Isolation and Study of the Chemical Structure of Low Molecular Weight Glycopeptides from Micrococcus lysodeikticus Cell Walls*

(Received for publication, February 23, 1967)

DAVID MIRELMAN† AND NATHAN SHARON
From the Department of Biophysics, The Weizmann Institute of Science, Rehovoth, Israel

SUMMARY

A preparative method for the isolation of oligosaccharide peptides from lysozyme digests of Micrococcus lysodeikticus cell walls is described. The method involves chromatography on Dowex 1, gel filtration on Bio-Gel P-4 columns, and chromatography on Dowex 50. Four glycopeptides designated as GP-1, GP-1a, GP-2, and GP-4 were isolated and their structures investigated by chemical and enzymatic techniques. GP-2 which comprises about 2% of the weight of the wall, is a disaccharide pentapeptide of the following structure, where Mur stands for muramyl.

\[
\text{GlcNAc} (1 \rightarrow 4) \text{MurNAc-L-Ala-D-Ala-Glu-Gly}
\]

GP-4, present in smaller amounts, possesses the same structure as GP-2, with the exception of the acetyl group on muramic acid. GP-1a, present in very small amounts, is apparently a tetrasaccharide pentapeptide, the tetrasaccharide moiety of which is identical with the cell wall tetrasaccharide GlcNAcβ(1 \rightarrow 4) MurNAcβ(1 \rightarrow 4) GlcNAcβ(1 \rightarrow 4) MurNAc; the peptide moiety linked to the muramic acid forming the terminal reducing end of this tetrasaccharide is identical with the peptide moiety of GP-2. In addition, small amounts of an unusual disaccharide, GlcNAcβ(1 \rightarrow 4) Mur, have also been isolated.

Bacteria are enclosed in a rigid cell wall, the principal structural components of which are glycopeptides (peptidoglycans)

* This investigation was supported by Grant AI 03528 from the National Institutes of Health, United States Public Health Service.
† This paper is part of a thesis to be submitted by David Mirelman to the Feinberg Graduate School of the Weizmann Institute of Science, in partial fulfillment of the requirements for a Ph.D. degree.

(1, 2). These are highly insoluble polymeric substances usually composed of two amino sugars, N-acetyl-D-glucosamine (2-acetamido-2-deoxy-D-glucose) and N-acetylmuramic acid (2-acetamido-2-O-(\text{-carboxyethyl})-2-deoxy-D-glucose) together with a limited number of amino acids, most commonly D-glutamic acid, L-lysine, D- and L-alanine, and glycine.

Knowledge of the chemical structure of bacterial cell wall glycopeptides is derived mainly from studies of fragments obtained by digestion of the isolated cell walls with lysozyme or other lytic enzymes. In previous studies (1) the fragments were isolated by paper chromatography and paper electrophoresis, yielding small amounts of material which were often insufficient for complete chemical characterization. The development of a method for the preparation of gram quantities of Micrococcus lysodeikticus cell walls (3), and application of ion exchange chromatography to the fractionation of lysozyme digests of these walls, led to the isolation of the disaccharide GlcNAcβ(1 \rightarrow 4) MurNAc and a tetrasaccharide GlcNAcβ(1 \rightarrow 4) MurNAcβ(1 \rightarrow 4) GlcNAcβ(1 \rightarrow 4) MurNAc in quantities sufficient for detailed studies of their chemical structure. Some properties of the natural disaccharide, for which a β(1 \rightarrow 6) linkage had originally been proposed, were different from those of an authentic GlcNAcβ(1 \rightarrow 4) MurNAc disaccharide synthesized by Flowers and Jeanloz (4), and it was therefore concluded that the natural disaccharide is β(1 \rightarrow 4) (5-7). The tetrasaccharide, which can serve as a substrate for lysozyme, was found to be a dimer of this disaccharide most likely linked by a β(1 \rightarrow 4) linkage (8).

In the case of Micrococcus lysodeikticus, the di- and tetrasaccharides are the principal components of the dialyzable fraction obtained from lysozyme digests of the cell wall. The other components present in the dialyzable fraction are low molecular weight glycopeptides. Several of these were isolated in 1960 by Ghuyseen and Salton (9). Subsequently Ghuyseen (10) studied two of the compounds in some detail and proposed partial structures for them.

In this communication, we describe a preparative method for the isolation of homogeneous oligosaccharide peptides from lysozyme digests of M. lysodeikticus cell walls, by using a sequence...
of steps which involve chromatography on columns of Dowex 1 (5), gel filtration on Bio-Gel P-4, and chromatography on columns of Dowex 50. Detailed chemical and enzymatic studies were carried out on several of the compounds thus isolated. Two of them were shown to be disaccharide pentapeptides and a third a disaccharide pentapeptide dimer. In addition, two other substances were isolated in small amounts and also investigated. One of them is a tetraeaccharide pentapeptide, and the other a disaccharide, GlcNAc-\(\beta\)(1 \(\rightarrow\) 4) Mur, in which the amino group of muramic acid is not acetylated. The structures assigned to these compounds are presented, and their relation to the chemical structure of the intact wall is discussed. Preliminary communications describing part of this report have appeared (11, 12).

**Materials and Methods**

Cell walls were prepared from cells of *M. lysodeikticus* obtained from Miles Laboratories, Elkhart, Indiana, with the method of Sharon and Jeanloz (3). Egg white lysozyme (twice crystallized) was a product of Worthington Laboratories; Bio-Gel P-4, Dowex 1-X8 (AG 1-X 8, 200 to 400 mesh), and Dowex 50-X2 (AG 50 W-X2, 200 to 400 mesh) were obtained from Bio-Rad Laboratories, Richmond, California.

Spartomyces amylase (N-acetylmuramyl-L-alanine amidase) (10) was a gift of Dr. J. M. Ghuysen; almond emulsin was a product of Koch Light Laboratories (England), hog \(\eta\)-amino acid oxidase was kindly supplied by Dr. Z. Bohak, and lysostaphin (13) was a gift of Dr. P. A. Tavormina. \(\alpha\)-N-Benzoylcarbonyl-L-glutamic acid \(\alpha\)-N-hydroxysuccinimidyl-\(\gamma\)-tert-butyl diester was a gift of Mr. A. Carmel. \(\eta\)-Gluconosamine hydrochloride was purchased from Pfannstiel Laboratories and \(N\)-acetyln-glucosamine was a gift of Parke Davis Company. The latter compound was recrystallized from ethanol before use. \(N\)-acetylmuramic acid was a gift of Dr. R. W. Jeanloz; muramic acid was prepared from *M. lysodeikticus* cell walls by the method of Park and Matsushima (14). Acetic anhydride, tritium-labeled (100 mC per mmole), or acetic-\(1\) \(^{3}\)C anhydride (150 mC per mmole) was obtained from the Radiochemical Centre, Amersham, England. All other materials used were of the highest purity available.

**Dialyzable Fraction**

This fraction was prepared by repeated digestion (three times) of cell walls by lysozyme. In a typical experiment, 10 g of cell walls were incubated for 20 hours at 37°C in the presence of 10 mg of lysozyme in 400 ml of 0.05 M ammonium acetate (pH 6.8) under a thin layer of toluene. The solution was dialyzed against 5 liters of distilled water at 4°C for 24 hours. Dialysis was repeated three times, and the dialyzable fraction freeze dried in a vacuum. The nondialyzable material was reincubated in the presence of lysozyme (20 mg per g of material) under the above conditions, and the solution was dialyzed three times against distilled water. Incubation with lysozyme under the same conditions, followed by dialysis, was repeated a third time. The total dialyzable fractions accounted for approximately 35% of the original weight of the cell walls. Dialysable material (6 g) was fractionated on a Dowex 1-X8 column (2.2 \(\times\) 110 cm) (6, 15) to give a disaccharide (0.98 g), tetrasaccharide (0.78 g), and two glycopeptide mixtures, one emerging with the front (F1, 2.4 g) and the other fraction emerging between F1 and the disaccharide (F2, 0.17 g).

