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Choline Metabolism in Pneumococci

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Phosphorylcholine and cytidine diphosphocholine as well as two enzyme activities, a choline kinase and a cytidine diphosphocholine pyrophosphorylase, were identified in pneumococcal extracts. It is suggested that cytidine diphosphocholine may be a biosynthetic precursor of the choline moiety in the teichoic acids of pneumococcus.

Choline is a structural component of the cell wall teichoic acid (3, 12) and of a membrane teichoic acid (Forssman antigen) (2) of *Streptococcus* (*Diplococcus*) *pneumoniae*. Over 95% of all the macromolecular choline is present in these two polysaccharides (11); the phospholipids of this bacterium, however, do not seem to contain choline (12).

The physiological importance of the choline residues in the pneumococcal teichoic acids is well documented. Pneumococci have an absolute nutritional requirement for choline (12). In addition, biosynthetic replacement of the choline in teichoic acid by structural analogues such as ethanolamine causes multiple physiological defects, including aberrant cell division, inability to undergo genetic transformation, resistance to autolysis (13), and phage resistance (10). Ethanolamine-grown pneumococci are also resistant to the lytic action of penicillin and other cell wall inhibitors (14). The biochemical basis of these phenomena seem to be multiple defects in the autolytic system of ethanolamine-grown pneumococci (11, 15). The essential role of teichoic acid choline residues as ligands for the catalytic activity and regulation of the autolysin has been demonstrated (8, 9).

In an attempt to learn about the biosynthetic origin of the teichoic acid choline residues in pneumococcus, we have examined the metabolism of choline in these bacteria. Two observations are reported. First, phosphorylcholine (P-choline) and cytidine diphosphocholine (CDP-choline) were identified as the major low-molecular-weight choline-containing components in pneumococcal extracts. Second, two enzymatic activities were detected in crude extracts of pneumococci: one of the enzymes converts choline to P-choline by using adenosine 5'-triphosphate as a phosphate donor (choline kinase); the other enzyme catalyzes the reaction between P-choline and cytidine 5'-triphosphate (CTP) to yield CDP-choline (CDP-choline pyrophosphorylase). The experimental evidence for these conclusions is described below.

Experiment 1. A culture of *Diplococcus pneumoniae* strain R36A clone R6 was grown at 37°C in a synthetic medium containing 0.1 μCi (in 5 μg) of [1,2-^{14}C]choline per ml. At the midpoint of the exponential phase of growth, the whole culture was poured onto three times its volume of crushed ice and was centrifuged in the cold. The cells were suspended in one-tenth volume of cold 10% trichloroacetic acid and extracted for 15 min. After centrifugation, trichloroacetic acid was removed from the extract by repeated shaking with ethyl ether, and final traces of ether were removed from the water phase by bubbling nitrogen gas through it.

The extract was analyzed by ion-exchange chromatography. Of a total 1×10^6 cpm of radioactivity, about one-third (3×10^5 cpm) remained adsorbed when applied to a column (0.8 by 30 cm) of Dowex-1 ($HCOO^-$) (X8), representing, presumably, nucleotide-linked material. The rest of the 7×10^5 cpm of radioactive material did not adsorb to the anion exchanger or was washed off the column with water. Virtually all of this latter material was adsorbed to a column (0.8 by 15 cm) of Dowex-50 (H^+) (X8) and was subsequently eluted with 1.5 N HCl in two major peaks, the elution properties of which coincided with those of P-choline and choline (Fig. 1). Fractions representing peak A were pooled, desalted by passing through a Sephadex G-25 column, and concentrated by lyophilization. A portion of this material was hydrolyzed with 6 N HCl at 110°C in a sealed tube for 12 h. Determination of total phosphate (5) and choline (from specific radioactivity) revealed the presence of equimolar quantities of these components. A separate portion of the concentrated peak A material was treated with intestinal phosphatase (Worthington) in tris(hydroxymethyl)aminomethane (Tris) buffer at pH 8.8 for 12 h and was then rechro-

