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The Enzymatic Aminohydrolysis of 4-Aminopyrimidine Deoxyribonucleotides*

III. PURIFICATION AND PROPERTIES OF 2′-DEOXYRIBOSYL 4-AMINOPYRIMIDONE-2,5′-PHOSPHATE AMINOHYDROLASE FROM MONKEY LIVER†

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Previous papers described the finding that extracts of tissues from warm-blooded animals catalyze specifically the aminohydrolysis of 2′-deoxyribosyl cytosine 5′-phosphate to 2′-deoxyribosyl uracil 5′-phosphate and of 2′-deoxyribosyl 5-methylcytosine 5′-phosphate to 2′-deoxyribosyl thymine 5′-phosphate (1, 3–6). The aminohydrolysis of dCMP by tissue extracts from warm-blooded animals has been reported also by Maley and Maley (7, 8) and by Fiala *et al.* (9, 10).

A highly purified enzyme preparation that catalyzes the aminohydrolysis of dCMP to dUMP, CH_3-dCMP to dTMP, and CH_2OH-dCMP to CH_2OH-dUMP was obtained from sea urchin eggs (1). Inasmuch as extensive purification failed to resolve separate aminohydrolases, it seemed likely that one enzyme with 2′-deoxyribosyl 4-aminopyrimidone-2,5′-phosphate aminohydrolase activity accounts for the aminohydrolysis of the three deoxyribonucleotides. The enzyme has been referred to as 2′-deoxyribosyl 4-aminopyrimidone-2,5′-phosphate aminohydrolase. Since dPAase[1] seems to be involved in the metabolic pathway to dTMP synthesis in eggs and in embryos of sea urchins (1), it is of interest to investigate whether or not the enzyme is present also in tissues of warm-blooded animals.

The present paper deals mainly with the preparation of highly purified dPAase from monkey liver and with some properties of the purified enzyme. It is shown also that the purification procedure worked out with monkey liver also permits the preparation of highly purified dPAase from rabbit liver. Finally evidence is reported that dPAase accounts for the aminohydrolysis of dCMP and CH_3-dCMP by the extracts of all tissues from warm-blooded animals that have been investigated in this laboratory.

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† In the previous papers of this series, the pyrimidine ring was numbered according to the Fischer system, whereas in the present paper the *Chemical Abstracts* system is used. The name aminohydrolase instead of deaminase is used after the suggestion of the Report of the Commission on Enzymes of the International Union of Biochemistry, E. C., 3. 5. 4. (2).

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[1] The abbreviations used are: dPAase, 2′-deoxyribosyl 4-aminopyrimidone-2,5′-phosphate aminohydrolase. (Note by Editor: because of the great length of the full name of this enzyme, this abbreviation has been permitted, in spite of the usual rule of the Journal that names of enzymes are not to be abbreviated.)

EXPERIMENTAL PROCEDURE

The reagents, the chemical determinations, and the methods for ion exchange chromatography and for paper chromatography were the same as in previous work (1, 5). dCDP-choline was kindly provided by Dr. R. L. Potter.

Proteins were determined as previously described (1), following the procedure of Lowry *et al.* (11) except in a few cases, indicated in the text, in which the protein concentration was determined spectrophotometrically (12).

Enzyme Assay—The purification procedure was routinely followed by assaying the CH_3-dCMP aminohydrolase activity of the fractions. Two assays were used. In Assay 1, absorbancy determinations were made on deproteinized aliquots of the reaction mixture (5). The reaction mixture, incubated at 38°, was: 40 mM CH_3-dCMP, 0.025 ml; 0.1 M phosphate buffer, pH 7.3, 0.150 ml; enzyme from 60 to 120 units; H_2O to a final volume of 0.5 ml. The final concentration of CH_3-dCMP was 2 mM. At zero time and after 5 and 10 minutes, 0.1-ml aliquots were removed from the reaction mixture and were deproteinized by the addition of 0.1 ml of 10% $HClO_4$. After high speed centrifugation (14,000 × *g* at 4° for 10 minutes), 0.1 ml of the supernatants was diluted with 2.4 ml of 0.01 N HCl, and the absorbancy of these solutions at 273 mμ and at 290 mμ was determined. One unit of enzyme was defined as the amount of enzyme causing a decrease in the absorbancy at 290 mμ of 0.001 per 10 minutes under the conditions of the assay. When Assay 1 was used for determining the dCMP aminohydrolase activity of the fractions, 3 mM dCMP was present in the reaction mixture and the absorbancy readings were taken at 267 mμ and at 280 mμ. Assay 1 was used up to Step 4.

In Assay 2, the rate of CH_3-dCMP aminohydrolysis was followed as the decrease in absorbancy at a wave length of 295 mμ by determining at intervals of 30 seconds the absorbancy of the reaction mixture at 23° ± 2° in quartz microcells (d = 1.0 cm) in the Beckman model DU spectrophotometer. The composition of the reaction mixture was as follows: 0.1 M phosphate buffer, pH 7.3, 0.1 ml; 0.14 M 2-mercaptoethanol, 0.01 ml; 6 mM

CH_3-dCMP, 0.025 ml; enzyme from 80 to 160 units; H_2O to a final volume of 0.3 ml. The final concentration of CH_3-dCMP was 0.5 mM. One unit of enzyme was taken as the amount of enzyme causing a decrease in the absorbancy at 295 mμ of 0.0005 per minute under the conditions of the assay. The units as defined for the two assay methods are equivalent for the purpose of the purification procedure. Under the conditions of Assay 2, the aminohydrolysis of 0.1 μmole of CH_3-dCMP per ml gives a ΔA at 295 mμ of −0.280; an isosbestic point at a wave length of 274.5 ± 0.5 mμ is observed.

When Assay 2 was used for determining the dCMP aminohydrolase activity of the fractions, the decrease in absorbancy was measured at a wave length of 285 mμ in quartz microcells (d = 0.10 cm). The composition of the reaction mixture was: 0.1 M phosphate buffer, pH 7.3, 0.1 ml; 60 mM dCMP, 0.025 ml; 0.14 M 2-mercaptoethanol, 0.010 ml; enzyme from 300 to 700 units; H_2O to a final volume of 0.3 ml. The final concentration of dCMP was 5 mM. The aminohydrolysis of 0.1 μmole of dCMP per ml gives a ΔA per 1.0-cm light path at 285 mμ of −0.310; an isosbestic point at a wave length of 270 ± 0.5 mμ is observed.

In Assay 2, the reference cuvette contained a solution absorbing in the ultraviolet that permitted suitable initial absorbancy readings of the experimental cuvettes.

