[Chem. Pharm. Bull. 19(5)1011—1016(1971)] UDC 547.963.32.07 : 577.15.074

Synthesis of a Nucleotide Coenzyme, CDP-Choline

KIYOMI KIKUGAWA and MOTONOBU ICHINO

Research Laboratory, Division of Fermentation and Chemical Products, Kohjin Co., Ltd.[1]

(Received December 14, 1970)

Cytidine diphosphate choline (CDP-choline), one of the nucleotide coenzymes, is known to be a precursor of phospholipid and play an important role in the living organisms. The coenzyme was synthesized in a fairly good yield by direct condensation of cytidine-5′ monophosphate (5′-CMP) and choline phosphate (P-choline) by the use of *p*-toluenesulfonyl chloride or methanesulfonyl chloride combined with dimethylformamide.

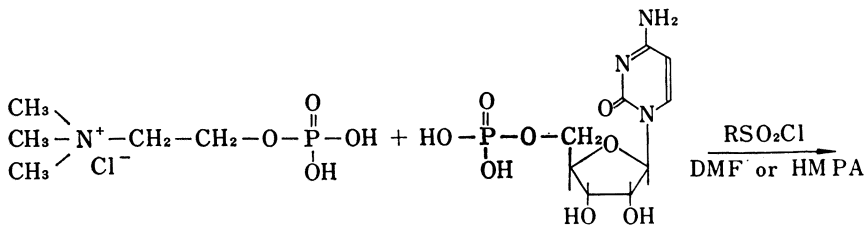
Cytidine diphosphate choline (CDP-choline) is a nucleotide coenzyme playing an important role in the metabolism of phospholipids. The coenzyme was discovered and the mechanism of the metabolism was clarified in 1956 by Kennedy and Weiss.[2]

The chemical synthesis of this nucleotide coenzyme was first investigated in 1956 by Kennedy,[3] who obtained it by condensation of cytidine-5′ monophosphate (5′-CMP) with choline phosphate (P-choline) in hydrous pyridine in the presence of a large amount of dicyclohexylcarbodiimide (DCC). There have been reported other methods to prepare the coenzyme including the intermediates such as cytidine diphosphate ethanolamine[4] and P^1-cytidine-5′,P^2-diphenyl phosphoric anhydride.[5] Another improved method to synthesize the coenzyme was the reaction of P-choline with 5′-CMP-morpholidate prepared by the treatment of 5′-CMP with morpholine in the presence of DCC.[6] The method which gave the coenzyme in an overall yield of about 50% against 5′-CMP is of practical use, although including two step processes starting from 5′-CMP.

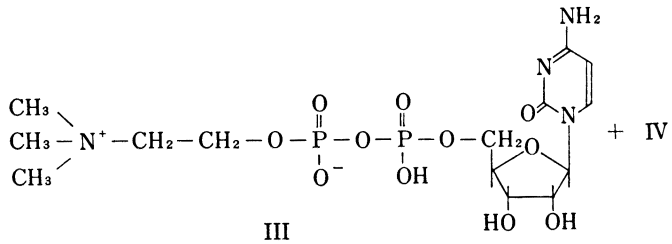
Diesters of pyrophosphoric acid, to which class the nucleotide coenzymes belong, have been synthesized (a) by direct condensation of phosphoric acids with DCC,[7] or (b) *via* intermediates of nucleoside phosphorochloridates,[8] phosphoromorpholidates,[9] α-pyridylphosphates,[10] diphenyl phosphoric anhydrides,[11] and S-ethyl phosphorothioates.[12]

This time, we attempted a direct condensation of two phosphate groups, 5′-CMP (I) and P-choline (II), using *p*-toluene(or methane)sulfonyl chloride as a condensing agent. Although sulfonyl chlorides have been frequently used for the synthesis of phosphodiester linkages,[13] there have been no attempt to prepare a pyrophosphate group using these chlorides. This paper will show that these reagents combined with N,N′-dimethylformamide (DMF) are conveniently used for the preparation of a nucleotide coenzyme such as CDP-choline (III).

