The Isolation and Structure Identification of a Disaccharide Containing Manno-muramic Acid from Micrococcus lysodeikticus Cell Wall*

(Received for publication, June 14, 1971)

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SUMMARY

The dialyzable fraction obtained after degradation of Micrococcus lysodeikticus cell wall with egg white lysozyme was fractionated on columns of Dowex 50 and Dowex 1 ion exchange resins. In addition to the diand tetrasaccharides and disaccharide peptides previously reported, a new tetrasaccharide and a new disaccharide were isolated in low yields, about 3 to 4% and 2%, respectively, of the total carbohydrate chain of the peptidoglycan. The tetrasaccharide contains 2 residues of 2-acetamido-2-deoxy-D-glucose (N-acetylglucosamine), 1 residue of N-acetylmuramic acid, and 1 residue of muramic acid, probably as the internal amide. The disaccharide contains 1 residue of 2-acetamido-2-deoxy-D-glucose (1 → 4)-linked to 1 residue of manno-muramic acid. The latter component gave by drastic hydrolysis 2-amino-2-deoxy-D-mannose (p-mannosamine) and was found, by gas-liquid chromatography of the per(trimethylsilyl) ether of the latter component, to be identical with synthetic manno-muramic acid.

The main component of bacterial cell walls is an insoluble peptidoglycan backbone having a glycan moiety composed of repeating units of N-acetyl-D-glucosamine (2-acetamido-2-deoxy-D-glucose) and N-acetylmuramic acid [2-acetamido-3-O-(p-1-carboxyethyl)-2-deoxy-D-glucose] (3) β-p-(1 → 4)-linked at C-4 of both units (4, 5). Despite the observation that a microheterogeneity of the components and of the chemical structure is well established for complex carbohydrates, no other carbohydrate components have been detected in all bacterial cell walls investigated until now, except for derivatives resulting from the deacetylation of N-acetylmuramic acid to give muramic acid having a free amino group (6) or internal amide (7). Extensive investigation of low molecular weight oligosaccharides obtained by degradation with egg white lysozyme of Micrococcus lysodeikticus cell wall showed the presence of additional oligosaccharides, besides the known di- and tetrasaccharides previously described (3). The isolation and structure identification of one of these oligosaccharides is the subject of the present paper.

METHODS AND MATERIAL

General—Optical rotations were determined in semimicro or micro (for amounts smaller than 3 mg) tubes with lengths of 100 or 200 mm, with the use of a Rudolph photoelectric polarimeter attachment model 200: the chloroform used was analytical reagent grade and contained approximately 0.75% of ethanol. Infrared spectra were determined on a Perkin-Elmer spectrophotometer, model 237. Evaporations were carried out under reduced pressure with an outside bath temperature kept below 45°. Amounts of volatile solvent smaller than 20 ml were evaporated under a stream of dry nitrogen. The microanalyses were performed by Dr. M. Mannor, Zurich, Switzerland.

Chromatography and Electrophoresis—Ion exchange chromatography was performed on Dowex 1 (X-8, 200 to 400 mesh, AcO− form) and on Dowex 50W (X-8, 200 to 400 mesh, H+ form). Paper chromatographies were performed ascending on Whatman No. 3MM paper previously washed with water. The following solvent systems were used unless otherwise mentioned: 1-butanol-pyridine-water (9:4:3 and 4:1:3; Solvents A and B, respectively), 1-pentanol-pyridine-water (1:1:1; Solvent C), 1-butanol-acetic-acid-water (3:1:1, 5:2:2, and 25:6:25; Solvents D, E, and F, respectively), and 1-butanol-ethanol-water (4:3:1; Solvent G).

Paper electrophoresis was performed on samples of 0.1 to 0.2 mg of substance on Whatman No. 3MM paper, previously washed with water, at 25 volts per cm, 1.0 to 2.0 ma per cm for 50 to 90 min. The solvents were 1-butanol-pyridine-acetic acid-
water (40:5:1:954, pH 5.8; Solvent H), 1-butanol-pyridine-acetic acid-water (20:1:15:474, pH 4.5; Solvent I), 0.10 M borate (pH 9.0; Solvent J), 0.25 M acetic acid (pH 2.0; Solvent K), and 0.2 M pyridine acetate (pH 6.5; Solvent L).

For two-dimensional combined paper chromatography and electrophoresis, the samples (0.5 to 0.6 mg) were deposited according to the method of Hoshino (8).

The compounds were detected on paper chromatograms and electrophoretograms by the benzyne-trichloroacetic and the ninhydrin reagents, and, additionally on paper chromatograms, by the silver nitrate and the benzidine-periodate reagents, or by treatment with alkali and observation under ultraviolet light, as described by Sharon and Seifter (9).

**Determination of Oligosaccharides and Oligosaccharide Peptides in Chromatographic Fractions.**—The reducing power of the fractions obtained by column chromatography was determined by the method of Park and Johnson (10), while the reducing groups adjacent to a 2-acetamido-2-deoxy group was measured by the Morgan-Elson test, as modified by Sharon et al. (5). The color formed by peptide-containing oligosaccharides with ninhydrin was determined by the method of Rosen (11).

**Preparation of Dialyzable Fraction of Lysozyme Digest of M. lysodeikticus Cell Walls.**—Commercially available cells (30 g) of *M. lysodeikticus* ATCC 4698, harvested in the late logarithmic growth phase and dried by spraying (Miles Laboratories, Inc., Elk hart, Indiana), were treated according to the procedure of Sharon and Jeanloz (15) in a 400-ml stainless steel mixing chamber. The homogenization was performed for 90 min, and the yield of cell walls was 4.6 to 4.8 g. The dialyzable fraction of the lysozyme digest was obtained according to the method of Sharon et al. (5) starting with batches of 10 to 20 g of lyophilized cell walls. The yield was 2.5 to 3.0 g of dialyzable material and 6.0 to 6.5 g of nondialyzable material from 10 g of cell wall.

