Enzymatic Synthesis of Deoxyribonucleotides. VII. Studies on the Hydrogen Transfer with Tritiated Water*

Agne Larsson

ABSTRACT: The enzymatic conversion of cytidine diphosphate to deoxycytidine diphosphate with purified enzymes from *Escherichia coli* B shows an absolute requirement for a dithiol as the hydrogen donor. This requirement is met by a specific protein (thioredoxin) or by reduced lipoate. The mechanism of hydrogen transfer during the enzymatic reduction of cytidine diphosphate was studied in the presence of tritiated water. Under these conditions the SH groups of the hydrogen donor are rapidly labeled. About 0.3 atom of tritium was found to be incorporated nonexchangeably into the deoxyribose of deoxycytidine diphosphate. The position of the label was determined by degrading deoxyribose 5-phosphate. After treatment with deoxyribose aldolase and alcohol dehydrogenase the hydrogens of positions 1 and 2 were obtained as ethanol, while those of positions 3 to 5 were recovered as glyceraldehyde 3-phosphate. All isotope was found in ethanol. In a second degradation deoxyribose 5-phosphate was oxidized with bromine to deoxyribonic acid 5-phosphate. This gave no loss of tritium, demonstrating that the hydrogen in position 1 was unlabeled.

The combined degradations thus showed that hydrogen was introduced exclusively into position 2′ during the formation of deoxycytidine diphosphate. The results exclude the formation of an intermediate containing a double bond in the sugar and favor a reaction mechanism involving the replacement of the hydroxyl group at position 2′ in CDP by a hydride ion.

In crude extracts of *Escherichia coli* B the enzymatic formation of deoxycytidine diphosphate (deoxyCDP)[1] from CDP required the presence of reduced triphosphopyridine nucleotide (TPNH), adenosine triphosphate (ATP), and magnesium ions (Reichard *et al.*, 1961). A partially purified enzyme fraction (fraction B) from the same organism, however, was no longer active with TPNH but, instead, showed an absolute requirement for reduced lipoate as the hydrogen donor (Reichard, 1962). Further work demonstrated that reduced lipoate acted as a model substance by replacing the physiological hydrogen donor, which had been removed during the purification of fraction B. This hydrogen donor was identified as a low molecular weight protein, called thioredoxin (Laurent *et al.*, 1964). It contains a single disulfide group which is reduced with TPNH by a specific enzyme, thioredoxin reductase, resulting in the formation of two sulfhydryl groups (Moore *et al.*, 1964). Reduced thioredoxin then functions as hydrogen donor in the formation of deoxyCDP from CDP. The scheme shown in Figure 1 was proposed to explain the roles of the different factors in the CDP reductase system of *E. coli* (Laurent *et al.*, 1964).

In the presence of thioredoxin reductase and substrate amounts of TPNH thioredoxin acts in a catalytic fashion. In the test tube the thioredoxin system can be replaced by reduced lipoate in relatively high concentrations. In both cases the functional groups of the hydrogen donor are sulfhydryl groups.

In the present work the enzymatic reduction of CDP was carried out in the presence of tritiated water in order to study the mechanism of the hydrogen transfer. In tritiated water the functional groups of the hydrogen donor are labeled, since hydrogen atoms of sulfhydryl groups are known to exchange rapidly with the protons of water (Fisher *et al.*, 1953). The incorporation of tritium into nonexchangeable positions in the sugar moiety of deoxyCDP was then studied. The position of the label was determined by degrading the deoxyribose 5-phosphate obtained from deoxyCDP. Evidence is presented for a mechanism of the reduction of CDP involving the replacement of the hydroxyl group at position 2′ in CDP by a hydride ion.

Experimental Procedures

Materials. Nucleotides were obtained from Sigma Chemical Co., St. Louis, Mo., and California Corp. for Biochemical Research, Los Angeles, Calif. All ribonucleotides used were shown to contain less than 0.1% of deoxyribonucleotides. Deoxyribose 5-phosphate was

* From the Department of Chemistry II, Karolinska Institutet, Stockholm, Sweden. *Received May 21, 1965.* This work was supported by grants from the Medical Faculty of the Karolinska Institutet and Svenska Sällskapet för Medicinsk Forskning, and also by grants to Professor Peter Reichard from the U.S. Public Health Service (CA 07897-02), the Damon Runyon Foundation (DRG 470-F), the Swedish Medical Research Council, and the Swedish Cancer Society. A preliminary report of this work was presented at the 2nd Meeting of the Federation of European Biochemical Societies in Vienna, April 1965.

[1] Abbreviations used in this work: CDP, cytidine diphosphate; ATP, adenosine triphosphate; TPNH, reduced triphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide.

prepared from deoxyAMP as described by Lampen (1957). DL-Glyceraldehyde 3-phosphate and dihydroxyacetone phosphate were purchased from Sigma Chemical Co. Tritiated water was obtained from The Radiochemical Centre, Amersham, Buckinghamshire, England.

Reduced DL-lipoate (Gunsalus and Razzell, 1957), thioredoxin (Laurent *et al.*, 1964), thioredoxin reductase (Moore *et al.*, 1964), and a partially purified CDP reductase preparation from *E. coli* B, called fraction B (Reichard, 1962), were prepared in this laboratory. Deoxyribose aldolase was purified from *Lactobacillus plantarum* as described by Pricer and Horecker (1960). Deoxycytidine triphosphatase was prepared according to Zimmerman and Kornberg (1961); the material after the first DEAE-cellulose chromatography was used. Yeast alcohol dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, and glycerol 1-phosphate dehydrogenase were obtained from C. F. Boehringer & Soehne GmbH, Mannheim, Germany. Yeast alcohol dehydrogenase preparations from Sigma Chemical Co., Worthington Biochemical Corp., Freehold, N. J., and Nutritional Biochemicals Corp., Cleveland, Ohio, were also used.

Rhodium catalyst (5% rhodium on alumina) was purchased from Baker & Co., Newark, N. J. Sephadex G-100 was obtained from Pharmacia, Uppsala, Sweden; DEAE-cellulose (0.9 mequiv/g) from Serva Entwicklungslabor, Heidelberg, Germany; Dowex 2-X8 (200–400 mesh) and Dowex 50 W-X8 (200–400 mesh) from California Corp. for Biochemical Research; Carbowax 1540 and Diatoport W (acid washed, 60–80 mesh) from F & M Scientific Corporation, Avondale, Pa.; and ultrafilters from Membrangesellschaft, Göttingen, Germany.

Assays. Deoxyribonucleotides were determined by microbiological assays with *Lactobacillus acidophilus* (Hoff-Jørgensen, 1952), as described earlier (Larsson, 1963).

Deoxyribose 5-phosphate was measured by the diphenylamine method of Dische (1930) using the absorbance index at 595 mμ of 3.87×10^3 published by Domagk and Horecker (1958). Deoxyribose 5-phosphate was also determined enzymatically by measuring the acetaldehyde formed by the action of deoxyribose aldolase (Pricer and Horecker, 1960). D-Glyceraldehyde 3-phosphate was measured by the oxidation of DPNH with glyceraldehyde 3-phosphate dehydrogenase (Beisenherz *et al.*, 1953). Total, inorganic, and ester phosphate was determined according to King (1932). Ethanol was determined by gas chromatography according to Bonnichsen and Linturi (1962). Protein was analyzed as described by Bücher (1947).