In experiments in which compounds with high molar absorbancy in the ultraviolet were tested as inhibitors of the enzyme, the dCMP aminohydrolase activity was measured by colorimetric determination of the ammonia set free with the Nessler reagent. The composition of the reaction mixture was: 0.2 M phosphate buffer, pH 7.1, 0.150 ml; 60 mM dCMP, 0.05 ml; enzyme from 120 to 480 units; H_2O to a final volume of 0.5 ml. The final concentration of dCMP was 6 mM. The incubation was made at 38° and the ammonia was determined on aliquots of the reaction mixture after 1, 4, and 7 minutes of incubation. No previous deproteinization was necessary when enzyme preparations from the ethanol II step and subsequent steps were used. Under the indicated conditions, the formation of ammonia was a linear function of time and a linear function of the concentration of enzyme. When 350 units of enzyme were used, 0.3 μmole of ammonia was formed in 3 minutes.

Purification Procedure

All operations, with the exception of the ethanol fractionations, were performed at about 0°. The ethanol fractionations were carried out in a freezing bath at −15°.

Livers of monkeys of the species *Cercopithecus* from Ethiopia were obtained from animals from which the kidneys were used for the preparation of the Salk antipoliomyelitis vaccine. The livers were chilled at 0°, approximately one-half hour after the death of the animals; and after approximately another 45 minutes, the purification procedure was started.

Step 1. Ammonium Sulfate Fractionation—The livers, freed of the gall bladders and of the principal biliary ducts, were minced with scissors; 330 g of minced tissue were suspended in 960 ml of 0.1 M phosphate buffer, pH 7.3. The suspension was homogenized in a Waring Blendor at low speed for 15 minutes. The homogenate was centrifuged in a Spinco preparative ultracentrifuge (head No. 21 at 19,000 r.p.m. for 60 minutes), and the sediment was discarded. Finely powdered ammonium sulfate was added slowly to the supernatant solution with mechanical stirring in the proportion of 209 g per liter. After being stirred for an additional 30 minutes, the mixture was centrifuged in a Spinco preparative ultracentrifuge (head No. 21, at 19,000 r.p.m. for 45 minutes), and the precipitate was discarded. Ammonium sulfate, 129 g per liter, was added slowly, with mechanical stirring, to the supernatant solution. After an additional stirring for 30 minutes, the precipitate was collected by centrifugation, as above, and the clear supernatant was discarded. The precipitate was dissolved in approximately 130 ml of 0.02 M phosphate buffer, pH 7.3; the protein concentration was about 40 mg per ml.

A second method for Step 1 was worked out in which the homogenization was done in ammonium sulfate solution so as to eliminate one centrifugation and also to minimize the contact of the experimenter with probably infectious material. Minced liver, 330 g, suspended in 970 ml of a solution containing 209 g of ammonium sulfate per liter of H_2O, was homogenized in a Waring Blendor at low speed for 8 minutes. Finely powdered ammonium sulfate, 51.5 g, was added slowly to the suspension, and the homogenization was continued for an additional 10 minutes. The suspension was centrifuged in a Spinco preparative ultracentrifuge (head No. 21, at 19,000 r.p.m. for 60 minutes), and the precipitate was discarded. Very often, the livers were fatty, and a thick layer of lipid material separated on the top of the centrifuge tube. This thick layer was easily removed mechanically before the supernatant was collected. The supernatant was passed through four layers of cotton gauze. Ammonium sulfate, 129 g per liter, was added to the supernatant, and the subsequent operations were as described previously.

The two procedures for Step 1 give equivalent enzyme preparations, which also do not differ significantly in subsequent steps of the purification.

The second method for Step 1 has been used for the purification described in Table I and Table VII, while the first method for Step 1 has been used in the experiments of identification of dPAase in tissue extracts.

In the preparation described in Table I, 1600 g of liver tissues were used, working up 330 g at a time during the first step.

Step 2. Ethanol Fractionation I—To the solution from the preceding step, absolute ethanol, previously chilled to −18°, was added slowly with continuous stirring to achieve a final concentration of 32% (volume for volume). During the addition of the ethanol, the suspension was in a freezing bath at −15°, and cooled to −10°. After 10 minutes, the suspension was centrifuged at −15° in an International model PR-2 refrigerated centrifuge (head No. 855 at 9800 r.p.m. for 20 minutes), and the precipitate was discarded. The clear supernatant was brought to 50% (volume for volume) with absolute ethanol previously chilled at −18°, at a rate such that the temperature stayed at −10°. After 10 minutes, the suspension was centrifuged, as above, and the clear supernatant was discarded. The precipitate was suspended in 0.2 M phosphate buffer, pH 7.3, homogenized in a glass homogenizer and centrifuged at 0° in an International model PR-2 refrigerated centrifuge (head No. 855 at 9800 r.p.m. for 20 minutes). The precipitate was discarded, and the clear supernatant was saved for the next step.

Step 3. Removal of Inactive Proteins by Adsorption on Alumina Gel—The pH of the solution from the preceding step was adjusted to 5.7 by slow addition, with mechanical stirring, of 1 N acetic acid. A suspension of alumina gel (20 mg per ml) was added slowly, with mechanical stirring, in the proportion of 1

TABLE I

Purification of 2'-deoxyribosyl 4-aminopyrimidone-2,5'-phosphate aminohydrolase from monkey liver

Step	Volume	Units per ml	Total units $\times 10^{-3}$	Proteins	Total proteins	Specific activity	Yield
	ml			*mg/ml*	*mg*	*units/mg protein*	%
1. $(NH_4)_2SO_4$ fractionation	680	3,380	2,298	38	25,840	89	100
2. Ethanol fractionation I	400	4,200	1,680	4.7	1,880	890	73
3. Alumina gel supernatant	440	3,500	1,540	2	880	1,750	67
4. Ethanol fractionation II	40	22,000	880	5.4	216	4,100	38
5. DEAE-cellulose chromatography	300	2,275	683	0.03	9	75,830	30
6. Ethanol precipitation I	27.5	14,000	385	0.15	4.1	93,300	17
7. Ethanol precipitation II	9.35	31,000	290	0.24	2.2	129,200	13
8. $(NH_4)_2SO_4$ fractionation	1.5	116,000	174	0.48	0.7	241,600	8

ml/10 ml of the solution. After being stirred for another 15 minutes, the gel was removed by centrifugation and discarded. The supernatant solution was brought to pH 7 by addition of a solution of Tris saturated at 0°.