1) Location: *Higashihama, Saiki, Oita.*
2) E.P. Kennedy and S.B. Weiss, *J. Biol. Chem.*, **222**, 193 (1956).
3) E.P. Kennedy, *J. Biol. Chem.*, **222**, 185 (1956).
4) Y. Sanno and K. Tanaka, *Chem. Pharm. Bull.* (Tokyo), **10**, 231 (1962).
5) R. Letters and A.M. Michelson, *Bull. Soc. Chim. Biol.*, **45**, 89 (1963).
6) T. Tanaka, H. Tanaka, T. Saito and T. Ishida, *Yakugaku Zasshi*, **85**, 863 (1963).
7) H.G. Khorana and A.R. Todd, *J. Chem. Soc.*, **1954**, 3733.
8) S.M.H. Christie, G.W. Kenner and A.R. Todd, *J. Chem. Soc.*, **1954**, 46.
9) J.G. Moffatt and H.G. Khorana, *J. Am. Chem. Soc.*, **84**, 649 (1961).
10) W. Kampe, *Chem. Ber.*, **98**, 1031 (1965).
11) A.M. Michelson, *Biochim. Biophys. Acta*, **91**, 1 (1964).
12) A.F. Cook, M.J. Holman and A.L. Nussbaum, *J. Am. Chem. Soc.*, **91**, 1522 (1969).
13) T.M. Jacob and H.G. Khorana, *J. Am. Chem. Soc.*, **86**, 1630 (1964).



II I



III + IV

R = CH_3–⟨benzene⟩–, CH_3–

DMF = dimethylformamide, HMPA = hexamethylphosphoroamide

Chart 1

As a preliminary experiment, 5′-CMP (I) and P-chloine (II) were shaken with the mixture of *p*-toluenesulfonyl chloride and DMF at room temperature for an hour. The molar ratio of 5′-CMP (I), P-choline (II) and *p*-toluenesulfonyl chloride was 1:3.2:3.2. No reaction was observed when the amount of DMF was 10 ml per 1 mmole of P-choline (II), whereas direct paper chromatography and paper electrophoresis of the reaction mixture revealed a new spot corresponding to CDP-choline (III) when the amount of DMF was reduced to 2 ml per 1 mmole of P-choline (II).

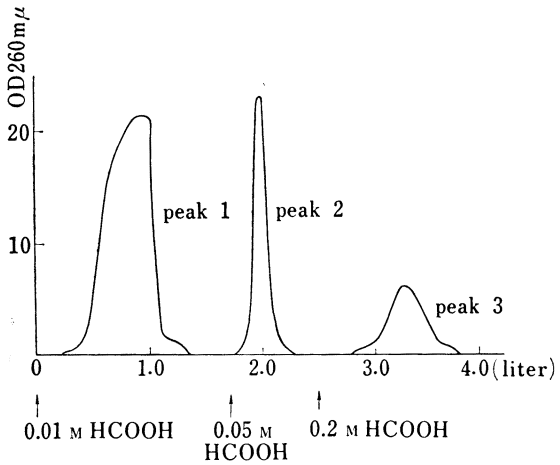


Fig. 1. Ion Exchange Column Chromatography

Column chromatography on Dowex 1 (formate) of the products by treating 5′-CMP (I) with P-choline (II) in the presence of *p*-toluenesulfonyl chloride and DMF. Details of the chromatography are given in Experimental.

The reaction mixture containing the product (III) was diluted with water, and after adjusting the pH of the aqueous mixture to 9.5 with ammo niacal water, it was subsequnetly applied onto a column of Dowex 1 (formate) to separate 5′-CMP (I), P-choline (II) and the reaction products. The column which was washed well with water to remove P-choline (II) was eluted with formic acid and three peaks containing ultraviolet absorbing substances were obtained. A typical elution profile is shown in Fig. 1. The ratios of the optical density at 280 mμ and pH 1 to that at 260 mμ and pH 1 of these peaks were around 2.0, which showed that all of these peaks contained 5′-CMP de-

