The Biosynthesis of a Choline Nucleotide by a Cell-free Extract from *Streptococcus pneumoniae*

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SUMMARY

Choline, a component of the wall teichoic acid of *Streptococcus pneumoniae*, was converted to cytidine diphosphocholine via choline phosphate by enzymes which were identified in cell-free extracts of the pneumococcus. The first enzyme, choline kinase, was investigated in some detail. It appeared to have a pH optimum of 7.3 to 7.4 and was stimulated by Mg^{2+} . Kinetic studies gave an apparent Michaelis constant (K_m) for ATP of 1 mM, and for choline of 0.19 mM, with V_{max} values of 3 nmol min⁻¹ (mg protein)⁻¹ and 0.5 nmol min⁻¹ (mg protein)⁻¹ respectively. The second enzyme, CDPcholine pyrophosphorylase was specific for CTP and had a requirement for Mg^{2+} with an optimum at 7 mM.

INTRODUCTION

Choline, a nutritional requirement of *Streptococcus pneumoniae*, is essential for the normal functioning of the bacterium (Rane & Subbarow, 1940; Tomasz, 1968). It is utilized as a structural component of the wall (C-) teichoic acid (Tomasz, 1967; Brundish & Baddiley, 1968) and of the pneumococcal Forssman antigen (Briles & Tomasz, 1973) but not in phospholipid biosynthesis. Structural analogues of choline, such as ethanolamine, can replace it as a growth requirement but several properties of the organism then become altered, e.g. (i) the daughter cells become unable to dissociate completely, forming chains of several hundred cells, (ii) the bacterium becomes resistant to both autolysis and detergent-induced lysis, and (iii) it is unable to undergo genetic transformation (Tomasz, 1968; Tomasz *et al.*, 1975).

This study reports the biosynthesis of cytidine diphosphocholine (CDPcholine) from choline in a cell-free system of *S. pneumoniae*. A similar pathway has been proposed in brewers' yeast which utilizes choline as a component of phosphatidylcholine (lecithin), one of the major phospholipids of this organism. This first enzyme of the pathway in yeasts, the choline kinase, has been investigated in some detail (Brostrom & Browning, 1973) and is compared here with the pneumococcal enzyme.

METHODS

Materials. [methyl-³H]Choline chloride was purchased from The Radiochemical Centre, Amersham. [methyl-³H]Choline phosphate was synthesized chemically from [methyl-³H]-choline chloride by the method of Riley (1944), except that it was purified by preparative electrophoresis on Whatman 3MM paper in buffer B instead of by precipitation and

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recrystallization of the calcium salt. Unlabelled choline chloride and CDPcholine were from Sigma, and choline phosphate from Boehringer.

Growth of organism. *Streptococcus pneumoniae* ATCC12213 was maintained on blood agar slopes at 4 °C and subcultured at monthly intervals.

For preparation of the crude enzyme, batches of the organism (15 l) were grown in a laboratory fermenter (L.H. Engineering, Stoke Poges, Berkshire) in a medium containing 450 g brain heart infusion and 35 g neopeptone (both Difco). The inoculum was 50 ml of an overnight culture. Incubation was at 37 °C with gentle stirring (1.5 rev. min⁻¹) and automatic addition of 5 M-NaOH to maintain the pH at 7.2. After 16 h, sterile glucose was added to give a final concentration of 1 % (w/v), and incubation was then continued with stirring at 3.5 rev. min⁻¹. Growth was monitored spectrophotometrically at 600 nm and cultures were harvested at an E_{600} of 2.0 in a Sorvall continuous flow centrifuge at 4 °C.

Cultures (100 ml) were also grown in the synthetic medium of Tomasz (1964) using 0.5 % (w/v) casein hydrolysate instead of individual amino acids.

Preparation of crude enzyme. Harvested bacteria were washed with 0.7 % (w/v) NaCl in 10 mM-Tris/HCl buffer, pH 7.5, and disrupted with no. 11 Ballotini beads in a Braun homogenizer in 0.9 % NaCl. Walls and unbroken cells were removed by centrifuging at 6000 g for 10 min at 4 °C. The supernatant fluid was used as the crude enzyme.

Analytical methods. The protein content of cell-free extracts was determined by the method of Lowry *et al.* (1951). Choline phosphate and CDPcholine were detected on paper by the Dragendorff spray reagent (Bregoff, Roberts & Delwiche, 1953). High voltage electrophoresis was performed in a Shandon Southern HVE apparatus model L24 (Shandon Southern Instruments, Camberley, Surrey) on Whatman no. 1 or 3MM papers using the following buffers: buffer A, 8.0 % (w/v) formic acid, pH 1.8; buffer B, pyridine/acetic acid/water (5:5:190, by vol.), pH 4.7. Descending paper chromatography on Whatman no. 1 paper was carried out in solvent C: ethanol/0.5 M-ammonium acetate, pH 3.8 (15:6, v/v).

