The Purification and Properties of Deoxycytidylate Deaminase from Chick Embryo Extracts

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The discovery of the enzyme deoxycytidylate deaminase (1, 2) has aroused interest in the role this enzyme may play in the synthesis of DNA thymidine. As shown in studies with extracts containing both deoxycytidylate deaminase and thymidylate synthetase (3), deoxycytidylate is an efficient precursor of deoxythymidylate, via the reaction sequence

\[ d\text{CMP} \rightarrow d\text{UMP} \rightarrow d\text{TMP} \]  

(1)

However, as shown by Bertani, Häggmark, and Reichard (4) with a partially purified enzyme system from *Escherichia coli* B, uridine nucleotides may also give rise to dTMP through the reaction sequence

\[ \text{UDP} \rightarrow d\text{UDP} \rightarrow d\text{UTP} \rightarrow d\text{UMP} \rightarrow d\text{TMP} \]  

(2)

The importance of this pathway to *E. coli* for the synthesis de novo of dTMP becomes apparent from studies indicating only a dubious contribution toward the formation of dTMP from Reaction Sequence 1, because of the low levels of deoxycytidylate deaminase in this organism (5). It remains to be seen whether an analogous pathway to Reaction Sequence 2 can be observed in animal tissues. Some support has been obtained from the finding that uridine-2-14C can be incorporated into the thymine of chick embryo DNA in the presence of sufficient 6-diazo-5-oxo-L-norleucine to prevent the conversion of uridine to cytidine (6).

Since as discussed previously (7, 8), dTMP synthesis may be the rate-limiting step in DNA synthesis, it becomes essential to determine the extent to which each pathway contributes to the formation of dTMP, providing both are present within an organism. It would also be of interest to determine whether these pathways are subject to similar regulatory mechanisms. It is possible that the role of deoxycytidylate deaminase in the synthesis of dTMP may be only a minor one; however, studies showing the variation of this enzyme with the degree of mitosis in regenerating liver (8), the association of the deaminase with the synthesis of DNA thymidine. As shown in studies with extracts containing both deoxycytidylate deaminase and thymidylate synthetase (3), deoxycytidylate is an efficient precursor of deoxythymidylate, via the reaction sequence in a DNA-synthesizing system *in vitro* (12) are suggestive of a metabolic function of some significance for this enzyme. Further evidence for this assumption was presented by the finding (13, 14) that thymidine triphosphate exerts a potent inhibitory effect on the deaminase, one subject to reversal by equally low concentrations of deoxythymidine triphosphate. The manner in which this feedback control process is effected could be studied only with purified enzyme, and thus a highly purified preparation was obtained from 6-day chick embryos. The preparation of the enzyme and the mechanisms that may be involved in the regulation of deoxycytidylate deaminase by dCTP and dTTP are the subject of this report. A preliminary account of these studies has been presented (15). Similar findings and conclusions were recently described by Scarano, Geraci, Polizza, and Campanile (16) with a spleen deaminase preparation.

**EXPERIMENTAL PROCEDURE**

**Materials and Methods**

Most of the nucleosides and nucleotides used were purchased from the Sigma Chemical Company, St. Louis, Missouri; Schwarz BioResearch, Inc., Mount Vernon, New York; and California Corporation for Biochemical Research, Los Angeles, California. The method of Chang and Welch (17) was used to prepare 5-iododeoxyctydine. The 5-fluoro derivative of deoxyctydine was a generous gift of Dr. Robert Duschinsky of Hoffmann-La Roche, Inc., Nutley, New Jersey. Schwarz BioResearch, Inc., was the source of 5-bromodeoxyctydine. The above halo-genated deoxyribonucleosides were converted to the corresponding 5′-nucleotides by the cyanoethyl phosphate procedure of Tener (18). The corresponding deoxyribonucleoside 5′-triphosphates, including 5-methyl-dCTP, were synthesized according to the method of Smith and Khorana (19).

CMB2 was purchased from the Sigma Chemical Company; EM from Schwarz BioResearch, Inc.; and iodoacetamide from the California Corporation for Biochemical Research. The latter was recrystallized twice prior to use, as was urea. Coarse cellulose phosphate with an exchange capacity of 0.9 meq per g was purchased from the Sigma Chemical Company, and Sephadex G-20 was from Pharmacia Fine Chemicals, Inc., Rochester, Minnesota.

**Radioisotopes**—dCTP labeled in the α position was prepared by chemical phosphorylation (19) of dCMP. The latter

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* The suggested name for the enzyme by the Commission on Enzymes of the International Union of Biochemistry is 4-amino-pyrimidinone-2,5′-phosphate aminohydrolase, EC 3.5.4.5. However, to preserve continuity and to avoid confusion in referring to previous publications of this series, the trivial name of deoxycytidylate deaminase will be employed.