TABLE I. Characterization and Yield of Each Peak

	$TOD^{pH\ 1}_{280m\mu}$	$TOD^{pH\ 1}_{280m\mu}$	$\frac{TOD^{pH\ 1}_{280m\mu}}{TOD^{pH\ 1}_{260m\mu}}$	Yield (%)[a]
Reaction mixture	23400	39700	1.70	
Peak 1	11970	24180	2.02	60.9
Peak 2	2230	4570	2.05	11.5
Peak 3	2810	5680	2.02	14.3
Total	17010	34430		86.7

a) The yield of each peak was calculated from $TOD^{pH\ 1}_{280m\mu}$ of the peak against that of the reaction mixture.

rivatives and were not contaminated with the substances derived from *p*-toluenesulfonyl chloride. Paper chromatography of the peak 1, peak 2 and peak 3 showed that the *Rf* values of the compounds from these peaks corresponded to those of CDP-choline (III), 5′-CMP (I) and P^1,P^2-dicytidine-5′ pyrophosphate (IV).

Reaction conditions to obtain the highest yield of III were investigated. As the amount of DMF greatly influenced the yield of the product (III), 0.3—0.4 ml of DMF was used per 1 mmole of P-choline (II) which was the minimum amount of the solvent to maintain the reaction mixture homogeneous. The molar ratio of 5′-CMP (I) to P-choline (II) was 1:3.2. The reaction was performed at room temperature for one hour, for the conditions were most practical and easily controled. The effects of the amount of the reagent were examined. Yields of the product (III) estimated by optical density were 30, 50, 60, 49, 44 and 37% respectively, when 0.8, 1.0, 1.15, 1.6, 2.1 and 2.6 molar excess of the reagent to P-choline (II) were used. Thus, 1.15 molar excess of the reagent to P-choline (II) was most efficient to afford the product (III). The reaction temperature did not influence the yield of III so long as it was maintained between 20° and 60°.

Under the best reaction conditions, peak 1 (III) was obtained in a yield of 60%, whereas peak 2 and peak 3 were obtained in a yield of 12 and 14% respectively (Fig. 1 and Table I). Fractions of the peak 1 were evaporated to afford crystalline powder of the product (III) in a yield of 60%, and it could be further converted into crystalline monosodium salt.

Paper chromatography and paper electrophoresis of the product (III) showed that it was not contaminated with other kind of compounds. It is certain that the compound (III) is a condensed product of 5′-CMP (I) and P-choline (II) in view of the fact that acid hydrolysis[3] of III gave 5′-CMP (I) and P-choline (II) and two gram atoms of the phosphorus were found in one mole of the compound (III). Physical and chemical properties of the product (III) were identical with those of the authentic CDP-choline which were synthesized according to the morpholidate method.[6]

In the above mentioned reaction, *p*-toluenesulfonyl chloride could be replaced by methane sulfonyl chloride, and the reaction with methanesulfonyl chloride afforded CDP-choline (III) in a yield of 50%.

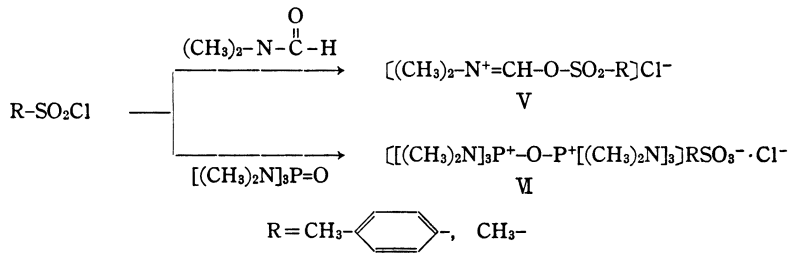


Chart 2

The solvent, DMF, could also be replaced by hexamethylphosphoroamide (HMPA) and CDP-choline (III) was obtained in a lower yield of about 10%. Other solvents tested did not give the product at all. It has been reported that sulfonyl chlorides can react with DMF to form iminium complexes (V)[14] and with HMPA to form phosphonium complexes (VI),[15] and these have been widely used for many synthetic works.[14—16] In our condensation reaction these complexes were presumed to be the active species of the reagents.

We believe that as well as Vilsmeier–Haack reagent[17] these iminium and phosphonium complexes would be conveniently utilized for the synthesis of pyrophosphate derivatives.