Step 4. Ethanol Fractionation II—To the enzyme solution from the previous step, absolute ethanol, chilled to −18°, was added slowly with continuous stirring, to achieve a final concentration of 15% (volume for volume). During the addition of the ethanol, the suspension was kept in a freezing bath at −15° and cooled to −10°. After 5 minutes in the freezing bath at −15°, the suspension was centrifuged at −15° in an International model PR-2 refrigerated centrifuge (head No. 855 at 9800 r.p.m. for 15 minutes), and the precipitate was discarded. In a freezing bath at −15°, the clear supernatant was brought to 35% (volume for volume) with absolute ethanol at −18°, at such a rate that the temperature stayed at −10°. After 10 minutes in the freezing bath at −15°, the suspension was centrifuged as above, and the clear supernatant was discarded. The precipitate was suspended in 0.01 M phosphate buffer, pH 7.3.

Step 5. Chromatography on DEAE-cellulose—DEAE-cellulose

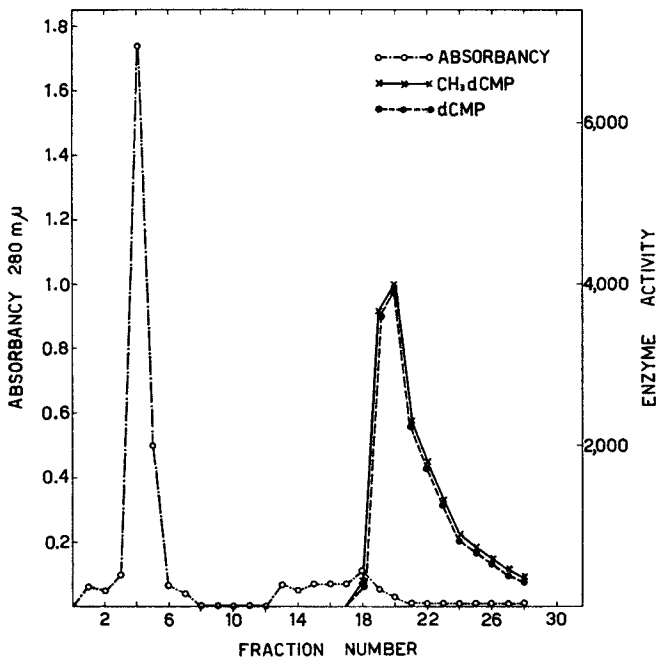

FIG. 1. Elution diagram on DEAE-cellulose of the enzyme from monkey liver. The enzyme activities are per ml of eluate. Details are given in the text.

(1), 30 g, was suspended in 1.5 liters of H_2O and light particles, which did not settle within 15 minutes, were removed by aspiration. The procedure for removing light particles was repeated by resuspending the settled resin in 1.5 liters of H_2O. The cellulose was resuspended in H_2O, 1.5 liters, and degased under a vacuum. This suspension was packed into a column, 4.5 × 22 cm, and washed in succession with 3 liters of 1 N HCl, with H_2O until the effluent was neutral, with 3 liters of 1 M Na_2CO_3, with H_2O until the effluent was neutral, with 3 liters of 1 N NaOH, and then with H_2O until the effluent was neutral. The column was equilibrated with 0.01 M phosphate buffer, pH 7.3, containing 2.8 mM 2-mercaptoethanol. Throughout the washings and the subsequent chromatography, the pressure on the top of the column was 27 ± 1 cm of Hg. A rate of 50 ml per 30 minutes was maintained for the first 100 ml of effluent after the enzyme solution had been applied to the column; the subsequent fractions were collected at a rate of 50 ml per 15 minutes. The rate of the effluent was regulated by adjusting the dropping from the tip of the column, and 50-ml fractions were collected throughout the whole column chromatography.

The enzyme solution from the previous step was passed through the column. The column was washed in succession with 400 ml of 0.01 M phosphate buffer, pH 7.3, with 250 ml of 0.05 M phosphate buffer, pH 7.3, and the enzyme was eluted with 0.1 M phosphate buffer, pH 7.3. The pattern of the chromatography is described in Fig. 1. The elution of protein was followed spectrophotometrically by determining the absorbancy at 280 mμ. Enzyme activity determinations were made on each fraction by Assay 2.

The data reported in Table I for the DEAE step were obtained from the pooled Fractions 19 to 24. The protein content was determined spectrophotometrically (12), and since the absorbancy readings were low, the protein determination and consequently the specific activity for the fractions of the DEAE step have only an indicative value.

Step 6. Ethanol Precipitation I—The effluents of the tubes from the DEAE column containing the enzyme were pooled in two groups, each of three successive tubes. To each group, consisting of 150 ml of effluent, were added 40 mg of EDTA and absolute ethanol (chilled to −18°), to achieve a final concentration of 55% (volume for volume). The addition of the ethanol was done as described in Step 4. When the addition of the ethanol was completed, the suspension was kept under continuous mechanical stirring at −15°, for 30 minutes. The suspension was centrifuged at −15° in an International model PR-2 refrigerated centrifuge (head No. 855 at 9800 r.p.m. for 30 minutes). The

supernatant was discarded, and the precipitate was suspended in as small a volume as possible of 0.1 M phosphate buffer, pH 7.3, containing 2.8 mM 2-mercaptoethanol. The suspension was centrifuged at 0° at 18,000 × *g* for 30 minutes. The precipitate was washed twice with the phosphate buffer. Supernatant and washings were pooled. The proteins of this enzyme solution were determined spectrophotometrically (12).

Step 7. Ethanol Precipitation II—To the solution of the enzyme from the previous step, absolute ethanol, chilled to −18°, was added to achieve a final concentration of 60% (volume for volume). The addition of the ethanol and the collection of the precipitate was done as described in Step 4. The precipitate was suspended in 0.1 M phosphate buffer, pH 7.3, containing 2.8 mM 2-mercaptoethanol. The suspension was centrifuged at 0° at 18,000 × *g* for 30 minutes. The precipitate was discarded and the supernatant kept for the subsequent step. The protein content of the enzyme fraction was determined spectrophotometrically (12). Steps 5, 6, and 7 must be carried out in rapid succession since the enzyme in the effluent from the DEAE column is very unstable. The EDTA that was added before the two ethanol precipitation steps seemed to increase the recovery of enzyme.

