The Uptake of Choline by *Streptococcus pneumoniae*

By A. MAVIS THOMAS,* PETER A. LAMBERT† AND
IAN R. POXTON‡

*Microbiological Chemistry Research Laboratory, The University,
Newcastle upon Tyne NE1 7RU*

(Received 17 March 1978; revised 14 June 1978)

Uptake of choline, a structural component of pneumococcal C- and F-teichoic acids, into bacteria growing in a defined medium was very efficient with an uptake constant ($[S]_{0.5}$) of $3.2 \mu\text{M}$. It was inhibited by iodoacetate, dinitrophenol and oligomycin but not by structural analogues of choline. Ethanolamine, however, was transported in the absence of choline but with a reduced affinity ($[S]_{0.5} 71.4 \mu\text{M}$). The same constitutive system was probably used by both ethanolamine and choline. It is suggested that this system required ATP and probably involved choline kinase.

INTRODUCTION

We recently demonstrated that choline, a nutritional requirement of *Streptococcus pneumoniae*, was metabolized into CDPcholine via choline phosphate by a cell-free extract of this organism (Poxton & Leak, 1977). This has also been shown independently by Bean & Tomasz (1977). Choline is utilized as a structural component of the wall teichoic acid or C-substance (Tomasz, 1967; Brundish & Baddiley, 1968) and of the membrane teichoic acid or F-antigen (Briles & Tomasz, 1973) of the pneumococcus.

We suggested that the first step in the metabolism of choline, i.e. its conversion to choline phosphate by the ATP-dependent choline kinase, may be involved in the transport mechanism for choline, as no pool of free choline was found in the cell. In the present study we have investigated this suggestion and attempt to suggest the mechanism whereby choline is taken up into the cell.

The uptake of ethanolamine, an analogue of choline, was also investigated. It is known that ethanolamine can replace choline in the pneumococcal cell envelope but that this alters some properties of the organism; for example, the daughter cells become unable to dissociate forming long chains, which are resistant to autolysis, detergent-induced lysis and infection by a bacteriophage, and also become unable to undergo genetic transformation (Tomasz *et al.*, 1975).

METHODS

Materials. [methyl- ^3H]Choline chloride and [1- ^3H]ethanolamine hydrochloride was purchased from The Radiochemical Centre, Amersham; the unlabelled compounds were from Sigma. Hemicholinium-3 was obtained from The Aldrich Chemical Co., Milwaukee, Wis., U.S.A.

Growth of organism. *Streptococcus pneumoniae* ATCC 12213 was periodically subcultured from freeze-dried stocks and maintained on blood agar plates at 4°C for up to 14 d. It was grown statically in 25 ml Universal bottles at 37°C with 20 ml filter-sterilized medium, similar to that used by Tomasz (1964) but

* Present address: Glaxo Research Ltd, Biochemistry Department, Sefton Park, Buckinghamshire.

† Present address: Glaxo Research Ltd, Microbiology Division, Greenford, Middlesex.

‡ Present address: Bacteriology Department, University Medical School, Teviot Place, Edinburgh EH8 9AG.

containing 0.5% (w/v) casein hydrolysate instead of individual amino acids. This medium contained choline at 28 μM . Cultures were routinely inoculated from plates into liquid medium, grown overnight at 37 °C, without shaking, then diluted into fresh medium to give an A_{600} of 0.05. After further incubation at 37 °C for approximately 1.5 h, A_{600} reached 0.1 (mid-exponential phase). At this density, cells were used for uptake experiments. Viable counts on blood agar plates indicated that 4.5×10^7 colony-forming units (c.f.u.) ml^{-1} were present. If ethanolamine, at the same concentration, was used instead of choline, 9.1×10^8 c.f.u. ml^{-1} were present at an A_{600} of 0.1. Total counts with a haemocytometer chamber showed that there were approximately twice as many individual cocci in ethanolamine medium as in choline medium for a given absorbance, with chains of, on average, about 30 cells in ethanolamine and 1 to 6 cells in choline.