Experimental

Methods——Paper chromatography was performed on Toyō Roshi No. 51A paper using solvent systems, (1) iso-PrOH–conc. NH_4OH–H_2O (7:1:2, V/V), (2) EtOH–0.5M NH_4OAc (5:2, V/V), (3) iso-AmOH–HCOOH–H_2O (3:2:1, V/V). Paper electrophoresis was performed on Toyō Roshi No. 51A paper using the following conditions, (A) in 0.02 M phosphate buffer (pH 7.5) at 1000 V/25 cm, (B) in 0.05 M acetate buffer (pH 4.5) at 1000 V/25 cm. The spot was detected by ultraviolet ray unless otherwise mentioned. Hanes–Isherwood (H-I) reagent,[18] Dragendorff reagent[19] and HIO_4–benzidine reagent[20] were used for the detection of the presence of phosphorus atom, quarternary ammonium salt and *cis* diol group respectively.

Spectrophotometric assay was carried out by using Hitachi Recording Spectrophotometer, EPS-3T, and optical rotation was determined by JASCO automatic polarimeter Model DIP-SL.

Phosphorus determination was performed according to the method of Allen[21] and the determination of sodium was perfomed by flame photometry.

P-Choline (II)——P-Choline (II) was prepared from its calcium salt according to the reported method.[5,6] P-Choline calcium salt tetrahydrate (3.3 g, 10 mmole) was dissolved in 10 ml of hot water, and to this was added 2.2 g (10 mmole) of oxalic acid dihydrate. The precipitated calcium oxalate was filtered off and the filtrate was evaporated *in vacuo* at 50° to afford the viscous residue. DMF (8 ml) was added to the residue and was evaporated at 70°. The gummy residue (3.1 g) which contained 10 mmoles of P-choline (II) and 0.9 g of DMF was obtained, and it was directly used in the next synthetic procedures. The amount of P-choline (II) in the mixture was determined by phosphorus assay.

CDP-Cholihe (III)——Method A, with *p*-Toluenesulfonyl Chloride and DMF: A mixture of 2.2 g (11.5 mmole) of *p*-toluenesulfonyl chloride and 3 ml of DMF was added to the gummy mixture containing 10 mmole of P-choline (II). It was shaken at room temperature for 10 min, to obtain the viscous solution. 5′-CMP (I) (1.0 g, 3.1 mmole) was added to the viscous solution and the mixture was stirred at room temperature for one hour. The reaction mixture was then added with 100 ml of water. Paper chromatography (solvent 1) and paper electrophoresis (buffer system A) of the aqueous mixture revealed new spots having *Rf* of 0.22 and mobility of +7.0 cm, both corresponding to those of CDP-choline.

After the pH of the aqueous mixture was adjusted to 9.5 with conc. NH_4OH, it was directly submitted to a column (1.7×50 cm) of Dowex 1×4 (formate) (200—400 mesh). The column was washed well with water to remove P-choline (II) and was eluted with 0.01 M formic acid to obtain peak 1, and successive elutions of the column with 0.05 M and 0.2 M formic acid gave peak 2 and peak 3 respectively. The elution profile was shown in Fig. 1. The ratios of the optical density of these three peaks were around 2.0, which reflected that the peaks contained 5′-CMP derivatives and not contaminated with another kind of UV-absorbing substances. The yield of each peak was listed in Table I. Paper chromatography and paper electrophoresis showed that the compounds from the peak 1 and the peak 2 were CDP-choline and 5′-CMP (I) respectively. The compound from the peak 3 seemed to be P^1,P^2-dicytidine-5′ pyrophosphate (IV) analyzed by paper chromatography. In this column chromatography substances derived from *p*-toluenesulfonyl chloride and another kind of polymeric substances of 5′ -CMP were eluted when the column was washed with 2 N NaOH.

The first peak obtained in a yield of 60% estimated by the total optical density (TOD) at 280 mμ and pH 1 against that of the initial mixture was coevaporated with ethanol to dryness and afforded 1.00 g (yield, 59% against 5′ -CMP (I)) of white crystalline powder of the product (III).

