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Production and Release of Peptidoglycan and Wall Teichoic Acid Polymers in Pneumococci Treated with Beta-Lactam Antibiotics

HELENE FISCHER AND ALEXANDER TOMASZ*
The Rockefeller University, New York, New York 10021

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Autolysin-defective pneumococci treated with inhibitory concentrations of penicillin and other beta-lactam antibiotics continued to produce non-cross-linked peptidoglycan and cell wall teichoic acid polymers, the majority of which were released into the surrounding medium. The released cell wall polymers were those synthesized by the pneumococci after the addition of the antibiotics. The peptidoglycan and wall teichoic acid chains released were not linked to one another; they could be separated by affinity chromatography on an agarose-linked phosphorylcholine-specific myeloma protein column. Omission of choline, a nutritional requirement and component of the pneumococcal teichoic acid, from the medium inhibited both teichoic acid and peptidoglycan synthesis and release. These observations are discussed in terms of plausible mechanisms for the coordination between the biosynthesis of peptidoglycan and cell wall teichoic acids.

The cell walls of *Streptococcus pneumoniae* contain two covalently linked carbohydrate polymers, peptidoglycan and teichoic acid (11). Although the structure of the pneumococcal peptidoglycan was found to resemble that of other characterized gram-positive bacteria (11), the wall teichoic acid is complex and unusual and contains choline as a structural component (13).

It is not known at what point during biosynthesis the nascent peptidoglycan and teichoic acid polymers become covalently linked to one another, forming a segregating unit that has been shown to remain intact during subsequent cell divisions of pneumococci (15). The existence of coordination between the biosyntheses of the two wall polymers has been suggested by work done with *Bacillus licheniformis* (16, 17, 18); no information is available concerning the effects of selective interference with teichoic acid metabolism on peptidoglycan synthesis.

To gain some insight into these complex questions, we have begun to develop an experimental system for pneumococci, and we are reporting here some of our findings. The availability of autolysin-defective transformants and the nutritional requirement of pneumococci for choline, a unique component of the teichoic acid, make these bacteria an ideal experimental system for such studies. The choline requirement allows selective interference with teichoic acid biosynthesis. The two observations described here are: (1) that penicillin-treated pneumococci continue to produce (and release) both peptidoglycan and wall teichoic acid polymers which are not linked covalently to one another, and (ii) that depriving the bacteria of choline inhibited both teichoic acid and peptidoglycan synthesis and release.

MATERIALS AND METHODS

Bacterial strains. *S. pneumoniae* R6 is a derivative of the Rockefeller University strain R36A. Other strains included R6ly4-4, a strain of pneumococcus with defective *N*-acetylmuramyl-L-alanine amidase (autolysin) activity, which was used in most of the experiments. This strain was constructed by introducing the *lyt*$^-$ marker of an autolysin-defective mutant into the parental (R6) strain via genetic transformation. Penicillin-resistant pneumococci were also constructed by genetic transformation with DNA from an intrinsically penicillin-resistant isolate.

Materials. M1 muramidase was a gift from K. Yokogawa, Dainippon Pharmaceuticals, Ltd., Osaka, Japan. Trypsin, egg white lysozyme, DNase, and RNase were obtained from Worthington Diagnostics, Freehold, N.J. Murein ascites containing the TEPC-15 myeloma protein was supplied via the National Cancer Institute (grant NCI-CB-25584) to M. Potter, National Institute of Allergy and Infectious Diseases, Bethesda, Md.; the myeloma protein was purified by the method of Chesebro and Metzger (4) and coupled to Sepharose 4B CNBr (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) by the method of Cuatrecasas (5). Penicillin G was obtained from Eli Lilly & Co., Indianapolis, Ind. Radiolabels L-*N*-[4,5-^{3}H]lysine, [*methyl*-^{3}H]choline, and $H_3[^{33}P]O_4$ were purchased from New England Nuclear Corp., Boston, Mass. All other chemicals (reagent grade) were purchased from commercial sources.

Production of released [^{3}H]lysine-labeled wall polymer. Cells were grown for four generations in C medium (14) supplemented with 0.1% yeast extract (Difco Laboratories, Detroit, Mich.). Upon reaching a density of 10^8 viable cells per ml, the cells were membrane filtered (0.45-μm pore diameter; Millipore Corp., Bedford, Mass.), washed, and resuspended in an equal volume of the chemically defined medium C_{den} (A. Tomasz, Bacteriol. Proc., p. 29, 1964), modified by the removal of leucine to inhibit further protein synthesis, and by the reduction of the lysine concentration from 230 to 8 μg/ml (C_{den} leu$^-$ low lys). There was no further protein synthesis after a 30-min incubation in this defined medium at 37°C, after which time the cells were again filtered, washed, and resuspended in half the original volume of fresh C_{den}-leu$^-$-low lys. Penicillin G was then added, and the cells were incubated for 15 min at 37°C before the addition of [^{3}H]lysine (3 μCi/ml; 100 Ci/mmol). Samples were removed at times indicated, and tubes were kept at 0°C, halting further incorporation of the radioactive label.

