THE ENZYMATIC SYNTHESIS OF CYTIDINE DIPHOSPHATE CHOLINE*

BY LOUISE FENCIL BORKENHAGEN AND EUGENE P. KENNEDY
(From the Department of Biochemistry, University of Chicago, Chicago, Illinois)
(Received for publication, February 25, 1957)

The function of cytidine coenzymes in the enzymatic synthesis of lecithin and of phosphatidylethanolamine has been described in previous papers (1–3). The synthesis of lecithin has been shown to take place according to the following equations

\[ \text{CTP} + \text{P-choline} \rightleftharpoons \text{CDP-choline} + \text{pyrophosphate} \]

\[ \text{CDP-choline} + \text{n-} \alpha,\beta-\text{diglyceride} \rightleftharpoons \text{lecithin} + \text{CMP} \]

In similar reactions, phosphatidylethanolamine is synthesized from P-ethanolamine via cytidine diphosphate ethanolamine. Since the reaction shown in Reaction 1 is essentially a transfer of P-choline to the cytidyl portion of CTP, the enzyme catalyzing the reaction has been named PC-cytidyl transferase (1). The enzyme catalyzing Reaction 2 has been called the PC-glyceride transferase. The analogous enzymes involved in the synthesis of phosphatidylethanolamine are termed PE-cytidyl transferase and PE-glyceride transferase, respectively.

The present report describes the preparation and some properties of the PC-cytidyl transferase of guinea pig liver.

Materials and Methods

The synthesis of CDP-choline and CDP-ethanolamine has been previously described (4). Other nucleotides were products of the Pabst Laboratories. P-choline and P-ethanolamine were synthesized by a modification of the method of Plimmer and Burch (5) and were purified and isolated by chromatography on Dowex I formate ion exchange resin.

The development of assay procedures based on the use of Norit for the quantitative adsorption of nucleotides was suggested by the previous work of Crane and Lipmann (6).

* Aided by grants from the Nutrition Foundation, Inc., and from the Life Insurance Medical Research Fund.

1 The following abbreviations are used in this paper: ATP = adenosine 5′-triphosphate; UTP = uridine 5′-triphosphate; GTP = guanosine 5′-triphosphate; ITP = inosine 5′-triphosphate; CTP = cytidine 5′-triphosphate; CDp = cytidine 5′-diphosphate; CMP = cytidine 5′-monophosphate; CDP-choline = cytidine diphosphate choline; P-choline = phosphorylcholine; Tris = tris(hydroxymethyl)aminomethane; P-ethanolamine = phosphorylethanolamine.
ENZYMATIC SYNTHESIS OF CDP-CHOLINE

EXPERIMENTAL

Preparation of Enzyme—Liver from normal adult male guinea pigs, stored at -15° for several weeks, was thawed at 5° and homogenized in 10 volumes of ice-cold 0.25 M sucrose in a Waring blender for 45 seconds. All subsequent operations were carried out at 0–5°. The homogenate was centrifuged for 5 minutes at 500 × g in an International refrigerated centrifuge and the precipitate was discarded. The pH of the supernatant fluid was adjusted to 5.2 by the dropwise addition of 2 N acetic acid, and the suspension was stirred for 30 minutes. The precipitate was removed by centrifugation and the supernatant solution discarded. The precipitate was taken up in 0.02 M Tris buffer, pH 8.0, containing 0.001 M Versene, the volume of buffer being equal to that of the original volume of the whole homogenate. The suspension was then dialyzed thoroughly against several changes of buffer of the same composition.

The dialyzed enzyme preparation was rapidly warmed to 55° by stirring it in a large bath maintained at that temperature and held at 55° for 20 minutes. The enzyme was cooled in an ice bath and centrifuged at about 18,000 × g in an angle head centrifuge at 0° for 20 minutes and the supernatant solution was discarded. The residue was again suspended in the Tris-Versene dialysis medium, and at this point could be frozen and stored for several weeks. Just prior to use, a portion of the enzyme was thawed and dispersed by treatment in a Raytheon sonic disintegrator for 10 minutes. Such preparations usually contained 5 to 10 mg. of protein per ml.

Attempts to prepare the enzyme in soluble form, suitable for fractionation by conventional methods, have thus far proved unsuccessful.

