Long, C. N. H. (1947), *Recent Progr. Hormone Res. 1* 99.
Rosenfeld, G. (1955), *Endocrinology 56*, 649.
Saffran, M., and Schally, A. V. (1955), *Endocrinology 56*, 523.
Savard, K. (1954), *Recent Progr. Hormone Res. 9*, 185.
Savard, K., and Casey, P. J. (1964), *Endocrinology 74*, 599.
Savard, K., and Mason, N. R. (1963), Program of the Meeting of the Endocrine Society, Atlantic City (Abstract).
Schwenck, E., and Werthessen, N. T. (1952), *Arch. Biochem. Biophys. 40*, 334.
Shimizu, K., Gut, M., and Dorfman, R. I. (1962), *J. Biol. Chem. 237* 699.
Shimizu, K., Hayano, M., Gut, M., and Dorfman, R. I. (1961), *J. Biol. Chem. 236*, 695.
Solomon, S., Levitan, P., and Lieberman, S. (1956), *Rev. Can. Biol. 15*, 282.
Stone, D., and Hechter, O. (1954), *Arch. Biochem. Biophys. 51*, 457.
Werbin, H., and Chaikoff, I. L. (1961), *Arch. Biochem. Biophys. 93*, 476.
Zaffaroni, A., and Burton, R. B. (1951), *J. Biol. Chem. 193*, 749.

Structure of the Cell Wall of *Staphylococcus aureus*, Strain Copenhagen. III. Further Studies of the Disaccharides*

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ABSTRACT: Two disaccharides have previously been obtained in high yield from the cell wall of *Staphylococcus aureus* by the use of hydrolytic enzymes. Disaccharide 1 is *N*-acetylglucosaminyl-*N*-acetylmuramic acid and disaccharide 2 is *N*-acetylglucosaminyl-*N*,*O*-diacetylmuramic acid. After hydrolysis of disaccharide 2 with β-acetylglucosaminidase, *N*,*O*-diacetylmuramic acid was prepared. Data obtained from periodate oxidation of these compounds coupled with their susceptibility to β-acetylglucosaminidase indicate that both disaccharides are β-1,4- linked and that the *O*-acetyl group of disaccharide 2 is on the 6- position of *N*-acetylmuramic acid.

The high reducing power and positive Morgan-Elson reaction of the disaccharides are due to their unusual susceptibility to hydrolysis at alkaline *p*H. These and other anomalous properties of the compounds are discussed in relation to data previously obtained (J.-M. Ghuysen and J. L. Strominger (1963), *Biochemistry 2*, 1119).

By means of enzymatic degradation two disaccharides were isolated in high yield from the cell wall of *Staphylococcus aureus* (Ghuysen and Strominger, 1963b). These compounds contained *N*-acetylglucosamine and either *N*-acetylmuramic acid or *N*,*O*-diacetylmuramic acid with the muramic acid residue at the reducing end. The data obtained suggested that the disaccharides were β-1,6- linked. A similar linkage had been proposed for a disaccharide obtained in lower yield from the cell wall of *Micrococcus lysodeikticus* (Salton and Ghuysen, 1959, 1960; Perkins, 1960). Jeanloz *et al.* (1963) have recently synthesized β-1,6-*N*-acetylglucosaminyl-*N*-acetylmuramic acid. Its properties were different from those of the disaccharide from *M. lysodeikticus* and they suggested that the natural disaccharide was β-1,4- linked. As a consequence, the structures of the two disaccharides from *S. aureus* have been reinvestigated, and the data obtained are presented in this paper.

Materials and Methods

Cell Walls and Enzymes. Cell walls of *S. aureus* were prepared as described previously except that after isolation by differential centrifugation (Ghuysen and Strominger, 1963a) they were further purified by treatment with trypsin and ribonuclease in 0.05 M potassium phosphate buffer, *p*H 7.0. *Chalaropsis* B enzyme (Hash, 1963) was kindly donated by Dr. J. C. Hash of Lederle Laboratories, and *Streptomyces* acetylmuramyl-L-alanine amidase was prepared as previously described (Ghuysen *et al.*, 1962). D-Glucosamine-6-phosphate *N*-acetylase was prepared from yeast according to the method of Brown (1962). β-Acetylglucosaminidase was prepared from pig epidi-

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dymis as previously described (Sanderson *et al.*, 1962).

