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# The Fermentative Production of CDP-Choline by Yeasts*

## (I) Identification of CDP-Choline

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**Abstract**

Large amounts of a cytidine derivative were formed when air-dried cells of brewer's yeast (100 mg/ml) were incubated with 5′-CMP (20 μmoles/ml) and phosphorylcholine. The substance was determined to be CDP-choline. On acid hydrolysis, it gave phosphorylcholine and 5′-CMP, which was demonstrated by paper chromatography and by treatment with 5′-nucleotidase. The phosphorylcholine gave an $R_f$ value identical with that of an authentic sample on paper chromatography. It was further identified, after treatment with non-specific phosphatase, by measurement of the molar ratio of phosphorus and choline. CDP-choline was additionally confirmed on the basis of NMR spectrum data and other physicochemical properties.

## Introduction

CDP-Choline was first found by Kennedy to be an important intermediate in the biosynthetic pathway of lipids[1,2]. This compound is widely distributed among animals, plants, and microorganisms. Though some strains of yeast contained large amounts of this compound, it was only endogenously synthesized. By the application of the fermentative method for the production of sugar nucleotides[3-5], the authors have succeeded in converting 5′-CMP to CDP-choline in good yields.

This paper deals with the identification of CDP-choline formed by fermentation of brewer's yeast.

## Materials and Methods

1. Materials 5′-CMP sodium salt was kindly supplied by Takeda Chemical Industries, Ltd. All other chemicals were commercial products. 5′-Nucleotidase was prepared from bull seminal plasma[6]. Prostatic non-specific phosphatase was kindly supplied by Dr. Y. Sugino of the Institute for Virus Research, Kyoto University.

2. Microorganism The microorganism used was brewer's yeast (*Saccharomyces*

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Abbreviations: 5′-CMP, cytidine-5′-monophosphate; CDP, cytidine diphosphate; CTP, cytidine triphosphate; CDP-choline, cytidine diphosphate choline.

*carlsbergensis*), which was obtained from Kirin Brewery Co., Ltd. The wet yeast was dried by an electric fan for 12 hr at room temperature. The air-dried cells were completely dried over $P_2O_5$ for 24 hr *in vacuo*. The dried cells thus prepared were kept at −20°C and used as the enzyme source throughout this work.

3. Analyses CMP, CDP, CTP, and CDP-choline were determined by measuring their optical densities at 260 mμ after extracting their spots with 0.1 N HCl from paper chromatograms. Paper chromatography was carried out on Toyo filter paper No. 53 with a solvent system containing 95% ethanol and 1 M ammonium acetate (2:1, pH 7.5).

Reaction products were separated by column chromatography with Dowex 1×2 ($Cl^-$ form), which was carried out by the method of Cohn and Carter[7]. CDP-choline was eluted with 0.001 N HCl, and 5′-CMP with 0.002 N HCl. Inorganic phosphate was determined by the method of Fiske and Subbarow[8] after the decomposition of samples by Nakamura's method[9], or by other methods with various enzymes.

CDP-choline was identified by the results of various enzyme reactions, which were carried out at 37°C for 2 hr.

4. Reaction system The composition of the reaction mixture is shown in Table 1. The total reaction mixture of 2.0 ml was shaken at 28°C for several hours. The reaction was terminated by immersing the reaction tube in boiling water for 3 min. After addition of 2.0 ml of water, cell debris was removed by centrifugation at 3,500 rpm for 5 min and the resulting supernatant solution was subjected to analysis.

Table 1. Composition of reaction mixture.

| | per ml |
|---|---|
| Glucose | 400 μmoles |
| 5′-CMP sodium salt | 20–30 μmoles |
| Phosphorylcholine calcium salt | 50 μmoles |
| Potassium phosphate buffer (pH 7.0) | 200 μmoles |
| $MgSO_4 \cdot 7H_2O$ (or $MgCl_2 \cdot 6H_2O$) | 12 μmoles |
| Air-dried cells of brewer's yeast | 100 mg |

The total reaction mixture of 2.0 ml was put in a test tube and reaction was carried out 28°C with shaking.