**Chromatography and Electrophoresis**

**Paper Chromatography**—Descending chromatograms were run on Whatman No. 1 paper by using 1-butanol-acetic acid-water (4:1:5, v/v, upper phase), Solvent I; 1-butanol-pyridine-water (6:4:3), Solvent II; chloroform-methanol-acetic acid (95:5:1), Solvent III; isobutyric acid-ammonium hydroxide solution (25% NH\(_3\), w/w)-water (66:1:33), Solvent IV; and 1-propanol-ammonium-hydroxide solution (25% NH\(_3\), w/w) (7:3), Solvent V.

**Thin Layer Chromatography**—This was carried out on glass plates with silica gel (Kieselgel G, Merck), by the ascending method. Rates of migration on paper chromatography and thin layer chromatography are given relative to alanine (\(R_{A\ell}\)) or relative to dinitrophenol (\(R_{DNP}\)).

**Paper Electrophoresis**—This was carried out on Whatman No. 3MM paper under a constant voltage of 50 volts per cm for 1 hour with the following buffer systems: (a) acetic acid, 1.2 M, adjusted with formic acid to pH 1.8; (b) acetic acid, 1 M-pyridine-water, pH 3.5; (c) pyridine, 1.2 M-acetic acid-water, pH 6.5. Rates of migration on paper electrophorograms are given relative to glutamic acid at pH 6.5 (\(M_{GA}\)) and to glucosamine at pH 3.5 (\(M_{G5}\)).

**Amino Acids and Amino Sugars**—These were detected on the paper chromatograms and electrophorograms with ninhydrin (0.5% in acetone). Reducing substances were detected with the alkaline silver nitrate reagent (16), and \(N\)-acetylaminos were detected as fluorescent spots by the method of Sharon and Seifter (17).

**Analytical Techniques**

\(N\)-Acetylamino sugars were estimated by the modified Morgan-Elson reaction (5, 18) with the use of heating times at 100°C of 3 and 35 min, with \(N\)-acetylglucosamine as standard. Reducing sugars were estimated by the ferricyanide method of Park and Johnson (19) with glucosamine hydrochloride as standard, and amino groups by the colorimetric ninhydrin method of Moore and Stein (20), with leucine as standard. Color densities were determined in the Klett-Summerson photoelectric colorimeter using Filter No. 66 for reducing sugars and Filter No. 56 for the ninhydrin test and for \(N\)-acetylaminos sugars. For the colorimetric assay of fractions from columns, aliquots were dried in a vacuum over KOH and H\(_2\)SO\(_4\), to remove interfering acids and buffers. Protein was estimated by measuring the absorbance at 280 m\(\mu\) with a Beckman DU spectrophotometer. Amino acids were determined on the Beckman-Spinco amino acid analyzer after samples had been hydrolyzed for 20 hours in 6 N HCl at 100°C in sealed ampules. Amino sugars were found to be degraded to a large extent under these conditions of hydrolysis and were therefore determined in parallel runs in which hydrolysis was carried out in 2 N HCl for 2 hours at 100°C. Hydrolysates were evaporated under reduced pressure over KOH and H\(_2\)SO\(_4\), before analysis. The amino acid analyzer integration constants for glucosamine and muramic acid were calculated from the colorimetric ninhydrin reaction for samples which had been hydrolyzed under the conditions used for the assay of amino sugars. The integration constants were 12.5 for glucosamine and 17.2 for muramic acid, as compared to 22.85 for glutamic acid.

**Determination of Molecular Weights**—Solutions, 1%, of samples...
in 0.2 M NaCl were run in a Spinco ultracentrifuge at 35,000 rpm at 21°. Calculations were based on the Yphantis equilibrium method (22). Partial specific volumes were calculated from the chemical composition of the compounds as proposed by McMeekin and Marshall (23). The value used for the glycopeptides in this study was \( \bar{v} = 0.69 \).

Nuclear magnetic resonance spectra were measured in D_2O solution with a Varian A 60 spectrometer by using tetramethylsilane as external standard and the chemical shifts are expressed in parts per million.

Radioactive spots on paper were revealed with the Vanguard strip scanner.

Potentiometric titrations were carried out with 0.25 N KOH and a Radiometer titrator at 23° (24).

**Dinitrophenylation**

Samples of glycopeptides (1 to 2 mg) were dissolved in 1 ml of 0.1 M NaHCO_3. A solution of dinitro-1-fluorobenzene, 2:4, in absolute ethanol (0.2 ml, 1%, \( \bar{v}/\bar{v} \)) was added, and the mixture was kept in the dark with shaking overnight at room temperature. After dilution with 7 ml of water, the mixture was extracted with ether in order to remove dinitrophenol. The aqueous layer was acidified with 0.2 ml of 6 \( \times \) HCl and extracted with ether once more. The aqueous phase was then evaporated to dryness and the residue was dissolved in 1 ml of water. An aliquot (0.5 ml) was hydrolyzed in a sealed tube (5 \( \times \) HCl, 6 hours, 100°); the hydrolysate was evaporated to dryness and samples were analyzed (a) by thin layer chromatography with Solvents III and V; (b) by paper chromatography with Solvents I and V; (c) by paper electrophoresis at pH 6.5. The remaining 0.5 ml of DNP-glycopeptide was hydrolyzed in 2 \( \times \) HCl for 2 hours at 100°. This hydrolysate was evaporated to dryness and part of this material was analyzed on the amino acid analyzer; another part was analyzed for DNP-muramic acid by descending paper chromatography in Solvents I and III and by paper electrophoresis at pH 6.5.

Peptide fragments isolated from enzymatic or partial acid hydrolysates of the homogeneous glycopeptides were dinitrophenylated and analyzed in the same manner as described for the intact glycopeptides. All chromatographic and electrophoretic separations were carried out by using as reference compounds commercial DNP-amino acids (Mann Research Laboratories, New York). DNP-glucosamine and DNP-muramic acid used as reference compounds were prepared in the laboratory from the corresponding amino sugars. Whole cell walls were dinitrophenylated according to the method of Ingram and Salton (25).

**Hydrazinolysis of Glycopeptides**

Glycopeptide (2 mg) in anhydrous hydrazine (Fluka, 1 ml) was heated for 7 hours at 100° in an evacuated sealed tube (26). The tube was opened and hydrazine was removed by evaporation to dryness under reduced pressure over H_2SO_4 for 24 hours. The resulting material was applied to the long column on the Beckman-Spinco amino acid analyzer. The terminal amino acids of the glycopeptides which did not react with hydrazine were eluted at their usual place and their quantity was estimated. The other amino acids which were converted to the hydrazide derivatives were not eluted from the column. Control experiments were run with a synthetic dipeptide L-Ala-Gly.

Glutamic acid \( \alpha \)-hydrazide was obtained by reacting anhydrous hydrazine (3 ml) with \( \alpha \)-N-benzoyl-L-glutamic acid (100 mg) under the same conditions as used for the \( \alpha \)-hydrazide. Glutamic acid \( \gamma \)-hydrazide was purified by paper electrophoresis at pH 6.5 (27) and found to give a single spot on paper chromatography (R_A 0.78 in Solvent I). The \( \alpha \) and \( \gamma \)-hydrazides differ significantly in their electrophoretic mobility at pH 3.5 as a result of the difference in the pK values of their COOH groups (28).

**Reduction of Glycopeptides with NaBH_4**

Glycopeptide (2 mg) was dissolved in 1 ml of 0.04 M potassium tetraborate. NaBH_4 (1 mg) was added, and the solution was kept for 2 hours at 4°. To the reaction mixture, 4 \( \times \) HCl (1 ml) was added, and the solution was heated for 2 hours at 100°. The hydrolysate was evaporated to dryness and dissolved in water (0.5 ml). The products were separated on high voltage electrophoresis at pH 3.5 and the spots were detected on the paper with the silver nitrate reagent and with ninhydrin.

