STUDIES ON THE IMMUNOCHEMISTRY OF STREPTOCOCCAL MUCOPEPTIDE*

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The cell walls of Gram-positive bacteria are complex structures which contain somatic antigens such as teichoic acids (1–3), polysaccharides (4–6), and teichuronic acids (7), and in many instances complete extraction of these antigens yields an insoluble residue composed almost entirely of mucopeptide (8). The chemical composition of mucopeptide is primarily limited to N-acetylglucosamine, N-acetylmuramic acid, and a few amino acids including alanine, glutamic acid, glycine, lysine, and diaminopimelic acid, although some variation in amino acid content is noted among the different species of bacteria (9, 10). The macromolecular structure of mucopeptide consists of a polymer of repeating units of N-acetylglucosamine and peptide-linked N-acetylmuramic acid. The peptides of the repeating units are further cross-linked by peptide bridges which connect the separate backbone hexosamine polymers to form the rigid structure of bacterial cell walls (11, 12).

Although the immunochemical features of the cell wall antigens have been studied in detail, little attention has been directed to the immune response to mucopeptides or to their immunochemical features. In view of the widespread occurrence of mucopeptide among all species of bacteria, it is conceivable that mucopeptide antibodies may be an important element in various aspects of the host response to infections, such as immunity and hypersensitivity. Although previous reports have demonstrated antibodies in rabbits hyperimmunized with bacterial vaccines (13, 14), the immunochemical basis for the antigenic specificity has not been elucidated. This report describes a precipitin reaction between streptococcal mucopeptide and mucopeptide antibodies and defines in immunochemical terms an antigenic basis for the specificity of this reaction.

Materials and Methods

Strains of Streptococci.—Streptococcal strains were obtained from Dr. R. C. Lancefield, the Rockefeller University.

Preparation of Cell Walls.—Cell walls were prepared from Groups A (S43/100), A-variant, (T27A), B (090R), C (H46A), D (D76), F (H127), and G (B549) streptococci according to the method of Bleiweis et al. (15).

Preparation of Mucopeptide.—Mucopeptides were extracted and prepared from cell walls by the hot formamide method of Fuller (16) as described by Krause and McCarty (8).

Cell Wall Lytic Enzymes.—Egg-white lysozyme was obtained from Mann Research Laboratories, New York. The *Streptomyces albus* enzymes were prepared as described by McCarty (17).

Analytical Methods.—Quantitative analysis of hexosamines and amino acids were determined by the method of Moore et al. (18, 19) and employed the Beckman, Spinco model 120B amino acid analyzer. 10 mg of a dried sample was placed into an acid-cleaned pyrex tube (8 × 125 mm) and dissolved in 5.0 ml of 4 N HCl. The tube was sealed and hydrolyzed at 105°C for 14 hr in a constant temperature chromatographic oven. The hydrolyzed content was then transferred to a 50 ml beaker and evaporated to dryness at room temperature in a vacuum dessicator containing NaOH pellets. The dried sample was rehydrated with distilled water and again evaporated to dryness. After this procedure was repeated three times to remove the remainder of the HCl, the dried sample was dissolved in 0.2 M sodium citrate, pH 2.2. A 1 ml (1 mg/ml) sample was employed for analysis. Correction for the minimal degradation of hexosamines which was encountered during the HCl hydrolysis of mucopeptide was achieved by determining the rates of decomposition of both amino sugar standards under identical hydrolysis conditions. A correction value was employed to calculate the muramic acid and glucosamine content of the mucopeptide.

Ion-Exchange Chromatography of S. albus Enzymes Lysate.—Diethylaminoethylcellulose (DEAE, Gallard-Schlesinger Chemical Manufacturing Corp., New York, 0.68 meq/g) columns were prepared by the method previously described by Young for the fractionation of bacterial cell wall components (20). DEAE-cellulose was regenerated by washing with a solution containing 0.125 M NaOH and 0.125 M NaCl. A slurry was employed to construct a 300 × 25 mm column which was packed with 5 lb./sq in. nitrogen pressure at room temperature. The column was washed with six volumes of 0.4 HCl and equilibrated with 0.02 M (NH₄)₂CO₃, pH 8.6, and stored at 4°C.

Between 50 and 100 mg of dialyzable material obtained from an *S. albus* enzymes digest of cell walls was dissolved in 10 ml of 0.2 M (NH₄)₂CO₃ and applied to the DEAE-cellulose column which was then eluted at 4°C with 0.02 M (NH₄)₂CO₃ buffer at a flow rate of 30 ml/hr. The eluate was collected in 3 ml fractions, and the eluted material was detected in the fractions by measuring the optical density at 220 mμ in a spectrophotometer. After fractions were obtained which exhibited an absorbency of less than 0.10, the next elution step with a higher concentration of buffer was initiated. The successive elution steps were 0.02, 0.05, 0.075, 0.10, 0.15, 0.20, 0.30, and 0.50 M (NH₄)₂CO₃ buffer.