## Results and Discussion

**1. Formation of cytidine derivative** After incubation with 5′-CMP and phosphorylcholine, the reaction mixture was analyzed by paper chromatography. Three UV-absorbing spots having $R_f$ values different from that of 5′-CMP were detected on a paper chromatogram (Fig. 1). Two of them were identified, by their $R_f$ values on the paper chromatogram, as CDP and CTP. The other showed the largest $R_f$ value, which was identical with that of authentic CDP-

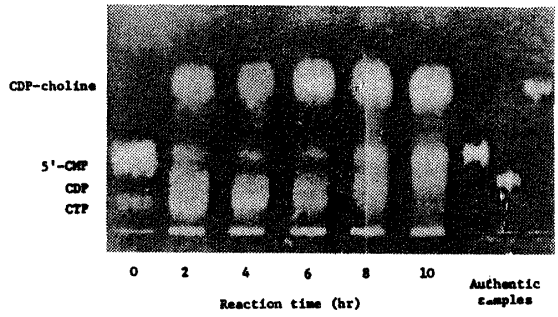


Fig. 1. Formation of CDP-choline from 5′-CMP by dried cells of brewer's yeast.

choline. The third cytidine derivative was not synthesized when any one of 5′-CMP, phosphorylcholine, or the air-dried cells was omitted from the complete system (Fig. 2).

A typical time course for the fermentative reaction is shown in Fig. 3. In the earlier stages of the reaction, 5′-CMP is consumed rapidly in accordance with the formation of CDP and CTP. On longer incubation, CDP and CTP decrease, with formation of the third cytidine derivative. The cytidine derivative accumulated was rather stable even after 12 hr incubation.

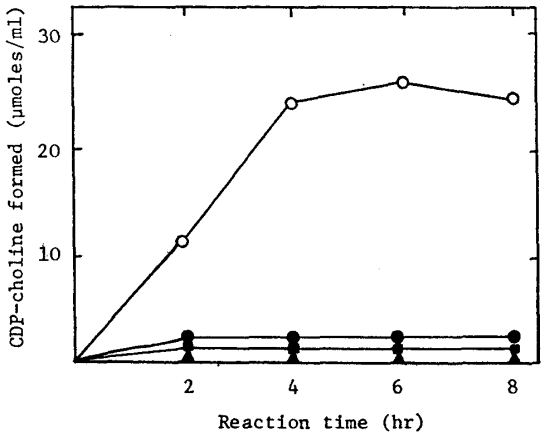


Fig. 2. Formation of CDP-choline.
○—○: complete system shown in Table 1
●—●: phosphorylcholine omitted
■—■: 5′-CMP omitted
▲—▲: dried cells omitted

**2. Isolation and identification of third cytidine derivative** In order to isolate the third cytidine derivative, the reaction was carried out on a scale ten times lager than previously (Fig. 3). The reaction mixture consisted of 400 μmoles of 5′-CMP, 2 mmoles of phosphorylcholine, 4 mmoles of potassium phosphate buffer (pH 7.0), 12 mmoles of glucose, 200 μmoles of $MgCl_2$, and 2 g of dried brewer's yeast. Total volume was 20 ml. After incubation at 28°C for 10 hr, the cells were centrifuged out. The supernatant solution was acidified with 1 N HCl to pH 3.8, and the precipitated protein was centrifuged and discarded. Activated carbon was added to the supernatant solution to absorb nucleotides, which were eluted with ethanol — ammonium hydroxide — water (50 : 5 : 45) solution. The eluate was concentrated to half its volume under reduced pressure at 30°C. The concentrated eluate, after pH was adjusted to 8.0 with 1 N ammonium hydroxide, was treated on a column of Dowex 1×2 ($Cl^-$ form). Elution was carried out according to the method of Cohn and Carter[7].