Assay of [methyl-³H]choline phosphate. Reactions in incubation mixtures (see Results) were terminated by adding 3 vol. ethanol, mixing thoroughly and cooling in ice, and the insoluble material was removed by centrifugation. The supernatant fluid, which contained almost all of the ³H, was dried by rotary evaporation in a small tube. The dried material was redissolved in 30 µl ethanol/water (1:2, v/v) and 10 µl samples were applied to Whatman no. 1 paper. Electrophoresis in buffer A was carried out for 30 min at 45 V cm⁻¹. Areas of the paper corresponding to standard choline phosphate were cut out and assayed for radioactivity in a scintillation fluid [containing 0.4 % (w/v) 2,5-diphenyloxazole (PPO) and 0.01 % (w/v) 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene (dimethyl POPOP) in toluene] using a Packard Tri-Carb model 3385 liquid scintillation spectrometer.

Assay of [methyl-³H]CDPcholine. Incubation mixtures were treated as for the choline phosphate assay except, instead of electrophoresis, samples were chromatographed in solvent C and areas corresponding to standard CDPcholine were assayed for radioactivity.

RESULTS

Metabolites of [³H]choline in whole bacteria

The defined medium of Tomasz (1964) (100 ml, containing 10 µCi [methyl-³H]choline chloride) was inoculated with one loopful of *S. pneumoniae* and incubated statically for 16 h at 37 °C. The bacteria were then washed twice with ice-cold 0.9 % NaCl, once with distilled water and then extracted with 20 ml 5 % (w/v) trichloroacetic acid (TCA) for 30 min at 4 °C. The extract was separated by centrifuging at 10000 g for 15 min and the

Fig. 1. (a) pH profile of choline kinase. Incubation mixtures (1.0 ml) containing 0.5 ml crude enzyme (8 mg protein), 0.2 ml 80 mM-veronal/HCl buffer, and (final concentrations) 0.04 % (w/v) 2-mercaptoethanol, 10 mM- Na_2HPO_4 and 10 mM-ATP, were incubated at 37 °C with 0.1 ml 40 mM-[methyl- ^3H]choline chloride ($0.5 \mu\text{Ci } \mu\text{mol}^{-1}$). After 20 min, duplicate 0.2 ml samples were assayed for choline phosphate and the pH value of the remainder was determined. (b) Magnesium ion dependence of choline kinase. MgCl_2 was added at (final concentrations) 0, 10, 15, 30 and 50 mM to 0.25 ml of an incubation mixture similar to that in (a). The whole incubation mixture was assayed for choline phosphate.

TCA was removed by six extractions with an equal volume of ether. Finally the extract was concentrated by rotary evaporation and chromatographed as a 2×0.5 cm band on Whatman no. 1 paper in solvent C. The distribution of radioactivity on the chromatogram was investigated by cutting the paper into 1 cm strips and measuring their radioactivity in a scintillation spectrometer. Results showed that 85 % of the radioactivity co-chromatographed with an authentic choline phosphate standard, 11 % co-chromatographed with CDPcholine and 4 % remained at the origin. No free choline was observed.

Cell-free studies on the enzymes of choline metabolism

Choline kinase (EC 2.7.1.32). In a preliminary experiment, choline, ATP and the crude enzyme were incubated together in a buffered system under conditions similar to those described in Fig. 1. Samples, taken at intervals over 60 min, showed a plateau for choline phosphate formation after about 30 min. This incubation mixture was then used as a basis for determining the optimum reaction conditions for the choline kinase. The enzyme had a pH optimum of between 7.3 and 7.4 (Fig. 1a), and its activity reached a maximum when incubated with about 30 mM- Mg^{2+} (Fig. 1b).

The kinetic properties of the pneumococcal choline kinase were examined for comparison with the corresponding yeast enzyme. Under the previously determined optimum conditions, the reaction $\text{choline} \rightarrow \text{choline phosphate}$ did not proceed any further (i.e. to the choline nucleotide). The enzyme's dependence on choline concentration was therefore investigated as follows: [methyl- ^3H]choline chloride, at between 0 and 3 mM, was incubated with the crude enzyme suspension for 20 min at 37 °C with 3 mM-ATP. Choline phosphate was assayed and the enzyme activity was related to the substrate concentration by both direct and double reciprocal plots (Fig. 2a). A V_{max} of $0.5 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$ was obtained, with an apparent K_m of 0.19 mM. Using a constant choline concentration, and varying the ATP between 0 and 20 mM, the two plots of enzyme activity (Fig. 2b) showed that the substrate (ATP) strongly inhibited the enzyme at above 2.5 mM. Identical results were obtained with either 5 or 20 mM- Mg^{2+} , so the effect was not due to magnesium