Analytical methods. Descending paper chromatography was carried out on Whatman no. 1 paper using either ethanol/0.5 M-ammonium acetate, pH 3.8 (15:6, v/v) or ethanol/1.0 M-ammonium acetate, pH 7.5 (15:6, v/v) as the solvent. High voltage electrophoresis was performed on a Shandon electrophoresis apparatus (model L24) using Whatman no. 1 paper with either 8% (v/v) formic acid (pH 1.8) or pyridine/acetic acid/water (5:5:190, by vol.; pH 4.7) as buffer.

Detection of compounds on paper. Choline and choline phosphate were detected by the Dragendorff spray reagent (Bregoff *et al.*, 1953). Ethanolamine was detected by ninhydrin and nucleotide derivatives by ultra-violet light.

Determination of incorporation of radioactive label. Radioactivity in choline phosphate and CDPcholine from cell-free extracts was determined as previously described (Poxton & Leak, 1977). To determine radioactivity in whole cells, 1 ml of the culture was filtered through a cellulose acetate membrane (13 mm diam., 0.45 μm pore size) held in a Swinnex holder, and the membrane was air-dried for 10 min at 37 °C. Instead of washing the membrane, which would introduce inconsistent results due to cell lysis, a background reading of medium containing the same concentration of label, without cells, was subtracted. Radioactivity was measured by counting the membranes in toluene scintillation fluid [8 g 2,5-diphenyloxazole and 200 mg 1,4-di-2-(4-methyl-5-phenyloxazolyl)benzene in 2 l toluene] using a liquid scintillation spectrometer.

Determination of $[S]_{0.5}$ values. The value of $[S]_{0.5}$ is the solute concentration giving one half of the maximum rate of uptake. This constant is preferred to the Michaelis-Menten constant for the measurement of uptake by whole cells as there are many metabolic controls on the transporting proteins in the system (Rose, 1976). Lineweaver-Burk graphs were constructed to calculate $[S]_{0.5}$. In addition, linear regressions were calculated to obtain the equation of the straight line ($y = mx + c$) of the Lineweaver-Burk plots; $1/[S]_{0.5}$ is the value of x when $y = 0$.

RESULTS AND DISCUSSION

When *S. pneumoniae* was grown overnight in a medium containing [methyl- ^3H]choline chloride there was no free choline present in the intracellular pool of choline metabolites (Poxton & Leak, 1977). To confirm that no choline was free inside the bacterium, even in an actively growing culture with excess choline still present extracellularly, an overnight culture was inoculated into fresh medium with 28 μM -[^3H]choline chloride (5 μCi) to give an A_{600} of 0.05 and incubated statically until A_{600} reached 0.1 (about 1.5 h). The culture was then harvested and extracted with trichloroacetic acid as described by Poxton & Leak (1977). After removal of the trichloroacetic acid into ether, all of the ^3H was found in the aqueous phase. The chromatogram of the extract was similar to that obtained previously (except the amount of label at the origin, possibly due to polymeric teichoic acids, was much lower) with no trace of free choline. This confirmed that there was no free choline inside the cell.

In a similar experiment, bacteria were incubated in a medium containing 1% (v/v) toluene to make the membrane permeable to choline and other small molecules up to a few thousand molecular weight (Schrader & Fan, 1974). Again there was no pool of choline in the cells and no stimulation in production of choline phosphate or CDPcholine.

Choline uptake by whole cells

Preliminary experiments to find the optimum specific activity of [^3H]choline to add to the incubations indicated that choline concentrations up to 1 μM containing 0.5 $\mu\text{Ci mmol}^{-1}$ were suitable and the rate of uptake was linear over 30 min. Cells grown in the defined choline medium were harvested in mid-exponential phase (A_{600} 0.1) and resuspended in fresh medium without choline to give an A_{600} of about 0.05. After 30 min at 37 °C, when

Fig. 1. Effect of inhibitors on the uptake of choline. Mid-exponential phase bacteria were suspended in synthetic medium containing [*methyl*-³H]choline chloride (0.25 μ M, 0.125 μ Ci ml⁻¹) and the following additions were made: A, 1 mM-iodoacetate; B, 1 mM-dinitrophenol; C, no addition; D, 0.25 mM-dinitrophenol; E, 0.25 mM-oligomycin; F, no addition. At intervals, 1 ml samples were taken and assayed for uptake of choline.

