Choline-containing Teichoic Acid As a Structural Component of Pneumococcal Cell Wall and Its Role in Sensitivity to Lysis by an Autolytic Enzyme*

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SUMMARY
The cell wall of Diplococcus pneumoniae was isolated and purified and its macromolecular components were characterized in two ways: (a) by selective extractions and (b) by solubilization of the wall with the pneumococcal autolytic enzyme followed by fractionation of the enzyme products. Two major macromolecular components could be identified. One, the peptidoglycan, is composed of glucosamine, muramic acid (plus muramic acid phosphate), lysine, alanine, and glutamic acid in the molar ratios of 1.0:1.2:1.5:3.8:2.0. The presence of additional amino acids, namely, aspartic acid, serine, glycine, and threonine in the molar ratios (with respect to glucosamine) of 0.9:1.0:1.0:0.5 was also detected. The other major component, teichoic acid, is rich in galactosamine, phosphate, and choline which occur in the molar ratios 1.0:1.65:0.9. These three constituents together make up 23% of the cell wall mass.

After treatment of cell walls with cell-free pneumococcal autolytic enzyme, most of the cell wall material can be recovered in the form of two soluble macromolecular fractions which are separable by gel filtration. Fraction I is of high molecular weight and contains teichoic acid polymers plus what appears to be the polysaccharide backbone of the peptidoglycan. Fraction II contains material of lower molecular weight and is rich in the amino acids lysine, glutamic acid, and alanine; these amino acids appear to represent cross-linked dimers and trimers of the peptide portion of the peptidoglycan. The teichoic acid and the polysaccharide components of Fraction I can be separated after sequential degradation with nitrous acid and periodate.

It has already been proposed in the literature that the antigenic C-polysaccharide of pneumococcus is a teichoic acid. Our studies further indicate that this teichoic acid contains choline and is a major structural component of pneumococcal cell wall.

The major autolytic activity in pneumococcal extracts appears to be an amidase which splits the bond between muramic acid and alanine in the peptidoglycan portion of the cell wall. Experiments are described which indicate that the lysis of pneumococci by deoxycholate occurs through the participation of this enzyme.

The choline component of the teichoic acid plays a key role in determining sensitivity to the autolytic enzyme, since walls prepared from pneumococci in which cell wall choline was replaced by ethanolamine were found to be totally resistant to the action of the autolytic enzyme.

A novel form of choline in nature was described recently; this substance was identified as a component of some macromolecular structure localized in the cell wall of the bacterium, Diplococcus pneumoniae (2).

At least 40 to 50% of the radioactive choline incorporated into the wall from the growth medium could be recovered as a component of the purified C-polysaccharide (2), one of the major antigenic components of pneumococcal cell walls, if the preparative methods of Goebel et al. (3) or Liu and Gotschlich (4) were followed. A varying fraction of the incorporated choline was also extractable with cold trichloracetic acid (2), a solvent often used for preparing teichoic acids (5) and which has been used to extract material with C-antigen activity from pneumococci (6). A quantitative release of choline in the form of macromolecules could also be achieved by treating live pneumococci with cell-free pneumococcal autolytic enzyme (7).

The physiological importance of the choline-containing macromolecules is indicated by the fact that pneumococci have a nutritional requirement for choline (8). In addition, when the analogue ethanolamine replaces choline in the cell walls of bacteria, the bacteria develop a number of physiological abnormalities; daughter cells do not separate at the end of cell division, the cells lose their ability to undergo genetic transformation, they do not autolyse in the stationary phase of growth, and they become resistant to detergent-induced lysis (9). These observations offer an opportunity to examine the relationship between cell wall primary structure and several properties involving the bacterial surface. Of particular interest is the relationship between wall structure and cellular lysis. A possible role of an autolytic enzyme in detergent-induced lysis has been suggested (10). With these thoughts in mind, we have begun to...
characterize pneumococcal cell walls and an autolytic enzyme present in cell lysates.

**EXPERIMENTAL PROCEDURE**

Pneumococci (strain R 36A) were grown in a chemically defined medium (11) buffered at pH 7.6 or 8.0. For the preparation of cell walls containing ethanolamine, choline was replaced in the growth medium with 20 μg per ml of ethanolamine. All chemicals used were commercially available analytical grade products. Purchased from New England Nuclear Corporation were 3H- and 14C-labeled radioactive compounds; 32P was obtained from Union Carbide Corporation. The isotopic tracers were added to the growth medium at 0.1 to 0.5 μCi per ml. The following compounds were used: choline (2H- or 2H-methyl or 2H-uniformly labeled), ethanolamine and lysine (both 14C uniformly labeled), and 32P04. Radioactive samples were either dried on glass fiber filters (Whatman, GFA, 2.4 cm) and placed in toluene scintillation fluid or liquid samples were added to ethanol-toluene scintillation fluid or Bray's solution and counted in a scintillation spectrometer (Nuclear Chicago Unilux or Mark I). Double label counting of 2H and 14C was performed by the discriminator-ratio method (12).

Sephadex gels were obtained from Pharmacia. All columns were prepared as recommended, and void volumes were determined using solutions of blue dextran. The conditions for the individual cases are described in the legends to figures.

**Preparation of Cell Walls**—Cell walls were prepared from exponentially growing cells according to a modification of a published procedure (2). The cells were washed two to four times (centrifugation, 12,000 × g, 10 min) with 0.15 M NaCl (in about 0.01 of original culture volume) and resuspended in the same volume of water (or occasionally in 0.15 M NaCl). After heating at 65–70°C for 15 min to inactivate the autolytic enzyme, the cells were washed once, resuspended in water, and disrupted by shaking in a Mickle cell disintegrator or in a Braun tissue homogenizer with an equal volume of washed glass beads (Ballotini No. 13) for 15 min, a treatment which left no recognizable bacterial forms. The broken cells were separated from the glass beads and the suspension was centrifuged at low speed (3,000 × g, 5 min) to remove any unbroken cells. The walls were then washed twice with acetone, twice with 0.15 M NaCl containing 0.01 M K2HPO4 (saline-phosphate buffer) and resuspended in the same. DNase (10 μg per ml) and RNase (60 μg per ml) (Worthington Biochemicals) and a few drops of chloroform were added and the suspension was incubated at 37°C for 4 to 12 hours with constant mixing with a magnetic stirrer. Trypsin, 50 μg per ml (Worthington Biochemicals), and 0.1 mM CaCl2 were added and incubation was continued for 12 hours. The suspension was then incubated with a second portion of trypsin for an additional 12 hours. The cell walls were washed six times in saline-phosphate buffer and six times in water. This number of washes was sufficient to reduce the counts released from isotope-labeled cell walls virtually to zero.

