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# Structure of the Cell Wall of *Staphylococcus aureus*, Strain Copenhagen. II. Separation and Structure of Disaccharides

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The disaccharides, β-1,6-*N*-acetylglucosaminyl-*N*-acetylmuramic acid and β-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 acetylmuramidase and an amidase from *Streptomyces albus* G. *N*-acetylmuramic acid and *N*,4-*O*-diacetylmuramic acid were formed from these compounds after hydrolysis with a β-acetylglucosaminidase. 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 (Ghuysen 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" (Ghuysen *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 (Ghuysen *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 (Ghuysen and Strominger, 1963). A second determination of acetylamino sugars, which is useful in distinguishing between *N*-acetylglucosamine and *N*-acetylmuramic acid or other 3-substituted acetylamino sugars and has been employed in earlier work (Strominger, 1958), was carried out as follows: To the dried sample (0.01–0.05 μmole of acetylamino sugar), 100 μl of 0.1 M 2-amino-2-methyl-1,3-propanediol ($AMP_2$) hydrochloride buffer, *p*H 9.15, was added.[1] After 7 minutes in a boiling water bath, 500 μl of a solution containing 4 parts of glacial acetic acid and 1 part of Morgan-Elson reagent was added. The chromogen was developed during 20 minutes at 37° and was measured at 585 mμ. This procedure differs from the first method in substitution of $AMP_2$ 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 $AMP_2$ buffer. The procedure employing borate buffer has been described previously (Strominger *et al.*, 1959). The procedure employing $AMP_2$ buffer was carried out as follows: To 0.01–0.05 μmole of amino sugar in 30 μl of $H_2O$ were added 10 μl of saturated $NaHCO_3$ and 10 μl 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 μl 0.1 M $AMP_2$ buffer, *p*H 9.2, was added,

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[1] Abbreviation used in this paper: $AMP_2$, 2-amino-2-methyl-1,3-propanediol.

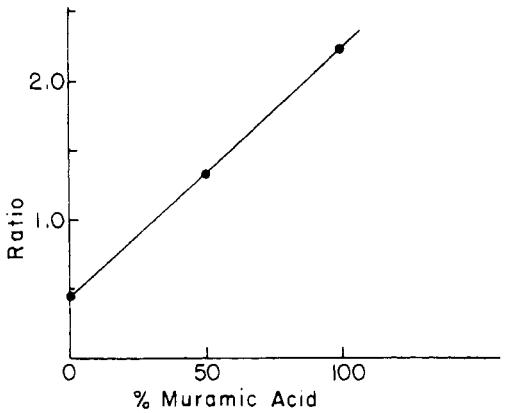


FIG. 1.—Determination of proportions of *N*-acetylglucosamine and *N*-acetylmuramic acid in a mixture. The ratio of absorbancies in the color tests carried out in borate and in $AMP_2$ buffers at *p*H 9.2 is the ordinate.

and without further heating[2] 500 $\mu$l of a solution of 4 parts of glacial acetic acid and 1 part of Morgan-Elson reagent was added. Color was again developed in 20 minutes at 37° and measured at 585 m$\mu$. The extinction coefficients observed were: for glucosamine, in borate buffer $\epsilon$ = 12,000 and in $AMP_2$ buffer $\epsilon$ = 5,500, and for muramic acid, in borate buffer $\epsilon$ = 11,000 and in $AMP_2$ buffer $\epsilon$ = 23,000. The ratio, absorbancy in $AMP_2$ 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 $AMP_2$ and borate buffers with reference to the standard curve (Fig. 1).[3]

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

*O-acetyl* groups were measured by direct reaction with alkaline hydroxylamine (Hestrin, 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-$\beta$-D-arabinohexose 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) isobutyric acid–0.5 N $NH_4OH$ (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 (Trevelyan *et al.*, 1950).

