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Characterization of Cell Wall Polymers Secreted into the Growth Medium of Lysis-Defective Pneumococci During Treatment with Penicillin and Other Inhibitors of Cell Wall Synthesis

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Autolysin-defective pneumococci secrete large quantities of choline-containing cell wall polymers into the growth medium during treatment with inhibitors of peptidoglycan synthesis. The secreted polymers were separated into three fractions by a combination of gel filtration on agarose and sodium dodecyl sulfate-gel electrophoresis. Fraction I had a high apparent molecular size and contained the Forssman antigen in complex with material exhibiting properties of cell wall teichoic acid. Choline-containing polymers of as yet uncharacterized structure were present in both fractions IIA and IIB, and fraction IIA also contained peptidoglycan components.

Autolysis-defective pneumococci secrete choline-containing cell wall components into the growth medium during treatment with penicillin and other cell wall inhibitors (24, 26). Secretion occurs in the complete absence of cellular lysis, and it is specifically induced by inhibitors of peptidoglycan synthesis only. Among the secreted cell components is an autolysin-inhibitory complex, and it has been proposed that the loss of such an inhibitor from the bacteria may be related to the triggering of autolysin activity, which plays an important role in the penicillin-induced lysis of pneumococci (24).

In an attempt to clarify the mechanism of this phenomenon, we undertook a more detailed biochemical characterization of the choline-containing macromolecules that are secreted into the growth medium of autolysin-defective pneumococci during treatment with cell wall inhibitors that are known to interfere either with a late step (benzylpenicillin) or with an early step (combination of D-cycloserine plus beta-fluoro-D-alanine) of peptidoglycan biosynthesis. As an additional variable, we examined the secretion of both biosynthetically new and old choline-containing macromolecules during the antibiotic treatments.

MATERIALS AND METHODS

Bacterial strains and growth conditions. An autolysin-defective derivative of *Streptococcus pneumoniae* strain R36A was used throughout the experiments (16). Bacteria were grown at 37°C without aeration in a modified, enriched, chemically defined medium ($Cd^{\cdot}_{en}$; A. Tomasz, Bacterial Proc., p. 29, 1964) supplemented with the dialyzable components of yeast extract (Difco) at a final concentration of 0.1% in the culture medium. Growth was measured by determination of the light scattering with a Coleman nephelometer (22).

Antibiotics. Benzylpenicillin (Squibb) was used at a concentration of 0.05 μg/ml. 3-Fluoro-2-deutero-D-alanine and D-cycloserine {D-4-[(2-oxo-3-pentene-4-yl)amino]-3-isoxazolidinone, sodium salt} were obtained from Merck Research Laboratories, Rahway, N. J. These drugs were used in combination in the following final amounts per milliliter of culture medium: 10 μg of fluoro-D-alanine plus 20 μg of D-cycloserine.

Biosynthetic labeling of the pneumococcal cell wall with radioactive isotopes. Pneumococcal cultures were labeled by growth in the $Cd^{\cdot}_{en}$ medium supplemented with the following radioactive compounds: [*methyl*-^{3}H]choline (4.22 Ci/mmol); *N*-acetyl-D-[1-^{3}H]glucosamine (5 Ci/mmol); or L-[4,5-^{3}H(N)]lysine (7.3 Ci/mmol). The amounts of the various isotopes per milliliter of culture medium were as follows: 1 μCi and 5 μg (choline); 2 μCi and 0.09 μg (*N*-acetylglucosamine); and 1 μCi and 11 μg (lysine). Before labeling with *N*-acetylglucosamine, cells were transferred into a medium containing a reduced amount of glucose (100 μg/ml). This concentration is enough to support growth during the labeling period. Two types of isotope labeling methods were used.

(i) Labeling of biosynthetically new material. Radioactive compound was added to the medium of an exponentially growing culture 10 min after the addition of antibiotics. Figure 1 illustrates the general design and typical results of such a labeling experiment. Radioactive choline was added to the pneumococcal cultures 10 min after addition of the antibiotics. At the particular doses used, neither benzylpenicillin (0.05 μg/ml) nor the combination of D-cycloserine (20

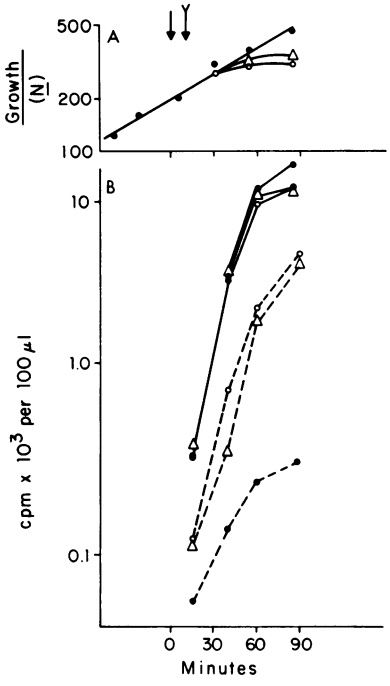

FIG. 1. *Growth, [^{3}H]choline incorporation, and release of isotope-labeled macromolecular components into the medium of Streptococcus pneumoniae during treatment with inhibitors of cell wall biosynthesis. Cells were grown in 10-ml cultures. At time zero (↓), penicillin (0.05 µg/ml) (○) or D-cycloserine (20 µg/ml) plus beta-fluoro-D-alanine (10 µg/ml) was added (△). The control culture (●) received no drug. Radioactive choline (1 µCi and 5 µg/ml) was added 10 min after drug addition (↓). (A) Cell growth, measured in light scattering units (N). (B) Incorporation of radioactive choline into macromolecular material in the whole-cell suspension (solid lines) and macromolecular material secreted into the culture medium (dashed lines).*

µg/ml) plus fluoro-D-alanine (10 µg/ml) had any appreciable effect on the rate of choline incorporation into macromolecular (i.e., cold trichloroacetic acid-precipitable) material for at least 1 h after the addition of the antibiotics. On the other hand, a very substantial portion (40 to 50% in the experiment illustrated and up to 80 to 90% in some other experiments) of the macromolecular material synthesized in the presence of the antibiotics had escaped into the growth medium.