Gel Filtration of Cell Wall Products.—The cell wall materials recovered in the fractions from the DEAE column were subjected to gel filtration (21). A distilled water slurry of Sephadex G-25 or G-50 (Pharmacia Fine Chemicals, Uppsala, Sweden) was poured into a 700 × 10 mm column and washed with approximately 12 volumes of distilled water. A 1 ml sample was added to the column and eluted with distilled water. The optical density of 3 ml fractions was measured in the spectrophotometer at 220 mμ. Samples were also eluted off the Sephadex column with 0.02 M tris buffer pH 7.0.

Serological Methods.—Quantitative precipitin analyses were performed according to a modification of a method of McCarty (22) as described by Abdulla and Schwab (14). The antigen-antibody precipitate after washing with cold saline was dissociated with 0.1 N NaOH and assayed photometrically at 280 mμ. Correction for the slight opalescence of the dissolved precipitate which is derived from the mucopeptide was achieved by subtracting the 320 mμ value from the 280 mμ value. With most sera at antigen-antibody equivalence, the optical density at 320 mμ was approximately 20% of the value at 280 mμ.

The capillary precipitin test for the detection of mucopeptide antibodies was performed in a manner similar to that employed by Swift, Wilson, and Lancefield for the detection of group-specific antibodies (23).

Antimucopeptide sera were obtained from rabbits which had been immunized intravenously with particulate cell wall mucopeptide. The mucopeptide, free of nearly all group carbohydrate, was obtained by acid extraction of cell walls at pH 0.5 at 100°C for 30 min. The immunization schedule was similar to that employed by McCarty and Lancefield (24) to raise streptococcal group-specific antibodies.

Certain streptococcal grouping antisera which contained mucopeptide antibodies were generously supplied by Dr. R. C. Lancefield, the Rockefeller University.

Mucopeptide which had been solubilized by ultrasonic treatment was employed in the precipitin tests. The mucopeptide, suspended in distilled water, was cooled by an ice-bath during treatment for 15 min in a 20 kc sonic oscillator.

Lysozyme digestion was also employed to solubilize the mucopeptide antigen. Mucopeptide, suspended in 0.07 M phosphate buffer (pH 7.4) containing 125 μg/ml of lysozyme, was incubated at 37°C for 24 hr. After incubation, the lysate was deproteinized with chloroform according to a method described by Heymann et al. (25). The final product was employed in mucopeptide-precipitin reactions.

RESULTS

Chemical and Antigenic Properties of Mucopeptide.—The mucopeptides extracted by the hot formamide method from cell walls of various groups of streptococci exhibited a similar chemical composition. Depicted in Table I are the results of chemical analyses of Groups A-variant, B, C-variant, F, and G streptococcal mucopeptide residues. It is to be noted that the mucopeptides isolated from the several different groups possessed identical chemical constituents, and the mole ratios are consistent with the previous findings of other investigators (26–29). In each case there are approximately 4 moles of alanine for each mole of glutamic acid and lysine. In general, the mole concentration of glucosamine is somewhat greater than that of muramic acid.

An antibody response to mucopeptide was achieved by the immunization of rabbits with mucopeptides of Groups A and C streptococci. Mucopeptide antibodies were detected in the sera collected at 7-day intervals by the capillary precipitin method. During the course of immunization an increase in precipitin activity of the rabbits' sera corresponded to an increase in proteins in the γ-globulin region of the electrophoretic pattern. Immunoelectrophoresis studies confirmed the γG-globulin nature of the antimucopeptide precipitins. Immune serum was placed into the central well of an immunodiffusion slide and, after

electrophoresis, solubilized mucopeptide was placed into one trough and specific rabbit anti-γG into the other. A distinct precipitin line developed between the mucopeptide and antiserum in an area comparable to that which developed between anti-rabbit γG and rabbit γ-globulin.

Depicted in Fig. 1 are the results of quantitative precipitin tests obtained between Groups A, B, C, and F mucopeptides and Group A-variant serum rich in mucopeptide antibodies. It is immediately apparent that mucopeptides of the various groups react with antibodies raised against Group A-variant mucopeptide. This finding suggests that an antigenic determinant is shared by the different mucopeptides and is consistent with the fact that these mucopeptides have a similar chemical composition.