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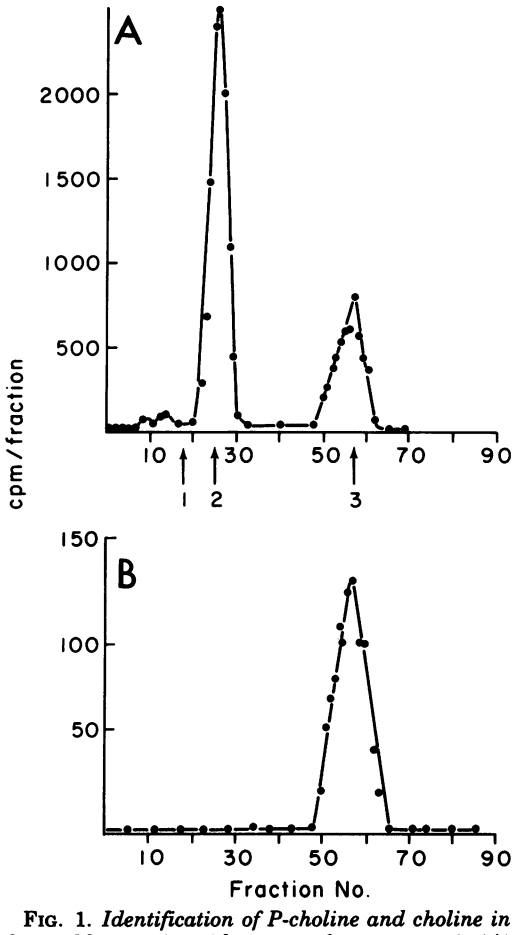

FIG. 1. *Identification of P-choline and choline in the trichloroacetic acid extract of pneumococci. (A) Trichloroacetic acid extract of [^{14}C]choline-labeled cells was preadsorbed to Dowex-1 ($HCOO^-$); material that did not adsorb to this anion-exchange column was concentrated and analyzed by cation-exchange chromatography on Dowex-50 (H^+) as shown. Arrows indicate elution positions for the standards: 1, CDP-choline; 2, P-choline; and 3, choline. (B) Material from peak A (P-choline) of (A) was rechromatographed on the same Dowex-50 (H^+) column after treatment with phosphatase. See text for details.*

matographed on a column of Dowex-50 (H^+), with 1.5 N HCl as eluent. The fractions were assayed for total and inorganic phosphate and for radioactivity. Figure 1 shows that the phosphatase treatment converted the radioactive material originally eluting at the position of P-choline to a substance eluting from the column as choline. The same treatment also liberated inorganic phosphate.

Experiment 2. This experiment was quite similar to experiment one except that the pneumococcal culture was labeled with two isotopes: [*methyl*-^{14}C]choline (0.1 μCi, 17 μg/ml) and [6-^{3}H]uracil (2 μCi, 2 μg/ml). The concentrated trichloroacetic acid extract was first analyzed by thin-layer chromatography (TLC) in three solvents. Three choline-containing substances were identified by radioautography. The R_f values corresponded to those of choline, P-choline, and CDP-choline, representing 2%, 81%, and 17%, respectively, of the total ^{14}C-labeled material in the extract (Table 1). The bulk of the concentrated trichloroacetic acid extract was then mixed with charcoal (Darco S-51; activated by washing first with concentrated formic acid and then with water and heating at 100°C) at low pH. The supernatant was decanted, and the charcoal was washed with 0.1 N formic acid. Material bound to charcoal was eluted with strongly alkaline ethanol (95% ethanol-concentrated ammonia-water; 40:2:58) and concentrated by evaporation at reduced pressure. A test with TLC revealed that all the P-choline remained unadsorbed, whereas the CDP-choline was quantitatively removed by the charcoal. The material eluted from charcoal was applied to a column (0.8 by 20 cm) of Dowex-1 ($HCOO^-$) (X8). Virtually all the ^{14}C-containing material eluted from the column as a single peak, during elution with a water-to-4 N HCOOH gradient at the position expected for CDP-choline. This peak also contained a constant ratio of ^{3}H-to-^{14}C radioactivity throughout the fractions. Analysis of this material provided further evidence that the doubly labeled substance present was [^{3}H]CDP-[^{14}C]choline. In three chromatographic solvents (TLC), the ^{14}C-containing material had R_f values identical to those of CDP-choline. Treatment with crude snake venom (*Ancistrodon halys* venom, Sigma, 80 μg; $MgCl_2$, 0.5 μM; Tris buffer, pH 8, 4 μM; total volume, 100 μl) for 3 h at 37°C converted the material to a ^{14}C-containing substance with the R_f of choline and a ^{3}H-containing substance with the R_f of cytidine. (Control experiments demonstrated powerful P-choline phosphatase activity in this venom.) Mild acid hydrolysis (1 N HCl, 100°C, 45 min) also yielded two substances; one labeled with ^{14}C migrated like P-choline, and another containing ^{3}H migrated like cytidine 5'-monophosphate. The hydrolytic products were separated by TLC in three solvent systems, and the position of the radioactive spots was determined by cutting the chromatogram into small sections and determining the radioactivity (^{3}H and ^{14}C) in a liquid scintillation spectrometer (Fig. 2).