**Preparation of Autolytic Enzyme**—Exponentially growing cells were harvested by centrifugation (as above), washed and resuspended in 0.01 volume of saline-phosphate buffer containing 0.002 M 2-mercaptoethanol. To a suspension of 5 × 109 bacteria per 5 ml, deoxycholate (0.03 to 0.1%) and pancreatic DNase (0.5 to 1 μg per ml) were added. The suspension was incubated at 37°C until maximum clearing occurred, usually in 10 to 15 min. The clarified suspension was centrifuged (12,000 × g, 10 min) to remove large particulate matter. The supernatant was passed through two glass fiber filters on top of a Millipore filter (type HA, 2.5 cm) held in a Millipore filter apparatus. The filtrate was filtered through a Sephadex G-75 column (3 × 40 cm, eluent saline-phosphate buffer containing 0.002 M 2-mercaptoethanol) in the cold to remove deoxycholate. “Autolytic” enzymatic activity eluted in the void volume of the column. The active fractions could be stored at 4°C for several months with no detectable loss of activity.

**Autolytic Enzyme Assay**—An 0.1 ml aliquot of the enzyme preparation (containing about 1010 cell equivalents of bacterial lysate) was added to 0.9 ml of a suspension of (saline-phosphate buffer containing 0.002 M 2-mercaptoethanol) cell walls (0.1 to 50 mg, dry weight, prepared from bacteria grown on choline) labeled either with radioactive choline or with radioactive lysine or both. After incubation at 37°C, samples were removed and centrifuged (International Micro-Capillary centrifuge, model MB, 14,000 × g, 10 min) and the amount of released radioactive label was determined. Occasionally, when enzymatic hydrolysis was carried out on a large scale with more concentrated suspensions of cell walls, a precipitate formed during the course of the reaction. The choline- and lysine-labeled components of the cell wall were always quantitatively released to the soluble fraction.

**Nitrous Acid and Periodate Treatment**—To a 1 ml aqueous solution of the choline-labeled fraction of the enzyme products from about 35 mg of cell walls, were added 1 ml of 5% NaNO2 and 1 ml of 5.8 N acetic acid (13). The solution was incubated at 25°C for 20 min and then adjusted to pH 5 to 6 by the addition of Na2CO3 solution. The extent of fragmentation was ascertained by filtering the treated material through a column (1 × 50 cm) of Sephadex G-100 with water as the eluent. After assay ing aliquots of each fraction, all fractions were pooled and concentrated by lyophilization. This material, as well as the untreated choline-containing enzyme products, was treated with periodate as follows. To about 1 ml of aqueous solution of the sample containing 10 to 20 mg of material was added an equal volume of 0.1 N sodium metaperiodate, and the solution was incubated at 37°C for 1 hour in the dark at pH 7. Unreacted periodate was destroyed by the addition of glycerol or ethylene glycol and incubation for an additional hour. Periodate reduction products were removed by filtration through a column (1 × 50 cm) of Sephadex G-10 in water as the eluent; the fragmented enzyme products were excluded from the gel, while the reduction products of periodate were retained. The treated sample was concentrated by lyophilization.

Dinitrophenylation was carried out by a standard procedure (14).

**Analytical Procedures**—Choline was estimated by bioassay using Neurospora crassa as the test organism (15). Samples were hydrolyzed for 12 to 16 hours in 6 N HCl, flash evaporated, and the residue was taken up in water for assay. Phosphate was determined by the method of Chen, Toribara, and Warner (16). Amino sugars and amino acids were determined with a Beckman model 120B automatic amino acid analyzer. Samples were hydrolyzed in 6 N HCl in tubes which had been sealed under vacuum. Cell walls were hydrolyzed at 110°C, while other samples were hydrolyzed at 100°C. After hydrolysis, samples were flash evaporated and the residue was taken up in 0.2 N citrate buffer (pH 2.2) for analysis. Decomposition curves for the amino sugars were determined separately for each material analyzed, since quite marked variations in decomposition rates in different materials were observed. Glucosamine, muramic acid,
TABLE I

Distribution of radioactive material during preparation of cell walls

Cell walls were prepared from bacteria grown in choline medium containing \(^{14}C\)-choline or \(^{14}C\)-lysine and in ethanolamine medium containing \(^{14}C\)-ethanolamine or \(^{14}C\)-lysine.

| Treatment                        | Label released from | Choline-containing cells | Ethanolamine-containing cells |
|----------------------------------|---------------------|--------------------------|------------------------------|
|                                  |                     | Choline | Lysine | Choline | Lysine |
| Heating of washed suspension     |                     | 12.7    | 40.1   | 25.5    | 20.0   |
| 65-70°, 15 min                   |                     |         |        |         |        |
| Mechanical disruption (Mickle disintegrator) |       | 39.5    | 45.4   | 1.5     | 49.0   |
| DNase, RNAse followed by trypsin |                     | 4.8     | 45.4   | 1.5     | 49.0   |
| Washes                           |                     | 1.9     | 0.4    | 4.7     | 6.5    |
| Final cell walls                 |                     | 41.1    | 14.1   | 68.3    | 24.5   |

Table II

Differential extraction of choline and lysine labels from purified cell walls

The extraction with 10% trichloracetic acid at 0° was performed with \(^{14}C\)-choline-labeled cell walls; \(^3H\)-choline- and \(^{14}C\)-lysine-labeled walls mixed in approximately equal proportions were used for the other extractions. After the treatments indicated, total and nonsedimentable \(^3H\) and \(^{14}C\) counts were determined. Most of the treatments were based on published procedures: trichloroacetic acid, 0° (6); formamide (18); sodium hydroxide and hydrochloric acid (19).

| Treatment                        | %       | Part of Fig. 2 showing gel filtration properties |
|----------------------------------|---------|-------------------------------------------------|
| Trichloroacetic acid             |         |                                                 |
| 5%, 100°, 1 hr                    | 100     | A                                               |
| 10%, 100°, 1 hr                   | 100     | A                                               |
| 10%, 0°, 6 days                   | 48      | A                                               |
| Formamide, 100%, 170°, 20 min     | 100     | B                                               |
| Periodate                        |         |                                                 |
| 0.05 M, 37°, 45 min              | 100     | C                                               |
| 0.05 M, 0°, 45 min               | 100     | C                                               |
| Sodium hydroxide, 0.1 M, 37°, 40 hr | 70     | D                                               |
| Hydrochloric acid, 0.1 M, 37°, 65 hr | 67 | D                                               |
| Phenol, liquefied, 37°, 17 hr     | 6       |

Fig. 1. Distinct classes of choline labeled molecules obtained during preparation of cell walls. Aliquots of radioactive material released from cells or cell fragments at the indicated stages of the procedure were filtered through a column (1 × 50 cm) of Sephadex G-100 using 0.15 M NaCl as eluent. •, wash before heating; +, heating; ○, mechanical disintegration; Δ, DNase, RNAse, trypsin. Horizontal arrow indicates fractions in which blue dextran eluted. Fraction volume was about 1 ml.