14) J.D. Albright, E. Benz, A.E. Lanzilotti and L. Goldman, *Chem. Commun.*, **1965**, 413.
15) G. Gawne, G.W. Kenner and R.C. Sheppard, *J. Am. Chem. Soc.*, **91**, 5669 (1969).
16) K. Kikugawa and M. Ichino, *Tetrahedron Letters*, **1971**, 87.
17) K. Kikugawa, M. Ichino and T. Kawashima, *Chen. Pharm. Bull.* (Tokyo), *in press.*
18) C.S. Hanes and F.A. Isherwood, *Nature*, **164**, 1107 (1949).
19) R. Munier and M. Macheboeuf, *Bull. Soc. Chim. Biol.*, **33**, 846 (1951).
20) J.A. Cifonelli and F. Smith, *Anal. Chem.*, **26**, 1132 (1954).
21) R.J.L. Allen, *Biochem. J.*, **34**, 858(1940).

The product (III) was identified with CDP-choline as follows.

(1) Paper chromatography and paper electrophoresisof the product (III) were performed, simultaneously with 5′-CMP (I), P-choline (II) and the authentic CDP-choline obtained by method D. It showed a single spot in every solvent or buffer system when 300 μg of the product (III) was developed and detected by UV ray, H-I reagent, Dragendorff reagent and HIO_4-benzidine reagent. The spots obtained from the compound (III) showed the same properties as those from the authentic sample.

Table II. Paper Chromatographic *Rf* Values and Paper Electrophoretic Mobilities

	Solvent or buffer system	Product (III)[a]	Authentic[b] CDP-choline	5′-CMP	P-Choline
Paper chromatography (*Rf*)	(1)	0.22[c]	0.22[c]	0.16[d]	0.21[e]
	(2)	0.17[c]	0.17[c]	0.09[d]	0.17[e]
	(3)	0.43[c]	0.43[c]	0.43[d]	0.65[e]
Paper electrophoresis (mobility)	(A)	+6.3 cm[f]	+6.3 cm[f]	+15.3 cm[f]	—
	(B)	+1.0 cm[f]	+1.0 cm[f]	+ 3.5 cm[f]	—

a) obtained by method A
b) obtained by method D Detection of the spot was performed by *c*) UV-ray, H–I reagent, Dragendorff reagent and HIO_4-benzidine reagent.
d) UV ray and H–I reagent
e) H-I reagent and Dragendorff reagent, and *f*) UV ray

(2) UV spectrum of the product (III) was measured in 0.1 N HCl and 0.05 M phosphate buffer(pH 7.0). λ_{max} (pH 1) 280, λ_{min} (pH 1) 242, λ_{max} (pH 7) 272, λ_{min} (pH 7) 250 mμ. ε 280 (pH 1)/ ε 260 (pH 1) =2.10, ε 250 (pH 1)/ε 260 (pH 1)=0.43. The absorption maximum, minimum and the ratios of the molecular extinctions were identical with those of the authentic CDP-choline and 5′-CMP (I)

(3) Phosphorus content of the product (III) was measured, and 2.05 gram atoms of phosphorus were found in 1.00 mole of the cytosine nucleus, the amount of which was estimated by the molecular extinction coefficient, ε 280 (pH 1); 12800, and it was near to the theoretical value of 2.00.

(4) The product (III) (10 mg) was dissolved in 0.5 ml of 1 N HCl and heated at 100° for one hour. Paper chromatography (solvent 1,2) of the hydrolysate revealed that the product was degradated into 5′-CMP (I) and P-choline (II). Kennedy[3] demonstrated that CDP-choline was degradated by acid into 5′-CMP (I) and P-choline (II).

(5) Water content of the product (III) was determined by drying it at 100° for 4 hours *in vacuo*. It contained 10.0% of water indicating that the product is trihydrated.

(6) Optical rotation of the compound (III) was quite identical with that of the authentic CDP-choline. $[\alpha]_D^{25°}=+19.3°$ (C; 0.86 in H_2O, based on the dried matter).

(7) NMR and IR spectrum of the compound (III) were quite identical with those of the authentic sample.

