Biochemistry

Volume 6, Number 7 July 11, 1967

The Deoxycytidylate Deaminase Found in *Bacillus subtilis* Infected with Phage SP8*

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ABSTRACT: The deoxcytidylate deaminase found in *Bacillus subtilis* infected with phage SP8 has been partially purified by fractionation on ion-exchange resin and by polyacrylamide gel electrophoresis. The enzyme deaminates 5-methyldeoxycytidylate at approximately one-fourth the rate of deoxycytidylate. It does not deaminate 5-hydroxymethyldeoxycytidylate. The SP8 dCMP deaminase is not inhibited by deoxythymidine triphosphate or 5-hydroxymethyldeoxyuridine triphosphate and it is not stimulated by deoxycytidine triphosphate, magnesium ions, or mercaptoethanol. The enzyme found in *B. subtilis* infected with phage SP8 is thus unique since all other known deoxycytidylate deaminases are regulated by feedback control. The marked increase in deoxycytidylate deaminase after infection of *B. subtilis* with phage SP8 is also found after infection with phage SP5C, another phage having hydroxymethyluracil in its deoxyribonucleic acid (DNA). Infection with phage SP3 whose DNA contains thymine and not hydroxymethyluracil does not result in the induction of a similar deoxycytidylate deaminase.

Deoxycytidylate deaminase,[1] an enzyme found in rapidly proliferating cells having an increased rate of DNA synthesis (Maley and Maley, 1959, 1961, 1964; Scarano *et al.*, 1960; Potter *et al.*, 1960), has also been detected after infection of *Escherichia coli* with phage T_2 (Keck *et al.*, 1960) and *Bacillus subtilis* with phage SP8 or ϕe (Marmur *et al.*, 1963; Roscoe and Tucker, 1964). This enzyme has been proposed as one of the enzymes responsible for the regulation of deoxythymidine 5′-monophosphate synthesis and, therefore, the regulation of DNA synthesis (Friedkin, 1959; Maley and Maley, 1960). In the case of the well-characterized dCMP deaminases from spleen and chick embryo, dCTP is an allosteric stimulator and dTTP an allosteric inhibitor (Maley and Maley, 1964, 1965; Scarano *et al.*, 1963).

SP8, a phage infectious for *B. subtilis*, has hydroxymethyluracil in place of thymine in its DNA (Kallen *et al.*, 1962). Infection of *B. subtilis* with phage SP8 results in the appearance of a new enzyme, deoxy-

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[1] The trivial name, dCMP deaminase, is used for this enzyme. The name suggested by the Commission on Enzymes of the International Union of Biochemistry is 4-aminopyrimidinone 2,5′-phosphate aminohydrolase. Other abbreviations used are: dHMUTP, 5-hydroxymethyldeoxyuridine triphosphate; dHMUMP, 5-hydroxymethyldeoxyuridine monophosphate; HMU, 5-hydroxymethyluracil; dHMCMP, 5-hydroxymethyldeoxycytidine monophosphate; dMeCMP, 5-methyldeoxycytidine monophosphate; dC, deoxycytidine; BPA, bovine plasma albumin; GSH, glutathione; AMP, CMP, and GMP, adenosine, cytidine, and guanosine monophosphates; ATP, adenosine triphosphate; dTMP, dAMP, dGMP, and dIMP, deoxythymidine, -adenosine, -guanosine, and -inosine monophosphates; dTDP, dADP, and dGDP, deoxythymidine, -adenosine, and -guanosine diphosphates; dUTP, dATP, and dGTP, deoxyuridine, -adenosine, and -guanosine triphosphates.

thymidylate-5′-nucleotidase, that catalyzes the hydrolysis of dTMP to deoxythymidine and P_i (Aposhian, 1965; Aposhian and Tremblay, 1966). An activity in such infected cells has also been reported to cleave dTTP to dTMP (Marmur *et al.*, 1964). Since either one or both of these activities could result in a diminished dTTP concentration in the cell, we wished to know whether the dCMP deaminase purified from cells infected with phage SP8 is subject to feedback control. Is it inhibited by dTTP and stimulated by dCTP like all other known dCMP deaminases? Or is it inhibited by dHMUTP? While our studies were in progress, the dCMP deaminases induced after infection of *E. coli* with phages T_2 or T_6 were shown to be inhibited by dTTP and stimulated by dCTP or dHMCTP (Fleming and Bessman, 1965; Maley and Maley, 1966).

In the present paper, the purification and some properties of phage SP8 induced dCMP deaminase are reported. Two protein components having dCMP deaminase activity but different electrophoretic mobilities have been isolated by preparative polyacrylamide gel electrophoresis. The enzyme is not inhibited by dTTP or dHMUTP nor stimulated by dCTP. Thus, the dCMP deaminase induced by infection of *B. subtilis* with phage SP8 appears to be unique among other known dCMP deaminases.

Experimental Procedure

Materials and Methods

B. subtilis SB19 and phage SP8, which corresponds to SP8* of Brodetsky and Romig (1965), were used in these studies. The growth medium used throughout is TY broth, modified as described by Trilling and Aposhian (1965). For phage propagation, $CaCl_2$ was added to a concentration of 2.5×10^{-3} M. Phages SP3 and SP5C were prepared as previously described (Aposhian and Tremblay, 1966; Trilling and Aposhian, 1965).

Hydroxymethyldeoxyuridine monophosphate was prepared using the procedures of Cline *et al.* (1959) and Maley (1962). The pooled fractions from the Dowex 50-formate column were identified as dHMUMP by the ratios of absorbancies at 250 to that at 260 mμ (280/260 = 0.55 and 250/260 = 0.68 at pH 2).