Fig. 2. Kinetics of pneumococcal choline kinase. Incubation mixtures (0.25 ml) contained 125 μ l crude enzyme (2 mg protein) and (final concentrations) 40 mM-veronal/HCl buffer, pH 7.4, 0.01 % 2-mercaptoethanol, 2 mM- Na_2HPO_4 and 10 mM- MgCl_2 . (a) [*methyl*-³H]Choline chloride was varied between 0 and 3 mM with ATP constant at 3 mM. After 20 min at 37 °C, the whole mixture was assayed for choline phosphate. Results are plotted directly and as a double reciprocal plot (inset). (b) and (c) Similar incubation mixtures were used except the concentration of [*methyl*-³H]-choline chloride was kept constant at 10 mM and ATP was varied between 0 and 20 mM (Fig. 2b) and 0 and 1 mM (Fig. 2c).

Fig. 3. Magnesium ion dependence of CDPcholine pyrophosphorylase. MgCl_2 was varied between 0 and 50 mM in incubation mixtures (0.25 ml) containing 60 μl crude enzyme, 120 μl 80 mM-veronal/HCl buffer, pH 8.4, and (final concentrations) 0.01 % 2-mercaptoethanol, 2 mM- Na_2HPO_4 , 10 mM-CTP and 10 mM-[methyl- ^3H]choline phosphate (0.06 $\mu\text{Ci } \mu\text{mol}^{-1}$). After 20 min incubation at 37 °C, the whole incubation mixture was assayed for CDPcholine.

limitation. In a further experiment, in which ATP was varied between 0 and 1 mM, more accurate determinations gave $V_{\max}$ as 3 to 3.2 nmol min⁻¹ (mg protein)⁻¹ and K_m as 1 mM (Fig. 2c).

Although no steps have been taken to purify the choline kinase in this study, we found that if the crude enzyme was centrifuged at 100000 *g* for 1 h, about 90 % of the enzyme activity per unit protein remained in the supernatant fluid.

CDPcholine pyrophosphorylase (EC 2.7.7.-). The experiment summarized above indicated that a choline nucleotide, with the same R_f as CDPcholine, was one of the metabolic products of choline. A preliminary experiment showed that CDPcholine could be synthesized in a cell-free system from choline phosphate and CTP: a mixture (1.0 ml) containing 0.5 ml crude enzyme, 20 mM-veronal/HCl buffer, pH 8.4, 0.01 % 2-mercaptoethanol, 2 mM- Na_2HPO_4 , 10 mM- MgCl_2 , 10 mM-[methyl- ^3H]choline phosphate (0.06 $\mu\text{Ci } \mu\text{mol}^{-1}$) and 10 mM-CTP was incubated for 50 min at 37 °C and 0.2 ml samples were taken at intervals and assayed for CDPcholine. The level of CDPcholine was observed to increase, reaching a plateau after about 30 min incubation.

CTP was a specific requirement of this enzyme. When it was replaced in the above incubation mixture by UTP, GTP or ATP at 10 mM and, after 30 min incubation, the whole mixture was assayed for choline nucleotides by chromatography in solvent C, no radioactivity was detected in the region between the origin and choline phosphate (where a choline nucleotide would be expected to run).

Using the incubation mixture described in Fig. 3 and varying Mg^{2+} between 0 and 50 mM, the maximum activity of the CDPcholine pyrophosphorylase was found with 7 mM- Mg^{2+} (Fig. 3).

No kinetic experiments were carried out on the CDPcholine pyrophosphorylase. Preliminary experiments on the crude enzyme system indicated that the reaction was not a simple one-step reaction. A variable amount of radioactivity, which could possibly be polymeric material, was always found at the origin. However, no free choline was found indicating the absence of a phosphatase.

DISCUSSION

The pneumococcus appears unique among bacteria in having choline in its cell wall. Choline is found widely in eukaryotic organisms as part of the phospholipid phosphatidylcholine (lecithin). This phospholipid is, however, relatively uncommon among bacteria (Hagen, Goldfine & Williams, 1966). Phosphatidylcholine is synthesized by two pathways in eukaryotes (Kennedy, 1962): (i) from CDP-D-1,2-diglyceride via phosphatidyl serine and phosphatidyl ethanolamine; or (ii) from choline via choline phosphate and CDPcholine plus D-1,2-diglyceride. There is evidence that both pathways occur in yeasts (Hunter & Rose, 1971; Brostrom & Browning, 1973), while the latter seems to be predominantly found in mammalian and plant systems. The metabolism of choline in the pneumococcus appears to be similar to the first two steps in pathway (ii). Tomasz (1967) has shown that ethanolamine cannot be methylated to choline by the pneumococcus.

