Isolation and Study of the Chemical Structure of a Disaccharide from Micrococcus lysodeikticus Cell Walls*

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SUMMARY

A large scale isolation of a disaccharide from a dialyzable fraction of Micrococcus lysodeikticus cell wall is described. This fraction was obtained by degradation of isolated walls with egg white lysozyme. After purification by chromatography on charcoal-Celite and Dowex 1, the compound was characterized by a crystalline heptaacetyl methyl ester. Comparison with synthetic O-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-2-acetamido-3-O-(p-1-carboxyethyl)-2-deoxy-D-glucose and with synthetic 2-acetamido-1,4-di-O-acetyl-2-deoxy-3-O-[p-1-(methyl carboxylate)ethyl]-6-O-(2-acetamido-3,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)α-D-glucopyranose, respectively, showed these compounds to be not identical, and a (1→4) linkage is proposed for the natural disaccharide.

EXPERIMENTAL PROCEDURE

Methods—Melting points were taken on a hot stage, equipped with a microscope, and correspond to "corrected melting point." 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% ethanol. Infrared spectra were determined on a Perkin-Elmer spectrophotometer, model 237. The mass spectra were determined on an AEI NS 9 mass spectrometer, with direct insertion and a resolution of 1000. Chromatograms on silica gel were made with the following method on "silica gel Davison," from the Davison Company, Baltimore (Grade 950; 60 to 200 mesh), used without pretreatment. When deactivation by contact with moist air occurred, reactivation was obtained by heating to 170-200° (manufacturer's instructions). The sequence of eluents was benzene or dichloromethane, ether, ethyl acetate, acetone, and methanol individually or in binary mixtures. The proportion of weight of substance to be adsorbed to weight of adsorbent was 1:50 to 1:100. The proportion of weight of substance, in grams, to volume of fraction of eluent, in milliliters, was 1:100. The ratio of diameter to length of the column was 1:20. 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 done by Dr. M. Manser, Zurich, Switzerland.

Paper Chromatography—Unless specified otherwise, paper chromatography was carried out by the descending method on
Whatman No. 1 paper with a mixture of 1-butanol, acetic acid, and water (4:1:5, v/v, upper phase). The sugars were revealed with the silver nitrate reagent, the amino sugars and amino acids with ninhydrin, and the disaccharides with the Morgan-Elson reagent, as modified by Salton (3).

Colorimetric Determination of N-Acetylglucosamine Sugars—Color formation of N-acetylglucosamine sugars in the Morgan-Elson test (12) was determined with the modifications of Aminoff, Morgan, and Watkins (13) and Reissig, Strominger, and Leloir (14) and with an additional modification of the latter procedure. Ghuyens and Salton (4) used the modification of Reissig et al. (14), applying various heating times, and they observed that the disaccharide gave a maximum color yield at 40 min of heating time. In our hands, no such maximum was observed, the color still increasing after 45 min of heating time; a time of 35 min was therefore arbitrarily selected. The color values were calculated on a molar basis.

RESULTS

Preparation of Dialyzable Fraction of Lysozyme Digest of M. lysodeikticus Cell Walls—Commercially available cells of M. lysodeikticus were disintegrated in the Sorvall Omni-Mixer high speed homogenizer, and the walls were isolated according to the method of Sharon and Leloir (15). The amino acid composition, after hydrolysis in 6 N hydrochloric acid for 18 hours at 110°C, was determined with an automatic amino acid analyzer. The results are reported in Table I. Glucosamine (2-amino-2-deoxyglucose) and muramic acid (2-amino-2-deoxy-3-(N-L-carboxyethyl)-N-glucose) were shown by paper chromatography to be present in the approximate molar ratio of 1:1 but were not determined quantitatively.

Table I

<table>
<thead>
<tr>
<th>Amino acid composition of isolated cell walls of M. lysodeikticus</th>
<th>mmoles/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>1.50</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.58</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.73</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.58</td>
</tr>
</tbody>
</table>

*The determination was performed by Dr. G. L. Mechanic.