Analytical Procedures. Acetylamino sugar, total organic phosphate, reducing power, *O*-acetyl groups, free amino groups, and formaldehyde were determined as previously described (Ghuysen and Strominger, 1963a,b) except for diminution in scale in some instances. Hydrolysis for measurement of total amino sugar was performed at 95° in 30 μl of 3 N HCl in sealed tubes. After 5–7 hours, the contents were neutralized with 3 N NaOH (30 μl). The rest of the procedure was as previously described (Strominger *et al.*, 1959). A similar hydrolytic procedure was used in the measurement of glucosamine with the specific D-glucosamine-6-phosphate *N*-acetylase of yeast, as described by Lüderitz *et al.* (1964).

Periodate Oxidations. Materials (100–150 mμmoles) were oxidized in 30–45 μl of 0.01 M periodate and 0.01 M acetate, *p*H 4.5, at room temperature in the dark. Periodate consumption was determined by measurement of decrease in absorbancy at 224 mμ of aliquots (1 μl) diluted to 100 μl. The calculation was based on the differential extinction coefficient, $\Delta\epsilon^{1cm}_{224m\mu} = 8730$, for periodate and iodate (Dixon and Lipkin, 1954) and was checked in each experiment employing ethylene glycol standards. The standard for formaldehyde determination was either oxidized ethylene glycol or oxidized *N*-acetylglucosaminitol.

Reduction of Sugars and Alkaline Hydrolysis of Acetyl Ester Groups. Neutral solutions of reducing sugars were reduced at room temperature with a 10-fold molar excess of a fresh unbuffered sodium borohydride solution. Measurement of the reducing power of acidified and reneutralized aliquots was made to determine the proportion of unreduced sugar. During this treatment, the *p*H increased from 7 to about 10.5 as borate was produced and some hydrolysis of the alkali-labile *O*-acetyl groups occurred. When desired, *O*-acetyl groups were quantitatively removed by heating the solution for 30 minutes at 60° after reduction was complete. The solutions were then brought to *p*H 5 with acetic acid.

Optical Rotations. The optical activity of solutions (10 mg/ml) were measured using a 1-dm cell of 1.8 ml capacity and a Rudolph Model 200 photoelectric polarimeter.

Chromatography. Sephadex G-50, medium grade, and G-25, fine grade, were obtained from Pharmacia, Inc., Rochester, Minn. ECTEOLA- and CM-celluloses were obtained from BioRad Laboratories, Richmond, Calif. All columns were run at room temperature.