The dHMUMP was used to synthesize dHMUTP by the procedure of Smith and Khorana (1958). The nucleoside triphosphate was purified on a Dowex 1-chloride column using increasing concentrations of LiCl in 0.01 N HCl to elute the nucleotide. The pooled fractions of dHMUTP, which contained 0.2 M LiCl in 0.01 N HCl, were neutralized with KOH and concentrated by lyophilization. The concentrated material was desalted on a Bio-Gel P-2 column (100–200 mesh). The nucleotide was located in the fractions passing through the Bio-Gel column by absorbancy measurements at 260 mμ. The approximate salt concentrations in the tubes were determined with a Barnstead conductivity meter (Model PM-3). The fractions with high conductivity readings were excluded from the final pool of dHMUTP. The pooled solution was concentrated by lyophilization. The ratio of micromoles of phosphorus to micromoles of base was 3.3:1 for the dHMUTP preparation. The molar extinction of 10.2×10^3 at 264 mμ and at pH 2 was used for hydroxymethyldeoxyuridine nucleotide.

Unlabeled nucleotides were obtained from Sigma Chemical Co. and from California Corp. for Biochemical Research. [^{14}C]dCMP and [^{14}C]dTTP were obtained from Schwarz BioResearch. Tris and other buffers were obtained from Sigma Chemical Co. The pH values indicated for buffers are those of 0.05 M solutions at 25° unless otherwise stated. Crystalline BPA was obtained from Armour and Co. DEAE-cellulose (Whatman powder, DE-50) and DEAE paper (Whatman anion exchanger, DE-81) were purchased from W. R. Balston, Ltd.

Protein concentrations were determined by the method of Lowry *et al.* (1951) after precipitation of the protein with cold 5% trichloroacetic acid. Crystalline bovine plasma albumin was used as the standard. Phosphorus was determined by the method of Chen *et al.* (1956). Absorbancy measurements were made with a Zeiss PMQII spectrophotometer unless otherwise stated.

Analytical or preparative gel electrophoresis was performed at 0° as described by Jovin *et al.* (1964) with the following exceptions. The preparative gel electrophoresis apparatus and the current regulated power supply produced by the Buchler Instrument Co. were used; the multiphasic Tris buffer system in the concentrating (upper) gel consisted of 0.0446 M Tris and 0.032 M phosphoric acid, and in the upper buffer of 0.0445 M Tris and 0.0464 M glycine. The concentrating gel contained 0.15% *N,N′*-methylenebisacrylamide and 0.025% *N,N,N′,N′*-tetramethylethylenediamine.

To assay for dCMP kinase, a mixture and conditions identical with the dCMP deaminase assay 2 were used. After incubation an aliquot was placed on Whatman 3MM paper and the di- and triphosphates were separated by chromatography using 1 M ammonium acetate–ethanol (30:70) as solvent. Deoxythymidylate-5′-nucleotidase was assayed as previously described (Aposhian, 1965).

Assay of dCMP Deaminase

Assay 1. Spectrophotometric Measurement of the Disappearance of dCMP. This assay is similar to assay 1 described by Maley and Maley (1964). The reaction mixture (0.25 ml) contains 0.05 M Tris-HCl buffer (pH 8.7), 2.2×10^{-3} M dCMP, and enzyme. Mg^{2+} is omitted. Tris-HCl buffer (pH 8.7) is used in assays of all fractions except for the gel fractions. These are assayed in 0.1 M potassium glycinate buffer (pH 9.4). The reaction mixture is incubated at 37° for 15 min and chilled, and 0.75 ml of cold 0.6 N perchloric acid is added. The absorbancy at 290 mμ is determined in a cuvet with a 2-mm light path using diluted perchloric acid as a blank. The amount of dCMP converted to dUMP after incubation with the enzyme is determined by subtraction of the A_{290} of a given sample from that of the control. A decrease of 0.1 in absorbancy at

290 mμ using a 2-mm light path corresponds to the disappearance of 0.05 μmole of dCMP. The control is identical with the experimental reaction mixture except that the enzyme is added after the incubation and after the addition of cold perchloric acid. Under the conditions of this assay, linearity with respect to enzyme concentration is maintained up to the conversion of 20% of the substrate.

Assay 2. The Conversion of [^{14}C]dCMP to [^{14}C]dUMP. The reaction mixture (0.10 ml) contains 0.05 M Tris-HCl buffer (pH 8.7), 2.5×10^{-3} M [^{14}C]dCMP (3×10^5 cpm/μmole), and an amount of enzyme capable of deaminating 10% or less of the substrate during the 15-min incubation period. For the gel fractions 0.1 M potassium glycinate buffer (pH 9.4) is used. After incubation at 37° for 15 min, the assay tubes are chilled and 5 μl of a carrier solution of unlabeled dCMP and dUMP is added to each reaction mixture. The carrier solution contained 80 μmoles of each nucleotide/ml. An aliquot (50 μl) of each reaction mixture is then streaked on strips of DEAE paper, formate form (2 × 25 cm). Drying of the streaks is not necessary before irrigation. The paper is irrigated with 0.05 M formic acid (Jacobson, 1964) until the solvent front moves 80% the length of the strip. After drying the paper, the spots are located by viewing under an ultraviolet light. The area corresponding to the product is cut out and the radioactivity determined in a Nuclear-Chicago scintillation counter using a scintillation fluid consisting of 4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene per l. of toluene.

With either assay a solution containing 0.5 mg of BPA/ml of 0.05 M Tris-HCl buffer (pH 8.7) is used as diluent of the enzyme prior to assay. One unit of enzyme is defined as the amount required to deaminate 1 μmole of dCMP/hr.

The results obtained using the two assays agree. The advantages of assay 2 include high sensitivity, relatively great rapidity (about 40 min for the separation on paper), and the ease of handling many separations simultaneously as compared with other *direct* assays. The disadvantage of this method is that other substances (nucleic acids, dTMP, nucleoside di- and triphosphates) migrate in the proximity of the [^{14}C]-dUMP spot. Such substances do not appear to be a problem in purified preparations.

