Structure of the Cell Wall of Staphylococcus aureus, Strain Copenhagen

II. Separation and Structure of Disaccharides

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The disaccharides, \( \beta\)-1,6-N-acetylglucosaminyl-N-acetylmuramic acid and \( \beta\)-1,6-N-acetylglucosaminyl-N,4-O-diacetylmuramic acid, have been identified as products of hydrolysis of the cell wall of \( S. \) aureus by an acetylmuraminidase and an amidase from \( S. \) aureus. N-Acetylmuramic acid and N,4-O-diacetylmuramic acid were formed from these compounds after hydrolysis with a \( \beta\)-acetylmuraminidase. The data obtained do not exclude the presence of a small percentage of disaccharides with other linkages, however. Several bases for the resistance of cell walls of \( S. \) aureus to hydrolysis by egg white lysozyme have been discussed.

In the preceding paper (Ghuyzen and Strominger, 1963) the preparation of fragments of the cell wall of \( S. \) aureus, strain Copenhagen, following enzymatic hydrolysis was described. A glycopeptide and a teichoic acid–glycopeptide complex were obtained through the action of the “32 enzyme” (Ghuyzen et al., 1962) which catalyzes the hydrolysis of one of the glycosidic linkages in the cell wall polysaccharide. Further treatment of each of these materials with an enzyme which catalyzes the hydrolysis of the linkage between acetylmuramic acid and L-alanine (Ghuyzen et al., 1962) was carried out and resulted in formation of a high molecular weight peptide and an oligosaccharide fraction from the glycopeptide. These two compounds, in addition to a teichoic acid, were also formed on similar treatment of the teichoic acid–glycopeptide complex. In the present paper the separation of the oligosaccharide fraction into two disaccharides will be reported. The structures of these two disaccharides have been elucidated.

MATERIALS AND METHODS

Acetylamino sugar was determined by a modified Morgan-Elson procedure using sodium borate buffer as described in the preceding paper (Ghuyzen and Strominger, 1963). A second determination of acetyl-

Amino sugars, which is useful in distinguishing between \( N\)-acetylmuramic and \( N\)-acetylmuramic acid or other 3-substituted acetylamino sugars and has been employed in earlier work (Strominger, 1968), was carried out as follows: To the dry sample (0.01–0.05 \( \mu \)mole of acetylamino sugar), 100 \( \mu \)l of 0.1 \( \mu \)m 2-amino-2-methyl-1,3-propanediol (AMP) hydrochloride buffer, \( \text{pH} \) 9.15, was added. After 7 minutes in a boiling water bath, 500 \( \mu \)l of a solution containing 4 parts of glacial acetic acid and 1 part of Morgan-Elson reagent was added. The chromogram was developed for 30 minutes at 37° and was measured at 550 \( \mu \)m. This procedure differs from the first method in substitution of AMP buffer for borate buffer and in the proportions of glacial acetic acid and Morgan-Elson reagent employed in the final step.

Amino sugars were determined by \( N\)-acetylation, followed by a similar estimation of the \( N\)-acetylamino sugars in either borate or AMP buffer. The procedure employing borate buffer has been described previously (Strominger et al., 1959). The procedure employing AMP buffer was carried out as follows: To 0.01–0.05 \( \mu \)mole of amino sugar in 30 \( \mu \)l of \( \text{H}_2\text{O} \), saturated with NaHCO\(_3\), and 10 \( \mu \)l of freshly prepared 5% acetic anhydride in ice-cold water. After 10 minutes at room temperature, the samples were placed in a boiling water bath for 3 minutes. Then 100 \( \mu \)l of 0.1 \( \mu \)m AMP buffer, \( \text{pH} \) 9.2, was added.

Abbreviation used in this paper: AMP, 2-amino-2-methyl-1,3-propanediol.

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and without further heating. 500 ml of a solution of 4 parts of glacial acetic acid and 1 part of Morgan-Elsdon reagent was added. Color was again developed in 20 minutes at 37° and measured at 585 m. The extinction coefficients observed were: for glucosamine, in borate buffer t = 12,000 and in AMP buffer t = 5,500, and for muramic acid, in borate buffer t = 11,000 and in AMP buffer t = 23,000. The ratio, absorbancy in AMP buffer/absorbancy in borate buffer, was 0.46 for glucosamine and 2.1 for muramic acid. A linear relationship exists between this ratio and the proportion of glucosamine and muramic acid in the sample (Fig. 1). Therefore, since the extinction coefficients of these two amino sugars in borate buffer are nearly identical, total amino sugar can be estimated from the reaction carried out in borate buffer, and the per cent of each sugar present can be determined from the ratio of absorbancies in AMP and borate buffers with reference to the standard curve (Fig. 1).3

Formaldehyde, formed after periodate oxidation, was measured with chromotropic acid (MacPadyen, 1945) using a micro procedure (Suzuki and Strominger, 1960). For measurement of total acetyl, methyl acetal was formed in methanolic HCl, distilled, and measured as the hydroxamate using a micro modification of the method of Ludowig and Dorfman (Ludowig and Dorfman, 1960) and ethyl acetate as the standard.