Preparation of Disaccharides. S. aureus cell walls (4000 mg) were suspended in 50 ml of 0.03 M triethylamine acetate buffer, *p*H 4.7, containing 10 mg of *Chalaropsis* B enzyme (Hash, 1963). Aliquots (0.4 μl) were removed for measurement of reducing power, which, after 5 hours at 37°, reached a maximum of 0.47 μmole/mg of cell wall, approximately equal to the muramic acid content of the walls. After 12 hours, insoluble material (240 mg) was removed by centrifugation,[1] and the supernatant was lyophilized (3760 mg). A sample (100 mg) of this material was filtered on a column of Sephadex G-50 (medium grade, 3 × 50 cm) eluted with water at a flow rate of 0.5 ml/minute. Fractions (2.5 ml) were analyzed for reducing power, free amino groups, and total phosphate. The elution pattern was found to be very similar to that of the "32" enzyme digest of cell walls previously described (Ghuysen and Strominger, 1963a) except that there was much less low molecular weight material (4 mg). The rest of the soluble digestion product (3660 mg) was dissolved in water (20 ml) and applied to a column of ECTEOLA-cellulose (3 × 23 cm) which had previously been equilibrated with 0.5 M LiCl, *p*H 5, and washed with water until the eluate was free of chloride ions. The column was eluted with water at 1 ml/minute, and after the collection of thirty 10-ml fractions the eluent was changed to 0.3 M LiCl, *p*H 5. Aliquots of the fractions were analyzed for reducing power, free amino groups, and total phosphate. All of the phosphate, together with material containing the bulk of the free amino groups, was eluted in a single peak by LiCl. This material was the teichoic acid–glycopeptide complex which will be described in the following paper of this series (Ghuysen *et al.*, 1965). After removal of salt the material weighed 1732 mg. The bulk of the material with reducing power was eluted with water, and weighed 1590 mg. It yielded analyses identical to the glycopeptide fraction isolated from the "32" enzyme digest of *S. aureus* cell walls (Ghuysen and Strominger, 1963a). A portion (500 mg) of this glycopeptide was incubated with 7 mg of *Streptomyces* acetylmuramyl-L-alanine amidase (Ghuysen *et al.*, 1962) in 5 ml of 0.05 M acetate buffer, *p*H 5.2. After 30 minutes, the release of free amino groups was maximal, and the digest was diluted to 15 ml and applied to a column of CM-cellulose (3 × 23 cm) which had previously been equilibrated with 0.4 M NaOAc, *p*H 6.4, and washed with water until the eluate was salt free. The column was eluted at 1 ml/minute with water (300 ml) and then with 0.4 M NaOAc, *p*H 6.4. Aliquots of the fractions (10 ml) were analyzed for reducing power, free amino groups, and total phosphate. A complete separation of nonadsorbed reducing material (disaccharides, 200 mg after lyophilization) from adsorbed material with free-amino groups and no reducing power (peptide) had been achieved. The peptide fraction was desalted by filtration on Sephadex G-25, fine grade, and then lyophilized (yield, 250 mg).

A portion (174 mg) of this mixture of disaccharides was separated by paper chromatography in butanol–acetic acid–water (3:1:1) as previously described (Ghuysen and Strominger, 1963b). The two components (disaccharide 1, R_F 0.25 and disaccharide 2, R_F 0.56), detected on marker strips with diphenylamine–trichloroacetic acid spray (Hough *et al.*, 1950), were eluted with water. The lyophilized eluates were separately chromatographed on a column of Sephadex G-25 (fine grade, 0.9 × 60 cm) in order to remove impurities

[1] In previous experiments, in which cell walls that had not been previously treated with trypsin and ribonuclease were employed, the amount of insoluble material was considerably greater (Ghuysen and Strominger, 1962a).

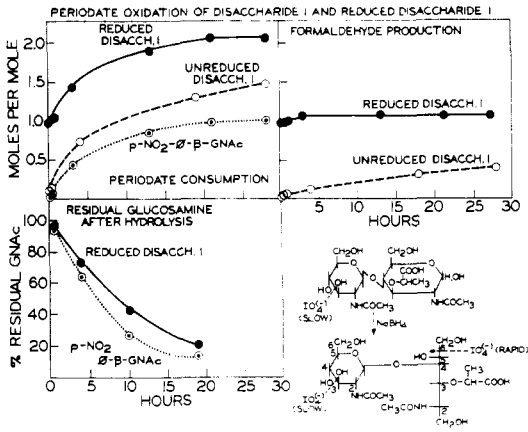

FIGURE 1: Periodate oxidation of disaccharide 1 and reduced disaccharide 1. Samples of disaccharide 1 or reduced disaccharide 1 (160 mμmoles) were oxidized in 0.01 M periodate (45 μl) containing 0.01 M acetate, *p*H 4.5, at room temperature. At the indicated times, aliquots were withdrawn for the determination of periodate consumption, formaldehyde production, and residual glucosamine (after hydrolysis).

derived from the chromatography paper. The column was eluted with water at 0.25 ml/minute, and fractions (1.5 ml) were analyzed for reducing power. The single sharp peak was pooled and lyophilized in each case. The yield of disaccharide 1 was 69 mg, and that of disaccharide 2 86 mg. As described elsewhere (Tipper *et al.*, 1964), the *Chalaropsis* B enzyme is an acetylmuramidase similar in specificity to the "32" enzyme from *Streptomyces*. Disaccharides 1 and 2 obtained here after lysis of cell walls with the *Chalaropsis* B enzyme are therefore identical with disaccharides 1 and 2 obtained after lysis with the *Streptomyces* "32"-acetylmuramidase (Ghuysen and Strominger, 1963b), followed in each case by further hydrolysis with acetylmuramyl-L-alanine amidase. They are both β-*N*-acetylglucosaminyl-*N*-acetylmuramic acids, differing only in the presence of an *O*-acetyl residue on the muramic acid residue of disaccharide 2.