Results

Purification of SP8 dCMP Deaminase (Table I). PREPARATION OF INFECTED CELLS.[2] To 280 l. of TY broth was added 6 l. of an overnight culture of *B. subtilis* SB19. The cells were grown with vigorous aeration at 37° to a titer of approximately 2×10^8 cells/ml (A_{600} = 1.0, using a Bausch and Lomb Spectronic 20). Phage SP8 was added at an input multiplicity of approximately 3. Twenty minutes after infection, the culture was rapidly chilled and the cells were collected by centrifugation in a DeLaval PX207 continuous-flow centrifuge and then in a Sharples centrifuge. The yield of cells was 350 g wet weight.

TABLE I: Purification of SP8 dCMP Deaminase.

Step	Units: Per ml	Units: Total	Protein (mg/ml)	Sp Act. (units/mg)
Cell extract	78.6	25,724	22.3	3.5
DEAE-cellulose[a]	3.9	16,205	0.032	122

[a] This step was performed many times on a smaller scale (see text) and these values are calculated for the large-scale procedure.

PREPARATION OF THE EXTRACT. Extracts were prepared as described by Aposhian and Tremblay (1966) except that 0.05 M glycylglycine (pH 8.0)–0.002 M GSH was used as buffer and EDTA was omitted. The volume of an extract prepared from 128 g of cells was 340 ml.

DEAE COLUMN CHROMATOGRAPHY. A column of DEAE-cellulose (3.4 × 14 cm) was prepared and equilibrated with 0.05 M Tris-HCl (pH 8.0)–0.002 M GSH. To 133 ml of cell extract was added an equal volume of cold distilled water. The diluted extract was applied to the column at the rate of 10 ml/min. The column was washed with 1 l. of 0.05 M Tris-HCl buffer (pH 8.0)–0.002 M GSH. For elution of the enzyme, a linear gradient was used between 0.05 and 0.50 M Tris-HCl buffer (pH 8.0). Each buffer contained 0.002 M GSH and had an initial volume of 4 l. At 1-min intervals, 17-ml fractions were collected. To locate the fractions that contain the dCMP deaminase activity, assay 1 was used. The enzyme was eluted in a volume of 1–2 l. after the buffer concentration had reached about 0.2 M. The active fractions were pooled and frozen at −80° within 5 hr of the time of elution. This material is stable for several months at −80° and can be frozen and thawed at least three or four times without loss of activity.

Attempts to elute dCMP deaminase from the column in a more concentrated solution were unsuccessful. Batch elution with 0.50 M Tris-HCl buffer (pH 8.0) or the addition of increasing amounts of NaCl or KCl to a 0.10 M Tris-HCl buffer did not yield a more concentrated enzyme fraction.

CONCENTRATION OF THE ENZYME. The DEAE eluate was placed in dialysis tubing and concentrated by covering the bag with dry Aquacide II (Calbiochem). This procedure reduced the volume from 1650 to 77 ml with approximately 75% recovery of dCMP deaminase activity. For comparing the properties of various fractions (Table II), a portion of the above DEAE fraction was further concentrated and stabilized

[2] The large-scale preparations of infected cells were performed at the New England Enzyme Center at Tufts University.

TABLE II: Substrate Specificity for SP8 dCMP Deaminase.[a]

Nucleotide	Relative Activity[b] of Enzyme Fraction DEAE Fraction	Gel I	Gel III
dCMP	100	100	100
dMeCMP	27	23	20
dCTP	12	<3	<3
dHMCMP	–	<3	<3
dAMP	6	<3	<3
dC	<3	–	–
CTP	6	–	–
CDP	<3	–	–
CMP	5	–	–

[a] The compound listed was substituted for dCMP in assay 1. The decrease in absorbancy at 284 and 265 mμ was used for determining the activity on dHMCMP and dAMP, respectively (Lee, 1963). For all others 290 mμ was used. The amount of protein used was 1.9, 4.5, and 4.3 μg of the DEAE fraction, gel fraction I, and gel fraction III, respectively. The assay was also performed at double these concentrations of protein. The total volume of the assay was reduced to 0.15 ml for experiments with gel fractions I and III. [b] The numbers in this table represent the activity of the compound as a substrate relative to the activity of dCMP which has been set at 100% for the particular enzyme fraction.

by dialysis against a 20% solution of sucrose (Mann, enzyme grade) in 0.2 M Tris-HCl buffer (pH 8.0). This concentrated enzyme solution was stable to storage at −80° and to repeated freezing and thawing for at least 15 times.

POLYACRYLAMIDE GEL ELECTROPHORESIS. Since all other attempts to increase the specific activity of the dCMP deaminase were unsuccessful, the criterion of purification was changed to the lowering of the number of protein bands on polyacrylamide gel electrophoresis. This has been accomplished as seen below.

Preparations that were used for polyacrylamide gel electrophoresis consisted of the DEAE eluate which was first concentrated with Aquacide II as described above and then dialyzed against aqueous 50% sucrose. Immediately prior to application on the gel, Tris-phosphate was added to a final concentration identical with that of the concentrating gel.

Electrophoresis of the concentrated DEAE fraction on analytical polyacrylamide gels and subsequent staining with Buffalo Black (naphthol blue black) showed nine bands (Figure 1). The bands corresponding to the dCMP deaminase activity were identified as described by Chrambach (1966). Duplicate analytical gels were subjected to electrophoresis at 0°; one was stained, and the other was cut into sections that were assayed for enzymatic activity; the relative mobilities (R_F) of the stained bands and of the activity were matched to reveal the protein bands carrying the activity. The results of the dCMP deaminase activity assay are shown in Figure 2. Two peaks with dCMP deaminase activity were found. The R_F values characteristic for these peaks and for the corresponding stained bands are listed in Table III.

