Bull. Inst. Chem. Res., Kyoto Univ., Vol. 54, No. 3, 1976

Review

Biological Activity of *Hansenula jadinii* with Regard to Large Scale Fermentative Production of CDP-Choline. (Phosphorylation of Choline and CMP and Inhibition of Choline Kinase by CTP.)

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Received April 15, 1976

INTRODUCTION

The fermentative production of CDP-choline by *Hansenula jadinii* from CMP and choline under the condition of high levels of inorganic phosphate added has been reported.[1)]

In this paper deals with the efficiency of energy sources on the phosphorylation of CMP and choline, and biological activities of *Hansenula jadinii* with regard to large scale fermentative production of CDP-choline and deals with the inhibition of choline phosphorylation by CTP.

An improved reaction system for large scale fermentative production of CDP–choline is described.

MATERIALS AND METHODS

Microorganisms The microorganisms used were *Hansenula jadinii* IFO 0987, baker's yeast and brewer's yeast. Air-dried cell preparations of these yeasts were prepared in the same manner described in the previous paper.[1)] Brewer's yeast was kindly donated by Kirin Brewery Co. Ltd., Kyoto Branch.

Chemicals Sodium salts of CMP and CTP were donated by Kyowa Hakko Kogyo Co. Ltd., Tokyo. Some CTP was produced fermentatively from CMP using dried cell preparation of *H. jadinii*. Purified preparation was obtained by column chromatography on Dowex 1, X–2 (Cl^-)[2)]. All other chemicals used were commercial products. Aminoethanols were neutralized to pH 8.0 by adding concentrated HCl

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** 相阪和夫，木村　光，栃倉　辰六郎：Laboratory of Industrial Microbiology, Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto. The following abbreviations have been used: CMP, Cytidine 5′-monophosphate; CDP, Cytidine 5′-diphosphate; CTP, Cytidine 5′-triphosphate; CDP-choline, Cytidine diphosphate choline; FBP, Fructose 1, 6–*bis*phosphate; P-choline, Phosphorylcholine; P-aminoethanol, Phosphorylaminoethanols.

prior to use.

Analysis. Phosphorylcholine and P-aminoethanols and cytidine nucleotides were determined as described in the previous paper.[1] Inorganic pyrophosphatase activity was assayed by the method of Heppel.[3] Choline kinase activity was assayed by coupling with NADH oxidation.[4~7] Crude enzyme activity was determined by the formation of phosphorylcholine. The reaction mixture contained 250 mM glycylglycine (pH 8.0), 10 mM choline, 25 mM ATP, 50 mM $MgCl_2$, 1 mM cysteine and the enzyme in a total volume of 2.0 ml. Reaction was carried out at 25°C. After the reaction had been stopped by heating in a boiling water bath, aliquots of 200–300 μl of supernatant spun at 3,000 rpm for 10 min were spotted on two filter papers (Toyo roshi No. 53, 20×60 cm), and chromatographed at the same time by electrophoresis with the solvent system of formate-acetate mixture at 3,000 V, 40 mA for 120 min.[8] After drying, one of the papers was sprayed with coloring reagent[9] to detect P-choline. The area corresponding to P-choline was cut out of the second uncolored paper and transferred to a test tube. To the tube was added 3 ml of 100 mM Tris-HCl (pH 7.5) and 50 μl of alkaline phosphatase of *Escherichia coli*[10] (50 μg protein per ml). The inorganic phosphate liberated after 3 hr incubation at 30°C was determined colorimetrically.[11]

Standard Reaction System. The standard reaction system for CDP-choline formation contained 20 mM CMP, 80 mM choline chloride, 600 mM glucose, 300 mM phosphate buffer (pH 8.0), 30, M $MgSO_4 \cdot 7H_2O$ and 100 mg per ml of dried cells in a total volume of 2.0 ml, shaken at 28°C. The contents of the standard reaction mixture were changed according to the experiment.

Protein was determined by phenol reagents.[12] To follow column fractionation, the protein content of the effluent was evaluated by the absorption at 260 and 280 nm.[13]

RESULTS

Effect of energy sources on CDP-choline formation, CMP phosphorylation and choline phosphorylation Using glucose and FBP as energy sources, the effect of energy source on CDP-choline formation and on phosphorylation of CMP and choline was investigated.

Figure 1–A shows that little CDP-choline was formed at the early stage of incubation in the reaction mixture with glucose, either in the choline system or in the P-choline system, while in the reaction mixture with FBP, 1.5 mM and 4 mM of CDP-choline respectively was found in the choline and P-choline systems. In the reaction mixture containing glucose, an apparent lag phase of CDP-choline formation was observed, but production of CDP-choline commenced soon after and increased further incubation for 1 hr in both choline and P-choline systems. The amount of CDP-choline produced in the glucose system exceeded that in the FBP system after 2 hr incubation. As shown in Fig. 1–B, the amount of CDP+CTP formed in the FBP system at 0.5 hr incubation was more than 4 times that of the glucose system. It is evident that FBP surpasses glucose as an energy source for CMP phosphorylation and for CDP-choline for-