and muramic acid phosphate were estimated by extrapolation to zero hydrolysis while galactosamine was estimated by the maximum amount of galactosamine plus galactosamine 6-phosphate. (The maximum amount of galactosamine after longer hydrolysis gave the same result.) The rate of disappearance of galactosamine 6-phosphate and the appearance of galactosamine in the hydrolysates indicated that all the galactosamine was present as the phosphate. The identification and quantitation of the amino sugars and amino acids were based on elution positions and color constants determined with commercially available standards. (The one exception to this was muramic acid-phosphate, which

Fig. 2. Gel filtration properties of choline-labeled molecules extracted from purified cell walls. The column volume in each case was about 1 × 50 cm. The eluent in A was 0.15 M NaCl; in B, C, and D, distilled water. The fraction volume was in all cases about 1 ml. The inset to D shows percentage of \(^3H\)-choline extracted as a function of time.
TABLE III
Composition of pneumococcal cell walls (Column 1) and fractions prepared from them by autolytic enzyme (column k') and subsequent nitrous acid and periodate treatments (Columns 3 and 4)

Derivation of the fractions is shown in Fig. 3. Molar ratios are expressed with respect to galactosamine. The dry weights of these fractions obtained from the indicated amount of cell walls is included. The data for C-polysaccharide (Column 5) are included for comparison. The absence of a numerical figure indicates that the component was not detected.

| Cell walls          | Choline-labeled enzyme products | After nitrous acid and periodate: retained by Sephadex G-25 | After nitrous acid and periodate: excluded by Sephadex G-25 | Composition of C-polysaccharide
|---------------------|--------------------------------|----------------------------------------------------------|----------------------------------------------------------|-------------------------------|
|                     | 
| µ moles/mg | molar ratios | % by weight | µ moles/mg | molar ratios | % by weight | % recovered | µ moles/mg | molar ratios | % by weight | % recovered | µ moles/mg | molar ratios | % by weight | % recovered |
| Choline             | 0.40 | 0.90 | 4.8 | 0.70 | 0.83 | 7.2 | 66.4 | 0.92 | 1.10 | 9.4 | 58.3 | 0.30 | 1.00 | 2.9 | 4.0 |
| Phosphate           | 0.85 | 1.65 | 8.2 | 1.18 | 1.40 | 11.3 | 60.9 | 1.35 | 1.61 | 13.0 | 47.3 | 0.28 | 1.00 | 5.6 | 6.1 |
| Galactosamine       | 0.53 | 1.00 | 10.5 | 0.84 | 1.00 | 17.0 | 72.0 | 0.84 | 1.00 | 17.0 | 48.2 | 0.66 | 2.38 | 13.3 | 36.2 |
| Glucosamine         | 0.21 | 0.40 | 4.2 | 0.30 | 0.36 | 6.1 | 63.7 | 0.06 | 0.07 | 1.2 | 8.5 | 0.67 | 2.38 | 13.3 | 36.2 |
| Muramic acid        | 0.21 | 0.40 | 6.8 | 0.34 | 0.40 | 11.0 | 72.2 | 0.20 | 0.24 | 7.5 | 28.3 | 0.87 | 3.10 | 10.9 | 47.7 |
| Muramic acid phosphate | 0.04 | 0.08 | 1.7 | 0.05 | 0.06 | 2.1 | 55.9 | 0.01 | 0.01 | 0.5 | 6.8 | 0.16 | 0.50 | 6.5 | 16.0 |
| Lysine              | 0.32 | 0.62 | 4.2 | 0.21 | 0.25 | 2.7 | 20.2 | 0.04 | 0.04 | 0.5 | 3.7 | 0.20 | 0.73 | 2.6 | 7.3 |
| Alamine             | 0.79 | 1.52 | 5.6 | 0.33 | 0.39 | 2.3 | 18.6 | 0.10 | 0.12 | 0.7 | 3.8 | 0.60 | 2.16 | 4.3 | 8.8 |
| Glutamic acid       | 0.43 | 0.83 | 5.5 | 0.36 | 0.43 | 4.6 | 37.3 | 0.17 | 0.20 | 2.1 | 11.8 | 0.68 | 2.45 | 8.8 | 15.8 |
| Aspartic acid       | 0.19 | 0.37 | 2.2 | 0.28 | 0.35 | 3.2 | 65.7 | 0.11 | 0.13 | 1.3 | 5.8 | 0.59 | 1.91 | 6.1 | 32.0 |
| Threonine           | 0.11 | 0.21 | 1.2 | 0.11 | 0.19 | 1.2 | 44.6 | 0.05 | 0.06 | 0.5 | 12.5 | 0.20 | 0.74 | 3.6 | 21.8 |
| Serine              | 0.20 | 0.38 | 1.9 | 0.11 | 0.13 | 1.1 | 24.5 | 0.05 | 0.06 | 0.5 | 12.5 | 0.20 | 0.74 | 3.6 | 21.8 |
| Glycine             | 0.21 | 0.40 | 1.2 | 0.16 | 0.19 | 0.9 | 34.0 | 0.09 | 0.11 | 0.5 | 12.8 | 0.20 | 0.72 | 3.4 | 11.0 |
| Valine              | 0.19 | 0.37 | 1.9 | 0.17 | 0.20 | 1.7 | 39.9 | 0.07 | 0.08 | 0.7 | 11.0 | 0.10 | 0.36 | 1.0 | 6.0 |
| Leucine             | 0.15 | 0.29 | 1.7 | 0.09 | 0.11 | 1.0 | 26.8 | 0.04 | 0.05 | 0.5 | 7.9 | 0.04 | 0.32 | 1.0 | 6.9 |
| Leucine             | 0.22 | 0.42 | 2.5 | 0.13 | 0.16 | 1.0 | 26.3 | 0.05 | 0.06 | 0.5 | 6.8 | 0.14 | 0.50 | 1.6 | 7.3 |
| Histidine           | 0.08 | 0.15 | 1.1 | Trace | Trace | Trace | Trace | Trace | Trace | Trace |
| Arginine            | 0.07 | 0.13 | 1.1 | Trace | Trace | Trace | Trace | Trace | Trace |
| Proline             | 0.08 | 0.10 | 1.0 | Trace | Trace | Trace | Trace | Trace |
| Tyrosine            | 0.07 | 0.13 | 0.7 | Trace | Trace | Trace | Trace | Trace |
| Phenylalanine       | 0.13 | 0.19 | 2.0 | Trace | Trace | Trace | Trace | Trace |
| Methionine          | 0.04 | 0.08 | 0.6 | Trace | Trace | Trace | Trace | Trace |

% dry weight accounted for: 70.3, 74.4, 66.2, 94.9, 86.0

Dry weights: 48.8 mg, 21.8 mg, 14.5 mg, 5.6 mg

* Calculated from data of Liu and Gotschlich (see Reference 4).
* Percentage of component recovered based on total amount of that component in the starting cell walls.
* A choline bioassay was done on C-polysaccharide kindly supplied by Dr. Emil Gotschlich of The Rockefeller University.
* Correction of the published value in Reference 4 (personal communication from Dr. Emil Gotschlich).
was identified on the basis of published elution properties (1) and for which we have used the color constant experimentally determined for muramic acid.) Since the amino sugars and amino acids in similar material have been identified (4), we have not isolated the individual components from the amino acid analyzer column to check their identity.

