Enzymatic Synthesis of Deoxyribonucleotides*

I. FORMATION OF DEOXYCYTIDINE DIPHOSPHATE FROM CYTIDINE DIPHOSPHATE WITH ENZYMES FROM ESCHERICHIA COLI

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Extracts from Escherichia coli catalyze the formation of deoxyriboylated phosphates from cytidine 5'-phosphate (1). Optimal formation of deoxyriboylates required the addition of adenosine triphosphate and Mg²⁺ ions to the extract, and, after treatment with Dowex 2, a strong stimulation of the reaction by reduced triphosphopyridine nucleotide was observed. Similar results were obtained in extracts from the chick embryo (3), and from different mammalian tissues (4). Triphosphopyridine nucleotide was also required for the reduction of ribonucleotides to deoxyribonucleotides. This latter reaction required the addition of dCDP. This latter reaction required the addition of TPNH (in place of DPNH) and Mg++. ions, and reduced lipoic acid. TPNH was not active.

Evidence was obtained with the enzyme system from Escherichia coli that dCDP was the primary product of the reaction, and it was suggested that the reduction of the ribonucleotide to the deoxyribonucleotide occurred at the diphosphate level.

Recently we reported briefly results obtained with partially purified enzyme fractions from Escherichia coli (3). The formation of deoxyriboylated phosphates from CMP required the participation of two protein fractions. One was shown to catalyze the phosphorylation of CMP to CDP, whereas the other fraction catalyzed the reduction of the ribonucleotide diphosphate to dCDP. This latter reaction required the addition of ATP, Mg²⁺ ions, and reduced lipoic acid. TPNH was not active with the purified preparation. The present paper is a full report of these studies.

EXPERIMENTAL PROCEDURE

The sources of the nonlabeled nucleotides and the tritium-labeled CMP, the preparation of the tritium-labeled CDP, and the preparation of the bacterial extract were described earlier. DEAE-cellulose was obtained from Serva-Entwicklungslabor, Heidelberg, Germany.

d-Lipoic acid (disulfide form) was obtained from Hoffman-La Roche and Company, A.G., Basel, Switzerland, from L. Light and Company, Ltd., Coinbrook, Bucks, England, and from Merck and Company, Inc., New York. Samples of α-(+)- and α-(−)-lipoic acid were generous gifts from Dr. K. Folker, Merck, Sharp and Dohme, Rahway, New Jersey, and from Dr. L. J. Reed, The University of Texas, Department of Chemistry, Austin 12, Texas. The reduction of the lipoic acids was carried out as described by Gunsalus and Razzell (6). Our early experiments and the experiments with the optically active lipoic acids were carried out with nondistilled substances. Most of the experiments recorded in this paper were obtained with a distilled preparation of L(SH)₂ (Merck). A comparison was made of the stimulatory effects of nondistilled, distilled, and residual L(SH)₂. Essentially identical results were obtained in all three cases.

The oily reduced lipoic acid was dissolved in water by the addition of 2 m NaOH, final pH 7.5. Portions of 1 ml, final concentration of L(SH)₂ approximately 0.05 m, were stored frozen under argon in Thunberg tubes. Each Thunberg tube was routinely used for the experiments of 1 week only, because LS₂ was found to be inhibitory and on several occasions our solutions of L(SH)₂ had lost activity after repeated thawing and freezing.

Reduction of Flavin Nucleotides—Two different methods were used for the reduction of riboflavin, FMN, and FAD. In one case, oxidized flavins were added to the reaction mixture and the tubes were repeatedly evacuated and filled with argon. A solution of Na₂S₂O₄, 2 μmole per ml, was added under anaerobic conditions from an Agla syringe. The amounts of Na₂S₂O₄ added to each tube varied between 2 and 50 μmoles. These amounts of dithionite did not result in an appreciable inhibition of the reaction in the presence of L(SH)₂.