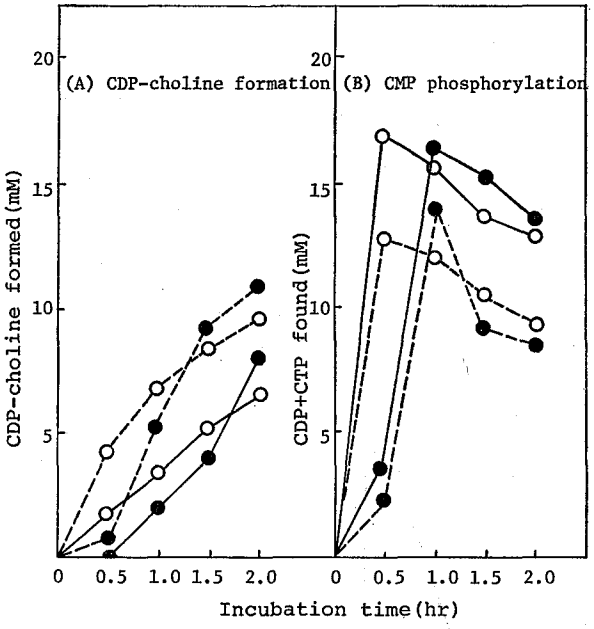


Fig. 1. Difference in chemical changes in CDP-choline formation as a function of energy source. The reaction mixture contained (mM): CMP, 20; choline, 80 (——) or P-choline, 50 (– – –); phosphate buffer (pH 8.0), 400; $MgSO_4$, 30; glucose, 400 (—●—) or FBP, 200 (—○—). Dried cells, 100 mg per ml. Total volume 2.0 ml.

mation in the early stage of incubation. The rate of phosphorylation of CMP was compared with that of choline in the reaction systems of glucose and BFP which were devoid of either choline or CMP.

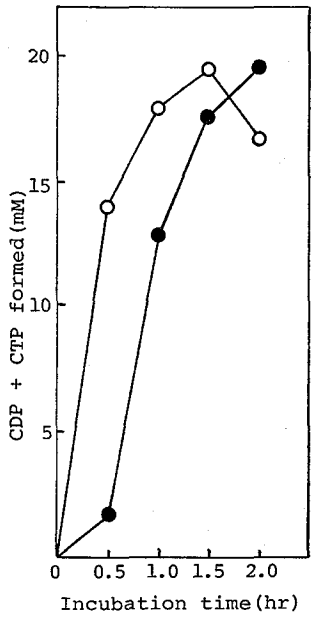


Fig. 2. Phosphorylation of CMP.
The reaction mixture was the same as the reaction mixture of Fig. 1–A except that choline or P-choline was omitted. Shaken at 28°C. Glucose system (—●—); FBP system (—○—).

As shown in Fig. 2, at the early stage of reaction of 0.5 hr incubation, about 65% of CMP was phosphorylated in the FBP reaction mixture, and 90% of CMP was phosphorylated within 1 hr incubation. The phosphorylation of CMP in the FBP system was almost complete within 1 hr incubation and further incubation resulted in the degradation of the product. On the other hand, the percentage of CMP phosphorylation in the reaction system of glucose was only about 10% and 60% at 0.5 hr and 1 hr incubation respectively. Phosphorylation of CMP in glucose system continued until 2 hr without degradation of the product.

The rate of phosphorylation of choline was also compared with regard to the energy sources. As shown in Fig. 3, P-choline formation in the FBP system proceeded more quickly than in the glucose system. About 2 mM of P-choline was formed in the FBP system at 0.5 hr incubation, but no detectable amount of P-choline was formed in the glucose system. P-choline was detected in the glucose reaction system after 1 hr

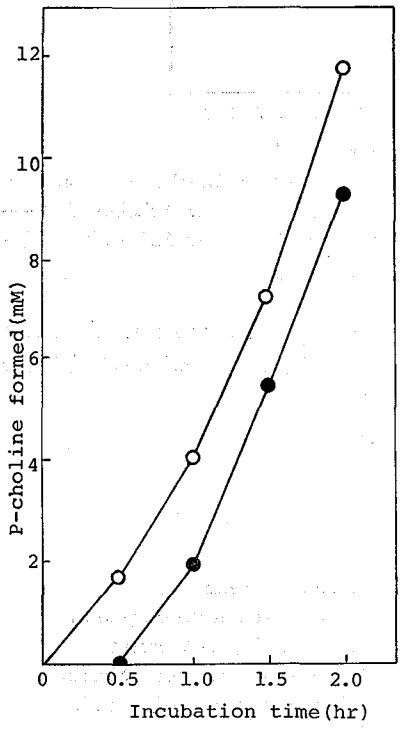


Fig. 3. Effect of energy source on choline phosphorylation.
The reaction mixture was the same as that of Fig. 1, except that CMP and P-choline were omitted. Energy sources: glucose (—●—); FBP (—○—). Shaken at 28°C.

incubation, and phosphorylation of choline proceeded parallel to the incubation time.

Biological activity of H. jadinii with respect to CDP-choline formation To produce large amounts of CDP-choline, it is necessary to use high concentration of CMP in the reaction mixture. Using dried cells of *H. jadinii*, phosphorylation of high concentrations of CMP and degradation of CMP and CTP were examined.