TABLE III: R_F Values of SP8 dCMP Deaminase in Polyacrylamide Gels Using a Discontinuous Buffer System.

Gel Fractions	R_F Values: Activity	R_F Values: Stain
Analytical gel fraction I[a]	0.62	0.65
Analytical gel fraction III[a]	0.52	0.54
Preparative gel fraction I	0.63	–
Preparative gel fraction III	0.52	–
Rerun of Preparative Gel Fractions		
Analytical gel fraction I	–	0.64
Analytical gel fraction III	–	0.53

[a] Duplicate analytical gels were subjected to electrophoresis at 0°; one was stained, and the other was cut into sections that were assayed for enzymatic activity (see Figure 2); the relative mobilities (R_F) of the stained bands and of the activity were matched to reveal the protein components carrying the activity. R_F is defined as a mobility of a protein band relative to the mobility of bromophenol blue.

Since the separation of the two active bands from each other and from their neighbors was satisfactory, a fractionation by preparative gel electrophoresis was performed. Concentrated DEAE fraction (56 ml) containing 24 mg of protein was applied to a column that consisted of 70 ml of resolving gel (7% monomer) and 100 ml of concentrating gel (2.5% monomer). Electrophoresis was conducted at a constant current of 50 ma for a total of 20 hr. The flow rate of buffer used for elution was maintained at 1 ml/min. The eluate from the gel was mixed continuously with 2 M Tris-HCl buffer (pH 8.0) so that the final concentration of Tris-HCl in the collected fractions (5 ml) was 0.2 M. The activity of the collected fractions was determined by assay 2. In Figure 3 is shown the activity profile of the eluate from the preparative gel electrophoresis. Again two peaks are apparent. Each peak of dCMP deaminase was eluted in a volume of about 50 ml. Each was concentrated by dialysis against a solution of 50% sucrose in 0.2 M Tris-HCl buffer (pH 8.0, w/v).

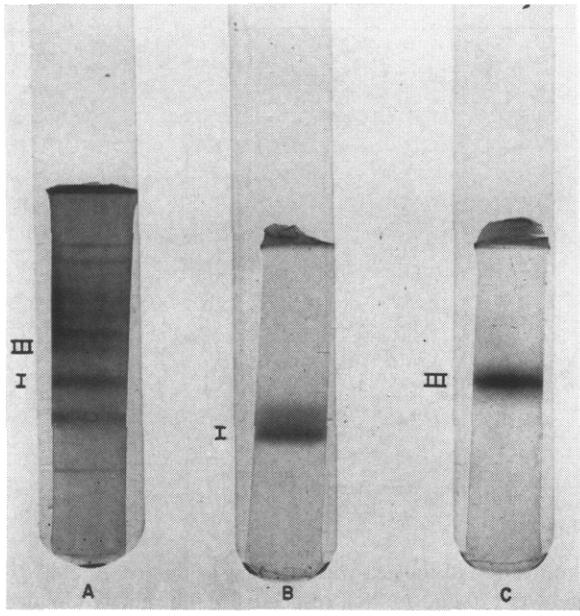

FIGURE 1: Analytical polyacrylamide gels. (A) DEAE fraction; (B) peak I of Figure 3; and (C) peak III of Figure 3. Conditions as described in Figure 2.

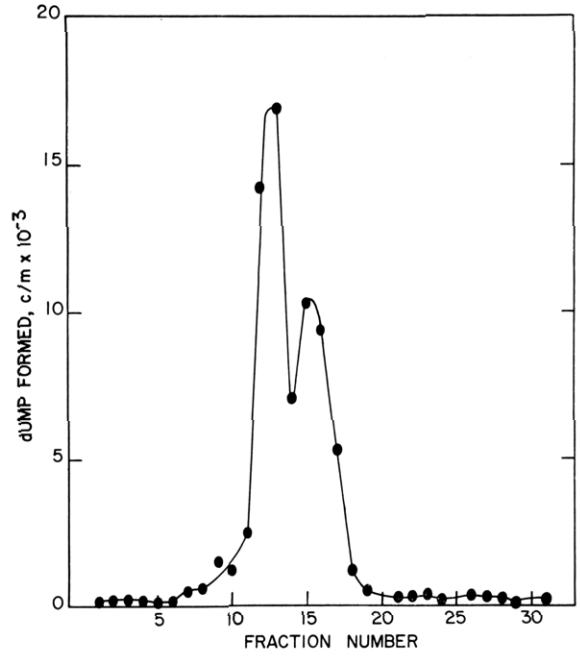

FIGURE 2: Analytical gel electrophoresis of SP8 dCMP deaminase. Electrophoresis was performed as described by Jovin *et al.* (1964). The 0° Tris buffer system and an acrylamide concentration of 7.0% in the lower gel were used. The amount of protein loaded on the gel was 130 μg of the concentrated DEAE fraction. The location of the bands and slicing of the gel into 30 slices were performed as described (Chrambach, 1966). Each slice of the gel was placed into a tube containing 0.1 ml of 0.5 M Tris-HCl (pH 8.7). After mixing briefly, 0.04 ml from each tube was assayed by assay 2. Incubation was for 30 min at 37°.

The concentrated fractions of peaks I and III (gel fractions I and III) of the preparative gel electrophoresis were subjected to electrophoresis on an analytical scale. Gel fractions I and III (Figure 3) appear as homogeneous proteins characterized by their R_F (Table III and Figure 1). The presence of a diffuse trailing edge of the stained band I may be indicative of some residual heterogeneity. Total recovery was low (25%), indicating denaturation may have occurred.