any choline intermediates would have been utilized, [³H]choline was added up to 1 μ M (0.5 μ Ci ml⁻¹). After a further 30 min the incorporation of choline into whole cells was measured. Results of uptake versus substrate concentration were plotted directly and as double reciprocal plots; these gave an $[S]_{0.5}$ value of 3.2 μ M for choline and an uptake capacity ' V_{max} ' of about 3 nmol choline min⁻¹ (ml cell suspension)⁻¹.

Effect of choline analogues on choline uptake

Seven identical cultures, in choline-free medium as above, were preincubated for 30 min at 37 °C and then either hemicholinium-3, betaine hydrochloride, *N,N*-dimethylethanolamine, *N*-methylethanolamine, choline chloride (each at 5 μ M) or an equivalent volume of choline- or analogue-free medium was added. Immediately after these additions, [³H]choline chloride was added to give 0.5 μ M (0.25 μ Ci ml⁻¹). After 30 min incubation at 37 °C, three 1 ml samples were assayed for incorporated ³H. None of the analogues had inhibited the uptake of choline. Only with unlabelled choline was there a decrease of 50% due to dilution of the label.

These results indicate that there is a very specific transport mechanism for choline, with no inhibition when the analogues were present at 10 times the concentration of choline. This should be compared with the uptake of choline by a marine pseudomonad (apparently having no use for choline) which is inhibited by more than 50% in the presence of betaine hydrochloride and *N,N*-dimethylethanolamine (Snipes *et al.*, 1974).

Effect of metabolic inhibitors on the uptake of choline

Using mid-exponential phase cells harvested and suspended in choline-free medium as above, the uptake of [³H]choline at 0.25 μ M over 70 min was inhibited completely by iodoacetate (1 mM) and inhibited by 64% by dinitrophenol at 1 mM and 31% at 0.25 mM. Oligomycin (0.25 mM) inhibited uptake by only 12% (Fig. 1).

Iodoacetate is a general metabolic inhibitor which acts by alkylating thiol groups in a large number of enzymes. Oligomycin interferes with membrane ATPase and dinitrophenol reduces the proton-motive force across the cell membrane by destroying the proton gradient. The proton-motive force provides the energy for transporting certain sugars and amino acids and produces ATP in conjunction with membrane ATPase. The complete inhibition of choline uptake by iodoacetate and the comparative lack of sensitivity to dinitrophenol and oligomycin suggests that choline uptake is not dependent solely on the amount of ATP or the maintenance of the proton-motive force. We have, however, previously shown a requirement for ATP by the choline kinase (Poxton & Leak, 1977).

Fig. 2. Effect of chloramphenicol on the uptake of choline by choline-grown (A, B) and ethanolamine-grown (C, D) bacteria. Mid-exponential phase bacteria were suspended in medium free from choline and ethanolamine and 1 mM-chloramphenicol was added to tubes B and D. All tubes were incubated for 30 min before adding [*methyl*- ^3H]choline chloride ($0.25 \mu\text{M}$, $0.125 \mu\text{Ci ml}^{-1}$). At intervals, 1 ml samples were taken and assayed for uptake of choline.

Ethanolamine uptake by whole cells

We investigated the uptake of ethanolamine as it led to altered properties of the organism when it replaced choline in the growth medium (see Introduction). *Streptococcus pneumoniae* was grown in medium containing ethanolamine ($28 \mu\text{M}$) overnight at 37°C . The culture was diluted into fresh medium containing ethanolamine and harvested in mid-exponential phase. The kinetics of uptake were followed as for choline-grown organisms. The uptake constant was found to be much higher for ethanolamine than for choline ($[\text{S}]_{0.5} 7.14 \mu\text{M}$) and the uptake capacity was correspondingly lower [$V_{\text{max}} 4 \text{ nmol ethanolamine min}^{-1} (\text{ml cell suspension})^{-1}$]. This suggests that the organism has a much greater affinity for choline than for ethanolamine.