**RESULTS**

**Distribution of Choline-containing Molecules during Cell Wall Preparation**—During the preparation of cell walls from cells labeled with radioactive choline, it became apparent that not all the choline which had been incorporated by cells remained associated with sedimentable wall structures. In order to determine if the choline released during the different steps of cell wall preparation represented specific subfractions of the choline-containing molecules, we measured the amount released by each treatment and examined their elution properties on Sephadex G-100 (Table I and Fig. 1). We have included in the table similar measurements of the release of radioactive lysine from choline-grown cells and the release of radioactive ethanolamine and lysine from ethanolamine-grown cells (9). Note that about 70% of the ethanolamine but only 40% of the choline originally associated with the cell suspension remained in the final wall preparation. It is possible that the action of endogenous autolytic enzyme or enzymes during harvesting of the bacteria contributes to the loss of choline from the cell wall.

The gel filtration properties of choline-labeled material released in the various steps of the wall preparation (Fig. 1) suggest that they may represent distinct classes of molecules. These fractions have not been further characterized, but it is likely that they contain biosynthetic intermediates, as well as breakdown products, of wall polymers. One must therefore bear in mind that isolated cell walls probably do not contain a complete complement of all the choline-containing molecules of the cell.

**Extraction of Choline-containing Molecules from Cell Walls**—Purified cell walls were treated with reagents previously used either to characterize or to extract various types of bacterial cell wall polymers or to do both (Table II). The choline-labeled molecules are solubilized to a much greater extent than the lysine-labeled ones by all the solvents which are known to be selective for cell wall polymers or to do both (Table II). The choline-labeled fraction contained biosynthetic intermediates, as well as breakdown products, of wall polymers. One must therefore bear in mind that isolated cell walls probably do not contain a complete complement of all the choline-containing molecules of the cell.

**Composition of Pneumococcal Cell Walls**—The composition of cell walls prepared by the methods described above is given in Column 1 of Table III. Molar ratios of the components present are expressed with respect to galactosamine. The constituents listed account for 70.3% dry weight, of the preparation.

**Fig. 3. Fractionation of cell wall components with autolyte enzyme, nitrous acid, sodium periodate, and gel filters. See text for detailed description.**

by cell-free autolytic enzyme preparations as shown in Fig. 4A. Cell wall mixtures consisting of about 50 mg of carrier cell walls and tracer amounts of 1H-choline- and 14C-lysine-labeled cell walls were hydrolyzed with the autolytic enzyme (total of 5 x 10**5** "cell equivalents"; see "Experimental Procedure") in a volume of about 150 ml. At the indicated times, aliquots of the reaction mixture were removed, heated at 65° for 10 min to inactivate the enzyme, centrifuged, and nonsedimentable "H and "C counts were determined. A precipitate which formed during the course of hydrolysis under these conditions was removed by the centrifugation. Choline- and lysine-labeled components were exclusively in the soluble fraction.

The choline- and lysine-labeled products can be readily separated on Sephadex G-25 (Fig. 4B). Only two peaks are obtained. Peak I contains all the choline label and is excluded from the matrix of the gel. Peak II contains only lysine label and is retained by the gel. A fraction of the lysine label varying in quantity from experiment to experiment elutes in Peak I, probably reflecting different extents of hydrolysis. The main part of Fig. 4B is a plot of the results from Experiment 3. For convenience, Peaks I and II will be referred to as the "choline-labeled" and "lysine-labeled" enzyme products, respectively.

**Characterization of Lysine-labeled Enzyme Products**—After concentration of the lysine-containing enzyme products by lyophilization, the material was passed through gel filters in order to obtain an estimate of its molecular weight. Sephadex G-10 and G-15 and Bio-Gel P-4 columns were used, with 1 m sodium chloride solution as eluent to minimize adsorption to the gels. The results of these experiments (Fig. 5) indicate an approximate molecular weight between 700 and 1500.

The amino sugar and amino acid composition of this material was determined with an automatic amino acid analyzer after acid
Control (no enzyme)

FIG. 4. A, hydrolysis of \(^{3}H\)-choline- and \(^{14}C\)-lysine-labeled cell walls with autolytic enzyme. Ordinate indicates percentage of label released. B, separation of choline-labeled enzyme products (I) from lysine-labeled enzyme products (II) on column (3 \(\times\) 40 cm) of Sephadex G-25. Eluent was distilled water; fraction volume was about 1 ml. The horizontal arrow marks the elution position of blue dextran. The inset shows the percentage of \(^{14}C\)-lysine present in Peak I in four separate experiments.

Fig. 5. Size and composition of lysine-labeled fraction of autolytic enzyme products. Gel filtrations were performed with columns (1 \(\times\) 50 cm), using 1 M NaCl as eluent. The fraction volume in each case was about 1 ml. The amino acid composition was determined with a Beckman amino acid analyzer, model 120B, after 6 N HCl hydrolysis for 13 hours at 110\(^\circ\)C. Inset: a, calculated on the basis of radioactivity \((^{14}C\)-lysine) per mg of cell walls in initial suspension and radioactivity in sample analyzed; b, with respect to lysine; c, on the basis of the absence of \(^{3}H\)-choline label from this fraction. d, no amino sugars were detected. From the decomposition curves of the amino sugars in whole cell walls under these conditions, galactosamine (as galactosamine-6-phosphate) would not be significantly destroyed, glucosamine 8% destroyed, muramic acid plus muramic acid phosphate 44% destroyed.

| Amino Acid         | molar ratio \(^a\) | molar ratio \(^b\) |
|--------------------|---------------------|---------------------|
| Choline            | 0.0                 | 0.0                 |
| Galactosamine      | 0.0                 | 0.0                 |
| Glucosamine        | 0.0                 | 0.0                 |
| Muramic acid +     | 0.0                 | 0.0                 |
| Muramic acid +     | 0.0                 | 0.0                 |
| Lysine             | 0.317               | 1.00                |
| Alanine            | 0.073               | 0.23                |
| Glutamic           | 0.252               | 1.11                |
| Aspartic           | 0.036               | 0.11                |
| Threonine          | 0.010               | 0.03                |
| Serine             | 0.007               | 0.21                |
| Glycine            | 0.054               | 0.17                |
| Valine             | 0.017               | 0.05                |
| Isoleucine         | 0.011               | 0.03                |
| Leucine            | 0.017               | 0.06                |

