bated at 37° for 5 min. To the preincubated solution, 0.1 cc. of the enzyme solution (the suspension of 105,000 *g* fraction in 10 cc. of isotonic KCl solution, prepared from 10 g. of the liver of a guinea pig) was added and further incubated at 37° for 15 min. with occasional shaking. One cc. of a mixture of 1.25*M* TCA and 1*M* H_3PO_4 (pH 2) was added to the resulting solution, the mixture was centrifuged, and 1.0 cc. of the supernatant was diazotized with 0.2 cc. of 0.05% $NaNO_2$ solution. The excess HNO_2 was decomposed with 0.2 cc. of 0.5% ammonium sulfamate solution, 0.2 cc. of 0.1% naphthylenediamine hydrochloride solution was added to the reaction mixture, and the whole was allowed to stand at 37° for 2 hr. If *o*-aminophenolglucuronide is formed, the reaction mixture turns pink.

The authors are grateful to Dr. S. Kuwada, Director of the Laboratories, and Dr. S. Tatsuoka, Vice-Director of the Laboratories, for their generosity in permitting the publication of this paper. Thanks are also due to Prof. O. Hayaishi and Dr. K. Hatanaka of the Biochemical Department, School of Medicine, Kyoto University, for their kindness in giving a sample of UDPGA prepared enzymically and the aid of enzymic assay. The authors are also indebted to Dr. Y. Asahi for the measurement of pKa and to Mr. M. Kan and his associates for microanalyses.

Summary

Uridine 5′-phosphoramidate (I) was allowed to react with α-glucuronic acid 1-phosphate (II), and α-uridine diphosphate glucuronic acid (α-UDPGA) (III) was isolated from the reaction mixture by ion exchange chromatography. Likewise, β-UDPGA (V) was produced from (I) and β-glucuronic acid 1-phosphate (IV). Formation of (III) was also observed by the oxidation of α-uridine diphosphate glucose (VI) in the presence of platinum oxide catalyst. Ability of (III) and (V) to form *o*-aminophenolglucuronide was examined with the transferase in microsomes of a guinea pig liver ane only (III) was found to be active. This fact indicated that natural UDPGA takes the same configuration as (III).

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38. Yasushi Sanno and Kuniyoshi Tanaka : A New Method for Synthesis of Cytidine Diphosphate-Ethanolamine and Cytidine Diphosphate-Choline.*1

(*Research Laboratories, Takeda Chemical Industries, Ltd.**2)

Cytidine diphosphate (CDP)-choline (IX) and cytidine diphosphate (CDP)-ethanolamine (IV) are coenzymes playing an important rôle in the metabolism of phospholipids.

Both compounds were discovered and the mechanism of their metabolism was clarified in 1956 by Kennedy, *et al.*[1] In the same year, Kennedy, *et al.* synthesized[2] (IX) and (IV) by condensation of cytidine 5′-monophosphate (5′-CMP) (VII) with choline phosphate (X) or with ethanolamine phosphate[3] (VI) in hydrous pyridine in the presence of dicyclohexylcarbodiimide (DCC) (V). This DCC method was first duplicated[2] (Chart 1) and it was found that although CDP-choline (IX) could be obtained in a relatively good yield as

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1) E. P. Kennedy, S. B. Weiss : J. Biol. Chem., **222**, 193 (1956).
2) E. P. Kennedy : *Ibid.*, **222**, 185 (1956).
3) V. Ferrari, G. Ferrari : Arch. sci. biol. (Bologna), **37**, 1 (1953); (C. A., **47**, 11534e (1953)).

given in the literature, the synthesis of CDP-ethanolamine (IV) produced various by-products because of the presence of a primary amino group in ethanolamine phosphate (VI), and therefore even repeated purification of the product did not afford simple CDP-ethanolamine (IV).

According to the reports of Kennedy, *et al.*,[2,4] the yield of (IV) was lower than 10%, and Schneider, *et al.* reported[5] that the yield of deoxy-CDP-ethanolamine prepared by the DCC method was 3.5%.