Step 8. Ammonium Sulfate Fractionation II—Finely powdered ammonium sulfate, 296 mg, twice recrystallized from 0.1 mM EDTA, was added slowly to each milliliter of the enzyme solution from the preceding step. The resulting suspension, after 24 hours at 0°, was centrifuged in an International model PR-2 refrigerated centrifuge (head No. 296 at 13,800 r.p.m. for 60 minutes), and the precipitate was discarded. To each milliliter of the clear supernatant, 135 mg of ammonium sulfate were added, and the suspension was kept for 48 hours at 0°. The precipitate was collected by centrifugation as mentioned and was dissolved in 0.1 M phosphate buffer, pH 7.3.

Reproducibility of Purification Procedure—All the steps of the purification procedure except Step 4 are easily reproducible; slight changes in the procedure of the ethanol fractionation II cause the enzyme to precipitate at an ethanol concentration higher than the one described here.

Sometimes enzyme preparations at Step 8 with specific activity as low as 120,000 were obtained. The best preparations resulted when fatty livers were used.

The homogeneity of the enzyme preparation has not yet been studied because of the limited amount of the purified enzyme available.

Properties of Enzyme

Stability of Enzyme Preparations—The centrifuged homogenate at 4° loses 15% of its activity in 20 hours. The ammonium sulfate fraction, kept as a paste at −20°, retained its full activity for a period of 10 months; whereas if it was kept in solution, it lost all its activity in the same period of time. The enzyme fractions from Steps 2 and 3 are stable at −20°; a preparation at Step 3, containing 15 mg of protein per ml, was kept at −20° for 10 months without any loss of activity; a preparation at Step 3, containing 4 mg of protein per ml, was kept at −20° for a month without any loss of activity. The enzyme fraction at Step 4 seems to be less stable than the enzyme fractions at Steps 2 and 3; a preparation at Step 4, kept at −20° for a month, lost 20% of its activity. The enzyme in the DEAE-cellulose fractions was completely stable for 1 hour at 0°, but after 12 hours it was found to be inactive. The enzyme fractions at Steps 7 and 8 are stable; preparations have been kept at −20° for 2 months without any loss of activity.

TABLE II

First order rate constants of thermal inactivation of 2′-deoxyribosyl 4-aminopyrimidone-2,5′-phosphate aminohydrolase

Enzyme with a specific activity of 21,400 was incubated at the indicated temperatures in 0.1 M phosphate buffer, pH 7.3, containing 2.7 mM 2-mercaptoethanol; the protein concentration was 1.5 mg per ml. At suitable time intervals, aliquots were taken and assayed for aminohydrolase activity. Each value is the average of 10 determinations.

E_a = 62.5 kilocalories mole^{-1}; standard deviation, 14.8.

Temperature	k min^{-1}	Standard deviation
34.8	0.0167	0.0047
38.9	0.102	0.023
41.0	0.154	0.021
43.2	0.310	0.05

When assaying the enzyme from Step 5, care must be taken to add the substrate before the enzyme in the incubation mixture; if the enzyme is diluted in absence of the substrate, very fast inactivation ensues at room temperature. The inactivation of the enzyme at Step 4 was studied as a function of temperature, in absence of substrate. The inactivation follows first order rate kinetics. The first order rate constants, k in Table II, were calculated by using the equation

$$k = \frac{1}{t} \ln \frac{a}{a_t}$$

in which a is equal to the initial amount of enzyme, and a_t is equal to the amount of enzyme at time t in minutes. From the data of Table II, the energy of activation, E_a, of dPAase inactivation was calculated by use of equation

$$E_a = R \frac{T_2 \cdot T_1}{T_2 - T_1} \ln \frac{k_2}{k_1}$$

in which R is the gas constant, k_1 is the rate constant at the absolute temperature T_1, and k_2 is the rate constant at the absolute temperature T_2; a value of 62.5 kilocalories mole^{-1} with a standard deviation of 14.8, was obtained. A straight line with deviations from linearity, within the limits of the experimental errors, resulted by plotting ln k against $1/T$; the slope of the line was equal to $-E_a/R$. The first order rate of the thermal inactivation, of dPAase and the E_a value of the same inactivation, indicate that the enzyme is inactivated as a consequence of thermal protein denaturation.

The inactivation of the aminohydrolase following incubation at 38° was prevented by addition of dCMP, dUMP, CH_3-dCMP, dTMP, and 2′,3′-GMP. 5′-CMP and deoxycytidine did not protect the enzyme. It seems that, in general, all compounds that were competitive inhibitors of dPAase, did also exhibit a protective effect on the thermal inactivation of the enzyme. Fig. 2 illustrates the protective effect of dTMP on the thermal inactivation of dPAase. Myers (13) reported stabilization by nucleotides of dPAase in centrifuged homogenate of rat thymus.

Stoichiometry, Equilibrium, and Identification of Products—The stoichiometry of the aminohydrolysis of dCMP and CH_3-dCMP was studied with enzyme from monkey liver (specific activity 4200). The spectrophotometric determination of the amino-

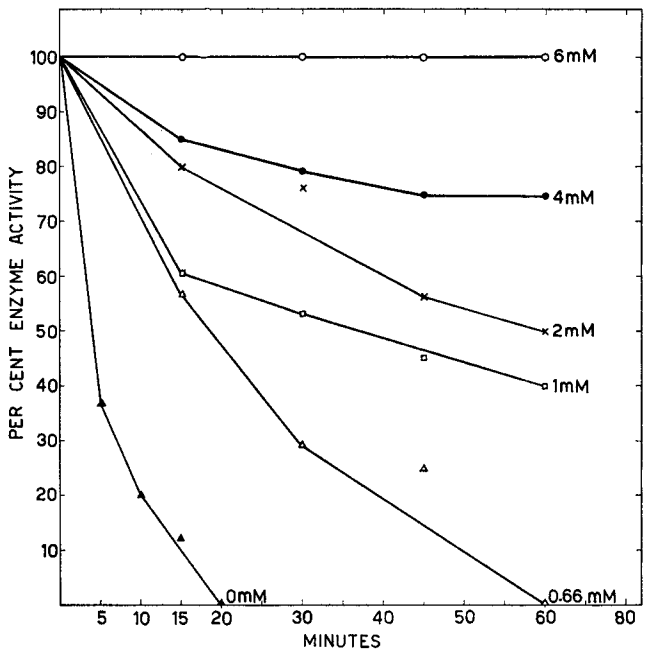

FIG. 2. Protection of thermal inactivation of dPAase by dTMP. Enzyme (specific activity, 1700) was incubated at 38° in 0.1 M phosphate buffer, pH 7.3, containing the indicated amount of dTMP; the protein concentration was 19 mg per ml. At suitable time intervals, aliquots were taken and assayed for dPAase activity with Assay 2.