Alcohol dehydrogenase activity was measured by the oxidation of DPNH in the presence of excess acetaldehyde according to the principles given by Theorell and Bonnichsen (1951). The following reagents were present in a final volume of 1 ml in a 1-cm cuvet: 0.5 μmole of freshly distilled acetaldehyde, 0.1 μmole of DPNH, and 50 μmoles of Tris-Cl^-, pH 7.6.

Triose phosphate isomerase was measured by the oxidation of DPNH in the presence of an excess of glyceraldehyde 3-phosphate and glycerol 1-phosphate dehydrogenase essentially as described by Beisenherz (1955). The following reagents were present in a final volume of 1 ml in a 1-cm cuvet: 0.4 μmole of DL-glyceraldehyde 3-phosphate, 0.1 μmole of DPNH, 10 μg of commercial glycerol 1-phosphate dehydrogenase (free from triose phosphate isomerase), and 50 μmoles of Tris-Cl^-, pH 7.6.

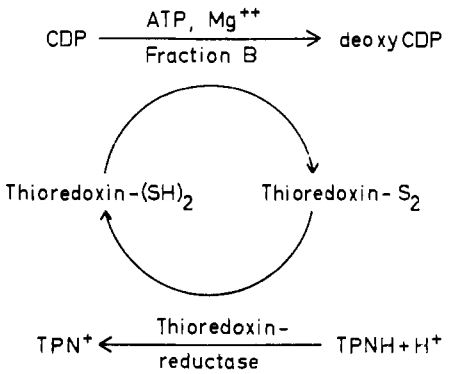


FIGURE 1: The CDP reductase system from *E. coli*.

The assays of alcohol dehydrogenase and triose phosphate isomerase were performed at room temperature and started by addition of the enzyme preparation to be tested. The reaction was monitored by absorbance readings at 340 mμ in a Zeiss spectrophotometer at half-minute intervals. Parallel controls to which no enzyme was added showed no decrease in absorbance. One unit of the respective enzyme activities is defined as that amount of protein which catalyzes the oxidation of 1 μmole of DPNH/min under the conditions of the assays. The calculations were based on the assumption that 1 μmole of DPNH gives an absorbance of 6.22 in these tests. Specific activity is defined as units of enzyme per mg of protein.

Radioactivity Measurements and Calculations. Radioactivity was measured in a Packard Tri-Carb liquid scintillation counter. Two different scintillation fluids were used, one described by Bray (1960), the other by the Packard Instrument Company (1959), using an ethanol–toluene medium (1:2). Tritium samples were counted by standard methods with counting efficiencies of 12.0 and 7.0%, respectively, for the two liquids. The counts presented in this paper have been corrected for background. All tritium determinations were performed under conditions where no quenching was observed except for the data shown in Figures 6 and 7C, which are presented without correction.

In the experiments in which the incubations were performed in water enriched with tritium, the specific activity of the hydrogen pool was calculated assuming that the whole volume of the volatile fraction of the incubation mixture was occupied by water, since the contribution of the other components to the hydrogen pool was considered to be insignificant. For this purpose the amount of radioactivity in 1 ml was determined

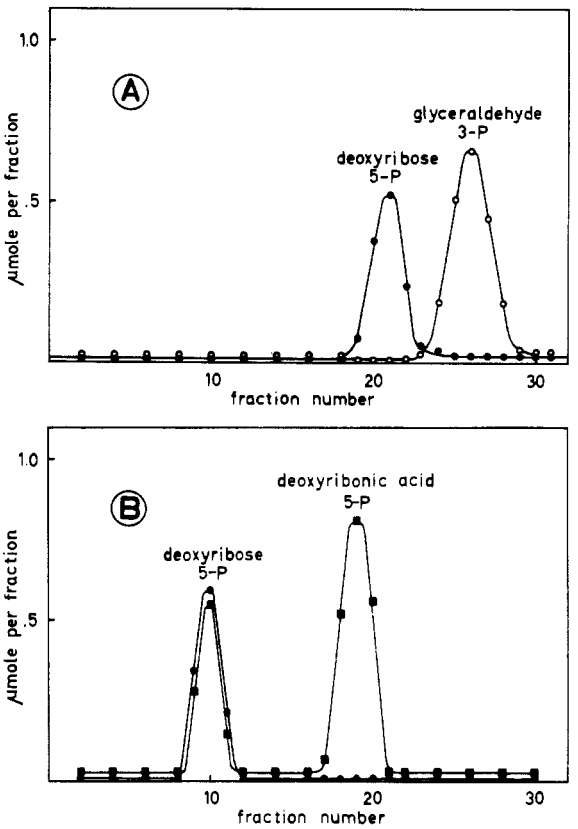


FIGURE 2: Separation of phosphate esters on Dowex 2-acetate columns (length 4.0 cm, diameter 0.4 cm) using convex gradients of ammonium acetate, pH 5.0, at +4°. Fractions of 1.0 ml were collected at a flow rate of 2.0 ml/hr. (A) Separation of deoxyribose 5-phosphate, 1.5 μmoles (●—●), and D-glyceraldehyde 3-phosphate, 3.0 μmoles (○—○). The closed mixing chamber contained 8 ml of water and the reservoir chamber 0.3 M ammonium acetate, pH 5.0. (B) Separation of deoxyribose 5-phosphate, 1.5 μmoles (●—●), and deoxyribonic acid 5-phosphate, 2.0 μmoles (■—■, ester phosphate). The closed mixing chamber contained 8 ml of water and the reservoir chamber 0.5 M ammonium acetate, pH 5.0.

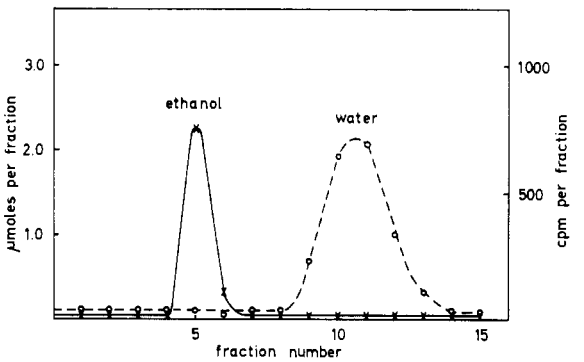


FIGURE 3: Separation of ethanol and water by gas-liquid partition chromatography. A sample containing ethanol, 3.0 μmoles, in 0.3 ml of tritiated water, 3000 cpm, was applied to a column of 25% (w/w) Carbowax 1540 and 75% (w/w) Diatoport (length 2 m, diameter 0.8 cm). A Perkin-Elmer vapor fractometer Model 116 E was used with nitrogen as carrier gas (0.4 atm, about 75 ml/min). The column temperature was 105°. Fractions were collected as described in the text and analyzed for ethanol (×—×) and tritium (○—○).

and the specific activity, expressed as cpm/μg-atom of hydrogen, was calculated assuming the presence of 1.11×10^5 μg-atoms of hydrogen in the same volume.