Synthesis of CDP-ethanolamine (IV) was attempted by new method shown in Chart 1 and it was found that the yield was improved and the product was readily separated from by-products. Synthesis of CDP-choline (IX) was also effected by methylation of the terminal amino group in (IV).

A well-known method[6,7] for preparing ethanolamine phosphate (VI) is the ring cleavage of ethyleneimine (III) with phosphoric acid. It was assumed that, if cytidine diphosphate (CDP) (II) was used instead of phosphoric acid for opening the ring of ethyleneimine (III), CDP-ethanolamine (IV) would be produced. Therefore dicyclohexylguanidinium salt of cytidine 5'-phosphoramidate[8] (5'-CMP-NH_2) (I) was first condensed[9] with orthophosphoric acid and the reaction mixture was subjected to ion exchange chromatography (Fig. 1) to separate CDP (II) as colorless crystals. A solution of CDP (II) dissolved in

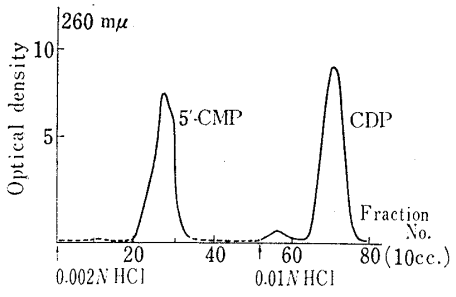

Fig. 1. Ion Exchange Chromatogram of CDP Synthesis

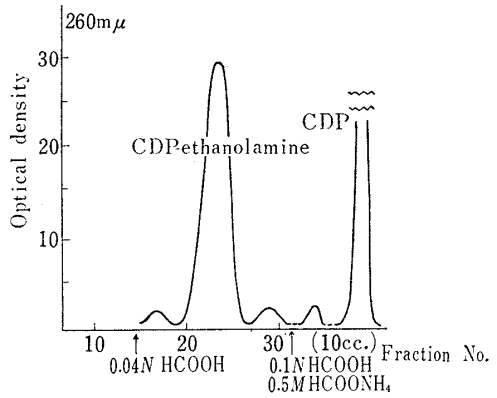

Fig. 2. Ion Exchange Chromatogram of CDP-ethanolamine Synthesis

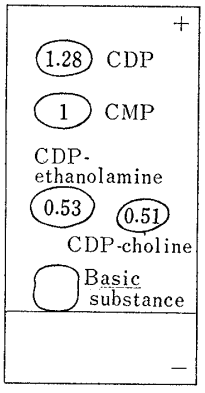

Fig. 3.*3 Paper Ionophoresis in Phosphate Buffer (pH 7.5)

*3 Figures show R_{CMP} (Ratio of the migration distance of the sample divided by that of CMP) of the substances.

4) E. P. Kennedy : J. Biol. Chem., **234**, 1998 (1959).
5) W. S. Schneider, J. Rotherhama : *Ibid.*, **233**, 48 (1958).
6) H. N. Christensen : *Ibid.*, **135**, 399 (1940).
7) D. M. Brown, *et al.* : J. Chem. Soc., **1957**, 2590.
8) K. Tanaka, M. Honjo, Y. Sanno, H. Moriyama : This Bulletin, **10**, 220 (1962).
9) R. S. Chamber, P. Shapiro, V. Kurkov : J. Am. Chem. Soc., **82**, 970 (1960).