Assay of released, SDS-insoluble, and total pools of [^{3}H]lysine-labeled wall polymer. Total [^{3}H]lysine-labeled polymer was determined by addition of an equal volume of ice-cold 20% trichloroacetic acid (TCA) to each sample of the cell suspension, followed by vigorous mixing. Material was then

* Corresponding author.

allowed to precipitate at 4°C for at least 30 min. Precipitates were filtered on Whatman GF/A glass fiber filters which were then washed thoroughly with additional 10% TCA, dried, and counted in 4 ml of toluene-based scintillation fluid in a Mark II Nuclear-Chicago scintillation counter.

To determine the amount of released [^{3}H]lysine-labeled polymer, cells were first pelleted at 3,000 × *g* for 10 min, and an equal volume of ice-cold 20% TCA was added only to the supernatant fraction. Material was allowed to precipitate at 4°C for at least 30 min before filtering and counting as described above.

The amount of sodium dodecyl sulfate (SDS)-insoluble material was determined as follows. An equal volume of ice-cold 10% TCA was added to the sample of the cell suspension, followed by vigorous mixing. The extracted cells were pelleted at 3,000 × *g* for 10 min and then resuspended in 0.1 M potassium phosphate (pH 7.0) containing 2% SDS. The samples were boiled for 5 min, followed by filtration through membrane filters (Millipore Corp.) and extensive washing with water. The filters were dried and counted as described above.

Production and assay of [^{3}H]choline-labeled wall polymer. [^{3}H]choline-labeled wall polymer was produced and assayed as described above, except labeling was in C_{den} leu$^-$ low choline, modified by the reduction of the choline concentration in the medium from 5 to 1 μg/ml. Such cultures received 3 μCi of [^{3}H]choline per ml (80 Ci/mmol) 15 min after the addition of penicillin. In experiments in which proportions of [^{3}H]choline and [^{3}H]lysine-labeled polymer production from parallel experiments were compared directly, C_{den} leu$^-$ low lys low choline medium was used.

Production and assay of polymer from growing cultures. Pneumococci were grown and filtered as described above, except they were resuspended in C_{den} medium modified by the reduction of lysine (to 8 μg/ml) or choline (to 2.5 μg/ml), but fully supplemented with leucine (250 μg/ml). When we labeled with [^{3}H]choline, the assays for released, total, and SDS-insoluble pools of wall polymer were identical to those described previously. When we labeled with [^{3}H]lysine, a trypsinization step was added (to eliminate [^{3}H]lysine incorporated into protein) as follows: for released [^{3}H]lysine-labeled polymer, the supernatants were digested with 50 μg of trypsin per ml (6 h, 37°C) before precipitation in cold 10% TCA as described above; for SDS-insoluble polymer, the TCA-extracted pellet was resuspended in digestion buffer (0.01 M potassium phosphate [pH 8.0] containing 500 μM calcium chloride and 0.05% sodium azide) and digested with trypsin (50 μg/ml, 6 h, 37°C) before boiling in 2% SDS and filtering as described above.

Preparation of cell sacculi. Cells were grown and transferred to C_{den} leu$^-$ low lys as described above and labeled for 3 h with [^{3}H]lysine (3 μCi/ml) at 37°C. Cultures were then cooled on ice, and TCA was added to a final concentration of 5%. After being swirled for several seconds, the sacculi were centrifuged (3,000 rpm, 4°C) and resuspended in 0.10 M potassium phosphate (pH 7.0) containing 0.15 M sodium chloride (phosphate-buffered saline) and 2% SDS. After being boiled for 15 min, the sacculi were centrifuged and washed several times with phosphate-buffered saline. The sacculi were then resuspended in 0.10 M potassium phosphate (pH 8.0) containing 0.2 mM calcium chloride and 0.05% sodium azide; trypsin (20 μg/ml) was added, and digestion proceeded for 12 h at 37°C. The SDS extraction was repeated as described above. Walls were determined to be protein free by the method of Lowry et al. (9).

Labeling wall polymer with $H_3[^{33}P]O_4$. Cells were grown as described above for labeling with [^{3}H]lysine, except the cultures were resuspended in low phosphate (reduction of phosphate from 38 to 2 mM), and 1 M Tris [pH 8.0] was added at a concentration of 36 mM. Labeling was done with 4 μCi of $H_3[^{33}P]O_4$ per ml.

Choline deprivation. Cells were grown as described above, filtered, washed, and resuspended in an equal volume of C_{den} leu$^-$ choline low lysine (complete removal of both leucine and choline from the medium and reduction of lysine from 230 to 8 μg/ml). Cells were incubated at 37°C for 60 min, followed by filtration, washing, and resuspension in half the original volume of fresh C_{den} leu$^-$ choline$^-$ low lysine. Penicillin was added, followed by 15-min of incubation at 37°C and addition of [^{3}H]lysine (3 μCi/ml). Total and released [^{3}H]lysine-labeled polymer was assayed as described above. Subsequent additions included choline (15 μg/ml), ethanolamine (120 μg/ml), CDP-choline (120 μg/ml), and choline-phosphate (60 μg/ml). Labeling with $H_3[^{33}P]O_4$ was done in C_{den} leu$^-$ choline low phosphate (described above). Supernatants were treated with DNase and RNase (both at 50 μg/ml, 5 h, 37°C) and extracted with an equal volume of butanol before precipitation (10% TCA) and filtration.