(ii) Labeling of biosynthetically old material. Pneumococci were labeled by exponential growth in the medium containing the radioactive compound for several cell generations; next the bacteria were transferred to isotope-free growth medium for 15 to 20 min (i.e., a period of about one-fourth or one-third of a generation time) in order to deplete cellular pools of the radioactive choline. After this period, antibiotics were added to the culture.

Antibiotic treatment. Susceptibility of bacterial cultures to cell wall inhibitors is known to depend on the growth rate (15) and, in some species, even on the cell concentration (18). For these reasons, the pneumococcal cultures to be treated with antibiotics were grown under the following, carefully controlled conditions: a 100-µl portion of a bacterial stock culture (frozen, stored at −70°C in growth medium at a cell concentration of about 2×10^7 to 5×10^7 viable units/ml) was inoculated into 10 ml of $Cd^{\cdot}_{en}$ medium in an 18-mm-wide standard glass culture tube. After growth at 37°C to a cell concentration of about 1×10^8 viable units/ml, 100 µl of this culture was diluted back into 10 ml of fresh $Cd^{\cdot}_{en}$ medium prewarmed at 37°C. Growth of this culture at 37°C was monitored by a Coleman nephelometer (calibrated to give the viable titer of the bacteria), and antibiotics were added to the exponentially growing cultures at the cell concentration of 2.5×10^7 to 5×10^7 viable cells/ml.

Transfer of bacteria from one medium to another was done either by centrifugation at room temperature (in a Sorvall centrifuge at 30,000 × *g* for 1 min) or by filtration on membrane filters (0.45-µm pore size; Millipore Corp., Bedford, Mass.) in vacuo followed by resuspension of the bacteria in prewarmed growth medium.

Determination of radioactivity incorporated into macromolecular material inside the bacteria or in the growth medium. (i) Cell-associated macromolecular material. Portions (100 µl) of the isotope-labeled bacterial culture were precipitated with 4 ml of cold 10% trichloroacetic acid; after 15 to 30 min at 0°C, the precipitates were collected by filtration (in vacuo) onto filter disks (Millipore membranes, 0.45-µm pore size). The filters were dried at 100°C for 10 min and transferred to scintillation vials. Five milliliters of scintillation fluid [3 g of 2,5-diphenyloxazole plus 0.1 g of 1,4-bis-2-(5-phenyloxazol)benzene (POPOP) per liter of toluene] was added, and radioactivity was counted in a Nuclear-Chicago (model Mark II) scintillation spectrometer.

(ii) Macromolecular material secreted into the growth medium. Portions (500 µl) of the isotope-labeled cultures were centrifuged at 12,000 × *g* for 10 min at room temperature in an Eppendorf microcentrifuge (Brinkman Co.); 100-µl portions of the supernatant solution were precipitated with cold trichloroacetic acid, and the radioactivity in the precipitates was determined as described above.

Preparation of crude secreted material. After 90 min of antibiotic treatment, cells were removed by centrifugation; the supernatant was dialyzed in the cold against distilled water and lyophilized. Samples were kept at −20°C, and before further fractionation procedures were redissolved in distilled water.

Agarose column chromatography. Crude secreted material (maximum amount used per column was about 4 to 5 mg of dry material) was put through an agarose column (Bio-Gel A5-m; 0.9 by 55 cm) and eluted with 0.05 M tris(hydroxymethyl)aminomethane (Tris)-maleate buffer, pH 6.9, at 4°C. Fractions of 0.85 ml were collected, and portions were assayed for radioactivity using 5 ml of Ready-Solv scintillation fluid (Beckman).

Sucrose density gradient centrifugation. Redissolved crude secreted material was layered on top of a linear sucrose density gradient (5 to 25% [wt/vol]

sucrose in 0.9% NaCl solution) with a 0.3-ml cushion of 60% sucrose at the bottom of the nitrocellulose tubes. Gradients were centrifuged in an SW50.1 rotor at 35,000 rpm for 16 h (Spinco model L3-50 ultracentrifuge). Fractions were collected after puncturing the bottom of the tube, and radioactivity was determined after adding small portions (50 to 100 μl) of the fractions to mixtures of 0.5 ml of water plus 5 ml of Ready-Solv scintillation liquid (Beckman).

SDS-gel electrophoresis. Electrophoresis was carried out in 10% acrylamide gels in the presence of sodium dodecyl sulfate (SDS) according to the method of Weber and Osborn (28). Gels were sliced into 1.2-mm slices, solubilized with 0.5 ml of 90% (vol/vol) NCS tissue solubilizer in water (Amersham/Searle) at 50°C for 2 h, and counted after addition of 10 ml of toluene scintillation fluid.