[2] Formation of the cyclic derivative essential for chromogen formation takes place in $NaHCO_3$ buffer during the earlier 3-minute heating, originally employed to destroy acetic anhydride. The *p*H of this mixture during heating (*p*H 8.3–8.5) is critical. Further heating in $AMP_2$ under these conditions diminished the color yield.

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

[4] We are extremely grateful to Drs. J. W. Pratt and N. K. Richtmeyer of the National Institutes of Health for providing these samples.

TABLE I

CHROMATOGRAPHIC MOBILITIES OF VARIOUS COMPOUNDS[a]

| Compound | Solvent A | Solvent B |
|---|---|---|
| *N*-acetylglucosamine | 0.28 | 0.55 |
| Diacetylchitobiose | 0.14 | 0.50 |
| Disaccharide 1 | 0.25 | 0.42 |
| Disaccharide 2 | 0.54 | 0.60 |
| *N*-acetylmuramic acid | 0.53 | — |
| *N,O*-diacetylmuramic acid | 0.72 | — |

[a] $R_F$ values are given.

## 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, Ghuysen and Strominger, 1963), and the third was obtained from the teichoic acid–glycopeptide complex (Figure 5b, preceding paper, Ghuysen 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 (Ghuysen and Strominger, 1963), the compounds behaved identically on the column. The effluents from

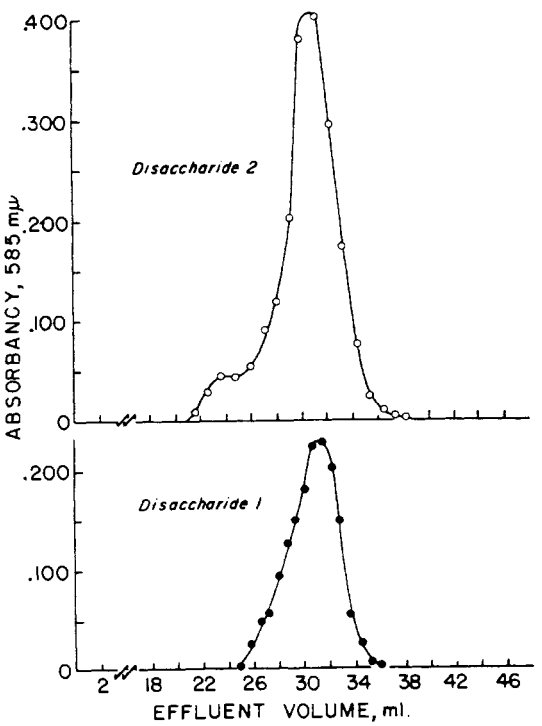


FIG. 2.—Filtration of the two disaccharides on a column of Sephadex G-25. Absorbancies were measured in the modified Morgan-Elson reaction using a 30-minute heating time in 1% borate buffer.

TABLE II

AMINO SUGARS PRESENT IN DISACCHARIDES 1 AND 2
Data are expressed as μmoles of amino sugar per ml of solution.

| Sample | Total Amino Sugar | Glucosamine | Muramic Acid |
|---|---|---|---|
| | Disaccharide 1 | | |
| As prepared | 12.0 | 6.0 | 6.0 |
| After $NaBH_4$ reduction | 6.4 | 6.4 | 0 |
| | Disaccharide 2 | | |
| As prepared | 17.0 | 8.5 | 8.5 |
| After $NaBH_4$ reduction | 8.4 | 8.4 | 0 |

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 $NaBH_4$ (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 mμmoles) was treated with 3.52 μl of the enzyme preparation[5] in presence of 15 μl of 0.01 M phosphate buffer, *p*H 5.6, 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 ($R_F$ = 0.28; greenish color with the diphenylamine-TCA spray). Compounds colored pink with the spray reagent with $R_F$ = 0.53 (referred as to $Y_1$) and $R_F$ = 0.72 (referred as to $Y_2$) 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 mixture 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 $R_F$ = 0.28 and only muramic acid in $Y_1$ and $Y_2$. Reduction of these compounds with $NaBH_4$ followed by acid hydrolysis resulted in total abolition of the color reactions, indicating that each of the compounds was a monosaccharide.