Experiment 3. Crude cell-free extracts of pneumococci were prepared by alumina grinding in 0.05 M Tris buffer, pH 7.4 (extract of 0.5 g of wet-weight bacteria in a final volume of 5

TABLE 1. *TLC R_f values for choline compounds*

Solvent[a]	Choline	CDP-choline	P-choline
A 0.25 M Tris, pH 8, in 50% ethanol (3)[b]	0.25	0.37	0.11
B 0.2 M ethylenediaminetetraacetic acid-isobutyric acid-1 M NH_4OH (1:62:37) (4)[b]	0.38	0.22	0.11
C 0.1 M K_2HPO_4 buffer, pH 6.8, concentrated (600 g/liter) $(NH_4)_2SO_4$–*n*-propanol (50:1)	0.20	0.26	0.08
D 1 M CH_3COONH_4-95% ethanol (30:75), saturated with $Na_2B_4O_7$, adjusted to pH 10 with NH_4OH (5)[b]	0.90	0.51	0.56
E Isopropanol-concentrated NH_4OH-water (70:1:29)[b]	0.46	0.25	0.22

[a] A, B, and C, Run on Eastman Chromagram sheet no. 6060, silica gel. D and E, Run on Brinkman MN-polygram cellulose sheets.

[b] Number in parentheses indicates reference.

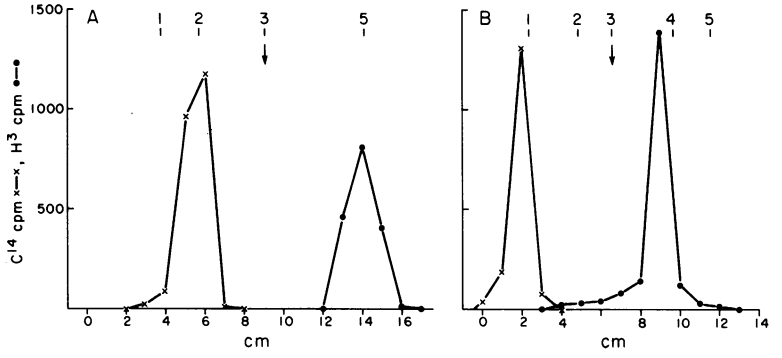

FIG. 2. *Identification of CDP-choline. The ^3H and ^{14}C double-labeled material from the anion-exchange column was hydrolyzed with snake venom (A) and with dilute HCl (B), and the reaction products were separated by TLC in solvent A (see Table 1). This solvent can clearly separate the nucleosides of uracil and cytosine from one another. Crosses show ^{14}C counts per minute and circles show ^3H counts per minute per segments (1 by 2.5 cm) of each chromatogram. Quantitative degradation of CDP-choline by these treatments is indicated by the lack of radioactivity at the position of the starting material (arrows). Positions of standards: 1, P-choline; 2, choline; 3, CDP-choline; 4, cytidine 5'-monophosphate; 5, cytidine.*

TABLE 2. *Conversion of choline to CDP-choline in vitro*[a]

Reaction	Conditions	Resulting mixture (cpm/10 μl)		
		Choline	P-choline	CDP-choline
Choline kinase	1 No enzyme	146,000	0	0
	2 No adenosine 5'-triphosphate	145,500	507	0
	3 Complete	112,400	32,110	1,490
CDP-choline pyrophosphorylase	1 No enzyme	0	68,100	0
	2 No CTP	2,080	65,770	250
	3 Complete	430	1,840	65,830

[a] Assay mixtures contained, in a total of 50 μl: Tris-hydrochloride buffer, pH 7.4, 5 μmol; $MgCl_2$, 0.2 μM; $MnCl_2$, 0.05 μM; [^{14}C]choline, 0.12 μM or [^{14}C]P-choline, 0.002 μM (containing 0.2 μCi); adenosine 5'-triphosphate or CTP, 0.5 μM; and 5 μl of enzyme. Mixtures were incubated at 37°C for 90 min and frozen. Portions of 6 to 15 μl were spotted on TLC sheets for development in appropriate solvent systems (see Table 1). Spots were precisely located by X-ray film autoradiography, cut out, and quantitated by liquid scintillation counting.

ml). Debris was removed by centrifugation, and assays for choline kinase and CDP-choline pyrophosphorylase were performed as described in the legend of Table 2. The results summarized in Table 2 demonstrate the presence of enzymes catalyzing the following reactions: (i) choline + adenosine 5'-triphosphate → P-choline, and (ii) P-choline + CTP → CDP-choline.

No attempts were made to identify further possible cofactor requirements.

