure 3. In the presence of 2 mM dCMP, at the initial stages of the reaction, the effect of dCTP-Mg, the allosteric activator of the enzyme (Scarano *et al.*, 1963), is negligible. When the substrate concentration decreases a marked difference appears in the time dependence of the reaction between the assay mixture without dCTP-Mg and the assay mixture containing the activator. In both cases, however, the reaction reaches the same final optical density, corresponding to 100% aminohydrolysis of dCMP.

The chromatographic assay procedure, modified as follows, was used for the determination of the extent of the reaction and for the identification of dUMP. Enzyme (1 unit) was added to 0.1 ml of incubation mixture containing 1 μmole of dCMP-2-^{14}C, 1.3 × 10^5 cpm/μmole and 10 μmoles of phosphate, pH 7.5, at 38°. The incubation time was 1 hr. Cold dCMP was added as carrier for the chromatographic separation. More than 99.7% of the radioactivity was in the dUMP spot and less than 0.3% in the dCMP spot. The amount of ammonia formed was in perfect agreement with the dUMP found. This indicates that within the limits of experimental error the reaction goes to completion. Ammonia was measured directly in the incubation mixture without previous deproteinization.

Recovery of dCTP from Incubation Mixtures after

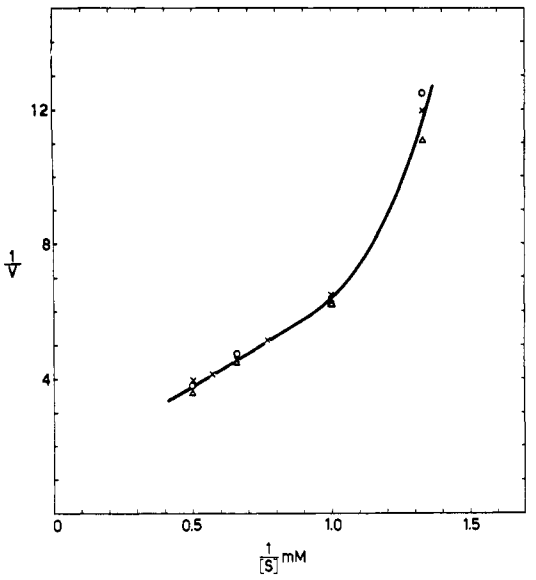

FIGURE 4: Dependence of the rate of aminohydrolysis upon dCMP concentration (Lineweaver–Burk plot). Enzyme assays were performed at 38° employing the spectrophotometric assay II; 0.82 unit of enzyme in 0.3 ml of incubation mixture.

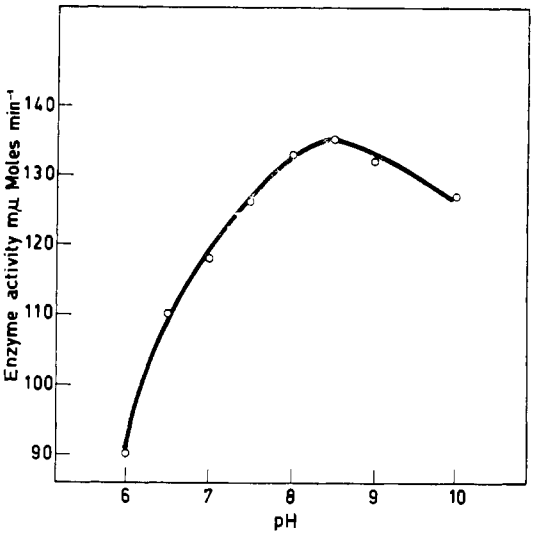

FIGURE 5: Activity of dCMP-aminohydrolase as a function of pH. Chromatographic assay; enzyme 0.13 unit/ml of incubation mixture.

100% Aminohydrolysis of the Substrate. dCTP-^{14}C (20 μM, 13 μc/μmole) was incubated under the conditions of Figure 3 with the exception that dCMP-^{12}C was used. The incubation mixture (25 μl) was spotted on a DEAE thin layer and chromatographed with 0.04 M HCl. The dUMP and dCTP spots were scraped off the layer and counted with the method already described. The counts (100% of them) were found in the dCTP spot and no radioactivity was detectable in the dUMP spot.

Substrate Specificity. dCMP and 5-CH_3-dCMP are substrates of the enzyme. No activity was observed with dCR, 3′-dCMP, dCDP, dCTP, CMP, 2′,3′-CMP, GMP, dGMP, AMP, and dAMP.

Effect of Substrate Concentration. In Figure 4 is plotted the reciprocal of the velocity of aminohydrolysis of dCMP against the reciprocal of dCMP concentration in 0.1 M phosphate buffer, pH 7.5, at 38°. The curve has two parts, a linear one at high substrate concentrations and a parabolic one at low substrate concentration. The K_m calculated from the linear part of the curve is 3.7 mM.