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The abbreviations and terminology used are: CMB, p-chloromercuribenzoate; EM, N-ethylmaleimide; Duponol, sodium lauryl sulfate.
compound was isolated from the DNA of E. coli grown in the presence of $^{32}$P, by the enzymatic phosphorylation of dCDP (21) with ATP, prepared by the method of Pressman (22). dCMP-2-14C, purchased from Schwarz BioResearch, Inc., was diluted to a specific activity approximating 1.0 x 10^6 c.p.m. per µg more or higher and chromatographed by concave gradient elution on a column of Dowex 1-formate (1 x 10-cm). The gradient consisted of a 500-ml mixing chamber containing water initially, and a reservoir of 1 N formic acid. The eluted product was collected by lyophilization.

Preparation of N-4-Hydroxy-dCMP—A modification of the procedure of Brown and Schell (23) for N-4-hydroxycytidine was employed. Hydroxylamine (5 x 3 ml) at pH 8.0 was added to 100 mg of dCMP. After 48 hours at room temperature, the solution was diluted with 3 ml of H$_2$O, and 3 ml of saturated Ba(OH)$_2$ were added, followed by 1 ml of 25% BaBr$_2$. The precipitate that formed on the addition of 4 volumes of 95% ethanol was centrifuged, washed twice with 95% ethanol, and dissolved in 10 ml of 0.1 N HCl. The increase in optical density was followed on aliquots of the concentrated solution at 280 µm. On reaching a maximum (24 to 48 hours), the solution was neutralized and passed through a Dowex 1-formate column (2 x 10 cm). The column was eluted with a formic acid gradient with 500 ml of H$_2$O in the mixing chamber and 4 N formic acid in the reservoir. The major ultraviolet-absorbing area that was eluted between the dCMP and dUMP regions in the elution pattern was collected and lyophilized. The best yields of N-4-hydroxy-dCMP obtained were about 50%. Absorption spectra were taken with a Cary model 14 recording spectrophotometer; pH 2.0, $\lambda_{max}$, 280, 223; $\lambda_{min}$, 248; pH 7.5, $\lambda_{max}$, 273, 237; $\lambda_{min}$, 263. These values differed only slightly in the acid spectra from those reported by Fox et al. (24) for N-4-hydroxycytidine; pH 2.11, $\lambda_{max}$, 280, 228; $\lambda_{min}$, 262.

Purification of Enzyme

The embryos (120 g) from 60 dozen fertile eggs, incubated at 37°C for 6 days, were homogenized with an equal volume (120 ml) of 0.154 M KCl in a glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged for 30 minutes at 35,000 X g, and the resulting supernatant (220 ml) was stirred for 10 minutes with 2 g of calcium phosphate gel. The gel was prepared according to the method of Kellin and Hartree (25), and concentrated by centrifugation just prior to use. Longer contact with the gel caused adsorption of the enzyme, which could be eluted by stirring with 200 ml of 0.1 M potassium phosphate (pH 7.5) for 30 minutes. However, the negative gel step, in which inert protein was adsorbed by the gel, but not the enzyme, was much more effective and less time-consuming. After removal of the gel by centrifugation at 35,000 x g for 10 minutes, 22.5 ml of 2% protamine sulfate were added dropwise to the supernatant (225 ml). The mixture was stirred for 10 minutes and then centrifuged. This step removed the bulk of the contaminating nucleic acids.

Ammonium Sulfate Precipitation—The supernatant (230 ml) from the preceding protamine step was fractionated with solid ammonium sulfate. The concentration of ammonium sulfate was raised from 0 to 0.35 saturation by the addition of 44.6 g and the resulting precipitate was centrifuged and discarded. The level of ammonium sulfate was then raised from 0.35 to 0.60 (34.7 µ) saturation, and the precipitate formed was centrifuged and dissolved in 0.02 M phosphate buffer (pH 7.5) containing 0.04 mM dCTP and 0.002 mM mercaptoethanol.

Second Negative Calcium Phosphate Gel—The 0.35 to 0.60 ammonium sulfate precipitate, in a volume of approximately 15 ml, was stirred for 10 minutes with 250 mg of calcium phosphate gel that had been previously centrifuged. After centrifugation at 35,000 x g for 10 minutes, the supernatant was desalted by passage through a column of Sephadex G-25 (2.5 cm in diameter x 21 cm in length). The column was prepared by mixing 20 g of Sephadex G-25 with 0.02 M phosphate (pH 7.5) containing 0.04 mM dCTP and 0.002 mM mercaptoethanol. The enzyme was eluted from the column with a solution similar to that described previously, in a volume of about 20 ml.

Phosphocellulose Column—The enzyme was absorbed on a phosphocellulose column (1 x 18 cm). The phosphocellulose was prepared by washing with 1 N NaOH and then with water until neutrality was attained. After this treatment, the phosphocellulose was equilibrated with 0.1 M potassium phosphate buffer (pH 7.5) followed by 0.02 M phosphate (pH 7.5). It was finally stored in 0.02 M phosphate at 4°C. Just prior to use, the phosphocellulose was stirred with sufficient dCTP and mercaptoethanol to yield 0.04 mM and 0.002 mM concentrations, respectively. After the crude enzyme solution had passed onto the phosphocellulose, the column was washed with four 5-ml fractions of 0.02 M phosphate containing 0.04 mM dCTP and 0.002 mM mercaptoethanol, followed by 5-ml fractions of 0.05 M phosphate containing dCTP and mercaptoethanol. The enzyme routinely appeared in Fractions 6 through 11 (Fig. 1). Since the enzyme was most concentrated in tubes 7 and 8, these fractions were usually pooled and lyophilized for further physical and kinetic experiments. The succeeding more dilute fractions were lyophilized separately as they usually appeared to be contaminated by a colored component.