Measurement of Enzymatic Synthesis of CDP-choline—Since Reaction 1, catalyzed by PC-cytidyl transferase, is readily reversible, the activity of the enzyme can be studied either by measuring the amount of CDP-choline synthesized from CTP + P-choline or by following the pyrophospholytic cleavage of synthetic CDP-choline to CTP + P-choline. Experiments in which the synthesis of CDP-choline was measured will be described first. In such experiments, P-choline-\(^{32}\)P was used as isotopic tracer. At the end of the enzymatic incubation, usually carried out in a final volume of 1.0 ml., the reaction was stopped by the addition of 5 ml. of 5 per cent trichloroacetic acid and the precipitate removed by centrifugation. A 5 ml. aliquot of the supernatant solution was treated with 2.0 ml. of an aqueous suspension of Norit A charcoal (10 mg. per ml.). After 10 minutes, during which the tubes were occasionally stirred, the charcoal was removed by centrifugation. CDP-choline is quantitatively adsorbed on the charcoal under these conditions (4), but P-choline is not adsorbed to any measurable extent. The charcoal precipitate was washed four
times with 5 ml. portions of water and finally suspended in 3 ml. of a 1 per cent solution of casein. 2 ml. aliquots of the charcoal suspension were then dried and counted in a gas flow counter of conventional design. The casein is included in the suspension medium simply as a binder to improve the plating and counting characteristics of the charcoal.

Zero time controls were included in each run to check the effectiveness of this procedure in removing the original labeled P-choline. Experiments in which varying amounts of synthetic CDP-choline-P32 were added to the enzyme incubation mixture after the addition of trichloroacetic acid indicated that the recovery of CDP-choline-P32 was quantitative.

| TABLE I |
| Stability of PC-cytidyl Transferase toward Heating |

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Conditions of heat treatment</th>
<th>CDP-choline synthesized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>°C.</td>
<td>min.</td>
</tr>
<tr>
<td>1</td>
<td>55</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>55</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>55</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>55</td>
<td>80</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>70</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

During the heat treatment, the conditions of which were varied as shown, each tube contained 10 μmoles of MgCl2, 50 μmoles of Tris buffer of pH 7.5, 10 μmoles of cysteine, 5 μmoles of P-choline-P32, and 0.10 ml. of guinea pig enzyme, which had not previously been heat-treated. The final volume was 1.0 ml. After heating, each tube was chilled in an ice bath, and 0.2 μmole of CTP was added. The tubes were then incubated at 37° for 1 hour.

The amount of CDP-choline synthesis was calculated by dividing the total number of counts recovered on the charcoal precipitate by the specific activity of the P-choline-P32, which was usually about 50,000 counts per micromole. The results are expressed as millimicromoles of CDP-choline formed.

Evidence as to the identity of enzymatically produced and synthetic cytidine diphosphate choline has been presented in a previous paper (1). Stability of Enzyme to Heat—The stability of the PC-cytidyl transferase toward heat was studied in the experiment shown in Table I. An enzyme preparation which had been carried through the precipitation at pH 5.2 and subsequent dialysis, but which had not yet been heat-treated, was used for this work. It will be seen that the enzyme is quite resistant to heating at 55°; in fact, the activity is almost doubled by heating the en-
zyme at this temperature for 10 minutes. This effect was consistently observed, and is probably due to the preferential destruction of hydrolytic and other interfering enzymes. If the enzyme is heated even briefly at 60° or higher, rapid inactivation takes place. Prolonged heating at 55° also leads to slow loss of activity.

In preparing the enzyme for routine use, advantage was taken of its stability at 55°, since by this means the preparation can be completely freed from the PC-glyceride transferase which catalyzes Reaction 2 and from the PE-cytidyl transferase which catalyzes the synthesis of CDP-ethanolamine from CTP and P-ethanolamine by a reaction closely similar to Reaction 1. In addition, the enzyme which carries out the hydrolytic cleavage of CDP-choline to CMP and P-choline is destroyed, and other degradative enzymes are considerably diminished in activity.

Requirement for Divalent Cations—The PC-cytidyl transferase is completely dependent upon the addition of divalent cations for activity (Fig. 1). Either magnesium or manganese ions will activate the enzyme.