As shown in Fig. 2, *H. jadinii* has sufficient ability to phosphorylate CMP in quantities, and the rate of CMP phosphorylation and stability of CMP and CTP were excellent compared to the other yeasts. The reaction mixture used was standard reaction mixture without choline.

Figure 4 shows the higher activity of CMP phosphorylation of *H. jadinii* than the other two yeasts. When 50 mM of CMP was added to the reaction mixture, 47 mM of CDP+CTP was accumulated within 2 hr incubation and almost all CDP+CTP formed in the reaction mixture remained for further 12 hr incubation without degradation.

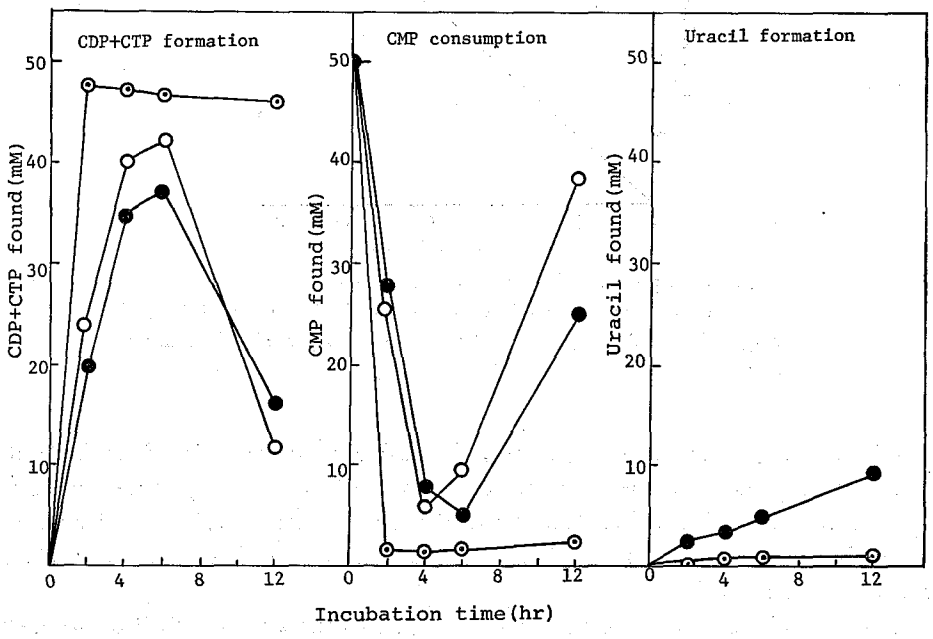


Fig. 4. Phosphorylation of CMP by yeasts and stability of CTP in the reaction mixture of fermentation.

The reaction mixture contained (mM): CMP, 50; phosphate buffer (pH 8.0), 300; glucose, 300; $MgSO_4$, 30 and 100 mg per ml of dried cells in a total volume of 2.0 ml. Shaken at 28°C. Brewer's yeast (—●—); baker's yeast (—○—); *Hansenula jadinii* (—◉—).

In the reaction mixtures of baker's yeast and brewer's yeast, comparative amounts of CDP+CTP were formed in 6 hr incubation, but soon after they were degraded to CMP. In the reaction mixture of brewer's yeast, the rate of CDP+CTP formation was less than 1/3 that of *H. jadinii* and a considerable amount of uracil was formed. In 12 hr incubation, uracil production amounted to 20% of CMP originally added.

The degradation activity of cytidine nucleotides (CMP, CDP and CTP) of *H. jadinii* is weaker than that of the other two yeasts.

Inorganic pyrophosphatase activity of *H. jadinii* was examined. Figure 5 shows the high activity of inorganic pyrophosphatase of *H. jadinii*. Using 1 mg per ml of dried cells, 4 mM of inorganic pyrophosphate was hydrolyzed within 1 min incubation at 30°C. The amount of hydrolyzable pyrophosphate is 240 mM per mg dried cells per hr.

Differences in CDP-choline formation at high concentration of CMP between P-choline system and choline system Since cells of *H. jadinii* have high activity of CMP phosphorylation

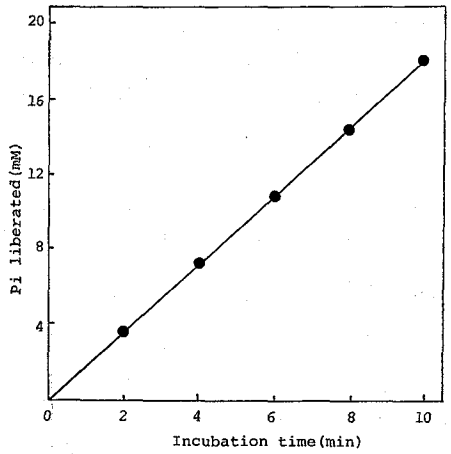


Fig. 5. Inorganic pyrophosphatase of *hansenula jadinii*.
The reaction mixture contained (mM): Tris-HCl buffer (pH 7.2), 67; $Na_2P_2O_7$, 16.7; $MgCl_2$, 16.7. Dried cells, 67μg per ml. Reaction was carried out at 28°C.

and of inorganic pyrophosphatase, large scale CDP-choline formation from CMP and choline was expected by use of *H. jadinii*. Although a relatively large amount of CDP-choline was produced from CMP and P-choline, a considerably smaller amount of CDP-choline was formed from CMP and choline, in spite of large amount of CDP and CTP being formed.