In the other method, the flavins were reduced first with hydrogen gas and palladium-asbestos catalyst. Different amounts, 1 to 20 μmoles, of the reduced flavins were then added from an Agla syringe to the incubation mixture under anaerobic conditions.

Incubations with reduced flavins were carried out under argon.³

Assay of Fraction A—The incubation mixture contained 0.03 ml of purified Fraction B, 0.5 μmole of ATP, 1.5 μmoles of MgCl₂, 0.05 μmole of tritium-labeled CMP, 0.3 μmole of L(SH)₂, and 1.5 μmoles of Tris buffer, final pH 8.0. Different aliquots of the enzyme fraction to be analyzed were always used, and care was taken to assure that the assay was carried out on the linear part of the curve (Fig. 1). Incubation was carried out at 37° for 15 minutes, and the formation of deoxyribonucleotides was determined as described earlier (1).

Assay of Fraction B—During the purification of Fraction B, the following assay was used. Different amounts of the fraction to be tested were incubated at 37° for 15 minutes together with

³ The abbreviations used are: LS₂, oxidized lipoic acid; L(SH)₂, reduced lipoic acid; BAL, British anti-Lewisite (2,3 mercaptopropanol); FMN, flavin mononucleotide.

² We wish to thank Dr. A. Ehrenberg, Nobel Medical Institute, Stockholm, Sweden, for helping us with the reduction of flavins.
of radioactivity present in the CMP, CDP, and CTP spots, respectively, were determined. The relative amount of radioactivity in the sum of CDP and CTP spots was taken as a measure of CMP kinase activity.

Protein was determined according to Lowry et al. (8).

RESULTS

Separation of Fractions A and B—Moore and Hurlbert (2) separated two enzyme fractions by adjustment of the pH of an extract from Novikoff hepatoma to approximately 5.0 with acetic acid. Both the resulting precipitate and the supernatant solution were required for the formation of deoxyctydine phosphates from CMP. A similar separation could now be obtained with the extract from E. coli.

Pressurized, frozen E. coli cells (60 g) were homogenized with 300 ml of 0.05 M Tris buffer, pH 8.0 (1). The suspension was stirred for 30 minutes in a bath and then centrifuged for 25 minutes at 10,000 × g. Of a 5% solution of streptomycin sulfate, 35 ml were added slowly with stirring to 220 ml of the supernatant solution. This addition took approximately 15 minutes, and the solution was further stirred vigorously for another 15 minutes and centrifuged at 10,000 × g for 10 minutes. The precipitate contained most of the DNA from the extract and was discarded. To 225 ml of the supernatant solution were added slowly 9 ml of m acetic acid. This resulted in a pH value of 4.7 to 4.9.

After brief centrifugation, the precipitate was rapidly suspended in 50 to 60 ml of 0.05 M Tris buffer, pH 7.75. The final pH of the suspension should be 6.2 to 6.4. The suspension was centrifuged for 5 minutes at 10,000 × g, and the pH of the supernatant solution was adjusted to 6.8 to 7.2 by addition of m ammonia, usually 0.2 to 0.4 ml. The enzyme activity of this fraction will be referred to as crude Fraction B.

The supernatant solution after the precipitation with acetic acid was neutralized with approximately 9 ml of m ammonia, final pH 7.5 to 8.0. The enzyme activity in this fraction will be referred to as crude Fraction A.

A summary of the procedure is given in Table I. There it appears that either of Fraction A or B alone was relatively ineffective in the formation of deoxyctydine phosphates from CMP, whereas the combined fractions showed good activity. This point is further illustrated by Fig. 1, which also demonstrates that within a limited range of enzyme concentration each enzyme fraction could be assayed in the presence of an excess of the other fraction.