**RESULTS**

**Separation of Oligosaccharides and Oligosaccharide Peptides by Chromatography of Dialyzable Fraction on Dowex 50.**—To a column (1.6 cm × 50 cm) of Dowex 50 (H⁺ form) was added a solution of the dialyzable material (2.90 g) in water (6 to 8 ml). The elution started with 2 liters of water. Fractions of 18 ml were collected at a rate of 72 ml per hour. After 100 fractions had been collected, the column was connected to a mixing chamber containing 2 liters of water, to which a reservoir containing 2 liters of 0.1 M hydrochloric acid was attached. Volume and rate were kept the same, and 200 fractions were collected.

The reducing power was determined on aliquots (0.1 ml) of alternate tubes, while aliquots (0.5 ml) were analyzed with the Reissig, Strominger, and Leloir (16) modification of the Morgan-Elson test, with a heating time of 35 min (5). Free amino groups were determined on aliquots of 0.1 to 0.2 ml with the ninhydrin method (11). The hydrochloric acid concentration was determined, on a 1-ml sample of every 10th tube, by titration with 0.1 M sodium hydroxide in the presence of phenolphthalein as indicator. The pattern of elution is shown in Fig. 1 and the yields are reported in Table 1.

A similar experiment (Experiment 2, Table 1) was performed on 4.36 g of dialysate using the same column of Dowex 50, but prolonging the water elution to 150 tubes and using a less steep gradient of hydrochloric acid. This resulted in a better separation of the third fraction (W₃) eluted with water and of the fourth fraction (P₄) eluted with hydrochloric acid, but the main fractions were essentially the same. The properties of fractions W₃ and P₄ are reported in Tables III and IV.

**Isolation of Oligosaccharides by Chromatography on Dowex 1-Acetate.**—The separation was performed as previously described (3). Fraction W₁ (1.28 g) obtained from the Dowex 50 column was dissolved in water (5 ml). The solution was applied to a column of Dowex 1 (AcO⁺ form; 2.2 cm × 60 cm; bed volume approximately 220 ml). Elution was performed with 0.8 M acetic acid introduced into a mixing flask containing 2 liters of water. Fractions of 18 ml were collected at a rate of 72 ml per hour. The fractions were examined for reducing power and by the Morgan-Elson test. The concentration of acetic acid was determined by titrating the solution of every 10th tube with 0.1 M sodium hydroxide in the presence of phenolphthalein. The elution pattern is shown in Fig. 2. The material from the six peaks was recovered by lyophilization of the solutions and analyzed by paper chromatography and paper electrophoresis. The yields are reported in Table 2.

The material recovered in the six fractions represented more than 70% of the material applied to the column. A similar experiment (Experiment 2, Table 1) was performed on 1.93 g of Fraction W₁, and gave similar results, but the overloading of the column resulted in a relatively smaller Fraction D₃, contaminated in part by Fraction D₄.

**Properties of Fractions Isolated from Dowex 1 Columns.**—Only the fractions which were homogeneous on paper chromatography or paper electrophoresis in various solvent systems and in various buffers using a wide pH range were investigated. The properties of the fractions are reported in Tables III and IV and in Figs. 3 and 4.

The first peak (D₁) consisting of 2-acetamido-2-deoxy-β-D-glucose and a small amount of neutral oligosaccharides, and the second peak (D₂) consisting of a small amount of weakly acidic oligosaccharides, were not further investigated.

**Fraction D₃.**—The material obtained from the third peak (D₃) showed only one spot on paper chromatography and electrophoresis (Table IV). On paper chromatography in the Solvent systems B and E, and on paper electrophoresis at pH 5.8 and 2.6, it moved slower than the known disaccharide O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-2-acetamido-3-O-(n-carboxyethyl)-2-deoxy-β-D-glucopyranoside (D₃). Its reducing power was about equivalent to that shown by the known tetrasaccharide O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-O-(2-acetamido-3-O-(n-carboxyethyl)-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-2-
The chromatographic properties of Da. These results suggest the acetic anhydride and Dowex 1 (CO\textsubscript{2}- form) (18) did not change on one of the muramic acid units. Finally, treatment withride unit, indicating that no unsubstituted amide group was pressure of the tetrasaccharide unit, showing less than 0.1 mole of ammonia liberated per tetrasaccharide unit, determined as described by Kirk (17).

The potentio-acyl groups per tetrasaccharide unit were detected. The potentio-
samine and muramic acid were obtained, but only three acetyl

duced by De. After acid hydrolysis, equimolar amounts of gluco-
hydrates, and acid and enzymic degradation (see subsequent paragraphs) (Tables V and VI). They were shown to be identical with the known Disaccharide D\textsubscript{5} and Tetrasaccharide D\textsubscript{4}, respectively, previously isolated under similar conditions (5).