Enzyme digestions. Preparation of released polymer for enzymatic analysis was done as described above, except that, instead of removing samples, the concentrated cell suspension was incubated with penicillin and radioactive label at 37°C for 2 h. The cells were pelleted at 3,000 × *g* for 10 min, and the supernatants were dialyzed exhaustively against distilled water at 4°C; the dialysate was then concentrated by vacuum dialysis in a Collodion Bag apparatus (Schleicher & Schuell, Keene, N.H.). Aliquots of the concentrated material (each in a final volume of 0.5 ml) were digested in the following enzyme buffer solutions: (i) 100 μg of egg white lysozyme per ml in 10 mM ammonium acetate (pH 6.5) containing 0.05% sodium azide; (ii) 40 μg of M1 muramidase per ml in 10 mM potassium phosphate (pH 7.0) containing 100 μM magnesium chloride, 100 M calcium chloride, and 0.05% sodium azide; (iii) 50 μg of trypsin per ml in 10 mM potassium phosphate (pH 8.0) containing 100 μM calcium chloride and 0.05% sodium azide.

Paper chromatography. Supernatants with labeled polymer prepared as described above were dialyzed against distilled water, concentrated to dryness, and resuspended in 75 μl of distilled water. Enzyme digests for chromatographic analysis were done in a final volume at 100 μl and applied without further concentration. Samples were applied to Whatman 3MM chromatography paper and developed for 72 h in 4:1:5 (vol/vol) butanol-acetic-acid-water (upper phase) or 5:3 (vol/vol) isobutyric acid–0.5 M ammonium hydroxide. The chromatograms were air dried, cut into 1-cm strips, and counted as described above for filters. Disaccharide-peptide monomer and dimer standards (20) were the generous gift of Thomas J. Dougherty.

Gel filtration. Samples prepared as described above were applied in a 1-ml volume with 0.6 mg of dextran blue (Pharmacia) to identical columns of Sephadex G-100 (2.5 by 72 cm), one column equilibrated with 0.15 M sodium chloride containing 0.05% sodium azide and the other with 0.15 M sodium chloride containing 0.2% SDS and 0.05% sodium azide. Samples (0.15 ml) of 1-ml fractions were counted in Ultrafluor (National Diagnostics, Somerville, N.J.) The elution pattern of dextran blue was measured spectrophotometrically at a wavelength of 600 nm.

Affinity chromatography. TEPC-15 Sepharose (5 ml) was equilibrated with 0.1 M sodium borate (pH 8.0) containing

0.15 M sodium chloride and 0.05% sodium azide (borate-buffered saline [BBS]). The sample (0.5-ml volume) was loaded, and the column was washed with 25 ml of BBS. Specific elution was performed with 2 mM choline-phosphate in BBS. Samples (0.15 ml) of 0.5-ml fractions were counted in Ultrafluor as described above. The column was regenerated by washing extensively with BBS and stored at 4°C in 0.1 M potassium phosphate (pH 7.0) containing 0.15 M sodium chloride and 0.05% sodium azide when not in use.

Determination of MICs. Duplicate series of tubes containing growth medium (C medium with 0.1% yeast extract) and drug dilutions were inoculated with 10^4 cells per ml and grown overnight at 37°C. The MIC was defined as the lowest drug concentration at which no growth was observed.

RESULTS

Cell wall synthesis in non-growing pneumococci. Inhibition of pneumococcal growth by the removal of a required amino acid (leucine) from the growth medium resulted in a rapid drop in the rates of incorporation of both peptidoglycan and teichoic acid into the cell wall (Fig. 1). Omission of leucine from the medium allowed the use of radioactive lysine as a label for peptidoglycan, and this system of nongrowing pneumococci was used in most of the experiments. Addition of penicillin to such a system resulted in a further decrease in the incorporation of both types of polymers but an increase in the total amount of polymeric (acid-precipitable) peptidoglycan and teichoic acid. Most of this excess material was released into the surrounding medium (Table 1).

Release of soluble cell wall polymers. The penicillin-induced release of cell wall polymers was examined in more detail. Pneumococci released a minimal amount of polymer, when amino acid starved alone (Fig. 2). When penicillin was added to these cultures, a significant amount of both lysine- and choline-containing polymers was released into the medium. The concentrations of penicillin needed to bring about this phenomenon were related to the MIC rather than to the absolute concentration of the drugs (Table 2). There was no decrease in culture turbidity during the period of penicillin treatment. Growing pneumococci were also capable of releasing both choline- and (trypsin-insensitive) lysine-containing polymers when exposed to penicillin, in amounts far exceeding that observed during antibiotic-free growth (data not shown). The nature of the labeled polymers released from growing cultures were not analyzed in the present study.