Purification of Fraction A—Ammonium sulfate, 24.3 g, was added to 100 ml of crude Fraction A to give a final saturation of 40%. The solution was stirred for 30 minutes and centrifuged. The precipitate was discarded. To 108 ml of the supernatant solution were added 8.64 g of ammonium sulfate to give a final saturation of 52.5%. After 30 minutes, the solution was again centrifuged. The precipitate was dissolved in 3 ml of 0.05 M Tris buffer, pH 7.6, and the supernatant solution was discarded. Ammonium sulfate was removed from the protein solution by treatment with Sephadex, column size = 2 × 15 cm. The protein was eluted in a volume of 6 ml.

Further purification of Fraction A was achieved by chromatography on DEAE-cellulose. For this purpose 2 ml of the ammonium sulfate fractionated enzyme solution were adsorbed

\* Enzyme units: millimicromoles of dCMP formed under standard conditions.
\* Specific activity: enzyme units per mg of protein.
\* In this experiment 0.05 ml of each of Fractions A and B was used.

A different assay was used with the most purified preparations of Fraction B. In this case the incubation mixture, final volume 0.1 ml, contained: 0.5 μmole of ATP, 1.5 μmole of MgCl₂, 0.05 μmole of TPNH, and 0.65 μmole of tritium-labeled CMP (approximately 5 × 10⁴ c.p.m. per μmole). The amounts of deoxyctydine phosphates formed were determined as described earlier (1).

Assay of CMP Kinase—CMP kinase was measured in the different chromatographic fractions from the DEAE-cellulose chromatogram of Fraction A by the following method. Of each chromatographic fraction, 0.005 ml was incubated together with 0.05 μmole of tritium-labeled CMP, 0.5 μmole of ATP, 2.5 μmole of MgCl₂, and 1.5 μmole of Tris buffer, pH 8.0, in a final volume of 0.1 ml for 10 minutes at 37°. The reaction was stopped by placement of the vessels in a water bath at 100°. After centrifugation, 0.01 ml of the supernatant solution was spotted on a paper sheet and chromatographed with isobutyric acid-ammonia (7) as described earlier (1). The total amounts of radioactivity present in the CMP, CDP, and CTP spots, respectively, were determined. The relative amount of radioactivity in the sum of CDP and CTP spots was taken as a measure of CMP kinase activity.

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The supernatant solution after the precipitation with acetic acid was neutralized with approximately 9 ml of m ammonia, final pH 7.5 to 8.0. The enzyme activity in this fraction will be referred to as crude Fraction A.

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\* Enzyme units: millimicromoles of dCMP formed under standard conditions.
\* Specific activity: enzyme units per mg of protein.
\* In this experiment 0.05 ml of each of Fractions A and B was used.
P. Reichard

TABLE II

<table>
<thead>
<tr>
<th>pH 4.8 supernatant</th>
<th>Ammonium sulfate</th>
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<th>DEAE fraction</th>
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<tr>
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<td>mg protein/ml</td>
<td>Units/ml</td>
<td>Total units</td>
</tr>
<tr>
<td>100</td>
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<td>1.62</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>21</td>
<td>0.58</td>
<td>920</td>
</tr>
<tr>
<td>28</td>
<td>0.17</td>
<td>0.61</td>
<td>450</td>
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</table>

* Enzyme units: micromoles of dCMP formed under standard conditions.
† Specific activity: enzyme units per mg of protein.

Fig. 2. Chromatography of Fraction A on DEAE-cellulose. The absorbancy at 280 mp was determined for each chromatographic fraction, and aliquots of each fraction were analyzed for Fraction A activity. Elution was carried out with phosphate buffer, pH 7.4. The molarities of the different steps are indicated by the arrows.

Fig. 3. Identity of Fraction A activity with CMP kinase. Aliquots of each chromatographic fraction were analyzed for CMP kinase (○-○) and Fraction A activity (□-□).

Fig. 4. Chromatography of Fraction B on DEAE-cellulose. Elution was started with 0.15 M phosphate buffer, pH 7.0. At the arrow, the molarity was changed to 0.3 M.