The lyophilized cell walls were then digested with crystalline egg white lysozyme (1 to 2 mg of enzyme per g of walls in 40 ml of 0.05 N ammonium acetate solution). The digestion was carried out under toluene for 24 hours at 37°C. The resulting clear solution was dialyzed against 3 to 4 volumes of distilled water at 3-4°C for 24 hours, and the dialysate was lyophilized. The dialysis was repeated three more times against similar volumes of fresh water, and each of the dialysates was lyophilized. The resulting white powders were combined, dissolved in a small volume of water, filtered, and lyophilized again. The solution retained in the dialysis bag was centrifuged at 9000 x g for 15 min to remove a small amount of suspended impurities, and was lyophilized. From 1 g of cell walls, 250 to 300 mg of dialyzable material and 650 to 700 mg of nondialyzable material were obtained.

Preparation of Disaccharide by Charcoal Chromatography of Dialyzable Fraction—A column (20 x 2.5 cm) of charcoal (Daro G-60) and Celite 535, 1:1 (w/w), was prepared according to Whistler and Durso (17). It was washed extensively with 3 N hydrochloric acid, followed by distilled water. A solution of 500 mg of the dialyzable fraction in 2 ml of water was added to the column, and the elution was started with 500 ml of water. This eluate was lyophilized, giving 52 mg, which consisted essentially of ammonium acetate and of a small amount of monosaccharides which were not further investigated. The column was then connected to a mixing chamber with 500 ml of water, to which a reservoir containing a mixture of ethanol and water, 2:8 (v/v), was attached. Fractions of 15 ml were collected at a rate of 2.5 ml per hour, and 140 fractions (approximately 1800 ml) were collected in this fashion. Aliquots of 0.5 ml of every other fraction were assayed by the Reissig et al. (14) modification of the Morgan-Elson procedure, with a heating time of 35 min; crystalline 2-acetamido-2-deoxyglucose was used as standard. The alcohol concentration was measured gravimetrically in selected tubes. A typical elution curve is given in Fig. 1. The fractions giving a positive Morgan-Elson reaction were pooled and lyophilized; after elution of 6 mg of material (tubes 1 to 45), a first peak (C-1) weighing 98 mg was eluted at an ethanol concentration of 12 to 15%; a second peak (C-2) emerging at an ethanol concentration of 16 to 18% weighed 30 mg; and a third peak (C-3) emerging at an ethanol concentration of 18 to 19% gave an additional 20 mg, which was followed by 13 mg of material. Further elution with 500 ml of ethanol and water (1:1, v/v) gave 107 mg of material, and a final elution with 500 ml of pyridine-ethanol-water (2:49:49, v/v) gave 5 mg. The total recovery of material from the column was thus 330 mg (66%).

Composition of Fractions from Charcoal Chromatography—Samples of 10 mg of the pooled fractions eluted from the charcoal column were hydrolyzed with 1 ml of 2 N hydrochloric acid for 4 hours at 100°C for analysis of the carbohydrate components, and with 6 N hydrochloric acid for 18 hours at 110°C for the amino acids. In all cases the acid was removed under vacuum in the presence of potassium hydroxide after completion of the hydrolysis. The dried samples were dissolved in 0.2 ml of water and examined by paper chromatography. Fractions C-1 and C-2 gave mainly spots corresponding to 2-amino-2-deoxyglucose and 2-amino-3-O-(1-carboxyethyl)-2-deoxy-D-glucose, with only trace amounts of amino acids. Later fractions contained the above two amino sugars together with lysine, glutamic acid, glycine, and alanine.

Fraction C-1 showed on paper chromatogram one major component with Rf 1-acetamido-2-deoxyglucose 1.0, together with several

Fig. 1. Isolation of the natural disaccharide by charcoal chromatography of the dialyzable fraction. Details of chromatographic techniques are reported in “Experimental Procedure.”
other contaminants. This major component did not give a reaction with ninhydrin, and reacted only weakly with the silver nitrate reagent. It gave a positive Morgan-Elson reaction on paper, however, and its chromatographic properties and color reactions suggested that it was the cell wall disaccharide previously described by Salton and Ghuysen (3,4) and by Perkins and Rogers (5-7). Since this disaccharide contained a free carboxyl group, purification on ion exchange resins was attempted.