The relative specific activities of deoxyribose 5-phosphate and deoxyCMP (atoms of tritium/molecule) were calculated by dividing the actual specific activity of the compound by the specific activity of the hydrogen pool.

General Procedure for Experiments with Tritiated Water. Enzyme fraction B was incubated with curie quantities of tritiated water together with substrates and cofactors in volumes of 3 to 5 ml. The reaction was stopped by freezing the mixture to −20°. Tritiated water was then sublimed in an evacuated closed system and collected with a liquid nitrogen trap. This volatile fraction was used for determination of the specific activity of the hydrogen pool. The nonvolatile fraction was dissolved in 5 ml of ice-cold water, and the enzymes were inactivated by boiling for 10 min. The denatured protein was removed by centrifugation. The easily exchangeable tritium was removed from this fraction by lyophilization. The residue was again dissolved in water and lyophilized. This procedure was repeated until the distillate was free from tritium.

Isolation of Deoxyribose 5-Phosphate from DeoxyCMP. Deoxyribose 5-phosphate was prepared from the tritiated deoxyCMP by hydrolysis with weak acid after labilization of the bond between the base and the sugar (Grossman and Greenlees, 1961). For this purpose rhodium catalyst (about 0.2 mg/μmole of deoxyCMP) was added to the solution containing approximately 20 μmoles of deoxyCMP/ml at pH 7. Hydrogen gas was lead through the solution at 37°. The conversion of the cytosine residue to the dihydropyrimidine was followed by a decrease in the absorbance at 280 mμ. The hydrogenation was stopped after 30 min, at which time more than 95% of the original absorbance at 280 mμ had disappeared. The catalyst was removed by centrifugation, HCl was added to a final concentration of 0.1 M, and the solution was boiled for 10 min. The mixture was then cooled, neutralized, and applied to a column of Dowex 2-acetate. Deoxyribose 5-phosphate was eluted with a gradient of ammonium acetate, pH 5.0, and precipitated as the barium salt as described by Pricer and Horecker (1960).

Chromatographic Procedures. Deoxyribose 5-phosphate, glyceraldehyde 3-phosphate, and deoxyribonic acid 5-phosphate were separated by chromatography on Dowex 2-acetate columns by gradient elution with

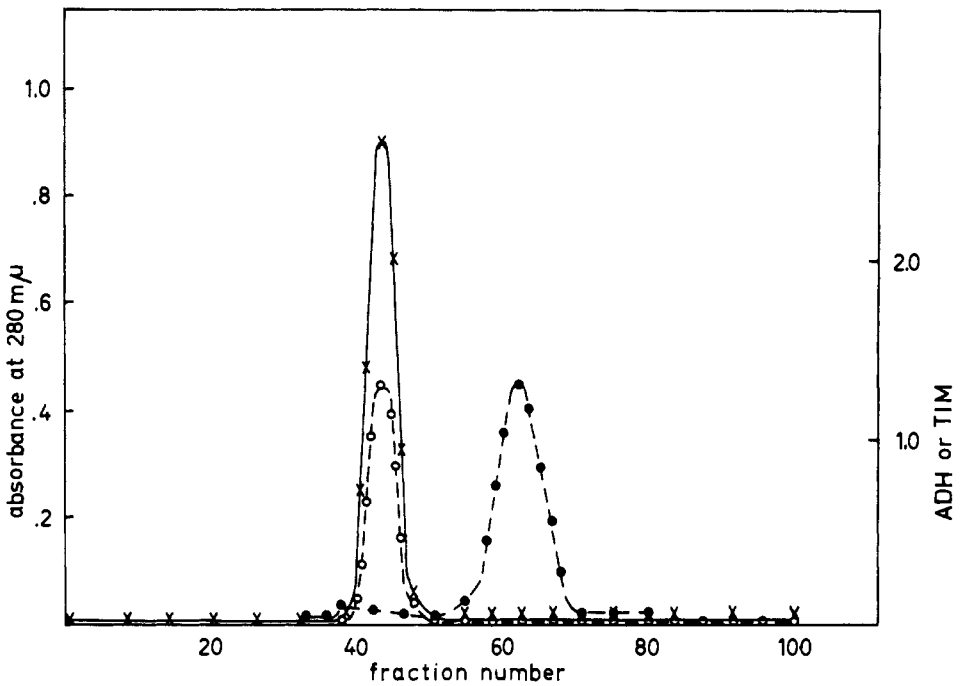


FIGURE 4: Chromatography of yeast alcohol dehydrogenase on a Sephadex G-100 column (length 100 cm, diameter 2 cm) after the heat inactivation step described in the text. The buffer used was 0.1 M potassium phosphate 0.05 M in EDTA, pH 8.5; temperature +4°. Fractions of 2.5 ml were collected at a flow rate of 7.5 ml/hr. Absorbance at 280 mμ (X—X); ADH = alcohol dehydrogenase activity, units × 10^{-3}/fraction (O--O); TIM = triose phosphate isomerase activity, units/fraction (●---●)

ammonium acetate, pH 5.0. The details of the procedures are given in the legend of Figure 2.

Ethanol obtained by cleavage of deoxyribose 5-phosphate with deoxyribose aldolase and alcohol dehydrogenase was separated from the water of the incubation mixture by gas chromatography. The conditions are given in the legend of Figure 3. Samples containing 1–3 μmoles of ethanol in 0.3 ml of water were applied to the column with the standard injection technique. The effluent from the column was collected in small tubes containing 0.3 ml of ice-cold water. Fractions were collected at 2-min intervals and analyzed for ethanol and tritium. The recovery of ethanol was about 80%.

The formation of dihydroxyacetone phosphate from glyceraldehyde 3-phosphate was demonstrated by thin layer chromatography on cellulose (Randerath, 1961) with phenol–water (90:10). The chromatograms were developed according to Bandurski and Axelrod (1951). The mobilities of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate relative to inorganic phosphate were 2.0 and 0.5, respectively.

Purification of Commercial Crystalline Alcohol Dehydrogenase from Yeast. Alcohol dehydrogenase was used to remove continuously the acetaldehyde formed by the action of deoxyribose aldolase on deoxyribose 5-phosphate. Crystalline preparations of alcohol dehydrogenase from two different sources were commercially available, one from horse liver, the other from yeast. van Eys (1961) showed that the horse liver enzyme is associated with a DPN-dependent aldehyde–ketone isomerase activity. In the presence of isomerase, glyceraldehyde 3-phosphate formed from deoxyribose 5-phosphate would be converted to dihydroxyacetone phosphate, and the hydrogen atom at position 4 in deoxyribose 5-phosphate would be lost to the water. Therefore crystalline alcohol dehydrogenase from yeast was used for our purpose. However, all available commercial preparations of this enzyme were found to contain varying amounts of triose phosphate isomerase activity as determined by the standard assay for the enzyme. Furthermore, the formation of dihydroxyacetone phosphate from glyceraldehyde 3-phosphate could be demonstrated directly by thin layer chromatography in an experiment in which 0.5 μmole of D-glyceraldehyde 3-phosphate and 10 μg of yeast alcohol dehydrogenase (Boehringer, Lot No. 6034211) in 0.1 ml of 0.05 M Tris-Cl^-, pH 7.6, were incubated at 37° for 10 min.