water was neutralized with ethyleneimine (Ⅲ) to pH 7~7.5 and allowed to stand at 37° for 40 hours, and the reaction mixture was submitted to ion exchange chromatography (Fig. 2) to give CDP-ethanolamine (Ⅳ) in 31~34% yield (against CDP (Ⅱ)). The reaction conditions were examined by paper ionophoresis (Fig. 3) in phosphate buffer (pH 7.5) of the reaction mixture and the above-mentioned conditions were thought to be the best, because it was found that (1) at a high temperature such as 65°, CDP (Ⅱ) is partly decomposed, (2) insufficient ethyleneimine (Ⅲ) turns the mixture into acid pH and prevents the progress of the reaction, and (3) excess ethyleneimine (Ⅲ) turns the mixture into alkaline pH and produces a basic polymer. As shown in Fig. 2, the by-product in the fraction eluted with 0.04*N* formic acid was very small in quantity and readily separated. Further, from the effluent and the washing, an ultraviolet-absorbing basic by-product was obtained in 27~33% yield (against CDP (Ⅱ)), and from the fraction eluted with 0.1*N* formic acid + 0.5*N* ammonium formate, CDP (Ⅱ) was recovered in 29~30% yield. Formation of a structural isomer (Ⅷ) was feared, but the product was confirmed as CDP-ethanolamine (Ⅳ) by the following methods. The product was decomposed into ethanolamine phosphate (Ⅵ) and 5′-CMP (Ⅶ) by hydrolyzing with *N* hydrochloric acid or with purified nucleotide pyrophosphatase obtained from a snake venom, the products migrated reasonably in paper ionophoresis in acetate buffer (pH 4.5) (Fig. 4), and the amino group in the

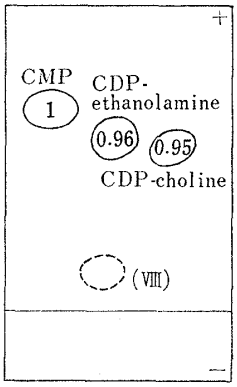

Fig. 4.*3 Paper Ionophoresis in Acetate Buffer (pH 4.5)

ethanolamine portion was detected by azotometry. Some by-products including 5′-CMP (Ⅶ) were found in the fraction eluted with 0.04*N* formic acid, but their amount, calculated from their optical density at 260 mμ, was only 1%, though they varied more or less with reaction conditions.

Methylation of ethanolamine is a well known method[10] for the preparation of choline (Ⅻ). With the assumption that application of this method to CDP-ethanolamine (Ⅳ) will produce CDP-choline (Ⅸ), the following experiment was carried out. As a preliminary experiment, a solution of ethanolamine phosphate (Ⅵ) in 50% methanol was shaken with methyl iodide at room temperature in the presence of potassium hydroxide, when a reaction set in with evolution of heat. Paper chromatography, ((a) solvent; 60% ethanol containing 0.02*N* acetic acid; paper, Toyo Roshi No. 7) of the product showed the same Rf value as that of authentic choline phosphate (Ⅹ). Therefore, (Ⅳ) was methylated under the same conditions, the reaction mixture was subjected to activated carbon chromatography to eliminate salts and 5′-CMP (Ⅶ), and the effluent was concentrated to give CDP-choline (Ⅸ) as a white powder. Paper ionophoresis at various pHs (Figs. 3 and 4) and paper chromatography (a) of the product gave a single spot with the same Rf as that of CDP-choline (Ⅸ) prepared by the DCC method,[2] and its yield was almost quantitative. To confirm the structure of the product, it was hydrolyzed with *N* hydrochloric acid,[2] and

10) G. Trier: Z. physiol. Chem., Hoppe-Seyler's, **80**, 409 (1912).

gave choline phosphate (X) and 5′-CMP (VII). When the hydrolysate was treated with the human prostatic phosphomonoesterase, cytidine (X) and choline (XII) were produced. (IX) was deaminated[11] with nitrous acid and the product, after treatment with carbon powder, showed the same ultraviolet spectrum as that of uridine 5′-monophosphate (5′-UMP) (XIV). It was therefore assumed that the product is uridine diphosphate (UDP)-choline (XIII) and in fact its hydrolysis with *N* hydrochloric acid afforded 5′-UMP (XIV). This fact shows that the amino group at C_4 in the cytosine ring in (IX) was not methylated with methyl iodide. It is certain that N_3 in the cytosine ring was not methylated from the fact that the product exhibited the same ultraviolet spectrum at various pHs as that of the CDP-choline (IX) prepared by the DCC method.[2] Studies on the structure of the product were all carried out with CDP-choline (IX) prepared by the DCC method[2] as a control and the two were found to show the same physical and chemical properties. Kennedy stated[2] that CDP-choline (IX) could be purified by treatment with activated carbon to remove the