The penicillin-induced release of [^{3}H]lysine-labeled polymer continued for more than 2 h after protein synthesis had ceased. Cells of the autolysin-defective mutant R6ly-4-4 prelabeled with [^{3}H]lysine released virtually no labeled polymer upon subsequent penicillin treatment (0.06 μg/ml), indicating that it was the nascent polymers synthesized during the period of penicillin exposure that were released by the cells (Fig. 3). Not all of the [^{3}H]lysine-labeled material synthesized during penicillin treatment was released; about

TABLE 1. Synthesis and release of peptidoglycan from growing and nongrowing cultures in the presence and absence of penicillin G[a]

Culture	(cpm)			
	Peptidoglycan		Teichoic acid	
	Cell wall	Released	Cell wall	Released
Leucine$^+$	17,300	ND	31,700	ND
Leucine$^+$ penicillin	7,300	9,900	20,000	13,800
Leucine$^-$	2,008	ND	4,200	ND
Leucine$^-$ penicillin	1,600	4,700	3,860	3,000

[a] Nongrowing cultures were held at a turbidity of N = 300 in C_{den}-leu$^-$-low lysine-low choline medium. When exponentially growing cultures (in C_{den} leu$^-$ low lysine low choline medium) reached N = 300, label (3 μCi of [^{3}H]lysine or [^{3}H]choline per ml, respectively) was added at time 0. Cells were labeled for 10 min, at which time samples of 0.3 ml were removed and brought to 0°C to halt further incorporation. Cell wall and released polymer were determined as described in the text. Values for cell wall and released polymer represent averages of triplicate samples. ND, Not done.

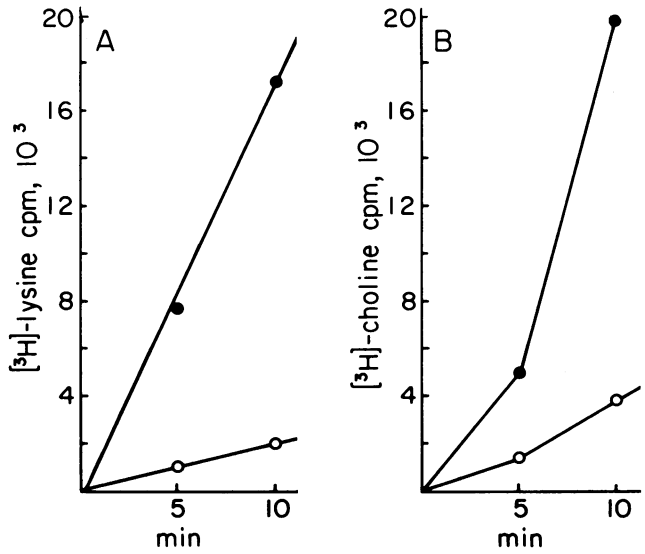

FIG. 1. Incorporation of peptidoglycan and teichoic acid into cell walls in growing (with leucine) and nongrowing (without leucine) cultures. For experimental description, see Table 1 and the text. (A) Incorporation of [^{3}H]lysine-labeled peptidoglycan in growing (●) and nongrowing (○) cultures; (B) incorporation of [^{3}H]choline-labeled teichoic acid. Points represent averages of triplicate samples.

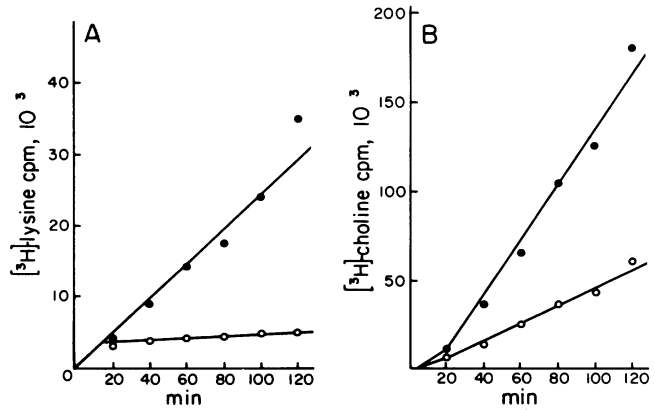

FIG. 2. Release of [^{3}H]lysine-labeled (A) and [^{3}H]choline-labeled (B) acid precipitable polymers from R6ly4-4 in the presence (●) or absence (○) of 0.06 μg of penicillin G per ml in non-growth medium. Released polymers were assayed as described in the text.

TABLE 2. Release of peptidoglycan from a penicillin-resistant strain and with other beta-lactam antibiotics[a]

Strain	Antibiotic (MIC [μg/ml])	Concn used (μg/ml)	Peptidoglycan released (cpm)	Released/total
R6ly4-4	Penicillin G (0.006)	0.0006	2,477	0.08
		0.006	5,458	0.20
		0.06	24,364	0.55
		6.0	34,718	0.76
	Ampicillin (0.025)	0.5	15,330	0.60
	Methicillin (0.80)	16.0	13,523	0.53
R6 Pen^r lyt$^-$ 4-4	Penicillin G (6.0)	0.006	2,113	0.17
		0.06	2,783	0.20
		60	8,228	0.58
		600	10,466	0.64

[a] Production and assay of released and total peptidoglycan polymer was accomplished as described in the text. In experiments with ampicillin and methicillin, these beta-lactams were used in place of penicillin. Values were determined from an average of triplicate samples.

30% remained with the cell wall fraction (insoluble in boiling 2% SDS), suggesting that it was covalently linked to the preexisting cell wall matrix (22).

The release of [^{3}H]choline-labeled polymer also continued for more than 2 h after protein synthesis had ceased. Both the wall and membrane teichoic acids of pneumococci have choline-containing carbohydrate components; the contribution of the lipid-containing membrane teichoic acid (Forssmann antigen) to the pool of released choline-labeled polymers could be assessed by gel filtration in the presence and absence of detergent (3) (Fig. 4). Although the detergent (0.2% SDS) caused a decrease in the molecular size of the Forssman antigen micelles, the elution position of the wall teichoic acid polymers remained unchanged. Bacteria prelabeled with [^{3}H]choline released only the membrane teichoic acid and no wall teichoic acid during subsequent penicillin treatment.