Autolysin-inhibitory activity. Partially purified pneumococcal *N*-acetylmuramyl-L-alanine amidase was incubated in 0.2 ml of 0.05 M Tris-maleate buffer (pH 6.9) with [^{3}H]choline-labeled cell wall substrate (200 μg and 10^6 cpm/ml) and with small portions of the secreted material, and the activity of amidase was determined as described previously (14).

Phenol extraction of crude secreted material. Radioactive secreted material was dissolved in water (up to 5 mg/ml), added to an equal volume of phenol, and stirred in the cold for 30 min. Phase separation was achieved by centrifugation and radioactivity was determined in both phases.

Chloroform extraction of crude secreted material. Crude secreted material, dissolved in distilled water (up to 5 mg/ml), was added to an equal volume of chloroform. The sample was shaken at room temperature for 1 h, the water phase was removed, and the chloroform phase was washed twice with water. The combined aqueous phases were lyophilized and kept at −20°C before further fractionation.

Precipitation with anti-polysaccharide C antiserum. Antiserum against the polysaccharide C of pneumococcal cell wall was obtained from Emil Gotschlich of this university. Secreted macromolecules (isolated from 2 ml of medium of cells containing radioactive choline in biosynthetically new material) were separated into the two radioactive fractions by gel filtration through an agarose column; the combined peaks were lyophilized and resuspended in 200 μl of distilled water. The assay (11) was carried out in a total volume of 50 μl, containing fraction I (4×10^2 cpm in 10 μl), fraction II (2.2×10^3 cpm in 20 μl), or purified [^{3}H]choline-labeled Forssman antigen (5×10^3 cpm in 5 μl); 25 μl of phosphate-buffered saline (PBS) (0.02 M sodium phosphate in 0.15 M NaCl); and a final concentration of antiserum diluted $1{:}10^{-5}$, $1{:}2.5 \times 10^{-3}$, or $1{:}5 \times 10^{-3}$ in PBS. Each test was done in duplicate. After incubation at 37°C for 30 min, samples were further incubated at 4°C for 40 h. After centrifugation for 5 min in an Eppendorf centrifuge, sediments were washed twice with PBS, and the radioactivity of the combined supernatants (as well as the resuspended sediments) was determined in Ready-Solv scintillation fluid.

Labeling of pneumococci with radioactive *N*-acetylglucosamine. Attempts were made to introduce isotope labels other than choline into the macromolecules secreted during inhibition of cell wall synthesis. Ten-milliliter cultures of pneumococci were grown in the modified, enriched, chemically defined ($Cd^{\bullet}_{en}$) medium containing radioactive *N*-acetylglucosamine (2 μCi and 0.09 μg/ml). To increase the incorporation of the radioactive *N*-acetylglucosamine, the concentration of glucose in the medium was also changed, from the normal 2 mg/ml to 100 μg/ml.

The bacteria were collected by centrifugation and washed with nonradioactive medium to remove extracellular isotope, and the whole-cell pellet was hydrolyzed with 6 N HCl in sealed tubes at 100°C for 20 h. The hydrolysates were concentrated to dryness in vacuo, and the residues were taken up in a few microliters of water and analyzed by two methods: paper chromatography and ion-exchange chromatography.

Paper chromatography was performed on sheets of Whatmann 3MM paper, using the solvent *n*-butanol–ethanol–water (10:1:2) in a descending system for 96 h. The paper was dried and cut into 0.5-cm pieces, and the positions of radioactive components were determined by scintillation counting. The three radioactive components detected had R_f values corresponding to those of glucosamine (and galactosamine) (41%), muramic acid (37%), and an unidentified substance (possibly a degradation product of 2,4,6-trideoxy-2,4-diaminohexose) (22%) (4).

Since galactosamine and glucosamine were only poorly separated by this system, the combined hexosamine material was also analyzed on a Dowex 50 column (Bio-Rad; Dowex AG 50W-X8, hydrogen form, 200/400 mesh; 0.9 by 46.5 cm; eluted with 0.3 N HCl) by the method of Gardell (9). On the column, two major radioactive components were identified as glucosamine (30%) and galactosamine (53%). An additional, unknown radioactive compound (13%) was also detected. About 2% of the total radioactivity incorporated by normally growing cells could be extracted with chloroform-methanol and was identified as glycolipid. In a few experiments, pneumococci were grown in the $Cd^{\bullet}_{en}$ medium modified by lowering the lysine and/or phenylalanine concentrations (to 5 μg/ml) so as to allow biosynthetic labeling with the radioactive amino acids ([^{3}H]lysine or [^{3}H]phenylalanine; 5 μCi per ml of medium).

RESULTS

Biochemical nature of choline-containing macromolecules secreted into the growth medium during antibiotic treatment. Sucrose density gradient centrifugation or gel filtration on agarose columns resolved the choline-containing polymers into two major fractions (Fig. 2 to 4). Qualitatively similar results were obtained with either benzylpenicillin or the combination of D-cycloserine plus beta-fluoro-D-alanine, irrespective of whether biosynthetically new or old material had been labeled with the radioactive choline.