Differences in CDP-choline formation at high concentration of CMP between choline system and P-choline system were investigated.

The reaction mixture contained 50 to 100 mM of CMP and 200 or 400 mM of choline in the choline system, and 100 or 200 mM of P-choline in the P-choline system. The other components were the same as the standard reaction mixture.

As shown in Fig. 6, in the choline system, 22–25 mM of CDP-choline was formed from 50 and 100 mM of added CMP, while in the P-choline system, 35 and more than 70 mM respectively of CDP-choline was formed from 50 and 100 mM of CMP. The amount of CDP-choline formed in the P-choline system was twice that formed in the choline system.

The effect of the concentration of CMP and CTP on CDP-choline formation and choline phosphorylation was compared in the reaction systems.

From the results shown in Fig. 1, A and B, and Fig. 2, it was evident that the rate of CMP phosphorylation, which was calculated from the ratio of ATP consumption, was 10 to 15 times more than that of choline phosphorylation in the early stage of incubation. It was assumed that choline phosphorylation was inhibited competitively by CMP phosphorylation. The competition of CMP phosphorylation with choline phosphorylation was investigated in the reaction mixture containing CTP by the rate of CDP-choline formation.

As shown in Fig. 7, in the reaction mixture initially containing 20 mM of CTP, CDP-choline production was not affected negatively by addition of CMP, but increased

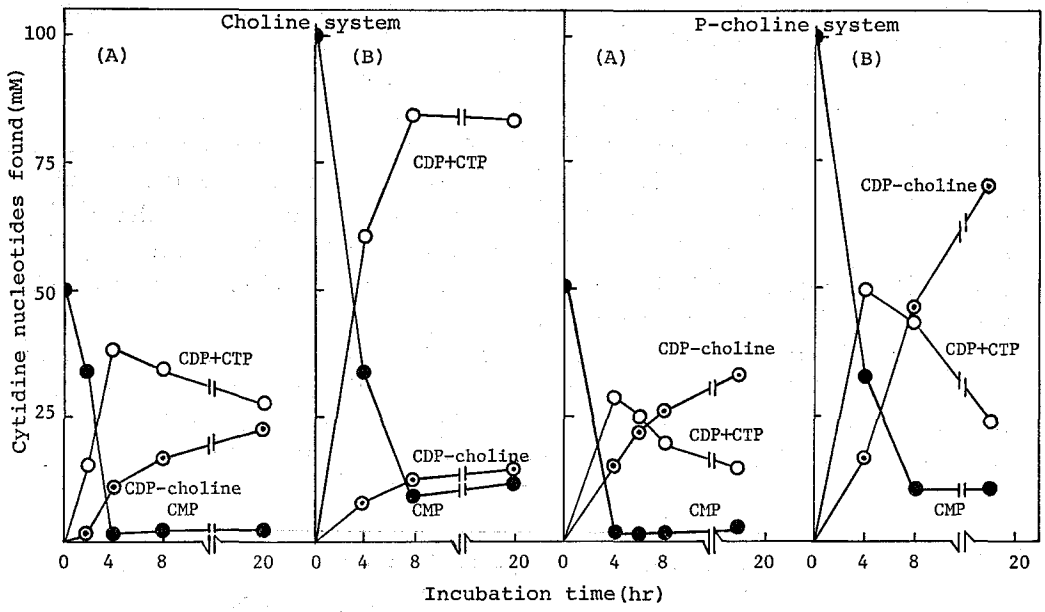


Fig. 6. Chemical changes in CDP-choline formation at high concentration of CMP.
The reaction mixture contained (mM): glucose, 1200; phosphate buffer (pH 8.0), 300 in (A) or 400 in (B); $MgSO_4$, 30; choline, 200 in choline system; P-choline in P-choline system. Dried cells, 200 mg per ml. Total volume 2.0 ml. Reaction was carried out by shaking at 28°C.