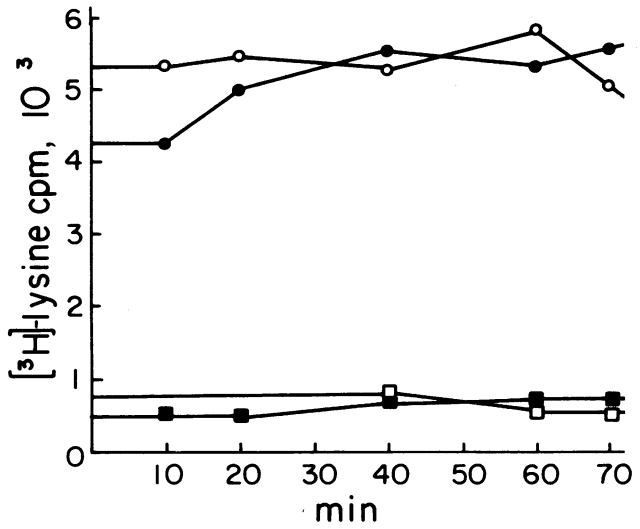

FIG. 3. Penicillin-induced release of peptidoglycan polymers from cells labeled with [^{3}H]lysine (3 μCi/ml) before the addition of antibiotic. After being labeled for 60 min in the absence of penicillin, cells were resuspended in label-free C_{den} medium. After another 60-min incubation at 37°C to chase intracellular [^{3}H]lysine into cellular polymers, penicillin (0.06 μg/ml) was added to half the culture at time 0. ○, □, No penicillin; ●, ■, 0.06 μg of penicillin per ml. Total polymers (○, ●) and released polymers (□, ■) were determined from averages of triplicate samples removed at the times indicated and assayed as described in the text.

Characterization of released polymers. The [^{3}H]lysine-labeled polymer was insensitive to treatment with mild alkali (0.1 N NaOH, 16 h, 37°C) and to trypsin (50 μg/ml, 16 h, 37°C), and only a small percentage was lost after exposure to acetic acid at pH 4 (0.1 N, 15 min, 100°C). In contrast, the polymer was digested by egg white lysozyme (100 μg/ml 16 h, 37°C) and by M1 muramidase (40 μg/ml, 16 h, 37°C; Table 3); the nature of the remaining, resistant material is unknown. Undigested polymer was chromatographically immobile in both butanol-acetic acid-water and isobutyric acid-ammonia mobile phases (data not shown). This result indicates that the precipitable [^{3}H]lysine-labeled polymer is not the bactoprenol-linked peptidoglycan precursor, despite the observed slight sensitivity to mild acid (6). Lipid-linked material would chromatograph with the solvent front in either of these solvent systems (1). M1 muramidase digestion resulted in conversion to a substance that is chromatographically mobile in both solvent systems. This information suggests that this polymer is similar to the linear, non-cross-linked peptidoglycan released from *Micrococcus luteus* (10) and *B. licheniformis* (16) upon treatment with beta-lactam antibiotics. Muramidase digestion had no effect on the chromatographic profile of [^{3}H]choline-labeled polymers; as expected, 95% of the labeled material remained at the origin both before and after enzyme treatment.

Our results suggest that the peptidoglycan and the teichoic acid polymers released from the penicillin-treated cells were not linked to one another. Digestion with M1 muramidase caused conversion of [^{3}H]lysine-labeled peptidoglycan into chromatographically mobile material; a teichoic acid chain covalently linked to the peptidoglycan backbone would have rendered the muramidase digestion product immobile in these solvent systems. More direct evidence for the lack of association between the released peptidoglycan and teichoic acid polymers was obtained by affinity chromatography on TEPC-15 Sepharose (Table 4). The [^{3}H]lysine-labeled peptidoglycan chains did not interact with the TEPC-15, a myeloma protein with a specific affinity for choline-phosphate residues (12), and they eluted with the initial buffer wash. The [^{3}H]choline-labeled teichoic acids were bound to the column quantitatively; addition of 2 mM choline-phosphate to the buffer permitted the specific elution of the bulk of the polymer. (Hydrophobic interaction between the column resin and the released teichoic acid might account for the incomplete recovery.) The TEPC-15 column did not retain any of the [^{3}H]lysine-labeled peptidoglycan material.

Attachment of nascent peptidoglycan and teichoic acid. The affinity columns of TEPC-15 were also used to analyze the effect of penicillin on the smaller portion of peptidoglycan and teichoic acid polymers (ca. 30% of the total polymers produced) that became attached to the cell wall sacculi of the nongrowing pneumococci. Bacteria in the leucine-free medium were labeled with [^{3}H]lysine in the presence or absence of penicillin (10× the MIC). Afterwards, sacculi were isolated and treated with M1 muramidase, and the digest was passed through TEPC-15 columns (Table 5).