Fig. 1. Requirement of divalent cation for PC-cytidyl transferase activity. Each tube contained 10 μmoles of cysteine, 50 μmoles of Tris buffer of pH 7.5, 0.2 μmole of CTP, 8 μmoles of P-choline-P³², and 0.2 ml. of enzyme in a final volume of 1.0 ml. The added divalent cation was varied as indicated. The tubes were incubated at 37° for 1 hour.
Since high concentrations of manganese are decidedly inhibitory, magnesium was routinely used in these experiments. Barium ions can activate the enzyme to a slight extent, but calcium ions have very little effect. The PC-cytidyl transferase reaction is not inhibited by calcium or barium ions when tested in the presence of magnesium ions, in contrast to the severe inhibition of the PC-glyceride reaction by calcium or barium (7, 1).

**Effects of Varying Concentrations of CTP and of P-choline**—An experiment in which the concentration of CTP was varied over a considerable range is shown in Fig. 2. Half the maximal rate was observed at concentrations of CTP of about $3 \times 10^{-4}$ M. The results of this experiment must be regarded as only approximate, since the CTP is attacked by hydrolytic enzymes still present in the heated preparation. When the concentration of P-choline was varied (Fig. 3), the concentration for half maximal rate was found to be about $0.5 \times 10^{-3}$ M.

**Specificity of Requirement for CTP**—The PC-cytidyl transferase enzyme is completely specific for CTP. An experiment in which UTP, ATP, GTP, and ITP were tested under identical conditions with CTP is shown in Table II. No detectable activity was displayed by any nucleotide except CTP. Under the conditions of the experiment, activity of about 2 per cent of that of CTP could have been detected. Further, of the cytidine nucleotides tested, only the triphosphate is active. The inac-
**Fig. 3.** Effect of varying P-choline concentration. The experimental conditions were identical with those shown in Fig. 1, except that 10 μmoles of MgCl₂ and 20 μmoles of KF were added to each tube and the P-choline concentration was varied as indicated. The time of incubation was 20 minutes at 37°C.

**Table II**

*Nucleotide Specificity of PC-cytidyl Transferase*

<table>
<thead>
<tr>
<th>Nucleotide added</th>
<th>P-choline converted to nucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
</tr>
<tr>
<td>CMP</td>
<td>0.20</td>
</tr>
<tr>
<td>CDP</td>
<td>0.20</td>
</tr>
<tr>
<td>CTP</td>
<td>0.20</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
</tr>
<tr>
<td>CTP</td>
<td>0.50</td>
</tr>
<tr>
<td>ATP</td>
<td>0.50</td>
</tr>
<tr>
<td>GTP</td>
<td>0.50</td>
</tr>
<tr>
<td>UTP</td>
<td>0.50</td>
</tr>
<tr>
<td>ITP</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Each tube contained 10 μmoles of MgCl₂, 8 μmoles of P-choline-P₃₂, 20 μmoles of KF, 50 μmoles of Tris buffer of pH 7.5, 10 μmoles of cysteine, and 0.20 ml of enzyme. The final volume was 1.0 ml. The added nucleotide was varied as shown. The tubes were incubated at 37°C for 1 hour.
tivity of CDP may be regarded as evidence that there is no significant cytidylate kinase in the heated enzyme preparation.

Measurement of Pyrophosphorolytic Cleavage of CDP-choline—The pyrophosphorolytic cleavage of CDP-choline was studied in experiments in which synthetic CDP-choline labeled with choline-1, 2-C\textsuperscript{14} was used as the tracer. At the end of the enzymatic incubation, the reaction was stopped by immersing the tubes in a boiling water bath for 5 minutes. 3 ml. of a suspension of Norit A (10 mg. per ml.) were then added to each tube, the contents of which were stirred for 10 minutes and then centrifuged. Aliquots of the supernatant solution were plated and counted. In control tubes which were boiled before the addition of CDP-choline, no significant radioactivity could be detected in the supernatant solution.

When known amounts of P-choline-1, 2-C\textsuperscript{14} were added to similar controls, the recovery of radioactivity in the supernatant solutions after charcoal treatment averaged 91 per cent. The values given in Figs. 4 and 5 are corrected to account for this factor, which is due principally to self-absorption.

Effect of Pyrophosphate Concentration—When CDP-choline labeled with choline-1, 2-C\textsuperscript{14} was incubated with the heat-treated enzyme under the conditions described in Fig. 4, little cleavage was noted in the absence of inorganic pyrophosphate, in contrast to experiments with unheated enzyme preparations, which catalyze a fairly rapid hydrolysis of the choline nucleotide (1). The effect of adding varying amounts of pyrophosphate is shown in Fig. 4. Half the maximal rate of pyrophosphorolysis takes place at concentrations of about 0.5 to 1.0 \times 10^{-3} \text{ M} pyrophosphate.