Since the presence of the isomerase during the cleavage of deoxyribose 5-phosphate would result in loss of hydrogen originally bound to carbon 4 in this molecule, it was important to eliminate the enzyme from the alcohol dehydrogenase preparation. This was done by heat inactivation in the presence of DPN which protected alcohol dehydrogenase (Sekuzu *et al.*, 1957), followed by column chromatography on Sephadex G-100.

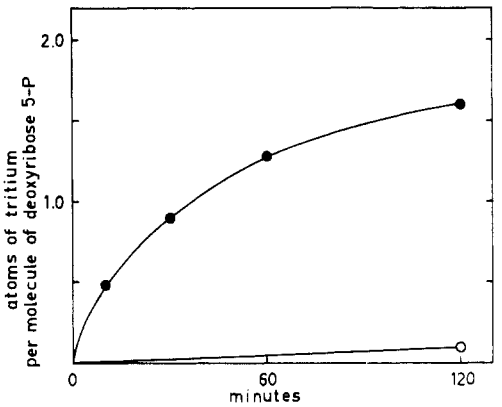


FIGURE 5: Incorporation of tritium from tritiated water into deoxyribose 5-phosphate in the presence (●—●) and in the absence (○—○) of deoxyribose aldolase.

Crystalline alcohol dehydrogenase from yeast (40 mg, Boehringer, Lot No. 6034211) containing 160 units of alcohol dehydrogenase activity and 2.9 units of triose phosphate isomerase activity per mg of protein was dialyzed against 0.05 M Tris-Cl$^-$, pH 7.6 (two 2-l. portions), for 4 hr. The effect of the heat treatment was strongly dependent on the ionic strength, but prolonged dialysis should be avoided, since this resulted in considerable inactivation of alcohol dehydrogenase. The enzyme preparation together with DPN (final concentration 6×10^{-2} M) was heated for 10 min at 65° in a final volume of 1.8 ml. Denatured protein was removed by centrifugation. In the supernatant fraction 65% of the original alcohol dehydrogenase activity was left, whereas only about 5% of triose phosphate isomerase activity remained. The mixture was then chromatographed on a column of Sephadex G-100 equilibrated with 0.1 M potassium phosphate, 0.05 M in EDTA, pH 8.5 (Figure 4). Fractions 40–47 which contained the alcohol dehydrogenase activity were pooled and concentrated by ultrafiltration against 0.05 M potassium maleate, pH 6.3. This material contained 160 units of alcohol dehydrogenase activity and 0.005 unit of triose phosphate isomerase activity per mg of protein. The procedure gave a 600-fold reduction of the contaminating isomerase activity relative to the alcohol dehydrogenase activity, the total recovery of which was 30%. This material was stored at −20° and was repeatedly thawed and frozen without loss of activity. When the purified yeast alcohol dehydrogenase preparation was used for trapping the acetaldehyde formed in the cleavage of deoxyribose 5-phosphate by deoxyribose aldolase no conversion of glyceraldehyde 3-phosphate to dihydroxyacetone phosphate occurred.

Results

Studies of the Reaction Mechanism of Deoxyribose Aldolase from Lactobacillus plantarum

Preliminary experiments demonstrated that isotope from tritiated water was incorporated into deoxyCDP formed from CDP by the CDP reductase system and that all of the label was localized in the sugar moiety of deoxyCDP. Enzymatic cleavage of deoxyribose 5-phosphate to acetaldehyde and glyceraldehyde 3-phosphate with deoxyribose aldolase was considered to be a suitable method for the further localization of tritium within the molecule. In this connection it was necessary to find conditions that would not result in an uncontrollable loss of tritium originally bound to deoxyribose 5-phosphate. For this purpose the mechanism of the deoxyribose aldolase reaction was studied by incubating deoxyribose 5-phosphate with deoxyribose aldolase in tritiated water and measuring the introduction of isotope into the sugar.

Deoxyribose 5-phosphate (8.0 μmoles), 50 μmoles of potassium maleate buffer, pH 6.3, and 80 μg of deoxyribose aldolase (320 units) in a final volume of 0.5 ml of water enriched with tritium to give a specific activity of 8700 cpm/μg-atom of hydrogen were incubated at 37° for 10, 30, 60, and 120 min, respectively. A control without enzyme was incubated at 37° for 120 min. The enzyme was inactivated by boiling for 2 min, and denatured protein was removed by centrifugation. The control sample was also boiled for 2 min. The solutions were then applied separately to Dowex 2-acetate columns. Tritiated water and easily exchangeable tritium were removed by washing the columns with 100 ml of 0.05 M acetic acid. Deoxyribose 5-phosphate was then eluted as described in the Experimental Section. In all cases a peak of diphenylamine-positive material coincided with a peak of radioactivity. The relative specific activities of the deoxyribose 5-phosphate samples obtained at each incubation time were determined (Figure 5). The results show that in the presence of enzyme the relative specific activity of deoxyribose 5-phosphate approached a value corresponding to the incorporation of 2 protons from water, whereas the nonenzymatic incorporation was neglible.

Studies of the Mechanism of DeoxyCDP Formation with Reduced Lipoate as Hydrogen Donor

Enzyme Incubation. The following mixture was incubated at 37° for 1 hr: 7 mg of fraction B, 4.0 μmoles of CDP, 10 μmoles of reduced DL-lipoate, 15 μmoles of ATP, 60 μmoles of $MgCl_2$, 200 μmoles of Tris-Cl$^-$, pH 8.5, 5 μmoles of EDTA, 30 μmoles of mercaptoethanol, and 1 curie of tritiated water (specific activity 4.25×10^5 cpm/μg-atom of hydrogen) in a final volume of 4.1 ml. The reaction was stopped, and water was removed as described in the Experimental Section. During the incubation 0.39 μmole of deoxyribonucleotides was formed as determined by the microbiological assay.

Isolation of Deoxycytidine Phosphates as DeoxyCMP. After removal of the exchangeable tritium the nonvolatile fraction of the incubation mixture was dissolved in 5 ml of 1 M HCl and boiled for 10 min in order to hydrolyze deoxyCTP and deoxyCDP to deoxyCMP. After a renewed removal of exchangeable tritium the residue, containing a total of 1.6×10^6 cpm, was applied to a column of Dowex 50-H$^+$ and eluted with 0.2 M acetic acid (Figure 6). DeoxyCMP (0.30 μmole) was