Assay Procedures

Assay t—The routine assay mixture employed for the enzyme purification and kinetic studies contained the following components (in micromoles): dCMP, 1.0; dCTP, 0.04; MgCl$_2$, 1.0; Tris-Cl buffer (pH 8.0), 50; H$_2$O; and enzyme added to a final volume of 0.50 ml. It was found convenient, as well as more efficient, to add the reaction components in a volume of 0.20 ml from a prepared mixture. Incubation was conducted for 10 minutes at 37°C, after which 1.5 ml of 0.6 M HClO$_4$ were added to stop the reaction. When necessary, the precipitated protein was removed by centrifugation. A 0.5-ml aliquot was diluted to 3.0 ml with H$_2$O, and the optical density of this solution was determined at 290 µm in a Beckman model DU spectrophotometer. A unit of activity was defined as the amount of enzyme required to deaminate 1 µmole of dCMP (equivalent to a decrease in 10 optical density units at 290 µm) under the above described reaction conditions. Although the reaction velocity at this substrate concentration was only about 60% of the maximal velocity (see "Assay 2"), this spectrophotometric assay procedure was found to be much more sensitive than those utilizing saturating substrate levels of dCMP. Complications resulting from the apparent pseudo (water is most probably the other reactant in addition to the substrate) first order nature of the kinetics were not encountered, as the deamination was essentially linear.
for 40% of the total reaction and proportional to the enzyme concentration.

To ensure further the latter effect, an amount of enzyme was assayed giving an optical density change of about 0.100 from each step of the purification scheme. For a comparison of the substrate specificity of the enzyme (Table I), the following micromolar extinction values were used: 5-methyl-dCMP, ε280 = 10.1; 5-bromo-dCMP, ε280 = 7.48; 5-fluoro-dCMP, ε280 = 8.68; 5-iodo-dCMP, ε280 = 6.78. The values for the halogenated nucleotides were determined from a comparison of the respective absorbances at the indicated wave lengths, with total phosphate analyses. The obtained micromolar extinctions at these wave lengths, and those at the maxima, were comparable to the values reported in the literature for the corresponding nucleosides (17, 26).

Assay 2—To obtain a measurement of maximal activity, the reaction mixture contained 5.0 μmoles of dCMP, 50 μmoles of Tris·Cl (pH 8.0), water, and enzyme to a final volume of 0.50 ml. In some cases, dCTP and MgCl₂ were added and these are noted in the appropriate tables and figures. The experimental conditions were the same as those in Assay 1, except that an aliquot of 0.10 ml was diluted to 5.0 ml.

Assay 3—To determine enzyme activity at the 2-mM level of substrate, in the absence of dCTP and MgCl₂, it was necessary to employ dCMP-2-¹⁴C as the substrate. The reactions in this case were stopped by heating at 100° for 2 minutes. Water (2 ml) was added to the reaction tubes and the contents passed through columns of Dowex 50-H⁺ (1 cm in diameter × 3 cm in length). The columns were washed with water until a total volume of 10 ml of eluate was collected. The radioactivity in the eluate, caused primarily by dUMP-2-¹⁴C, was determined by plating 0.5 ml and measuring the activity in an automatic Nuclear-Chicago thin window gas flow counter. By this method, accurate Km values for dCMP could be determined in the presence and absence of dCTP and Mg++. Enzyme preparations with a specific activity of 40 or greater were used for all of the substrate specificity, kinetic, and inhibition studies.

Charcoal Treatment of Enzyme—Acid-washed Norit was thoroughly washed with 0.1 M EDTA (pH 7.5), followed by glass-redistilled water, and dried. Approximately 10 mg of charcoal were stirred with 0.3 mg of enzyme in 2 to 3 ml of 0.05 M phosphate (pH 7.5) containing 0.04 M dCTP and 0.0015 M MgCl₂. This treatment effectively removed the dCTP, as evidenced by the complete absence of enzyme activity, when assayed at the 2-mM level of substrate.

RESULTS

Enzyme Purification—In our initial studies with the deaminase, the extreme lability of the enzyme militated against any attempt at purification. However, the finding that the combination of dCTP and Mg++ (15, 27) stabilized the enzyme enabled the purification scheme in Table I to be developed. To date this outline has been followed approximately 20 times with excellent reproducibility. The only step that may present some difficulty is the absorption and elution from phosphocellulose, as different components have on the enzyme at low substrate concentrations, is not believed to be caused by the stabilizing effect that these components have on the enzyme at low substrate concentrations, but rather by an alteration in the structure of the enzyme with an attendant change in the affinity of the enzyme for its substrate.