The peptidoglycan incorporated into the cell wall in the presence of penicillin contained markedly fewer covalently

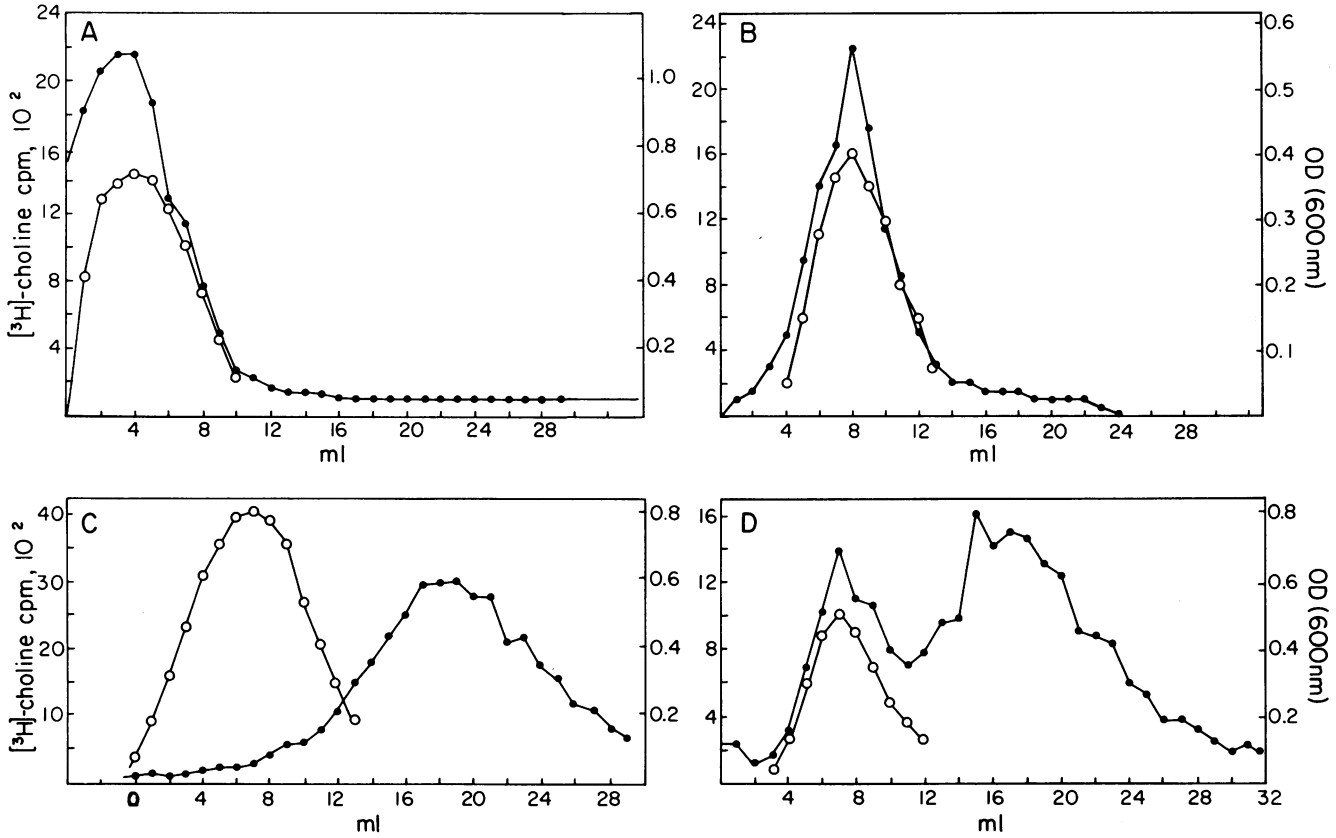

FIG. 4. G100 gel filtration (column, 2.5 by 76 cm) of [^{3}H]choline-labeled polymer released from penicillin-treated cells in absence (A and B) or presence (C and D) of 0.2% SDS in the elution buffer (0.15 M sodium chloride and 0.05% sodium azide). (A) and (C) Polymer labeled (old label) with [^{3}H]choline (3 μCi/ml) before penicillin treatment. Nongrowing bacteria were suspended at 37°C in C_{den} leucine$^-$ low choline medium containing 3 μCi of [^{3}H]choline per ml for 2 h. The cells were transferred to fresh, prewarmed, nonradioactive medium and incubated at 37°C for an additional 2 h before the addition of penicillin (0.06 μg/ml) and collection of released labeled polymers. (B) and (D) Production of [^{3}H]choline-labeled (new label) polymer during penicillin treatment was measured as described in the text. ○, Blue dextran; ●, cpm of [^{3}H]choline.

linked teichoic acid residues per disaccharide than did the control (no penicillin) cultures. In both growing and nongrowing cultures, 30% of the peptidoglycan fragments solubilized by muramidase were substituted with a choline-phosphate-containing teichoic acid.

Effect of choline starvation on peptidoglycan synthesis. The observations described so far indicate that beta-lactam inhibitors of peptidoglycan synthesis also have profound effects on the biosynthesis of the pneumococcal teichoic acids. The unique choline requirement of pneumococci allowed us to test the reverse situation: the effect of selective teichoic acid inhibition on the synthesis of peptidoglycan. When pneumococci in the basic experimental system were starved of choline for 1 h before the addition of penicillin, the synthesis and release of both the teichoic acid and the peptidoglycan polymer, the latter containing no choline, were suppressed. (In this experiment, release of teichoic acid was measured by following the incorporation of radioactive phosphate into acid-precipitable material that was resistant to nuclease treatment and extraction by lipid solvents [see Materials and Methods].) Subsequent readdition of choline permitted rapid resumption of the synthesis and release of [^{3}H]lysine-labeled peptidoglycan (Fig. 5). Addition of the choline analog ethanolamine had a similar effect. Renewed synthesis was not seen upon addition of CDP-choline or choline-phosphate, neither of which are internalized by the cell.

