THE SYNTHESIS OF CYTIDINE DIPHOSPHATE CHOLINE,
CYTIDINE DIPHOSPHATE ETHANOLAMINE,
AND RELATED COMPOUNDS*

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The work of Kennedy and Weiss (1-3) has shown that CDP-choline1 and
CDP-ethanolamine are naturally occurring coenzyme forms of P-choline
and P-ethanolamine, and that these nucleotides are precursors of
lecithin and phosphatidylethanolamine, respectively. This paper will
describe the synthesis by chemical procedures of CDP-choline, CDP-
ethanolamine, and some closely related nucleotides, ADP-choline, UDP-
choline, and GDP-choline. These syntheses have been accomplished by
the condensation of P-choline or P-ethanolamine with the appropriate
5'-ribotide in the presence of DCC.

The elegant work of Khorana and his collaborators (4-6) has shown the
usefulness of DCC as a reagent for the preparation of diesters of pyrophos-
phoric acid.

\[
2R-O-P-OH + R'N=\text{C}=NR' \rightarrow R-O-P=O-P-O-R + R'NHCONHR'
\]

(1)

The carbodiimide method is specially suited for the synthesis of nucleo-
tide pyrophosphates, since no protective groups are needed and the reac-

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from the American Cancer Society, recommended by the Committee on Growth of
the National Research Council.

1 The following abbreviations are used in this paper: CDP-choline = cytidine di-
phosphate choline = PI-cytidine-5'-P2-choline pyrophosphate; CDP-ethanolamine =
cytidine diphosphate ethanolamine = PI-cytidine-5'-P2-O-ethanolamine pyrophos-
phate; ADP-choline = adenosine diphosphate choline = PI-adenosine-5'-P2-choline
pyrophosphate; UDP-choline = uridine diphosphate choline = PI-uridine-5'-P2-
choline pyrophosphate; GDP-choline = guanosine diphosphate choline = PI-gua-
nosine-5'-P2-choline pyrophosphate; AMP = adenosine-5'-phosphate; CMP = cyti-
dine-5'-phosphate; UMP = uridine-5'-phosphate; GMP = guanosine-5'-phosphate;
DCC = N,N'-dicyclohexylcarbodiimide; P-choline = phosphorylcholine; P-ethanol-
amine = phosphorylethanolamine; Tris = tris(hydroxymethyl)aminomethane.
tion can be carried out under mild conditions, such as in aqueous pyridine at room temperature.

**Materials and Methods**

Choline-1,2-C\(^{14}\) bromide was purchased from Tracerlab, Inc. Ethanolamine-1,2-C\(^{14}\) was the gift of Dr. I. Zabin. \(\text{P-choline}\) and \(\text{P-ethanolamine}\) were synthesized as previously described (7).

AMP was a crystalline product of the Ernst Bischoff Company, Inc. The CMP used in the first attempts to synthesize CDP-choline was a generous gift of the Pabst Laboratories. Larger amounts of CMP, together with UMP and GMP, were prepared from ribonucleic acid (California Foundation for Biochemical Research) by treatment with the diesterase from the venom of *Crotalus adamanteus* (Ross Allen Reptile Farm). The diesterase was freed of 5'-nucleotidase activity by repeated chromatography on powdered filter paper by the method of Hurst and Butler (8). The mixture of mononucleotides from the ribonucleic acid was separated by chromatography on Dowex 1 formate resin by using increasing concentrations of formic acid in the procedure described by Hurlbert et al. (9). The nucleotides were eluted in the order reported by these workers, and were well separated. Each band was concentrated to dryness and again chromatographed. The products so obtained had the absorption spectra of pure CMP, GMP, and UMP, respectively (10), and consisted entirely of 5'-nucleotides, as shown by quantitative cleavage with the specific 5'-nucleotidase of *C. adamanteus* venom.

DCC was prepared by the method of Schmidt et al. (11). A partially purified nucleotide pyrophosphatase was obtained from potatoes by a method based on that of Kornberg and Pricer (12). Carbon, hydrogen, and nitrogen analyses were carried out by the Micro-Tech Laboratories, Skokie, Illinois, total phosphorus being determined by the method of Gomori (13). Measurements of absorption spectra were made in a Beckman model DU spectrophotometer, and C\(^{14}\) determinations by drying small aliquots in aluminum cups, which were then counted in a windowless gas flow counter under conditions of negligible self-absorption.

**EXPERIMENTAL**

The details of the preparation of CDP-choline will be given to exemplify the methods employed.

120 mg. of CMP (free acid) and 58.7 mg. of \(\text{P-choline-1,2-C}^{14}\) (free acid) were dissolved in a mixture of 2 ml. of water and 14 ml. of pyridine in a 125 ml. Erlenmeyer flask fitted with a ground-glass stopper and an efficient magnetic stirrer. To this solution, 3.6 gm. of DCC were added and the mixture was stirred at 37° for 2 days, after which a further addition of 1.8
gm. of DCC was made and stirring continued for another 2 days. A final addition of 1.8 gm. of DCC was then made and the stirring continued for 3 days more. This prolonged reaction time was found necessary to assure complete reaction of the CMP.