| Tube number | Material recovered | Tube number | Material recovered |
|-------------|--------------------|-------------|--------------------|
| W\textsubscript{1} | 1-5 | 1.28 | 1-16 | 1.94 | 45.5 |
| W\textsubscript{1} | 6-40 | 0.11 | 19-30 | 0.10 | 2.3 |
| W\textsubscript{1} | 41-80 | 0.11 | 31-70 | 0.22 | 5.1 |
| W\textsubscript{2} | 71-90 | 0.01 | 0.9 |
| W\textsubscript{3} | 110-116 | 0.04 | 1.4 | 91-130 | 0.10 | 2.3 |
| P\textsubscript{1} | 130-150 | 0.02 | 0.7 | 188-196 | 0.01 | 0.2 |
| P\textsubscript{2} | 140-185 | 0.10 | 3.4 | 206-220 | 0.16 | 3.7 |
| P\textsubscript{3} | 160-170 | 0.01 | 0.3 | 230-245 | 0.01 | 0.2 |
| P\textsubscript{4} | 332-388 | 0.01 | 0.2 |
| Total | 1.68 | 57.5 | 2.59 | 60.4 |

Fig. 1. Fractionation on a column of Dowex 50 (1.6 cm X 50 cm) (H\textsuperscript{+} form, 200 to 400 mesh) of the dialyzable fraction (2.90 g) of a digest of M. lysozyme cell wall with egg white lysozyme. The fractions (18 ml) were eluted at a rate of 72 ml per hour. The elution was performed at first with water (Fractions 1 to 100), and then with a gradient of hydrochloric acid (Fractions 101 to 290). The reducing power (---) and color formation in the Morgan-Elson test (-----) and in the ninhydrin test (-----) were determined as described under "Methods and Materials." The yields are reported in Table I.

**Table 1**

Yields of fractionation on Dowex 50 column of dialyzable fraction of digest by egg white lysozyme of M. lysodeikticus cell walls.

The digest (2.90 g in Experiment 1 and 4.86 g in Experiment 2) was adsorbed on a Dowex 50 column and eluted with water (W\textsubscript{1} to W\textsubscript{10}) and with a gradient of hydrochloric acid (P\textsubscript{1} to P\textsubscript{10}) (see Fig. 1).

**Table II**

Yields of fractionation on Dowex 50 column of Fraction W\textsubscript{1}.

| Fraction | Experiment 1 | | | | Experiment 2 | | |
|----------|--------------|---|---|---|---|---|---|
| Tube number | Material recovered | | | | Tube number | Material recovered | | |
| D\textsubscript{4} | 5-10 | 0.009 | 0.4 | | 5-10 | 0.026 | 1.3 |
| D\textsubscript{5} | | | | | 27-37 | 0.012 | 0.6 |
| D\textsubscript{6} | 40-55 | 0.044 | 3.4 | | 50-65 | 0.075 | 3.9 |
| D\textsubscript{7} | 66-74 | 0.028 | 2.2 | | 80-91 | 0.034 | 1.8 |
| D\textsubscript{8} | 78-100 | 0.719 | 56.2 | | 98-138 | 1.097 | 56.8 |
| D\textsubscript{9} | 125-145 | 0.143 | 11.2 | | 150-225 | 0.280 | 11.9 |
| Total | 0.939 | 73.4 | 1.49 | 76.3 |

The color formed in the Elson-Morgan test was less than that produced by muramic acid. The reducing power (---) and the color formation in the Morgan-Elson test (-----) were determined as described under "Methods and Materials" and "Results." The yields are reported in Table II.

The results suggest the presence of a tetrasaccharide having the carboxylic group and the amino group of one of the muramic acid units linked through an internal amide linkage.

Fraction D\textsubscript{4}—The material isolated from the fourth peak (D\textsubscript{4}) was shown to be homogeneous by paper chromatography and electrophoresis (Table IV), and generally moving slightly slower than the known Disaccharide D\textsubscript{5}. Its reducing power was almost identical with that of D\textsubscript{5}. After acid hydrolysis, it gave two compounds, one showing on paper chromatograms in various solvent systems a speed of migration identical with that of glucosamine. On paper chromatography, the second component was similar to, but not identical with muramic acid [2-amino-3-O-(1-carboxyethyl)-2-deoxy-\textalpha-D-glucose] and the color produced with the silver nitrate reagent developed much more slowly than that produced by muramic acid.

Fractions D\textsubscript{5} and D\textsubscript{6}—The materials isolated from the fifth peak (D\textsubscript{5}) and from the sixth peak (D\textsubscript{6}) were investigated by paper chromatography and electrophoresis, elementary analysis, optical rotation, infrared spectra, reducing power, analysis of the carbohydrate components, and acid and enzymic degradation (see subsequent paragraphs) (Tables V and VI). They were shown to be identical with the known Disaccharide D\textsubscript{5} and Tetrasaccharide D\textsubscript{6}, respectively, previously isolated under similar conditions (5).
Acetamido-2-deoxy-\(\text{manno}\)-oligosaccharides D\(n\) of Tetrasaccharide D\(3\) showed the formation, in increasing order, by paper electrophoresis. The solution obtained from the treatment of Tetrasaccharide D\(3\) showed only the starting material. The mixtures were also examined by paper chromatography (19). The reducing power is expressed as the ratio of the weight of 2-acetamido-2-deoxy-d-glucose giving an equivalent color to the reducing power of the sample.

2-Amino-3-O-(d-1-carboxyethyl)-2-deoxy-d-glucose, determined by the method of Kent and Strange (14), before and after hydrolysis with 4 M HCl for 4 hours.