TABLE III

Stoichiometry of aminohydrolysis by 2'-deoxyribosyl 4-aminopyrimidone-2,5'-phosphate aminohydrolase

The experiments were performed at 38° in phosphate buffer at pH 7.3 for 120 minutes; enzyme, 2250 units per ml, except for the experiment with CH_2OH-dCMP, for which see the text.

Compounds	Initial concentration	Final concentration
	mM	
dCMP	1.74	0
dUMP	0	1.53
NH_3	0	1.70
CH_3-dCMP	1.56	0
dTMP	0	1.56
NH_3	0	1.56
CH_2OH-dCMP	1.0	0
CH_2OH-dUMP	0	1.0

hydrolysis of the nucleotides, the colorimetric determination of NH_3 production, and the quantitative isolation of the products on a Dowex 1-Cl^- column were carried out on suitable aliquots of a single incubation mixture. Table III summarizes the experiments. dUMP and dTMP, isolated from the incubation mixtures, were identified as previously described (1, 5).

For the identification of CH_2OH-dUMP as the product of the aminohydrolysis of CH_2OH-dCMP, 1 μmole of CH_2OH-dCMP was incubated at 20° in the presence of 0.1 M phosphate buffer, pH 7.3, and 3,300 units of enzyme (specific activity, 60,000) for 3 hours. The final volume was 1.2 ml. CH_2OH-dUMP, 1 μmole, was isolated on a Dowex 1-Cl^- column as described previously for the isolation of dUMP (1, 5). The nucleotide was identified by the position in the elution chromatogram and by paper chromatographic identification of CH_2OH-uracil obtained by acid hydrolysis of the isolated nucleotide. The acid hydrolysis of CH_2OH-dUMP was carried out in HCOOH, 98% at 175° for 30 minutes.

The data in Table III indicate that in the presence of large amounts of dPAase and after prolonged incubation the aminohydrolysis of CH_3-dCMP, dCMP, and CH_2OH-dCMP go to completion within the limits of experimental error.

The aminohydrolysis of CH_3-dCMP and of dCMP as a function of time are shown in Fig. 3. With 700 units of enzyme per ml of incubation mixture, the aminohydrolysis of 0.125 μmole of CH_3-dCMP is complete in 8 minutes, while the aminohydrolysis of 0.097 μmole of dCMP is still only 93% complete after 160 minutes.

Effect of Substrate Concentration—Fig. 4 is a plot, after Lineweaver and Burk (14), of the velocity of aminohydrolysis of CH_3-dCMP as a function of substrate concentration. Inhibition by high substrate concentration is indicated by the *open circles* on the left of Fig. 4. The *open circles* on the right of the same figure indicate inhibition by product at low substrate concentration. The equation of the line in the interval of linearity, calculated by the method of least squares (15), is

$$\frac{1}{V} = 2.750 + 0.629\,\frac{1}{S}$$

The Michaelis-Menten constant, K_m, calculated according to Lineweaver and Burk (14), is 0.23 mM.

The K_m values for dCMP and CH_2OH-dCMP are 2.15 mM and 1.31 mM, respectively. In the experiments from which the K_m for dCMP was derived, 2500 units of enzyme per ml, a sub-

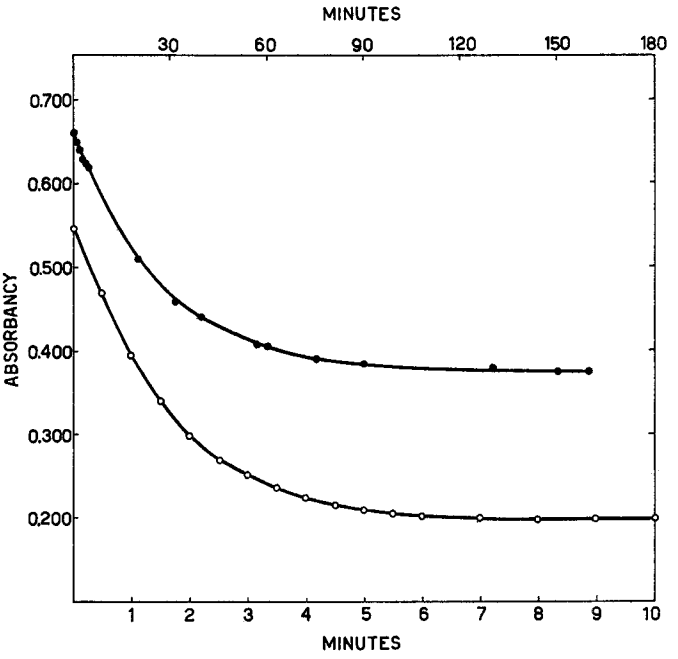

FIG. 3. Time dependence of aminohydrolysis of CH_3-dCMP (○——○) and of dCMP (●——●). For CH_3-dCMP, the absorbancy refers to 295 mμ, and the time is indicated at the *bottom*, whereas for dCMP, the absorbancy refers to 285 mμ, and the time is indicated at the *top* of the figure. CH_3-dCMP, 0.125 μmole, or dCMP, 0.097 μmole, was incubated with 700 units of enzyme (specific activity, 15,400). The final volume was 1 ml, and other conditions were as in Assay 2.

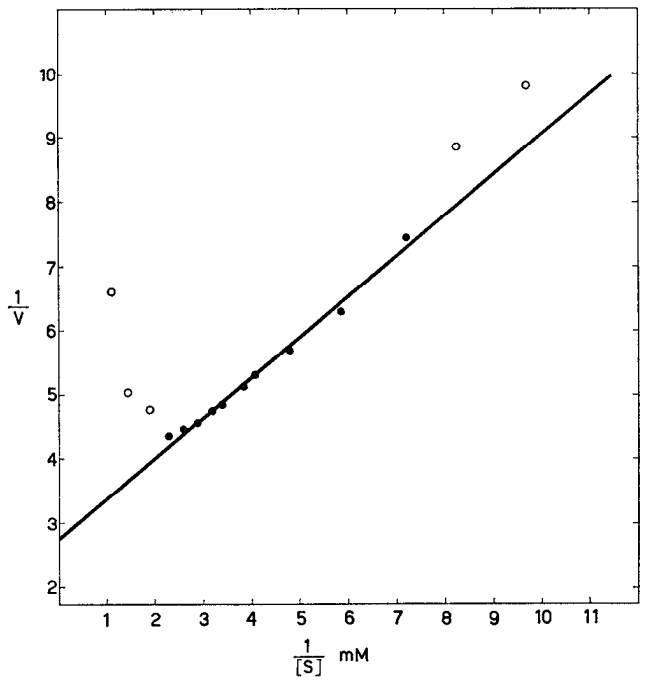

FIG. 4. Relation between CH_3-dCMP concentration and rate of aminohydrolysis. Each cuvette contained 1.5 ml of 0.1 M phosphate buffer, pH 7.2, 3000 units of enzyme (specific activity 3800), and the indicated quantity of substrate in a total volume of 2 ml. The temperature was 23° ± 2°. The velocity is expressed as ΔA at 295 mμ per minute and per 10-mm light path. The experimental points indicated by ○ have not been considered in the calculation of the equation of the line.