Method B, with Methanesulfonyl Chloride and DMF: A mixture of 1.3 g (11.5 mmole) of methanesulfonyl chloride and 3 ml of DMF was added to the gummy mixture containing 10 mmole of P-choline (II). It was shaken at room temperature for 10 min, and 1.0 g (3.1 mmole) of 5′-CMP (I) was added to the viscous solution. It was then stirred at room temperature for one hour. Paper chromatography and paper electrophoresis of the reaction mixture showed that CDP-choline (III) was a major reaction product. The separa tion, isolationand identification of the product (III) were same as in method A. Crystalline white powder of CDP-choline was obtained in a yield of 50.0%.

Method C, with *p*-Toluenesulfonyl Chloride and HMPA: A mixture of 2.2 g (11.5 mmole) of *p*-toluene–sulfonyl chloride and 3 ml of HMPA was added to the gummy mixture containing 10 mmole of P-choline (II). 5′-CMP (I) (1.0 g, 3.1 mmole) was reacted under the same condition as in method A, and isolation was performed similarly. Crystalline powder of CDP-choline (III) was obtained in a yield of about 10%.

Method D according to the Morpholidate Method[6]: 5′-CMP-Morpholidate (4-morpholine-N,N′-dicyclo-hexylcarboxamidinium salt) (1.28 g, 2 mmole) was reacted with 8 mmole of P-choline (II) according to the method of Tanaka, *et al.*[6] Separation and isolation of the product were similarly performed as in method A. Crystalline powder of the authentic CDP-choline was obtained in a yield of 55%.

CDP-Choline Monosodium Salt——Monosodium salt of CDP-choline (III) was prepared from the product (III) obtained by method A. Thus, 200 mg of CDP-choline (III) was dissolved in 1.0 ml of water, and after the pH of the solution was adjusted to 6.0 with 2N NaOH, 3 ml of ethanol was added. Crystallization occurred after standing at room temperature overnight to afford plates of 130 mg of CDP-choline monosodium salt.

Determination of the Yield of CDP-Choline (III) in the Condensation with *p*-Toluenesulfonyl Chloride and DMF——In the condensation reaction using *p*-toluenesulfonyl chloride and DMF, the effects of the

amount of *p*-toluenesulfonyl chloride and the reaction temperature were examined. 5′-CMP (I) (1.0 g, 3.1 mmole) was added to the mixture of 10 mmole of P-choline (II), 3 ml of DMF and *p*-toluenesulfonyl chloride which were previously mixed and treated at room temperature for 10 min.

The reaction mixtures were stirred at 25° for one hour with the varying amounts of *p*-toluenesulfonyl chloride of 1.5 g (7.9 mmole), 1.9 g (10 mmole), 2.2 g (11.5 mmole), 3 g (15.8 mmole), 4 g (21.0 mmole) and 5 g (26.3 mmole). The yields of the compound (III) estimated were 30, 50, 60, 49, 44 and 37% respectively.

The effects of the reaction temperature were examined at 20°, 25°, 30°, 40°, 50°, 60° and 70° using 11.5 mmole of *p*-toluenesulfonyl chloride, and no significant effects were found at the reaction temperature between 20° and 60°, but at 70° small decrease of the yield (about 50%) was observed.

The determination of the yield of the compound (III) was as follows. Each reaction mixture was added with water to make the total volume 500 ml. One tenth of the aqueous mixture ($TOD_{280\ m\mu}$(pH 1),4000) was made alkaline(pH 9.5) with NH_4OH and it was subsequently applied onto a column (1.7×18 cm) of Dowex 1×4 (formate)(200—400 mesh). The column was eluted with 0.01M formic acid to obtain the peak 1. The fractions of the peak 1 (corresponding to III) were pooled and the $TOD_{280\ m\mu}$(pH 1) was determined. The yield of the compound (III) was estimated by the $TOD_{280\ m\mu}$(pH 1) of the peak 1 against that of the initial $TOD_{280\ m\mu}$(pH 1) of 5′-CMP (I).

Acknowledgement The authors wish to express their sincere thanks to Mr. Y. Higuchi and Miss N. Yoshiuchi for their technical assistances. Thanks are also due to Mr. T. Itai, the Director, the Manager of the Division and Mr. T. Nakamura, the Manager of the Laboratory, for their generosity in permitting the publication of this paper.