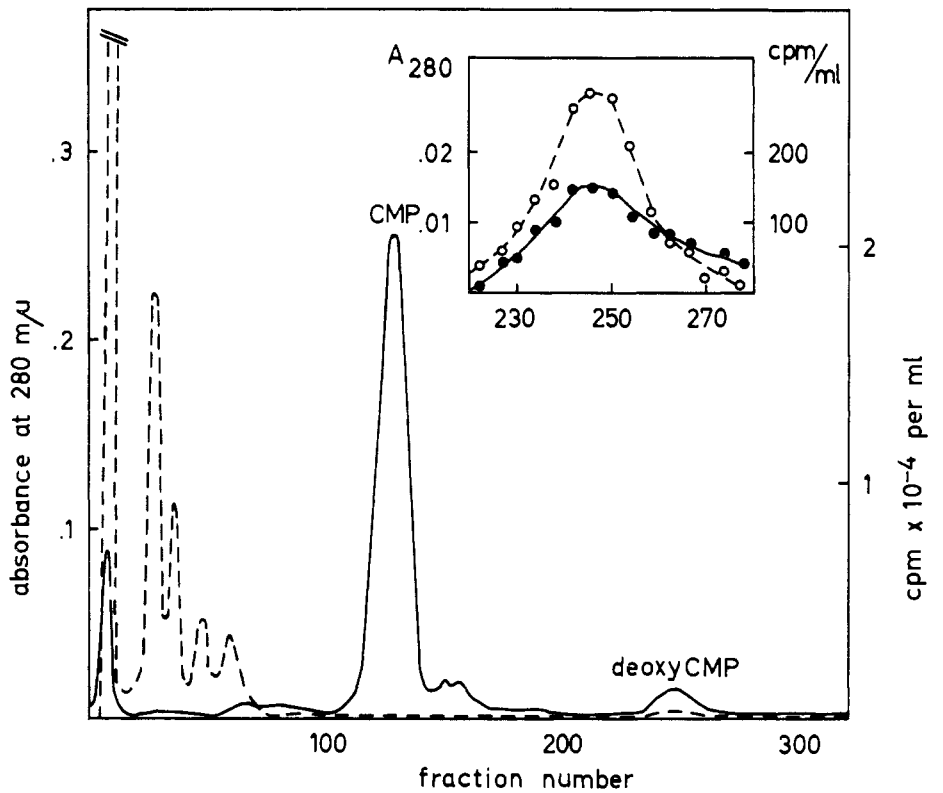


FIGURE 6: Isolation of deoxycytidine phosphates formed in the presence of tritiated water by the CDP reductase system with reduced lipoate as hydrogen donor. Deoxycytidine phosphates were converted to deoxyCMP by acid hydrolysis, and deoxyCMP was purified by chromatography on a Dowex 50-H^+ column (length 26 cm, diameter 2 cm) using 0.2 *M* acetic acid. Fraction volumes 10 ml, flow rate 40 ml/hr. Absorbance at 280 mμ (●—●) and radioactivity (O- - -O) were measured. The insert is an enlargement of the region of the deoxyCMP peak.

isolated with a specific activity of 1.24×10^5 cpm/μmole corresponding to the incorporation of 0.29 atom of tritium/molecule of deoxyCMP.

Isolation of Deoxyribose 5-Phosphate from DeoxyCMP. To 0.30 μmole of labeled deoxyCMP was added 69 μmoles of nonlabeled deoxyCMP to give a specific activity of 535 cpm/μmole. The linkage between the base and the sugar was split as described in the Experimental Section, and deoxyribose 5-phosphate was isolated by chromatography on a column of Dowex 2-acetate. The chromatogram showed only one peak of radioactivity, which coincided completely with deoxyribose 5-phosphate. After precipitation as the barium salt the material had a specific activity of 540 cpm/μmole of deoxyribose 5-phosphate. This demonstrates that all tritium originally present in deoxyCMP was located in the deoxyribose moiety.

Degradation of Deoxyribose 5-Phosphate with Deoxyribose Aldolase. Deoxyribose 5-phosphate (1.9 μmoles, specific activity 495 cpm/μmole[2]), 12 μg of deoxyribose aldolase (50 units), 3.6 μmoles of DPNH, 140 μg of purified yeast alcohol dehydrogenase (22 units), and 25 μmoles of potassium maleate buffer, pH 6.3, in a total volume of 0.3 ml were incubated at 37° for 10 min. This resulted in the cleavage of 1.5 μmoles of deoxyribose 5-phosphate as measured by the oxidation of DPNH. The reaction was stopped by freezing the mixture. The volatile fraction, containing ethanol and water, was then sublimed from the frozen mixture in an evacuated closed system and collected with a trap immersed in liquid nitrogen. The distillate contained 1.3 μmoles of ethanol which was separated from the water of the incubation mixture by preparative gas chromatography. The specific activity of the ethanol isolated was found to be 430 cpm/μmole.

The nonvolatile fraction of the incubation mixture was chromatographed on a column of Dowex 2-acetate. D-Glyceraldehyde 3-phosphate (1.1 μmoles) was isolated and found to contain less than 5 cpm/μmole.

Oxidation of Deoxyribose 5-Phosphate to Deoxyribonic Acid 5-Phosphate. Deoxyribose 5-phosphate (1.9 μmoles, specific activity 410 cpm/μmole[2]) was treated for 2 hr at 20° in darkness with 50 μmoles of bromine in a total volume of 0.3 ml containing 8 μmoles of sodium benzoate, pH 6 (Hudson and Isbell, 1929). This resulted in the complete oxidation of deoxyribose 5-phosphate to deoxyribonic acid 5-phosphate as

[2] The degradation experiments were performed with separate preparations of deoxyribose 5-phosphate obtained under identical conditions. This explains the slight differences in the specific activities of the starting materials.