Aliquots of $Y_1$ and $Y_2$ were hydrolyzed in 2 N HCl and chromatographed in solvent A. Only one spot was revealed with ninhydrin. It had an $R_F$ identical to that of muramic acid. Moreover, ninhydrin degradation (Stoffyn and Jeanloz, 1954) of the acid-hydrolyzed $Y_1$ and $Y_2$ 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. $Y_2$ 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 reacetylation, its extinction coefficient was increased by 40% while the extinction coefficient of $Y_1$ was unchanged by this procedure.

*Acetyl analyses and periodate oxidation of the disaccharides and monosaccharides.*—Gas chromatography of acid hydrolysates of 0.1 μmole each of $Y_1$ and $Y_2$ was carried out as employed recently (Sanderson *et al.*, 1962; Erwin *et al.*, 1961).[6] The only volatile acid present in each case was acetic acid, but the yield of acetic acid appeared to be greater in $Y_2$ than in $Y_1$ (Fig. 3).

Total acetyl analysis of disaccharide 1 and $Y_1$ 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. Dissaccharide 2, however, contained 3 moles, and $Y_2$, 2 moles (Table III). Analysis for *O*-acetyl groups by direct treatment with alkaline hydroxylamine indicated that disaccharide 2 and $Y_2$ each contained one *O*-acetyl group while no *O*-acetyl groups were found in disaccharide 1 or $Y_1$ (Table III).

The *O*-acetyl group was also detected in infrared spectra of disaccharide 2 and $Y_2$ (Fig. 4). These spectra differed from the spectra of disaccharide 1 and $Y_1$ 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 (Bellamy, 1958).

Formaldehyde determination after periodate oxidation revealed that virtually no formaldehyde was produced from either disaccharide, while about 1 mole was formed from $Y_1$ and from $Y_2$ (Table III).

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

[5] 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 diacetylchitobiose.

[6] The assistance of Dr. Gino Marco and Mr. Andrew Bybell, Monsanto Chemical Co., St. Louis, is gratefully acknowledged.