Ultracentrifuge patterns on the isolated protein with the synthetic boundary cell showed it to possess a low sedimentation coefficient (1 to 2 S), and as a result, it is difficult to ascertain at present whether the apparent homogeneity that develops after 32 minutes at 59,780 r.p.m. is caused by the presence of different aggregate states of the protein, back-diffusion effects, or a general lack of homogeneity of the sample. Of interest is the finding that sucrose gradient centrifugation (28) of the protein in the presence of dCTP and Mg++ yields a much higher sedimentation coefficient (about 7 S) than that obtained with the ultracentrifuge, and may indicate that the rate of sedimentation varies with the conditions employed. A more detailed discussion of the sucrose gradient data is presented below.

Cofactor Requirements—As revealed earlier (13, 15), the measurement of enzyme activity by the spectrophotometric assay at substrate concentrations of 2 mM and less requires the presence of dCTP in crude extracts and dCTP + Mg++ in purified deaminase preparations. Since the apparent inability to detect the enzyme in the absence of dCTP and Mg++ is caused by the insensitivity of the assay at the lower substrate concentrations, dCMP-2-¹⁴C was utilized as described in Assay 3 in “Experimental Procedure.” With this substrate it was possible to determine apparent Km values for dCMP in the presence and absence of the cofactors. Thus, Fig. 2 shows that the combination of dCTP and Mg++ decreases the Km for dCMP from 4.1 mM to 0.69 mM. Even more striking is the marked dependence of the enzyme on the cofactor combination at substrate concentrations that are less than 2 mM.

The dCTP requirement is more readily observed in Fig. 3; dUDP could not replace dCTP, as was observed earlier in cell-free extracts of chick embryo homogenates (13). The Mg++ requirement was first encountered on observing a marked loss in activity after elution of the enzyme from the phosphocellulose column and to a lesser extent after passage of enzyme solutions through Sephadex G-25. In both cases, the activity could be effectively restored by the addition of Mg++ to the assay mixtures or to the depleted enzyme solutions. An almost complete dependence on Mg++ could be effected by prior treatment of the phosphocellulose column with EDTA. A study of the metal ion requirement of enzyme eluted from such a column is presented in Table II, where it is seen that Mn++ and Ca++ could substitute for Mg++. The addition of EDTA to the complete assay solution again eliminated the activity of the deaminase. As discussed below, the dependence upon dCTP and Mg++ at suboptimal substrate concentrations is not believed to be caused by the stabilizing effect that these components have on the enzyme at low substrate concentrations, but rather by an alteration in the structure of the enzyme with an attendant change in the affinity of the enzyme for its substrate.

Studies with α- and γ-Labeled dCTP—The possibility that dCTP was promoting the reaction in the following manner

\[ \text{dCMP} + \alpha\text{-dCTP} \rightarrow \alpha\text{-dUMP} + \text{dCTP} + \text{NH}_3 \]
was ruled out with the indicated labeled compound, as no radioactivity was found in the isolated dUMP. Neither was radioactivity found in added carrier dCDP, suggesting that activation did not occur by phosphate transfer to the enzyme. The loss of phosphate from dCTMP was measured in the course of an enzyme assay and found to be negligible. The above evidence would support the role of dCTP as being more in the nature of a cofactor than of a coenzyme. A cofactor is distinguished in this case by its apparent noninvolvement as a participant in the reaction.

Substrate Specificity—The initial studies of Scarano and Maggio (1) indicated that 5-methyl-dCMP is deaminated by extracts of sea urchin eggs in addition to dCMP. Later studies (29) with purified rabbit liver deaminase yielded similar results and also revealed 5-hydroxymethyl-dCMP to be deaminated. It was thus not too surprising to find 5-fluoro- and 5-bromo-dCMP to be deaminated by rat embryo extracts (30) used as a source of the enzyme. Recently, the 5-iodo derivative of dCMP was also found to be deaminated by tissue extracts (31). The activity of the purified chick embryo enzyme with these various substrates was compared and apparent $K_m$ values obtained are presented in Table III.

The similarity in apparent $K_m$ values for all of the compounds except 5-fluoro-dCMP suggests that the enzyme can accommodate these compounds equally well despite the divergent van der Waals' radii of the substituting groups: 1.35 Å for fluoro; 1.90 Å for bromo; 2.0 Å for methyl; and 2.15 Å for iodo (32). From a comparison of the $V_{max}$ of the various compounds, it would appear that inductive effects from the substituted groups may be a major factor involved in determining the rates of deamination. However, both steric and inductive effects may be involved in the deamination of 5-fluoro-dCMP.