**N-Acetylation**

N-Acetylation was performed by a modification of the method of Gilvarg and Katchalski (29). Glycopeptide (3 mg) was dissolved in 0.6 ml of 0.05 M phosphate buffer at pH 6.5 and 2 drops of acetic anhydride were added. The solution was shaken and kept at room temperature overnight, then lyophilized to yield the partially N-acetylated glycopeptide. N-Acetylation was similarly performed with tritium or C\(^{14}\)-labeled acetic anhydride.

**Optical Configurations**

Glutamic Acid—Glycopeptide (30 mg) was hydrolyzed in a sealed tube with 6 \( \times \) HCl for 20 hours at 100°. The hydrolysate was evaporated to dryness, and the amino acids were dinitrophenylated as before. DNP-glutamic acid was isolated from the mixture by chromatography on thin layer plates. The bands corresponding to DNP-glutamic acid (R_f 0.12) in Solvent III were taken off the plates and eluted with glacial acetic acid. The concentration of DNP-glutamic acid in the eluate was determined from measurements of the absorption at 345 m\( \mu \) (e = 17,400) with a Cary spectrophotometer, and optical rotation was measured with a Bendix polarimeter by using 2-cm cells and sodium D line.

Afamine—Samples of peptide (1 mg) isolated from enzymatic or partial acid hydrolysates were hydrolyzed in 6 \( \times \) HCl for 24 hours at 100°, evaporated to dryness, and chromatographed on...
Digestion with Almond Emulsion

Glycopeptide (1 mg) was dissolved in 0.5 ml of 0.05 M ammonium acetate (pH 6.8) and 1 mg of enzyme was added. The mixture was incubated at 37°C for 24 hours; it was then separated by electrophoresis at pH 3.5 and the compounds were detected as fluorescent spots (17). GlcNAcβ(1 → 4) MurNAc served as a control.

Digestion with Streptomyces Amidase

Glycopeptide (1 mg) was dissolved in 0.5 ml of 0.05 M ammonium acetate-acetic acid buffer, pH 5.4, and 0.2 ml of the amidase solution. The mixture was incubated at 37°C for 2 hours, and, following incubation, an aliquot was analyzed by paper electrophoresis and paper chromatography. Under these conditions, 1 ml of the amidase solution releases the disaccharide moiety from 10 μmoles of disaccharide peptide (GP-2).

Digestion with Lysostaphin

Glycopeptide (1 mg) was incubated at 37°C for 20 hours with lysostaphin (0.1 mg, 200 units per mg) in 0.05 M Tris buffer, pH 7.5 in 0.145 M NaCl (13). Digestion mixtures were analyzed by paper electrophoresis and chromatography.

Partial Acid Hydrolysis

Glycopeptides were hydrolyzed in 2 N HCl at 100°C for 15 min. The products were separated by paper electrophoresis at pH 6.5, or pH 3.5, followed by paper chromatography in Solvent I. Peptides were eluted from the paper by water, checked for homogeneity in several electrophoretic and chromatographic systems, and analyzed for composition on the Beckman-Spinco amino acid analyzer.

RESULTS

The main starting material for this study was the glycopeptide mixture which emerges as the first fraction (F1) upon chromatography on a Dowex 1 acetate column of the dialyzable material from lysosome digest of M. lysodeikticus cell walls (5). In some of the experiments, F1 (1 g in 40 ml of 0.05 M ammonium acetate) was incubated under toluene with lysozyme (50 mg) for 24 hours at 37°C. This treatment was found to release from F1 small amounts of di- and tetrasaccharides.

Analysis of F1 by paper chromatography and paper electrophoresis revealed the presence of a number of ninhydrin-positive spots, some of which did not react with the reagent for N-acetylamino sugars. Removal of F1 of the latter compounds, together with other low molecular weight impurities, was effected by gel filtration.

Fractionation on Bio-Gel P-4

F1, 500 mg, previously incubated with lysozyme, was dissolved in 5 ml of water, and applied to a column (110 × 2.3 cm) of Bio-Gel P-4 which had been washed with water. The column was eluted with water and fractions of 4.5 ml were collected at a rate of 50 ml per hour. Aliquots were assayed for N-acetylamino sugars (0.2 ml) by using 35-min heating time, for free amino groups (0.1 ml), and for protein. The results of a typical experiment are given in Fig. 1. It can be seen that protein (lysozyme) emerges first; it is accompanied by some material which gives positive ninhydrin and Morgan-Elson reactions and is thus of glycopeptide nature. The bulk of the glycopeptide emerges after the protein (effluent volume 210 to 250 ml) and the fractions corresponding to this peak (lightly shaded area in Fig. 1) were pooled and used for further studies. Lyophilization of these fractions yielded 160 mg of BG II (32% of F1). Larger yields of BG II (up to 50% of F1) could be obtained by combining some of the earlier fractions as well, but these were contaminated by increasing amounts of lysozyme.

Fractions emerging after the glycopeptide peak (BG III, effluent volume 255 to 310 ml, Fig. 1) contained mainly di- and tetrasaccharides (yield 35 mg, 7% of F1). These compounds were further purified by chromatography on a Dowex 1 acetate column (5) and shown to be identical with the original cell wall di- and tetrasaccharides, GlcNAcβ(1 → 4) MurNAc and its β(1 → 4) dimer, respectively, by their rate of migration on paper chromatograms and electropherograms, and by their quantitative color reactions in the Morgan-Elson test (5).

The last two peaks contained only ninhydrin-positive material and were devoid of acetylamino sugars; together they accounted for approximately 30% of the weight of F1. Analysis of the peaks before and after acid hydrolysis revealed that they contained a variety of free amino acids and ammonia (the latter most likely from the ammonium acetate used in the lysozyme digests).

Fractionation of F1, which had not been incubated with lysozyme gave a pattern which differed from that presented in Fig. 1 in the following respects. (a) Protein was absent; (b) no oligosaccharide peak (BG III in Fig. 1) was observed. In other respects, however, lysozyme-treated and untreated F1 behaved similarly upon fractionation on Bio-Gel P-4.

Analysis of BG II by paper chromatography and paper electro...
This was followed by a linear gradient of increasing pH and buffer started with 200 ml of pyridine-formic acid buffer (0.2 M, pH 2.9).

Tetrasaccharide

prior to analysis by the Morgan-Elson method, 0.2-ml aliquots were dried overnight over KOH. Morgan Elson, O--O; ninhydrin, O—O.

**TABLE I**

Recoveries by weight of fractions from cell walls of *Micrococcus lysodeikticus* digested repeatedly (three times) with lysozyme

Lysozyme digests of *M. lysodeikticus* cell walls were prepared as described in text. F1, disaccharide, and tetrasaccharide were isolated by chromatography of the dialyzable fraction of the digest of a Dowex 1 column (3). The isolation of the compounds GP-1 to GP-4 from F1 is described in the text. Percentages are calculated on the basis of weight of lyophilized crude fraction relative to the weight of lyophilized walls.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Cell wall</th>
<th>Dialyzable material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialyzable material</td>
<td>35</td>
<td>%</td>
</tr>
<tr>
<td>F1 (glycopeptide mixture)</td>
<td>13</td>
<td>37</td>
</tr>
<tr>
<td>GP-1</td>
<td>1.8</td>
<td>5.1</td>
</tr>
<tr>
<td>GP-1a</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>GP-2</td>
<td>2.0</td>
<td>5.7</td>
</tr>
<tr>
<td>GP-2a</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>GP-4</td>
<td>1.0</td>
<td>2.8</td>
</tr>
<tr>
<td>Disaccharide</td>
<td>8</td>
<td>22.9</td>
</tr>
<tr>
<td>Tetrasaccharide</td>
<td>3.2</td>
<td>9.1</td>
</tr>
<tr>
<td>Total isolated compounds</td>
<td>16.2</td>
<td>46.2</td>
</tr>
</tbody>
</table>

phoresis revealed that it is a complex mixture of glycopeptides. Isolation of the single homogeneous glycopeptides from the above mixture could be achieved by ion exchange chromatography on columns of Dowex 50-X2, by using a pyridine-formic acid buffer for elution.