TABLE I

Chemical Composition of Formamide-Extracted Mucopeptide of Groups A-variant, B, C-variant, F, and G Streptococci

Components	Group				
	A-variant	B	C-variant	F	G
	μmole/mg				
Glucosamine	0.537	0.704	0.558	0.663	0.557
Muramic acid	0.383	0.664	0.531	0.573	0.390
Alanine	2.476	2.530	2.564	2.541	2.480
Glutamic acid	0.640	0.762	0.784	0.659	0.624
Lysine	0.602	0.666	0.605	0.659	0.616
Glycine	0.028	0.024	0.034	0.022	*
Ammonia	1.034	1.420	1.149	1.488	1.254

* Trace amounts.

Mucopeptides are made up of three principal moieties: the hexosamine polymer consisting of repeating units of N-acetylglucosamine and N-acetylmuramic acid; peptides linked to the polymer through the muramic acid residues; and amino acid or peptide bridges which cross-link the peptides associated with adjacent hexosamine polymers. It is conceivable that any one of these components, or a combination of them, may function as an antigenic determinant. Methods were devised to isolate an inhibitory haptenic fraction from purified mucopeptide which was enzymatically digested by the *S. albus* enzymes. These enzymes have been shown to solubilize the mucopeptide with the release of the hexosamine fractions and peptides (17).

Degradation Products Released from Cell Walls by Enzymatic Treatment.—Previous studies have shown that, in the case of *Micrococcus lysodeikticus*, lysozyme solubilizes mucopeptide by hydrolyzing the 1,4 glycosidic linkage of the hexosamine polymer with the release of glucosamine–muramic acid–peptide

units of varying sizes (30). In the work reported here, mucopeptide solubilized with lysozyme retained serologic reactivity with mucopeptide antiserum.

In contrast to these results achieved with lysozyme, *S. albus* enzymes completely destroyed the serological reactivity of the mucopeptide. Furthermore, gel filtration studies indicate that the bulk of the mucopeptide following *S. albus* enzymes digestion consists of heterogeneous fragments considerably smaller than the mucopeptide solubilized by lysozyme treatment or sonic treatment. The results of gel filtration on Sephadex G-50 of sonically disrupted mucopeptide and lysozyme and *S. albus* enzymes digests of mucopeptide are illustrated in Fig. 2. Mucopeptide solubilized by sonication was eluted off the Sephadex G-50 column at void volume. Although the bulk of the mucopeptide treated
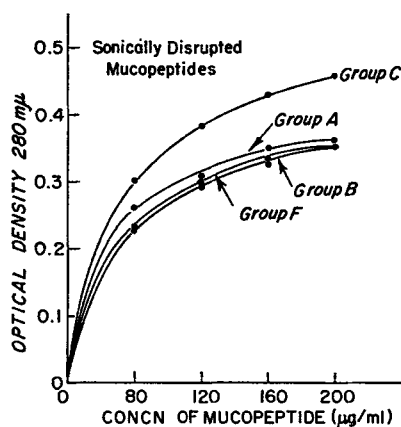

FIG. 1. Quantitative precipitin reaction between sonically disrupted mucopeptides of Groups A, B, C, and F streptococci, and mucopeptide antiserum.

with lysozyme was eluted at void volume, appreciable material was retarded on the column. Thus a portion of the mucopeptide has been digested to heterogeneous units which are smaller than the sonically disrupted mucopeptide. In contrast to these results, all mucopeptide hydrolyzed with *S. albus* enzymes was eluted after void volume. The broad peak of the retarded elution pattern indicates a marked degree of fragmentation of the mucopeptide into small heterogeneous components which are retarded to a greater degree than those obtained with lysozyme.

The presence of a haptenic inhibitor of the mucopeptide precipitin reaction in the *S. albus* enzymes digest of mucopeptide was detected by quantitative precipitin-inhibition tests. The mucopeptide fractions from Groups B and G, collected after void volume off a Sephadex G-50 column as depicted in Fig. 2, were employed as inhibitors of the precipitin reaction between Group B mucopeptide