The semisolid reaction mixture was shaken vigorously with 40 ml. of water and filtered on a Büchner filter. The crystalline dicyclohexylurea was washed with several portions of water on the filter. The turbid filtrate was extracted with an equal volume of ether and the ether phase discarded. The aqueous phase was freed of traces of ether by warming to 40° under a jet of air. The pH was adjusted to 8 to 9 by the addition of 0.5 N KOH and the solution diluted with water to a volume of 100 ml.

The solution was passed over a column of Dowex 1 formate (2 per cent cross-linked), 15 cm. high and 1 cm. in diameter, which was then washed with 200 ml. of water in several portions. The adsorbed material was eluted with an arrangement of apparatus similar to that described by Hurlbert et al. (9). The mixing chamber initially contained 300 ml. of water, and the upper reservoir 0.04 N formic acid. Three fractions of about 15 ml. each were collected per hour with the aid of a fraction collector.

Each fraction was analyzed for cytidine compounds by measurement of the optical density at 280 and 260 m\(\mu\) of an aliquot in 0.01 N HCl. Aliquots of each fraction were also plated, dried, and counted. The results are shown in Fig. 1. A band of radioactive material appears in Tubes 6 to 9.
inclusive; this is the unchanged P-choline-1,2-C\textsuperscript{14}. A second peak of radioactive material appears in Tubes 12 to 16 and is exactly coincidental with a cytidine compound as shown by measurement of optical density at 280 \textmu m; this is CDP-choline.

The material in Tubes 12 to 16 was concentrated under a vacuum almost to dryness, and then frozen and lyophilized. The yield was 80.5 mg., or 46 per cent of theory based on CMP.

A small amount of unchanged CMP appears in Tube 17. The expected by-product of the reaction, P\textsuperscript{1},P\textsuperscript{2}-dicytidine-5'-pyrophosphate, may be obtained in yields about equal to that of CDP-choline if the elution is prolonged for another ten to fifteen fractions. Although it was expected that dicholine pyrophosphate would also be formed, this compound has not in fact been detected among the reaction products. It may also be noted that the P-choline does not react nearly as completely as the CMP. The unchanged P-choline in Tubes 6 to 9 may easily be recovered and used again.

Properties of CDP-choline—CDP-choline, prepared in the manner described, is a white, amorphous, somewhat hygroscopic powder.

\[
\text{C}_{15}\text{H}_{37}\text{N}_{10}\text{O}_{11}\text{P}_{2}\cdot 3\text{H}_{2}\text{O}. \quad \text{Calculated. N 10.31, P 11.4, H 6.13, C 30.94 (943.47) Found.} \quad \text{N 10.23, P 11.2, H 6.22, C 31.88}
\]

It dissolves readily in water to form an acidic solution. The absorption spectrum in the ultraviolet region 245 \textmu m to 300 \textmu m is identical with that of highly purified CMP. The millimolar extinction coefficient of CDP-choline in 0.01 N HCL at 280 \textmu m is 13.7, based on absorbancy per 2 atoms of total P. Under identical conditions, CMP has a millimolar extinction coefficient of 13.3. The 280/260 ratio for CDP-choline is 2.17, while that for CMP is 2.15.

Rechromatography of the CDP-choline on Dowex 1 formate, with either 0.04 N formic acid in the upper reservoir or 0.2 M ammonium formate at pH 9.4, failed to reveal any significant contamination. When chromatographed on paper in two different solvents, CDP-choline migrated as a single spot containing all the radioactivity and ultraviolet absorbancy (Table I).

CDP-choline is quantitatively adsorbed on charcoal (Norit A) under conditions in which P-choline is not absorbed at all. This affords a convenient method for estimating the possible degree of contamination of the CDP-choline with P-choline. When 0.20 \textmu mole of CDP-choline (10,160 c.p.m.) was shaken with an aqueous suspension of 30 mg. of Norit A at neutral pH and then centrifuged, only 72 counts remained in the supernatant solution. Under these conditions, radioactive P-choline could be recovered quantitatively in the supernatant solution. Thus it may be
TABLE I