Fraction B (68 ml = 3.100 absorbancy units at 280 mp) was allowed to percolate into the column by gravity. Elution was started with 0.15 M phosphate buffer, pH 7.0, at a rate of 12 ml per 6 minutes. When the absorbancy at 280 mp in the effluent had decreased below 0.4, the molarity of the eluent was changed to 0.3 M phosphate. A second protein peak appeared immediately (Fig. 4). The 73 ml of protein in this peak contained Fraction B activity and were precipitated by addition of 41 g of solid ammonium sulfate. The precipitate obtained after centrifugation was dissolved in 2 to 3 ml of 0.05 M Tris buffer, pH 7.4, and desalted by passage through a 2 x 15-cm Sephadex column. The column was previously equilibrated with 0.05 M

to a DEAE-cellulose column of 1.4 x 6.7 cm. Elution was started with 0.02 M phosphate buffer, pH 7.4, with a fraction size of 2 ml per 6 minutes. When 30 ml had been eluted, the molarity of the buffer was changed to 0.05 M. At this molarity the enzyme activity was eluted after the major protein peak (Fig. 2) and the procedure resulted in a considerable purification of Fraction A. A summary of the results of the purification procedure is given in Table II. The high yields in both steps make it apparent that interfering substances were removed during the purification procedure. It will be shown below that Fraction A activity was identical with CMP kinase, and it seems reasonable that the interfering substances at least in part were phosphatases and pyrophosphatases.

Identity of Purified Fraction A with CMP Kinase—In crude extracts of E. coli the first product of the reduction of cytidine phosphates was found to be dCDP (1). It seemed therefore very likely that CDP was a more direct substrate for the reaction than CMP. A possible function for Fraction A could thus be to transform CMP to CDP.

During the course of the purification of Fraction A, it was indeed found that CMP kinase activity paralleled Fraction A activity. This could most clearly be demonstrated during the chromatographic step. It appeared that in different chromatographic fractions, a close parallelism was observed between enzyme A activity and CMP kinase activity (Fig. 3).

The most direct demonstration of the fact that purified Fraction A was required for the formation of CDP from CMP came from the findings described below that the requirement for Fraction A disappeared, when CDP was used as substrate for the reaction in place of CMP.

It should be stressed that during the purification of Fraction A activity, L(SH)₂ was included in the assay system, and that the requirement for L(SH)₂ was not apparent with crude Fraction A. Crude Fraction A therefore contained not only CMP kinase activity but also a second compound, for which L(SH)₂ could be substituted.

Purification of Fraction B—Crude Fraction B was further purified by chromatography on DEAE-cellulose. A 4 x 4-cm column of DEAE-cellulose was first washed with 5 liters of 0.1 M phosphate buffer, pH 7.0, and then with 50 ml of water. Crude

![Graph](http://www.jbc.org/)

![Graph](http://www.jbc.org/)

![Graph](http://www.jbc.org/)
The precipitate was washed twice with 2 ml of 0.05 M Tris buffer, pH 7.0, and the washings were discarded. A second enzyme fraction was then eluted from the alumina with 1 ml of 0.03 M phosphate buffer, pH 7.0. The elution procedure was repeated twice with 0.5 ml of the same buffer. This fraction will be referred to as Fraction B2.

Table III gives the purification data for Fraction B and also shows the requirements for both Fractions B1 and B2.

**Requirements for Formation of dCDP with Fraction B**—Fig. 5 shows the dependence of the reaction on the concentration of Fraction B with CDP as substrate. Linearity was observed only at higher enzyme concentrations. The time curve of the reaction (Fig. 6) showed a brief lag period in the beginning, followed by a period of linearity. The reaction showed a linear course up to at least 15 minutes, sometimes up to 1 hour. A 15-minute period was used routinely in all the following experiments.