OTHER ATTEMPTS AT PURIFICATION. Phase partition separation of nucleic acids by the method of Okazaki and Kornberg (1964) may be used to remove 80–90% of the DNA in the extract either before or after the DEAE step. This procedure was discontinued when difficulty was encountered in removing the polyethylene glycol 6000 from the enzyme fraction. The residual polyethylene glycol interfered with subsequent purification procedures. Attempts to increase the specific activity of the DEAE fraction by rechromatography on DEAE cellulose or fractionation using phosphocellulose, hydroxylapatite, Sephadex, Bio-Gel, or ammonium sulfate were unsuccessful.

Properties of the Enzyme. pH OPTIMUM. The enzyme had maximum activity at approximately pH 9.4 using potassium glycinate buffer (Figure 4). Potassium phosphate and sodium carbonate appear to be inhibitory. No significant difference was found for the pH–activity relationships between gel fractions I and III as shown in Figure 4.

CATION REQUIREMENT. Neither Mn^{2+} nor Mg^{2+} is required for activation of SP8 dCMP deaminase. This is true for all fractions including gel fraction I. Sodium versenate is without effect on the activity or the stability of the enzyme.

SUBSTRATE SPECIFICITY. The substrate specificity of the DEAE fraction, gel fraction I, and gel fraction III is summarized in Table II. The enzyme is not a nonspecific deaminase as shown by its failure to catalyze the deamination of dAMP. No differences of substrate specificity were found between gel fractions I and III.

The activity of the DEAE fraction on dCTP might conceivably be caused by the presence of a nonspecific pyrophosphatase converting dCTP to dCMP. Since this activity is not found in the gel fractions, dCTP is probably not a substrate for the dCMP deaminase.

It has been reported that d5MeCMP is not deaminated by crude extracts of phage SP8 infected *B. subtilis* even though such extracts deaminate dCMP (Marmur *et al.*, 1964). We have also confirmed this lack of activity in crude extracts. However, when a partially purified preparation of SP8 dCMP deaminase is used, d5MeCMP is deaminated at approximately one-fourth the rate found for deamination of dCMP. Crude cell extracts do not appear to deaminate d5MeCMP because this nucleotide is destroyed by deoxythymidylate-5′-nucleotidase as shown previously (Aposhian and Tremblay, 1966).

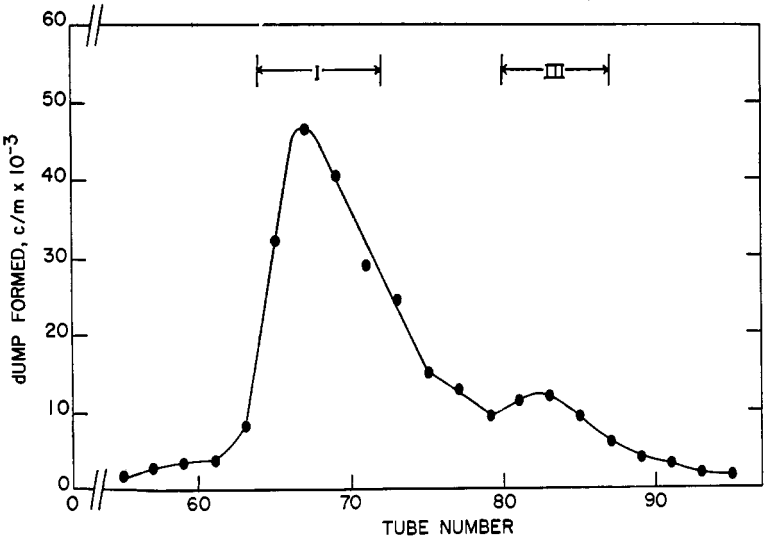

FIGURE 3: Preparative gel electrophoresis of SP8 deaminase. Composition of the gels was the same as that of the analytical gels. The volumes of the resolving gel and concentrating gel were 70 and 150 ml, respectively. The gel was loaded with 24 mg of the concentrated DEAE fraction in a volume of 56 ml.

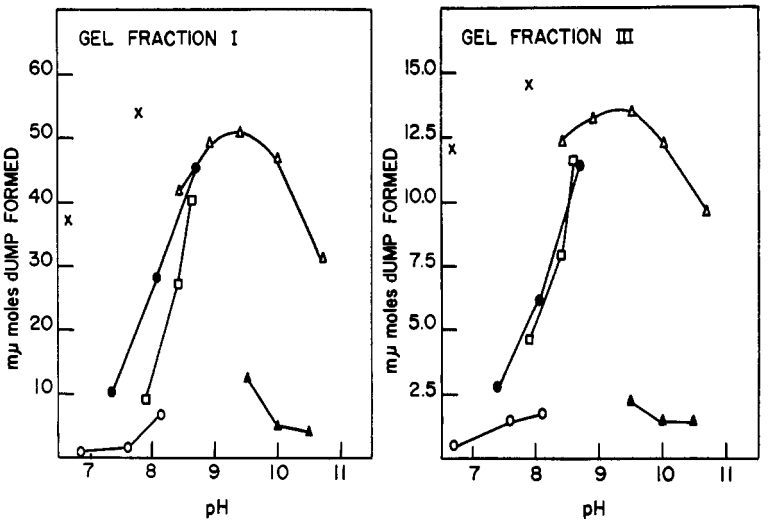

FIGURE 4: Comparison of the two gel fractions of SP8 dCMP deaminase as to pH optima and buffers. The buffers used were Tris-HCl (●——●), potassium glycinate (△——△), potassium phosphate (○——○), sodium carbonate (▲——▲), 2-amino-2-methyl-1,3-propanediol (□——□), and glycylglycine (×-×). The pH of the buffers was determined on 1 M solutions at 25°. Assay 2 was used with a buffer concentration of 0.05 M. The enzyme preparations were added in approximately 2 μmoles of Tris-HCl (pH 8.0) containing 5 mg of sucrose.