and mucopeptide antiserum. As depicted in Fig. 3, Groups B and D mucopeptide fractions in concentrations as low as 6 mg/ml markedly inhibited the reaction. Although a preparation of *S. albus* enzymes contains a lysozymelike enzyme, it is unlikely that this enzyme destroys the reactivity of the mucopeptide because egg-white lysozyme is ineffective in this respect. On the other hand, another of the *S. albus* enzymes, *N*-acetylmuramyl L-alanine amidase, splits off the
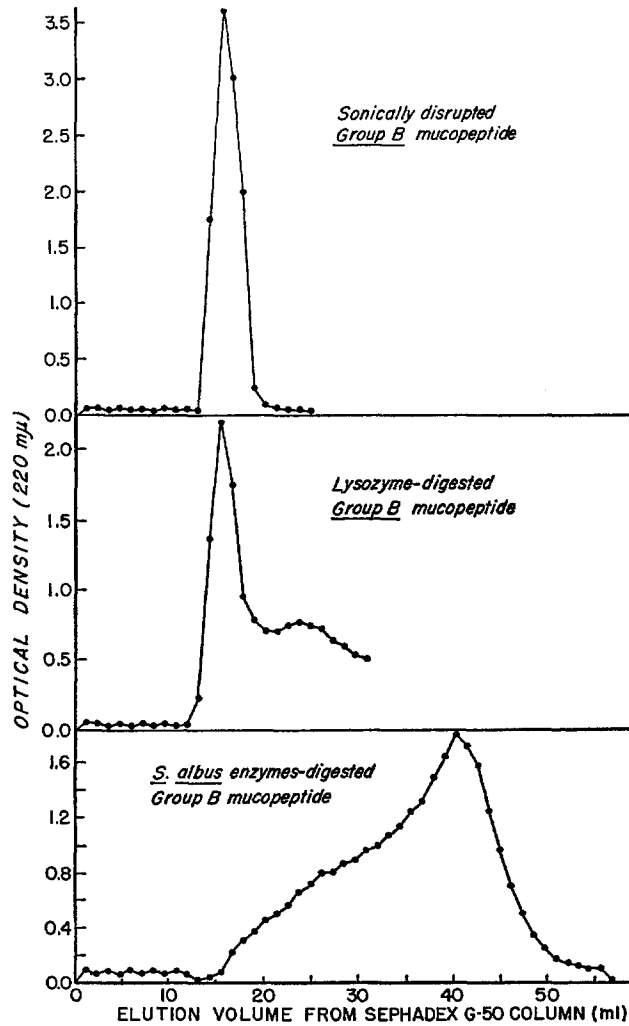

FIG. 2. Gel filtration on a Sephadex G-50 column of sonically disrupted, lysozyme-digested, and *S. albus* enzymes-digested Group B mucopeptide preparations. Column dimensions: 700 × 10 mm.

peptide moiety linked to the muramic acid (31). It is thus conceivable that this amidase may have released the peptide from the hexosamine polymer and eliminated the precipitin reactivity of the mucopeptide. This possibility was investigated in the following experiment.

Isolation of Inhibitory Hapten from an S. albus Enzymes Digest of Mucopeptide.—900 mg of Group D cell walls, after treatment with 10% trichloroacetic acid at 4°C to remove the bulk of the group and type antigens, were suspended in 25 ml of 0.005 M ammonium acetate (pH 8.0). 25 ml of concentrated *S. albus* enzymes preparation was added to the cell wall suspension and the mixture was incubated at 45°C for 24 hr according to the method of McCarty (17). The digest was dialyzed in cellophane tubing against frequent changes of distilled
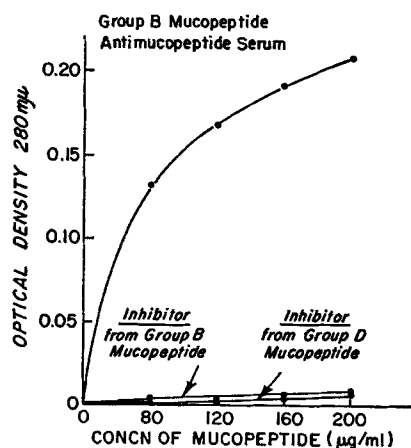

FIG. 3. Quantitative precipitin inhibition of the Group B mucopeptide reaction by dialyzable *S. albus* enzymes digests of Groups B and D mucopeptide.

water. The dialysates were pooled, concentrated, and lyophilized. In the majority of the experiments the lyophilized dialyzable product was resuspended in a small volume of 0.02 M (NH$_4$)$_2$CO$_3$ and added to the DEAE-cellulose column. Twelve fractions were obtained using (NH$_4$)$_2$CO$_3$ buffer with increasing molarities from 0.02 to 0.5 M. However, upon analyzing the fractions for inhibitory activity by the capillary precipitin method, it was found that only fractions II and III, which were eluted off the DEAE-cellulose column with 0.02 M (NH$_4$)$_2$CO$_3$, possessed mucopeptide inhibitory properties. Depicted in Fig. 4 is the elution pattern from the DEAE-cellulose column with 0.02 M (NH$_4$)$_2$CO$_3$. The three major fractions eluted at this buffer concentration were designated fractions I, II, and III.