### Table III
Physical and chemical properties and components of fractions obtained from digest with egg white lysozyme of M. lysodeikticus cell wall

| Fraction | \[^{13}C, (0.5, in water)^a\] | Reducing power\(^b\) | Muramic acid\(^d\) | Carbohydrates\(^d\) | Amino acids\(^d\) |
|----------|------------------------------|---------------------|------------------|------------------|------------------|
|          |                              |                     | Before hydrolysis | After hydrolysis |                  |
|          |                              |                     | %                | %                |                  |
| W4       | 0.29                         | 10.0                | MA, GN          | Ala, Gly, Glu |                  |
| F4       | 0.28                         | 5.0                 | 31.0            | MA, GN          | Ala, Gly, Glu |
| D5       | +0.8 \(-\) +0.6 \(+\)        | 47.5                | 49.0            | MA, GN          | None            |
| D4       | +15.0 \(-\) +12.6 \(+\)      | 48.0*               | 50.0*           | MA, GN          | None            |
| D3       | +6.0 \(-\) +3.0 \(+\)        | 50.0                | 51.0            | MA, GN          | None            |
| D2       | \(-11.0 \(-\) +12.0 \(+\)    | 48.5                | 50.0            | MA, GN          | None            |

\(a\) Determination in cominicro or micro tubes (1 dm) with a Rudolph photoelectric polarimeter model 200.

\(b\) Determination with the Park and Johnson procedure (10).

\(c\) Muramic acid.

\(d\) Determined by paper chromatography.

### Table IV
Paper chromatography and paper electrophoresis of fractions obtained from digest with egg white lysozyme of M. lysodeikticus cell wall

| Fraction | Paper chromatography\(^a\) | Paper electrophoresis\(^b\) |
|----------|----------------------------|----------------------------|
|          | Solvent B | Solvent E | Solvent B | Solvent E | Solvent K (pH 2.6) |
|          | \(R_F\) | \(R_GNA\) | \(R_F\) | \(R_GNA\) | \(R_M\) | \(R_K\) |
| W4       | 0.14     | 0.21     | 0.16     | 0.38     | +0.51    | -0.55 |
| F4       | 0.18     | 0.27     | 0.28     | 0.36     | +0.45    | -1.00 |
| D5       | 0.40     | 0.61     | 0.66     | 0.86     | +0.51    | -0.47 |
| D4       | 0.41     | 0.66     | 0.46     | 1.09     | +0.66    | +0.53 |
| D3       | 0.48     | 0.73     | 0.50     | 1.19     | +0.69    | +0.96 |
| D2       | 0.32     | 0.48     | 0.32     | 0.76     | +0.77    | +0.60 |
| 2-Acetamido-2-deoxy-d-glucose | 0.06 | 1.00 | 0.42 | 1.00 | +0.12 | +0.30 |

\(a\) Ascending on Whatman No. 3 MM paper relative to 2-acetamido-2-deoxy-d-glucose (GNAc).

\(b\) Whatman No. 3 MM paper. The detection was performed as described under "Methods and Material."

\(c\) Determined by amino acid analyzer.

\(d\) Determined by paper chromatography.

### Lysozyme Degradation of Tetrasaccharides D\(4\) and D\(5\)—Samples (5 mg) of Tetrasaccharides D\(4\) and D\(5\) in water (10 ml) were incubated separately with egg white lysozyme (0.2 mg) at 37\(^\circ\). The solutions were examined at various time intervals with the Mor-{
online}Gan-Elsom test after stopping the reaction by freezing in an ice-acetone bath. The results are reported in Fig. 4.

The mixtures were also examined by paper chromatography and paper electrophoresis. The solution obtained from the treatment of Tetrasaccharide D\(4\) showed the formation, in increasing order, of the disaccharide D\(3\), whereas the digest containing Tetrasaccharide D\(5\) showed only the starting material.

### Incubation of Disaccharides D\(4\) and D\(5\) with \(\beta\)-N-Acetylglucosaminidase—Disaccharides D\(4\) and D\(5\) (0.3 mg) were incubated separately for 24 hours at 37\(^\circ\) with 40 \(\mu\)l of a solution of \(\beta\)-N-acetylglucosaminidase composed of 0.2\% bovine serum albumin, 50 \(\mu\)l of a solution of \(\beta\)-N-acetylglucosaminidase (pH 4.1), and pig epididymis extract (1:5:4) (10). The incubation mixtures were directly adsorbed on paper and examined by electrophoresis in Solvent L and 37 volts per cm, and by descending chromatography in Solvent F. The results are reported in Table VII.

### Treatment of Disaccharides D\(4\) and D\(5\) with Boron Tribromide—In order to elucidate the hexosamine skeleton of the acidic component of D\(4\), the other linkage was cleaved with boron tribromide, as described by Bonner, Bourne, and McNally (20) for the hydrolysis of methyl ethers. The method was tested simultaneously on Disaccharide D\(4\).

Samples (10 mg) of Disaccharides D\(4\) and D\(5\), respectively, were hydrolyzed with 4 M hydrochloric acid for 4 hours at 100\(^\circ\). After evaporation of the solution in a vacuum, an ethanol solution of the residuum was streaked on Whatman No. 3 MM paper, and the chromatogram was developed with Solvent E. The substances were localized by cutting thin strips on the edges which were tested with ninhydrin and the silver nitrate reagent. From the remaining parts of the paper, the bands migrating at the same speed as muramic acid were cut off and eluted with water. The eluates were evaporated to give, each, about 4 mg of material.

An aliquot (2 mg) of each of the residuum was suspended in 0.5 ml of dry dichloromethane, and the solution was cooled in an ice bath. To the suspension was added 0.25 ml of boron tribromide cooled with ice. Synthetic (gluco)-muramic acid was also similarly treated as standard. The reaction mixture was kept for 48 hours at room temperature under moisture exclusion. The substances gradually dissolved, and, after 48 hours, the colored reaction mixture was evaporated in a vacuum. Methanol (2 ml) was added to the glassy residue and evaporated, and this procedure was repeated twice.