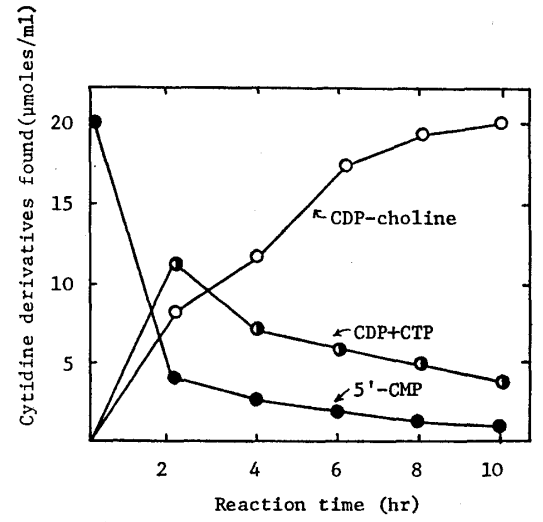


Fig. 3. Time course of formation of cytidine derivatives.
The reaction mixture consisted of 400 μmoles of 5′-CMP, 2 mmoles of phosphorylcholine, 4 mmoles of potassium phosphate buffer (pH 7.0), 12 mmoles of glucose, 200 μmoles of $MgCl_2$, and 2 g of dried cells of brewer's yeast in a total volume of 20 ml. The reaction was carried out at 28°C in a 300 ml-flask with shaking.

The elution pattern for a typical small-scale reaction mixture is shown in Fig. 4. The main cytidine derivative was eluted with 0.001 N HCl. By the addition

of activated carbon to the effluent, the nucleotide was absorbed; it was then eluted with ammoniacal ethanol solution. The eluate was concentrated to a small volume under reduced pressure at 30°C, then lyophilized. About 25 mg of the purified cytidine derivative was obtained as its ammonium salt.

The sample decomposed at 190—198°C. Analysis showed: C, 31.66; H, 6.19; N, 12.23; P, 9.12 and O, 40.80%. Calculated values for $C_{14}H_{26}O_{11}N_4P_2(NH_4) \cdot 3H_2O$ are: C, 30.00; H, 6.43; N, 12.50; P, 10.10 and O, 40.00%.

Paper chromatograms of the isolated cytidine derivative and its components are shown in Fig. 5. The $R_f$ value of the derivative was identical with that of authentic CDP-choline. The hydrolysate (1 N HCl, 80 min) of the sample did not contain free inorganic phosphate, but contained 5′–CMP and phosphorylcholine. The former was confirmed by paper chromatography and by treatment with 5′-nucleotidase, and the latter was proved by spraying with Dragendorf reagent on paper chromatograms. On treatment with non-specific phosphatase the isolated phosphorylcholine released one mole of phosphorus per mole of choline, which was determined as reineckate by the method of Ackerman and Salmon[10]. Total phosphorus was determined after decomposition with sulfuric acid[9]. The derivative contained two moles of phosphorus per mole of cytidine. On treatment of the hydrolysate with 5′-nucleotidase the derivative released one mole of phosphorus per mole cytidine, whereas by treatment with non-specific phosphatase it released two moles of phosphorus. The derivative liberated one mole of phosphorus when treated with snake venom (Agkistrodon halys, Mamushi), which is known to contain pyrophosphatase and 5′-nucleotidase. These experiments showed that in this cytidine derivative 5′–CMP and phosphorylcholine are bound through pyrophosphate linkage. The experimental results with these various enzymes are summarized in Table 2.

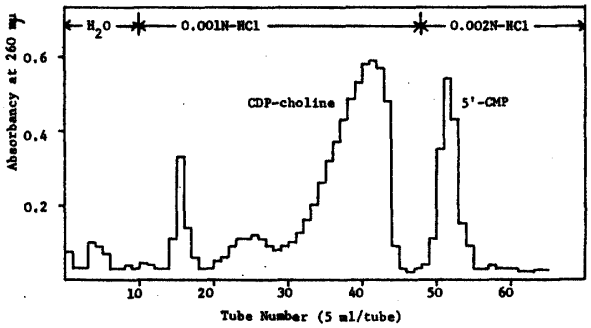


Fig. 4. Separation of CDP-choline by Dowex 1×2 ($Cl^-$ form) column chromatography.
Column: 1×19 cm