In the case of several selected mucopeptide antisera, fraction I, which represents the fall-through peak, possessed no inhibitory activity, whereas fraction
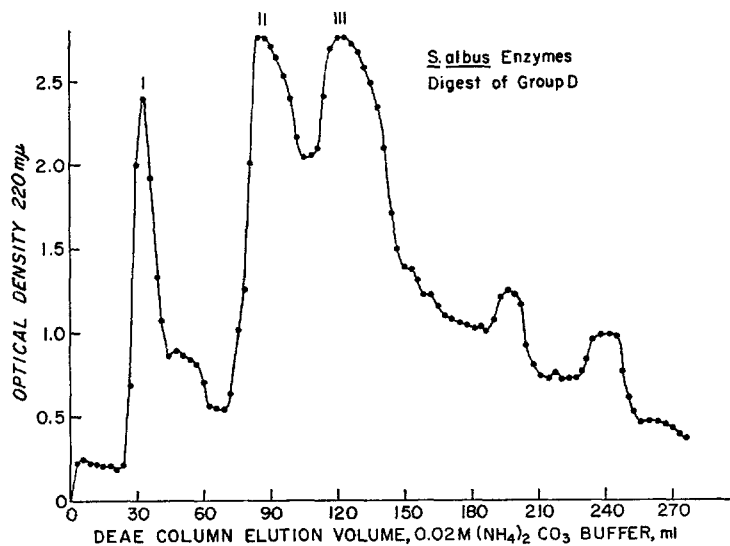
Fig. 4. DEAE-cellulose column chromatography of an *S. albus* enzymes digest of Group D cell walls. Column dimensions: 300 × 25 mm. Buffer: 0.02 M ammonium carbonate (NH₄)₂CO₃, pH 8.6 at 4°C.
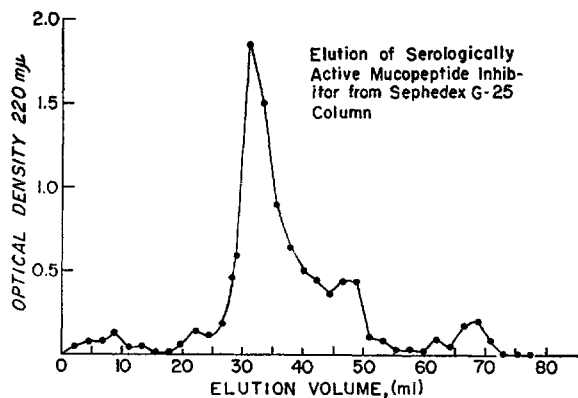

Fig. 5. Gel filtration on a Sephadex G-25 column of the fraction III eluted from DEAE-cellulose column depicted in Fig. 4. This fraction was an inhibitor of the mucopeptide precipitin reaction. Column dimensions: 700 × 10 mm.

III was a very potent inhibitor. Fraction II was inhibitory, but less active than fraction III. Fraction III was further purified by gel filtration. Illustrated in Fig. 5 is the elution pattern of fraction III on a Sephadex G-25 column, which had a void volume of 19.0 ml as determined by the elution of lysozyme (mol wt 14,700). This elution pattern clearly suggests that the molecular weight was

less than 5000. Depicted in Fig. 6 is the pattern obtained when the pooled product from the column of Fig. 5 is subjected to repeated DEAE chromatography. Fraction III was noted to be the major peak which was eluted by 0.02 M
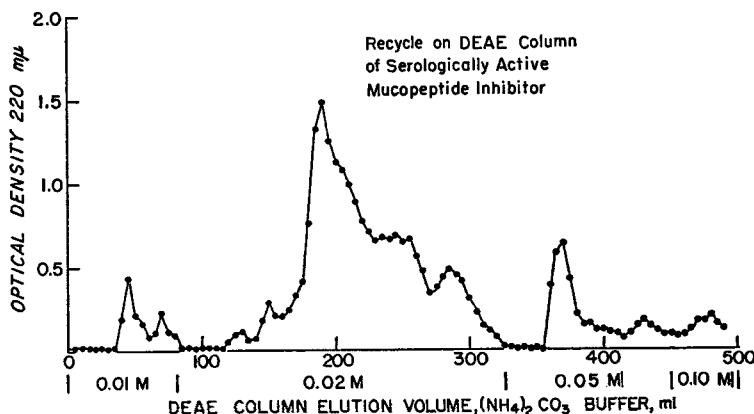