A solution of each residuum in water (0.1 ml), was chromatographed on Whatman No. 1 paper, treated with 0.1 M barium chloride in Solvent A according to the method of Heyworth, Perkins, and Walker (21). Both the products obtained from synthetic (gluco)-muramic acid and from the acidic component of...
Disaccharide D₅ migrated at the same speed, different from that of the product obtained from the acidic component of Disaccharide D₄. The three spots, however, migrated at a speed faster than that of any of the known hexosamines, and it was assumed that the lactyl group had migrated, under the anhydrous conditions of the reaction, to the amino group or to one of the hydroxyl groups. Consequently, the residue was hydrolyzed with 1 M hydrochloric acid (0.1 ml) for 1 hour at 100°. After evaporation, the residue was chromatographed according to the method of Heyworth et al. (21). The products obtained from synthetic (gluco)-muramic acid and from the acidic component of the known Disaccharide D₄ migrated at the same speed as n-glucosamine, whereas the product obtained from the acidic component of Disaccharide D₅ migrated at the same speed as synthetic D-mannosamine.

**Reduction of Disaccharides D₄ and D₅ with Sodium Borohydride**—The reducing component of Disaccharide D₄ was identified by sodium borohydride reduction and iodine oxidation. The experiments were performed, in addition, on the known Disaccharide D₅ as standard.

To solutions of the Disaccharides D₄ or D₅ (0.5 mg), respectively, in water (0.15 ml), cooled to 0°, was added sodium borohydride (1 mg). The solution was kept for 2 hours at 0°, and then concentrated hydrochloric acid (0.1 ml) was added. After the

**Table V**

Elementary analysis of fractions isolated from digest by egg white lysozyme of *M. lysodeikticus* cell wall

| Fraction | C     | H     | N     | Acetyl |
|----------|-------|-------|-------|--------|
| W₁       | 47.77 | 7.01  | 11.45 |        |
| P₁       | 44.82 | 6.25  | 10.90 |        |
| D₁       | 49.09 | 6.39  | 14.82 |        |
| D₂       | 45.88 | 6.45  |       |        |
| D₃       | 45.73 | 6.61  |       |        |
| D₄       |       |       | 17.64 |        |

| Calculated for | C₆H₁₆N₂O₃⁴⁺ | C₆H₁₆N₂O₃⁻ | C₆H₁₆N₂O₃⁴⁺ | C₆H₁₆N₂O₃⁻ |
|----------------|-------------|-------------|-------------|-------------|
|                | 46.86       | 6.42        | 5.75        | 17.67       |
|                | 47.31       | 6.40        | 6.13        | 14.13       |
|                | 46.39       | 6.49        | 6.01        | 13.54       |
|                | 45.94       | 6.50        | 5.84        | 17.03       |

° Determinations by Dr. W. Manser, Zurich, Switzerland.

- Tetrasaccharide D₅ (see Scheme 1).
- Disaccharide D₄.
- Tetrasaccharide D₅ + one molecule of water.
- Disaccharide D₅ and D₆.

Fig. 3. Infrared spectra of Disaccharides D₄ (II, 0.8 mg) and D₅ (III, 0.7 mg) and Tetrasaccharides D₁ (I, 0.9 mg) and D₂ (IV, 0.6 mg), in potassium bromide (100 mg) discs, determined with a Perkin-Elmer spectrophotometer, model 237.

Fig. 4. Degradation of Tetrasaccharides D₁ and D₅ (5.0 mg each) with egg white lysozyme (0.2 mg) as described under "Methods and Material" and "Results." Determination by reducing power (Tetrasaccharide D₁, △—△; Tetrasaccharide D₅, △—△) and Morgan and Elson test (Tetrasaccharide D₁, ○—○; Tetrasaccharide D₅, ○—○).

TABLE V

| Fraction | Elementary analysis⁶  |
|----------|-----------------------|
|          | C     | H     | N     | Acetyl |
| W₁       | 47.77 | 7.01  | 11.45 |        |
| P₁       | 44.82 | 6.25  | 10.90 |        |
| D₁       | 49.09 | 6.39  | 14.82 |        |
| D₂       | 45.88 | 6.45  |       |        |
| D₃       | 45.73 | 6.61  |       |        |
| D₄       |       |       | 17.64 |        |

Calculated for

| C₆H₁₆N₂O₃⁴⁺ | C₆H₁₆N₂O₃⁻ | C₆H₁₆N₂O₃⁴⁺ | C₆H₁₆N₂O₃⁻ |
|-------------|-------------|-------------|-------------|
| 46.86       | 6.42        | 5.75        | 17.67       |
| 47.31       | 6.40        | 6.13        | 14.13       |
| 46.39       | 6.49        | 6.01        | 13.54       |
| 45.94       | 6.50        | 5.84        | 17.03       |

⁶ Determinations by Dr. W. Manser, Zurich, Switzerland.

⁷ Tetrasaccharide D₅ (see Scheme 1).

⁸ Disaccharide D₄.

⁹ Tetrasaccharide D₅ + one molecule of water.

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tion was kept overnight at 0°C, and then saturated with carbon dioxide. After acidification with concentrated hydrochloric acid the Disaccharides Dd or Dq (0.5 mg), respectively, in water (0.05 ml), were added at 0°C. 5 mM iodine (0.2 ml) and 0.05 ml of 0.1 M sodium carbonate-sodium hydrogen carbonate (1:1). The solution was evaporated at 5°C in a vacuum desiccator and examined by electrophoresis or by descending chromatography and compared to markers in the same buffer as described under "Methods and Material" and "Results."