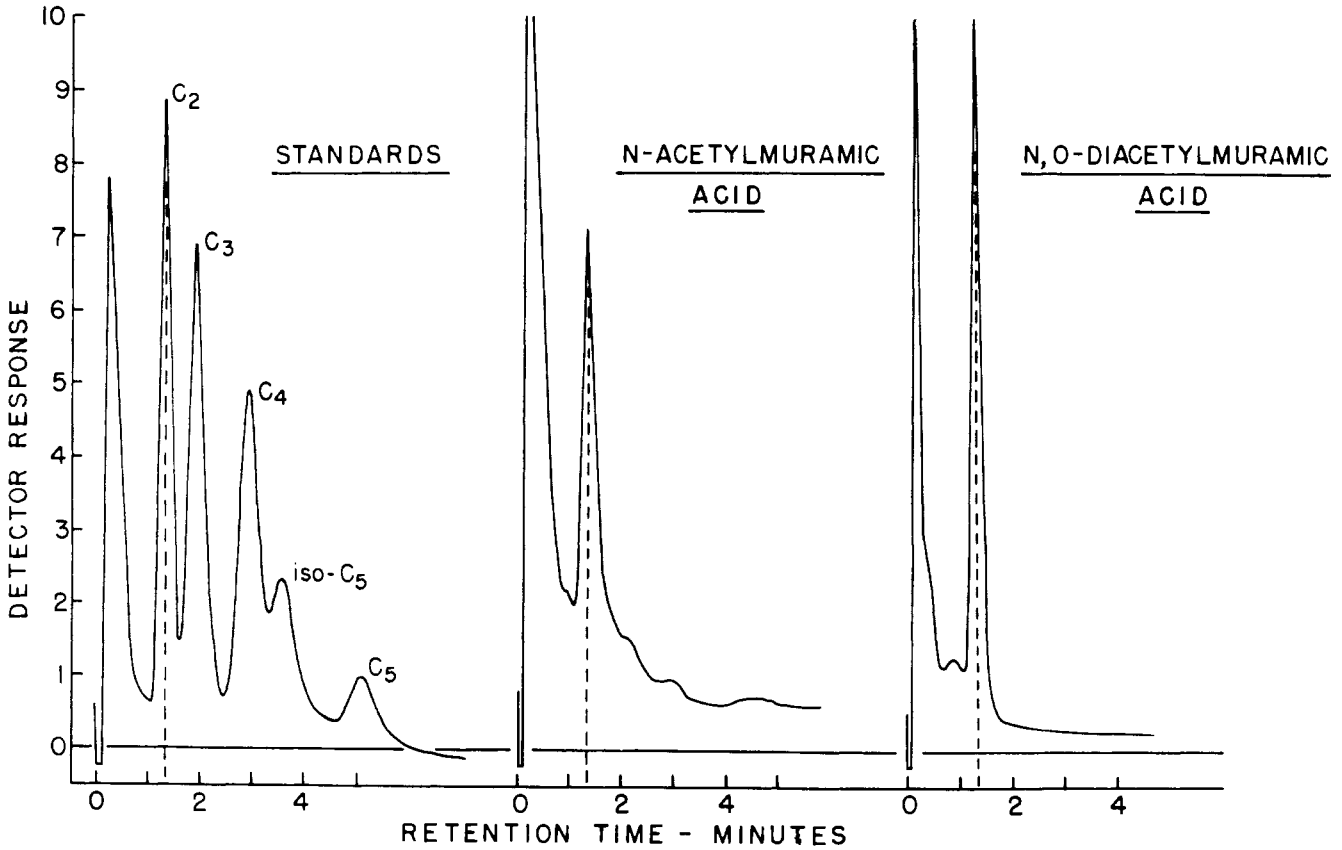


FIG. 3.—Gas chromatography of *N*-acetylmuramic acid and *N,O*-acetylmuramic acid after acid hydrolysis. Approximately equivalent amounts of the samples, derived from disaccharides 1 and 2, were employed. The standards were fatty acids of chain lengths indicated. Note that the retention times of the acids in the two sugars were the same as the retention time of acetic acid.

in borate or in $AMP_2$ buffers. In 1% borate the minimum time of heating required for full development of the color for $Y_1$ was 4–5 minutes, and for $Y_2$ 10 minutes (Fig. 5). The delay observed with $Y_2$ 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 $Y_1$ and $Y_2$ after 10 minutes of heating were identical to *N*-acetylglucosamine with characteristic maxima at 545 and 585 mμ. The molar extinction coefficient at 585 mμ in borate buffer was 23,800 for *N*-acetylmuramic acid ($Y_1$) and 21,000 for its *O*-acetyl derivative ($Y_2$). In $AMP_2$ buffer, full color development was not obtained after 30 minutes of heating (Fig. 5). The chromogens formed after heating in $AMP_2$ had the same absorption spectra as those formed after heating in borate. The molar extinction coefficients were 24,540 for the *N*-acetylmuramic acid ($Y_1$) but only 13,000 for its *O*-acetyl derivative ($Y_2$).

TABLE III
ANALYSES OF VARIOUS COMPOUNDS[a]

| Compound | Total Acetyl[b] | *O*-Acetyl[c] | Formaldehyde[d] |
|---|---|---|---|
| Disaccharide 1 | 1.78 | 0.00 | 0.06 |
| Disaccharide 2 | 2.94 | 0.82 | 0.10 |
| *N*-acetylmuramic acid[e] | 0.98 | 0.00 | 0.79 |
| *N,O*-diacetylmuramic acid[e] | 2.05 | 1.12 | 0.75 |

[a] Data are expressed as μmoles per μmole of compound. [b] Measured after hydrolysis in methanolic HCl and distillation of methyl acetate (Ludowieg and Dorfman, 1960). [c] Measured by direct reaction with alkaline hydroxylamine (Hestrin, 1949). [d] Measured after periodate oxidation at *p*H 4.6 (MacFadyen, 1945; Suzuki and Strominger, 1960). [e] The samples employed were prepared from disaccharides 1 and 2 after hydrolysis with β-acetylglucosaminidase.