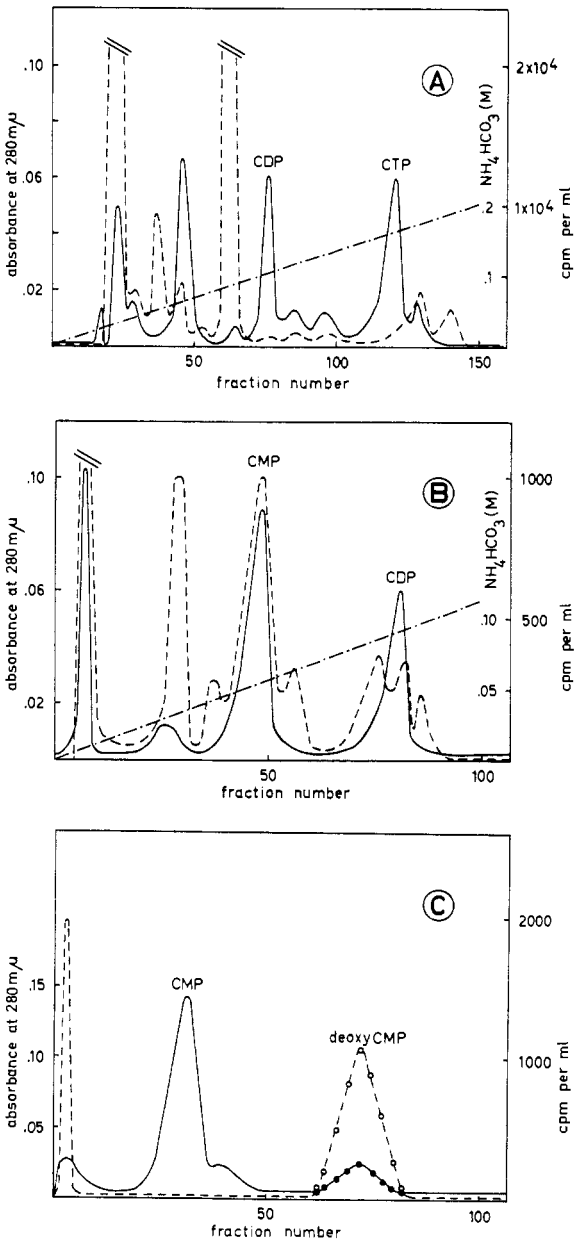


FIGURE 7: Isolation of deoxycytidine phosphates formed in the presence of tritiated water by the CDP reductase system with reduced thioredoxin as hydrogen donor. (A) Purification of cytidine phosphates from the incubation mixture by chromatography on a DEAE-cellulose column (length 60 cm, diameter 2 cm) using a linear gradient of ammonium bicarbonate, pH 8.6 (-·-·-). Fraction volumes 15 ml, flow rate 45 ml/hr. (B) Rechromatography on a column of DEAE-cellulose (length 46 cm, diameter 0.9 cm) of the pooled CDP and CTP fractions from chromatogram A after treatment with deoxycytidine triphosphatase. A linear gradient of ammonium bicarbonate, pH 8.6, was used (-·-·-). Fraction volumes 7 ml, flow rate 21 ml/hr. (C) Isolation of deoxyCMP from the pooled CMP fractions of chromatogram B on a column of Dowex 50-H^+ (length 20 cm, diameter 0.9 cm) using 0.2 M acetic acid. Fraction volumes 3 ml, flow rate 21 ml/hr. Absorbance at 280 mμ (———) and radioactivity (- - -) were measured in the three chromatograms.

measured by the disappearance of the diphenylamine-positive material. The mixture was then adsorbed onto a column of Dowex 2-acetate and eluted with a gradient of ammonium acetate as described in the Experimental Section. Deoxyribonic acid 5-phosphate (1.5 μmoles) was isolated and found to have a specific activity of 415 cpm/μmole. The results of the degradation of deoxyribose 5-phosphate from deoxyCDP obtained with reduced lipoate as hydrogen donor are summarized in Table I.

TABLE I: Summary of the Results of the Degradation of Deoxyribose 5-Phosphate from DeoxyCDP obtained with Different Hydrogen Donors.[a]

	Hydrogen donor	
	Reduced lipoate	Reduced thioredoxin
Deoxyribose 5-phosphate	495	485
↓		
Ethanol	430	460
+		
Glyceraldehyde 3-phosphate	<5	<5
Deoxyribose 5-phosphate	410	485
↓		
Deoxyribonic acid 5-phosphate	415	500

[a] The figures given are specific activities (cpm/μmole).

Studies of the Mechanism of DeoxyCDP Formation with Reduced Thioredoxin as Hydrogen Donor

Enzyme Incubation. The following mixture was incubated at room temperature for 3 hr: 11 mg of fraction B, 2.4 μmoles of CDP, 0.006 μmole of thioredoxin, 1.8 μmoles of TPNH, 0.08 mg of thioredoxin reductase (the preparation obtained after chromatography on DEAE-cellulose was used), 4.0 μmoles of ATP, 36 μmoles of $MgCl_2$, 85 μmoles of Tris-Cl^-, pH 8.5, 10 μmoles of EDTA, 15 μmoles of mercaptoethanol, and 4 curies of tritiated water (specific activity 3.1×10^6 cpm/μg-atom of hydrogen) in a total volume of 3.1 ml. The amount of deoxyribonucleotides formed was 0.41 μmole as determined by the microbiological assay.

Isolation of Deoxycytidine Phosphates as DeoxyCMP. DeoxyCMP was now isolated by an alternative method to the one used in the previous experiment. The principal

difference was that all initial operations were performed between pH 8.5 and 9.1 instead of under the acid conditions used in the previous procedure.

An aliquot of the nonvolatile fraction of the incubation mixture containing 0.28 μmole of deoxyribonucleotides and 10^7 cpm of tritium in nonexchangeable positions was chromatographed on a column of DEAE-cellulose by gradient elution with ammonium bicarbonate according to Staehelin (1961) (Figure 7A). The pooled CDP and CTP fractions contained 0.10 μmole of deoxyCDP and deoxyCTP. This material was concentrated and desalted by evaporation to dryness and dissolved in a small volume of water. The solution was then incubated for 2 hr at 37° with 150 μg of deoxycytidine triphosphatase, 14 μmoles of $MgCl_2$, 150 μmoles of Tris acetate, pH 9.1, and 30 μmoles of mercaptoethanol in a final volume of 2.7 ml. The reaction was stopped by boiling for 5 min. Denatured protein was removed by centrifugation, and the supernatant solution was rechromatographed on a column of DEAE-cellulose (Figure 7B). The ultraviolet absorbing peak in the position of CMP contained 0.05 μmole of deoxyCMP and coincided with a peak of radioactivity. The specific activity of deoxyCMP in this peak was found to be 0.96×10^6 cpm/μmole, which was equivalent to the incorporation of 0.31 atom of tritium/molecule, assuming that all the label in this pool resided in deoxyCMP. The material was concentrated, desalted by evaporation to dryness, and dissolved in a small volume of water.

DeoxyCMP was finally isolated free from CMP by chromatography on a column of Dowex 50-H^+ (Figure 7C). Two radioactive peaks were obtained: a minor peak which was shown to contain nonvolatile tritiated material in the beginning of the chromatogram and a major one, coinciding with the ultraviolet absorbing peak of deoxyCMP. From the second peak 0.05 μmole of deoxyCMP was obtained. It had a specific activity of 0.80×10^6 cpm/μmole, corresponding to the incorporation of 0.26 atom of tritium/molecule.

Isolation of Deoxyribose 5-Phosphate from DeoxyCMP. Labeled deoxyCMP (0.014 μmole) was diluted with 13.4 μmoles of nonlabeled deoxyCMP to give a specific activity of 835 cpm/μmole. Deoxyribose 5-phosphate was obtained from deoxyCMP as described above and had a specific activity of 840 cpm/μmole. This material was further diluted with nonlabeled deoxyribose 5-phosphate so that the material used for the subsequent degradations had a specific activity of 485 cpm/μmole.

Degradation of Deoxyribose 5-Phosphate with Deoxyribose Aldolase. Deoxyribose 5-phosphate (3.8 μmoles, specific activity 485 cpm/μmole) was incubated at 25° for 70 min with 10 μg of deoxyribose aldolase (40 units), 7.7 μmoles of DPNH, 250 μg of purified yeast alcohol dehydrogenase (40 units), and 37 μmoles of potassium maleate buffer, pH 6.3, in a total volume of 0.44 ml. Complete cleavage of deoxyribose 5-phosphate was obtained as measured by oxidation of DPNH. The reaction was stopped by freezing the mixture. Ethanol and water were collected as in the previous

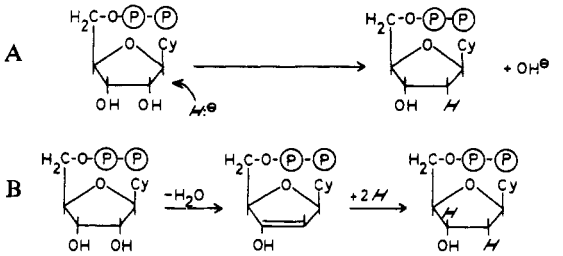


FIGURE 8: Hypothetical mechanisms for the reduction of CDP.

experiment. The distillate contained 1.8 μmoles of ethanol in about 0.3 ml of water. The ethanol was separated from the water by gas chromatography. Ethanol (1.2 μmoles) was isolated with a specific activity of 460 cpm/μmole.