FIG. 6. DEAE-cellulose column chromatography of the inhibitory material collected from the pooled peak fractions depicted in Fig. 5. Column dimensions: 300 × 25 mm.
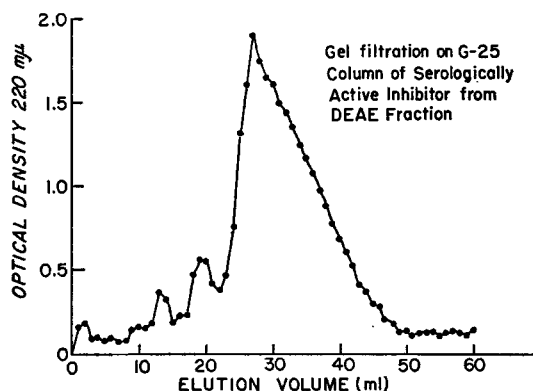

FIG. 7. Gel filtration on a Sephadex G-25 column of the inhibitory material collected in the peak fractions eluted from the DEAE column depicted in Fig. 6 with 0.02 M $(NH_4)_2CO_3$. Column dimensions: 700 × 10 mm. The peak fractions off this Sephadex column were pooled and designated purified fraction III. This material was employed in subsequent chemical and immunological analyses.

$(NH_4)_2CO_3$ buffer and only minimal material was eluted at volumes similar to that for fractions I and II. The buffer was removed from the pooled material containing fraction II, and the product, again purified by G-25 gel filtration, is illustrated in Fig. 7. The peak fractions illustrated in this figure were pooled and

employed in subsequent chemical and immunological analyses. In a similar manner, DEAE fractions I and II were also purified by G-25 gel filtration and recycled through DEAE-cellulose and again on a Sephadex G-25 column. The purified fractions, I, II, and III, were tested for serological inhibitory activity. Fraction I had minimal inhibitory activity, fraction II was inhibitory, but fraction III was a markedly active inhibitor.

Depicted in Table II are the results of the chemical analyses of Group D cell

TABLE II

Chemical Composition of Dialyzable Fractions of Group D Cell Walls Treated with S. albus Enzymes

Components	Cell walls	Elutions of DEAE-cellulose column		
		Fraction I	Fraction II	Fraction III
μmole/mg				
Glucosamine	0.533	1.080	0.118	0.030
Muramic acid	0.239	0.990	0.111	0.026
Alanine	1.360	0.174	4.934	5.267
Glutamic acid	0.330	0.052	1.308	1.448
Lysine	0.328	0.052	1.389	1.372
Aspartic acid	0.029	0.006	0.008	—
Threonine	0.015	—	0.009	—
Serine	0.015	0.011	0.007	—
Glycine	0.024	0.010	0.022	0.022
Valine	0.019	—	0.011	—
Isoleucine	0.011	—	—	—
Leucine	0.020	—	0.006	—
Tyrosine	0.008	—	—	—
Phenylalanine	0.009	—	—	—
Galactosamine	0.278	—	—	—
Ammonia	0.485	1.381	2.009	1.613
Total recovery, %	44.9*	50.3*	92.8	92.6

* These recovery values do not include rhamnose, glucose, and galactose.