**Purification of Disaccharide by Chromatography on Dowex 1**—Dowex 1 was converted into the acetate form, and then poured onto a column 1.6 × 50 cm (bed volume, approximately 100 ml). Fractions C-1 (418 mg) pooled from several charcoal columns were dissolved in 2 ml of water and added to the column. A mixing chamber containing 2 liters of water, into which was passed 0.8 N acetic acid from a reservoir, was attached to the column. Fractions of 13 ml were collected, at a rate of 80 ml per hour. Aliquots (1 ml each) of every other fraction were dried, under vacuum, in the presence of potassium hydroxide and analyzed for their N-acetylglucosamine content; a heating time of 35 min was used. For determination of the acetic acid concentration, 1 ml samples of every 10th tube were titrated with 0.1 N sodium hydroxide with phenolphthalein as indicator.

The elution pattern is shown in Fig. 2. The major peak (tubes 75 to 105, about 0.3 N acetic acid) was lyophilized to give 242 mg (58%) of substance which was homogeneous on paper chromatograms. The material obtained by lyophilization of tube 80 was identical on a paper chromatogram with that from tube 100. Acid hydrolysis of a sample of this peak with 2 N hydrochloric acid for 4 hours at 100°, followed by paper chromatography, revealed the presence of only glucosamine and muramic acid. The total recovery from the column was 265 mg (70%). Similar purification of Peak C-2 of the charcoal-Celite column showed that it contained only 3% of disaccharide.

**Direct Isolation of Disaccharide by Chromatography of Dialyzable Fraction on Dowex 1**—The method described above for the isolation of the disaccharide is tedious, since it involves two chromatographic separations, first on charcoal-Celite and then on Dowex 1. Accordingly, the possibility of isolating the pure disaccharide from the dialyzable fraction directly by chromatography on Dowex 1 was investigated.

To a column (1 × 32 cm) of Dowex 1-acetate, prepared as above, a solution of 150 mg of dialyzable material in 1 ml of water was added, and a gradient elution with acetic acid was applied. The mixing chamber contained 1 liter of water, and the reservoir, 0.8 N acetic acid. Fractions of 10 ml were collected at a rate of 20 ml per hour and assayed as described above. The results are shown in Fig. 3. Three major Morgan-Elson-positive peaks were isolated and analyzed by paper chromatography before and after acid hydrolysis. Before hydrolysis, the first two peaks gave several slowly moving spots which reacted positively in the ninhydrin and Morgan-Elson tests and reacted weakly with the silver nitrate reagent. After acid hydrolysis, spots corresponding to glucosamine, muramic acid, lysine, glutamic acid, glycine, and alanine appeared. The third peak (tubes 52 to 64, about 0.3 N acetic acid) gave essentially a single spot showing \( R_F = 0.4 \text{acetamido-2-deoxyglucose} \) on paper chromatograms, with traces of slowly moving impurities. Upon acid hydrolysis, equimolar amounts of glucosamine and muramic acid, as judged by visual estimation of paper chromatograms, were liberated. The material obtained from this peak (20 mg,
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Calculated: C 45.94, H 6.50, N 5.64
Found: C 45.79, H 6.68, N 5.58

The infrared adsorption spectrum is shown in Fig. 4. Acid hydrolysis gave only glucosamine and muramic acid. On paper chromatograms, the compound behaved identically with the product described by Sutton and Ghuysen (3, 4) and by Perkins and Rogers (5-7) (Table III). It also showed on paper chromatograms the same speed of migration in two different solvent systems as the disaccharide described by Hoshino (8). The color developed in various modifications of the Morgan and Elson reaction is reported in Table II.