Stability Studies—It appears that in the process of activating the deaminase at suboptimal substrate concentrations, dCTP and Mg$^{++}$ confer a more stable configuration on the enzyme structure. This effect was most readily shown by incubating the enzyme at 37° in the presence and absence of dCTP and Mg$^{++}$ (Fig. 4). In contrast to the instability of the enzyme observed in our initial experiments (2), purified preparations have been kept for at least 2 months in the presence of dCTP and Mg$^{++}$ without loss in activity. The specificity of the stabilization was further studied by the substitution of various nucleotides for dCTP, but, as can be seen, dCTP was the most effective of the deoxyribonucleotides tested. dCDP, which is not presented, was as ineffective as dGTP and dATP. These latter results are in contrast to those reported originally by Myers (33) on the stabilizing effect of various nucleotides on the deoxycytidylate deaminase in thymus extracts. However, dGMP was found to be almost as effective as dCTP as a stabilizing agent (27), which is in agreement with the findings of Myers (33). Evidence has been obtained suggesting that the stabilizing effects of dGMP and dCTP are related to their ability to cause aggregation of enzyme subunits (see "Sucrose Density Gradient Studies" for more detail). As expected, dCMP protected the enzyme against loss in activity as was also found by Scarano, Bonaduce, and De Petrocellis (29), but concentrations as high as 14 mM were required to give results comparable to those with dCTP and dGMP at concentrations of 0.20 mM and 0.48 mM, respectively. The addition of EDTA to the incubation mixture eliminated the stabilizing effect of dCTP, thus emphasizing a similar requirement for Mg$^{++}$ in both the activation and stabilization of the deaminase. It would appear at present that the protective influence of the previously mentioned compounds may be related to their ability to bind to the enzyme. Some support for this proposal comes from the finding that dGMP is an effective competitive inhibitor of the deaminase (29). Similarly, N-4-hydroxy-dCMP (15), another strong competitive inhibitor of the enzyme, was found to be a good stabilizing agent. Whether the competitive inhibitors protect the enzyme in a manner similar
It could be claimed that the activating effect of dCTP and Mg\(^{++}\) is in reality a reflection of their ability to prevent the inactivation of the enzyme during the 10-minute assay period. Thus, the stabilization of the deaminase would appear to be secondary to the observed activation effect.

Inhibition of Deaminase by dTTP and N-4-Hydroxy-dCMP—The finding that dTTP (13) is a potent inhibitor of deoxycytidylate deaminase has been further amplified in studies with the purified enzyme. Of significance is the finding that the inhibition is extremely specific. Neither dTDP nor any other ribo- or deoxyribonucleotide is effective at concentrations comparable to inhibitory levels of dTTP, with the exception of such analogues as dUTP and 5-fluoro dUTP. It was possible to obtain a Michaelis plot with the purified enzyme and, as indicated in Fig. 5, dTTP would appear to be a noncompetitive inhibitor of dCMP deamination, even though the lines do not meet at 1/ν = 0. The latter is possibly caused by experimental error or by the presence of dCTP. The apparent K\(_v\) of 0.1 mm does not present a valid picture of the inhibitory effectiveness of dTTP since the reactions were conducted of necessity in the presence of dCTP, which, as indicated previously (13, 15, 27), effectively reverses the inhibition caused by dTTP. A measure of the true K\(_v\) can possibly be obtained from an extrapolated plot of the apparent K\(_v\) versus the concentration of dCTP.

Inhibition studies with dTTP were also conducted at concentrations of dCMP (10 mm) that did not require the presence of dCTP and Mg\(^{++}\) for the demonstration of activity. Mg\(^{++}\) was apparently not required at these high substrate levels for the demonstration of inhibition by dTTP and presumably is not required at the lower substrate levels (2 mm). However, this will be difficult to prove as Mg\(^{++}\) is necessary in order to observe activity at the 2-mm level of dCMP. It should be noted, however, that the combination of dCTP and Mg\(^{++}\) reversed the inhibition caused by dTTP at high substrate levels (10 mm).

The finding that N-4-hydroxy-dCMP was an effective inhibitor of the deaminase held promise that this compound could be used in studies in vivo and in mince studies as a means of evaluating the metabolic importance of deoxycytidylate deaminase. As shown in Fig. 6, this nucleotide is a competitive inhibitor of the deaminase with an apparent K\(_v\) of 0.013 mm. The corresponding ribonucleoside derivative was completely inactive as an inhibitor. However, although the N-4-hydroxy derivative would have been an interesting inhibitor, it has since been found that N-4-hydroxy-dCMP-2-\(^{14}\)C is reconverted to dCMP in the cell, and thus eliminated as a useful metabolic tool.

pH Optimum of Deoxycytidylate Deaminase—Since, as indicated, the deamination of dCMP is effected at suboptimal substrate concentrations (2 mm) under different conditions than those at saturating levels of substrate (10 mm), it was of interest to determine whether the pH optimum was dependent on the conditions employed. As shown in Fig. 7, this was found to be the case. The optimal pH at the 10-mm level of dCMP appears to fall at about 7.6, whereas at the 2-mm level the optimal pH falls at about 8.2. Whether the differences in the pH optimum curves are caused by inherent structural differences in the protein under the described conditions, or to the distinct possibility that two different enzymes are involved, is not known as yet. As indicated in the sucrose gradient studies below, the former possibility would appear to be the most likely at present.

The optimal pH for the halogenated and \(5\)-methyl derivatives of dCMP was also determined at the 2-mm level of substrate, and results similar to those for dCMP were obtained, except for

4 F. Maley, unpublished observations.
slight differences in the broadness of the pH curve. In addition, the optimal pH for 5-iodo-dCMP appeared to be slightly lower than that for the other substrates (pH 8.0).