Fractions I and II, isolated from a sucrose gradient, were dialyzed and concentrated (by lyophilization), and portions of each were applied to an agarose column. All the radioactive

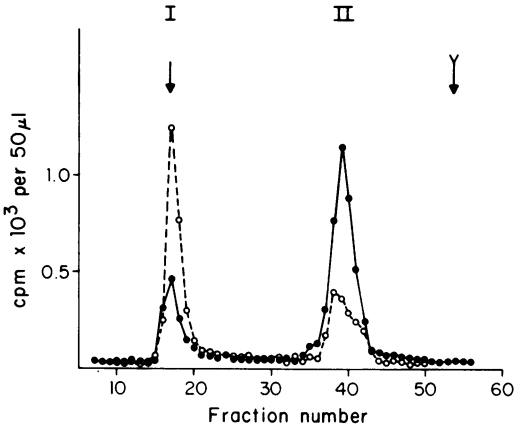

FIG. 2. *Agarose column chromatography of the crude, choline-labeled material secreted during penicillin treatment of pneumococci. A 10-ml culture was treated with benzylpenicillin in the presence of [³H]-choline as described in the legend to Fig. 1. After 90 min of drug treatment, the bacteria were removed by centrifugation and the supernatant was used as the source of crude secreted material, containing biosynthetically new isotope label (see Fig. 1). Another culture was grown in [³H]choline (1 µCi and 5 µg/ml) containing medium for several generations. The cells were transferred to fresh, isotope-free medium before the addition of penicillin. After 90 min of drug treatment, the supernatant was used for the isolation of crude secreted material, which in this case contained the isotope label in biosynthetically old material (see Materials and Methods). 100-µl portions of both preparations containing 4 × 10⁵ to 10 × 10⁵ cpm were passed through a Bio-Gel A5-m agarose column in 0.05 M Tris-maleate buffer, pH 6.9, as eluant, as described in Materials and Methods. Fractions (0.85 ml) were collected, and 50-µl portions were assayed for radioactivity. Two major radioactive fractions (I and II) were observed. Arrows indicate the elution of blue dextran (↓) and salt (Y). Biosynthetically new label (solid lines and solid circles); biosynthetically old label (dashed lines and open circles).*

material from the fraction I of the sucrose gradient eluted as fraction I (i.e., in the exclusion volume) of the agarose column, while material from fraction II of the sucrose gradient eluted from the agarose column at the position corresponding to the second agarose fraction (not shown).

The choline-containing macromolecules secreted into the medium were also analyzed by electrophoresis in SDS-gels. Two electrophoretic components were detected (A and B in Fig. 4). The position of B corresponds to the mobility of pneumococcal F-antigen.

In further experiments, the electrophoretic mobilities of the sucrose gradient (and agarose) fractions were tested: material from fraction I

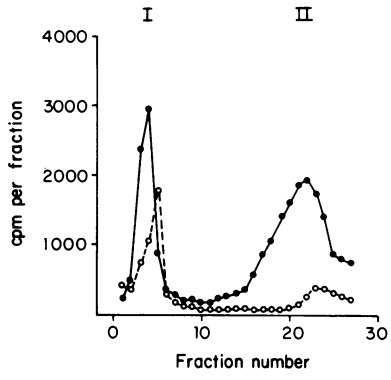

FIG. 3. *Sucrose density gradient analysis of the crude, choline-labeled material secreted during penicillin treatment. Biosynthetically new and old [³H] choline-labeled material was prepared from supernatants of penicillin-treated pneumococci as described in the legend to Fig. 2 and in Materials and Methods. The crude material was layered on top of a linear sucrose gradient (5 to 25% sucrose [wt/wt] in 0.15 M NaCl solution), and centrifugation was carried out in an SW50 rotor (for details, see Materials and Methods). Fractions were collected from the bottom of the tube and assayed for radioactivity. The two major radioactive fractions are represented by I and II. New label (solid lines and solid circles); old label (dashed lines and open circles).*

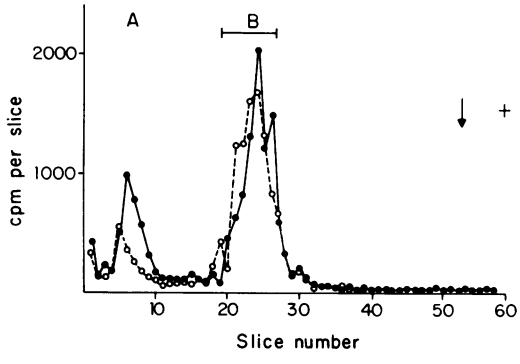

FIG. 4. *SDS-polyacrylamide gel electrophoresis of crude choline-labeled material secreted during penicillin treatment. Biosynthetically new and old [³H] choline-labeled material was prepared from supernatants of penicillin-treated pneumococci as described in the legend to Fig. 2. Electrophoresis of the crude secreted material was carried out according to the method of Weber and Osborn (28). Gels were sliced and assayed for radioactivity as described in Materials and Methods. Two major electrophoretic components were detected (A and B). Region B corresponds to the electrophoretic mobility of the pneumococcal Forssman antigen; the arrow indicates the position of the tracking dye. New label (solid lines and solid circles); old label (dashed lines and open circles); position of the anode (+).*

migrated exclusively as component B; material from fraction II gave a mixture of components A (60% of material) and B (about 40% of material) (Fig. 5).

We concluded that the choline-containing macromolecules may be separated into three fractions by the methods applied. Fraction I behaves as material with high molecular size both in sucrose gradients and in agarose chromatography. Fraction I migrates as component B in SDS-gel electrophoresis. Fraction II behaves as lower-molecular-weight material in sucrose gradients, and it may be further resolved by SDS-gel electrophoresis into fractions IIA and IIB. Addition of SDS to the gradients made all the radioactive material move to the top of the gradient. This behavior is reminiscent of the properties of F-antigen, which also tends to form micellar aggregates.