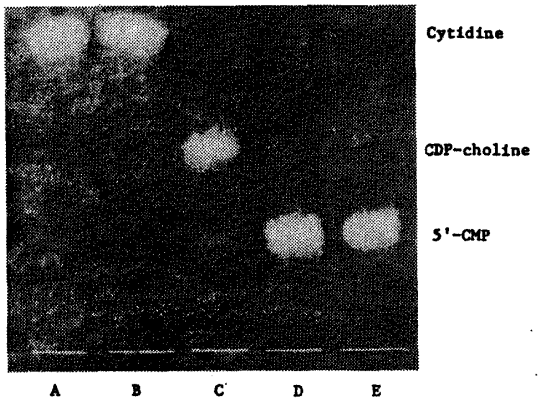


Fig. 5. Paper chromatogram for the identification of isolated CDP-choline.
Solvent: 95% ethanol–1 M ammonium acetate (2 : 1, pH 7.5)
A: authentic cytidine, B: acid-hydrolysate of isolated CDP-choline after treatment with non-specific phosphatase, C: isolated CDP-choline, D: acid-hydrolysate of isolated CDP-choline (1 N HCl, 100°C, 80 min), E: authentic 5′-CMP.

The identification of the derivative was further confirmed by physicochemical

Table 2. Identification of the sample.

(1) Total phosphates by Nakamura's method

| | Pi* (phosphorus) |
|---|---|
| 5′-CMP | 1.02 (1.00) |
| CDP-choline | 1.94 (1.92) |
| sample | 1.96 (1.92) |

*Ratio to 1 mole of cytidine

(2) Hydrolysis with 1 N-HCl (100°C, 80 min)

| | CMP liberated |
|---|---|
| CDP-choline | 1.01 (1.00) |
| sample | 0.93 (0.92) |

(2-a) Treatment with 5′-nucleotidase (37°C, 2 hours)

| | Pi* liberated |
|---|---|
| 5′-CMP | 0.93 (1.00) |
| CDP-choline | 1.05 (1.15) |
| hydrolysate | 1.08 (1.18) |

*Ratio to 1 mole of cytidine

(2-b) Treatment with non-specific phosphatase (37°C, 2 hours)

| | Pi liberated |
|---|---|
| P-choline | 1.03* |
| CDP-choline | 2.16** |
| hydrolysate | 1.84** |

*Ratio to the initial Pi

**Ratio to 1 mole of cytidine

(3) Treatment with snake venom (pyrophosphatase and 5′-nucleotidase)

| | Pi | Cytidine |
|---|---|---|
| | (μmole/μmole substrate) | |
| P-choline | 0 | — |
| 5′-CMP | 1.28 | 1.21 |
| CDP-choline | 0.91 | 1.18 |
| sample | 0.90 | 0.87 |

(4) Measurement of choline after the separation of P-choline

| | Pi/choline |
|---|---|
| P-choline | 1.220 (1.000) |
| sample | 1.225 (0.995) |

analyses. The IR and UV spectra of the derivative were identical with those of authentic CDP-choline. The NMR spectrum of the derivative is shown in Fig. 6, and shows the presence of the protons in positions 5 and 6 in the pyrimidine ring and of the protons of the methyl group attached to the quaternary ammonium in the choline. The ratio of the numbers of these prosons was 1:1:9, which indicates the presence of the pyrimidine ring and choline in the ratio of 1:1.

From these results, the cytidine derivative was identified as CDP-choline. The optimum conditions for the fermentative formation of CDP-choline will be reported in the following paper.

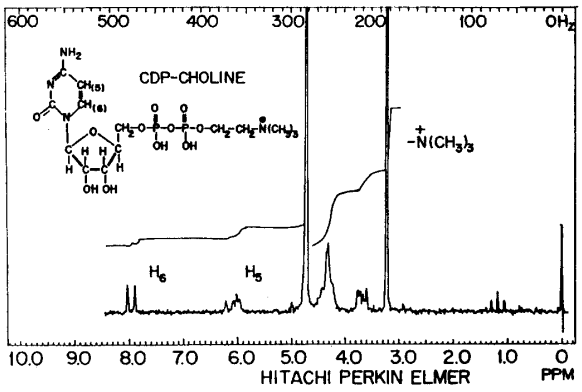


Fig. 6. NMR spectrum of isolated CDP-choline.

**Acknowledgement**

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