Heptaacetyl Methyl Ester Derivative of Natural Disaccharide—A solution of 13 mg of natural disaccharide in 0.5 ml of pyridine and 0.4 ml of acetic anhydride was left for 72 hours at room temperature. The solution was then poured into ice-water and extracted with chloroform. The organic layer was washed with water, dried, and evaporated to a sirupy residue which was freed from pyridine by codistillation with toluene. To a solution of

\[ C_{15}H_{22}N_{2}O_{3}\]

Color yields of natural and synthetic oligosaccharides in Reissig et al. and Aminoff et al. modifications of Morgan-Elson test

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Reissig et al. (14) modification</th>
<th>Aminoff et al. (13) modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Acetylmuramic acid</td>
<td>0.95</td>
<td>1.15</td>
</tr>
<tr>
<td>Natural disaccharide</td>
<td>0.85</td>
<td>0.95</td>
</tr>
<tr>
<td>Synthetic disaccharide</td>
<td>0.88</td>
<td>1.15</td>
</tr>
<tr>
<td>Natural tetraccharide</td>
<td>0.88</td>
<td>1.8</td>
</tr>
</tbody>
</table>

- Relative to an equimolar amount of 2-acetamido-2-deoxy-\(\beta\)-glucopyranosyl-\(1\rightarrow6\)-2-acetamido-3-\(O\)-(n-1-carboxymethyl)-2-deoxy-\(\alpha\)-\(\beta\)-glucopyranose (1); natural disaccharide (II); 2-acetamido-1,4-di-\(O\)-acetyle-2-deoxy-3-\(O\)-(n-1-carboxymethyl)-2-deoxy-\(\alpha\)-\(\beta\)-glucopyranose (III); heptaacetyl methyl ester derivative of natural disaccharide (IV).

Paper chromatography of natural and synthetic disaccharides

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Re-acetylated disaccharides in solvent mixtures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Natural disaccharide</td>
<td>1.05</td>
</tr>
<tr>
<td>Synthetic disaccharide</td>
<td>1.00</td>
</tr>
<tr>
<td>N-Acetylmuramic acid</td>
<td>2.0</td>
</tr>
</tbody>
</table>

- I, butanol-acetic acid-water, 4:1:5 (upper phase); II, butanol-acetic acid-water, 3:1:1; III, butanol-pyridine-water, 6:4:3; IV, pyridine-ethyl acetate-acetic acid water, 5:5:1:3.
- Reproducibility of results in this solvent mixture was not good, and lower values were sometimes observed (1).

- Admixture of this compound with synthetic 2-acetamido-1,4-di-\(O\)-acetyl-2-deoxy-3-\(O\)-(n-1-[methylcarboxylate]ethyl)-6-\(O\)-(2-
acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl) -α-D-glucopyranose (19) depressed the melting point to 210° to 220°.

The infrared adsorption spectrum is reported in Fig. 4, the x-ray diffraction data in Table V, and the mass spectrum in Fig. 5.

Synthesis of 2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 6)-2-acetamido-3-O-[α-1-(methyl carboxylate)ethyl]-6-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-α-D-glucopyranoside (Compound II)-Benzyl 2-acetamido-4-O-acetyl-2-deoxy-3-O-[α-1-

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Melting point</th>
<th>Optical rotation, [α]d</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural disaccharide</td>
<td>Amorphous</td>
<td>+10 → +4.5°</td>
<td>Water</td>
</tr>
<tr>
<td>Synthetic disaccharide</td>
<td>Amorphous</td>
<td>+16 → +14</td>
<td>Water</td>
</tr>
<tr>
<td>Derivative of natural disaccharide</td>
<td>235-236°</td>
<td>+40</td>
<td>Chloroform</td>
</tr>
<tr>
<td>Derivative of synthetic disaccharide</td>
<td>240-241°</td>
<td>+40</td>
<td>Chloroform</td>
</tr>
</tbody>
</table>

* O-2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 6)-2-acetamido-3-O-[α-1-(methyl carboxylate)ethyl]-2-deoxy-β-D-glucopyranoside.

**Heptaacetyl methyl ester.

2-Acetamido-1,4-di-O-acetyl-2-deoxy-3-O-[α-1-(methyl carboxylate)ethyl]-6-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-α-D-glucopyranoside (19).