INFLUENCE OF dCTP, Mg^{2+}, AND MERCAPTOETHANOL ON dCMP DEAMINASE ACTIVITY. The dCMP deaminases from other sources have been shown to require Mg^{2+} and to be activated by dCTP and mercaptoethanol (Maley and Maley, 1964, 1965, 1966; Scarano *et al.*, 1962, 1963; Fleming and Bessman, 1965; Scarano, 1960). In the case of SP8 dCMP deaminase, the addition of 10^{-2} M $MgCl_2$ and 5×10^{-5} M dCTP did not stimulate the activity. These additions also did not affect the deaminase activity found in the presence of 1×10^{-3} M dTTP.

Mercaptoethanol (10^{-4} M) alone or in combination with the above nucleotides was also ineffective. For these studies, the cell extract, DEAE fraction, gel fraction I, and gel fraction III were used.

Since there was no stimulation by the addition of dCTP to the assay mixture, the dCMP deaminase fractions were checked for dCMP kinase activity,

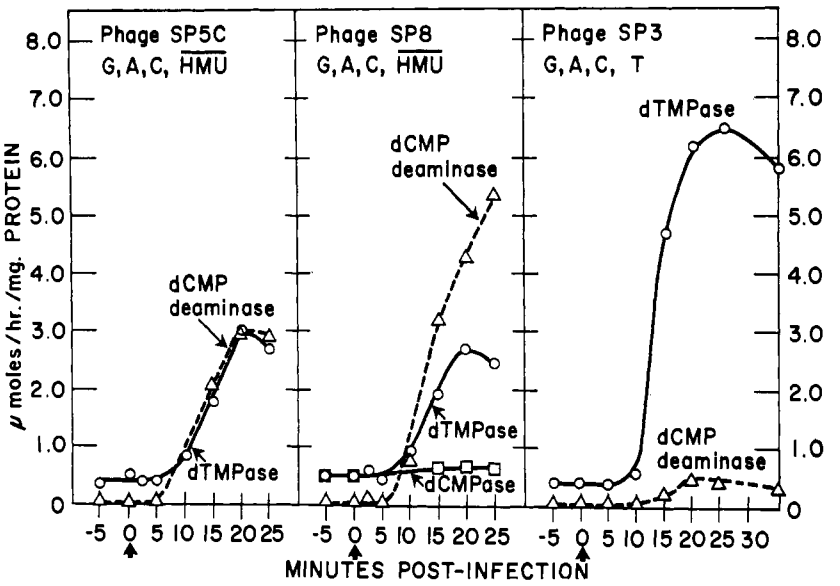

FIGURE 5: dCMP deaminase and dTMP-5′-nucleotidase activity after phage infection. The major bases in the DNA are indicated for each phage at the top of the figure. The bacteria were grown in TY broth at 37° with vigorous aeration to a cell titer of about 2×10^8/ml. Approximately four phages per cell were then added. At appropriate times, 200 ml of the culture was poured onto crushed ice and the infected cells were collected by centrifugation. The cells were resuspended in 2 ml of buffer containing: 0.05 M glycylglycine (pH 8.0)–0.002 M GSH. They were then disrupted for 1 min with a Mullard ultrasonic generator. After centrifugation at 8000*g* for 30 min, the extracts were immediately assayed for enzyme activity. Assay 1 was used for dCMP deaminase (△- - - -△). Deoxythymidylate-5′-nucleotidase (dTMPase, ○——○) was assayed as previously described (Aposhian, 1965). The dCMPase activity (□——□) described for SP8-infected cells is an indication of nonspecific phosphatase activity present in the host before infection (Aposhian, 1965).

which might convert dCMP to dCDP and dCTP. No kinase activity was detectable when assays for this enzyme were performed under conditions identical with those for the assay of dCMP deaminase. These findings do not support the possibility that maximum activation had taken place through the action of a contaminating enzyme that converted the substrate of the deaminase reaction into its activator.

LACK OF INHIBITION BY dHMUTP. The dCMP deaminase partially purified from SP8-infected cells is not inhibited by dTTP. In order to eliminate the possibility that the lack of effect of dTTP might be due to destruction of this nucleotide during the deaminase assays, [^{14}C]dTTP was incubated in place of [^{14}C]dCMP under conditions for the deaminase assay. No dTTPase activity was found in the DEAE fraction or gel fraction I.

Since hydroxymethyluracil is present in SP8 DNA as the major base instead of thymine, dHMUTP rather than dTTP was next considered as the possible feedback inhibitor. dHMUTP was synthesized and tested for any inhibitory or activating influence. At concentrations of 4×10^{-5} to 1×10^{-3} M, dHMUTP was neither inhibitory nor stimulatory when the DEAE, gel I, or gel III fraction was used.

INFLUENCE OF OTHER NUCLEOTIDES. Other nucleotides which were tested at concentrations ranging from 5×10^{-6} to 2.5×10^{-3} M were dTMP, dTDP, dUTP, dAMP, dADP, dATP, dGMP, dGDP, dGTP, dIMP, CMP, AMP, ATP, GMP, and dHMUMP. None of these significantly inhibited or stimulated gel fractions I and III. The product, dUMP, is inhibitory at concentrations equal to that of the substrate.

INFLUENCE OF SALTS. Potassium fluoride at 0.02 M does not inhibit the dCMP deaminase activity. Ammonium chloride and potassium chloride at concentrations of 0.1 M inhibit the deaminase approximately 80 and 50%, respectively, but do not inhibit it at 10^{-3} M.

dCMP DEAMINASE AFTER INFECTION WITH OTHER *B. subtilis* PHAGES. The increase in dCMP deaminase activity also occurs after infection of *B. subtilis* with SP5C (Figure 5), a phage which has hydroxymethyluracil in its DNA (Aposhian and Tremblay, 1966), and which differs from SP8 in that an increased amount of $CaCl_2$ must be added to the medium for phage propagation (Aposhian, 1965). The difference in the specific activities of this enzyme after SP5C and SP8 infection indicates another difference between the closely related phages SP5C and SP8. The extracts were prepared and assayed under identical conditions.