**TABLE VI**

| Compounds | Heating time |
|-----------|--------------|
|           | 3 min | 35 min |
| N-Acetylmannosamine<sup>a</sup> | 0.41 | 1.0 |
| N-Acetylmuramic acid<sup>d</sup> | 0.82 | 0.97 |
| N-Acetyl-manno-muramic acid<sup>d</sup> | 0.58 | 0.09 |
| Disaccharide Dq<sup>e</sup> | 0.032 | 0.21 |
| Disaccharide Dd<sup>e</sup> | 0.05 | 0.37 |

<sup>a</sup> Relative to an equimolar amount of 2-acetamido-2-deoxy-glucose (N-acetyl-n-glucosamine) tested under identical conditions. The measurements were made with a Zeiss spectrophotometer at 585 nm.

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**FIG. 5.** Fractionation on a Dowex 50 column (H<sup>+</sup> form, 200 to 400 mesh, 1.25 cm × 75 cm) of an hydrolysate (39.6 mg) of Disaccharide Dq. Elution with 0.3 M hydrochloric acid, fractions of 4.2 ml per 8 min. A, n-glucosamine hydrochloride; B, manno-muramic acid. Under these conditions, manno- and glucos- muramic acid emerge at the same volume.

(0.03 ml), the solution was saturated with carbon dioxide to remove the excess of iodine, the tube was sealed, and the content was hydrolyzed as just described. For both disaccharides the proportions of n-glucosamine remained unchanged, whereas the proportions of (gluco)-muramic acid and manno-muramic acid (calculated as n-glucosamine) were decreased to 33% and 45% for Disaccharides Dq and Dd, respectively.

**Isolation of Acidic Component of Disaccharide Dq and Preparation of N-Acetyl Derivative—**A solution of Disaccharide Dq (40 mg) in 8 ml hydrochloric acid (8 ml) was heated in a sealed tube for 4 hours at 100°C. After cooling, water (10 ml) was added, and the solution was evaporated in vacuo, the last traces of acid being removed in a desiccator, in the cold, in the presence of potassium hydroxide and concentrated sulfuric acid. The residue was dissolved in 0.3 M hydrochloric acid (2 ml) and the solution was fractionated on a Dowex 50 column according to the procedure of Gardell (22). The results are reported in Fig. 5.

Fractions 63 to 79 were pooled and lyophilized. The residue (16.8 mg) migrated on paper chromatography and paper electrophoresis as n-glucosamine. It crystallized from a mixture of water-methanol-acetic anhydride, and gave, after degradation with ninhydrin (23), a compound migrating as n-arabinose on paper chromatograms.

Fractions 85 to 88 were pooled and lyophilized. The residue was dissolved in water (2 ml) and the solution was treated with Amberlite IR-45 (OH<sup>-</sup> form) until pH 6.7 was reached. The solution was lyophilized; the residue (7.0 mg) could not be crystallized, it became yellow above 130°C, and melted at about 145-147°C

\[ C_7H_7NO_5 (251.25) \]

Calculated: N 5.28
Found: N 4.94 (Kjeldahl)

The color obtained in the Elson-Morgan reaction had a maximum absorbance peak at 523 nm. Degradation with ninhydrin (23) gave a product migrating on paper chromatogram at the same speed as the product obtained by ninhydrin degradation of muramic acid (Table VIII). Paper chromatography in Solvents E, B, and C and electrophoresis in Solvent H are reported in Table VIII.

**N-Acetylation of the product obtained from Fractions 85 to 98**

*a* The disaccharides (0.3 mg) were incubated separately for 24 hours at 38°C with 36 mg of an 1:1 mixture of n-glucosamine hydrochloride and of muramyl 2-acetamido-3-O-(D-1-carboxyethyl)-2-deoxy-N-mannosamine. The incubation mixtures were directly adsorbed on paper chromatograms.

**TABLE VII**

| Compounds | Electrophoresis mobility<sup>b</sup> | Paper chromatography<sup>c</sup> |
|-----------|-----------------|-----------------|
| 2-Acetamido-2-deoxy-n-glucose | 1.1 | 1.00 |
| N-Acetylmuramic acid | 20.8 | 1.56 |
| N-Acetyl-manno-muramic acid | 16.6 | 0.88 |
| Disaccharide Dq | 16.6 | 1.00 |
| Disaccharide Dd | 16.6 | 1.00 |
| Digest of Disaccharide Dq | 1.1; 16.6; 20.8 | 0.85; 0.99; 1.55 |
| Digest of Disaccharide Dd | 1.1; 21.3 | 0.85; 1.62 |

<sup>b</sup> Relative to an equimolar amount of 2-acetamido-2-deoxy-glucose (N-acetyl-n-glucosamine) tested under identical conditions. The measurements were made with a Zeiss spectrophotometer at 585 nm.

<sup>c</sup> 2-Acetamido-2-deoxy-0-n-glucopyranosyl (1 + 4) -2-acet- amido-3-O-(p-1-carboxyethyl)-2-deoxy-D-mannose.

<sup>d</sup> 2-Acetamido-3-O-(p-1-carboxyethyl)-2-deoxy-n-glucose.

<sup>e</sup> 2-Acetamido-3-O-(p-1-carboxyethyl)-2-deoxy-n-mannose.