Hydrolysis, and the results are included in Fig. 5. None of the cell wall amino sugars (see below) were detected. The amino acids lysine, alanine, and glutamic acid are the major components of this fraction, representing 85% of the amino acids detected. The molar ratios obtained were lysine-alanine-glutamic acid, 1.0:2.1:1.1. This is similar to the ratio of the same amino acids in whole cell walls (see Table III, Column 1), 1.0:2.5:1.3. Of the other seven amino acids detected in this fraction, the most abundant ones were aspartic acid, serine, and glycine. They were present in the ratios 0.1:0.3:0.2 per lysine residue. Up to 86% of the radioactive lysine associated with the purified cell walls was recovered in this fraction (see Fig. 4).

Choline-containing Enzyme Products—Fig. 6 shows the elution profile from Sephadex G-100 of the choline-containing polymers released from purified cell walls by autolytic enzyme treatment. In three of four such preparations, most of the material was excluded from the Sephadex G-100 gel; in one preparation, however, part of the material appeared fragmented to smaller sizes. Heterogeneity of size and of ionic character is indicated by the results of chromatography on Sephadex G-200 dextran gels and DEAE-Sephadex (Fig. 7, A and B). This fraction of the enzyme products represented 44.6% of the mass of the starting cell walls. Choline, phosphate, amino sugar, and amino acid analyses were performed on this material. About 74% of the dry weight could be accounted for in terms of the components listed in Table III, Column 2.

Pertinent data for the average composition of C-polysaccharide (4) have been included for comparison (Table III, Column 5). Although Liu and Gotschlich (4) did not analyze for choline in their preparations, we have subsequently determined the amount of this compound in a C-polysaccharide preparation provided by Dr. Emil C. Gotschlich and obtained a value of 0.74 ± 0.09 \(\mu\) mole per mg. We have included this value in Table III.

By comparing the composition of the enzyme products (Table III, Column 2) with that of the starting cell walls (Table III, Column 1), the enzyme products are seen to be enriched in choline, phosphate, galactosamine, glucosamine, muramic acid and mura-
mic acid phosphate, and aspartic acid. Between 60.9 and 72.2% of the amounts of these components in the starting cell walls was recovered in this fraction with very little change in the molar ratios. Relative to the starting cell wall material, the concentration of all amino acids except aspartic acid is decreased in the enzyme products, only 18 to 44% of the various amino acids remained in this fraction and their concentration could probably be further decreased by more extensive autolytic enzyme treatment.

A decrease in lysine, alanine, and glutamic acid is expected, since about 60 to 80% of these amino acids are recovered in the lysine-labeled fraction of the enzyme products. Of the amino acids remaining in the choline fraction, glutamic acid and aspartic acid show an increase and alanine a decrease relative to lysine. Thus, the ratio of lysine-alanine-glutamic acid-aspartic acid is 1.0:1.6:1.7:1.3 compared to 1.0:2.5:1.3:0.6 in the starting cell walls. The molar ratios of the other amino acids are not changed significantly. If one assumes that all the lysine and alanine could be obtained, under ideal conditions, in the lysine-labeled fraction, there would still remain an excess of 0.9 glutamic acid and 1.2 aspartic acid residues per lysine residue. We cannot account for the loss of the amino acids from valine downward in the table.

Since only 10 to 20% of these particular amino acids are recovered in the lysine-rich fraction, it seems that they are removed either in the precipitate which forms during enzymatic hydrolysis of the walls or during the Sephadex G-25 or G-100 fractionation steps.

Another conclusion from these analyses is that the average amino acid and amino sugar composition of the enzyme products is quite similar to that of Liu and Gotschlich's C-polysaccharide (Table III, Column 5).

**Fragmentation of Choline-containing Enzyme Products with Nitrous Acid and Sodium Periodate**—Treatment of the choline-containing polymers with sodium periodate results in fragmentation of the polymers. This occurs whether one treats whole cells (2), the cell walls derived from them (Fig. 2C), or the choline-labeled fraction of the autolytic enzyme products (Fig. 8A). Treatment with nitrous acid also causes fragmentation of the choline-containing polymers and periodate treatment applied after nitrous acid causes still further fragmentation (Fig. 8, B and C).

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**Fig. 6.** Elution of $^3$H-choline labeled fraction of autolytic enzyme products from column (1 X 50 cm) of Sephadex G-100. Eluent was 0.15 M NaCl, and the fraction volume was about 1 ml. The horizontal arrow marks the elution position of blue dextran.

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**Fig. 7.** Comparison of choline-labeled enzyme products from purified cell walls with choline-labeled products of deoxycholate-induced lysis of whole cells. A and B, doubly labeled $^3$H-choline- and $^{32}$P$_4$-containing products from purified cell walls. C and D, **C-choline-labeled products from deoxycholate-induced lysate. Column sizes were 1 X 50 cm. Eluents for the DEAE-Sephadex column were the following ammonium acetate buffers (22): I, 0.1 M, pH 8; II, 0.1 M, pH 8 -> pH 4; III, 0.1 M, pH 4 -> 0.5 M, pH 4; IV, 0.5, pH 4; V, 0.5 v, pH 4 -> 2.0 v, pH 4.5. Fraction volume was about 1 ml. The horizontal arrow marks the elution position of blue dextran.
Combination of these treatments was used for the further fractionation of the choline-labeled enzyme products. After treatment with 0.24 M nitrous acid and then with 0.05 M sodium periodate (see Fig. 3), filtration of the reaction mixtures through Sephadex G-25 yielded two subfractions (Fig. 8C): (a) a choline-labeled fraction which was retained by the column and (b) a fraction which was excluded. The position of the latter fraction in the elution profile was marked by a small peak of 14C-lysine in the exclusion volume of the column. A total of 92% of the dry mass of the starting material was recovered in these two fractions: 66.5% in the choline-rich fraction and 25.7% in the lysine-rich part.

The composition of the two fractions is shown in Table III. Fraction I (Column 3) contains 58.3% of the choline, 47.3% of the phosphate, 48.2% of the galactosamine, and 35.1% of the muramic acid (and muramic acid phosphate) of the cell wall and represents about 76% of the mass of choline-labeled enzyme products. The molar ratios of choline, phosphate, and galactosamine to muramic acid plus muramic acid phosphate of the cell wall and of all the amino acids are drastically reduced. The proportions of peptidoglycan components remaining in this fraction are quite different from those characteristic of peptidoglycan (21). For example, the ratio of glucosamine to muramic acid plus muramic acid phosphate is 1.0:3.5. The relatively small amount of amino acids left in this fraction have the proportions of 1.0 lysine, 2.5 alanine, 4.3 glutamic acid, 2.8 aspartic acid, 1.2 threonine, 1.2 serine, and 2.2 glycine.