TABLE 3. Enzyme and chemical sensitivity of released [^{3}H]lysine-labeled polymers[a]

Treatment	10% TCA-precipitated [^{3}H]lysine-labeled polymer (cpm)
None	34,000
M1 muramidase	9,210
Egg white lysozyme	7,700
Trypsin	31,800
None	1,785
Sodium hydroxide (0.1 N)	1,804
Acetic acid, pH 4 (0.1 N)	1,200

[a] Enzyme-treated samples were incubated at 37°C for 16 h in a final volume of 0.5 ml as follows: 100 μg of egg white lysozyme per ml in 10 mM ammonium acetate buffer (pH 6.5) containing 0.05% sodium azide; 40 μg of M1 muramidase per ml in 10 mM potassium phosphate buffer (pH 7.0) containing 100 μM magnesium chloride, 100 μM calcium chloride, and 0.05% sodium azide; 50 μg of trypsin per ml in 10 mM potassium phosphate buffer (pH 8.0) containing 100 μM calcium chloride and 0.05% sodium azide. Chemical sensitivities were determined in a final volume of 1.0 ml; alkaline hydrolysis was determined by 16-h incubation at 37°C; and the acid was determined by heating to 100°C for 15 min. [^{3}H]lysine-labeled polymers were prepared as described in the text.

TABLE 4. Affinity chromatography with TEPC-15 Sepharose[a]

Sample	cpm (% recovery)					
	Expt 1		Expt 2		Expt 3	
	[^{3}H]lysine	[^{3}H]choline	[^{3}H]lysine	[^{3}H]choline	[^{3}H]lysine	[^{3}H]choline
Loaded on TEPC-15 Sepharose	20,000	34,000	20,000	17,000	60,000	60,000
Eluted with BBS	18,600 (0.93)	0	12,800	0	33,500 (0.56)	0
Eluted with 2mM choline-phosphate in BBS	0	25,000 (0.74)	0	10,000 (0.58)	0	23,200 (0.39)

[a] Preparation of the column is described in the text; final column volume was 5 ml. Samples ([^{3}H]lysine- or [^{3}H]choline-labeled released polymer) were loaded in a 0.5-ml volume and washed with 25 ml of 0.10 M sodium borate (pH 8.0) containing 0.15 M sodium chloride and 0.05% sodium azide (BBS). Choline-phosphate (2 mM in BBS) eluted specifically bound material. Numbers in parentheses represent fractional recovery.

DISCUSSION

Several reports have described the continued production (and release) of peptidoglycan polymers by penicillin-treated, gram-positive bacteria (8, 10, 16) or reverting protoplasts (7). However, in these studies, concomitant production of teichoic acid polymers was not noted and, at least in some of the studies, the possible contribution of autolytic wall degradation to the observed peptidoglycan release could not be ruled out rigorously. In the studies described here, we used an autolysin-defective pneumococcus, and the stability of radioactive precursors incorporated into the cell walls of this strain is demonstrated by the lack of release of previously incorporated peptidoglycan material during treatment of the cells with penicillin (see Fig. 3).

When treated with penicillin, the autolysin-defective strain of pneumococcus released newly formed peptidoglycan into the medium. The soluble peptidoglycan was initially acid precipitable but became acid soluble when digested with either M1 muramidase or egg white lysozyme. The chromatographic mobility of the digestion products indicated that the released peptidoglycan was not cross-linked.

In addition to releasing nascent peptidoglycan, penicillin-treated autolysin-defective pneumococci also released teichoic acids, as judged by the appearance of [^{3}H]choline-labeled polymers in the medium. Analyses showed that about half of this material was the Forssman antigen, whereas the other half represented wall teichoic acids. Both preexisting and nascent Forssman antigen polymers were released; in contrast, only the newly synthesized wall teichoic acid was released with penicillin treatment. Bertram et al. (2) have shown that protoplasts of *Bacillus subtilis* W23 can synthesize wall teichoic acid (polyribitol phosphate) from nucleoside diphosphate-linked precursors and liberate these polymers into the medium. However, production of peptidoglycan in the same system was not studied.

Evidence described in this communication indicates that peptidoglycan and wall teichoic acid polymers that were produced and released into the medium from penicillin-treated cells were not linked to one another. Penicillin and

TABLE 5. TEPC-15 Affinity chromatography of wall fragments prepared from cells labeled with [^{3}H]lysine in the presence or absence of penicillin[a]

Expt	Recovery (cpm) of cell walls prepared:			
	Without penicillin		With penicillin	
	Adherent	Nonadherent	Adherent	Nonadherent
1	6,560 (0.34)	12,700	1,050 (0.12)	7,880
2	68,000 (0.29)	165,000	8,100 (0.10)	73,400

[a] Numbers in parentheses represent ratios of adherent to total fragments. Wall sacculi were prepared by TCA extraction, followed by boiling in SDS as described in the text. Isolated sacculi were resuspended in buffer (0.01 M potassium phosphate [pH 7.0] containing 1 mM magnesium chloride, 0.2 mM calcium chloride, and 0.05% sodium azide) and digested with M1 muramidase (100 μg/ml, 16 h, 37°C). Undigested material was removed by centrifugation, and the soluble material was chromatographed on TEPC-15 agarose, as described in the text. Adherent, fragments adherent to TEPC-15; Nonadherent, fragments not adherent to TEPC-15.