Reversal of CMB Inhibition by dCTP—The findings by Scarano, Geraci, Polzella, and Campanile (16) that CMB inhibition of a spleen deaminase could be reversed by dCTP has been further investigated with the purified chick embryo enzyme, with similar results being obtained. As expected, the inhibition was far more effective than originally reported by us for crude chick embryo extracts (2). The results suggest that sulfhydryl groups may be present in or near the active site, or that reaction with CMB results in a detrimental alteration of the enzyme structure. Since the inhibition is prevented by dCTP, as shown in Table IV, the reactive sulfhydryl groups must become masked or buried within the protein structure on the addition of dCTP, which would tend to support the thesis that the combination of dCTP and Mg$^{++}$ alters the conformation of the enzyme or causes an aggregation of the protein to occur. Either change or a combination of both could explain the decreased reactivity of CMB. As in the case of dTTP, the extent of inhibition was dependent on the amount of dCTP employed.

EM and iodoacetamide were far less effective than CMB as inhibitors (Table IV), but here, too, the inhibition was prevented by dCTP.

**Reversal of Urea and Duponol Inhibition by dCTP—**Further

**Fig. 4.** Stability of deoxycytidylate deaminase at 37°. Norit-treated enzyme (66 μg of protein) was incubated at 37° in the presence of 1 μmole of MgCl$_2$, 15 μmoles of phosphate (pH 7.5), and 0.2 μmole of the indicated nucleotides in a volume of 0.2 ml. Aliquots were removed from each tube at the indicated time intervals and assayed for deoxycytidylate deaminase activity with Assay 1.

**Fig. 5.** Effect of dTTP on dCMP deaminase. Assay 1 was used except that the concentration of dCMP was varied from 1 mM to 8 mM. The levels of dTTP were 0.18 mM (O—O) and 0.072 mM (●—●); no inhibitor (X—X).

**Fig. 6.** Effect of N-4-hydroxy-dCMP on dCMP deaminase. Assay 1 was used except that the concentrations of dCMP were varied from 1 mM to 8 mM. The levels of N-4-hydroxy-dCMP were 0.019 mM (●—●) and 0.058 mM (X—X); no inhibitor (O—O).

**Fig. 7.** Determination of pH optimum. Assays 1 and 2 were used, respectively, for the 2 mM (●—●) and 10 mM (O—O) dCMP reactions. The amount of enzyme protein employed in ●—● was 7 μg and in O—O 17 μg. The determinations of pH were made at 37° on a Radiometer TTTr pH meter with a single probe microelectrode after incubation for 10 minutes at 37°. Enzyme activity was measured after stopping the reactions as previously described. No more than 15 seconds elapsed between the pH determination and the addition of acid.

**Table IV**

**Effect of sulfhydryl inhibitors on deoxycytidylate deaminase and prevention of inhibition by dCTP**

The reaction conditions were those used in Assay 1.

<table>
<thead>
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<th>Compound</th>
<th>Concentration</th>
<th>dCTP Concentration</th>
<th>Inhibition</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>%</td>
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<td>CMB</td>
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<td>0.042</td>
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</tr>
<tr>
<td></td>
<td>0.002</td>
<td>0.042</td>
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<td>EM</td>
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<td>0.0016</td>
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</tr>
<tr>
<td></td>
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<td>0.04</td>
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<tr>
<td>Iodoacetamide</td>
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<td>0.0016</td>
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support for the possibility that dCTP and Mg$^{++}$ exert their effect through an alteration in enzyme structure is provided by the finding that the inhibition encountered by increasing concentrations of urea is reversed by the above cofactors (Fig. 8).
Similar results were obtained with Duponol (Table V). The ability of these substances to inhibit enzymes by disaggregation has been clearly shown in the case of muscle aldolase (34), but although an analogy can be drawn with the deaminase, the inhibition could possibly have been effected through less drastic means, such as an alteration in a helical region of the protein.

The nature of the inhibition reversal mechanism appears to be unrelated to that involving hydrophobic areas of the protein, as dCTP and Mg++ could not reverse the inhibition encountered with dioxane and ethylene glycol.

Sucrose Density Gradient Studies—The utility of gradient studies by the procedure of Martin and Ames (28) as a means of estimating changes in protein molecular weight has been amply shown by many investigators (35-38). Such studies were thus conducted with the deaminase under a variety of conditions to determine whether an alteration in the state of aggregation could be effected. Particular interest was directed toward those conditions under which a dependence on dCTP and Mg++ for activity was observed.