**Chromatography on Dowex 50x2**

A column (83 x 2.3 cm) of Dowex 50-X2 (200 to 400 mesh) was equilibrated with pyridine-formic acid buffer (0.2 M in pyridine, adjusted to pH 2.9 by the addition of formic acid). BG II, 1 gram, was dissolved in 5 ml of water, adjusted to pH 2.2 with formic acid, and added to the column. Elution was started with 200 ml of pyridine-formic acid buffer (0.2 M in pyridine, pH 2.9), and then a gradient with pyridine-formic acid buffer (1.0 M in pyridine adjusted to pH 5.3) was applied (mixing chamber, 1 liter of the starting buffer; reservoir, 1 liter of pyridine-formic acid buffer, 1 M in pyridine, pH 5.3). Fractions of 10.6 ml were collected at a rate of 60 ml per hour, and aliquots were assayed for N-acetylamino sugars (0.2 ml) with 35 min heating time and for ninhydrin-positive material (0.1 ml). The pH was measured in samples taken from every 20th tube.

The results of a typical separation are given in Fig. 2. Three major peaks (GP-1, GP-2, GP-4) and several minor ones are observed. The tubes corresponding to the various peaks were pooled and lyophilized, and the materials obtained dissolved in small amounts of water; the solutions were filtered and lyophilized again. Dissolution in water and lyophiilation were repeated twice in order to ensure the maximal removal of pyridine and formic acid.

The yields of lyophilized fractions at this stage were GP-1, 280 mg; GP-2, 300 mg; and GP-4, 150 mg. Table I summarizes the yields of crude glycopeptides with respect to the starting material, the cell wall. For comparison, the yields of disaccharide and tetrasaccharide obtained (15) are also given. Analysis of the three quantitatively predominant fractions by paper chromatography (Solvents I and IV) and paper electrophoresis revealed that GP-1 and GP-2 contained a major component which gave a positive reaction for amino groups and N-acetylamino sugars. Small amounts of contaminating materials were removed from GP-1 and GP-2 by preparative paper electrophoresis and are denoted as GP-1a and GP-2a, respectively. Their yields based on BG II (from F1 not treated with lysozyme) were approximately 1 to 2% each.

The purified compounds were checked for homogeneity on paper chromatography (Table II) and paper electrophoresis (Fig. 3) and were found to run as single spots. The analytical data for the five compounds isolated are given in Table III.

Small amounts of GP-1 and GP-1a were also isolated from F2 by chromatography on Dowex 50-X2 under the conditions used for chromatography of BG II

**Configuration of Glutamic Acid**

DNP-l-Glutamic acid was isolated following dinitrophenylation of acid hydrolysates of BG II and of GP-1. Optical rotations measured in glacial acetic acid solutions showed values of [M] = +242 and [M] = +233, respectively, as compared to [M] = -253 for DNP-l-glutamic acid in the same solvent (30).

**Color Yields**

In Table IV, the color yields for the various compounds in the modified Morgan-Elson reaction after 3- and 35-min heating time with N-acetylglucosamine as standard are given.
Nuclear Magnetic Resonance

These spectra of GP-1, GP-2, and GP-4 measured in D$_2$O solutions at the same concentrations (20 mg per ml) were similar (Fig. 4) except for the high field region (62 to 2.2 p.p.m.) in which the acetate protons absorb. As the peptide C-methylene protons also absorb in this region, accurate calculations of the number of protons in each peak could not be made, but the spectra could be compared qualitatively. In the spectra of GP-1 and GP-2, two sharp peaks superimposed on broad peaks (the latter presumably due to methylene protons) can be seen in this region, whereas in the spectrum of GP-4 only one such peak is observed. The areas under the sharp peaks in GP-1 and GP-2 on one hand, and in GP-4 on the other hand, were measured and found to be in the approximate ratio of 2:1. At the region of 1.5 p.p.m., three peaks can be seen in each of the three spectra. These peaks most likely belong to the protons of the methyl groups of the two alanines and of the lactyl residue of muramic acid found in these glycopeptides (32).

End Group Analysis

Potentiometric Titrations—From the potentiometric titration curves the number of ionizable groups per molecule and their pK values were calculated and are given in Table V. The nature of these groups was inferred from a correlation of the measured pK value with those given in the literature (28, 33) for the amino acids and amino sugar constituents of the glycopeptides (Table III).

Reduction with Sodium Borohydride—Analysis by paper electrophoresis at pH 3.5 of acid hydrolysates of the different glycopeptides which had been reduced by sodium borohydride showed the presence of only one reducing sugar, glucosamine. Comparison with paper electropherograms of hydrolysates of non-reduced glycopeptides revealed that the reducing spot of muramic acid has disappeared. A ninhydrin-positive spot still appeared in the same region in parallel runs and this could be ascribed to muraminitol. The same compound was found in hydrolysates of the reduced cell wall disaccharide (GlcNAcβ-(1 → 4) MurNAc).

Dinitrophenylation—e-DNP-Lys was found in acid hydrolysates of dinitrophenylated GP-1 and GP-2. Analysis of these hydrolysates on the amino acid analyzer revealed that all the lysine in GP-2 had disappeared and that a peak corresponding to e-DNP-lysine (eluted with ammonia on the short column) had appeared. In GP-1, only half the lysine had disappeared, and lysine and e-DNP-Lys was found in equimolar quantities

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent I</th>
<th>Solvent II</th>
<th>Solvent IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP-1</td>
<td>0.10</td>
<td>0.04</td>
<td>0.54</td>
</tr>
<tr>
<td>GP-1a</td>
<td>0.22</td>
<td>0.06</td>
<td>0.63</td>
</tr>
<tr>
<td>GP-2</td>
<td>0.42</td>
<td>0.16</td>
<td>0.81</td>
</tr>
<tr>
<td>GP-2a</td>
<td>0.89</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>GP-4</td>
<td>0.29</td>
<td>0.08</td>
<td>0.98</td>
</tr>
<tr>
<td>GlcNAcβ-(1 → 4) MurNAc</td>
<td>1.10</td>
<td></td>
<td>0.75</td>
</tr>
</tbody>
</table>

Table II
Rates of migration of compounds on paper chromatography in different solvents

Descending paper chromatography was on Whatman No. 1. Solvent I, 1-butanol-acetic acid-water (4:1:5, v/v, upper phase); Solvent II, 1-butanol-pyridine-water (6:4:3); Solvent IV, Isobutyric acid-ammonium hydroxide solution (25% NH$_3$, w/w) - water (66:1:33); Compounds were revealed by ninhydrin or by fluorescence (17). R$_{AM}$, mobility relative to alanine.

Fig. 3. Migration of glycopeptides on high voltage paper electrophoresis (1 hour, 50 volts per cm) in pyridine-acetate buffers at three different pH values. Spots revealed on paper by ninhydrin: No. 1, unfractionated glycopeptide mixture (BG II); No. 2, GP-1; No. 3, GP-2; No. 4, GP-3; No. 8, GP-4; No. 9, GP-2a.
Table III
Composition and molecular weights of compounds isolated from lysozyme digests of M. lysodeikticus cell walls

Materials were dried at 50° under reduced pressure before analysis.