Quantitative aspects of secretion. The chemical composition and the absolute amounts of the material secreted from the bacteria are not known at the present time. The distribution of material into biochemical fractions was evaluated on the basis of radioactivity. The relative amounts of choline-labeled macromolecules belonging to fractions I, IIA, and IIB have varied considerably, depending on the nature of antibiotic used and also as a function of the biosynthetic age of the isotope label.

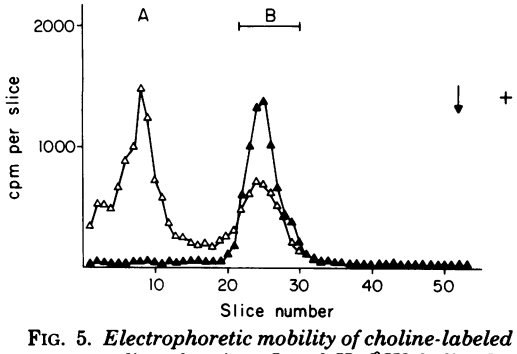

FIG. 5. *Electrophoretic mobility of choline-labeled sucrose gradient fractions I and II. [^{3}H]choline-labeled, biosynthetically new material secreted during treatment with penicillin was prepared and centrifuged in a sucrose density gradient as described in the legend of Fig. 3. The two major fractions (I and II) were isolated and dialyzed against water. After lyophilization, the fractions were dissolved in water. SDS-acrylamide gel electrophoresis was carried out according to the method of Weber and Osborn (28). Gels were sliced and radioactivity was counted as described in Materials and Methods. The graph indicates that fraction I migrated as a single electrophoretic component B (solid line and solid triangles). Fraction II was resolved into two electrophoretic components, A and B (solid lines and open triangles). The arrow indicates the position of the tracking dye.*

(i) Penicillin-induced secretion. The majority (70%) of the biosynthetically old macromolecules behaved as fraction I, and virtually all of this material migrated as component B during SDS-gel electrophoresis.

In contrast, the majority of biosynthetically new macromolecules (70%) belonged to fraction II; about half of this material belonged to fraction IIA (i.e., migrated in SDS-gel electrophoresis as component A), and the other half behaved as fraction IIB.

(ii) Secretion induced by treatment with D-cycloserine plus beta-fluoro-D-alanine. The majority of both biosynthetically old and new macromolecules (60 to 70%) secreted belonged to fraction I (Fig. 6) and migrated as component B in electrophoresis. About 10% of the secreted material belonged to fraction IIA, and about 20 to 30% belonged to fraction IIB (Fig. 7).

A summary of these data is presented in Table 1. Low amounts of choline-containing macromolecules were also detected in the growth media of control cultures (i.e., in the absence of antibiotic treatment). Such molecules belonged exclusively to peak II-type material (not shown).

Secretion of peptidoglycan-containing macromolecules. While choline incorporation may be used as a specific means of monitoring pneumococcal teichoic acid synthesis (21, 26), no satisfactory selective labeling method is avail-

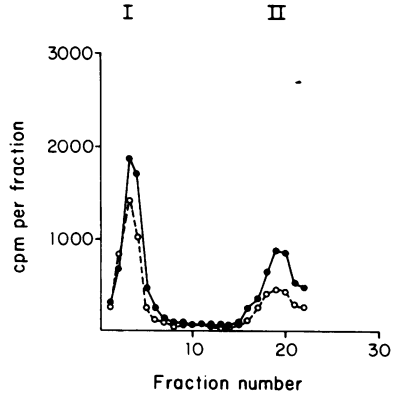

FIG. 6. *Sucrose density gradient analysis of choline-labeled material, secreted during treatment with beta-fluoro-D-alanine plus D-cycloserine. Crude secreted material (biosynthetically new and old [^{3}H] choline label) was isolated from the supernatants of pneumococcal cultures treated for 90 min with beta-fluoro-D-alanine plus D-cycloserine and centrifuged in a linear sucrose gradient (5 to 25% [wt/wt] sucrose in 0.15 M NaCl solution). For details, see Materials and Methods. New label (solid lines and solid circles); old label (dashed lines and open circles). Two major radioactive fractions are indicated by I and II.*

able at the present time for the measurement of peptidoglycan synthesis in this bacterium. After a number of preliminary experiments, we tested the release of radioactive macromolecules during penicillin treatment of pneumococci grown in the presence of radioactive *N*-acetylglucosamine. This compound seems to be utilized under the growth conditions used, mostly for polysaccharide (teichoic acid and peptidoglycan) synthesis; an additional small portion (about 5%) of the isotope incorporates into glycolipid material (3).

Figure 8 shows the rapid and extensive secretion of *N*-acetylglucosamine-labeled macromolecules synthesized during penicillin treatment of pneumococci with 40 to 60% of the newly made polymers exported to the medium. Over 90% of these macromolecules eluted from the agarose A5-m column as a broad fraction overlapping with the elution volume of fraction II of the choline-labeled macromolecules; the rest of the radioactive material was excluded from the gel (Fig. 9). Upon SDS-gel electrophoresis, the majority of labeled molecules (70%) migrated as component A; about 25% migrated as component B, and a few percent (2 to 4%) ran with the tracking dye. This latter component was soluble in lipid solvents (Fig. 10).