Preparation of N,O-Diacetylmuramic Acid from Disaccharide 2. Disaccharide 2 (19 mg) was incubated at 38° in 350 μl of 0.01 M citrate buffer, *p*H 4.2, containing 0.1 M NaCl, 0.01% bovine serum albumin, and 20 μl of β-*N*-acetylglucosaminidase (activity, 10^7 mμmoles of *p*-nitrophenyl-β-*N*-acetylglucosaminide hydrolyzed/μl per hour). Aliquots (0.27 μl) were removed for measurement of free *N*-acetylamino sugar. After 6 hours, 78% of disaccharide 2 had been hydrolyzed. The reaction mixture was then applied to a column of Sephadex G-25 (fine grade, 1 × 47 cm) and eluted with water at 0.3 ml/minute. Fractions (1.4 ml) were analyzed for reducing power, and chloride ion was detected with silver nitrate. Reducing power appeared as two peaks, that with the larger elution volume being 83% of the total. It was free of chloride ion. This peak was pooled and separated by paper chromatography in butanol–acetic acid–water (3:1:1) as previously described (Ghuysen and Strominger, 1963b) into acetylglucosamine. (R_F 0.30) and *N,O*-diacetylmuramic acid (R_F 0.69). The diacetylmuramic acid was further purified by chromatography on the same Sephadex G-25 (fine grade) column used before (yield after lyophilization, 6.5 mg). The yields in this isolation procedure are summarized in Table I.

TABLE 1: Yields of Fractions during the Isolation of Disaccharides from 3660 mg of Soluble Cell-Wall Digest.

	Yield (mg)	Theory[a] (mg)	Obtained (%)
ECTEOLA-cellulose column			
Teichoic acid–glycopeptide complex	1732	1620	107
Glycopeptide	1590	2040	78
Total	3322	3660	91
CM-cellulose column			
Peptide	250	286	88
Disaccharides	208	223	93
Total	458	509	90
Paper chromatography			
Disaccharide 1	69		
Disaccharide 2	86		
Total	155	174	89
N,O-Diacetylmuramic acid	6.5	10	65

[a] The theoretical yields were calculated on the basis of a glycopeptide having the repeating unit (GNAc-MurNAc-Ala_2-Glu-Lys-Gly_5) and a teichoic acid containing (GNAc-ribitol-phosphate)$_2$-Ala. The amounts of these two units in the cell wall are approximately equal on a molar basis. The apparent yield of teichoic acid is enhanced by the glycopeptide component of the teichoic acid–glycopeptide complex, which also reduces the apparent yield of glycopeptide.

Results

Periodate Oxidations of Disaccharide 1. A sample of the *N*-acetylglucosaminyl-*N*-acetylmuramic acid (disaccharide 1) was reduced with $NaBH_4$. Determination of reducing power showed that less than 2% remained unreduced. Both the reduced and the unreduced disaccharides were subjected to periodate oxidation.

The reduced disaccharide consumed 1 mole of periodate rapidly with formation of 1 mole of formaldehyde. A second mole of periodate was consumed slowly without formaldehyde formation (Figure 1). The rate of oxidation of *p*-nitrophenyl-β-*N*-acetylglucosaminide was identical to the slow rate of uptake