<table>
<thead>
<tr>
<th>Compound</th>
<th>GP-1</th>
<th>GP-1a</th>
<th>GP-2</th>
<th>GP-2a</th>
<th>GP-4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmoles/mg</td>
<td>µmoles/mg</td>
<td>µmoles/mg</td>
<td>µmoles/mg</td>
<td>µmoles/mg</td>
</tr>
<tr>
<td>Amino sugar</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucosamine</td>
<td>1.01</td>
<td>1.14</td>
<td>1.77</td>
<td>1.92</td>
<td>0.90</td>
</tr>
<tr>
<td>Muramic acid</td>
<td>0.95</td>
<td>1.08</td>
<td>1.74</td>
<td>1.91</td>
<td>0.88</td>
</tr>
<tr>
<td>Amino acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>1.93</td>
<td>2.19</td>
<td>1.66</td>
<td>1.61</td>
<td>1.76</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.88</td>
<td>1.00</td>
<td>0.92</td>
<td>1.00</td>
<td>0.89</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.87</td>
<td>0.99</td>
<td>0.86</td>
<td>0.93</td>
<td>0.91</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.85</td>
<td>0.96</td>
<td>0.82</td>
<td>0.88</td>
<td>0.93</td>
</tr>
<tr>
<td>Acetate</td>
<td>4.40</td>
<td></td>
<td>1.89</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Nitrogen found | 10.9% | 10.6% | 10.8% |
| Nitrogen calculated | 12.0% | 11.7% | 12.2% |
| Molecular weight |       |       |       |
| Found           | 1960  | 864   | 1050  |
| Calculated      | 1886  | 1440  | 952   | 454   | 910   |

Amino acid analysis of acid hydrolysates of DNP-GP-4 revealed that, in addition to the disappearance of all the lysine, muramic acid had also disappeared. A new DNP-derivative was detected in hydrolysates of DNP-GP-4 (paper chromatography: R₄nitrophenol 0.87, Solvent I; R₄nitrophenol 0.73, Solvent V; thin layer chromatography: R₄nitrophenol 0.11, Solvent III; electrophoresis: M₈Gluc 0.50, pH 0.5). In the various electrophoretic and chromatographic systems, this compound migrated at the same rate as authentic DNP-muramic acid. DNP-muramic acid was identified as the only DNP-derivative in acid hydrolysates of dinitrophenylated GP-2a.

Hydrazinolysis—Only alanine and glycine were found in glycopeptides which had been treated with hydrazine. The molar yields of glycine in the three glycopeptides analyzed was 35 to 38%. In a control experiment in which a synthetic dipeptide, L-Ala-Gly was hydrazinolized and analyzed, no alanine was detected, whereas 50% of the glycine was recovered. The molar ratios (uncorrected) of alanine and glycine obtained after hydrazinolysis, taking glycine as 1.00, are given in Table VI.

N-Acetylation

Acetylation of GP-1 and GP-2 under conditions in which only amino groups with pK below 8.0 react (29) did not appreciably affect these glycopeptides as shown by paper electrophoretic analyses of the acetylation mixtures. This indicated that under the conditions of acetylation the ε-amino group of lysine reacted to a small extent only. In the case of GP-4 a new compound was formed which could be separated from unreacted GP-4 by paper electrophoresis at pH 3.0 or pH 6.5. The new compound mi-
FIG. 4. Nuclear magnetic resonance spectra of glycopeptides in D₂O (20 mg per ml) measured with a Varian A-60 spectrometer by using tetramethylsilane (TMS) as external reference. a, GP-2; b, GP-1; c, GP-4.

**TABLE V**

**Ionization constants of glycopeptides obtained by potentiometric titrations**

The pK values were calculated from aqueous titrations with 0.25 N KOH with a Radiometer titrator (24). The theoretical molecular weights given in Table III were used in the calculations.

<table>
<thead>
<tr>
<th>Groups per mole</th>
<th>pK 1.18</th>
<th>pK 10.2</th>
<th>10.79</th>
<th>10.85</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP-1</td>
<td>GP-2</td>
<td>GP-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.18</td>
<td>10.2</td>
<td>1</td>
<td>10.79</td>
<td>10.85</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0.9</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td>2.81</td>
<td>3.60</td>
<td>1.9</td>
<td>3.58</td>
<td>3.43</td>
</tr>
<tr>
<td><strong>Assignment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>e-NH₂ of lysine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₂ of amino sugar</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peptide carboxyl group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

**TABLE VI**

**Molar ratios of amino acids released after hydrazinolysis**

Hydrazinolysis of glycopeptides was carried out as described in text. The dried reaction mixtures were then analyzed on the long column of the Beckman Spinco amino acid analyzer. Molar ratios were calculated taking glycine as 1.00. Yield of glycine was 55 to 38% of the theoretical (see also Reference 34).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>GP-1</th>
<th>GP-2</th>
<th>GP-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.43</td>
<td>1.27</td>
<td>1.00</td>
</tr>
</tbody>
</table>

---

By paper electrophoresis (at three pH values) and on paper chromatography (Solvent I) at the same rate as GP-2. The values of the color yield for this compound (isolated from paper) in the Morgan-Elson assay were close to those obtained for GP-2 (Table IV). GP-4 was also acetylated under the same conditions with C⁻ or tritium-labeled acetic anhydride. On paper chromatography and electrophoresis the spot giving fluorescence (17) and ninhydrin color was found to contain radioactivity as well.
Acetylation of GP-2a under the same conditions either unlabeled or labeled acetic anhydride afforded a product indistinguishable from the cell wall disaccharide GlcNAcβ(1 → 4)MurNAc in its rate of migration on paper chromatography (Solvent I and II) and paper electrophoresis (pH 6.5). The color yields of N-acetylated GP-2a in the Morgan-Elson test were identical with those of the cell wall disaccharide (Table IV). The second product reacted only with the ninhydrin reagent. It was homogeneous on paper chromatography (R_{A1} 0.27 in Solvent I) and paper electrophoresis (M_{OEN} 0.59 at pH 3.5). Amino acid analysis showed that this compound contained alanine, glutamic acid, glycine, and lysine in the molar ratio of 1.94:1.00:0.93:0.90, and can thus be considered as a pentapeptide. Dinitrophenylation followed by acid hydrolysis revealed the presence of ε-DNP-lysine and DNP-alanine.

Amidase digests of GP-1 contained a larger number of products than those of GP-2. One product was identical with the cell wall disaccharide; two other products, both ninhydrin-positive, were also released. One of these migrated on paper electrophoresis as a component obtained from amidase digests of GP-2; the other ninhydrin-positive spot with M_{OEN} 0.28 at pH 3.5 was probably a decapeptide. It was isolated from paper electropherograms (pH 3.5) and shown to be homogeneous by paper chromatography (R_{A1} 0.12 in Solvent I). Although migrating at a rate different from that of the pentapeptide moiety of GP-2, the molar ratios of the amino acid constituents of the above peptide (Ala:Glu:Gly:1, 205:1:00:093:091) were identical with those of the pentapeptide obtained from GP-2. Dinitrophenylation studies showed that alanine was the NH₂-terminal amino acid; ε-DNP-lysine was also found.

Incubation of GP-1a with the amidase yielded two products which were separated on paper electrophoresis at pH 6.5 and pH 3.5 (Fig. 5). One of the products was ninhydrin-negative and on paper gave a positive reaction for N-acetylamino sugars. On paper electrophoresis and paper chromatography (Solvent I), it migrated as the cell wall tetrascarbohydrate. The second product was only ninhydrin-positive and migrated on paper electrophoresis at pH 3.5 and on paper chromatography (Solvent I) as the pentapeptide released from GP-2 by the amidase.

GP-4 was not affected by the amidase but could be digested by the enzyme after it had been N-acetylated. The products of digestion, disaccharide and peptide, were identical with those obtained by incubation of GP-2 with the amidase. GP-4 which had been N-acetylated with ¹⁴C-acetic anhydride released upon incubation with the amidase a radioactive disaccharide having the same properties (chromatography, electrophoresis, quantitative color yield in the Morgan-Elson test) as the cell wall disaccharide.

Digestion with Lysozyme and Lysostaphin

Of the five compounds isolated, only GP-1a was affected by lysozyme and by lysostaphin under the conditions used. Incubation of GP-1a with lysozyme released two products, identified as the cell wall disaccharide and GP-2 (Fig. 5). Two other products were found in lysostaphin digests of GP-1a. One of these was an oligosaccharide migrating on electrophoresis and on paper chromatography as the trisaccharide GlcNAcβ(1 → 4)MurNAc obtained by digestion of the cell wall tetrascarbohydrate with lysostaphin (8, 13). The second product was presumably a N-acetylmuramyl pentapeptide. No free N-acetylmuramic acid and N-acetylglycosamine were released.