O-acetyl groups were measured by direct reaction with alkaline hydroxylamine (Heitman, 1949), using the same reagents. In the O-acetyl determination, tetra-O-acetyl-3-deoxy-D-ribohexose and 2,4-di-O-acetyl-1,6-anhydro-3-deoxy-D-glucosaminohexose were used as standards.4 The color yield of these O-acetyl sugars per mole of acetate was considerably higher than the yield obtained from methyl or ethyl acetate.

Paper chromatography was carried out by the descending technique on Whatman No. 1 paper using the following solvents: (A) n-butanol-acetic acid-water (3:1:1), (B) isobutyratic acid-0.5 N NH₄OH (5:3), (C) pyridine-water (4:1), and (D) n-butanol-ethanol-water (52:32:16). Sugars were detected with the diphenylamine-trichloroacetic acid reagent (Hough et al. 1950) or with alkaline silver nitrate (Truvelyan et al., 1950).

**RESULTS**

**Preparation of Disaccharide 1 and Disaccharide 2.**—Three oligosaccharide preparations have been studied. Two of these were obtained independently from the glycopeptide (Fig. 5a, preceding paper, Ghuyssen and Strominger, 1963), and the third was obtained from the teichoic acid-glycopeptide complex (Figure 5b, preceding paper, Ghuyssen and Strominger, 1963). These three preparations have given identical data.

Paper chromatography of these preparations in solvents A and B, followed by spraying the paper with the diphenylamine-trichloroacetic acid reagent, revealed that each of the oligosaccharides yielded two compounds, colored pink by the reagent (Table I). These two compounds, which will be referred to as disaccharide 1 (slower moving) and disaccharide 2 (faster moving), were separated by preparative paper chromatography in solvent A, then purified on a column of Sephadex G-25 (Fig. 2). As expected from previous data (Ghuyssen and Strominger, 1963), the compounds behaved identically on the column. The effluents from

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3 Formation of the cyclic derivative essential for chromogen formation takes place in NaHCO₃ buffer during the earlier 3-minute heating, originally employed to destroy acetic anhydride. The pH of this mixture during heating (pH 8.3-8.5) is critical. Further heating in AMP under these conditions diminished the color yield.

4 Since minor variations in extinction coefficients occur from time to time, it is necessary to run a standard curve with each set of analyses.

5 We are extremely grateful to Drs. J. W. Pratt and N. K. Richtmeyer of the National Institutes of Health for providing these samples.
TABLE II

**AMINO SUGARS PRESENT IN DISACCHARIDES 1 AND 2**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total</th>
<th>Glucose-</th>
<th>Muramic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amino Sugar</td>
<td>amine</td>
<td></td>
</tr>
<tr>
<td>Disaccharide 1</td>
<td>12.0</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>As prepared</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After NaBH₄ reduction</td>
<td>6.4</td>
<td>8.4</td>
<td>0</td>
</tr>
<tr>
<td>Disaccharide 2</td>
<td>17.0</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>As prepared</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After NaBH₄ reduction</td>
<td>8.4</td>
<td>8.4</td>
<td>0</td>
</tr>
</tbody>
</table>

28-36 ml containing the disaccharides, were pooled separately and lyophilized. The residues were then dissolved in 1 ml of water.

**Composition of Disaccharides 1 and 2.**—After hydrolysis in 2 N HCl for 3 hours at 95° in a sealed tube, HCl was removed in vacuo. Two-dimensional paper chromatography in solvents C and A indicated that each disaccharide contained only muramic acid and glucosamine. No trace of glucose was detected in these hydrolysates with D-glucose oxidase.

Accurate estimation of the muramic acid and of glucosamine present after acid hydrolysis was carried out after N-acetylation, as described under Methods. Both disaccharides contained equimolar amounts of glucosamine and muramic acid (Table II).