strate concentration from 0.7 to 2.3 mM, cuvettes with a light path of 0.10 cm and other conditions as in Assay 2 were used. The same conditions were used in the experiments from which the K_m value for CH_2OH-dCMP was derived, except that substrate concentrations of 0.22 to 1.65 mM and 2750 units of enzyme per ml were employed. The experimental deviations from linearity were negligible in both instances and the equations, the constants for which were determined by the method of least squares (15), are for dCMP and CH_2OH-dCMP, respectively:

$$\frac{1}{V} = 0.437 + 0.940\,\frac{1}{S}$$

$$\frac{1}{V} = 0.624 + 0.819\,\frac{1}{S}$$

in which V is expressed as ΔA at 285 mμ per 1.0-cm light path per minute, and S in millimoles per liter.

The V_{max} under the conditions in which the K_m were measured and referred to an enzyme concentration of 1500 units per ml of incubation mixture are, for CH_3-dCMP, dCMP, and CH_2OH-dCMP, 0.13, 0.44, and 0.24 μmole per minute per ml of incubation mixture, respectively.

Turnover Number—By measuring dPAase activity with ammonia formation under the conditions of the colorimetric assay described, it has been calculated that the enzyme preparation at Step 8 catalyzes the aminohydrolysis of 6,900 moles of dCMP per minute per 100,000 g of protein at pH 7.1 and 38°.

Effect of pH—The aminohydrolysis of dCMP as a function of pH was studied by using Assay 1 with the universal buffer of Teorell and Stenhagen (16) in place of the phosphate buffer. An enzyme of specific activity of 3800 was used. The incubation mixtures contained 120 units of enzyme. The incubation mixtures with which the pH optimum for CH_3-dCMP aminohydrolysis was measured, contained 0.33 mM CH_3-dCMP and 400 units of enzyme per ml. The absorbancy determinations were made at pH 2 on a 9.4 times diluted incubation mixture at 15-, 75-, and 135-second intervals. Fig. 5 shows the results.

With the use of 3mM CH_3-dCMP, a pH optimum of 9 was obtained, indicating that the pH optimum is dependent on the substrate concentration.

Substrate Specificity—The relative activities using CH_3-dCMP and dCMP as substrate did not change throughout the purification of the monkey liver dPAase. The purified enzyme is active on CH_3-dCMP, dCMP, and CH_2OH-dCMP. dPAase from monkey liver, tested at Step 4 of the purification procedure, does not deaminate 3′-dCMP, dCDP, dCTP, 5′-CMP, 2′,3′-CMP, deoxycytidine, 5-methyldeoxycytidine, cytidine, cytosine, dGMP, 5-hydroxymethylcytosine, 5-methylcytosine, dAMP, 5′-AMP, 2′,3′-AMP, deoxyadenosine, adenosine, dCDP-choline, and CDP-choline at concentrations of 0.1, 0.2, 1, and 2 mM in 0.1 M phosphate buffer, pH 7.3, and with a large excess of enzyme. These results, obtained by determining the absorbancy change at the appropriate wave lengths, were confirmed by colorimetry with Nessler's reagent. Under the conditions of incubation of Table III, 5′-CMP, deoxycytidine, and 5-CH_3-deoxycytidine were recovered by ion exchange chromatography in 100% yields, and no ammonia production was detected.

Effect of Pyrimidine and Purine Compounds and of $(NH_4)_2SO_4$—Table IV lists nucleotides that were found to inhibit the monkey liver enzyme. The following compounds, tested up to the concentrations indicated, did not affect the enzyme activity: 5′-UMP, 6.2 mM; 2′,3′-UMP, 7.5 mM; deoxyuridine, 10 mM. The experiments were done with enzyme at Step 4 and by Assay 1 with dCMP as substrate.

K_i values for dTMP, dUMP, and 5′-GMP of 0.45, 1.0, and 0.4 mM, respectively, were obtained under the conditions of

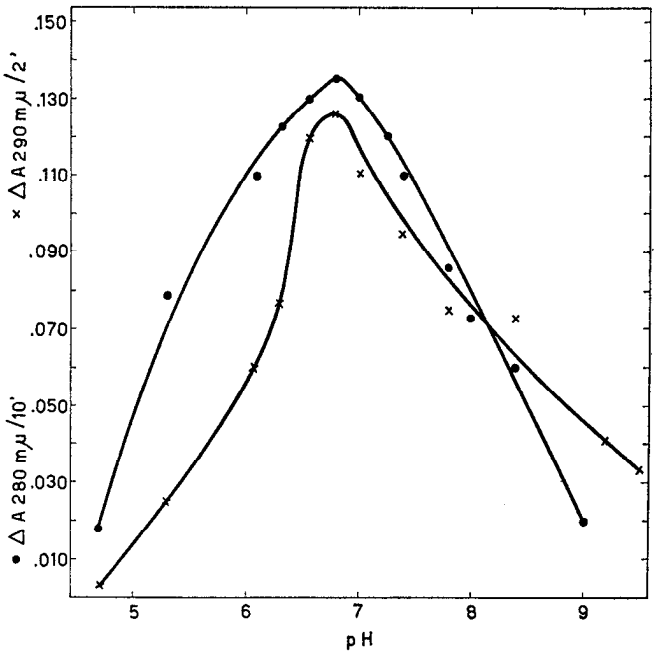

FIG. 5. Dependence of the rate of CH_3-dCMP aminohydrolysis (×——×) and of dCMP-aminohydrolysis (●——●) on the pH.

Assay 2 with CH_3-dCMP as substrate in concentrations ranging from 0.1 to 0.2 mM. The K_i values were calculated by plotting the experimental results after Dixon (17); the plots showed that the inhibitions were of the competitive type.

$(NH_4)_2SO_4$, tested up to a concentration of 30% saturation, did not inhibit dPAase.