Similar experiments were carried out with the disaccharides (Fig. 6). In borate, an optimum heating time of 25–30 minutes was found for both disaccharides. After this duration of heating the molar extinction coefficient at 585 mμ was 9000 for each compound. In $AMP_2$ buffer the optimum duration of heating was over 45 minutes, and the color yields were greatly decreased (Fig. 6).

## DISCUSSION

The materials examined in the present study are representative of the polysaccharide backbone of the basal layer of the cell wall. The over-all 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 $F_1$ acetylhexosaminidase of *Stretpomyces albus* G (Ghuysen and Salton, 1960; Ghuysen, 1960; Salton and Ghuysen, 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.

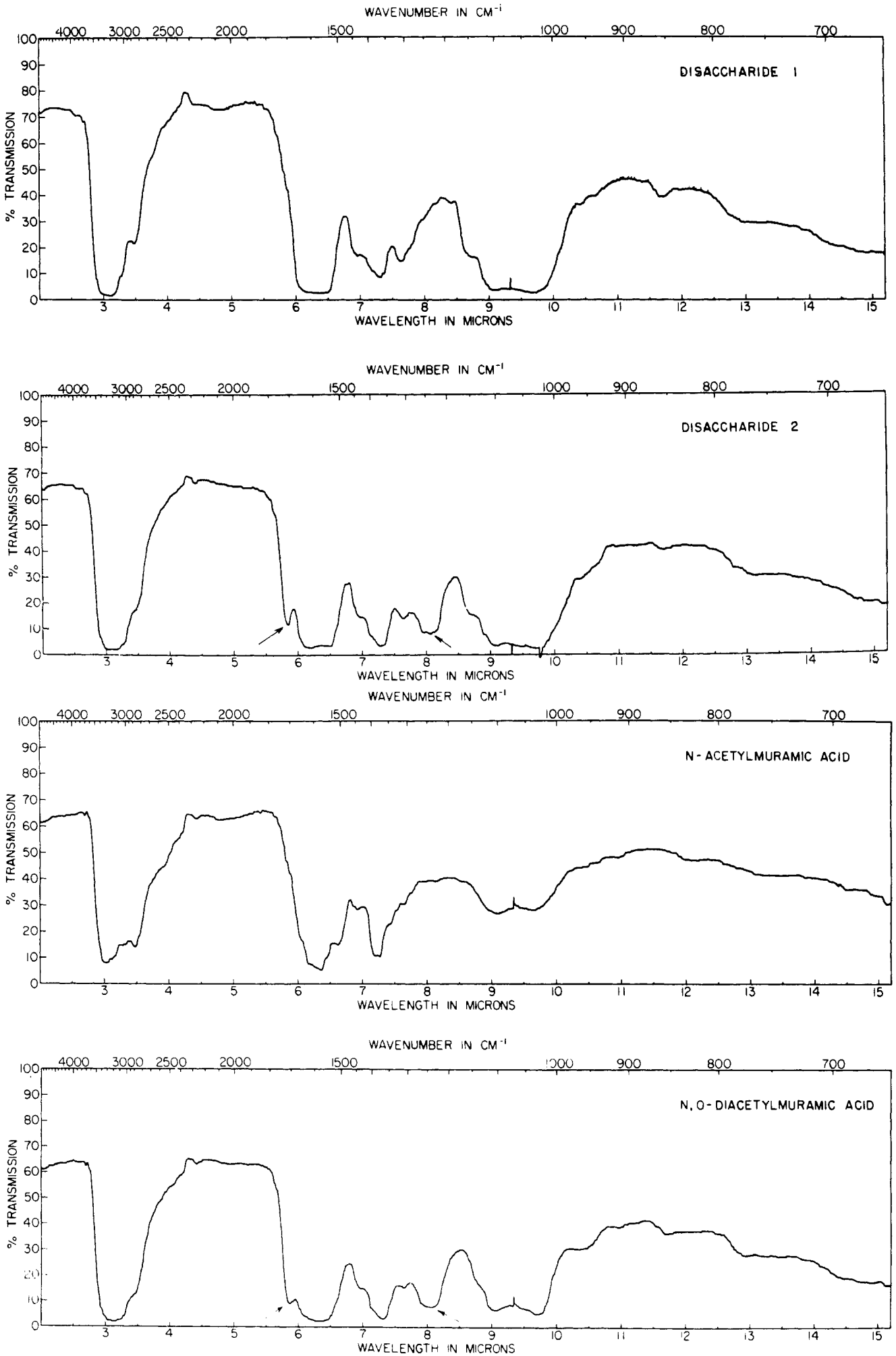


FIG. 4.—Infrared spectra. The absorption bands due to the *O*-acetyl groups are indicated by arrows.

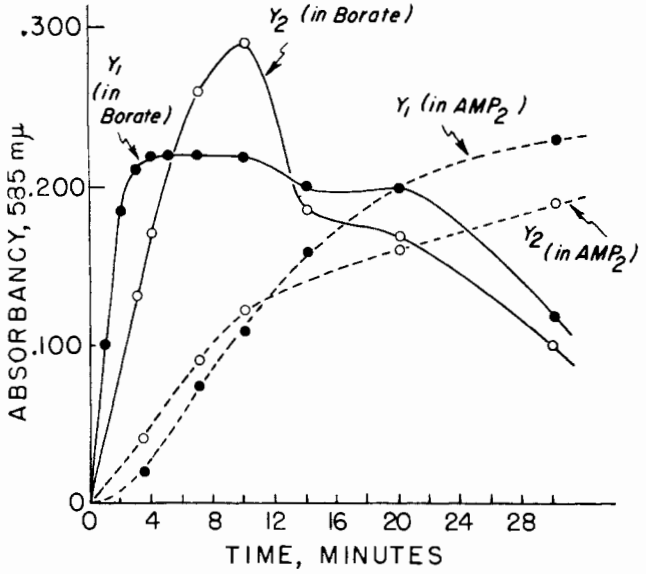


FIG. 5.—Influence of duration of heating at 100° on color development of the monosaccharides. $Y_1$ (*N*-acetylmuramic acid, 5.6 mμmoles) and $Y_2$ (*N,O*-diacetylmuramic acid, 8.7 mμmoles) were heated in borate or $AMP_2$ buffer in a modified Morgan-Elson method as described under Methods.

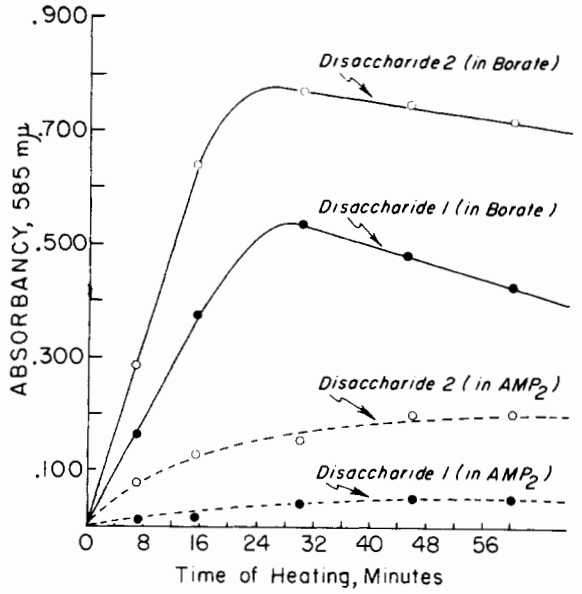


FIG. 6.—Influence of duration of heating at 100° on color development of disaccharides. Disaccharide 1 (36.6 mμmoles) and disaccharide 2 (52 mμmoles) were heated in borate or $AMP_2$ buffer in a modified Morgan-Elson method as described under Methods.