A comparison was made between CMP, CDP, and CTP as substrates for the reaction. With Fraction B alone and at low concentrations of the nucleotides, it was quite obvious that CMP was a very poor substrate and that CDP was superior to CTP (Fig. 7). When Fractions A and B were combined, however, very little difference was found among the three ribonucleotides. With Fraction B alone, optimal formation of dCDP required initial concentrations of CDP of above 0.4 mM (Fig. 8).
With CDP as substrate the reaction still showed an absolute requirement for both ATP and Mg²⁺ ions (Fig. 9). Optimal conditions were achieved with concentrations of approximately 1 mM ATP and 0.02 mM Mg²⁺. Higher concentrations were found to be inhibitory.

In all the experiments reported so far, reduced lipoic acid was added during the incubation. The requirement for this substance appeared as soon as the separation of Fractions A and B had been carried out. With purified Fraction B, optimal formation of dCDP required the addition of relatively large amounts of L(SH)₂ (Fig. 10).

Many attempts were made to replace L(SH)₂ with other reducing agents. The following substances could not replace L(SH)₂, nor did they give any further stimulation in the presence of L(SH)₂ (Table IV): TPNH, DPNH, FMNH₂, FADH₂, reduced riboflavin, glutathione, ascorbic acid, or 2,3-mercaptopropanol. The addition of dithionite at concentrations which, in the presence of L(SH)₂, did not inhibit the enzyme reaction showed no effect in the absence of lipoic acid. Oxidized lipoic acid could not replace the reduced form. Reduced dl-lipoamide or the optically active forms of L(SH)₂ showed approximately the same activity as dl-L(SH)₂.

In the crude bacterial extract, L(SH)₂ inhibited the formation of deoxycytidine phosphates. At 0.01 mM concentration, a 50% inhibition was observed. However, low concentration of arsenite and especially cadmium ions (Fig. 11) very strongly inhibited the reaction, and this inhibition could be reversed by the addition of L(SH)₂.

The reaction showed a pH optimum of approximately 8.0. Thus, in one experiment, the following amounts of dCMP (mmoles) were formed at different pH values, with Tris buffer: pH 7.0, 0.64; pH 7.4, 0.96; pH 7.8, 1.09; pH 8.2, 1.15; pH 8.6, 0.98; pH 9.0, 0.75.

**TABLE IV**

<table>
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<tr>
<th>Addition</th>
<th>mm</th>
<th>Omission</th>
<th>mmoles</th>
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<tr>
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* Incubations were carried out under argon under standard conditions.
† Reduced with Pd-asbestos catalyst + H₂ gas.
The enzyme reaction was inhibited by many different types of substances. Inhibition by deoxynucleotides has been described earlier (9). It was also found that neutral salts inhibited very strongly at concentrations above 0.01 M. Of interest was the fact that at concentrations up to 1 mM concentration did not inhibit.

Several of our purified enzyme B preparations were stimulated by low concentrations of EDTA. Higher concentrations were always inhibitory (Fig. 11). EDTA (5 × 10⁻⁴ M) was routinely included in the reaction mixture.

**DISCUSSION**

The results in the present paper describe some of the properties of an enzyme system that catalyzes the formation of a deoxyribonucleotide from a ribonucleotide. With partially purified enzymes (Fraction B), it could be shown that CDP was the preferred substrate for the formation of deoxyribonucleotides. This, together with the earlier finding (1) that dCDP was the first deoxyribosyl phosphate formed, clearly established that the reduction of cytidine phosphates to the corresponding deoxyribosyl compounds takes place at the diphosphate level.

The reaction showed an absolute requirement for reduced lipoic acid, ATP, and magnesium ions (or Mn⁺⁺, Fe⁺⁺, or Ca⁺⁺, see (1)). From these complex requirements it appeared probable that several enzymatic steps were involved in the over-all reaction. This was also borne out by the demonstration that Fraction B could be further separated into two protein fractions (Fractions B1 and B2), both of which were required for the over-all reaction. So far it has not yet been possible to carry out the reaction in a stepwise fashion, and it seems likely that further purification of Fractions B1 and B2 is required before this goal can be achieved.