Effect of Metals, Sulfhydryl-binding Agents and of Reducing Agents—As shown by the data of Table V, dPAase from monkey liver is inhibited by metals and by sulfhydryl-binding reagents. $MgCl_2$, $MnSO_4$, $FeCl_3$, $ZnSO_4$, $Co(NO_3)_2$, and $Pb(NO_3)_2$ tested up to a concentration of 3 mM did not inhibit the enzyme.

The inhibition by metals is counteracted by 2-mercaptoethanol (Table VI).

Reduced glutathione, 2-mercaptoethanol, and EDTA did not affect the enzyme activity. A protective effect by 2-mercaptoethanol and by EDTA on the storage of some preparations of dPAase was observed.

Identification of 2'-Deoxyribosyl 4-Aminopyrimidone-2,5'-phosphate Aminohydrolase in Tissues of Several Warm-blooded Animals—The purification procedure worked out with monkey liver was used up to Step 7 to purify dPAase from rabbit liver and comparable results were achieved. Rabbit liver, 320 g, was used for the preparation described in Table VII.

TABLE IV

Inhibition of 2'-deoxyribosyl 4-aminopyrimidone-2,5'-phosphate aminohydrolase by nucleotides

Nucleotide	Concentration	Inhibition
	mM	%
dGMP	1.0	30
	2.0	69
	3.0	100
dTMP	0.7	52
	1.4	67
	2.1	87
dUMP	1.0	21
	2.0	40
	3.0	80
dAMP	1.0	48
	2.0	73
	3.0	80
5'-AMP	6.6	13
2',3'-AMP	4.0	23
	8.0	33
5'-CMP	4.0	40
	8.0	47
2',3'-CMP	8.8	37
5'-GMP	0.3	20
	0.6	67
	1.2	100
2',3'-GMP	2.3	34
	3.4	70
	4.5	75

TABLE V

Inhibition of 2'-deoxyribosyl 4-aminopyrimidone-2,5'-phosphate aminohydrolase by metals and sulfhydryl reagents

The experiments were performed under the conditions of Assay 1 except for the presence of the indicated concentration of the inhibitor; 300 units per ml of enzyme (specific activity, 3800) were used in each incubation mixture.

Compounds	Concentration	Inhibition
	mM	%
$CuSO_4$	0.002	38
	0.005	66
	0.01	100
$AgNO_3$	0.002	15
	0.01	56
	0.05	100
$FeSO_4$	1.0	35
	2.0	68
$HgCl_2$	0.0005	17
	0.001	30
	0.005	100
$SnCl_2$	2.0	17
$K_3[Fe(CN)_6]$	0.5	10
p-Chloromercuribenzoate	0.005	11
	0.01	35
	0.015	75
	0.02	100
o-Iodosobenzoate	0.06	53
	0.13	63
Monoiodoacetate	1.0	0
	2.0	0

TABLE VI

Inhibition of 2'-deoxyribosyl 4-aminopyrimidone-2,5'-phosphate aminohydrolase by metals in the presence of 2-mercaptoethanol

Conditions as in Table V except for the presence of 1.4 M 2-mercaptoethanol.

Compounds	Concentration	Inhibition
	mM	%
$CuSO_4$	2.0	56
	4.0	68
$FeSO_4$	4.0	0
$AgNO_3$	2.0	0
$HgCl_2$	2.0	6

No change in the ratio, CH_3-dCMP aminohydrolase activity over dCMP aminohydrolase activity, was observed throughout the purification. The rabbit liver dPAase at Step 4 did not act on 5'-CMP, deoxycytidine, cytidine, or 5-CH_3-deoxycytidine; no other compounds were tested. The enzyme from rabbit liver was not tested with CH_2OH-dCMP.

TABLE VII

Purification of 2′-deoxyribosyl 4-aminopyrimidone-2,5′-phosphate aminohydrolase from rabbit liver by procedure worked out with monkey liver

Step	Volume	Units per ml	Total units $\times 10^{-3}$	Proteins	Specific activity	Yield
	ml			*mg/ml*	*units/mg protein*	%
1. $(NH_4)_2SO_4$ fractionation	180	3,200	576	52.4	61	100
2. Ethanol fractionation I	80	4,700	376	8.5	552	65
3. Alumina gel supernatant	97	3,400	330	3.7	920	57
4. Ethanol fractionation II	17.5	17,000	298	10.85	1,570	52
5. DEAE-cellulose chromatography	150	1,000	150			26
6. Ethanol precipitation I	18.3	8,000	146			25
7. Ethanol precipitation II	4.2	16,000	67	0.57	28,000	12

TABLE VIII

Specific activity of 2′-deoxyribosyl 4-aminopyrimidone-2,5′-phosphate aminohydrolase of centrifuged homogenates from tissues of warm-blooded animals

The determinations were made with Assay 1, with the use of CH_3-dCMP as substrate.

Source	Specific activity
	units/mg protein
Adult monkey liver	31
Chick embryo liver	35
Rat embryo liver	18
Embryonic rat tissues	29
Adult rabbit liver	13
Regenerating rabbit liver	24
Human embryo liver	65
Ehrlich ascites cells	66
Adult rat liver[2]	5
Dunning hepatoma[2]	6

In Table VIII are reported the specific activities of centrifuged homogenates of tissues from several warm-blooded animals and the specific activities of an enzyme fraction obtained from adult rat liver and Dunning hepatoma. Embryonic rat liver, embryonic chicken liver, embryonic human liver, and Ehrlich ascites cells were fractionated with ammonium sulfate as described in this paper in Step 1, Method 1, of the purification procedure. Adult rat liver and Dunning hepatoma were fractionated as previously described (18). In all instances, an enzyme fraction that exhibited the substrate specificity of dPAase was obtained; the isolation and the identification of the unreacted substrates and of the products, dCMP, CH_3-dCMP, dUMP, and dTMP, was made as previously described (1, 5); the compounds were recovered as nucleotides in yields ranging from 80 to 100%. The absence of phosphate donors in the incubation mixtures and substrate specificity experiments exclude the possibility of the sequence of reactions, NH_2-deoxynucleotide → NH_2-deoxynucleoside → OH-deoxynucleoside → OH-deoxynucleotide. The experiments of enzyme fractionation give direct evidence of the presence of dPAase in the tissues that have been examined and with the exception of adult rat liver and Dunning hepatoma support the quantitative significance of the data in Table VIII.

[2] After fractionation as previously described (18).