Solutions of disaccharides 1 and 2 (25 µl) were treated with 25 µl of a fresh solution of NaBH₄ (15 mg/ml) during 2 hours at room temperature. After this treatment, the Morgan-Elson reaction for acetylamino sugars (carried out in borate buffer) was negative in both cases although the original disaccharides gave this reaction. Three hundred µl of 2 N HCl was then added and the mixture heated for 3 hours at 95° in sealed tubes. After the mixture was desiccated in vacuo over NaOH pellets, 200 µl of methanol was added to the residues. The solutions were again evaporated in vacuo. This treatment with methanol (to remove boric acid) was repeated seven times. The residues were dissolved in 25 µl of water, and amino sugars were once more estimated. All the muramic acid (but none of the glucosamine) had been destroyed in both cases (Table II). Thus each of the compounds is a disaccharide in which the reducing group is the muramic acid residue. It should be noted that their identical behavior on Sephadex G-25 had suggested that the compounds had nearly identical molecular weights. Moreover, the reducing powers of the two disaccharides, measured by ferricyanide reduction (Park and Johnson, 1949), were identical, yielding a value of 1.1 moles reduced per mole, relative to a glucose standard.

**Hydrolysis by β-Acetylglucosaminidase.**—The β-N-acetylglucosaminidase from pig epididymis (Findlay and Levvy, 1960; Sanderson et al., 1962) hydrolyzed both compounds. Complete hydrolysis was readily obtained. Each solution (about 50 µm/moles) was treated with 3.52 µl of the enzyme preparation in presence of 18 µl of 0.01 M phosphate buffer, pH 5.6.

The preparation employed hydrolyzed about 1 mmole of p-nitrophenyl-N-acetyl-β-D-glucosaminide per minute per ml and had a protein content of 0.35 mg per ml. An enzyme, capable of hydrolyzing these disaccharides, is also present in the crude preparation from Streptomyces albus G, but its activity is much weaker. This enzyme is probably the same as that which is able to hydrolyze diacetylsclerobioses.

**For 2 hours at 37°.** Chromatography of these incubation mixtures in solvent A indicated that the original disaccharides had disappeared. From each of the disaccharides a compound with the mobility of acetylglucosamine was formed (R₆ = 0.28; greenish color with the diphenylamino- TCA spray). Compounds colored pink with the spray reagent with R₆ = 0.53 (referred to as Y₁) and R₆ = 0.72 (referred to as Y₂) were obtained from disaccharides 1 and 2, respectively (Table I). Larger amounts of these four compounds were prepared by paper chromatography from larger scale incubation mixtures containing 3-4 µmoles of each disaccharide. They were eluted from the chromatograms and concentrated to a small volume.

After acid hydrolysis of aliquots in 2 N HCl and application of the colorimetric method for distinguishing glucosamine and muramic acid, only glucosamine was found in the two compounds with R₆ = 0.28 and only muramic acid in Y₁ and Y₂. Reduction of these compounds with NaBH₄ followed by acid hydrolysis resulted in total ablation of the color reactions, indicating that each of the compounds was a monosaccharide.

Aliquots of Y₁ and Y₂ were hydrolyzed in 2 N HCl and chromatographed in solvent A. Only one spot was revealed with ninhydrin. It had an R₆ identical to that of muramic acid. Moreover, ninhydrin degradation (Stoffyn and Jeanloz, 1954) of the acid-hydrolyzed Y₁ and Y₂ gave rise in each case to a degradation product identical to that obtained from muramic acid (Strominger et al., 1959) on paper chromatography in solvent D. Y₂ appeared to give a low extinction coefficient in the Morgan-Elson reaction carried out after 7 minute heating in borate buffer. After acid hydrolysis in 2 N HCl and racemization, its extinction coefficient was increased by 40% while the extinction coefficient of Y₁ was unchanged by this procedure.

**Acetyl analysis and peridate oxidation of the disaccharides and monosaccharides.**—Gas chromatography of the acid hydrolysates of 0.1 mmole each of Y₁ and Y₂ was carried out as employed recently (Sanderson et al., 1962; Erwin et al., 1961). The only volatile acid present in each case was acetic acid, but the yield of acetic acid appeared to be greater in Y₂ than in Y₁ (Fig. 3).