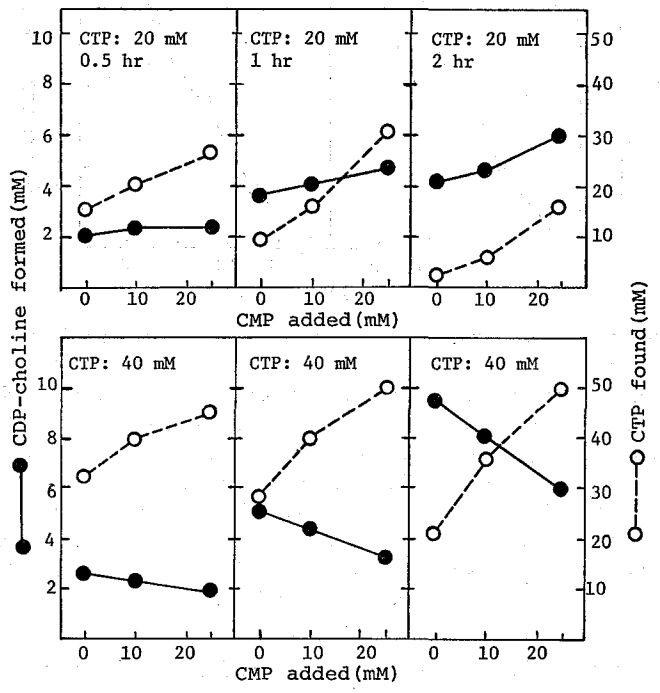


Fig. 7. Effect of supplied CMP on CDP-choline formation from CTP and choline.
The reaction mixture contained (mM): glucose, 100; phosphate buffer (pH 8.0), 300; $MgSO_4$, 30; choline, 80; CMP, indicated in the figures. Cells, 100 mg per ml. Shaken at 28°C in a total volume of 2 ml.

on further incubation, while in the CDP-choline production in the reaction mixture initially containing 40 mM of CTP, the amount of CDP-choline formed was reduced by addition of CMP at either 10 mM or 25 mM. Inhibition by added CMP was markedly observed on further incubation for 2 hr. The only difference between the reaction system containing 20 mM CTP and 40 mM CTP was the amount of CTP initially present. More than 30 mM of CTP was accumulated in the later reaction system in which CDP-choline formation was inhibited. It was thought that CDP-choline production was not inhibited by competition of CMP with choline for phosphorylation energy, but in high concentration of accumulated CTP. This hypothesis was proved by the following experiments.

The reaction mixture of Fig. 8 contained 10–80 mM of CTP, which was formed by preincubation of CMP in the reaction mixture as mentioned in the footnote of the figure. CDP-choline formation was initiated by addition of choline or P-choline, the amount of which corresponded to 4 times or twice that of CMP added.

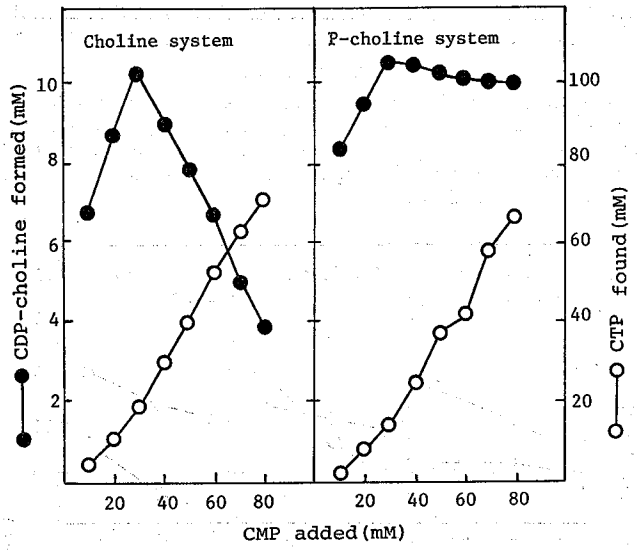


Fig. 8. Effect of prephosphorylation of CMP on CDP-choline formation from either choline or P-choline.

The reaction mixture for prephosphorylation of CMP contained (mM): glucose, 600; phosphate buffer (pH 8.0), 300; $MgSO_4$, 30; indicated amount of CMP, and 100 mg per ml of dried cells in a total volume of 1.8 ml. Prephosphorylation was carried out at 28°C for 3 hr by shaking. CDP-choline formation was initiated by addition of choline or P-choline and 100 mM of glucose. Choline added was 4 times as much as CMP in choline system and P-choline added was 2.5 times as much as CMP in P-choline system in final volume of 2.0 ml. Reaction was carried out for 4 hr (choline system) or 2 hr (P-choline system). Shaken at 28°C.

A remarkable reduction in CDP-choline formation was observed in choline system in which more than 30 mM of CTP was accumulated. The amount of CDP-choline formed from CTP in concentrations of 40 mM, 60 mM, and 80 mM was respectively 85%, 60%, and 40% of 20 mM of CTP added, while in the P-choline system, reduction

of CDP-choline formation on increasing the amount of CTP added was not observed. These results suggest that the inhibition of CDP-choline formation under high concentration of added CMP is caused by accumulation of high concentration of CTP in an early stage of incubation which may act as an inhibitor of choline phosphorylation.

Inhibition of choline kinase by CTP. The effect of high concentration of CTP on purified choline kinase activity was investigated. Choline kinase of *H. jadinii* was extracted and purified by the method of Brostrom.[6] Choline kinase was extracted from 200 g of air-dried cells which were ground with an equal weight of alumina for 3 hr with 500 ml of 10^{-2} M potassium phosphate (pH 8.0), and the extracts were fractionated by addition of ammonium sulfate. Further purification of the enzyme was performed by successive column chromatography on DEAE-cellulose and hydroxyapatite and gel filtration through Sephadex G–150.

The enzyme preparation obtained through these procedure was not a single protein, but the specific activity was elevated about 100-fold with a yield of 1.3%. A summary of the purification steps is shown in Table I.