walls and purified fractions I, II, and III. As illustrated in this table, fraction III was composed of alanine, glutamic acid, and lysine, with only trace amounts of hexosamines. Thus the major peptide moiety of the mucopeptide has been isolated in this fraction. The original amino acid chromatographic analysis of fraction III is illustrated in Fig. 8. It should be noted that only the major amino acids of mucopeptide were detected in this chromatogram. These amino acids plus ammonia represent approximately 92% of the total weight of the sample applied to the column. Fraction I was composed primarily of glucosamine and muramic acid in a mole ratio of 1:1 and had a minimal concentration of amino
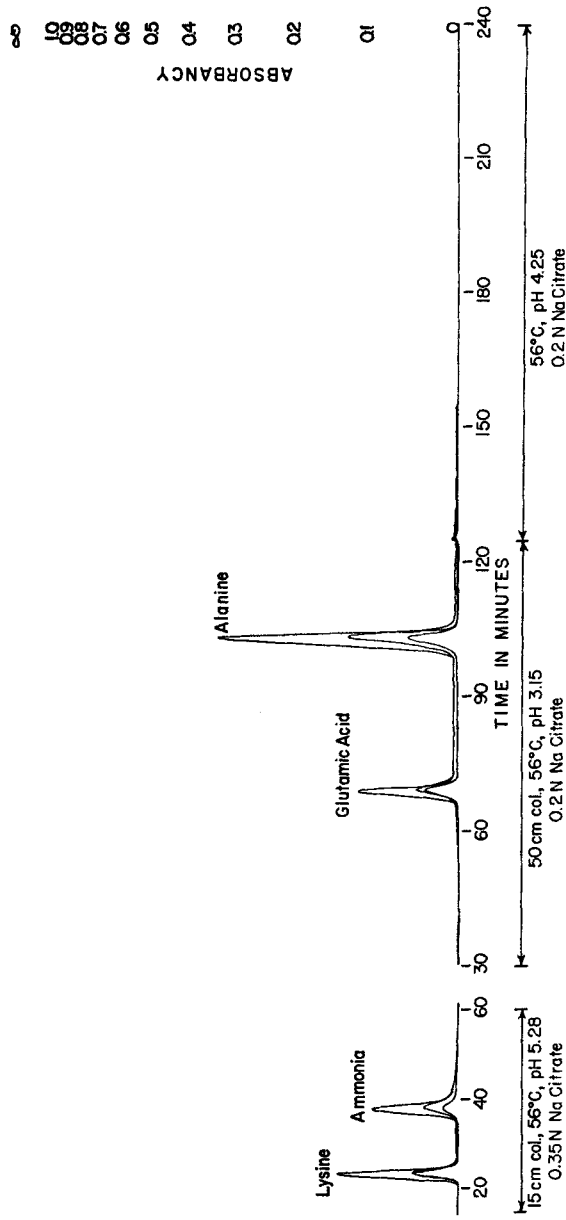
FIG. 8. Analysis of a hydrolysate of purified fraction III. Hydrolysis was carried out with 4 N HCl at 105°C for 14 hr. Columns were prepared with Beckman custom spherical research resin AA-27, and automatic recording equipment was employed.
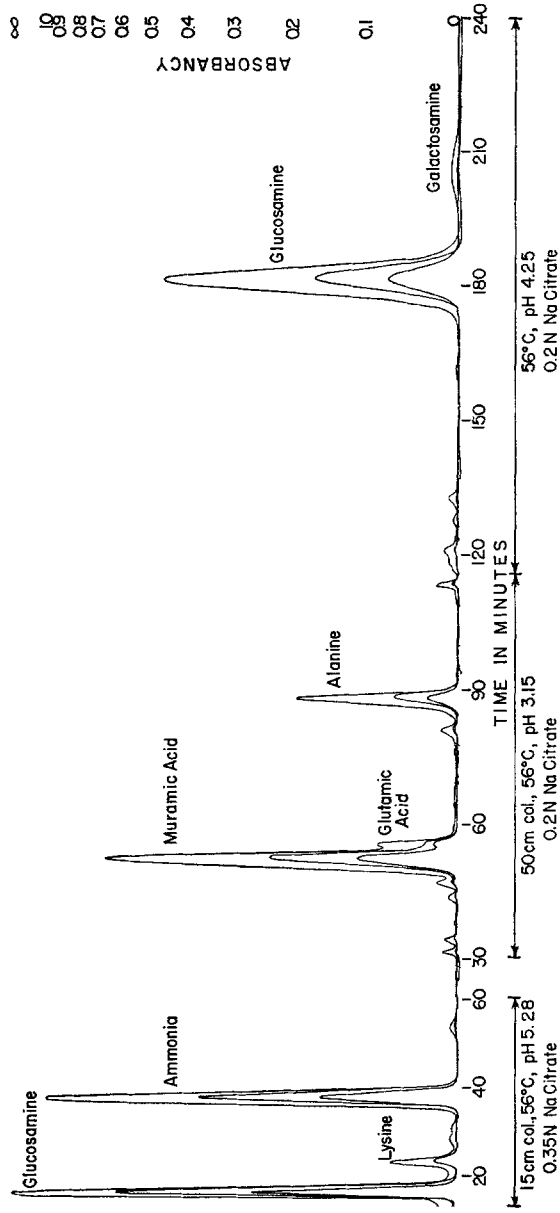
FIG. 9. Analysis of a hydrolysate of purified fraction I. Hydrolysis was carried out with 4 N HCl at 105°C for 14 hr. Conditions of chromatography were similar to those employed for fraction III.

acid residues. The original chromatographic analysis of this fraction is depicted in Fig. 9. This fraction had minimal inhibitory activity. The bulk of fraction II consists of amino acids, but there is an appreciable content of hexosamines. This fraction inhibits the precipitin reaction, but it is less active than fraction III.