The composition of Fraction II (Column 4, Table III) suggests that it consists primarily of molecules derived from the polysaccharide backbone of the peptidoglycan with some of the original complement of amino acids attached. It is apparent that the proportions of these substances also deviate from those normally found in peptidoglycan. For example, the ratio of glucosamine to muramic acid plus muramic acid phosphate is 1.0:3.5. The relatively small amounts of amino acids left in this fraction have the proportions of 1.0 lysine, 2.5 alanine, 4.3 glutamic acid, 2.8 aspartic acid, 1.2 threonine, 1.2 serine, and 2.2 glycine.

In spite of the fact that only 56% of the weight of the choline-containing material (Table III, Column 3) can be accounted for by the components listed indicates this also. This unaccounted for weight could represent degradation products of ribitol, a diaminotrideoxyhexose, and glucose (6) (see below).

Deoxycholate-induced Lysis and Autolysis—Fig. 7 shows the chromatographic and gel filtration properties of (a) choline-containing material prepared from purified cell walls with autolytic enzyme (Fig. 7, A and B) and (b) the choline-containing material prepared by deoxycholate lysis of whole cells (Fig. 7, C and D). There are several points to be made from this figure. We have already mentioned the heterogeneity of the choline-labeled enzyme products obtained from purified cell walls. The elution profiles in Fig. 7, A and B, were obtained with enzyme products prepared from cell walls doubly labeled with 14C-choline and 32P. The almost perfect overlap of the 3H and 32P in the elution profiles indicates that the isotopes label the same molecules. The elution profiles in Fig. 7, C and D, were obtained with a deoxycholate lysate of 14C-choline labeled cells. Although C and D are not identical with A and B, respectively, they are quite similar. Furthermore, the elution profiles of C-polysaccharide isolated from a deoxycholate-induced lysate on Sephadex G-200 and DEAE-Sephadex under the same conditions (22) are quite similar to Fig. 7, A and B, respectively. These findings together with the close similarity in chemical composition of our choline-containing enzyme products and that of C-polysaccharide (prepared and analyzed by Liu and Gotschlich (4), indicate a basic similarity between deoxycholate-induced lysis and the hydrolysis of purified cell walls by the isolated autolytic enzyme. This similarity suggests that deoxycholate-induced lysis can be explained by the action of an endogenous autolytic enzyme, as originally proposed by Dubos (10).

Treatment of Ethanolamine-containing Cell Walls with Autolytic Enzyme—In view of the similarity between deoxycholate-induced lysis and the lysis of purified cell walls by cell-free enzyme preparations, it was of particular interest to test the sensitivity of purified ethanolamine-containing cell walls to cell-free enzyme. It will be recalled that bacteria containing ethanolamine in their cell walls become resistant to deoxycholate-induced lysis (9). Cell walls labeled with 14C-ethanolamine and with 14C-lysine were pre-
Resistance of cell walls containing ethanolamine to hydrolysis by autolytic enzyme

| Label                        | Enzyme added | Sedimentable cpm/ml after 6 hr | Non sedimentable cpm/ml after 6 hr | % cpm sedimentable |
|------------------------------|--------------|-------------------------------|------------------------------------|---------------------|
| Cell walls containing ethanolamine in place of choline | Yes          | $2.0 \times 10^3$             | $1.8 \times 10^3$                  | 91                  |
| $^{14}$C-Ethanolamine        | Yes          | $1.9 \times 10^3$             | $4.3 \times 10^3$                  | 18                  |
| $^{14}$C-Lysine              | Yes          | $1.8 \times 10^3$             | $1.8 \times 10^3$                  | 100                 |
| $^{14}$C-Lysine              | No           | $1.8 \times 10^3$             | $2.6 \times 10^3$                  | 100                 |
| $^{14}$C-Lysine              | Yes          | $1.1 \times 10^3$             | $1.0 \times 10^3$                  | 100                 |
| $^{14}$C-Lysine              | No           | $1.1 \times 10^3$             | $0$                                | 0                   |

Discussion

Composition of Pneumococcal Cell Walls—To our knowledge, the cell walls of D. pneumoniae have not been characterized before. The walls contain large amounts of choline, phosphate, and galactosamine. In addition, the normal peptidoglycan components (aspartate, threonine, serine, and glycine) are also present in our wall preparations, although cytoplasmic contamination cannot be rigorously excluded, it seems unlikely from the following considerations. In preparations of cell walls from $^{14}$C-lysine-labeled cells, wall-bound lysine label can be reduced to an apparently minimum level, after which further treatment and washing cause no more loss of counts. Moreover, at least 86% of the lysine label remaining with the cell walls can be obtained in the form of small molecules with a composition characteristic of the peptidoglycan portion of the peptidoglycan. Thus, if the non-peptidoglycan amino acids are present in protein, that protein is deficient in lysine, alanine, and glutamic acid and therefore would not be indicative of cytoplasmic contamination. Another consideration is that the non-peptidoglycan amino acids which could be quantified in the fractions prepared from cell walls (Table III), valine, leucine, and isoleucine, show little change in their molar ratios with respect to each other, although, as a group their ratios with respect to galactosamine, for example, differ from fraction to fraction. It is interesting to speculate in this connection that these amino acids may be components of a wall protein such as an autolytic enzyme.

Fragmentation of Cell Walls—Dissolution of purified cell walls by treatment with autolytic enzyme and the separation of the solubilized products by gel filtration permit the recovery of about 72% of the original dry mass of cell wall material in the form of two main fractions: Fraction I, a large molecular weight, choline-rich fraction and Fraction II, a smaller molecular weight fraction rich in lysine, alanine, and glutamic acid. Extraction studies and chemical analyses indicate that Fraction I contains mainly components of a teichoic acid-like polymer plus the polysaccharide backbone of the peptidoglycan, whereas the size and composition of Fraction II show that it represents the peptide portion (cross-linked dimers or trimers of a tetrapeptide containing 1 lysine, 1 glutamic acid, and 2 alanine residues) of the peptidoglycan. The amount of peptide remaining in Fraction I varies from experiment to experiment but at least 86% of the peptide can be removed from this fraction. Interestingly, aspartic acid, serine, glycine, and threonine remain with Fraction I.