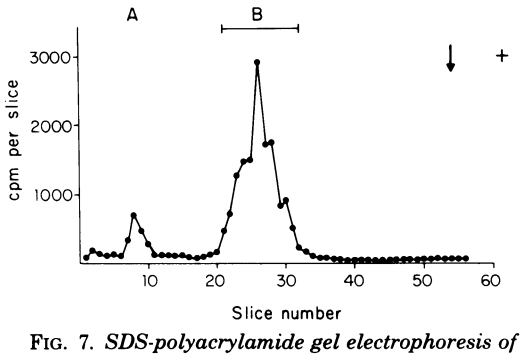

FIG. 7. *SDS-polyacrylamide gel electrophoresis of choline-labeled material secreted during treatment with beta-fluoro-D-alanine plus D-cycloserine. Biosynthetically new [^{3}H]choline-labeled material secreted during 90 min of treatment of pneumococci with the antibiotics was isolated (see Materials and Methods). The two major electrophoretic components are indicated by A and B. After electrophoresis of the crude material, gels were sliced and radioactivity was counted as described in the legend to Fig. 4. The arrow indicates the position of the tracking dye.*

A much smaller percentage (15%) of the *N*-acetylglucosamine-labeled macromolecules were secreted into the medium during treatment of the pneumococci with D-cycloserine plus 3-fluoro-D-alanine. These findings suggest that peptidoglycan material is also present among the material secreted during antibiotic treatment. Because of the difficulties with labeling peptidoglycan in pneumococci, no quantitative estimate of the amounts of peptidoglycan in the medium could be made.

This conclusion was supported by the results of lysine-labeling experiments also. Penicillin treatment induced the secretion of a significant portion (about 29%) of the newly synthesized lysine-labeled macromolecules into the medium (Table 2). (Under comparable conditions, only about 7% of phenylalanine-labeled [protein] macromolecules were found in the growth medium.) Similar to the case of the *N*-acetylglucosamine-labeled macromolecules, the lysine-labeled macromolecules synthesized and secreted during penicillin treatment were made up exclusively of fraction II-type material that migrated as component A during SDS-gel electrophoresis.

Further characterizations of choline-labeled fractions I and II. Larger quantities of fractions I and II were prepared from the supernatants of several liters of penicillin-treated (and radioactive choline-labeled) pneumococci after the removal of lipids by chloroform extraction. Fractions I and II of an agarose chromatography

TABLE 1. *Secretion of choline-labeled macromolecules during treatment with cell wall inhibitors*

Antibiotic used[a]	Biosynthetic age of label[b]	Distribution of the secreted macromolecular choline into fractions[c] (% of total secreted material)		
		I	IIA	IIB
Benzylpenicillin	Old	70	15	15
	New	30	35	35
D-Cycloserine + beta-fluoro-D-alanine	Old	70	5	25
	New	70	10	20

[a] Antibiotic treatment with benzylpenicillin (0.05 μg/ml) and the combination of D-cycloserine (20 μg/ml) plus beta-fluoro-D-alanine (10 μg/ml) was as described in Material and Methods.

[b] Isotope labeling of biosynthetically old or new cellular polymers was done by the procedures described in Materials and Methods.

[c] Fractions I, IIA, and IIB were separated by a combination of agarose gel filtration and SDS-gel electrophoresis (Materials and Methods). The results of several experiments are summarized.

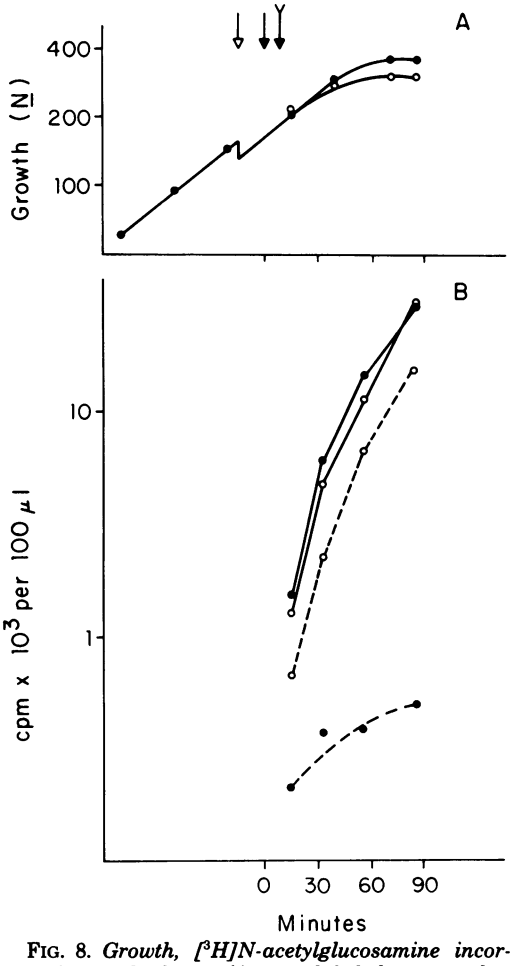

FIG. 8. *Growth, [^{3}H]N-acetylglucosamine incorporation, and release of isotope-labeled macromolecular components into the medium of Streptococcus pneumoniae during treatment with benzylpenicillin. A 20-ml culture was grown up to a cell titer of 3 × 10^7 cells per ml. After centrifugation, the bacteria were resuspended in 20 ml of medium containing only 100 µg of glucose per ml (⇩), and the culture was divided into two 10-ml portions. After 15 min (↓), benzylpenicillin was added to one culture (0.05 µg/ml; ○); the control culture (●) remained without antibiotic. [^{3}H]N-acetylglucosamine (2 µCi and 0.05 µg/ml) was added 10 min after drug addition (⇩). (A) Cell growth, measured in light scattering units (N). (B) Incorporation of [^{3}H]N-acetylglucosamine into trichloroacetic acid-precipitable material in the whole-cell suspension (solid lines) and labeled macromolecular material released into the culture medium (dashed lines).*