**X-ray diffraction data of crystalline heptaacetyl methyl ester derivatives of natural and synthetic disaccharides**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>X-ray diffraction data*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural disaccharide, heptaacetyl methyl ester</td>
<td>A</td>
</tr>
<tr>
<td>2-Acetamido-1,4-di-O-acetyl-2-deoxy-3-O-[α-1-(methyl carboxylate)ethyl]-6-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-α-D-glucopyranoside (19)</td>
<td>14.73M, 12.8M, 10.83W, 9.31W</td>
</tr>
<tr>
<td>Heptaacetyl methyl ester</td>
<td>3.04S, 3.00S, 2.98S, 2.72S</td>
</tr>
<tr>
<td>(1), 8.64M, 6.61M, 6.37M</td>
<td></td>
</tr>
<tr>
<td>5.01M, 4.96M, 4.46S (2), 4.27M</td>
<td></td>
</tr>
<tr>
<td>3.83W, 3.71W, 3.53W</td>
<td></td>
</tr>
<tr>
<td>3.44M, 3.30W, 2.75V, 2.49</td>
<td></td>
</tr>
<tr>
<td>V, 2.27V, 2.00W.</td>
<td></td>
</tr>
</tbody>
</table>

* Data given are interplanar spacings for CuKα radiation with relative intensities estimated visually. S, strong; M, moderate; W, weak; V, very; strongest lines are numbered in order of decreasing intensities (1, strongest).
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pmg) of 10% palladium on charcoal as catalyst. After filtration and evaporation, the residue was purified by adsorption on a column of Dowex 1-acetate, followed by elution with a gradient of acetic acid, as described for the natural disaccharide. The fractions showing a positive Morgan-Elson reaction were pooled to give, after lyophilization, 30 mg (67%) of an amorphous product which showed a slight mutarotation in water from $[\alpha]_D^{25} +16^\circ$ to $+14^\circ$ (after 4 hours, at equilibrium, $c = 1.06$). Prior to analysis, the product was dried for 2 hours at 60-70° in high vacuum in presence of phosphorus pentoxide; the results of chemical analysis showed the strong retention of 1 mole of water.

$$C_{13}H_{22}N_2O_7\cdot\text{H}_2\text{O}$$

Calculated: C 44.35, H 6.86, N 5.44

Found: C 44.22, H 6.86, N 5.00

The behavior of the product on paper chromatograms in four different solvent systems is reported in Table III, the color formation in the modified Morgan-Elson reactions in Table II, and the infrared absorption spectrum in Fig. 5.

DISCUSSION

The cell walls were prepared from commercially available cells of *M. lysodeikticus* as previously described (15). Attempts to prepare the walls by trichloroacetic acid extracts according to the method of Park and Hancock (21), which could be adapted to large scale, were not successful, since the *M. lysodeikticus* cell wall was unstable to hot trichloroacetic acid, as observed originally by Salton and Pavlik (22). An electron micrograph was similar to that of other gram-positive cell walls (23) and showed a homogeneous material. The relative proportion of amino acids found in our preparations is similar to that found by other investigators (22, 24), although the total content of these acids found previously was lower. Degradation with crystalline lysozyme was carried out in a fashion similar to that previously described (4) and the amount of dialyzable material recovered was of the same order as previously reported (4, 7).

In order to fractionate the large amount of material available, column chromatography was attempted. The first adsorbent investigated was a mixture of Darco G-60 charcoal and Celite 535 (17), the eluents being water, then water containing an increasing amount of ethanol, and finally a mixture of water, ethanol, and pyridine. The results are reported in Fig. 1. The total recovery of material was about 70%. Fraction C-1 (20% of the dialyzable material) showed one main spot on the paper chromatogram. After hydrolysis, it gave only 2-amino-2-deoxyglucose (glucosamine) and 2-amino-3-O-(1-carboxyethyl)-2-deoxyglucose (muramic acid). The behavior of the spot on paper chromatograms and the color reactions given by Fraction C-1 were identical with those of the disaccharide described by Salton (3), and Perkins (7). Fraction C-2 contained only a very small amount of the same disaccharide. Further purification was accomplished by chromatography on a column of Dowex 1-acetate, with a gradient of acetic acid, and the results are shown in Fig. 2. The major peak was the pure disaccharide obtained in this last purification in about 60% yield for a total recovery of 70% of the material placed on the column. The yield of pure disaccharide was about 12% of the dialyzable material and about 4% from the whole cell wall. Direct fractionation of the dialysate on Dowex 1-acetate with the same acetic acid gradient gave a peak of pure disaccharide, in addition to peaks showing the presence of amino acids (Fig. 3). The yield of disaccharide was of the same order of magnitude as that obtained in the purification with two absorptions, 13% from the dialyzable fraction.