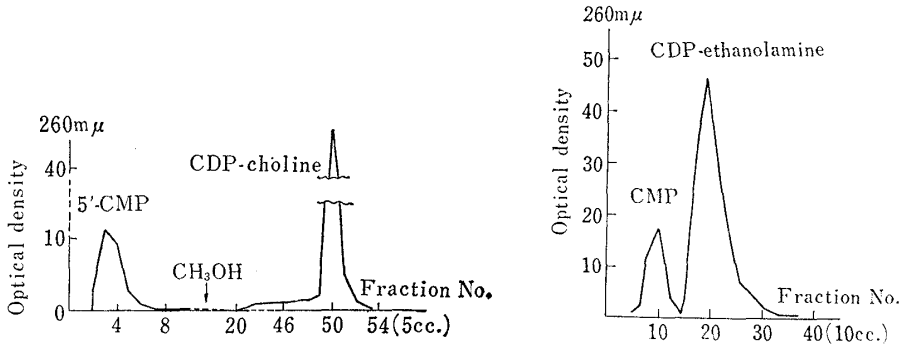

Fig. 5. Carbon Chromatogram

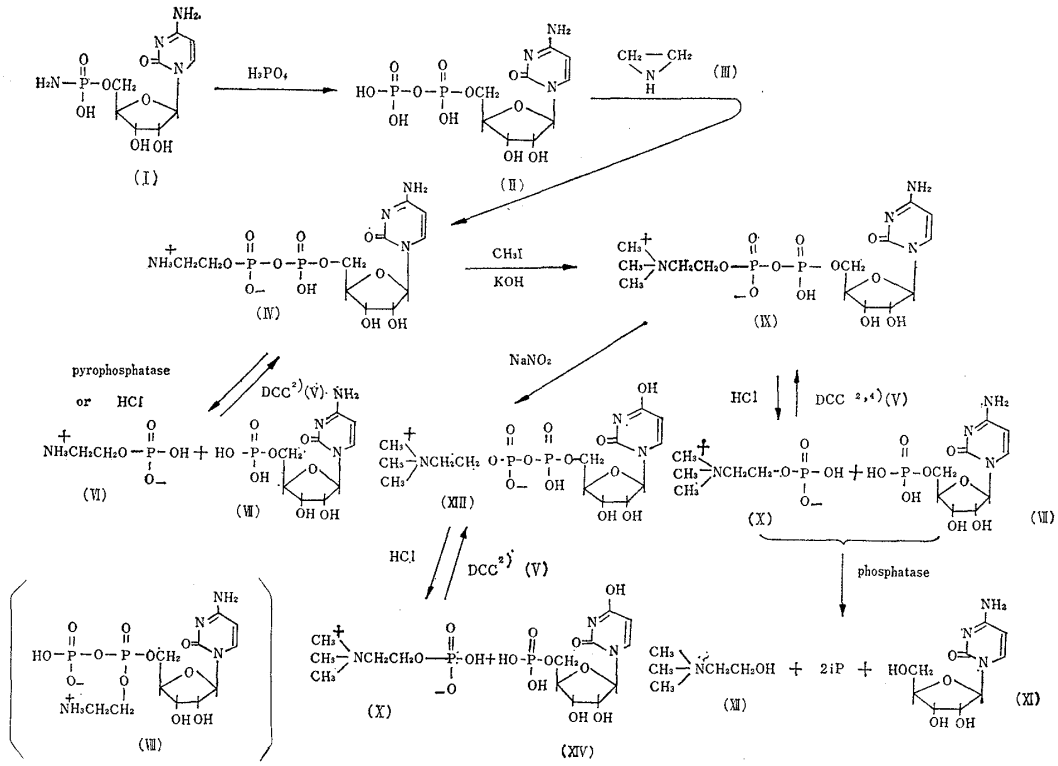

Chart 1.