**Total acetyl analysis of disaccharide 1 and Y₁, by distillation of methyl acetate following hydrolysis in methanolic HCl indicated that these compounds contained 2 and 1 mole of acetic acid, respectively, as expected.** Disaccharide 2, however, contained 3 moles, and Y₂, 2 moles (Table III). Analysis for O-acetyl groups by direct treatment with alkaline hydroxylamine indicated that disaccharide 2 and Y₂ each contained one O-acetyl group while no O-acetyl groups were found in disaccharide 1 or Y₁ (Table III).

The O-acetyl group was also detected in infrared spectra of disaccharide 2 and Y₂ (Fig. 4). These spectra differed from the spectra of disaccharide 1 and Y₁ in the presence of absorption bands at 1730 cm⁻¹ and 1230 cm⁻¹, characteristic of acyl esters and due, respectively, to the C=O and C-O—stretching vibrations of these esters (Bemmy, 1958).

Fochalddehyde determination after periodate oxidation revealed that virtually no formaldehyde was produced from either disaccharide, while about 1 mole was formed from Y₁ and from Y₂ (Table III).

**Morgan-Elson Reaction of the Disaccharides and of the Monosaccharides.**—Acetylamino sugar determinations were carried out after different times of heating酸 groups.

The assistance of Dr. Gino Marcro and Mr. Andrew Bybell, Monsanto Chemical Co., St. Louis, is gratefully acknowledged.
in borate or in AMP₃ buffers. In 1% borate the minimum time of heating required for full development of the color for Y₁ was 4-5 minutes, and for Y₂ 10 minutes (Fig. 5). The delay observed with Y₂ is compatible with the time necessary to remove the O-acetyl group in this compound under alkaline conditions (see Abrams, 1958, for example). The absorption spectra obtained from Y₁ and Y₂ after 10 minutes of heating were identical to N-acetylglucosamine with characteristic maxima at 545 and 585 mp. The molar extinction coefficient at 585 mp in borate buffer was 23,500 for N-acetylmuramic acid (Y₁) and 21,000 for its O-acetyl derivative (Y₂). In AMP₃ buffer, full color development was not obtained after 30 minutes of heating (Fig. 5). The chromogens formed after heating in AMP₃ had the same absorption spectra as those formed after heating in borate. The molar extinction coefficients were 24,540 for the N-acetyl-

<table>
<thead>
<tr>
<th>Compound</th>
<th>Acetyl</th>
<th>O-Acetyl</th>
<th>Formaldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disaccharide 1</td>
<td>1.78</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Disaccharide 2</td>
<td>2.94</td>
<td>0.82</td>
<td>0.10</td>
</tr>
</tbody>
</table>
| N-acetyl-

**Table III**

**ANALYSIS OF VARIOUS COMPOUNDS**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Acetyl</th>
<th>O-Acetyl</th>
<th>Formaldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disaccharide 1</td>
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<td>2.94</td>
<td>0.82</td>
<td>0.10</td>
</tr>
</tbody>
</table>
| N-acetyl-

* Data are expressed as moles per mole of compound.
* Measured after hydrolysis in methanolic HCl and distillation of methyl acetate (Ludwig and Dorfman, 1960). * Measured by direct reaction with alkaline hydroxylamine (Hestin, 1949). * Measured after periodate oxidation at pH 4.5 (MacFadyen, 1945; Suzuki and Strominger, 1960). * The samples employed were prepared from disaccharides 1 and 2 after hydrolysis with β-acetylglucosaminidase.

**DISCUSSION**

The materials examined in the present study are representative of the polysaccharide backbones of the basal layer of the cell wall. The overall yields of material were about 50%. Losses were due to manipulations and to use of material for analysis; no fractions were discarded. This study is thus distinguished from the previous studies of oligosaccharides obtained after digestion of the cell wall of *M. lysodeikticus* by egg white lysozyme or by the F1 acetylhexosaminidase of *Streptomyces albus* G (Ghuyesen and Salton, 1960; Ghuyesen, 1960; Salton and Ghuyesen, 1960). In those studies only the dialyzable oligosaccharides, two of which were not linked to peptide, were examined.