The kinetics of the yeast choline kinase have been investigated by Brostrom & Browning (1973). The results obtained for the pneumococcal choline kinase, however, differ to some extent; the apparent Michaelis constant (K_m) with respect to choline was $190\text{ }\mu\text{M}$ for the pneumococcus and $15\text{ }\mu\text{M}$ for the yeast, whereas K_m with respect to ATP was 1.0 mM for the pneumococcus and 0.14 mM for the yeast. The pH optima also differ, that for the yeast being 8.5 to 9.5 compared with 7.3 to 7.4 for the pneumococcus. Both enzymes require magnesium. It should be noted that the yeast enzyme was partially purified and that K_m for the pneumococcal enzyme, especially with respect to ATP concentration, will be inaccurate as ATP will be metabolized in other ways. The assay procedure for the pneumococcal enzyme was simple and perhaps more reproducible than that used for the yeast enzyme.

Possibly the uptake of choline by *S. pneumoniae* involves the action of the kinase as there is no pool of free choline inside the cell. This could, however, be due to the bacteria being in the stationary phase of growth.

We propose that CDPcholine, which is synthesized from choline phosphate, is the direct precursor of the choline which is incorporated into pneumococcal C-teichoic acid. As not every repeating unit of C-teichoic acid is substituted with choline (I. R. Poxton & J. Baddiley, unpublished results), the control of choline substitution is probably at the level of the choline kinase or the CDPcholine pyrophosphorylase, possibly in combination with the phosphorylcholine esterase described by Holtje & Tomasz (1974).

In an independent study, Bean & Tomasz have shown that choline phosphate and CDPcholine are metabolic products of choline in the pneumococcus (B. Bean & A. Tomasz, unpublished results).

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REFERENCES

- BREGOFF, H. M., ROBERTS, E. & DELWICHE, C. C. (1953). Paper chromatography of quaternary ammonium bases and related compounds. *Journal of Biological Chemistry* **205**, 565-574.
BRILES, E. B. & TOMASZ, A. (1973) Pneumococcal Forssman antigen - a choline containing lipoteichoic acid. *Journal of Biological Chemistry* **248**, 6394-6397.
BROSTROM, M. A. & BROWNING, E. T. (1973). Choline kinase from Brewers' Yeast. *Journal of Biological Chemistry* **248**, 2364-2371.
BRUNDISH, D. E. & BADDILEY, J. (1968). Pneumococcal C-substance, a ribitol teichoic acid containing choline phosphate. *Biochemical Journal* **110**, 573-582.

- HAGEN, P.-O., GOLDFINE, H. & WILLIAMS, P. J. LEB. (1966). Phospholipids of bacteria with extensive intracytoplasmic membranes. *Science* **151**, 1543-1544.
- HOLTJE, J. V. & TOMASZ, A. (1974). Teichoic acid phosphoryl-choline esterase. A novel enzyme activity in pneumococcus. *Journal of Biological Chemistry* **249**, 7032-7034.
- HUNTER, K. & ROSE, A. H. (1971). Yeast lipids and membranes. In *The Yeasts*, vol. 2, pp. 211-270. Edited by A. H. Rose and J. S. Harrison. London: Academic Press.
- KENNEDY, E. P. (1962). The metabolism and function of complex lipids. *Harvey Lectures, Series 57*, 141-171.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265-275.
- RANE, L. & SUBBAROW, Y. (1940). Nutritional requirements of the Pneumococcus. I. Growth factors for types I, II, V, VII and VIII. *Journal of Bacteriology* **40**, 695-704.
- RILEY, R. F. (1944). Metabolism of phosphoryl choline. I. Synthesis of calcium phosphorylcholine chloride containing the radioactive isotope P³². *Journal of the American Chemical Society* **66**, 512-513.
- TOMASZ, A. (1964). Studies on the competence (for genetic transformation) of *Diplococcus pneumoniae* in a synthetic medium. *Bacteriological Proceedings* **29**, 29.
- TOMASZ, A. (1967). Choline in the cell wall of a bacterium: novel type of polymer-linked choline in pneumococcus. *Science* **157**, 694-697.
- TOMASZ, A. (1968). Biological consequences of the replacement of choline by ethanolamine in the cell wall of pneumococcus. *Proceedings of the National Academy of Sciences of the United States of America* **59**, 86-93.
- TOMASZ, A., WESTPHAL, M., BRILES, E. B. & FLETCHER, P. (1975). On the physiological functions of teichoic acids. *Journal of Supramolecular Structure* **3**, 1-16.