Partial Acid Hydrolysis

Hydrolysis of the glycopeptides under mild conditions (2 N HCl, 100°, 15 min) gave a complex mixture of compounds as

---

**Enzymatic Hydrolysis**

Incubation of GP-1 and GP-2 with almond emulsin (which contains N-acetyl β-D-glucosaminidase activity) released N-acetylglycosamine, which was detected on paper electrophoresis (pH 6.5, 3.5). Even prolonged incubation (24 hours) with large amounts of the enzyme (1:1, w/w, ratio of enzyme to substrate) resulted only in partial digestion of GP-1 and GP-2. Under the same conditions, GP-4 was not affected by almond emulsin, but it was digested to a small extent after N-acetylation.

GP-1, GP-1a, and GP-2 were readily digested by the streptomycetes amidase. GP-2 gave two products which were separated on paper electrophoresis at pH 6.5 and purified further at pH 3.5. One of the products was Morgan-Elson-positive and ninhydrin-negative and was identified as GlcNAcβ(1 → 4)MurNAc by its rate of migration on paper electrophoresis (pH 6.5) and on paper chromatography (Solvent I) and its quantitative color yields in the Morgan-Elson reaction (Table IV). The second product reacted only with the ninhydrin reagent. It was homogeneous on paper chromatography (R_{A1} 0.27 in Solvent I) and paper electrophoresis (M_{OEN} 0.59 at pH 3.5). Amino acid analysis showed that this compound contained alanine, glutamic acid, glycine, and lysine in the molar ratio of 1.94:1.00:0.93:0.90, and can thus be considered as a pentapeptide. Dinitrophenylation followed by acid hydrolysis revealed the presence of ε-DNP-lysine and DNP-alanine.

Amidase digests of GP-1 contained a larger number of products than those of GP-2. One product was identical with the cell wall disaccharide; two other products, both ninhydrin-positive, were also released. One of these migrated on paper electrophoresis as a component obtained from amidase digests of GP-2; the other ninhydrin-positive spot with M_{OEN} 0.28 at pH 3.5 was probably a decapeptide. It was isolated from paper electropherograms (pH 3.5) and shown to be homogeneous by paper chromatography (R_{A1} 0.12 in Solvent I). Although migrating at a rate different from that of the pentapeptide moiety of GP-2, the molar ratios of the amino acid constituents of the above peptide (Ala:Glu:Gly:1, 205:1:00:093:091) were identical with those of the pentapeptide obtained from GP-2. Dinitrophenylation studies showed that alanine was the NH₂-terminal amino acid; ε-DNP-lysine was also found.

Incubation of GP-1a with the amidase yielded two products which were separated on paper electrophoresis at pH 6.5 and pH 3.5 (Fig. 5). One of the products was ninhydrin-negative and on paper gave a positive reaction for N-acetylamino sugars. On paper electrophoresis and paper chromatography (Solvent I), it migrated as the cell wall tetrascarbohydrate. The second product was only ninhydrin-positive and migrated on paper electrophoresis at pH 3.5 and on paper chromatography (Solvent I) as the pentapeptide released from GP-2 by the amidase.

GP-4 was not affected by the amidase but could be digested by the enzyme after it had been N-acetylated. The products of digestion, disaccharide and peptide, were identical with those obtained by incubation of GP-2 with the amidase. GP-4 which had been N-acetylated with ¹⁴C-acetic anhydride released upon incubation with the amidase a radioactive disaccharide having the same properties (chromatography, electrophoresis, quantitative color yield in the Morgan-Elson test) as the cell wall disaccharide.

**Digestion with Lysozyme and Lysostaphin**

Of the five compounds isolated, only GP-1a was affected by lysozyme and by lysostaphin under the conditions used. Incubation of GP-1a with lysozyme released two products, identified as the cell wall disaccharide and GP-2 (Fig. 5). Two other products were found in lysostaphin digests of GP-1a. One of these was an oligosaccharide migrating on electrophoresis and on paper chromatography as the trisaccharide GlcNAcβ(1 → 4)MurNAc obtained by digestion of the cell wall tetrascarbohydrate with lysostaphin (8, 13). The second product was presumably a N-acetylmuramyl pentapeptide. No free N-acetylmuramic acid and N-acetylglycosamine were released.

**Partial Acid Hydrolysis**

Hydrolysis of the glycopeptides under mild conditions (2 N HCl, 100°, 15 min) gave a complex mixture of compounds as
shown by analysis on paper in two dimensions, first by electrophoresis at pH 6.5, and then by chromatography in Solvent I (Fig. 6). Three of the compounds, originating from the partial acid hydrolysate of GP-2, were studied in detail. They were isolated and purified by a combination of paper electrophoresis and paper chromatography.

A pentapeptide \((M_{Glu} 0.59 \text{ at pH 3.5})\) was eluted with water from the electropherogram and purified on paper chromatography in Solvent I \((R_{Ala} 0.27)\). It was composed of Ala, Glu, Gly, Lys in the molar ratio of 1.82:1.00:1.05:0.91. Upon dinitrophenylation and hydrolysis, DNP-Ala and d-DNP-Lys were found. This peptide appears thus to be identical with the peptide released from GP-2 by digestion with the amidase.

A dipeptide with \(M_{Glu} 1.05 \text{ at pH 3.5}\) was eluted with water from the paper and purified on paper electrophoresis. Amino acid analysis indicated a composition of Ala, Glu, Gly with a molar ratio of 1.2:1:0.9. Dinitrophenylation experiments revealed the presence of DNP-Ala. On incubation of acid hydrolysates of this peptide with hog \(\alpha\)-amino acid oxidase, the alanine was found to belong to the \(\alpha\) series, since it was not affected by the enzyme. After reaction between this peptide and anhydro hydrazine, two products reacting as hydrazides were found by paper electrophoretic analyses at pH 3.5. One of these migrated at a rate identical with authentic glutamic acid \(\alpha\)-hydrazide \((0.82)\) and a second fast moving product migrated as alanine hydrazide. Free glycine was also detected on the paper.

Partial acid hydrolysates of GP-1, GP-4, and acetylated GP-4 were not investigated in detail but were compared with the corresponding hydrolysates of GP-2 under the conditions of paper chromatography and paper electrophoresis. A composite map of the partial acid hydrolysates of GP-1, GP-2, and GP-4 is given in Fig. 6. Four amino acids (alanine, glycine, glutamic acid, and lysine; Spots 2, 3, 9, and 7, respectively) and two amino sugars (muramic acid and glucosamine, Spots 1 and 6, respectively) were identified in the three hydrolysates. In addition, the hydrolysates contained several identical peptides, in approximately equal amounts, as judged by the intensity of the ninhydrin spots revealed by ninhydrin.

A tripeptide with \(M_{Glu} 0.62 \text{ at pH 6.5}\) was eluted from the paper and purified on paper chromatograms \((R_{Ala} 0.92, R_{Ala} 0.27, R_{Ala} 0.27)\). Amino acid analysis indicated a composition of Ala, Glu, Gly with a molar ratio of 1.2:1:0.9. Dinitrophenylation experiments revealed the presence of DNP-Ala. On incubation of acid hydrolysates of this peptide with hog \(\alpha\)-amino acid oxidase, the alanine was found to belong to the \(\alpha\) series, since it was not affected by the enzyme. After reaction between this peptide and anhydro hydrazine, two products reacting as hydrazides were found by paper electrophoretic analyses at pH 3.5. One of these migrated at a rate identical with authentic glutamic acid \(\alpha\)-hydrazide \((0.82)\) and a second fast moving product migrated as alanine hydrazide. Free glycine was also detected on the paper.

Partial acid hydrolysates of GP-1, GP-2, and acetylated GP-4 were not investigated in detail but were compared with the corresponding hydrolysates of GP-2 by paper chromatography and paper electrophoresis. A composite map of the partial acid hydrolysate of GP-1 \((2 \text{ N HCl, 15 min, 100°})\) of the three glycopeptides GP-1, GP-2, and GP-4. Spots revealed by ninhydrin.

Demarcated clear regions, spots found in partial acid hydrolysates of each one of the three glycopeptides. Hatched areas, additional substances present only in partial acid hydrolysates of GP-1.