Re Values of CDP-choline and Related Compounds

Solvent System 1 was 0.02 N acetic acid in 60 per cent ethanol; Solvent System 2 was 0.25 M Tris-HCl buffer of pH 8.0 in 50 per cent ethanol. Ascending chromatograms were run with Whatman No. 43 paper.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent System 1</th>
<th>Solvent System 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDP-choline</td>
<td>0.36</td>
<td>0.68</td>
</tr>
<tr>
<td>CDP-ethanolamine</td>
<td>0.33</td>
<td>0.59</td>
</tr>
<tr>
<td>ADP-choline</td>
<td>0.41</td>
<td>0.66</td>
</tr>
<tr>
<td>UDP-choline</td>
<td>0.46</td>
<td>0.71</td>
</tr>
<tr>
<td>GDP-choline</td>
<td>0.33</td>
<td>0.63</td>
</tr>
<tr>
<td>CMP</td>
<td>0.45</td>
<td>0.57</td>
</tr>
<tr>
<td>CDP</td>
<td>0.32</td>
<td>0.60</td>
</tr>
<tr>
<td>CTP</td>
<td>0.26</td>
<td>0.60</td>
</tr>
<tr>
<td>Dicytidine-5'-pyrophosphate</td>
<td>0.21</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Fig. 2. 1.0 μmole of CDP-choline-1,2-Cl\(^{14}\) (8000 c.p.m. per μmole) was hydrolyzed in 0.5 ml. of 1.0 N sulfuric acid for the times shown, and then treated with 30 mg. of Norit A suspended in 3.0 ml. of water. The unhydrolyzed CDP-choline was quantitatively absorbed on the charcoal, which was removed by centrifugation. An aliquot of the supernatant solution was exactly neutralized with barium hydroxide and the barium sulfate was removed by centrifugation. The radioactive P-choline in the supernatant solution was then estimated by plating and counting an aliquot. The recovery of radioactive P-choline from control tubes carried through the entire procedure, but to which no CDP-choline was added, was shown to be quantitative.
calculated that the maximal contamination of CDP-choline with P-choline is 0.7 per cent.

Acid Hydrolysis of CDP-choline—When CDP-choline is hydrolyzed in mineral acid (Fig. 2), cleavage occurs at the pyrophosphate linkage, liberating CMP and P-choline, which undergo further hydrolysis only slowly.

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

**Fig. 3.** Cleavage of CDP-choline by nucleotide pyrophosphatase. Each tube contained 1.0 μmole of CDP-choline-1,2-C\(^{14}\) (4100 c.p.m. per μmole), 50 μmoles of Tris buffer of pH 7.4, and 0.2 ml. of potato nucleotide pyrophosphatase in a total volume of 1.0 ml. The tubes were incubated at 37° for the time intervals shown. A suspension of 30 mg. of Norit A in 3.0 ml. of water was then added, and the unhydrolyzed CDP-choline was quantitatively absorbed on the charcoal. The charcoal was removed by centrifugation, and the P-choline-1,2-C\(^{14}\) in the supernatant solution was estimated by counting an aliquot.

After 40 minutes in 1.0 N sulfuric acid at 100°, only 7.5 per cent of the total phosphorus has been converted to inorganic orthophosphate. The pyrophosphate linkage of CDP-choline is somewhat more resistant to acid than the pyrophosphate linkages of ATP. About 18 minutes in 1.0 N sulfuric acid at 100° are required to bring about a 50 per cent hydrolysis (Fig. 2).

Enzymatic Hydrolysis of CDP-choline—CDP-choline is not attacked by the phosphomonoesterase of human semen nor by the 5'-nucleotidase of *C. adamanteus*. It is rapidly cleaved by the nucleotide pyrophosphatase of potatoes (Fig. 3).
CDP-ethanolamine, ADP-choline, UDP-choline, and GDP-choline—CDP-ethanolamine was made from CMP and phosphorylethanolamine-1,2-C\textsuperscript{14} by a method analogous to that used for CDP-choline. The yield of CDP-ethanolamine was smaller (10 to 20 per cent based on CMP).

ADP-choline, UDP-choline, and GDP-choline were synthesized from the corresponding 5'-ribotides and phosphorylcholine-1,2-C\textsuperscript{14} in satisfactory yield (30 to 40 per cent).

These compounds have not been isolated in substance but as chromatographic fractions, the identity and purity of which have been established by the following criteria: (a) absorption spectra in the region 250 to 300 nm; (b) ratio of choline or ethanolamine (based on content of C\textsuperscript{14}) to purine or pyrimidine (based on measurement of optical density); (c) content of total N and P; (d) homogeneity when rechromatographed on ion exchange resins and on paper (Table I).

**SUMMARY**

The following compounds have been synthesized by treating phosphorylcholine or phosphorylethanolamine with the appropriate 5'-ribotide in the presence of \(N,N'-\)dicyclohexylcarbodiimide: cytidine diphosphate choline, cytidine diphosphate ethanolamine, adenosine diphosphate choline, uridine diphosphate choline, and guanosine diphosphate choline. The properties of cytidine diphosphate choline are described in detail.

**BIBLIOGRAPHY**

THE SYNTHESIS OF CYTIDINE DIPHOSPHATE CHOLINE, CYTIDINE DIPHOSPHATE ETHANOLAMINE, AND RELATED COMPOUNDS
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