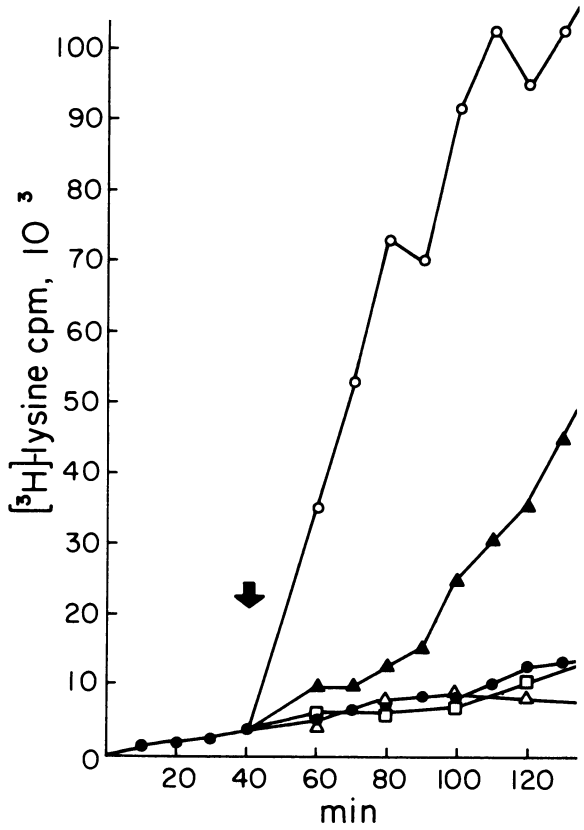

FIG. 5. Inhibition of peptidoglycan release as a result of choline deprivation. Cells were choline starved for 60 min before the addition of penicillin (0.06 μg/ml) and, 15 min later, [^{3}H]lysine (3 μCi/ml). Released peptidoglycan was assayed as described in the text. Additions made at the time indicated by arrow: choline, 15 μg/ml (○); ethanolamine, 120 μg/ml (▲); CDP-choline, 120 μg/ml (□); choline phosphate, 60 μg/ml (△). Synthesis of [^{3}H]lysine-labeled polymer covalently linked to the cell wall was similarly depressed by choline starvation (data not shown).

other beta-lactam antibiotics interfered with both the incorporation of these polymers into the cell wall sacculus and their covalent linkage to one another. Although the possibility that the attachment of teichoic acid units to peptidoglycan may occur by a penicillin-sensitive reaction cannot be ruled out at the present time, it seems more likely that the release of unlinked polymers is the consequence of inhibition of peptidoglycan cross-linking by a penicillin-sensitive transpeptidase. This suggestion is supported by the fact that the effective concentrations of beta-lactam antibiotics were related to their respective MICs. In wall membrane preparations from *B. licheniformis*, concomitant synthesis of cross-linked peptidoglycan was found to be necessary for incorporation of teichoic acid into an SDS-insoluble fraction (21). Our observations are consistent with this finding and support the suggestion that cross-linked peptidoglycan may be the substrate for the teichoic acid ligase reaction. Interestingly, the relatively small amounts of peptidoglycan attached to the cell wall sacculus during inhibition of transpeptidation (i.e., in the presence of penicillin) appeared also to be nearly free of teichoic acid chains. This finding may also be interpreted as further evidence for the need for cross-linked peptidoglycan for teichoic acid ligation to occur. A transpeptidase, with its ability to regulate simultaneously the incorporation of both peptidoglycan and teichoic acid, may function as a point of coordinate control of cell wall biosynthesis in pneumococci.

The inhibition of peptidoglycan synthesis and release by selective interference with teichoic acid synthesis (choline deprivation) represents, to our best knowledge, the first example of this type of metabolic interdependence.

The interpretation of the choline starvation experiment is not clear at the present time. Choline is a component of both the wall teichoic acid and the membrane teichoic acid (Forssmann antigen) of these bacteria, and it is not clear whether the observed effects of choline starvation are related to a perturbed membrane or wall teichoic acid metabolism or to some as yet undefined metabolic role of choline.

Previous work with *B. licheniformis* had established that bacitracin inhibited teichoic acid biosynthesis only when peptidoglycan biosynthesis was proceeding concurrently (19). Bacitracin, in blocking desphosphorylation of the undecaprenol pyrophosphate intermediate in peptidoglycan biosynthesis, prevented regeneration of the undecaprenol phosphate carrier molecule. The concomitant inhibition of teichoic acid biosynthesis implied that the undecaprenol carrier participated in the biosynthesis of both wall polymers. It is conceivable that the mechanism of peptidoglycan inhibition in the choline-deprived cells also involves the common bactoprenol carrier. A hypothetical, "incomplete" teichoic acid, lacking the choline-phosphate moiety but linked to bactoprenol, may accumulate and may "trap" the lipid carrier on the incomplete teichoic acid chains, unavailable for peptidoglycan metabolism. Experiments designed to test this hypothesis are now in progress.

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