When the enzyme was centrifuged in the presence of a 5 to 20% sucrose gradient containing 0.10 mM dCTP and 1.5 mM MgCl₂, a fairly sharp peak of activity was obtained from tubes 20 to 28 under the indicated conditions in Fig. 9. Comparison with catalase as a reference standard yielded an $s_{20,w}$ of about 7.5, which was far from agreement with the ultracentrifuge data (1 to 2 S). However, if the enzyme was centrifuged under conditions associated with high substrate concentration (5 mM dCMP), a considerable loss in activity was encountered, but an average $s_{20,w}$ value of about 2.0 was obtained, which compares favorably with the ultracentrifuge data. The loss in activity could have been anticipated from the stability studies. Gradient centrifugation of the enzyme in the presence of dCTP, Mg++, and dCMP (5 mM) yielded enzyme in both regions, thus suggesting that an equilibrium mixture of different aggregate forms was present. Similar results were obtained if the dCTP, Mg++ concentration was maintained, but the dCTP concentration lowered to 1 mM. In the hope of diminishing the considerable loss in activity associated with the centrifuge run in the presence of 5 mM dCMP, 1.5 mM MgCl₂ was added. The added Mg++ failed to prevent the loss in activity, but a small amount of activity was now found in the faster sedimenting area. An explanation was derived from the fact that a small amount of dCMP (0.001 mM) was present in the 0.05 ml of solution containing 1 μmole of dCMP, 1 μmole of Mg, and 50 μmoles of Tris (pH 8.0) and incubated at 37° for 30 minutes.

An interesting result was obtained by centrifuging the enzyme in a gradient containing 0.055 μmole of dGMP per ml as a stabilizing agent. As indicated above, dGMP has been found to be a stabilizing agent.

**Table V**

<table>
<thead>
<tr>
<th>dCTP concentration (mM)</th>
<th>Δ$Ad_{20}$</th>
<th>+ Duponol</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.008</td>
<td>0.306</td>
<td>0.014</td>
</tr>
<tr>
<td>0.02</td>
<td>0.320</td>
<td>0.140</td>
</tr>
<tr>
<td>0.04</td>
<td>0.320</td>
<td>0.260</td>
</tr>
<tr>
<td>0.08</td>
<td>0.320</td>
<td>0.260</td>
</tr>
</tbody>
</table>

An explanation for these results may come from the fact that the ratio of protein (micrograms) to dCTP (micromoles) in the ultracentrifuge studies was about 50,000:1, whereas in the sucrose gradient it was only 600:1. Also, Mg++ was not present in the former case and, as indicated in the gradient studies, Mg++ was necessary in addition to dCTP in order to show the elevated sedimentation rate of the protein. The low sedimentation values obtained with the sucrose gradient in the absence of dCTP would tend to bear these points out. Further studies with higher levels of dCTP and Mg++ comparable to those employed in the gradient studies may clarify this subject and are currently in progress.
competitive inhibitor (29) of the deaminase and was expected to yield similar results to those obtained with dCMP. However, the enzyme activity was now found to sediment in a manner analogous to that obtained with dCTP and Mg++. It would thus appear from the competitive inhibition studies (29) that the inhibitory and stabilizing effects of dGMP are distinct from one another. In contrast to these findings, preliminary studies in our laboratory indicate that dGMP and dCTP may compete for the same site at the 2-mm level of substrate, as the inhibition caused by the dGMP is effectively reversed by dCTP (Table VI). It is difficult to reconcile these conflicting results at present, unless dGMP exerts its effect at two sites on the enzyme.

The stabilizing influence of dCTP and dGMP on the enzyme may be related to the compactness of the aggregate formed in its presence. Thus, sulphydryl groups that are on the surface of the enzyme and others susceptible to oxidation in the absence of these nucleotides may now become buried within the aggregated structure of the enzyme and as a result be less susceptible to attack. This proposal is supported by the data illustrating the prevention of CMB inhibition by dCTP (Table IV). Further experiments will be necessary, however, to clarify these results.

As for the significance of dGMP as an inhibitor of the deaminase, the data indicate that it can effectively inhibit the enzyme, although not as well as dTTP (Table VI), and thus may exert a regulatory influence on the deaminase in vivo. The reversal of the inhibition by dCTP (Table VI) is in support of such an assumption. However, calculations on the competitive effectiveness of dGMP and dTTP as regulators of the deaminase in vivo will not be possible until the relative pool sizes of these compounds are known. If dGMP was a competitive inhibitor of dCMP, as found with higher levels of substrate (29), one would have anticipated that the affinity of the enzyme for dGMP would be increased by dCTP. The opposite was found to be the case. Thus, the reversal of dGMP inhibition by dCTP and the similarity in the sedimentation patterns obtained with the two nucleotides suggest that they exert their effects at closely related sites.

Attempts to measure a sedimentation rate of the enzyme in the presence of dTTP, Mg++, or dTTP, Mg++, and dCMP were unsuccessful because of the instability of the enzyme, under these conditions, over the length of time that the centrifuge run was conducted (11 hours).