Hydrolysis by a β-acetylglucosaminidase, which is devoid of activity on α-acetylglucosaminides, established that acetylglucosamine 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 acetylglucosamine 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-acetylglucosaminidase, 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*-diacetylmuramic 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*-acetylglucosaminyl-*N*-acetylmuramic acid and β-1,6-*N*-acetylglucosaminyl-*N*,4-*O*-diacetylmuramic 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 $F_1$ 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 (Abrams, 1958; Brumfitt *et al.*, 1958; Brumfitt, 1959) but their location in the cell wall has not previously been established. The proportion of *N*-acetyl- and *N,O*-diacetylmuramic acid, and the corresponding disaccharides, was about 2:3. The entire preparation and characterization of these compounds was carried out fortuitously between *p*H 4 and *p*H 6 (Ghuysen and Strominger, 1963). At higher *p*H's the *O*-acetyl groups are labile, and it may be noted that the walls themselves were prepared from the cells between *p*H 7 and 8. Some loss of *O*-acetyl groups could have occurred in this *p*H range, and the percentage of *N,O*-diacetylmuramic acid residues in the wall itself is, therefore, a matter of conjecture. The minimum percentage of the *N*-acetylmuramic acid residues which are *O*-acetylated is, however, 60%.

The resistance of cell walls of *S. aureus* to egg white lysozyme (Salton, 1957) has at least two bases. The presence of the highly negatively charged antigen, the teichoic acid, in the cell wall has previously been shown to be inhibitory to this enzyme (Mandelstam and Strominger, 1961), a strongly basic protein. Moreover, the presence of *O*-acetyl groups in walls of mutants of *M. lysodeikticus* has been implicated as a cause of lysozyme-resistance (Brumfitt *et al.*, 1958; Brumfitt, 1959) and the presence of *O*-acetyl groups in *S. aureus* walls therefore presumably contributes to its resistance to egg white lysozyme. The enzyme employed in the preparation of the disaccharides from the walls of *S. aureus*, the "32 enzyme," a protein far less basic than egg white lysozyme (Ghuysen *et al.*, 1962), can from these studies be characterized as a powerful acetylmuramidase, capable of cleaving linkages of *N,O*-diacetylmuramic acid as well as linkages of *N*-acetylmuramic acid, whether or not the teichoic acid antigen is present in the wall. An exact description of the hydrolytic activity of this enzyme, however, must await investigation by other means of the nature of the linkage between the acetylmuramic acid residues and the acetylglucosamine residues in the polysaccharide.

It should be noted that free disaccharide was not formed on treatment of the cell walls of *S. aureus* with the acetylmuramidase alone; these compounds were liberated only on subsequent hydrolysis with the ami-

[7] The formaldehyde formed on periodate oxidation of the two disaccharides was low but not zero. The small values 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.

dase. These facts indicate the virtual absence of acetylmuramic acid residues, unsubstituted by peptide, in these walls. The situation is thus quite different from the hydrolysis of walls of *M. lysodeikticus* by egg white lysozyme, which treatment alone resulted in liberation of a small percentage of di- and tetrasaccharide (Ghuysen and Salton, 1960; Ghuysen, 1960). The cell wall of *S. aureus* may therefore be a far more rigid structure than that of *M. lysodeikticus*. This fact could provide a further explanation for its resistance to egg white lysozyme.

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# 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 $2 \times 10^{-6}$ 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, those 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.*, 1956).

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; Shoolery *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 substituted for inositol in any concentration up to $10^{-4}$ g/ml, 100 times the maximally effective concentration of *myo*-inositol (Eagle *et al.*, 1956), and 1000 times the concentration ($10^{-7}$ g/ml) with a partial growth-promoting action. Further, when eight of these compounds were used at $10^{-3}$ g/ml in conjunction

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