<sup>f</sup> 2-Acetamido-2-deoxy-n-glucose (N-acetyl-n-glucosamine) tested under identical conditions. The measurements were made with a Zeiss spectrophotometer at 585 nm.
Table VIII

Paper chromatography and paper electrophoresis of manno-muramic acid isolated from Disaccharide Dq, of its N-acetyl derivative, and of its product of degradation with ninhydrin

| Compound                                              | Paper chromatography | Paper electrophoresis |
|-------------------------------------------------------|----------------------|------------------------|
|                                                       | Solvent E            | Solvent B             | Solvent C | R<sub>GN</sub> | R<sub>GN</sub> | R<sub>GN</sub> | M<sub>GN</sub> |
| Muramic acid                                           | 2.76                 | 1.00                   | 1.02       | 0.13           |
| Manno-muramic acid isolated from Disaccharide Dq       | 2.80                 | 1.00                   | 1.02       | 0.13           |
| Degradation product of muramic acid                   | 3.88                 |                        |            |                |
| Degradation product of manno-muramic acid              | 3.88                 |                        |            |                |
| N-Acetylmuramic acid                                   | 1.65                 | 0.70                   |            |                |
| N-Acetyl-manno-muramic acid                           | 1.71                 | 0.70                   |            |                |

* Whatman No. 1 paper, descending; revelation with ninhydrin.
* Whatman No. 3MM paper, Solvent H, 75 min, 35 volts per cm; revelation with ninhydrin.

Fig. 6. Comparison of the acid hydrolysates (2.5 M hydrochloric acid, 2 hours at 100°) of Disaccharides D<sub>1</sub> and D<sub>2</sub> (see "Methods and Material" and "Results" for details) by paper chromatography, descending, on Whatman No. 1 paper in Solvent E (A) and in Solvent A (B): 1 and 3, standards of synthetic glucosamine hydrochloride; 2, hydrolysate of Disaccharide D<sub>1</sub>; 4 and 6, standards of synthetic manno-muramic acid (2a); 5 and 7, hydrolysate of Disaccharide D<sub>2</sub>; 8 and 9, standards of N-glucosamine hydrochloride. The faster moving spot of glucosamine, of variable intensity, corresponds to the free base.

The separation of manno-muramic acid from (gluco)-muramic acid was performed according to the procedure described for muramic acid (2a). The resulting product could not be crystallized. It was examined by paper chromatography in Solvents E and C, and the results are reported in Table VIII.

Comparison of Acidic Component of Disaccharide D<sub>1</sub> with Synthetic Manno-muramic Acid [2-Amino-3-O-(2-carboxyethyl)-2-deoxy-D-mannose] by Paper and Gas-Liquid Chromatography—Solutions (0.1%) of Disaccharides D<sub>1</sub> and D<sub>2</sub> respectively, in 2.5 M hydrochloric acid were heated for 2 hours at 100° in sealed tubes. After evaporation in vacuo, traces of hydrochloric acid were removed by repeated addition of water and evaporation. The residues were examined by paper chromatography in Solvents A and E, and the results are reported in Fig. 6.

Since the separation of manno-muramic acid from (gluco)-muramic acid was difficult to obtain by paper chromatography, the separation by gas-liquid chromatography of the N-acetyl-4,6-bis-O-(trimethylsilyl) methyl ester methyl α-D-glucoside was performed as follows. Disaccharide D<sub>1</sub> (5.0 mg, contaminated with about 20 to 30% of Disaccharide D<sub>2</sub> as shown by paper chromato-
Treatment of Disaccharide D₅ with Dowex 1 (Acetate Form)—In order to test the possible epimerization of Disaccharide D₅ into Disaccharide D₆, a solution of pure Disaccharide D₅ (25 mg) in water (0.1 ml) was adsorbed on Dowex 1 (acetate form) as described for the isolation of the oligosaccharides. After being kept at room temperature (20-30°C) for 2 days, the disaccharide was eluted as previously described. Each fraction (1 to 2 ml) of the symmetrical peak that corresponds to D₅ was lyophilized, and then acetylated, methanolyzed, and per(trimethylsilyl)ated as just described, and finally examined by gas-liquid chromatography. No evidence for manno-muramic acid was found.

**DISCUSSION**

The chemical structure presently accepted for the peptidoglycan backbone of the cell wall of all bacteria and of many other microorganisms (3) consists of a carbohydrate chain composed of alternating β- (1 → 4) N-acetylglucosamine (2-acetamido-2-deoxy-α-glucose) and N-acetylmuramic acid [2-acetamido-3-O-(N-1-carboxyethyl)-2-deoxy-N-glucose] units attached to a peptide network. This simplified conception was modified after the isolation of muramic acid devoid of N-acetyl substitution, as such, from _M. lysodeikticus_ cell wall (6), or as internal amide from _B. subtilis_ spores (7), and after the recent isolations of the _N_-glycolyl derivative of muramic acid from _Bacillus_ bacteria cell walls (27, 28). Since various forms of bacterial cell walls are present and these, in addition, possess regions of growth, it may be assumed that variation in the physical structure could be reflected in changes in the chemical composition of the carbohydrate part of the backbone. It is with this objective in mind that a study of the minor components of the carbohydrate moiety of the peptidoglycan backbone of the _M. lysodeikticus_ cell wall was undertaken.

The cell wall was degraded with egg white lysozyme as in previous experiments. Adsorption on a Dowex 50 column retained most of the peptide material. From this material, two oligosaccharides were isolated in pure state; they are assumed to be identical with the two disaccharide peptides previously isolated by Mirelman and Sharon (6).

The material not retained on the Dowex 50 resin was adsorbed on Dowex 1 resin in the acetate form. A very minute fraction was not retained; it contained a trace of _α_-glucosamine, no more than 0.5% of the total _α_-glucosamine of the cell wall. This result illustrates the high specificity of egg white lysozyme as an endoenzyme.