Two disaccharides were separated from the oligosaccharide fraction. Each contained equimolar amounts of glucosamine and muramic acid, and the muramic acid in each was totally reduced by sodium borohydride, thus establishing that each was a disaccharide with muramic acid as the reducing end. Moreover, reducing powers of the two compounds were equivalent.
Fig. 4.—Infrared spectra. The absorption bands due to the O-acetyl groups are indicated by arrows.
Hydrolysis by a β-acetylgalactosaminidase, which is devoid of activity on α-acetylgalactosaminides, established that acetylgalactosamine is linked at the nonreducing end of each of the disaccharides in the β-configuration. The low yield of formaldehyde produced on periodate oxidation of the disaccharides contrasted with production of formaldehyde from the free sugars indicates that the aldehyde group of acetylgalactosamine is linked to C-6 of the acetylmuramic acid. Formaldehyde would be formed from these compounds if the linkage were at C-4, the only other carbon atom of acetylmuramic acid which would be available for the glycosidic linkage.7

One of the disaccharides, and the muramic acid residue derived from it after cleavage by the β-acetylgalactosaminidase, contained an O-acetyl as well as an N-acetyl residue. This O-acetyl group was detected chemically with alkaline hydroxylamine and by infrared spectroscopy. The formation of 1 mole of formaldehyde on periodate oxidation of the N,O-diacylmuramic acid established that the O-acetyl group is at C-4, and, moreover, provided evidence that this muramic acid residue was in the pyranose form. It is thus possible to assign the structures β-1,6-N-acetylgalactosaminyl-N-acetylmuramic acid and β-1,6-N-acetylgalactosaminyl-N,4-O-diacylmuramic acid to these disaccharides. The first of these is identical to a compound obtained from cell walls of *M. lysodeikticus* through the action of egg white lysozyme or of the F1 hexosaminidase from *Streptomyces albus* (Ghuysen and Salton, 1960; Ghuysen, 1960; Salton and Ghuysen, 1960). The presence of O-acetyl groups in bacterial cell walls has been observed several times previously.

1 The formaldehyde formed on periodate oxidation of the two disaccharides was low but not zero. The small amount observed (Table III) represented an optical density of about 0.050. Probably this small amount was derived from material eluted from the chromatograms with the disaccharides. The possibility that 10% of the disaccharides isolated might have the β-1,4-linkage, from which disaccharides formaldehyde would be formed, cannot be excluded, however. This point should be emphasized in considering the structure of the total polysaccharide in the cell wall.
Failure of 10 Congeners of myo-Inositol to Support or to Inhibit the Growth of a Cultured Human Cell

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Ten congeners of myo-inositol failed to support the growth of a human cancer cell (KB) even at $10^{-4}$ g/ml, 1000 times the minimal effective concentration of myo-inositol itself. Eight compounds tested at $10^{-3}$ g/ml failed to inhibit the growth-promoting activity of myo-inositol at $2 \times 10^{-3}$ g/ml, a ratio of analog to myo-inositol of 500:1.

Interest in the supporting or inhibiting effect of inositol congeners on human cell growth arises from the fact that ordinary (myo) inositol is one of the twenty-two organic compounds (including thirteen amino acids) which are necessary and sufficient for growth of cultured human cells. In the absence of myo-inositol, these defined components, supplemented with dialyzed serum, permit growth only on the addition of serum ultrafiltrate. Experiments in which ninety growth factors were examined showed that myo-inositol was able wholly to replace the ultrafiltrate, while none of the other eighty-nine factors, either separately or together, showed demonstrable activity (Eagle et al., 1955).

Most cultured mammalian cells can synthesize only a fraction of their myo-inositol requirement from glucose (Eagle et al., 1960). One cell line, a mouse fibroblast, not only produced enough for its own survival and growth but released sufficient inositol into the medium to permit the parabiotic growth of another and inositol-dependent line. Another cell, a variant of the Hela strain, synthesized marginal amounts, so that exogenous inositol became essential for survival only at inocula of less than 200,000-500,000/ml (Eagle and Piez, 1962). With most cultured mammalian cells, however, exogenous inositol was essential for survival and growth (Eagle et al., 1956), presumably because of the loss of the newly synthesized material to the medium in amounts which exceeded the biosynthetic capacity of the cell (Eagle and Piez, 1962).

A number of recently synthesized inositol analogs and derivatives (McCasland et al., 1954,1961,1963a,b,c; Shoelery et al., 1961) have now been tested both for their ability to support the growth of an inositol-requiring culture (human carcinoma strain KB) (Eagle, 1955), and for their possible antagonism to myo-inositol itself. The compounds are listed in Table I. None of these substances for inositol in any concentration up to $10^{-3}$ g/ml, 100 times the maximally effective concentration of myo-inositol (Eagle et al., 1956), and 1000 times the concentration ($10^{-3}$ g/ml) with a partial growth-promoting action. Further, when eight of these compounds were used at $10^{-3}$ g/ml in conjunction

References

Park, J. T., and Johnson, M. J. (1949), J. Biol. Chem. 181, 149.