**Fig. 6.** Fingerprints (high voltage electrophoresis at pH 6.5, origin at A, followed by chromatography in Solvent I) of the partial acid hydrolysates \((2 \text{ N HCl, 15 min, 100°})\) of the three glycopeptides GP-1, GP-2, and GP-4. Spots revealed by ninhydrin.
color on paper. These included the pentapeptide Ala–Glu–
(Gly)–Lys–Ala (Spot 4), the tripeptide Ala–Glu–Gly (Spot 10),
and the dipeptide Lys–Ala (Spot 8) which were identified by
comparison of their mobility on paper with the corresponding
purified peptides isolated from partial hydrolysates of GP-2.
A dipeptide of alanine and glutamic acid which has not been fully
identified (Spot 11) was also found in the three maps. Spot 5,
present only in the partial acid hydrolysate of GP-1, is presum-
ably a decapeptide, since material with the same paper chroma-
tographic and electrophoretic mobility was also found in amidase
digests of GP-1. Spot 12 in GP-1 appears to be a different penta-
peptide containing Ala, Glu, Gly, Lys in a molar ratio of 1.60:
1.00:1.01:0.78, but was not investigated in detail owing to the
small amounts of material obtained. Attempts to isolate mate-
rial corresponding to other spots were not successful. Peptide
maps of partial acid hydrolysates of acetylated GP-4 were ident-
elical with those of GP-2 and are not included in Fig. 6.

DISCUSSION

One limitation in the studies of the chemical structure of
bacterial cell wall glycopeptides has been the lack of suitable
methods for the isolation of sufficient quantities of low molecular
weight fragments from enzymatic digests of these walls.

In view of our earlier success in the separation of the acidic
compounds from cell wall digests on a basic ion exchanger
(Dowex 1) (5, 6), we attempted the fractionation of the amphi-
teric glycopeptides on acidic ion exchangers, since ion exchangers
of this type are extensively used for the fractionation of peptides
(35) and more recently have also been applied to the separation
of glycopeptides (36). The system described in this paper was
found to give good separation of glycopeptides from digests of
cell walls after preliminary fractionation on Dowex 1 and Bio-
Gel P-4. The compounds were obtained in good yield, and with
only small amounts of impurities. Total recoveries in column
experiments were about 70 to 80%. The procedure was used
for fractionation of gram quantities of glycopeptide mixture
but can be readily scaled up, or down, as necessary. It is now
being used successfully for the fractionation and isolation of
soluble glycopeptides from Bacillus licheniformis ATCC 9945.*

From the yields of the three major glycopeptides (Table I), it
can be seen that they account for approximately 5% of the weight
of the cell walls. The amino sugar content in these glycopeptides
is approximately 50% and that of the cell walls used in this study
is 27%. The amino sugars of the glycopeptides isolated by us
thus account for 9.4% of the total amino sugars of the cell walls.
This is considerably less than the proportion of amino sugars
which are devoid of peptide moieties, since the total yield of disac-
charide and tetrasaccharide amounts to 11.2% of the weight of
the wall (Table I) and thus to 42% of the total amino sugar
content of the walls. It appears, therefore, that nearly
half of the muramic acid residue in isolated M. lysodeikticus
cell walls are unsubstituted, a conclusion which is in
line with that put forward very recently by Ghuysen et al. (8).
Altogether we have been able to account for about 52% of the
cell wall amino sugars, which together with the peptide moieties
linked to some of the muramic acid residues correspond to 16% of
the weight of the walls or to 46% of the weight of dialyzable
material. Part of the dialyzable material is comprised of glyco-
peptides with a molecular weight higher than 2000 which have

* D. Mirelman and N. Sharon, in preparation.

not been investigated (BG 1 in Fig. 1) and of free oligosaccharides
(BG III). Another part is comprised of amino acids and pep-
tides (not bound to amino sugars BG IV, in Fig. 1) whose origin
is unknown. BG I, BG III, and BG IV together account for
some 20% of the dialyzable fraction.

A significant fact which has been repeatedly observed in studies
of lysozyme digests of M. lysodeikticus is that only a small part
of the peptide moieties of these walls are found in the dialyzable
materials. The amino acid contents of GP-1, GP-2, and GP-4
correspond to about 7% of total amino acid content of the wall
(which is 37% (5)) or 42% of the amino acids in the dialyzable
material, whereas the bulk of the amino acids is in the nondialyz-
able fraction, in a form still unidentified.

The compounds isolated from the three major peaks of the
Dowex 50 column after removal of small amounts of impurities
by rechromatography on Bio-Gel P-4, were shown to be homo-
geneous in paper chromatograms in three different solvent sys-
tems and on paper electropherograms at three pH values where
their rates of migration in the different systems differed from
each other (Table II and Fig. 3). Analysis of amino acids and
sugar showed that the three compounds are composed of
GlcN, Mur, Ala, Glu, Gly, Lys. The glutamic acid and one of
the alanines were found to be of the D configuration whereas lysine
was assumed to be of the L configuration since this is the only
form in which this amino acid is found in cell walls (1). Molec-
ular weight determinations showed that GP-2 and GP-4 are
disaccharide peptides. The molecular weight of GP-1 was
found to be twice that of GP-2. This, together with other evi-
dence, showed that GP-1 is a dimer of GP-2, or disaccharide-
decapeptide-disaccharide.

Since GP-2 was investigated in greater detail than the two other
glycopeptides, we shall discuss the evidence for its structure
first, and subsequently present the structure of the other
compounds. In the modified Morgan–Elson assay, both after
heating for 3 min and for 35 min, the molar color yield of GP-2
was identical with that of the cell wall disaccharide GlcNAcβ
(1→4) MurNAc, and different from the molar color values of the
synthetic GlcNAcβ(1→6) MurNAc. Indeed, a disaccharide
identical with GlcNAcβ(1→4)MurNAc was isolated from GP-2
after the glycopeptide had been digested with the streptomyces
amidase. This also indicates that the two acetyl groups found
per mole of GP-2 (Table III, Fig. 4) are linked to the amino
groups of glucosamine and muramic acid. Reduction by sodium
borohydride showed that muramic acid was the terminal reducing
group of the glycopeptide. The ε-NH₂ group of lysine was found
due to nitrophenylation experiments to be the only free amino
group in the glycopeptide and potentiometric titration gave
results in agreement with this finding (Table V). Two carboxyl
end groups were found by titration and hydrazinolysis experi-
ments, and these have been shown to belong to glycine and ala-
nine. The yield of glycine in the latter experiments was 35% to
38% of the theoretical value, comparable to yields reported
recently by Muñoz et al. (34). No other free amino acids were
found on analysis of GP-2 which had been treated with hydra-
inine.

Three peptides were isolated from partial acid hydrolysates
of GP-2. One of these proved to be the dipeptide Lys–Ala.
The configuration of the alanine moiety was established by the
use of D-amino acid oxidase, whereas the lysine was presumed to
possess the L configuration (see above). This peptide was also
identified by comparison with a synthetic L-Lys–L-Ala compound.
The second peptide isolated was identified as L-Ala-d-Glu-Gly. Dinitrophenylation experiments revealed that alanine was NH$_2$-terminal; this alanine belonged to the L series since it was not affected by d-amino acid oxidase. Upon hydrazinolysis, the α-hydrazide of glutamic acid was obtained. It was identified by comparison with synthetic α- and γ-hydrazides of glutamic acid. This indicates that, in the tripeptide, the α-COOH of glutamic acid is linked to glycine and the γ-COOH is free. The third peptide found in the partial acid hydrolysate of GP-2 was a pentapeptide, Ala$_2$-Glu-Lys-Gly, in which alanine was shown to be NH$_2$-terminal. On paper electrophoresis and paper chromatography, this pentapeptide migrated at a rate identical with the pentapeptide which is released from GP-2 by the action of amidas.

The structure proposed for GP-2 on the basis of this data is given in Fig. 7. This compound is most likely identical with DSP II isolated several years ago by Ghuysen (10), although the structure presented here is complete, and differs from the one given by him.