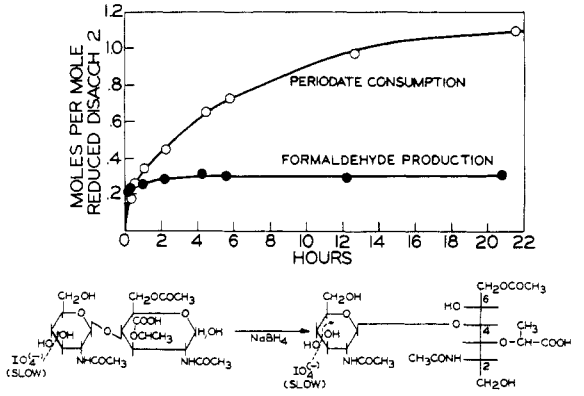

FIGURE 2: Periodate oxidation of reduced disaccharide 2. Sodium borohydride (1.5 μmoles) was added to a solution of disaccharide 2 (0.2 μmole) in water (30 μl). After 15 minutes at room temperature, reduction was terminated by the addition of acetic acid (3 μmoles). The product was oxidized in 0.01 M periodate (40 μl) containing 0.03 M acetate, *p*H 4.5. At the indicated times, aliquots were removed for the determination of formaldehyde and residual periodate.

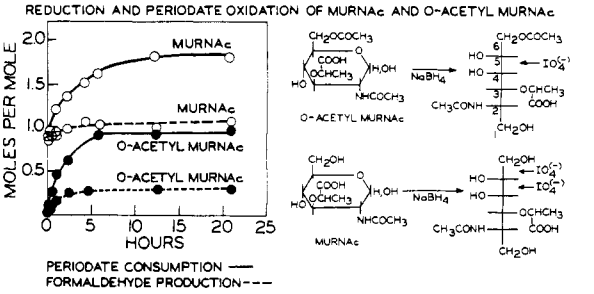

FIGURE 3: Periodate oxidation of reduced *N*-acetylmuramic acid and reduced *N,O*-acetylmuramic acid. Samples of *N*-acetylmuramic acid and *N,O*-diacetylmuramic acid (140 mμmoles) were reduced for 15 minutes at room temperature in 0.04 M sodium borohydride (30 μl). The products were oxidized in 0.01 M periodate (41 μl) containing 0.03 M acetate, *p*H 4.5. At the indicated intervals, aliquots were removed for the determination of formaldehyde and residual periodate.

of the second mole of periodate. Moreover, no glucosamine disappeared during uptake of the first mole, and its slow disappearance also corresponded to the rate of uptake of the second mole (Figure 1). The first mole of periodate was therefore consumed by *N*-acetylmuramic acid and the second by *N*-acetylglucosamine.

The unreduced disaccharide was also subjected to periodate oxidation (Figure 1). Periodate oxidation was slow and did not reach completion after 40 hours. The data indicate that the rate of uptake of periodate by *N*-acetylglucosamine in the unreduced disaccharide was the same as in the reduced disaccharide (as evidenced by the disappearance of glucosamine), but the oxidation of acetylmuramic acid, accompanied by formaldehyde production, was exceedingly slow.

Periodate Oxidations of Disaccharide 2. A sample of β-*N*-acetylglucosaminyl-*N,O*-diacetylmuramic acid (disaccharide 2) was completely reduced with $NaBH_4$, de-*O*-acetylated by alkaline hydrolysis, and oxidized with periodate. The data obtained were identical with those obtained for disaccharide 1, indicating that the presence of the *O*-acetyl group is the only difference between the two compounds.

A sample of disaccharide 2 was reduced with $NaBH_4$. During reduction, 24% of its *O*-acetyl groups were lost (measured with alkaline hydroxylamine) while its reducing power decreased by 93%. Periodate oxidation of this mixture (Figure 2) resulted in rapid uptake of about 0.2 mole of periodate with concomitant production of about the same amount of formaldehyde. The periodate consumption then increased slowly to 1.1 moles without further formaldehyde production. The small initial periodate consumption and formaldehyde production were presumably due to that portion of the material which had lost its *O*-acetyl group.

Periodate Oxidation of N-Acetylmuramic Acid and of N,O-Diacetylmuramic Acid. The rate of oxidation of these compounds under the standard conditions, as measured by formaldehyde production or periodate consumption, was extremely slow, being no more than 20% complete in 48 hours. The compounds were therefore reduced with $NaBH_4$ to products more easily oxidized. The reduction of *N,O*-diacetylmuramic acid was accompanied by a loss of 18% of the *O*-acetyl groups and a decrease in its reducing power of 90%. Periodate oxidation of this mixture (Figure 3) resulted in uptake of 0.95 mole of IO_4^- slowly with formation of 0.2 mole of formaldehyde. Reduced *N*-acetylmuramic acid consumed 1.75 moles of periodate. One mole was consumed rapidly with concomitant production of 1.0 mole of formaldehyde, while the remaining periodate was consumed slowly and resulted in production of only 0.1 mole of formaldehyde. Comparison of the behavior of *N*-acetylmuramic acid and *N,O*-diacetylmuramic acid in these experiments is compatible only with substitution of the *O*-acetyl group at C-6.