11) H. S. Loring, N. G. Luthy : J. Am. Chem. Soc., **73**, 4215 (1951).

coexisting choline phosphate (X). It was found that the most possible impurity, 5′-CMP (VII), could be separated from CDP-ethanolamine (IV) or from CDP-choline (IX) when the mixture was subjected to active-carbon chromatography. For example, a mixture of 5′-CMP (VII) and (IV) or a mixture of 5′-CMP (VII) and (IX) was adsorbed on a column of activated carbon at acid pH and eluted with 2% ammonia water in the case of the former mixture, when 5′-CMP (VII) was first eluted and then CDP-ethanolamine (IV), as shown in Fig. 5. In the case of the latter mixture, the column was first treated with aqueous am monia as above, but CDP-choline (IX) was not eluted until the column was treated with aqueous methanol containing ammonia. Each fraction was concentrated immediately and subjected to paper ionophoresis, giving a single spot at the expected Rf. Judging from the kind of the solvents used for elution, the adsorbability of CDP-choline (IX) on activated carbon is stronger than that of CDP-ethanolamine (IV), probably due to the methyl group at the amino portion of choline (XII). As mentioned above, it was found that 5′-CMP (VII), choline phosphate (X) and ethanolamine phosphate (VI) can all be eliminated by treatment with activated carbon.

Experimental[*4]

CDP (II)——To a solution of 4 g. of dicyclohexylguanidinium salt of 5′-CMP-NH_2 (I) (purity, 75%) in 70 cc. of *o*-chlorophenol 7 cc. of 85% H_3PO_4 was added and the mixture was left standing for 7 hr. with cooling in ice-water. After sometime, 200 cc. of $CHCl_3$ was added to the reaction mixture and allowed to stand in an ice-box overnight, when a syrupy substance was deposited, which was separated from the supernatant by decantation (or by centrifugation, if necessary). The syrupy substance was washed with 150 cc. and 100 cc. of Me_2CO, the resulting solid was dissolved in 100 cc. of H_2O, and the solution was shaken with Et_2O. The aqueous layer was concentrated *in vacuo* to remove the organic solvent, diluted to 1.5 L. with H_2O, adjusted to pH 8.5 with NH_4OH, and poured at a rate of S.V:3 on a column (3×21 cm.) of 115 cc. of Dowex-1, X8 (200～400 mesh, Cl- form). The column was washed with 2 L. of water and eluted with the following solvents.

Solvent	Volume of solvent (L.)	Total optical density pH 2, 260 mμ	Substance
0.002*N* HCl	5.16	27504	5′-CMP (VII)
0.01*N* HCl	2.65	27825	CDP (II)
		127	Others
		55456	(Recovery rate, 97%)

The CDP (II) fraction was poured as such on a column of 20 g. of activated carbon, the column was washed with water, and eluted with 50% MeOH containing 1% of NH_4OH. The eluate was concentrated *in vacuo* to eliminate MeOH and NH_4OH, diluted to 300 cc. with wrter, and passed through a column of 23 cc. of Amberlite IR-120 (acid form) to give a solution of free CDP (II). The solution was evaporated *in vacuo* to dryness at room temperature and the residue was recrystallized from 20 vols. of water to CDP (II) (1.6 g.) as colorless needles, m.p. 183°(decomp.), $[\alpha]_D^{22}$ +26°(c=0.5%, H_2O). *Anal.* Calcd. for $C_9H_{15}O_{11}N_3P_2 \cdot H_2O$: C, 25.64; H, 4.32; N, 9.97; P, 14.74. Found: C, 25.59; H, 4.32; N, 10.41; P, 14.53.