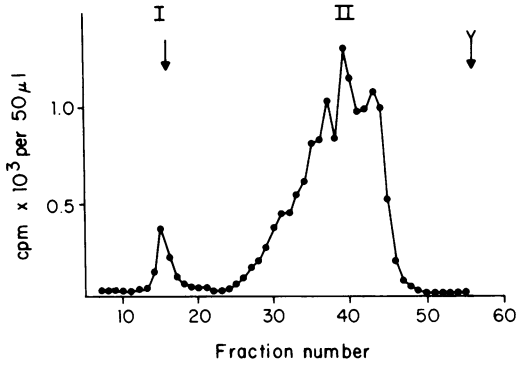

FIG. 9. *Agarose column chromatography of the crude N-acetylglucosamine-labeled material secreted during penicillin treatment. A 10-ml culture was treated with benzylpenicillin in the presence of [^{3}H]N-acetylglucosamine as described in the legend to Fig. 8. After 90 min of drug treatment, the crude secreted material (new label) was isolated. A 50-µl volume containing 3 × 10^6 cpm was passed through a Bio-Gel A5-m agarose column and eluted in 0.05 M Tris-maleate buffer, pH 6.9. Aliquots (50 µl) of the fractions (0.85 ml) were assayed for radioactivity. Arrows indicate the elution of blue dextran (↓) and salt (⇩).*

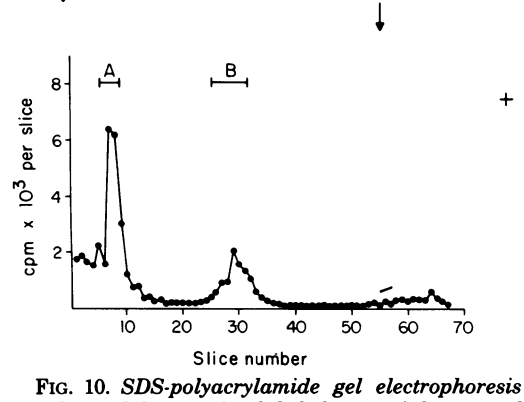

FIG. 10. *SDS-polyacrylamide gel electrophoresis of N-acetylglucosamine-labeled material secreted during treatment with benzylpenicillin. Crude secreted material, labeled with [^{3}H]N-acetylglucosamine as described in the legend to Fig. 8, was isolated from the medium of pneumococcal cultures treated with penicillin. Electrophoresis and assay for radioactivity were carried out as described in Fig. 4. Two major components are detectable (A and B). The arrow indicates the position of the tracking dye.*

were pooled, dialyzed (or desalted on a Sephadex G10 column), and lyophilized; this material was used for several types of assays (Table 3). Details of the assays are described in Materials and Methods. As a comparison, the corresponding properties of purified pneumococcal Forssman antigen are also included in the table. Fraction I showed several properties similar to those of purified Forssman antigen (2, 10). They both behaved in a similar manner during sucrose density gradient centrifugation, in elution from agarose columns, and in SDS-gel electrophoresis. Also, both fraction I and the Forssman antigen had autolysin-inhibitory activity (13), and both

TABLE 2. *Secretion of lysine- and N-acetylglucosamine-labeled macromolecules during treatment with cell wall inhibitors*

Isotope label[a]	Drug	Concn (μg/ml)	Macromolecular material in the medium[a] (% of total incorporated)
Lysine, new label	None		3.5
	Benzylpenicillin	0.06	29.2
	D-Cycloserine	100	5.5
N-acetylglucosamine, new label	None		1.7
	Benzylpenicillin	0.06	48.0

[a] [*U*-^{3}H]lysine (5 μCi and 10 μg per ml of medium) and [1-^{3}H]*N*-acetylglucosamine (10 μCi and 5 μg per ml of medium) were used for the biosynthetic labeling of newly made macromolecular material, which was quantitated by the procedure described in Materials and Methods.

TABLE 3. *Some properties of the macromolecules secreted during treatment with penicillin*

Determination[a]	Purified Forssman antigen[b]	Fraction[c]		
		I	IIA	IIB
Percent of material precipitable with cold trichloroacetic acid	35	60–70	(15)	
Percent soluble in phenol	100	100	100	100
Percent precipitable with antiserum against polysaccharide C	1.4	18	(1.3)	
Autolysin-inhibitory activity	+	+	0	0
Alternative complement pathway activation (%)[d]	<2	63	(2)	

[a] Details of the procedures used to determine precipitability in trichloroacetic acid, solubility in phenol, reactivity to antiserum and autolysin-inhibitory activity are described in Materials and Methods.

[b] Prepared and purified as described in reference 13.

[c] Fractions I, IIA, and IIB were prepared as described in Materials and Methods.

[d] Testing of the fractions' ability to activate the alternative complement pathway was performed in G. Winkelstein's laboratory (Johns Hopkins University School of Medicine, Baltimore, Md.) according to procedures described in reference 29.

were soluble in phenol. On the other hand, fraction I differed from the purified Forssman antigen in a set of other properties. In contrast to the Forssman antigen, a substantial portion of the fraction I-associated radioactivity (18%) was precipitated by antiserum against polysaccharide C (i.e., the pneumococcal cell wall teichoic acid) (11); a large portion (60 to 70%) of fraction I was precipitable by cold trichloroacetic acid, in contrast to the more limited (35%) precipitability of Forssman antigen. On a dry-weight basis, fraction I material was found to be a powerful activator of the alternative complement pathway. Recent studies have shown that pneumococcal cell wall can activate the alternative complement pathway (29). In addition, the activity has been found to reside in the wall teichoic acid; purified Forssman antigen had a low specific activity in this assay (J. A. Winkelstein and A. Tomasz, submitted for publication).