<table>
<thead>
<tr>
<th>TABLE VI</th>
<th>Reversal of dGMP and dTTP inhibition of deoxyxanthidylate deaminase by dCTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction conditions were those used in Assay 1 with additions as indicated in the table.</td>
<td></td>
</tr>
<tr>
<td>dCTP</td>
<td>Inhibition</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>μM</td>
<td>%</td>
</tr>
<tr>
<td>2.4</td>
<td>77</td>
</tr>
<tr>
<td>3.6</td>
<td>70</td>
</tr>
<tr>
<td>5.6</td>
<td>65</td>
</tr>
<tr>
<td>9.6</td>
<td>43</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Numerous examples of feedback control mechanisms effected by an end product of a metabolic pathway have been postulated for bacterial systems (39). However, the finding that deoxycytidylate deaminase, an enzyme localized at a juncture in the conversion of dCMP to dCTP and dTTP, is effectively regulated by low levels of dTTP makes it one of the first clear-cut examples of end product inhibition in an animal system. Of special significance is the demonstration that the product at the other end of the juncture, dCTP, can reverse the inhibition caused by dTTP at equally low concentrations. The cell is thus presented with an effective means of maintaining a balanced synthesis of dCTP and dTTP from pyrimidine precursors. Evidence that may support this proposition comes from the finding that thymidine can spare the utilization of d-cytidine in embryo mince preparations (13). However, there is no direct proof that this apparent sparing effect results from the above proposed mechanism.

The exact nature of the mechanism by which the combination of dCTP and Mg++ enhances the affinity of the enzyme for the substrate dCMP is not known, but it would appear from the sucrose gradient studies that a change in the state of aggregation of the enzyme and possibly conformation are involved. Further evidence comes from the observations that the inhibitions effected by CMB, urea, and Duponol are overcome by dCTP and Mg++, and that the latter cause a shift in the pH optimum of the deaminase. A recent report (42) revealed ADP to increase the affinity of threonine deaminase for its substrate in a manner similar to that described for dCTP and Mg++ in this study.

The alteration of enzyme structure, with attendant changes in activity by substances that are not substrates of the enzyme, that are not participants in the reaction, and that exert their effect at a site removed from the substrate site, has become a subject of growing magnitude and interest. Monod, Changeux, and Jacob (43) have recently reviewed this area rather extensively and have termed the site at which end product inhibition is effected as an allosteric site. The **allosteric** nature of the deaminase inhibition by dTTP is supported by the finding that this inhibition appears to be noncompetitive (Fig. 5). Because of the difficulty in maintaining enzyme stability in the presence of dTTP for the lengths of time required for the sucrose gradient studies, it has not been possible to determine whether this compound exerts its effect by causing a change in the state of aggregation of the enzyme, as would appear to be the case for dCTP and Mg++. Nor has it been possible to clearly differentiate the activation effects of dCTP and Mg++ at low substrate concentration from that at higher levels, though the data would tend to support the possibility that dCMP causes a disaggregation of the enzyme at the latter concentrations (Fig. 9) to a form with a lower affinity for the substrate. Since the activation effect by dCTP and Mg++ is not observed at the 10-mm levels of dCMP, it may be that a desensitization to activation results from the dissociation of the enzyme. That a change in protein structure probably occurs at the higher substrate levels is supported by the demonstration of a shift in the pH optimum at these concentr-
tions, as well as by the sucrose gradient studies. An analogous desensitization effect was observed for the inhibition of aspartate transcarbamylase (35) by CTP on heat dissociation of this enzyme. However, it has not been possible as yet to obtain a desensitization from the dTTP inhibition with deoxyxycytidylate deaminase.

Regardless of the numerous mechanistic interpretations that can be derived from the observed effects of dCTP and dTTP on deoxyxycytidylate deaminase, the extremely low levels of nucleotide required to regulate the activity of this enzyme cannot help but suggest an important role for the deaminase in the synthesis of dTTP.

SUMMARY

A procedure for the preparation of highly purified deoxyxycytidylate deaminase from 6-day chick embryos has been described. The purified enzyme has been found to be markedly dependent on the presence of deoxycytidine 5′-triphosphate (dCTP) and Mg++ at substrate concentrations of deoxycytidine 5′-monophosphate of 2 mM and less. However, at higher concentrations of the monophosphate (10 mM), maximal enzyme activity could be attained even in the absence of these cofactors.

Sucrose density gradient studies have shown that an aggregation of the enzyme protein in the presence of dCTP and Mg++ is associated with the apparent increase in affinity of the enzyme protein in the presence of dCTP and Mg++. This aggregation is found to be more readily detectable in the presence of urea and Duponol.

The synthesis of N-4-hydroxydeoxycytidine 5′-monophosphate and its competition with deoxycytidine 5′-monophosphate for the enzyme is also associated with the aggregated state of the protein. Deoxyguanosine 5′-monophosphate, an effective inhibitor of the enzyme, is reversibly inhibited by deoxycytidine 5′-triphosphate, is that of thymidine 5′-triphosphate, and is reversed by deoxycytidine 5′-triphosphate. Further evidence that a structural change is effected by dCTP and Mg++ comes from studies showing these cofactors to produce a shift in the pH optimum of the enzyme, a prevention of p-chloromercuribenzoate inhibition, and a reversal of inhibition by thymidine 5′-monophosphate.

The previously described inhibition of deoxycytidylate deaminase by thymidine 5′-triphosphate has been further amplified by studies showing these cofactors to produce a shift in the pH optimum of the enzyme, a prevention of p-chloromercuribenzoate inhibition, and a reversal of inhibition by thymidine 5′-monophosphate, suggest that thymidylate biosynthesis from deoxycytidylate is regulated by end product inhibition.

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