The nonvolatile fraction of the incubation mixture was chromatographed on a column of Dowex 2-acetate. D-Glyceraldehyde 3-phosphate (2.6 μmoles) was isolated with a specific activity of less than 5 cpm/μmole.

Oxidation of Deoxyribose 5-Phosphate to Deoxyribonic Acid 5-Phosphate. Deoxyribose 5-phosphate (3.8 μmoles, specific activity 485 cpm/μmole) was treated with 16 μmoles of bromine and 20 μmoles of sodium benzoate, pH 6, in 0.3 ml of water. After 40 min in darkness at room temperature 75% of the deoxyribose 5-phosphate was oxidized, as measured by the disappearance of diphenylamine-positive material. The reactants were then separated by chromatography on a column of Dowex 2-acetate. Deoxyribonic acid 5-phosphate (2.0 μmoles) and deoxyribose 5-phosphate (0.6 μmole) were isolated. The specific activities were found to be 500 and 460 cpm/μmole, respectively. The results of the degradation of deoxyribose 5-phosphate from deoxyCDP obtained with reduced thioredoxin as hydrogen donor are summarized in Table I.

Discussion

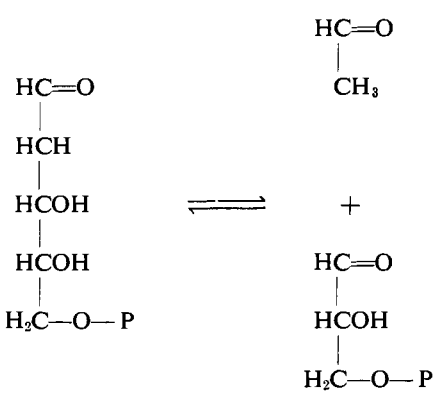
The conversion of CDP to deoxyCDP is fundamentally a reductive process in which the hydroxyl group at position 2′ in CDP is replaced by a hydrogen atom (Figure 8). A hypothetical mechanism for this enzymatic reaction was proposed by Reichard (1962). In the first step the hydroxyl group at position 2′ in CDP was thought to be eliminated, together with a hydrogen of an adjacent carbon atom. This would lead to the formation of an intermediate containing a double bond, which in a second step would be reduced to form deoxyCDP (mechanism B of Figure 8). An alternative mechanism would involve the replacement of the hydroxyl group at position 2′ in CDP by a hydride ion (mechanism A of Figure 8).

With purified enzymes the conversion of CDP to deoxyCDP shows an absolute requirement for a dithiol as the reductant. In *Escherichia coli* a specific protein, reduced thioredoxin, fulfills this requirement *in vivo*. *In vitro* reduced lipoate can act as a model substance

and can substitute for thioredoxin. Both substances participate in the reaction by means of their sulfhydryl groups. If the reduction of CDP *in vitro* is performed in tritiated water, the functional groups of either hydrogen donor are labeled, since hydrogen atoms of sulfhydryl groups are known to exchange rapidly with the protons of water (Fisher *et al.*, 1953). Label from water would thus be incorporated *via* the hydrogen donor into the deoxyCDP formed. Thereby it should be possible to distinguish between the two proposed mechanisms. DeoxyCDP formation according to mechanism B would give incorporation of tritium into positions *2 and 3* (or *2 and 1*), whereas mechanism A would give incorporation only into position *2*. The experiments presented in this paper were performed in order to distinguish between the two alternative mechanisms.

In preliminary experiments of this type with both hydrogen donors 0.3 atom of tritium was found to be incorporated into each molecule of deoxyCDP formed This low value was taken as evidence of an isotope effect and gave no information as to whether one or two hydrogen atoms was incorporated. On the basis of these results it was therefore impossible to distinguish between the two alternative mechanisms. This could be done, however, by determinating the location of the label in the sugar moiety of deoxyCDP.

For this purpose a procedure for the degradation of deoxyribose 5-phosphate was worked out. The method chosen involved the cleavage of deoxyribose 5-phosphate with deoxyribose aldolase from *Lactobacillus plantarum*. This enzyme catalyzes the following reaction, the equilibrium of which is reached when about 30% of the deoxyribose 5-phosphate is cleaved.



In the present investigation the mechanism of the deoxyribose aldolase reaction was studied by incubating deoxyribose 5-phosphate with enzyme in tritiated water. The incorporation of isotope in nonexchangeable positions in deoxyribose 5-phosphate was followed with time. After 2 hr 1.5 atoms of tritium/molecule of deoxyribose 5-phosphate had been incorporated by the enzyme. A final value of 2 would be expected if the three α-hydrogen atoms in acetaldehyde are in equilibrium with protons of the water. This equilibrium was, however, not reached in our experiment, which in all probability depended on an isotope effect. Our results thus confirm the mechanism for the deoxyribose aldolase reaction, proposed by Horecker (1959), entailing the reversible formation of a carbanion of acetaldehyde by liberation of an α-hydrogen atom.[3]

When deoxyribose aldolase was used for the degradation of deoxyribose 5-phosphate prepared from deoxyCDP equilibration of hydrogen atoms in position 2 with the protons of water had to be expected. If tritium was present in this position loss of isotope would occur. It appeared likely, however, that such a loss could be avoided to a large extent by coupling the aldolase reaction with a large excess of alcohol dehydrogenase at pH 6.3. The acetaldehyde formed from deoxyribose 5-phosphate would then rapidly be removed, since at pH 6.3 the equilibrium of the alcohol dehydrogenase reaction is completely shifted towards ethanol (Bäcklin, 1958).

By cleaving the tritiated deoxyribose 5-phosphate with deoxyribose aldolase in the presence of DPNH and purified yeast alcohol dehydrogenase, carbons 1 and 2 were recovered as ethanol and 3 to 5 as glyceraldehyde 3-phosphate. After isolation of the products of the cleavage and determination of their specific activities it could be determined whether the label was attached to positions 1 + 2, to positions 3 + 4 + 5, or both.

Position 1 could then be investigated separately by oxidation of the aldehyde group to a carboxyl group. The hydrogen attached to carbon 1 was thus recovered in the water. The deoxyribonic acid 5-phosphate formed could then be isolated and its specific activity determined.

The following procedure was planned for the further degradation of glyceraldehyde 3-phosphate (positions 3–5 of deoxyribose 5-phosphate): Oxidation with bromine would remove the hydrogen originally bound to position 3 of deoxyribose 5-phosphate. The hydrogen at position 4 would equilibrate with the protons of water on incubation of glyceraldehyde 3-phosphate with triose phosphate isomerase. Position 5 would then be obtained by difference. However, in the actual experiments no tritium was found in glyceraldehyde 3-phosphate and therefore no further degradation was required.