Table I. Purification of Choline Kinase

The reaction mixture contained (mM): choline, 2.5; ATP, 5; $MgCl_2$, 10; glycylglycine (pH 8.0), 50; cysteine, 1.0; phosphoenol pyruvate, 0.76; NADH, 0.32 and pyruvate kinase, 15 units; lactate dehydrogenase, 43 units and aliquots of enzyme in a total volume of 2.0 ml. Initial velocity was assayed at 25°C.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
Cell free ext.	8560	5990	0.7*
Amm–SO_4 (30–60%) fraction	3050	6100	2
DEAE-cellulose fraction	106	2480	23
Hydroxyapatite fraction	5	240	48
Sephadex G–150 gel filtrate	1.1	79	72

* Activity was assayed by P-choline formation by paper electrophoresis.

Table II shows the inhibition of choline kinase by CTP. About 50% of activity was lost on addition of 1.8 mM of CTP.

Effect of CMP feeding on CDP-choline formation Since high concentration of CTP inhibited CDP-choline production in quantity by inhibition of choline kinase, the reaction system was modified as follows: to keep the content of CTP formed at below in the early stage of incubation, the amount of CMP added initially was modified to 20 mM, and 5 mM of CMP and 100 mM of glucose were fed successively during the incubation. The details of the reaction conditions are described in the footnote of the figures.

As shown in Fig. 9, A–II, CDP-choline formation from choline and CMP was comparable to that in the P-choline system (Fig. 9, B–II), and the amount of CDP

Table II. Inhibition of Choline Kinase by CTP

The reaction mixture contained 4 μmol/ml of choline, 10 μmol/ml of ATP, 10 μmol/ml of $MgCl_2$, 100 μmol/ml of glycine buffer (pH 8.0), the indicated concentration of CTP and 0.625 mg protein/ml of choline kinase (hydroxyapatite fraction) in a total volume of 2.0 ml.

CTP concentration $\times 10^{-3}$	Relative activity* (%)
0	100
1.8	49.5
3.6	30
6.3	10
10	10

* ADP formation was assayed by the decrease of optical density at 340 nm in a system containing PEP, NADH, PK and LDH. Each reaction was carried out at 30°C under static conditions.

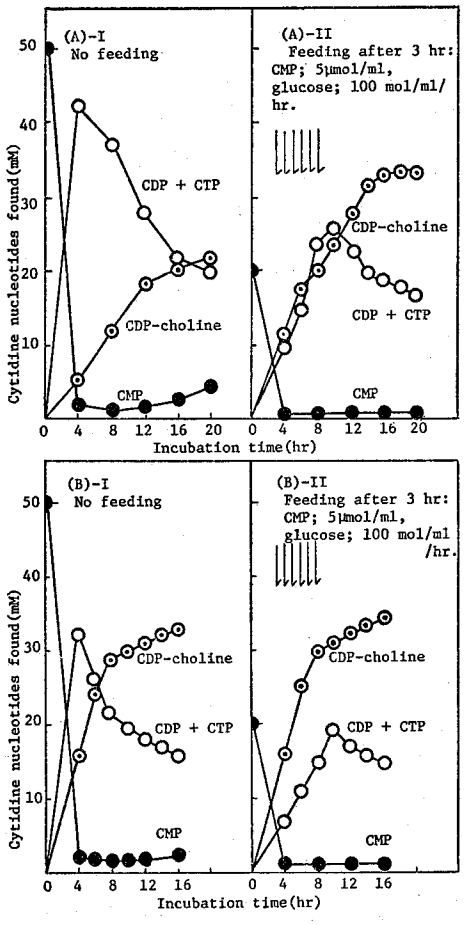


Fig. 9. Differences in chemical changes in CDP-choline formation between direct reaction and feeding of CMP and glucose.

The reaction mixture of A–I contained (mM): CMP, 50; glucose, 1200; choline, 200; phosphate buffer (pH 8.0), 300; $MgSO_4$, 30 and 100 mg per ml of dried cells in total volume of 2.0 ml. The reaction mixture of B–I was the same as that of A–I, except that choline was replaced by 100 mM of P-choline.

The reaction mixture of A–II initially contained (mM): CMP, 20; choline, 200 glu cose, 600; phosphate buffer (pH 8.0), 300; $MgSO_4$, 30 and 100 mg per ml of dried cells. After 3 hr, 5 mM of CMP and 100 mM of glucose were fed successively as indicated in the figure. Final concentration of CMP and glucose amounted to 50 and 1200mM respectively, in total volume of 2.0 ml. The reaction mixture of B–II was the same as that of A–II, except that choline was replaced by 100 mM of p-choline.

+CTP accumulated in the reaction mixture was kept at less than 30 mM. The amount of CDP-choline formed from choline by this method amounted to 33–35 mM and was about 1.5 times that formed in the system without feeding.

Effect of incubation time on CDP-choline formation Prolonged incubation to produce CDP-choline in quantity was ineffective, because the rate of CDP-choline formation decreased remarkably on prolonged incubation in both the choline and P-choline systems. The effect of preincubation time on CDP-choline formation was compared.