The formation of a deoxyribosyl derivative from a ribosyl derivative is principally a reductive process and therefore might be assumed to require the participation of a reducing agent. It therefore seems likely that the function of L(SH)₂ was to act as a hydrogen donor. However, it cannot yet be decided if lipoic acid participates directly in the reaction. Enzyme-bound lipoic acid acts as a prosthetic group during the oxidative decarboxylation of α-keto acids. This is the only demonstrated enzymatic function of lipoic acid, and in this type of reaction the enzyme-bound lipoic acid acts as an oxidizing agent. If lipoic acid is indeed a cofactor for the reduction of ribonucleotides, this would represent a completely new type of function for this substance.

As an alternative explanation, one might conceive that the added L(SH)₂ acted indirectly by reducing some other enzyme-bound cofactor which in turn reacted directly in the reduction of ribonucleotides. Many attempts were made to find out if the participation of a flavin nucleus during the reaction could be demonstrated. For this purpose the reaction was carried out under aerobic conditions with dithionite or FMNH₂ or both, FADH₂, or reduced riboflavin. None of these substances showed any activity in the absence of L(SH)₂. It was found that in the presence of L(SH)₂, dithionite did not inhibit the enzyme reaction, but that relatively small amounts of the reduced flavin nucleotides showed a pronounced inhibition. Against the participation of a flavoprotein was also the complete absence of inhibition of the reaction by atenin in concentrations up to 1 mM. All these results tentatively speak against the participation of flavin nucleotides during the reaction with purified Fraction B, but this possibility cannot be ruled out completely.

Several other possible reducing agents were investigated with respect to their ability to replace L(SH)₂ or to show a further stimulation in the presence of L(SH)₂, all with negative results. These substances included reduced pyridine nucleotides, ascorbate, and monothiols such as glutathione, cysteine, and mercaptoethanol. The earlier, demonstrated stimulatory effects of TPNH (1, 2) or DPNH (4) in crude extracts thus must have been indirect. Of special interest was the demonstration that 2,3-mercaptopropanol (BAL) was completely inactive with the purified Fraction B. This shows that the observed requirement for L(SH)₂ was not an unspecific effect characteristic for thiols. On the other hand no stereospecificity was observed, since both (+)- and (−)-L(SH)₂ showed equal activity. Furthermore, the optimal synthesis of dCDP required the addition of L(SH)₂ or reduced lipoamide at high concentrations. These results might indicate a nonenzymatic function of the added L(SH)₂.

In the crude bacterial extract, the reduction of CDP was strongly inhibited by low concentrations of cadmium or arsenite ions. Both substances are known to be relatively specific inhibitors of enzyme reactions that involve thiols. Since the enzyme reaction in the crude extract was carried out without added L(SH)₂, these inhibitory effects strongly suggest the direct participation of a dithiol in the reaction. It is possible that this dithiol during the enzyme purification was oxidized or destroyed otherwise, and that this is the reason for the requirement for L(SH)₂. Thus we visualize the function of the added L(SH)₂ to keep an enzyme-bound dithiol in the reduced form. This

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**Fig. 12. Possible intermediates in the formation of dCDP from CDP**
The finding of a requirement for L(SH)₂ is strongly reminiscent of an early report by Grossman (10), describing the stimulatory effect of BAL on the formation of different deoxyribonucleosides and deoxyribonucleotides from the corresponding ribosyl compounds. Grossman's report was actually a strong incentive for us to test the action of L(SH)₂ in our system, even though it was apparent at an early stage (11) that many dissimilarities exist between his and our system.

The formation of dCDP from CDP furthermore showed absolute requirements for the addition of ATP and Mg++ ions. AMP was inactive; and ADP was much less active than ATP. So far it has not yet been possible to work out the stoichiometry of the ATP requirement, but it seems clear that the reduction of CDP includes at least one energy requiring step. In principle, two different mechanisms may explain the ATP requirement.