Fraction I can be further resolved by degradation first with nitrous acid and subsequently with periodate. About 92% of the mass of this fraction can be recovered after treatment with these reagents in the form of two subfractions: one which is retained by Sephadex G-25 and contains all the choline and another which is excluded by Sephadex G-25 and contains glucosamine and muramic acid. Thus, this procedure seems to achieve the separation of the teichoic acid-like component (in degraded form) from the polysaccharide backbone of the peptidoglycan.

Nature of Macromolecular Choline Carrier—One of the main purposes of this study was to identify the macromolecular structure or structures to which choline is attached. It was already known from earlier work that choline is attached to some structure localized in the anatomical region of cell walls (2). The studies described here further specify this structure as a polysaccharide or teichoic acid which is rich in galactosamine and phosphate as well as choline, and which is a structural part of the pneumococcal cell wall. This classification of the macromolecular carrier of choline is based on the following observations. (a) Macromolecular substances rich in choline can be selectively extracted from purified cell walls (without solubilization of the peptidoglycan) by reagents which are known to extract teichoic acids and polysaccharides; (b) treatment of purified cell walls with autolytic enzyme prepared from pneumococci can solubilize the choline-containing macromolecules substantially “freed” of the peptide component of the cell walls; (c) recovery of the choline label after various degradative (enzymatic and chemical) treatments of cell walls invariably yielded molecules which also contained galactosamine and phosphate in addition to choline. The molar ratio of these components remained essentially unchanged in spite of the fact that these treatments caused drastic changes in molecular size; (d) the presence of choline and phosphate in the same molecules is also indicated by the cofractionation of $^3$H-choline and $^{32}$P04 label during ion exchange and Sephadex G-200 chromatography of the autolytic enzyme products.

C-Poly saccharide and Choline-containing Teichoic Acid—There are two series of recent publications which are relevant to our findings. Liu and Gotschlich (4, 22) and Brandish and Baddiley (6, 24) have isolated and analyzed the C-polysaccharide from...
pneumococci. The presence of covalently linked choline in C-polysaccharide prepared by the method of either Liu and Gotschlich (4) or Brundish and Baddiley (6) was reported earlier (2). Recently, the latter authors (6) have confirmed the presence of choline in C-polysaccharide prepared by repeated extraction (for a week) of defatted, dried pneumococci with cold trichloroacetic acid followed by purification with Cetavlon and anion exchange chromatography. Such preparations had high antigenic activity, were essentially free of amino acids, and contained choline, galactosamine, and phosphate in the molar ratio of 1.0:1.0:2.1, similar to the ratios of these same components in C-polysaccharide. Such preparations had high antigenic activity, were essentially free of amino acids, and contained choline, galactosamine, and phosphate in the molar ratio of 1.0:1.0:2.1, similar to the ratios of these same components in C-polysaccharide.

The material analyzed by these authors (6) contained, in addition, about 1 residue each of ribitol and glucose and probably about 1 residue of a diaminotrideoxyhexose per choline residue. We detected a faint spot corresponding to the dinitrophenylated derivative of diaminotrideoxyhexose upon electrophoresis of an acid hydrolysate of 1-fluoro-2,4-dinitrobenzene-treated cell walls (25). Furthermore, the direct Ehrlich test applied to periodate-positive materials of the teichoic acid gave strong positive reactions2 (25). On the basis of these observations, it is likely that the dinitrophenylated derivative of diaminotrideoxyhexose is also present in the teichoic acid component of our wall preparations. This conclusion is supported by the fragmentation of the teichoic acid by nitrous acid; Brundish and Baddiley (6) concluded that nitrous acid reacted with the dinitrophenylated derivative of d-aaminotrideoxyhexose in their trichloroacetic acid-extracted C-polysaccharide causing fragmentation of the teichoic acid polymer. We did not analyze our cell wall preparations for ribitol and glucose.

Liu and Gotschlich prepared their C-polysaccharide by a modification of the original procedure of Tillett, Goebel, and Avery (26); pneumococci were lysed with deoxycholate and the lysate was purified by fractional alcohol precipitations, treatment with nuclease and proteolytic enzymes, and extensive dialysis. Such preparations had high antigenic activity and contained covalently bound amino acids, galactosamine, and muramic acid as well as galactosamine and phosphate. Our own analyses on such preparations demonstrated the presence of choline.

The close similarity, in composition and in fractionation properties, between such C-polysaccharide preparations and our choline-rich autolytic enzyme products has already been pointed out. The choline to phosphate ratios are very similar, being 0.59 in our material and about 0.5 in the C-polysaccharide, although with respect to the amount of galactosamine present, the values for choline and phosphate in C-polysaccharide are somewhat lower. Such variations could be due to differences in the growth medium of the bacteria from which the materials were prepared. Alternatively, some selective loss of choline and phosphate from the polymeric material might be caused by a drastic early step (heating at 100° at pH 4) of the procedure used to prepare C-polysaccharide (4). Another difference is the smaller amounts of amino acids in the C-polysaccharide. However, as we have stated before, it is likely that the amount of lysine, alanine, and glutamic acid could be further reduced in our material by more extensive hydrolysis. We cannot explain the difference in the aspartic acid content of the two materials.

Free amino acids and the other containing some components of the peptidoglycan, are both derived from pneumococcal cell walls in which the teichoic acid and peptidoglycan chains form a single, covalently interlinked network. Apparently breaks can be introduced into this network at a number of different sites resulting in the release of the antigenically active galactosamine-rich macromolecules (22) in a manner capable of varying portions of the other polymers of the cell wall. Thus cold trichloroacetic acid appears to release teichoic acid substantially free of peptidoglycan. It should be added that this procedure also seems to introduce breaks in the backbone of teichoic acid, and, in our hands at least, trichloroacetic acid extraction never solubilized more than about 50% of choline from mid log phase bacteria. On the other hand, in deoxycholate-induced lysis, teichoic acids seem to be solubilized by autolytic enzyme or enzymes which split bonds between the peptide side chains and the polysaccharide backbone of the peptidoglycan, releasing high molecular weight complexes of teichoic acid and peptidoglycan backbone with some residual amino acids still attached.

It is interesting to point out that the presence of ribitol, glucose, and dinitrophenyldihexose in the cell wall in approximately the proportions suggested by Brundish and Baddiley (6) would enable us to account for almost all of the dry weight of the wall. For example, the presence of 1 residue each of ribitol, glucose, and dinitrophenyldihexose per 2.1 phosphate residues or per 1.0 galactosamine residue (which represent the limits of the disparity between results) would bring the percentage of the weight of the wall accounted for up to 89.4% or 94.9%, respectively. Similarly, the presence of these components in the same proportions in the choline-labeled fraction of the enzyme products brings the respective percentages of weight accounted for up to 100.8 or 114.3%. This seems also true for the C-polysaccharide preparations of Liu and Gotschlich.