Reducing Power of Disaccharides. The reduction of ferricyanide by the disaccharides, calculated on a molar basis, appeared to be anomalously high when measured after 15 minutes' heating in carbonate-cyanide buffer (Park and Johnson, 1949). The reaction was therefore investigated as a function of the time of heating. The reaction with *N*-acetylglucosamine was 90% complete at 8 minutes (Figure 4). The reaction with the disaccharides was slower, however, and after 30 minutes reached a maximum of 1.45 moles of ferricyanide reduced per mole of disaccharide (relative to an *N*-acetylglucosamine standard). These high values suggested that hydrolysis of the glycosidic linkages might be taking place under the alkaline conditions of the determination.

Susceptibility of the Glucosamine Moiety to Reduction

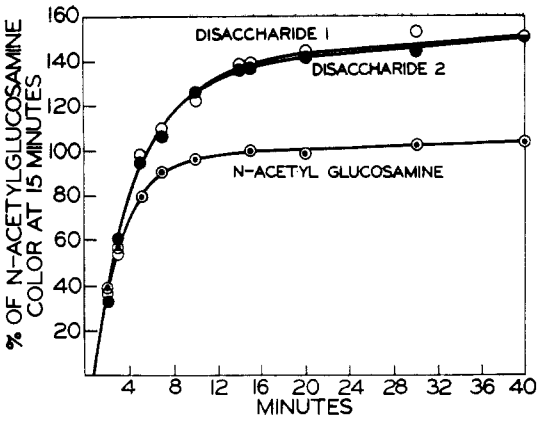

FIGURE 4: Reduction of ferricyanide by the disaccharides as a function of time of heating in carbonate-cyanide-ferricyanide. Samples of disaccharide or of *N*-acetylglucosamine (5 mμmoles) in 400 μl of the 1:1:2 mixture of reagent 1 (carbonate-cyanide), reagent 2 (ferricyanide), and water described by Park and Johnson (1949) were heated in a boiling-water bath for the indicated times. Reagent 3 (ferric ammonium sulfate and Duponol, 500 μl) was then added, and the optical density at 690 mμ was measured after 15 minutes at room temperature.

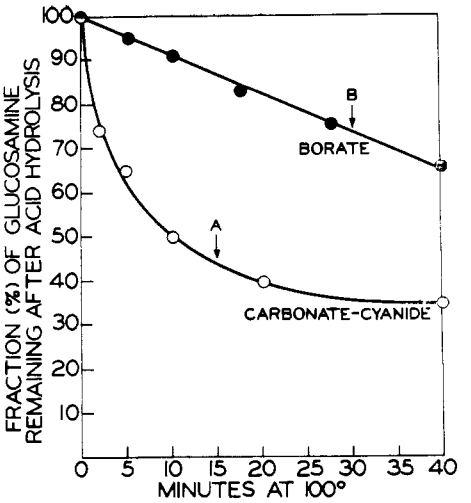

FIGURE 5: Alkaline hydrolysis of disaccharide 1. Samples of disaccharide 1 (45 mμmoles) were sealed in tubes with 30 μl of either 2% potassium borate or a 1:1:2 mixture of reagent 1, reagent 2, and water (Park and Johnson, 1949). The tubes were heated for the indicated times in a boiling-water bath. The products were reduced with sodium borohydride (300 mμmoles) and hydrolyzed in HCl, and the residual glucosamine was then determined.