The addition of orthophosphate (0.01 to 0.05 M) in other experiments was found to be without effect.

Incorporation of Pyrophosphate into CTP—To obtain further information about the function of pyrophosphate, P\textsuperscript{32} labeled pyrophosphate was prepared by the method of Kornberg and Pricer (8). The incorporation of labeled pyrophosphate into CTP was studied in the experiment described in Table III. The CTP was separated from the unchanged pyrophosphate by adsorption on charcoal by the procedure described above for determining the synthesis of CDP-choline. The results indicate that there is a detectable incorporation of pyrophosphate into the nucleotide form in the absence of added unlabeled CDP-choline, but the incorporation is stimulated almost 10-fold by the addition of CDP-choline.

The radioactive nucleotide formed from labeled pyrophosphate in this system was identified as CTP by paper chromatography. The experiment shown in Table III was repeated with labeled pyrophosphate of high specific activity (10\textsuperscript{6} counts per micromole) and with 1 \text{ pmole} of unlabeled CDP-choline and 2 \text{ pmoles} of carrier CTP. At the end of the incubation,
FIG. 4. Effect of varying pyrophosphate concentration on the hydrolysis of CDP-choline. Each tube contained 20 μmoles of KF, 10 μmoles of MgCl₂, 50 μmoles of Tris buffer of pH 7.5, 0.2 μmole of CDP-choline labeled with choline-1,2-C¹⁴ (46,000 counts per micromole), and 0.2 ml. of enzyme, in a final volume of 1.0 ml. The concentration of pyrophosphate was varied as indicated. The tubes were incubated for 20 minutes at 37°.

TABLE III
Conversion of P³²-Labeled Pyrophosphate to CTP

<table>
<thead>
<tr>
<th>Additions</th>
<th>Counts incorporated into nucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>0.20 μmol unlabeled CDP-choline</td>
<td>124</td>
</tr>
</tbody>
</table>

Each tube contained 10 μmoles of MgCl₂, 50 μmoles of Tris buffer, pH 7.4, 20 μmoles of KF, 2 μmoles of pyrophosphate labeled with P³² (specific activity 46,000 counts per micromole), 0.25 μmole of unlabeled CTP as carrier, and 0.2 ml. of enzyme in a final volume of 1.0 ml. Unlabeled CDP-choline was added as indicated. The tubes were incubated at 37° for 30 minutes. The reaction was stopped by the addition of 5 ml. of 5 per cent trichloroacetic acid and the precipitate removed by centrifugation. 4 ml. aliquots of the supernatant solution were then stirred with 30 mg. of Norit A, which was then removed by centrifugation and washed four times with 5 per cent trichloroacetic acid and once with water. The charcoal was suspended in 3 ml. of a 1 per cent solution of casein and 2 ml. aliquots of this suspension were counted.

The nucleotides were adsorbed on charcoal, washed four times with 5 ml. of cold 5 per cent perchloric acid and twice with 5 ml. of water, and were then eluted by being washed four times with 2 ml. portions of 50 per cent
alcohol containing 1 per cent of concentrated ammonia. The complete system (containing CDP-choline) had 24,050 counts in the nucleotide fraction, whereas the control (CDP-choline omitted) had only 3811 counts. The ammoniacal ethanol extracts were then concentrated to a small volume and an aliquot was chromatographed on Whatman No. 43 paper with 60 per cent ethanol containing 0.1 M acetate buffer, pH 4.8, as developing solvent. In this system, CTP has an $R_F$ of 0.14, CDP of 0.19, CMP of 0.36, and pyrophosphate of 0.30.

After the chromatograms were developed, the spots corresponding to the cytidine nucleotides were located by viewing with an ultraviolet lamp and were cut out, and the radioactivity was determined. The paper spots were then eluted with dilute hydrochloric acid and the concentration of cytidine nucleotide was determined spectrophotometrically. The CTP recovered from the complete system had a total radioactivity of 1657 counts and a specific activity of 27,600 counts per micromole, and the CTP recovered from the control (no CDP-choline added) had a total of 142 counts and a specific activity of 5070 counts. The CDP recovered from the extract of the complete system contained 897 counts with a specific activity of 10,080 counts per micromole. No radioactivity could be detected in the CDP spot from the control or in any other area of either strip.