According to this interpretation, therefore, one should regard the teichoic acid components in the trichloroacetic acid extracts of Brundish and Baddiley (6), in the material prepared from deoxycholate lysates by Gotschlich and Liu (22) and in our cell walls to be essentially identical. Gotschlich and Liu, however, proposed that the structure of the teichoic acid in their preparation was poly-N-acetylgalactosamine phosphate, which is not compatible with the above interpretation. The proposal by Gotschlich and Liu was based principally on two observations. (a) A polymer containing N-acetylgalactosamine and phosphate was isolated by cold trichloroacetic acid extraction of the "debris" of a pneumococcal autolysate. The material had C-antigen activity and 86% of its dry weight could be accounted for as N-acetylgalactosamine and phosphate, a composition clearly not permitting even an additional mole of choline per mole of galactosamine. A similar polymer in which 98% of the ninhydrin-positive material was accounted for by galactosamine was extracted with cold trichloroacetic acid from purified C-polysaccharide. (b) Gotschlich and Liu further reported loss of antigenic activity (measured by precipitin assay) and destruction of galactosamine residues by periodate oxidation of these polymers. Although it is possible that polymers of N-acetylgalactosamine phosphate exist in pneumococci, we would suggest that, instead of being typical pneumococcal teichoic acids, such molecules represent a special, minor class of wall components or that they may arise during the special conditions of prolonged toluene autolysis. It would be important to know what percentage of the total wall material such "80% pure" N-acetylgalactosamine phosphate polymers represent.

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2 A. Tomasz, unpublished data.
In addition, we could not observe destruction of the galactosamine residues in cell walls or in soluble teichoic acids by periodate oxidation, although the same treatment caused extensive fragmentation of the teichoic acid chains. These findings are in agreement with the report of Brundish and Baddiley (6) who described destruction of ribitol and glucose residues but survival of galactosamine during periodate oxidation of C-polysaccharide preparations.

The cause of the disagreement between the findings of Gotschlich and Liu on the one hand and the observations of Brundish and Baddiley and our own is not clear at present. However, it would seem important to call attention to the rather small size of the galactosamine-containing periodate products. Molecules of this size may have been removed by the gel filtration procedure used to purify the periodate-treated material of Gotschlich and Liu. In addition, such molecules may also be below the critical size which seems to be needed for a positive precipitin reaction (22).

Model of Pneumococcal Cell Wall—For the sake of clarity it seemed useful to sketch a tentative model of the basic features of the pneumococcal cell wall (Fig. 9). In the drawing we assumed that the basic structural principles of gram-positive cell walls (21) are also true for pneumococci. Within this framework the drawing is consistent with all the information discussed so far and it also incorporates data reported by Brundish and Baddiley (6) and Liu and Gotschlich (4, 22). The basic element of the structure is the alternating N-acetylglucosamine-N-acetylmuramic acid “backbone” of a typical peptidoglycan which has two kinds of substituents. Each muramic acid residue has the usual tetrapeptide (containing 2 alanine, 1 glutamic acid, and 1 lysine residues) attached to it. The tetrapeptide substituents seem to be further cross-linked to one another at least to the extent of forming dimers and trimers. Less frequently, the polysaccharide backbone is cross-linked to chains of a teichoic acid polymer which is rich in choline, galactosamine, and phosphate and probably contains ribitol, glucose, and the diaminotrideoxyhexose. The presence of two separate polymers, teichoic acid and peptidoglycan, in pneumococcal cell walls has already been suggested by Gotschlich and Liu. As we have just discussed, the structure of the teichoic acid has not been established, although the fragmentation by nitrous acid and periodate indicates some kind of repeating pattern, as proposed by Frundish and Baddiley.

Autolytic Enzyme and Deoxycholate-induced Lysis—Based on the composition of the autolytic enzyme products which we obtain from pneumococcal cell walls and on the proposed model of cell wall structure, a major activity in our enzyme preparations seems to be that of an amidase, cleaving the amide bond between alanine and muramic acid to yield the two classes of products which we have already discussed, namely, the large molecular weight, choline-containing fraction and the small molecular weight, peptide fraction.

Although we have been speaking of autolytic “enzyme” in the singular, we must emphasize that our enzyme preparation is a very crude extract of cellular contents and might contain several enzymes capable of acting on cell walls. It is possible, for example, that a deacetylase is present which removes acetyl groups from the amino groups of glucosamine, rendering the residues susceptible to the action of nitrous acid; this might explain the loss of glucoseamine during the nitrous acid-periodate treatment. It will be recalled that some differences were detected when the choline-containing products obtained by enzymatic hydrolysis of walls and deoxycholate-induced lysis of cells were compared on

![Fig. 9. Schematic diagram showing basic features of proposed pneumococcal cell wall structure. G, N-acetylgalactosamine; M, N-acetylmuramic acid. Arrows mark apparent sites of action of the autolytic enzyme and reagents used in the characterization of the cell walls: ↓, autolytic enzyme; →, trichloroacetic acid; →, sodium metaperiodate; →, nitrous acid; →, formamide; →, sodium hydroxide and hydrochloric acid. Sephadex G-200 and DEAE-Sepharose. Whether these differences are due to the presence of additional enzymes operating during deoxycholate-induced lysis or simply to different extents of hydrolysis cannot be answered at present.

The enzymatic activity in our enzyme preparations must be very nearly the same as that responsible for deoxycholate-induced lysis of whole cells, since the choline-containing fraction of enzyme products obtained from purified cell walls with cell-free autolytic enzyme is very similar to the choline-containing products of deoxycholate lysis. Our results provide a confirmation of the proposal by Dubos (10) that deoxycholate lysis results from the action of an autolytic enzyme.

Autolytic Sensitivity and Primary Structure of Teichoic Acid—One of the main purposes of this study was to gain more precise information concerning the role of choline-containing cell wall structures in cell lysis. Our results prove that a change in the primary structure of the teichoic acid of the cell wall, the substitution of ethanolamine for choline (9), renders the wall resistant to hydrolysis by the autolytic enzyme. Although we cannot describe in detail the mechanism of this resistance, it seems clear that a rather precise relationship between the teichoic acid and peptidoglycan components is required for normal functions.

Whether ethanolamine confers resistance by altering some feature of an enzymatic recognition site or by causing a gross distortion of the orientation of wall polymers, or perhaps both, cannot be decided on the basis of present information. As further examples of the importance of the structure of teichoic acids to cellular functions, one should recall the effect of alanine-containing teichoic acids on the streptolysin sensitivity of streptococci (27) and the requirement for glucosylation of teichoic acid for the adsorption of a Bacillus subtilis bacteriophage (28, 29).

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Choline-containing Teichoic Acid As a Structural Component of Pneumococcal Cell Wall and Its Role in Sensitivity to Lysis by an Autolytic Enzyme

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