As shown in Fig. 10, cells were preincubated with standard reaction mixture for

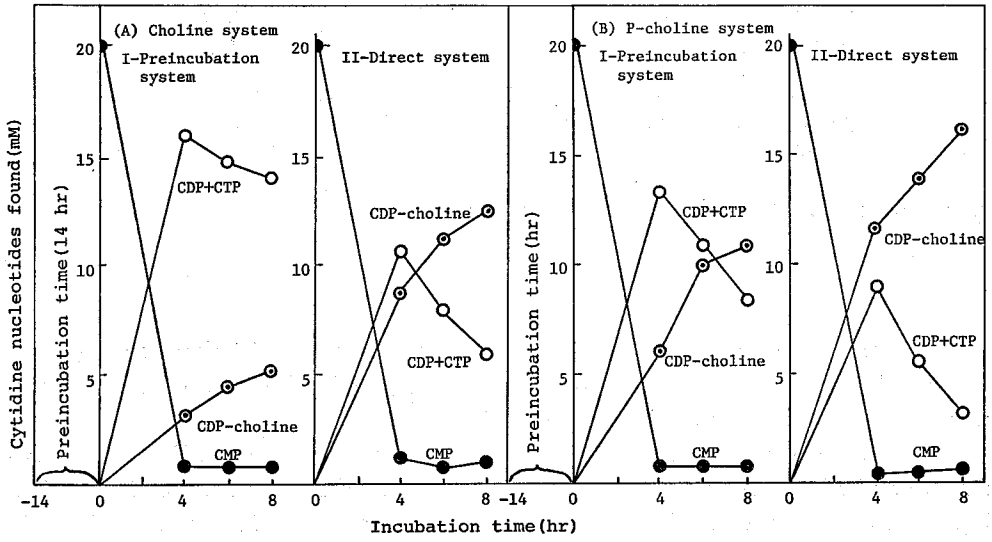


Fig. 10. Effect of preincubation on CDP-choline production from CMP and either choline or P-choline.

The reaction mixture of preincubation system (A–I, B–I) contained (mM): phosphate buffer (pH 8.0), 300; glucose, 600; Mg^{2+}, 30 and dried cells 200 mg in a total volume of 1.5 ml. After 14 hrs CMP, 20 (mM); glucose, 600 (mM); choline, 80 (mM) (A–I) or P-choline, 50 (mM) (B–I) were added in a final volume of 2.0 ml. Direct reaction system (A–II, B–II) contained (mM): CMP, 20; phosphate buffer (pH 8.0), 300; glucose, 600; Mg^{2+}, 30; choline (A–II), 80 (mM) or P-choline (B–II), 50 and 100 mg per ml of dried cells in a total volume 2.0 ml. Shaken at 28°C.

14 hr without addition of CMP and choline or P-choline. A remarkable decrease in CDP-choline formation was observed in the preincubated system. CDP-choline production after preincubation was reduced to 30% of that in the choline system without preincubation, and to about 50% of that in the direct reaction P-choline system. The rate of CMP phosphorylation was not decreased.

Physphorylation of aminoethanols Aminoethanols, N-substituted choline, were phosphorylated by choline kinase of *H. jadinii* under the condition of fermentation. Table III shows the rates of the phosphorylation of aminoethanols. More than 30 mM of P-choline, P-dimethylaminoethanol, P-monomethylaminoethanol and P-diethylaminoethanol were produced. Phosphorylation of monoethylaminoethanol was about one half that of dimethylaminoethanol, and the amount of P-ethanolamine

Table III. Phosphorylation of Aminoethanols by *H. jadinii*

	Aminoethanol phosphorylated (μ moles/ml)	
	Incubation time (hr)	
	6	12
Choline	27.0	32.0
Dimeth-laminoethanol	35.5	45.3
Monomethylaminoethanol	25.4	34.5
Ethylcholine	18.5	28.0
Diethylaminoethanol	31.5	40.0
Monoethylaminoethanol	15.4	23.3
Ethanolamine	9.1	14.6

The incubation mixture contained (mM): glucose, 600; potassium phosphate buffer (pH 8.0), 300; aminoethanol, 80; $MgSO_4$, 30 and 100 mg per ml of dried cells. Total volume 2.0 ml. Shaken at 30°C.

represented about 32% of the P-dimethylaminoethanol produced.

DISCUSSION

The effect of energy source on CDP-choline formation, on CMP phosphorylation and on the phosphorylation of choline was investigated. Important factors in formation of CDP-choline was also investigated.

Fructose 1, 6-*bis*phosphate is an excellent energy source for CDP-choline formation and phosphorylation of choline and CMP in the early stage of incubation, but glucose is effective in the further incubation. Since the amount of CDP+CTP (mainly CTP) formed was more than twice the amount of CDP-choline formed within 0.5 hr incubation in both FBP and glucose reaction systems, it was assumed that phosphorylation of CMP did not regulate the rate of CDP-choline formation in the early stage of incubation.

From the data presented in Fig. 2, it is evident that the rate of CDP-choline formation depends on the rate of choline phosphorylation rather than that of CMP phosphorylation. Fructose 1, 6-*bis*phosphate showed higher efficiency in the phosphorylation of CMP than glucose, because the rate of CMP phosphorylation at 0.5 hr incubation was about 7 times than that in the glucose system. Higher efficiency of FBP than glucose in the phosphorylation of choline was also observed.