Such a large increase in dCMP deaminase activity is not found after infection with phage SP3. This phage contains the four normal bases adenine, thymine, guanine, and cytosine in its DNA. To detect even this small amount of dCMP deaminase activity it was neces-

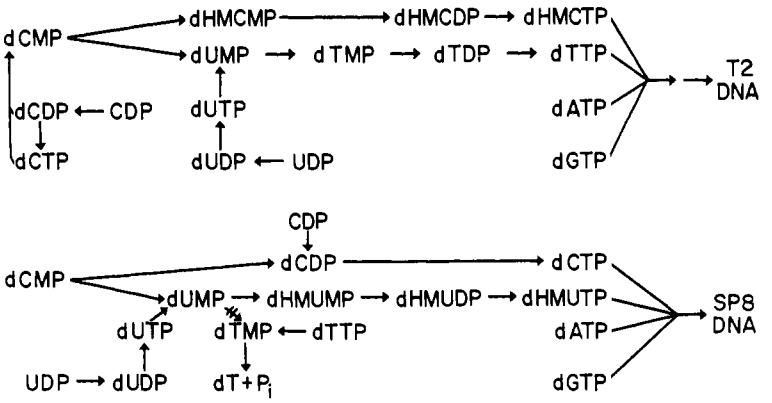

FIGURE 6: Nucleotide pathways for phage DNA synthesis.

sary to substitute 0.05 M glycylglycine (pH 8.0) for Tris-HCl in the assay. Experiments to determine the significance of the small amount of dCMP deaminase activity are in progress. The dTMP-5′-nucleotidase activity is an indication of an effective infection of *B. subtilis* by phage. The significance of this nucleotidase after SP3 and SP5C infection has been presented elsewhere (Aposhian, 1965; Aposhian and Tremblay, 1966).

Discussion

Two protein components with dCMP deaminase activity can be isolated by preparative polyacrylamide gel electrophoresis from *B. subtilis* infected with phage SP8. They cannot be distinguished from each other by changing the conditions of the enzyme assay, such as the pH or the nature of the buffers. Their substrate specificities are identical and both enzyme activities are equally unaffected by the presence of dCTP, dTTP, dHMUTP, or other nucleotides in the reaction mixture. The two enzymatically active components may be caused by irreversible alterations independent of the active site in the native conformation of dCMP deaminase. For example, the cleavage by proteolytic attack of one or more amino acids from the ends of the enzyme might be responsible for such a change. It is possible that two proteins with minor differences in primary structure but the same activity were synthesized in cells infected with phage SP8. Multiple forms having different electrophoretic mobilities and identical activities have been reported for the T_4 phage induced dCMP hydroxymethylase (Dirksen and Chrambach, 1965) and for chicken mitochondrial malic dehydrogenase (Kitto *et al.*, 1966). A third possibility is the suggestion, from studies with the latter enzyme, that such forms may represent different enzymatically active conformations of a single protein (Kitto *et al.*, 1966).

While conformational changes are characteristic of allosteric enzymes, the results of our experiments indicate that the dCMP deaminase purified from *B. subtilis* infected with phage SP8 uniquely differs from dCMP deaminases isolated from other sources by its insensitivity to nucleotide effectors. A hitherto common property of all dCMP deaminases from a variety of sources such as sea urchin eggs (Scarano, 1960), regenerating liver (Scarano *et al.*, 1962), spleen (Scarano *et al.*, 1963), chick embryo (Maley and Maley, 1964, 1965), and T-even phage-infected *E. coli* (Fleming and Bessman, 1965; Maley and Maley, 1966) has been the stimulation of their activity by dCTP plus Mg^{2+} and the inhibition of this activity by dTTP. These nucleotides, as well as dHMUTP, are without effect on the enzyme isolated from *B. subtilis* infected with phage SP8. Since the same results are found with crude extracts and partially purified fractions of the enzyme, desensitization of the enzyme does not seem a likely explanation for this phenomenon. We cannot, however, presently rule out a different protein conformation *in vivo*.

Since the dCMP deaminases found in *E. coli* infected with T-even phages behave like other known feedback-regulated dCMP deaminases (Fleming and Bessman, 1965; Maley and Maley, 1966) but the phage SP8 enzyme does not, the question arises as to the reason these phage-induced enzymes differ in their susceptibility to feedback control. An explanation is suggested by the manner in which the utilization of dCMP differs for the synthesis of the pyrimidine deoxynucleoside triphosphates required by each type of infected cell, as summarized in Figure 6.

The only reaction responsible for hydroxymethylation of a deoxycytidylate nucleotide in *E. coli* infected with T-even phages is the one catalyzed by dCMP hydroxymethylase (Flaks and Cohen, 1959). Deoxycytidylate, the substrate of this enzyme, is, however, also a substrate for dCMP deaminase. The product of the latter enzyme reaction is dUMP, a dTTP precursor. Uncontrolled dCMP deaminase activity in a T-even phage-infected cell might deprive the cell of dCMP, the only immediate precursor of the one nucleotide, dHMCMP, absolutely required for phage DNA production. It has been shown, however, that the T-even dCMP deaminase can be regulated (Fleming and Bessman, 1965; Maley and Maley, 1966). When the dTTP

level increases prior to phage DNA synthesis, the dTTP inhibits the dCMP deaminase induced after the infection of *E. coli* by T-even phage. The expected result of such an inhibition would be to increase the amount of dCMP available for hydroxymethylation. As the dHMCTP accumulates, a level is reached that stimulates phage dCMP deaminase so that an increased amount of dUMP is now available for dTTP synthesis. The potent dCTP pyrophosphatase found in the T-even phage-infected cell (Zimmerman and Kornberg, 1961; Koerner *et al.*, 1959) makes it unlikely that dCTP, the usual stimulator, would be present *in vivo* to cause a continuous stimulation. Thus in T-even phage infection, feedback regulation of dCMP deaminase activity serves as a sensitive means of adjusting and controlling the supply of dCMP available for the synthesis of the essential dHMCMP necessary for T-even phage DNA production.