The first peak of substance (D₅) to be eluted with a gradient of acetic acid consisted of a tetrasaccharide composed of an equimolar proportion of _α_-glucosamine and muramic acid as shown by its reducing power. Comparison with the known Tetrasaccharide D₅ and determination of the presence of one free carboxylic group (instead of two), of three acetyl groups (instead of four), of the absence of a primary amide group, and of the resistance to further lysozyme degradation suggest the structure of a tetrasaccharide similar to D₅. In this structure, however, the carboxylic and amino groups of the muramic acid unit further removed from the reducing end form an internal amide ring (see Scheme 1, Tetrasaccharide D₅). This structure agrees with the results of the paper chromatography and electrophoresis, and with a preliminary determination of the nuclear magnetic resonance spectrum at 100 MHz. It is probable that the structure of Tetrasaccharide D₅ is identical with that of the tetrasccharide isolated in high yield from the spores of _B. subtilis_ by Warth and Strominger (7). Complete identification of this structure will require larger amounts of material and comparison with synthetic internal amides of muramic acid (29). The very low proportion of these internal amide groups in a “normal” cell wall (as compared to the cell wall of a spore) suggests a definite biological role for this internal amide. Since Mirelman and Sharon (6) have demonstrated the presence of free amino groups in the muramic acid units of the peptidoglycan, it is possible that the internal amide isolated in this study result from a condensation between free carboxylic and amino groups. Such condensation has been shown to take place very easily (29), and could take place during the lyophilization process. Warth and Strominger (7), however, have presented good evidence for the presence of these groups in _B. subtilis_ spore cell walls before isolation.

The second new oligosaccharide to be isolated (D₆) was shown by determination of the reducing power to be a disaccharide composed in equimolar proportion of a 2-acetamido-2-deoxy-α-glucose unit and of a residue very similar to, but not identical with (gluco)-muramic acid. This structure was in agreement with the results of the paper chromatography and electrophoresis, and with the elementary analysis. After hydrolysis the product corresponding to muramic acid had a behavior very similar to that of (gluco)-muramic acid. The first evidence of a manno structure for this compound was the long time necessary for reduction of the silver nitrate reagent, a condition typical for manno derivatives. The structure of a manno derivative of muramic acid for the acidic component of Disaccharide D₅ was ascertained by hydrolysis, under strong acid conditions, of the lactyl ether chain and detection, in low yield, of 2-amino-2-deoxy-mannosamine (mannosamine) by paper chromatography and electrophoresis.

Since it was difficult to ascertain the chemical structure of this acid component, because of the small amount of unstable material available, the synthesis of manno-muramic acid [2-acetamido-3-O-(N-1-carboxyethyl)-2-deoxy-N-mannosamine] was accomplished (25). Comparison of the natural with the synthetic compound proved to be difficult. In most paper chromatography solvent systems, manno-muramic acid and (gluco)-muramic acid behaved similarly. In addition, manno-muramic acid gave, after hydrolysis, many secondary products, and in only two solvent systems was it

1 M. Halford, personal communication.
possible to obtain a clear separation between the two acids (Fig. 6). Identification by gas-liquid chromatography offered the best evidence for the identification of the structure of natural manno-muramic acid, when performed on the per(trimethylsilyl) ether of the methyl ester methyl α-D-glycoside (Fig. 7). The sample of the natural compound was obtained from a late fraction of Peak D1 of Experiment 2, and was heavily contaminated with Disaccharide D1. Based on the detector response (25), it can be assumed that at least two-thirds of this late fraction are composed of the manno derivative. Although not enough material was available for the preparation of a crystalline derivative of natural manno-muramic acid and for determination of its optical rotation, it is possible to conclude from the optical rotation of the disaccharide that both the lactyl chain and the mannose main chain belong to the D-series.

Disaccharide D4 was isolated in very low proportion (2% of the carbohydrate chain of the peptidoglycan) and it is possible that the disaccharide could have resulted from an artifact of the isolation, since 2-acetamido-2-deoxy-D-glucose is known to epimerize into 2-acetamido-2-deoxy-D-mannose under alkaline conditions (30), and a Dowex 1 resin in the acetate form was used for the separation. This isomerization was shown to be most unlikely under the conditions used, since treatment of Disaccharide D5 under more rigorous conditions of isolation showed no trace of epimerization.

The structure of Disaccharide D4 (see Scheme 1) was ascertained by comparison of its properties with those of Disaccharide D5, by determination of the reducing unit by oxidation and reduction, and by degradation with β-N-acetylglucosaminidase. The low color yield in the Morgan-Elson reaction supported a β-n-
(1 → 4) linkage. Although no quantitative data were obtained, it can be concluded that the manno structure confers to the disaccharide linkage a greater resistance toward β-N-acetylglucosaminidase degradation than does the dextro structure of Disaccharide D3 (Table VII).

The present study shows, in the enzymic hydrolysate of *M. lysodeikticus* cell wall, the presence in minor proportions of two new oligosaccharides, Disaccharide Dq and Tetrasaccharide Da. In addition, a disaccharide peptide Dz having a free amine group and being probably identical with the disaccharide peptide GP-4 isolated by Mirelman and Sharon (6) has been isolated also in small yield. The isolation of these fragments demonstrates the microheterogeneity of the structure of the carbohydrate chain of the peptidoglycan. Although it is possible that these fragments result from enzymic degradation during isolation, their isolation raises interesting problems of structure-function relationship which will require for their solution the development of methods for locating these minor components in the cell wall.

Acknowledgments—We thank Dr. Conchie for a gift of β-N-acetylglucosaminidase from pig epididymis, Miss Marjorie Rascher for technical assistance, and Mr. Keyes Linsley for the determination of the gas-liquid chromatograms.

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J. Biol. Chem. 1972, 247:381-390.

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