Effect of pH. The rate of aminohydrolysis of dCMP as a function of pH was studied at 38° by means of the chromatographic method employing dCMP-2-^{14}C (Figure 5). The enzyme activity decreases sharply at low pH values and very slowly at high pH values; a broad peak with a maximum at pH 8.5 is observed.

Inhibitors. In contrast with the specificity for the substrate, the enzyme is competitively inhibited by both pyrimidine and purine deoxynucleotides. Table III lists the competitive inhibitors of the enzyme and their K_i determined at 38° with the spectrophotometric assay II using dCMP as substrate. In addition to the competitive inhibitors the enzyme is strongly inhibited with a multimolecular kinetics by dTTP-Mg (Scarano *et al.*, 1963, 1964, 1967). dCTP-Mg reverts the inhibition by dTTP-Mg but has no effect on the inhibition by the competitive inhibitors.

TABLE III: Dissociation Constants of Complexes between dCMP-aminohydrolase and Competitive Inhibitors at 38°.

Inhibitor	Dissociation Constant (mM ± 0.02)
dTMP	0.12
dUMP	0.70
dGMP	0.14
dAMP	0.20

Activation by dCTP. The enzyme activity, as shown in Figure 3 and already reported (Scarano *et al.*, 1963, 1964), is enhanced by deoxycytidine triphosphate at low substrate concentrations in the presence of Mg^{2+}. The subsequent paper (Scarano *et al.*, 1967) reports a detailed study of the kinetic properties of dCMP-aminohydrolase.

Molecular Weight. The molecular weight of deoxycytidylate aminohydrolase was estimated by sucrose gradient centrifugation (Martin and Ames, 1961) and by Sephadex gel filtration (Andrews, 1964). From the data obtained with both methods and reported in Figures 6 and 7, a molecular weight of $1.2 \times 10^5 \pm 5 \times 10^3$ was found. Experiments with both methods demonstrated that the molecular weight of the enzyme

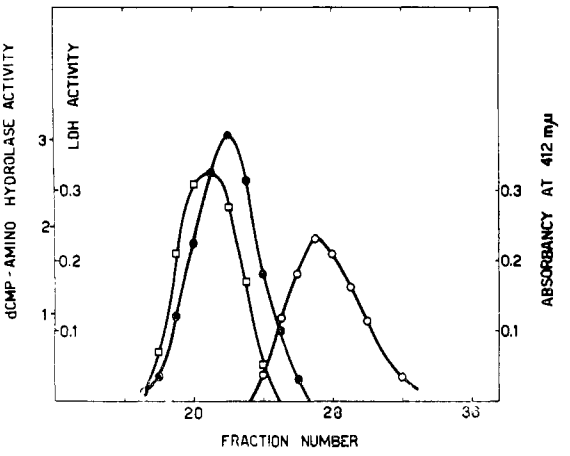

FIGURE 6: Sedimentation pattern of dCMP-aminohydrolase in a 5–20% sucrose gradient. LDH (25 μg), 0.5 mg of hemoglobin, and 4 μg of dCMP-aminohydrolase in 50 μl were layered on a 4.4-ml gradient. After 15 hr of centrifugation in a Spinco Model L 2 ultracentrifuge at 4° and 3.9 × 10^4 rpm in an SW39L rotor the fractions were collected. (□---□), LDH activity; (●—●), dCMP-aminohydrolase activity; (○—○), hemoglobin optical density at 412 mμ.

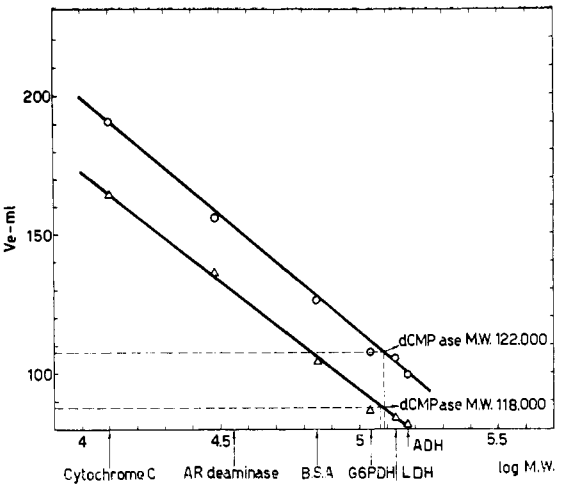

FIGURE 7: Determination of the molecular weight of dCMP-aminohydrolase by filtration on Sephadex G100. A mixture of 8 mg of bovine serum albumin, 25 μg of LDH, 50 μg of ADH, 40 μg of AR-deaminase, 1 mg of G-6-PDH, 1 mg of cytochrome *c*, and 55 μg of dCMP-aminohydrolase in 1.5-ml total volume was filtrated through a column of Sephadex G100, fine, beadform, equilibrated with 50 mM Tris–7 mM β-mercaptoethanol–0.8 M ammonium sulfate, pH 7.5. Albumin was determined following the absorbancy of the fractions at 280 mμ, the enzymes by activity measurements, and cytochrome *c* by following the absorbancy at 412 mμ. Fractions (2 ml) were collected. (△---△), 1.4 × 140 cm column; (○—○), 1.7 × 110 cm column.