Illustrated in Fig. 10 are the results of mucopeptide quantitative precipitin inhibition tests using purified mucopeptide fractions I and III. As depicted in this figure, as little as 2 mg/ml of fraction III inhibited 90% of the reaction between Group B mucopeptide and a mucopeptide antiserum. Fraction I, which was essentially devoid of peptide residues, gave minimal inhibition. These results clearly suggest that the peptide residue is a major antigenic determinant of mucopeptide.
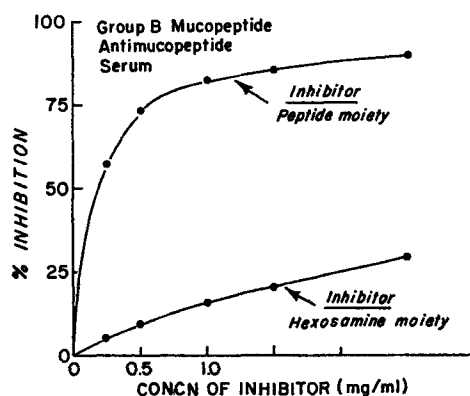

FIG. 10. Inhibition of quantitative mucopeptide precipitin reaction with purified fraction III, the peptide rich moiety, and fraction I, hexosamine rich moiety.

DISCUSSION

Streptococcal mucopeptide extracted from cell walls by the hot formamide method is composed of N-acetylglucosamine, N-acetylmuramic acid, alanine, glutamic acid, lysine, and small variable amounts of glycine. The ratio of glucosamine to muramic acid approaches one in mucopeptides derived from the various streptococcal groups. Although the amino acid composition may vary to some extent from strain to strain, alanine, lysine, and glutamic acid approach a mole ratio of approximately 4:1:1, a finding which is consistent with previous reports (26–29).

A general pattern has emerged for the macromolecular structure of mucopeptide, which is probably shared by most bacteria. Ghuysen and Salton (32) isolated a disaccharide composed of N-acetylglucosamine, N-acetylmuramic acid, and a peptide from *Micrococcus lysodeikticus* cell walls which had been digested with lysozyme. These and other studies have suggested that mucopeptide contains a hexosamine polymer which consists of repeating units of the disaccha-

ride. The peptide is linked to the backbone polymer through the muramic acid and, in turn, is cross-linked by an amino acid or peptide bridge.

Evidence from several sources indicates that the structure of the mucopeptide of hemolytic streptococci conforms to this general pattern. For example, Heymann et al. (25) isolated a disaccharide of the two amino sugars with an alanine substituent from Group A mucopeptide prepared by hot formamide extraction.

The studies described here focus attention on the relationship between the chemical structure and the antigenic specificity of the mucopeptide subunits. Digestion of cell walls of formamide-extracted mucopeptide yields, upon treatment with the S. albus enzymes, a peptide which has potent haptenic inhibitory properties when added to the mucopeptide precipitin reaction. This peptide consists of alanine, lysine, and glutamic acid in a mole ratio of 4:1:1. Release of this peptide from the mucopeptide is undoubtedly dependent upon the action of an N-acetylmuramyl L-alanine amidase, which was identified by Ghuysen (31) as one of the S. albus cell wall lytic enzymes.

While these findings identify the peptide as one antigenic determinant of the mucopeptide, preliminary studies indicate that the hexosamine polymer in certain instances is also antigenic. The precipitin reaction achieved with selected mucopeptide antisera is significantly inhibited by a mucopeptide fraction which contains a small percentage of peptides but is rich in hexosamines. Thus, immunization of animals with vaccines of bacteria or mucopeptide results in the production of antibodies directed against either the peptide moiety which is linked to the muramic acid, or against the hexosamine polymer.

The biological significance of the immune response to mucopeptide is not clear. There is, as yet, no evidence that mucopeptide antibodies have a protective effect against challenge with homologous or heterologous bacteria. Mucopeptide injected into the skin of rabbits induces severe necrotic lesions, but the pathogenesis may be dependent upon a toxic process rather than a hypersensitivity phenomenon (33). Rotta et al. (34) injected rabbits intravenously with solubilized mucopeptide and achieved a febrile response similar to that observed with endotoxin. It is conceivable that the tolerance induced in rabbits injected with repeated doses of mucopeptides (34, 35) may be dependent upon mucopeptide antibodies.

SUMMARY

Streptococcal mucopeptide, solubilized by either ultrasonic treatment or lysozyme, gave a precipitin reaction with rabbit antimucopeptide serum. A haptenic inhibitor of this reaction, which was composed of alanine, glutamic acid, and lysine in a mole ratio of 4:1:1, was isolated from a Streptomyces albus enzymes digest of Group D cell walls by ion exchange chromatography. When selected antisera were employed, greater than 90% inhibition of the mucopeptide quantitative precipitin reaction was achieved with 2 mg/ml of this inhibi-

tor, whereas a hexosamine fraction with minimal concentrations of amino acid residues was inactive in this respect. These results suggest that the peptide moiety is an antigenic determinant of mucopeptide.

Preliminary results indicate that the hexosamine polymer of the mucopeptide is a secondary antigenic determinant.

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