GP-4 appears to possess the same structure as GP-2 with the exception of the acetyl group on muramic acid. It was found that acetylation of GP-4 under conditions in which the ε-NH$_2$ of lysine reacts only to a small extent, gives a product indistinguishable from GP-2 in its color reactions, rates of migration on paper electrophoresis, and paper chromatography, and susceptibility to the streptomycose amidase. Partial acid hydrolysates of acetylated GP-4 gave peptide maps identical with those of acid hydrolysates of GP-2 (Fig. 6). The assigned structure is also clearly supported by nuclear magnetic resonance spectroscopy where the main difference between the spectrum of GP 2 and GP-4 was in the 2 p.p.m. region in which the acetylmethyl groups absorb (Fig. 4). In the spectrum of GP-4 only one sharp peak was observed, as compared to the two peaks found in the spectra of GP 2 and GP-1.

Furthermore, a compound GP-2a found as an impurity in the fraction of GP-2 eluted from the Dowex 50 column was identified as the disaccharide GlcNAcβ(1 → 4) Mur (Fig. 8). Dinitrophenylation studies of this compound gave DNP-muramic acid whereas acetylation with acetic anhydride, whether labeled or unlabeled, afforded a compound indistinguishable from the cell wall disaccharide GlcNAcβ(1 → 4) MurNAc, in electrophoretic or chromatographic mobility, and Morgan-Elson color yield.

The isolation of fragments with a free amino group in an amino sugar, shown in this case to be muramic acid, is of special interest since such cell wall fragments have not been isolated before and their biological significance is unknown. It is, however, pertinent to note that muramic acid residues with a free amino group have been previously detected by dinitrophenylation techniques in lysozyme digests of M. lysodeikticus cell walls (25). We have repeated these experiments and have also found DNP-muramic acid in hydrolysates of dinitrophenylated intact cell walls of M. lysodeikticus.

GP-1 appears to be a dimer of GP-2 in which the two disaccharide pentapeptides are linked through a peptide bond formed between the free carboxyl of the terminal d-alanine of one peptide chain and the ε-amino group of lysine in the other peptide moiety. Potentiometric titrations revealed three carboxyl terminal groups and only one ε-amino terminal (Table V). The free carboxyls were identified by hydrazinolysis as those of glycine (2 residues) and alanine (1 residue) and the ε amino group as that of lysine. A second ε-amino group is released upon hydrolysis of dinitrophenylated GP-1. Digestion with amidase releases a disaccharide, a pentapeptide (perhaps owing to an endoamidase impurity in this enzyme) and a decapeptide which had the same mobility in electrophoresis and chromatography (Solvent I) as the slowest moving peptide released in partial acid hydrolysate of GP-1 (Fig. 6). The structure proposed for GP-1 on the basis of these data is given in Fig. 9. This compound is most likely identical with DSP I also isolated by Ghuysen (10). It is of interest to note that cross-links between the carboxyl group of d-Ala in one peptide and the ε-NH$_2$ of lysine in another peptide have been recently indicated in cell walls of M. lysodeikticus by Muñoz et al. (34).

A fifth compound, GP-1a, found as an impurity in crude GP-1 fractions (Fig. 2) was obtained in small quantities after purification by paper electrophoresis and paper chromatography. This compound also appears in the F$_2$ peak of the Dowex 1 column. The appearance of GP-1 and GP-1a in both F$_1$ and F$_2$ eluted
has been shown to be present in cell walls of Staphylococcus aureus (2). Its structure has been established both by degradation to occur in nucleotide intermediates of bacterial cell wall synthesis in the presence of GP-2.3 was recently shown that a tetrasaccharide pentapeptide is formed catalyzed by lysozyme, in the same manner as suggested for the glycopeptide which may result from the transglycosylation reaction and the disaccharide. It is, however, possible that part of this present in the intact cell wall as evidenced by the isolation of GP-2, and a pentapeptide, shown to be the same as that released from GP-2 by the amidase (Fig. 5). Digestion of GP-1a with lysozyme afforded two compounds identified as GP-2 and the disaccharide GlcNAcβ(1 → 4) MurNAc (Fig. 5). These experiments show that GP-1a is a tetrasaccharide pentapeptide. Incubation of GP-1a with lysostaphin resulted in the formation of MurNAc-pentapeptide and a trisaccharide which was identified by comparison with the trisaccharide GlcNAcβ(1 → 4) MurNAcβ(1 → 4) GlcNAc obtained by digestion of the cell wall tetrasaccharide with lysozym. It may thus be concluded that in GP-1a the peptide moiety is attached to the N-acetylmuramic acid which forms the reducing end, and therefore the structure of this glycopeptide is most likely

\[
\text{GlcNAcβ(1 → 4) MurNAcβ(1 → 4) GlcNAcβ(1 → 4) MurNAc} \quad \text{pentapeptide}
\]

Structures corresponding to such a fragment are undoubtedly present in the intact cell wall as evidenced by the isolation of GP-2 and the disaccharide. It is, however, possible that part of this glycopeptide may result from the transglycosylation reaction catalyzed by lysozyme, in the same manner as suggested for the formation of part of the cell wall tetrasaccharide (6). Indeed, it was recently shown that a tetrasaccharide pentapeptide is formed upon incubation of the cell wall tetrasaccharide with lysozyme in the presence of GP-2.1

The peptide sequence L-Ala–γ-D-Glu–L-Lys–D-Ala is known to occur in nucleotide intermediates of bacterial cell wall synthesis (2). Its structure has been established both by degradation (37) and by chemical synthesis (38). The same peptide sequence has been shown to be present in cell walls of Staphylococcus aureus and Micrococcus roseus (34). A similar peptide sequence in which lysine has been replaced by diaminopimelic acid has been found in cell walls of Escherichia coli (39) and Aerobacter cloacae (40), although the linkage of glutamic acid was not ascertained.

In all glycopeptides of the various bacteria thus far investigated, the peptide chains are linked to a homopolymeric polysaccharide backbone of repeating units of GlcNAcβ(1 → 4) MurNAc via the carboxyl groups of muramic acid. The relative amount of these carboxyl groups which are linked to peptides is variable in different organisms. In the case of M. lysodeikticus, it appears to be rather low, at most 60% (cf. also Reference 8). The fragment GP-1 which is a disaccharide-decapeptide-disaccharide (Fig. 9) is presumably a representative of structures responsible for cross-linking between adjacent polysaccharide chains. Of special interest in these peptides is the presence of the rather uncommon γ-linked glutamic acid. A unique feature of the peptide in M. lysodeikticus is also that the ω-carboxyl group of glutamic acid is linked to glycine. The role of the free amino groups situated at some muramic acid residues is not fully understood; it may be to anchor by covalent bonds other polymers present in the cell wall, or to cross-link peptide chains.

The picture of the cell wall structure emerging from the glycopeptides isolated here is in good agreement with the general theory of cell wall structure in different bacteria. It would appear, however, that the structure of the M. lysodeikticus cell wall is rather irregular, especially in respect to the substitution of the carboxyl groups of the muramic acid moiety in its polysaccharide backbone. Part of these irregularities may be due to the action of autolytic enzymes which are known to degrade cell walls in the course of their isolation (41, 42).

Acknowledgments—We wish to thank Mr. V. Grisaro for the preparation of F, and Mrs. S. Ehrlich-Rogozinsky for the potentiometric titrations. Thanks are also due to Dr. J. M. Ghysen for the streptomyces amidase, to Dr. P. A. Tavormina for the lysostaphin, and to Dr. Z. Bohak for the hog d-amino acid oxidase.

References

Isolation and Study of the Chemical Structure of Low Molecular Weight Glycopeptides from *Micrococcus lysodeikticus* Cell Walls

David Mirelman and Nathan Sharon


Access the most updated version of this article at [http://www.jbc.org/content/242/15/3414](http://www.jbc.org/content/242/15/3414)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/242/15/3414.full.html#ref-list-1](http://www.jbc.org/content/242/15/3414.full.html#ref-list-1)