does not change in the presence of 0.13 mM dCTP-Mg, 0.13 mM dTTP-Mg, or 6 mM dTMP. The dCTP and the dTTP concentrations used are about ten times those at which the maximum regulatory effects occur. The recovery of the enzyme was 60% in the sucrose gradient experiments and 95% in the gel filtration experiments. A value of the $s_{20,w}$ of 5.8 ± 0.1 was determined by sedimentation velocity in the analytical ultracentrifuge using the absorbancy in the ultraviolet. The enzyme (0.6 mg/ml) was dissolved in 0.05 M phosphate–7 mM β-mercaptoethanol–1.2 mM $MgCl_2$–10 μM dCTP, pH 7.5.

Isoelectric Point. The isoelectric point of the enzyme was measured by starch gel electrophoresis performed as described under the paragraph on homogeneity. As demonstrated by the data of Figure 8 a value of 4.7 was found.

Stability. The stability of the purified enzyme was studied in imidazole, Tris, and phosphate buffer. In Figure 9 are reported experiments in imidazole buffer. dCTP-Mg, dTTP-Mg, ammonium sulfate, and glycerol protect the enzyme from inactivation. It is pertinent to notice that in the absence of Mg^{2+} no protection by dCTP and dTTP is observed.

Turnover Number. The turnover number of the enzyme at 30 mM dCMP has been measured by the titrimetric assay. On the basis of a molecular weight of 1.2 × 10^5, the enzyme catalyzes at 38° and pH 7.5, the aminohydrolysis of 1.10 × 10^5 moles of dCMP/mole of enzyme per min in water, 1.15 × 10^5 in 50 mM Tris, 1.30 × 10^5 in 50 mM phosphate, and 7.5 × 10^4 in 50 mM imidazole.

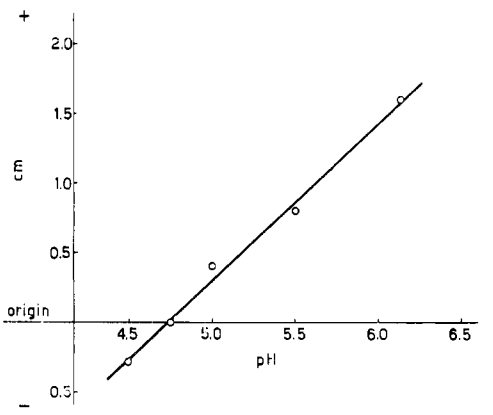

FIGURE 8: Isoelectric point of dCMP-aminohydrolase. The starch gel was prepared in citric acid–sodium citrate of ionic strength 0.05 at the indicated pH values; enzyme, 15 μg.

Discussion

Deoxycytidylate aminohydrolase was purified about 4.8 × 10^4-fold over the centrifuged homogenate from donkey spleen. The purified enzyme is at least 95% homogeneous on the basis of chromatography on