DISCUSSION

The results on the purification of the monkey and rabbit liver 2′-deoxyribosyl 4-aminopyrimidone-2,5′-phosphate aminohydrolase confirm the conclusion, drawn from the previous work on the sea urchin egg dPAase (1), concerning the failure to resolve separate aminohydrolases for dCMP, CH_3-dCMP, and CH_2OH-dCMP. A pertinent fact is that the increase in specific activity in the over-all purification of the monkey liver dPAase is 8000 times over the specific activity of the centrifuged homogenate. The experiments with tissues from warm-blooded animals reported in this paper also support the conclusion that a single enzyme is present.

Of practical interest is the fact that the purification procedure that was worked out with monkey liver produces similar results as well with the rabbit liver enzyme both as regards the increase in specific activity at each step and as regards the final yield.

It is important to point out the striking similarities of dPAase purified from such different sources as monkey liver and eggs of the sea urchin *Sphaerechinus granularis* (1); both enzymes have the same substrate specificity and their properties are similar.

dPAase is widely distributed in nature; the enzyme has been found in echinoderma (1), in eggs and embryos of the frog,[3] in fishes, in warm-blooded animals, and in bacteria (19). Unfertilized eggs of *Sphaerechinus granularis*, monkey liver, and rabbit liver contain, respectively, 7000, 1900, and 1800 units of the enzyme per g of fresh material.

The occurrence of dPAase may even be wider than our studies indicate, since experiments indicating absence of the enzyme should be interpreted with caution in view of its high lability. The lability of dPAase makes it also difficult to determine the functional role of the enzyme from experiments with homogenates; dPAase may be inactivated in the first few minutes of the incubation.

The time dependence of the aminohydrolysis of CH_3-dCMP and of dCMP is of interest. As indicated by the data in Fig. 3, the aminohydrolysis of dCMP is slowed down with time to a greater extent than the aminohydrolysis of CH_3-dCMP. The phenomenon may be explained by the fact that, although in both cases inhibition by product is effective, in the case of the aminohydrolysis of CH_3-dCMP the enzyme has higher affinity for the substrate than for the product, whereas in the case of

[3] B. De Petrocellis, P. Grant, and E. Scarano, unpublished experiments.

the aminohydrolysis of dCMP, it has higher affinity for the product than for the substrate. The kinetic properties of dPAase may be important in regulating the pattern of the pyrimidine deoxyribonucleotide pool in cells (1).

The data on the thermal inactivation of dPAase and its protection by nucleotides suggest another interpretation of the finding reported by Fiala and Fiala (20) concerning a microsomal inactivator of dPAase. The first order rate kinetics and the value of the activation energy of the inactivation of dPAase reported in the present paper point to protein denaturation as the mechanism of the inactivation. Furthermore, it is known (21) that a 5′-nucleotidase occurs in microsomal preparations; as a consequence, dCMP does not protect dPAase from inactivation at 38° in the presence of microsomes because it is converted to the nonprotecting nucleoside. Because of the likely presence of dCMP in the enzyme preparations of Fiala and Fiala (20), their inference of the occurrence in microsomes of a specific enzymatic inactivator of dPAase might require a reinterpretation.

The results reported in this paper and earlier (1) do not justify reference to dPAase as dCMP-aminohydrolase. The name dCMP-aminohydrolase is neither justified by substrate specificity nor indicated by what can now be deduced about the function of the enzyme. The name used in the present paper is indicative of the finding that of all the compounds tested, only the naturally occurring 4-aminopyrimidone-2 deoxyribonucleotides are substrates of the enzyme.

dPAase may be necessary for the biosynthesis of dTMP as has been discussed previously (1), and indeed several interesting papers by Maley and Maley (9, 22), by Sugino and Potter (23), and by Fiala *et al.* (9, 10) indicate such a function. The work by Eidinoff *et al.* (24), showing that CH_3-deoxycytidine is a very effective agent for reversing the effects of 5-fluorodeoxyuridine or 5-fluorodeoxycytidine, leads, although more indirectly, to the same conclusion. It seems reasonable to assume that in their experiments CH_3-deoxycytidine is phosphorylated to CH_3-dCMP, which is deaminated by dPAase to dTMP.

The formation of deoxyribonucleotides at the diphosphate level (25, 26) does not necessarily exclude the aminohydrolase step catalyzed by dPAase in the biosynthetic pathway to dTMP. The reactions involved may be the following.

$$2\ CDP \rightarrow 2\ dCDP \rightarrow dCMP + dCTP$$

dCTP would go directly into new DNA, while dCMP would be used for dTMP synthesis either through dUMP (27) or through CH_3-dCMP (1). On the other hand, dUDP, as Reichard *et al.* (26) have shown, is formed in *Escherichia coli* from UDP; dUDP could supply dUMP, and, as a consequence, the aminohydrolase step catalyzed by dPAase would be unnecessary for dTMP synthesis. dPAase until now has not been found in *E. coli* (5).

A necessary consideration at this point is the possibility of multiple alternative metabolic pathways, as emphasized by Potter (28); dPAase may catalyze an anabolic or catabolic alternative pathway of pyrimidine deoxyribonucleotide metabolism. Still another possibility is that dPAase while having no role in pyrimidine deoxyribonucleotide metabolism would function as a regulatory enzyme for DNA synthesis and as a consequence, for cell multiplication (29). Enzymes with no role in the sequence of a biosynthetic pathway may regulate that pathway by subtracting a necessary metabolite.

SUMMARY

An enzyme with 2′-deoxyribosyl 4-aminopyrimidone-2,5′-phosphate aminohydrolase activity has been purified from monkey liver.

The purified enzyme fraction from monkey liver catalyzes the aminohydrolysis of 6,900 moles of deoxycytidylic acid per minute per 100,000 g of protein at pH 7.1 and 38°. The following properties of the monkey liver enzyme have been studied: pH optimum, Michaelis-Menten constants, maximal velocities, substrate specificity, effect of pyrimidine and purine derivatives, of metals, of sulfhydryl-binding agents, and of reducing reagents. The thermal inactivation of the enzyme from monkey liver at Step 4 of the purification procedure has been investigated, and an activation energy for the reaction of inactivation of the enzyme of 62.5 kilocalories per mole with a standard deviation of 14.8 has been found. With the first seven steps of the purification procedure worked out with monkey liver, a 2′-deoxyribosyl 4-aminopyrimidone-2,5′-phosphate aminohydrolase has been purified 460-fold from rabbit liver.

Experiments that indicate the presence of 2′-deoxyribosyl 4-aminopyrimidone-2,5′-phosphate aminohydrolase in tissue extracts from warm-blooded animals have been reported.

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