While enzymes catalyzing the biosynthesis of the backbone structure of structurally simpler teichoic acids (such as polyribitol phosphate or polyglycerol phosphate) have been described (1, 4, 6, 7), nothing is known about the biosynthesis of the complex pneumococcal teichoic acids, which contain *N*-acetylgalactosamine, trideoxydiaminohexose, phosphate, and, apparently, glucose and ribitol, in addition to choline (3, 11). The presence of CDP-choline in pneumococcal extracts suggests (but does not prove) that a biosynthetic precursor of the macromolecule-linked choline may be CDP-choline.

When added in equimolar amounts to P-choline, several different triphosphates stimulated conversion to CDP-choline. However, the rate of CDP-choline formation was different with the various triphosphates; during a 90-min incubation period, the conversion of P-choline to CDP-choline was as follows (expressed as percentage of P-choline converted): 96.7 (CTP), 34 (deoxy-CTP), 20 (guanosine 5'-triphosphate), 18 (adenosine 5'-triphosphate), 14 (deoxyuridine 5'-triphosphate), 12 (with uridine 5'-triphosphate, deoxyuridine 5'-triphosphate, deoxythymidine 5'-triphosphate, deoxyguanosine 5'-triphosphate). Studies with an enzyme preparation purified of low-molecular-weight components by filtration on Sephadex G-75 showed CDP-choline synthesis that was stimulated by addition of CTP, but not by any other added nucleoside triphosphate. Thus, it seems that only CTP is the actual triphosphate substrate for this reaction, but the CTP can be generated from soluble pool compounds present in crude extracts when triphosphates other than CTP are added to the assay mixture.

In an independent effort, Poxton and Baddiley have also demonstrated the presence of CDP-choline and CDP-choline pyrophosphorylase in pneumococci (I. Poxton and J. Baddiley, submitted for publication).

LITERATURE CITED

1. **Baddiley, J., J. G. Buchanan, V. L. Rajbhandari, and A. R. Sanderson.** 1962. Teichoic acid from the walls of *Staphylococcus aureus* H. Biochem. J. 82:439.
2. **Briles, E. B., and A. Tomasz.** 1973. Pneumococcal Forssman antigen, a choline-containing lipoteichoic acid. J. Biol. Chem. 248:6394–6397.
3. **Brundish, D. E., and J. Baddiley.** 1968. Pneumococcal C-substance, a ribitol teichoic acid containing choline phosphate. Biochem. J. 110:573–582.
4. **Burger, M. M.** 1963. The enzymic glucosylation of polyglycerophosphate. Biochim. Biophys. Acta 71:495.
5. **Chen, P. S., Jr., T. Y. Toribara, and H. Warner.** 1956. Microdetermination of phosphorus. Anal. Chem. 28:1756–1758.
6. **Glaser, L.** 1964. The synthesis of teichoic acids. J. Biol. Chem. 239:3178–3186.
7. **Glaser, L.** 1973. Bacterial cell surface polysaccharides. Annu. Rev. Biochem. 42:91–112.
8. **Höltje, J. V., and A. Tomasz.** 1975. Lipoteichoic acid: a specific inhibitor of autolysin activity in pneumococcus. Proc. Natl. Acad. Sci. U.S.A. 72:1690–1694.
9. **Höltje, J. V., and A. Tomasz.** 1975. Specific recognition of choline residues in the cell wall teichoic acid by the *N*-acetyl-muramic acid-L-alanine amidase of pneumococcus. J. Biol. Chem. 250:6072–6076.
10. **McDonnell, M., C. R. Lain, and A. Tomasz.** 1975. "Diplophage," a bacteriophage of *Diplococcus pneumoniae*. Virology 63:577–582.
11. **Mosser, L. J., and A. Tomasz.** 1970. Choline-containing teichoic acid as a structural component of pneumococcal cell wall and its role in sensitivity to lysis by an autolytic enzyme. J. Biol. Chem. 245:287–298.
12. **Tomasz, A.** 1967. Choline in the cell wall of a bacterium: a novel type of polymer-linked choline in Pneumococcus. Science 157:694–697.
13. **Tomasz, A.** 1968. Biological consequences of the replacement of choline by ethanolamine in the cell wall of pneumococcus: chain formation, loss of transformability and loss of autolysis. Proc. Natl. Acad. Sci. U.S.A. 59:86–93.
14. **Tomasz, A., A. Albino, and E. Zanati.** 1970. Multiple antibiotic resistance in a bacterium with suppressed autolytic system. Nature (London) 227:138–140.
15. **Tomasz, A., and M. Westphal.** 1971. Abnormal autolytic enzyme in a pneumococcus with altered teichoic acid composition. Proc. Natl. Acad. Sci. U.S.A. 68:2627–2630.