In addition to the disaccharide and to the compounds containing amino acids, a product corresponding to the previously described tetrasaccharide (10) was obtained in both purifications. In the purification on Darco G-60 and Celite, an additional
amorphous compound was isolated in a small yield (0.1% of the weight of the cell wall); on paper chromatograms its speed of migration corresponded to that of a disaccharide and it showed the presence of only 2-amino-2-deoxyglucose after hydrolysis.

The glucosamine-muramic acid disaccharide was shown to be homogeneous on paper chromatograms in four different solvent systems (Table III). It migrated on paper in a manner identical with the major spot of a product kindly provided by Dr. O. Hoshino. It was amorphous, but showed a slight mutarotation in water. The elemental analysis corresponded to that of a disaccharide composed of 2-acetamido-2-deoxyglucose and 2-acetamido-3-O-(p-1-carboxyethyl)-2-deoxyglucose. It was compared with synthetic 0-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-2-acetamido-3-O-(p-1-carboxyethyl)-2-deoxy-D-glucose (Compound II). This compound was obtained as an amorphous product in 67% yield by alkaline hydrolysis followed by catalytic hydrogenolysis of benzyl 2-acetamido-4-O-acetyl-2-deoxy-3-O-[p-1-(methyl carboxylate)ethyl]-6-O-(2-acetamido-3, 4, 6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-a-D-glucopyranoside (Compound I) (20). Structure II had been proposed for the disaccharide by Salton and Ghuysen (9, 10) and by Perkins (11).

Both disaccharides showed similar speeds of migration on paper chromatograms in three solvent systems, but the natural product was slightly faster on development with the upper phase of butanol-acetic acid-water, 4:1:5 (Table III). The optical rotations at equilibrium were slightly different (Table IV), but the infrared spectra (Fig. 4) showed no significant discrepancy. Both products showed significant differences in the Morgan-Elson test, however, either in the modification of Reissig et al. (14) with a 3-min or a 35-min heating time or in the modification of Aminoff et al. (13). The {\( \lambda_{\text{max}} \)} (545 and 585 m\( \mu \)) and \( \lambda_{\text{min}} \) (565 m\( \mu \)) of the absorption curves were identical for the colors formed by both compounds. When the colors obtained for both products in the three modifications were compared on a molar basis with the colors formed by crystalline 2-acetamido-3-O-(p-1-carboxyethyl)-2-deoxy-α-D-glucose (N-acetylmuramic acid) (20), the intensity of the color developed by the natural disaccharide was approximately 5, 30, and 25%, whereas that of Compound II was 95, 125, and 200%, respectively (Table II). The low yield in color produced by the natural disaccharide has already been observed by other investigators (8-11).

Since both the natural disaccharide and Compound II were amorphous, the preparation of various crystalline derivatives was attempted. Finally the \( \alpha \) anomer of the heptaacetyl methyl ester derivative of the natural disaccharide was obtained in crystalline form after treatment with acetic anhydride in pyridine solution, diazomethane, and then acetic anhydride and zinc chloride. Since a sufficient amount of Compound II was not available to prepare the \( \alpha \) anomer of the heptaacetyl methyl ester by the same method, a new synthesis of 2-acetamido-1, 4-di-O-acetyl-2-deoxy-3-O-(p-1-(methyl carboxylate)ethyl)-6-O-(2-acetamido-3, 4, 6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-a-D-glucopyranoside (Compound III) was devised and is reported elsewhere (19). The optical rotations of both derivatives were identical (Table IV); their melting points (Table IV), and their infrared adsorption curves (Fig. 4) were nearly identical. In admixture, however, both products gave a sharp depression of the melting point. Further evidence for the nonidentity of the peracyetylated natural disaccharide and Compound III was obtained by comparing the x-ray powder diffraction patterns of both products recrystallized from the same solvent mixture (Table V) and their mass spectra (Fig. 5). In the latter case, because of the complexity of the starting materials, no attempt was made to identify the resulting fragments. The striking difference of the patterns of fragmentation clearly indicates that the natural and synthetic disaccharides are not identical.