One explanation could be that an "activated" CDP derivative is formed as an intermediate; e.g. CDP phosphorylated or pyrophosphorylated in position 2'. The purpose of such an activation could be to facilitate the introduction of a double bond between carbon atoms 2' and 3', since a phosphate or pyrophosphate group would be expected to be a better "leaving-group" than the OH group. The unsaturated compound could then finally be reduced to dCDP. A schematic outline of this hypothetical reaction sequence is given in Fig. 12. The active intermediate is written as cytidine 2'-pyrophosphate 5'-pyrophosphate. One alternative intermediate, cytidine 2'-phosphate 5'-pyrophosphate, was synthesized by Dr. J. P. Verheyden, Syntex Institute for Molecular Biology, Palo Alto, California. It was inactive in our enzyme system and thus is excluded as an intermediate.

An alternative explanation for the ATP requirement in the formation of dCDP could be an "activation" of L(SH)₂. It is known that protein-bound lipoic acid, and not free lipoic acid, functions as cofactor in the decarboxylation of α-keto acids. Reed and co-workers (12) discovered an enzyme, lipoyl-X hydrolase, which released protein-bound lipoic acid. A second enzyme then catalyzed the reincorporation of lipoic acid into the protein, resulting in a functional lipoic acid protein complex (13). This second enzyme required the addition of ATP and Mg++ ions for its action. It seems possible that the ATP requirement in our enzyme reaction might be explained on a similar basis.

In this paper the enzymatic reduction of CDP was studied, and this reaction was used as an assay during enzyme purification. The purified Fraction B, however, also catalyzed the reduction of UDP (5). Furthermore, in unpublished experiments together with Mr. A. Lawton we found that Fraction B catalyzed the formation of deoxyadenosine and deoxyguanosine phosphates, respectively, from the corresponding ribosyl compounds. It is not yet possible to decide whether the reduction of all four ribonucleotides was carried out by the same or different enzymes.

Nevertheless it appears that the reduction of ribonucleotides might represent a major pathway for the biosynthesis of deoxyribonucleotides in different types of cells. At first sight the low enzyme activity in the crude extract of E. coli might be considered to argue against the possibility that this reaction could be a major pathway for the biosynthesis of deoxyribose. However, there is a considerable amount of evidence that indicates that the enzyme activity exists in a strongly inhibited form in the crude bacterial extract. The inhibition can in part be explained by the presence of DNA and deoxyribonucleases (cf. (9)), and in part by the presence of an unknown inhibitor which is removed together with Fraction A. The latter substance is present in especially large quantities in bacteria grown on glucose as a source of carbon. Thus it was observed on several occasions that during the purification of Fraction B, a more than 3-fold increase of total enzyme activity could be obtained after streptomycin and acetic acid precipitation. Such considerations demonstrate that little significance can be attached to the purification factors obtained during the enzyme preparation, and that the finding of quite small amounts of enzyme activity in the crude bacterial extract does not necessarily argue against the concept that the formation of deoxyribonucleotides through a reduction of ribonucleotides represents a major metabolic pathway for the biosynthesis of deoxyribose.

**SUMMARY**

Two enzyme fractions were purified from extracts of Escherichia coli B; both were required for the formation of deoxyribonucleotides from cytidine 5'-phosphate. One of these fractions, Fraction A, catalyzed the phosphorylation of cytidine 5'-phosphate to cytidine 5'-diphosphate. The other enzyme, Fraction B, then carried out the reduction of cytidine diphosphate to deoxyribose diphosphate.

The reaction catalyzed by Fraction B showed absolute requirements for ATP, Mg++ ions, and reduced lipoic acid. Even though it cannot yet be decided with certainty whether or not reduced lipoic acid participated directly in the reaction, evidence for the direct participation of a dithiol was obtained.

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