with $NaBH_4$ *after Heating in Alkali.* Samples of disaccharide 1 (46 mμmoles) were sealed in tubes with 30 μl either of 2% borate (*p*H 9.15) employed in measurement of the Morgan-Elson reaction (Reissig *et al.*, 1955) or of the carbonate-cyanide-ferricyanide buffer (*p*H 10.3) employed in reducing power determination. These tubes were heated for varying times at 100°, cooled, acidified with 2 N HCl (3 μl), and, after 10 minutes at room temperature, made alkaline again by the addition of 2 N NaOH (3 μl). Fresh $NaBH_4$ solution (1 M, 3 μl) was then added to each solution, and after 5 hours at room temperature the solutions were acidified with 1 N acetic acid (6 μl) and dried. Boric acid was removed by repeated evaporations in the presence of absolute methanol. The residues were hydrolyzed in 2 N HCl (14 μl) for 6 hours at 95° and dried. The products of hydrolysis were analyzed for glucosamine. In both cases a progressive loss of glucosamine occurred with time of heating (Figure 5). These data indicated that hydrolysis of the disaccharide was occurring, thus exposing glucosamine residues for reduction by $NaBH_4$.

The hydrolysis rate in potassium borate buffer was lower than that in carbonate-cyanide buffer. Only 25% of the disaccharide was hydrolyzed at the time of maximum color yield in the modified Morgan-Elson reaction (Ghuysen and Strominger, 1963b). This would give rise to total acetylhexosamine equivalent to 50% of the disaccharide content, which is consistent with the color yield, which at this time is about 50% of that obtained from an equimolar amount of *N*-acetylglucosamine, heated for only 7 minutes. The subsequent drop in color yield probably reflects a competition between hydrolysis and destruction of the unstable chromogens produced from the products during heating in borate.

Under the conditions of reducing power determination, hydrolysis is apparently 55% complete at 15 minutes, but reaches a maximum of only 65% even on prolonged heating. It is probable, however, that the disaccharide is completely hydrolyzed at this time, but that a portion of the product, even after reduction and hydrolysis, is still able to give a positive Morgan-Elson reaction. When samples of disaccharides 1 and 2 and of *N*-acetylglucosamine were heated in carbonate-cyanide for 20 minutes, and then chromatographed on a thin layer of boric acid–buffered silica gel G in butanol-ethanol-water (11:3:6), spraying of the plate with anisaldehyde–sulfuric acid reagent (Stahl and Kaltenbach, 1961) caused immediate production at room temperature of bright blue spots (R_F 0.05) from all three compounds. These faded on heating at 140°, but this heating revealed other degradation products, all of which had higher mobilities than disaccharide 2, *N*-acetylglucosamine, or *N*-acetylmuramic acid, all of which were absent. Hydrolysis accompanied by degradation of the acetylamino sugars in the alkaline conditions of the first stage of reducing-power determination probably competes with oxidation by ferricyanide, and presumably explains why the reducing powers of the disaccharides do not reach a value of 2.

Optical Rotation. The specific rotations of disaccharides 1 and 2 at equilibrium (c = 1 in water, 23°) were +7.8° and +23.5°, respectively.

Discussion

These data, coupled with the fact that both disaccharides are hydrolyzed by β-*N*-acetylglucosaminidase (Ghuysen and Strominger, 1963b), are compatible only with a β-1,4- linkage for the disaccharides. The diacetylmuramic acid is *N*,6-*O*-diacetylmuramic acid. The data (2 moles uptake with formation of 1 mole of formaldehyde) obtained in periodate oxidation of the reduced *O*-acetyl-free disaccharides are compatible only with a 1,4- link, with the *N*-acetylglucosamine in pyranose ring form, or with a 1,6- link with *N*-acetylglucosamine in the furanose ring form. With the latter structure, however, the fast, first mole of periodate uptake accompanied by formaldehyde formation would occur at C_5-C_6 in *N*-acetylglucosamine and would also occur in the unreduced disaccharide, while the second slow oxidation would occur at C_4-C_5 in *N*-acetylmuramic acid. Clearly, however, the first mole of periodate taken up cannot be due to oxidation of *N*-acetylglucosamine, since glucosamine measured enzymatically disappeared slowly. These data eliminate the possibility of a 1,6- link.