CDP-Ethanolamine (IV)——A solution of 200 mg. of CDP (II) (total optical density 260 mμ, 3090 at pH 2) in 18 cc. of H_2O was adjusted to pH 7～7.5 with ethyleimine (III) and kept at 37° in a sealed tube for 40 hr. Since the yield of the product, CDP-ethanolamine (IV), varied markedly with reaction conditions such as temperature, pH, and time, the above conditions were decided after examining the reaction mixture by paper ionophoresis in phosphate buffer (pH 7.5). Impurities in the reaction mixture were unchanged CDP (II) and by-products such as CDP-polyethanolamine and a fluorescent substance. The reaction mixture was concentrated in a reduced pressure to remove the excess ethyleneimine (III), the residue was diluted to 120 cc. with H_2O, adjusted to pH 8.5 with NH_4OH and poured at a rate of S.V:1.5 into a column (2×16 cm.) of 40 cc. of Dowex-1, ×2 (200～400 mesh, formate form). The column was washed with 800 cc. of H_2O and eluted successively with 0.04*N* HCOOH, 0.1*N* HCOOH+0.1*N* $HCOONH_4$, and 0.1*N* HCOOH+0.5*N* $HCOONH_4$.

[*4] All melting points are uncorrected.

Fraction No.	Solvent	Total optical density pH 2, 260 mμ	Yield (%)	Substance
1	Effluent and washing	1032	33	Basic substance
2	0.04*N* HCOOH	37		Unknown
3		966	31	CDP-ethanolamine (IV)
4		47		5′-CMP (VII)
5	0.1*N* HCOOH+0.1*N* $HCOONH_4$	79		Unknown
6	0.1*N* HCOOH+0.5*N* $HCOONH_4$	897	29	CDP (II)
		13		Others
		3071		(Recovery rate, 99.5%)

Concentration of the CDP-ethanolamine (IV) fraction separated scaly crystals. The product, CDP-ethanolamine (IV), was identified by the following experiments.

1) The Rf value of the product in paper chromatography (a) was agreed with that given in the literature, the relation of its Rf value to those of 5′-CMP (VII) and CDP-choline (IX) was as expected, and migration of the product in paper ionophoresis was reasonable.

2) The purity ($\varepsilon_{280} \times 10^3 = 13.7$ at pH 2) of the product as such (containing some water) was 90.5%.

3) Determination of amine by azotometry: 100.1 % of nitrogen was detected by azotometry against the amount of (IV) calculated from its optical density at 280 mμ. Nitrogen values of 5′-CMP (VII) and ethanolamine phosphate (VI), which were employed as control, were 0% and 99.4%, respectively.

4) Hydrolysis with *N* HCl: A solution of 4.252 mg. of CDP-ethanolamine (IV) in 3 cc. of *N* HCl was heated at 100° in a boiling water bath for 40 min. The reaction mixture was examined by paper chromatography (a) and paper ionophoresis in acetate buffer (pH 5) or in borate buffer (pH 9.2) and 5′-CMP (VII) and ethanolamine phosphate (VI) were detected from their UV absorption and coloration with ammonium molybdate or with sulfosalicylic acid plus $FeCl_3$. The products gave the same Rf values as the authentic samples.

5) Decomposition with purified pyrophosphatase obtained from snake venom: To a salution of 3.1 mg. of the substance (CDP-ethanolamine) in Tris buffer (pH 9.0), $MgCl_2$ and the enzyme solution (100 γ protein) were added and the mixture was kept at 37° for about 1 hr. The reaction mixture was heated at 100° for 15 min. to inactivate the enzyme, filtered after cool to remove an insoluble substance (chiefly protein), and concentrated *in vacuo*, to form the test solution. The control was a solution of the authentic sample in the inactivated enzyme solution in Tris buffer. Paper chromatography (a) and paper ionophoresis in acetate buffer (pH 5) of the hydrolysate indicated the presence of 5′-CMP (VII) and ethanolamine phosphate (VI).