What then is the explanation for the uniqueness of SP8 deaminase in being insensitive to feedback control? In contrast to *E. coli* infected with T-even phages, dCMP in the SP8 system is the substrate for enzymic reactions whose products can be synthesized by other pathways.

In *B. subtilis* infected with phage SP8, none of these dCMP-requiring reactions can be considered essential. There thus appears to be no need for feedback control of SP8 dCMP deaminase. Uninhibited dCMP deaminase activity would produce dUMP, which has been suggested as the precursor of the unusual base, hydroxymethyluracil, in the phage DNA (Marmur *et al.*, 1963; Roscoe and Tucker, 1964, 1966). In addition, deoxycytidylate need not be an obligatory precursor of either dUMP or dCTP. Since the thymidylate synthetase reaction is blocked in cells infected with hydroxymethyluracil-containing phages (Roscoe and Tucker, 1964, 1966), there is no depletion of dUMP by conversion to dTMP. Additional dUMP might also be obtained from reduction of UDP to dUDP, conversion to dUTP, and subsequent hydrolysis to dUMP as believed to occur in T-even *E. coli* systems (Bertani *et al.*, 1963; Larsson and Reichard, 1966). The reduction of CDP to dCDP and the conversion of dCDP to dCTP, as reported for *E. coli* (Bertani *et al.*, 1963; Larsson and Reichard, 1966), might also be a source of the dCTP necessary for DNA synthesis. This alternate pathway would circumvent the need for dCMP as a substrate for a kinase reaction. Hence, maintenance of dCMP availability in the cell by feedback regulation of dCMP deaminase does not seem necessary and has not been found in SP8-infected *B. subtilis*.

Acknowledgment

We wish to acknowledge the generous assistance of Mr. Leo Shapiro during the early parts of this investigation.

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The Synthesis of Reagent Quantities of [2,3-^{3}H]*N*-(*n*-Propyl)hydroxylamine of High Specific Activity for Derivatizing Trace Amounts of Acyl Phosphates*

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ABSTRACT: [2,3-^{3}H]*N*-(*n*-Propyl)hydroxylamine hydrochloride was synthesized by reduction of 1-[2,3-^{3}H]-nitropropane with zinc dust in an aqueous ammonium chloride solution. The 1-[2,3-^{3}H]nitropropane was prepared by catalytic reduction of 3-nitropropene with palladium on carbon in tetrahydrofuran in the presence of approximately a stoichiometric amount of carrier-free tritium, followed by addition of carrier 1-nitropropane. The 1-[2,3-^{3}H]nitropropane was divided into a number of vials and stored in liquid nitrogen to minimize radiodecomposition, and the [2,3-^{3}H]*N*-(*n*-propyl)hydroxylamine was synthesized in batches and purified as needed. The [2,3-^{3}H]*N*-(*n*-propyl)hydroxylamine was purified by gradient elution from a Dowex 50 (Na) column followed by continuous-flow electrophoresis in a Brinkmann apparatus at two different pH values. The final product gave a single radioactive peak coinciding with the carrier propylhydroxylamine spot on paper electrophoresis and on chromatography in two solvent systems. The specific activity of the [2,3-^{3}H]*N*-(*n*-propyl)hydroxylamine prepared from different tritiated preparations ranged from 545 to 666 mc/mmole. *N*-(*n*-Propyl)-hydroxylamine was about 65% as effective as hydroxylamine in releasing inorganic phosphate from the acyl phosphate residue in a guinea pig NaK adenosine triphosphatase preparation. [2,3-^{3}H]*N*-(*n*-Propyl)hydroxylamine can be conveniently prepared in reagent quantities (0.1–0.2 mmole). It should find general use as a reagent for characterization of trace amounts of acyl derivatives.

A radioactive hydroxylamine derivative would be a valuable reagent in characterizing acyl phosphate compounds such as that found in the NaK ATPase[1] preparation incubated in the presence of [^{32}P]ATP, Mg, and Na (Nagano *et al.*, 1965; Hokin *et al.*, 1965; Bader *et al.*, 1966), and possibly that postulated to be an acyl phosphate intermediate in the ADP–ATP-exchange reaction in beef heart mitochondria (Colomb *et al.*, 1966).

Since hydroxylamine itself contains no atoms which can be made both radioactive and nonexchangeable with the atoms of water it was necessary to synthesize an *N*-alkylhydroxylamine containing radioactivity in stable atoms of the alkyl group. Inasmuch as some of the preparations containing acyl phosphates bound to protein are highly impure and contain very little acyl phosphate (the most active NaK ATPase preparations contain only a few hundred pmoles of acyl phosphate/mg of protein) it appeared that a hydroxylamine of high specific activity would be needed. Another problem was that the concentration of hydroxylamine required to react with acyl phosphates ranges from about 0.1 to 0.5 M. Thus it was necessary to obtain the hydroxylamine

* From the Department of Physiological Chemistry, University of Wisconsin, Madison, Wisconsin 53706. *Received February 2, 1967.* Supported by grants from the National Institute of Neurological Diseases and Blindness (No. NB-01730) and the Wisconsin Alumni Research Foundation.

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[1] Abbreviations used: NaK ATPase, sodium- + potassium-activated adenosine triphosphatase; PHA, *N*-(*n*-propyl)hydroxylamine; ATP, adenosine triphosphate; ADP, adenosine diphosphate.