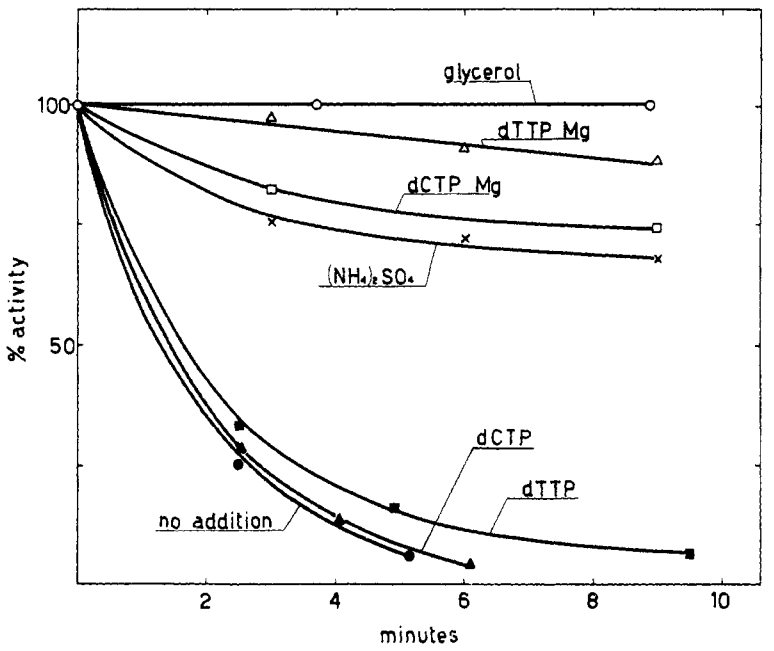

FIGURE 9: Protection of dCMP-aminohydrolase against thermal inactivation at 38° in 50 mM imidazole, pH 7.5. (○—○), 20% glycerol; (△—△), 20 μM dTTP + 2 mM $MgCl_2$; (□—□), 20 μM dCTP + 2 mM $MgCl_2$; (×—×), 0.25 M ammonium sulfate; (■—■), 20 μM dTTP, no $MgCl_2$; (▲—▲), 20 μM dCTP, no $MgCl_2$; (●—●), no addition. Enzyme concentration, 8 μg/ml. At the indicated times samples were collected and assayed for activity.

TEAE-cellulose, starch gel electrophoresis, and polyacrylamide gel electrophoresis. The enzyme has a specific activity about ten times higher than that of the monkey liver enzyme, which was the best enzyme preparation available.

Donkey spleen was chosen as a source of the enzyme because of the high initial specific activity and of the large amount of tissue available. The spleen of the horse contains about one-fifth as much enzyme as the donkey spleen. Centrifuged homogenates of spleen, liver, kidney, and thymus from the common slaughterhouse animals have one-tenth or less of the enzyme which occurs in the centrifuged homogenates of donkey spleen. The purification procedure up to step 4 worked out for the donkey spleen, can be used with satisfactory results to purify the enzyme from horse spleen.

The enzyme is highly specific for a pyrimidine deoxynucleoside 5′-monophosphate. Other authors tested the specificity of the aminohydrolase on partially purified preparations of the enzyme and found that analogs of dCMP halogenated on the 5 position of the pyrimidine ring are substrates for the enzyme (Maley and Maley, 1964) and that 6-aza-dCMP is also substrate for the enzyme partially purified from Ehrlich ascites cells (Kára and Šorm, 1964). 5-CH_2OH-dCMP is a substrate for the enzyme partially purified from monkey liver (Scarano *et al.*, 1962b).

No requirement of divalent cations and/or of dCTP has been found for the dCMP-aminohydrolase activity from the centrifuged homogenate up to the purified enzyme as demonstrated by the fact that the incubation mixtures to assay the enzyme throughout the whole purification contain neither dCTP nor Mg^{2+}. In addition, by using a dCMP concentration higher than 2 mM, no activatory effect by dCTP-Mg can be detected as demonstrated in a detailed study of metal requirement and of dCTP and dTTP action (Scarano *et al.*, 1967). The quantitative recovery of dCTP-^{14}C from incubation mixtures after 100% aminohydrolysis of the substrate demonstrates that dCTP does not participate in the reaction.

Maley and Maley (1964) have reported that at low substrate concentrations Mg^{2+} and dCTP are cofactors of dCMP-aminohydrolase from chick embryos. However, it is pertinent to remember that when measuring dCMP-aminohydrolase activity by a 10-min assay at 37° the stabilization of this very labile enzyme by dCTP-Mg^{2+} at low substrate concentration is the most critical factor. Requirement of dCTP and Mg^{2+} has never been observed with dCMP-aminohydrolase preparations from sea urchin embryos (Scarano *et al.*, 1960), monkey liver (Scarano *et al.*, 1962b), rabbit liver (Scarano *et al.*, 1962b), and donkey spleen.

The molecular weight of the homogeneous enzyme from donkey spleen as measured by sucrose gradient centrifugation and gel filtration, is not affected by dCTP-Mg, dTTP-Mg, or dTMP. Thus, no dissociation of the enzyme molecule into subunits occurs in the regulation of its activity.

Maley and Maley (1965) have reported a change of the $s_{20,w}$ of dCMP-aminohydrolase from chick embryo caused by the regulatory nucleotides, as measured by sucrose gradient centrifugation. However, the very low

recovery of the enzyme in the experiments reported (Maley and Maley, 1965) may have led to misinterpretation of the data.

The turnover number has been calculated on the basis of the molecular weight of 1.2×10^5. The turnover number of dCMP-aminohydrolase is about one-fourth that of the crystalline AMP-aminohydrolase from rabbit muscle reported by Lee (1957). The purified enzyme from chick embryos (Maley and Maley, 1964) has a specific activity less than 1/100 as that of the spleen enzyme. Thus, it seems unlikely that the chick enzyme is homogeneous as claimed by the authors.

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