Investigation of choline-induced transport system

Bacteria were cultured in both choline-based and ethanolamine-based media and harvested in mid-exponential phase. Each culture was then resuspended and preincubated in a choline- and ethanolamine-free medium with or without 1 mM-chloramphenicol. After 30 min, [^3H]choline ($0.25 \mu\text{M}$, $0.125 \mu\text{Ci ml}^{-1}$) was added and incubation was continued for 65 min at 37°C . Throughout this period samples (1 ml) were assayed for choline uptake (Fig. 2).

There was no evidence from this experiment of induction of a choline transporting system in the ethanolamine-grown cells as the choline-grown control cells were similarly affected by chloramphenicol. The fact that chloramphenicol inhibited the uptake of choline suggests that protein synthesis is required to maintain choline uptake capacity.

As there was no evidence for induction of choline transport, and as we had earlier shown a difference in affinity for choline and ethanolamine when the organisms were grown on the homologous substrate, we investigated the change in uptake constants and capacities when the cells had been grown on one substrate and then changed over to the other substrate to follow uptake.

When the uptake of choline was assayed after bacteria had been grown on ethanolamine, $[\text{S}]_{0.5}$ was $1.4 \mu\text{M}$ -choline and V_{max} was $12 \text{ nmol choline min}^{-1} (\text{ml cell suspension})^{-1}$. For the uptake of ethanolamine by choline-grown cells, $[\text{S}]_{0.5}$ was $14.2 \mu\text{M}$ -ethanolamine and V_{max} was $6 \text{ nmol ethanolamine min}^{-1} (\text{ml cell suspension})^{-1}$. These results suggest that a maximum amount of enzyme is present in cells grown on ethanolamine thus giving an increased uptake capacity. When cells are grown on choline, however, a 'normal' amount of enzyme is present but this has a lower affinity for the uptake of ethanolamine.

Metabolism of ethanolamine

In an attempt to show the intermediates in ethanolamine metabolism, cells grown in [³H]ethanolamine were extracted with trichloroacetic acid and treated as for choline-grown cells (Poxton & Leak, 1977). Regardless of incubation time and extraction procedure, we could not detect any intermediates. We therefore conclude that intermediates were being rapidly metabolized.

We conclude from this study that choline is transported into the pneumococcus by a highly specific constitutive enzyme which may be ATP-dependent. Choline kinase is probably involved. Choline analogues are transported by the same enzyme system but with a much lower affinity.

A. M. T. thanks the Science Research Council for an Advanced Course Studentship. This work was financed by a grant from the Medical Research Council to Professor Sir James Baddiley, FRS, in whose laboratory it was carried out.

REFERENCES

- BEAN, B. & TOMASZ, A. (1977). Choline metabolism in pneumococci. *Journal of Bacteriology* **130**, 571–574.
- 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.
- BRUNDISH, D. E. & BADDILEY, J. (1968). Pneumococcal C-substance, a ribitol teichoic acid containing choline phosphate. *Biochemical Journal* **110**, 573–582.
- POXTON, I. R. & LEAK, D. J. (1977). The biosynthesis of a choline nucleotide by a cell-free extract from *Streptococcus pneumoniae*. *Journal of General Microbiology* **100**, 23–29.
- ROSE, A. H. (1976). In *Chemical Microbiology*, ch. v, pp. 159–179. London: Butterworths.
- SCHRADER, W. P. & FAN, D. P. (1974). Synthesis of cross-linked peptidoglycan attached to previously formed cell wall by toluene-treated cell walls of *Bacillus megaterium*. *Journal of Biological Chemistry* **249**, 4815–4818.
- SNIPES, W., KEITH, A. & WANDA, P. (1974). Active transport of choline by a marine pseudomonad. *Journal of Bacteriology* **120**, 197–202.
- 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., WESTPHAL, M., BRILES, E. B. & FLETCHER, P. (1975). On the physiological functions of teichoic acids. *Journal of Supramolecular Structure* **3**, 1–16.