Since Structure II proposed for the natural disaccharide by Salton and Ghuysen (9, 10) and by Perkins (11) is not correct, it is necessary to re-examine their results and interpretations. The structure 0-2-acetamido-2-O-(p-1-carboxyethyl)-2-deoxy-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranosyl or N-acetylmuraminyl-(1→4)-N-acetylglucosamine, proposed by Hoshino (8), is not acceptable in view of the results of the alkaline iodine oxidation obtained by Perkins (11) and of the reduction with sodium borohydride obtained by Salton and Ghuysen (9, 10). The latter reduction is unfortunately not conclusive when applied to 2-acetamido-3-O-(p-1-carboxyethyl)-2-deoxy-D-glucose, since, in addition to a reduction, epimerization can take place at C-2 (25) as well as β elimination of the n-1-carboxyethyl group at C-3 under alkaline conditions (26). Attempts to obtain the pure 2-amino-3-O-(p-1-carboxyethyl)-2-deoxy-D-glucitol or its N-acetyl derivative by sodium borohydride reduction have not been successful as yet.¹

Since the 2-acetamido-2-deoxy-D-glucose is the nonreducing moiety of the natural disaccharide, the only possible structure is that of 0-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-3-O-(p-1-carboxyethyl)-2-deoxy-D-glucose (IV) and, for its heptaacetyl methyl ester derivative, Structure V.

Salton and Ghuysen (9, 10) based Structure II on the failure of the natural disaccharide to release formaldehyde after periodate oxidation. This behavior of a product possessing Structure IV can be explained by the disaccharide being in the pyranose form. In this case no formaldehyde is formed, as was shown in the oxidation of 2,3,4-tri-O-methyl-D-glucose (27) or of 2,4-di-O-methyl-D-galactose (28). Another possibility would be stabilization of the formyl ester at C-5 as a result of the oxidation of the C-1 to C-2 link in the 2-acetamido-3-O-(p-1-carboxyethyl)-2-deoxy-D-glucose moiety, and no further oxidation would take place between C-5 and C-6. This possibility was demonstrated in the oxidation of 3-O-methyl-D-glucose, 3,4-di-O-methyl-D-galactose, and 3,4-di-O-methyl-N-mannose (29). It is of interest that most of the derivatives of glucose, galactose, and mannose just mentioned are substituted at C-3 and C-4, as is the 2-acetamido-3-O-(p-1-carboxyethyl)-2-deoxy-D-glucose moiety of Compound IV.

A (1→6) linkage for the natural disaccharide was proposed by Perkins (11) on the basis of a positive Morgan-Elson reaction, since 2-acetamido-2-deoxyglucose derivatives substituted at C-4 had been shown to give no color in this reaction (20, 29). When the color formed by the natural disaccharide is compared with that formed by crystalline 2-acetamido-3-O-(p-1-carboxyethyl)-2-deoxy-D-glucose or by the synthetic disaccharide II, however, very low values are obtained under the usual conditions (Table II). The value was much higher after a 35-min heating time, but under these conditions N,N′-diacetylchitobiose, a disaccharide with (1→4) linkage, also shows a positive reaction as reported by Salton and Ghuysen (10). A C-4-substituted amino sugar, 2,4-diacetamido-2,4-dideoxy-D-glucose, has also been

¹ R. W. Jeanloz and E. Walker, unpublished results.
shown to give a positive Morgan-Elson reaction after a prolonged time of reaction (30).

It can thus be concluded that Structure IV is the most likely one for the disaccharide isolated from *M. lysodeikticus* cell wall after digestion with egg white lysozyme. Final evidence will have to await the synthesis of Compound IV, in which we are presently engaged.

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