CDP-Choline (IX)——To a solution of 8.8 mg. of KOH in 11 cc. of 50% MeOH, 11.3 mg. of CDP-ethanolamine (IV) (purity, 90.2%) and 12.5 mg. (0.0055 cc.) of MeI, were added and the mixture was shaken in a closed vessel, when MeI disappeared immediately. The mixture, after standing at 20° for 1 day, was adjusted to pH 1.5 with HCl and poured into a column of 1 g. of activated carbon. The column was washed with H_2O and treated first with 2% NH_4OH to elute 5′-CMP (VII) and then with 50% MeOH containing 2% NH_4OH to elute CDP-choline (IX). Concentration of the latter fraction gave a crystalline powder, which was confirmed as CDP-choline (IX) by the experiments shown below. In all the experiments, CDP-choline (IX) prepared by the DCC method[2] was used as a control.

1) Paper ionophoresis, paper chromatography and UV absorption spectrum: The Rf values in paper ionophoresis at various pHs and paper chromatography (a) and the UV absorption of the product agreed with those of the authentic sample.

2) Hydrolysis with *N* HCl: A solution of 3 mg. of the product in 3 cc. of *N* HCl was heated at 100° in a boiling water bath for 30 min. Paper ionophoresis (acetate buffer, pH 5) and paper chromatography (a) of the concentrated reaction mixture showed spots of 5′-CMP (VII) and choline phosphate (X).

3) Deamination with $NaNO_2$: To a solution of 4.2 mg. of the product and 10 mg. of $NaNO_2$ in 0.2 cc. of H_2O seven 0.003-cc. portions of AcOH were added at room temperature at intervals of 30 min. with stirring and the mixture was allowed to stand at 5° overnight. UV absorption of the reaction mixture at 250～260 mμ was disturbed because of the presence of excess HNO_2, and the mixture was treated with activated carbon to remove inorganic substances. The solution was again subjected to measurement of its UV absorption, giving almost the same value as that of 5′-UMP (XIV) as shown in the following Table.

	ε 250/ε 260		ε 280/ε 260		ε 290/ε 260	
pH	2	12	2	12	2	12
Deaminated product	0.75		0.38		0.05	
5′-UMP (XIV)	0.74		0.38		0.03	
5′-CMP (VII)	0.46	0.84	2.10	0.99	1.55	0.33
CDP-choline (IX)	0.456	0.86	2.05	0.96	1.54	0.36

Heating of a solution of the deaminated product in 2 cc. of *N* HCl at 100° for 30 min. yielded 5′-UMP (XIV).

4) Action of human prostatic phosphomonoesterase on the *N* HCl hydrolysate (confirmation of choline (XII): The hydrolysate and the enzyme solution[12] (2 units) were dissolved in acetate buffer (0.6 cc., pH 5.4) and kept at 37° for 2 hr. and at 100° for 15 min. The solution was filtered, concentrated, and submitted to paper chromatography. The Rf values of the products are shown in the following Table.

Substance	60% EtOH, 0.02*N* AcOH	H_2O-BuOH-AcOH (5:4:1)	Detected by
Choline (XII)		0.30, 0.29	Dragendorff reagent
Cytidine (XI)	0.64	0.24	Ultraviolet absorption

Carbon Chromatography——1) A solution of 200 mg. of CDP-ethanolamine (IV) and 20 mg. of 5′-CMP (VII) was poured into a column of 10 g. of activated carbon at pH 2 and eluted with 2% NH_4OH. 2) A solution of 30 mg. of CDP-choline (IX) and 15 mg. of 5′-CMP (VII) was adsorbed on 2 g. of activated carbon at pH 2 and eluted first with 2% NH_4OH and then with 50% MeOH containing 2% NH_4OH.

Paper Ionophoresis——Paper ionophoresis was carried out under following conditions:

1) 0.05*M* Phosphate buffer; pH 7.5; 11 v./cm.; 500 v.
2) 0.1*M* Acetate buffer; pH 4.5 or 5.0; 11 v./cm.; 500 v.; paper, Toyo Roshi No. 7.

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Summary

Reaction of cytidine diphosphate (CDP) (II) with ethyleneimine (III) produced CDP-ethanolamine (IV), and methylation of the product with methyl iodide yielded CDP-choline (IX).

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12) S. E. Kerr, F. Chernigoy: J. Biol. Chem., **228**, 495 (1957).