The amount of CDP+CTP formed was 5 to 7 times more than that of P-choline formed, and the amount of ATP consumed in their phosphorylation was calculated as 10 to 15 times more than for choline phosphorylation. It can be concluded that choline phosphorylation, which was inhibited by high concentration of CTP, is one of the rate determining factors in CDP-choline formation.

It has been reported that when high concentration of CMP was added, significant amount of side product (uracil or uridine) were formed in the reaction mixture of brewer's yeast.[14] However, appreciable amounts of side products, such as uracil or uridine were not formed in the reaction mixture of *H. jadinii*. Furthermore, higher apparent activity of CMP phosphorylation was observed in *H. jadinii* than in

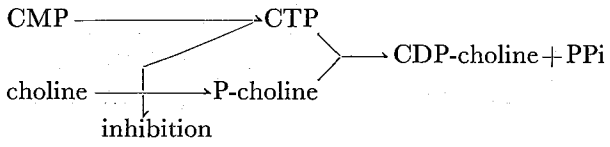
the other two yeasts.

The amount of pyrophosphate hydrolyzable by one mg dried cell of *H. jadinii* per hr was calculated as 240 μmoles. Owing to high activity of inorganic pyrophosphatase, there is no accumulation of inorganic pyrophosphate, and so the equation of CDP-choline formation of

$$\text{CTP} + \text{P-choline} \rightarrow \text{CDP-choline} + \text{PPi}$$

is thought to proceed toward CDP-choline formation. Fermentative production of CDP-choline was not so severely inhibited by addition of CMP to the reaction mixture, but was inhibited by large quantities of CTP formed in the reaction mixture in the early stage of incubation. Inhibition occurred at the step of choline phosphorylation.

Although inhibition of choline kinase by AMP,[6] P-choline and ADP[4~6] has been reported, that by CTP has not. Further experimental results on the choline kinase of *H. jadinii* will be presented in the following paper. The inhibition of choline kinase by CTP is considered to be one of the controlling factors in biosynthesis of CDP-choline.



That lower yield of CDP-choline was obtained from CMP and choline than from CMP and P-choline indicates that choline phosphorylation is an important key reaction in CDP-choline formation.

To produce high concentration of CDP-choline it is important to keep the CTP levels in the reaction mixture below 30 mM at which concentration CDP-choline formation was not negatively affected. For this purpose, a new method was developed in which small amounts of CMP and glucose were fed successively. The yield of CDP-choline by this method was 33–35 mM, which is comparable to that from P–choline. By this method P-choline could be replaced by choline chloride.

Biological activity of *H. jadinii* in producing large quantities of CDP-choline from choline and CMP was investigated. Efficiency of energy sources on the phosphorylation of choline and CMP was compared. It was evident that *H. jadinii* is more suitable for CDP-choline production than the other yeasts.

Among the aminoethanols dimethylaminoethanol was phosphorylated largely than the other aminoethanols. P-dimethylaminoethanol formed reached 1.5 times the amount of choline phosphorylated. This indicated, as also suggested by Ramasarma, that P-dimethylaminoethanol was less inhibitory toward choline kinase than was P-choline.[5]

SUMMARY

Fructose 1, 6-*bis*phosphate was more favorable energy source than glucose for the phosphorylation of both choline and CMP and for CDP-choline formation under

fermentative conditions.

In the reaction system using FBP as energy source, the yield of CDP-choline at an early stage of incubation was much higher than that of glucose, and phosphorylation rate of both CMP and choline was several times larger than in the glucose system. Under the fermentative conditions used here, phosphorylation of a small amount of CMP did not compete with the phosphorylation of choline for their energy requirement, but in the reaction mixture which contained more than 40 mM of CTP, the rate of CDP-choline formation was considerably decreased.

Hansenula jadinii exhibited high activity of phosphorylation of CMP to CTP under fermentative conditions. More than 40 mM of CMP were phosphorylated within 2 hr incubation by 100 mg per ml of the dried cells and the product CTP was stable on further incubation.

Activity of choline phosphorylation was lower than that of CMP phosphorylation. The ratio of choline phosphorylation to CMP phosphorylation was calculated as 1/10 to 1/15 on the rate of ATP consumption.

H. jadinii exhibited high inorganic pyrophosphatase activity. Two hundred and forty mM of inorganic pyrophosphates were hydrolyzed by one mg of cells per hr.

Choline phosphorylation was inhibited by high concentration of CTP, and inhibition of choline kinase by CTP was confirmed with purified choline kinase.

An improved method for CDP-choline formation in high yield from CMP and choline was investigated.

By feeding small amounts of CMP and glucose successively to the reaction mixture, the concentration of CTP accumulated in the reaction mixture could be maintained below the levels at which choline kinase was inhibited. By this method, more than 33 mM of CDP-choline was produced from the final concentration of 50 mM of CMP.

Inhibition of choline kinase by CTP was investigated by use of purified choline kinase.

Aminoethanols were phosphorylated by this yeast under the condition of glucose catabolism.

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