The previous erroneous conclusion that the disaccharides were 1,6- linked was based on three facts, the interpretation of each of which involved an assumption now known to be incorrect. In interpreting periodate oxidations it may usually be assumed that the sugar at the reducing end of a disaccharide is an open chain. It is obvious from the striking difference in rate of periodate uptake of the reduced and unreduced disaccharides (Figures 1 and 2) that in this instance this assumption was unwarranted. There are several other examples of pyranose sugars in which ring opening is exceedingly slow during periodate oxidation. For example, the production of formaldehyde by periodate oxidation of diacetylchitobiose required several days (Barker *et al.*, 1958).

The positive Morgan-Elson reaction obtained after 30 minutes of heating at *p*H 9.2 in borate buffer had also suggested that the disaccharide was 1,6- linked, since 4-substituted *N*-acetylamino sugars do not give this reaction (Kuhn *et al.*, 1954; Jeanloz and Trémège, 1956). The unusually long heating time required (30 minutes) compared to 7 minutes for *N*-acetylglucosamine was unexplained. It is now apparent that the disaccharide is unusually alkali labile, having a reducing power of 1.5, and that the positive Morgan-Elson reaction is presumably given by the products of borate-catalyzed hydrolysis.

Finally, the formation of formaldehyde from *N*,*O*-diacetylmuramic acid during periodate oxidation seemed to indicate that the *O*-acetyl group was at C-4 and hence that the glycosidic linkage must have been at C-6. It is apparent, however, that formation of formaldehyde from *N*,*O*-diacetylmuramic acid may have been caused by the removal of some of its *O*-acetyl groups during periodate oxidation. The presence of impurities in the original preparation, possibly derived from the filter paper employed in purification, also may have contributed to the erroneous formaldehyde values.

References

Barker, S. A., Stacey, M., and Webber, J. (1958), *J. Chem. Soc.*, 2218.

Brown, D. H. (1962), *in* Methoden der Enzymatischen Analyse, Bergmeyer, H. U., ed., Weinheim, Verlag Chemie, p. 151.

Dixon, J. S., and Lipkin, D. (1954), *Anal. Chem. 26*, 1092.

Ghuysen, J.-M., Leyh-Bouille, M., and Dierickx, L. (1962), *Biochim. Biophys. Acta 63*, 286.

Ghuysen, J.-M., and Strominger, J. L. (1963a), *Biochemistry 2*, 1110.

Ghuysen, J.-M., and Strominger, J. L. (1963b), *Biochemistry 2*, 1119.

Ghuysen, J.-M., Tipper, D. J., and Strominger, J. L. (1965), *Biochemistry 4*, 474 (this issue; following paper).

Hash, J. (1963), *Arch. Biochem. Biophys. 102*, 379.

Hough, L., Jones, J. K. N., and Wadman, W. H. (1950), *J. Chem. Soc.*, 1702.

Jeanloz, R. W., Sharon, N., and Flowers, H. M. (1963), *Biochem. Biophys. Res. Commun. 13*, 20.

Jeanloz, R. W., and Trémège, M. (1956), *Federation Proc. 15*, 282.

Kuhn, R., Gauhe, A., and Baer, H. H. (1954), *Chem. Ber. 87*, 1138.

Lüderitz, O. D., Simmons, A. R., Westphal, O., and Strominger, J. L. (1964), *Anal. Biochem. 9*, 263.

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

Perkins, H. R. (1960), *Biochem. J. 74*, 182.

Reissig, J. L., Strominger, J. L., and Leloir, L. F. (1955), *J. Biol. Chem. 217*, 1959.

Salton, M. R. J., and Ghuysen, J.-M. (1959), *Biochim. Biophys. Acta 36*, 552.

Salton, M. R. J., and Ghuysen, J.-M. (1960), *Biochim. Biophys. Acta 45*, 355.

Sanderson, A. R., Strominger, J. L., and Nathenson, S. G. (1962), *J. Biol. Chem. 237*, 3603.

Stahl, E., and Kaltenbach, U. (1961), *J. Chromatog. 5*, 351.

Strominger, J. L., Park, J. T., and Thompson, R. E. (1959), *J. Biol. Chem. 234*, 3263.

Tipper, D. J., Strominger, J. L., and Ghuysen, J. M. (1964), *Science 146*, 781.