Our interpretation of these data is that fraction I contains both Forssman antigen and newly made cell wall teichoic acids in some type of a complex.

The properties of fraction II (Table 3) are distinct from those of fraction I in two respects: while this material is also soluble in phenol, it has a molecular size lower than that of fraction I on agarose columns and in sucrose density gradients; and it may be resolved into two subfractions (IIA and IIB) by SDS-gel electrophoresis. The material in fraction II has a low degree (15%) of precipitability in cold trichloroacetic acid; it is poorly precipitated by antiserum against polysaccharide C; it has no autolysin-inhibitory activity; and it is a poor activator of complement via the alternative pathway. The biosynthetic labeling experiments with radioactive lysine and *N*-acetylglucosamine indicate that most of the secreted peptidoglycan material is present in fraction IIA.

DISCUSSION

Although the chemical characterization of the secreted polymers is preliminary and incomplete, the presence of several types of cell wall polymers in the medium of antibiotic-treated pneumococci is already evident. The detection of glucosamine, muramic acid, and lysine in fraction IIA indicates the presence of peptidoglycan material. Continued production of peptidoglycan polymers, and their secretion into the

growth medium, during treatment with penicillin has already been reported in several species of bacteria (5, 20, 25, 27), and our observations extend the validity of these findings to pneumococci. On the other hand, the penicillin-induced secretion of wall teichoic acids, described here and in the accompanying communication (26), seems to be a novel phenomenon. In fact, the absence of glycerol-labeled (teichoic acid) material from the culture medium of penicillin-treated *Bacillus licheniformis* has been briefly noted in the literature (5). It is conceivable that some bacterial species have a regulatory mechanism that shuts off teichoic acid biosynthesis when peptidoglycan synthesis is disturbed. In contrast, pneumococci continue producing both of the major cell wall polymers during penicillin treatment. The presence of wall teichoic acids in the culture medium of penicillin-treated pneumococci is evident from the rate and extent of secretion of choline-containing polymers synthesized in the presence of penicillin (26). In addition, a significant portion of the radioactive choline-containing macromolecules can be precipitated with antiserum against wall teichoic acids (polysaccharide C).

Interestingly, biosynthesis of choline-containing polymers seems to continue at the normal rate not only during penicillin treatment but also in pneumococci treated with D-cycloserine plus beta-fluoro-D-alanine, i.e., in the absence of peptidoglycan biosynthesis. The newly made choline-containing polymers produced under these conditions are secreted into the medium in total amounts comparable to those observed for penicillin-treated cells. This observation suggests that the physiological coordination between the biosyntheses of pneumococcal peptidoglycan and wall teichoic acids (23) is restricted to a relatively late reaction: the covalent attachment of teichoic acid chains to peptidoglycan oligomers.

In accordance with current models on the mechanism of action of penicillin and cell wall assembly (1, 8, 12, 19), we propose that in pneumococci, as in other bacteria, penicillin inhibits a transpeptidase that is responsible for the incorporation of murein precursors into the preexisting cell wall. Under these conditions, both newly made peptidoglycan and newly made cell wall teichoic acid polymers accumulate and, instead of attaching to the cell wall structure, are secreted from the cells into the growth medium.

We propose that the three classes of polymers (fractions I, IIA, and IIB) are related to terminal stages in the biosynthesis of cell walls. Even in their present, still heterogeneous form, these fractions are strikingly different from one another in several properties, and the relative amounts of radioactive polymers in fractions I, IIA, and IIB show wide fluctuations, depending on the design of the labeling procedure. In experiments in which the bacteria were treated with penicillin and the radioactive choline label was in biosynthetically old material, most of the secreted macromolecules accumulated as fraction I. In contrast, most of the biosynthetically new material (i.e., polymers made in the presence of the antibiotic) was made up of macromolecules that belonged to fractions IIA and IIB.

All these observations suggest a biosynthetic relationship between fractions I, IIA, and IIB. The composition, the sedimentation and gel filtration properties of fraction I, and its migration during SDS-gel electrophoresis are reminiscent of the corresponding properties of the "carrier" lipoteichoic acid wall teichoic acid complexes (1, 6, 7) first described by Glaser and his colleagues.

The properties of fractions IIA and IIB do not allow the assignment of any simple, tentative chemical structure. While all the observations suggest strongly that these fractions contain most of the newly made wall teichoic acid polymers, the poor reactivity of this fraction with antiserum against wall teichoic acids is puzzling. Possibly, some inhibitor of the serological precipitation reaction may be present, or these molecules may lack some unknown structural feature that is characteristic of the teichoic acid polymers already attached to the cell wall and that is essential for serological reactivity.

Fraction IIA contains peptidoglycan oligomers and choline-containing macromolecules of as yet undefined structure. The possibility that some of the peptidoglycan chains may already carry covalently attached wall teichoic acids is presently being investigated.

The continued production and secretion of different types of cell surface polymers by antibiotic-treated, lysis-defective bacteria may provide a useful experimental system for the elucidation of the complex terminal stages of cell wall assembly. Studies are in progress to establish the possible significance of these polymer secretion phenomena for the triggering of the irreversible effects (i.e., lysis and killing of the bacteria) of penicillin and other cell wall inhibitors (17).

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