These results make it clear that the radioactive pyrophosphate reacts with CDP-choline to yield CTP, which then may be hydrolyzed in part to CDP with a specific activity about half of the CTP, since one of the radioactive terminal phosphates has been removed. In addition, some unidentified side reaction not involving CDP-choline leads to the labeling of CTP at a rate about one-tenth of that of the PC-cytidyl transferase reaction.

Effect of CDP-choline Concentration—The rate of pyrophosphorolysis as a function of CDP-choline concentration is shown in Fig. 5. Half the maximal rate is observed at about 0.2 $\times$ 10$^{-3}$ M CDP-choline.

Other Properties of Enzyme—The enzyme is active over a pH range of 6.4 to 8.0, as measured by the pyrophosphorolytic cleavage of CDP-choline. The pH optimum is about 7.2.

The omission of cysteine from the incubation mixture had little or no effect on activity; it was nevertheless included in most experiments. The activity is unaffected by the addition of 0.02 M fluoride. Treatment of the enzyme preparation in a Raytheon sonic disintegrator for periods up to 40 minutes at about 5° diminished the activity only slightly. The enzyme can be stored for several weeks at -15° with no loss of activity.

Distribution of Enzyme—PC-cytidyl transferase is widely distributed throughout nature. It has been found in the kidney, liver, heart, and brain of the rat, in the liver of the hog and guinea pig, in the brain of the
calf, and in other mammalian tissues. It is found in liver of avian species, as shown by the high activity of the enzyme in chicken liver, and also in several strains of yeast and in carrot root, but has not yet been detected in microorganisms other than yeast. It is of interest that the enzyme from yeast has the same narrow specificity for cytidine nucleotides as does the enzyme from guinea pig liver.

**Fig. 5.** Effect of varying CDP-choline concentration on pyrophosphorolysis. The experimental conditions were the same as for Fig. 4, except that each tube contained 2 μmoles of inorganic pyrophosphate, and the concentration of CDP-choline-1,2-C^{14} was varied as shown.

This wide distribution of the enzyme leads to the conclusion that the same general mechanism of formation of lecithin prevails throughout this range of organisms.

**DISCUSSION**

The enzymatic synthesis of CDP-choline is another example of the basic pattern for the biosynthesis of substituted nucleotide pyrophosphates discovered by Kornberg (9)

\[ \text{Nucleoside--P--P--P + R--P} \rightleftharpoons \text{nucleoside--P--P--R + P--P} \]
The synthesis of uridine diphosphate glucose (10) and guanosine diphosphate mannose (11) also follows the same general scheme.

The discovery of CDP-choline and CDP-ethanolamine leads to the suggestion that cytidine diphosphate serine and cytidine diphosphate inositol may also be formed in analogous reactions and play similar roles in the biosynthesis of phosphatidylserine and monophosphoinositol, respectively, but no evidence in support of this hypothesis has as yet been presented. Two nucleotides from *Lactobacillus arabinosus* have been identified by Baddiley and his coworkers as cytidine diphosphate glycerol (12) and cytidine diphosphate ribitol (13). The finding of the glycerol compound in particular suggests a function in the realm of lipid metabolism.

The function of cytidine coenzymes in the biosynthesis of phospholipides has been recently confirmed in independent studies by McMurray et al. (14) with use of cell-free preparations of brain, and by Williams-Ashman and Banks (15) with enzymes derived from the seminal vesicle.

**SUMMARY**

The enzymatic synthesis of cytidine diphosphate choline takes place according to the following equations

\[
\text{Cytidine triphosphate} + \text{phosphorylcholine} \rightleftharpoons \text{cytidine diphosphate choline} + \text{pyrophosphate}
\]

The name **PC-cytidyl transferase** is suggested for the enzyme catalyzing this reaction. The reaction is readily reversible and methods are described for measurement of either the synthesis or pyrophosphorolysis of cytidine diphosphate choline. The PC-cytidyl transferase of guinea pig liver is stable to heat at 55°, requires magnesium or manganese ions for activity, and is completely specific for cytidine triphosphate. A separate enzyme, the PE-cytidyl transferase, catalyzes the synthesis of cytidine diphosphate ethanolamine from CTP and phosphorylethanolamine by an analogous reaction.

**BIBLIOGRAPHY**

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