The enzymatic reduction of CDP in tritiated water was performed either with the thioredoxin system or with reduced lipoate. In the first experiment (reduced lipoate as the hydrogen donor) acid hydrolysis was used to transform deoxyCTP and deoxyCDP to deoxyCMP, which was isolated by chromatography on a column of Dowex 50-H^+. It was found that 0.29 atom of tritium had been incorporated per molecule of deoxyCMP. In the second experiment (reduced thioredoxin as the hydrogen donor) an alternative method was used for the preparation of deoxyCMP. All initial operations were carried out at pH values between 7 and 9. In this experiment 0.26 atom of tritium to be nonexchangeably incorporated was found per molecule of deoxy CMP. Since deoxyCMP isolated by two independent

[3] After completion of this manuscript, Rosen *et al.* (1965) published evidence for the formation of a carbanion of acetaldehyde in the deoxyribose aldolase reaction.

methods showed approximately the same relative specific activities, it may be concluded that no loss of isotope occurred during the preparation of deoxyCMP. Deoxyribose 5-phosphate was then obtained from deoxyCMP with unchanged specific activity, demonstrating that all the label found in deoxyCMP was present in deoxyribose.

Deoxyribose 5-phosphate from both experiments was degraded using the methods discussed above. In both cases it was shown that during the formation of deoxyCDP from CDP hydrogen is exclusively incorporated into position 2 of deoxyribose.

In deoxyCDP two hydrogen atoms are bound to carbon atom 2′. From the results presented in this paper it cannot be concluded whether one or both of these atoms become labeled during the formation of deoxyCDP in tritiated water. Thomson *et al.* (1962) found that cytidine-2-^{14}C-ribosyl-^{3}H *in vivo* in the rat is converted to deoxycytidine without significant loss of the tritium from the sugar. This finding is supported by experiments[4] with CMP specifically tritiated at position 2′. When this nucleotide was enzymatically reduced to deoxyCDP with a crude extract of *E. coli* again no loss of tritium occurred.

These results thus demonstrate that the hydrogen atom at position 2′ of CDP is not lost during the enzymatic formation of deoxyCDP and indicate that only one of the two hydrogens in position 2′ of deoxyCDP is newly introduced during the reaction. Therefore it can be assumed that during the formation of deoxyCDP from CDP only one atom of hydrogen is introduced into position 2 of the deoxyribosyl moiety.

Since only *one* position was found to become labeled during the formation of deoxyCDP, mechanism B seems to be excluded. Instead the present results are consistent with a mechanism entailing replacement of the hydroxyl group at position 2′ in CDP by a hydride ion (mechanism A). In the enzyme system from *E. coli* it is, however, unknown how a hydride ion is created from the two sulfhydryl groups in the hydrogen donor. In lactobacilli recent work by Blakley and Barker (1964) and Abrams and Duraiswami (1965) strongly implicates the participation of a cobamide coenzyme in the reduction of cytidine phosphates. In the purified CDP reductase system from *E. coli*, however, no vitamin B_{12} was found (Holmgren *et al.*, 1965).

Acknowledgment

Thanks are extended to Professor R. Bonnichsen and Dr. A. Maehly, The Government Laboratory for Forensic Chemistry, Stockholm, Sweden, for placing their gas chromatography equipment at the author's disposal and for much valuable advice and discussion.

References

Abrams, R., and Duraiswami, S. (1965), *Biochem. Biophys. Res. Commun. 18*, 409.

Bäcklin, K.-I. (1958), *Acta Chem. Scand. 12*, 1279.

Bandurski, R. S., and Axelrod, B. (1951), *J. Biol. Chem. 193*, 405.

Beisenherz, G. (1955), *Methods Enzymol. 1*, 387.

Beisenherz, G., Boltze, H. J., Bücher, T., Czok, R., Garbade, K. H., Meyer-Arendt, E., and Pfleiderer, G. (1953), *Z. Naturforsch. 8b*, 555.

Blakley, R. L., and Barker, H. A. (1964), *Biochem. Biophys. Res. Commun. 16*, 391.

Bonnichsen, R., and Linturi, M. (1962), *Acta Chem. Scand. 16*, 1289.

Bray, G. (1960), *Anal. Biochem. 1*, 279.

Bücher, T. (1947), *Biochim. Biophys. Acta 1*, 292.

Dische, Z. (1930), *Mikrochemie 8*, 4.

Domagk, G. F., and Horecker, B. L. (1958), *J. Biol. Chem. 233*, 283.

Eys, J. van (1961), *J. Biol. Chem. 236*, 1531.

Fisher, H. F., Conn, E. E., Vennesland, B., and Westheimer, F. H. (1953), *J. Biol. Chem. 202*, 687.

Grossman, L., and Greenlees, J. (1961), *Anal. Biochem. 2*, 189.

Gunsalus, I. C., and Razzell, W. E. (1957), *Methods Enzymol. 3*, 941.

Hoff-Jørgensen, E. (1952), *Biochem. J. 50*, 400.

Holmgren, A., Reichard, P., and Thelander, L. (1965), Abstracts, 2nd Meeting of the Federation of European Biochemical Societies, Vienna, p. 44.

Horecker, B. L. (1959), *J. Cell. Comp. Physiol. 54 Suppl. 1*, 89.

Hudson, C. S., and Isbell, H. S. (1929), *J. Am. Chem. Soc. 51*, 2225.

King, E. J. (1932), *Biochem. J. 26*, 292.

Lampen, J. O. (1957), *Methods Enzymol. 3*, 186.

Larsson, A. (1963), *J. Biol. Chem. 238*, 3414.

Laurent, T. C., Moore, E. C., and Reichard, P. (1964), *J. Biol. Chem. 239*, 3436.

Moore, E. C., Reichard, P., and Thelander, L. (1964), *J. Biol. Chem. 239*, 3445.

Packard Instrument Company (1959), Operation Manual, La Grange, Ill.

Pricer, W. E., and Horecker, B. L. (1960), *J. Biol. Chem. 235*, 1292.

Randerath, K. (1961), *Biochem. Biophys. Res. Commun. 6*, 452.

Reichard, P. (1962), *J. Biol. Chem. 237*, 3513.

Reichard, P., Baldesten, A., and Rutberg, L. (1961), *J. Biol. Chem. 236*, 1150.

Rosen, O. M., Hoffee, P., and Horecker, B. L. (1965), *J. Biol. Chem. 240*, 1517.

Sekuzu, I., Yamashita, J., Nozaki, M., Hagihara, B., Yonetani, T., and Okunuki, K. (1957), *J. Biochem. (Tokyo) 44*, 601.

Staehelin, M. (1961), *Biochim. Biophys. Acta 49*, 11.

Theorell, H., and Bonnichsen, R. (1951), *Acta Chem. Scand. 5*, 1105.

Thomson, R. Y., Scotto, G. T., and Brown, G. B. (1962), *J. Biol. Chem. 237*, 3510.

Zimmerman, S. B., and Kornberg, A. (1961), *J. Biol. Chem. 236*, 1480.

[4] Unpublished experiments by C. Coutsogeorgopoulos, P. Reichard, and I. A. Rose.