Isolation and Characterization of β-1,4-*N*-Acetylmuramyl-*N*-acetylglucosamine and Its *O*-Acetyl Derivative*

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ABSTRACT: The disaccharide 4-*O*-β-*N*-acetylmuramyl-*N*-acetylglucosamine and its *O*-acetyl derivative have been isolated from cells walls of *Staphylococcus aureus* strain *Copenhagen* following hydrolysis with lysostaphin. The purified disaccharides comprised 85% of the glycan component and did not contain the subunits covalently linked to teichoic acid. The linkage in these disaccharides was characterized as β-1,4 by periodate oxidation and nuclear magnetic resonance spectroscopy, before and after reduction with $NaBH_4$. Since the complementary disaccharide, 4-*O*-β-*N*-acetylglucosaminyl-*N*-acetylmuramic acid, has previously been isolated in high yield from cell walls of the same organism, the glycan has a fully *N*-acetylated, β-1,4 linked structure. It is therefore a substituted form of chitin. Its conformation in the intact peptidoglycan is discussed.

The cell wall peptidoglycan of *Staphylococcus aureus* has the structure shown in Figure 1 (Mandelstam and Strominger, 1961; Ghuysen *et al.*, 1965a,b; Jarvis and Strominger, 1967; Tipper *et al.*, 1967; Tipper and Berman, 1969; Tipper, 1969). The glycan consists of alternating 1,4-linked residues of *N*-acetylglucosamine and *N*-acetylmuramic acid, and about two-thirds of the latter carry 6-*O*-acetyl residues (Tipper *et al.*, 1965). Every residue of muramic acid is linked to peptide, and teichoic acid is linked to glycan in an unknown manner. Lysostaphin, a staphylolytic enzyme mixture produced extracellularly by *Staphylococcus epidermidis* strain K-6-W1 (Schindler and Schuhardt, 1964), catalyzes the hydrolysis of this structure at the positions indicated (Browder *et al.*, 1965). It lyses only those organisms with cross-bridges formed by chains of glycine residues, *i.e.*, staphylococci and a few other organisms (Schindler and Schuhardt, 1966). It has been resolved into two fractions, a lytic peptidase and an endo-*N*-acetylglucosaminidase that is unable to lyse staphylococci (Browder *et al.*, 1965) Sequential action of the glycine endopeptidase, amidase, and endo-*N*-acetylglucosaminidase of the preparation on *S. aureus* cell walls results in the fragmentation of the peptidoglycan into neutral and basic peptide fragments[1] and fragmentation of the peptide-free glycan into the disaccharide *N*-acetylmuramyl-*N*-acetylglucosamine (MurNAc-GlcNAc) and its *O*-acetyl derivative (MurN,O-diAc-GlcNAc).[2] The purpose of the present paper is to report the isolation of these disaccharides in high yield and to report their characterization, thus establishing the complete structure of the glycan of *Staphylococcus aureus*.

Materials and Methods

Cell walls of *S. aureus* strain Copenhagen were prepared as previously described (Tipper and Berman, 1969). Lysostaphin was a gift from Drs. Peter Tavormina, H. P. Browder, W. Zygmunt, and J. Young, Mead Johnson Research Laboratories, Evansville, Ind. 4-*O*-β-*N*-Acetylglucosaminyl-*N*-acetylmuramic acid (GlcNAc-MurNAc) was isolated from *S. aureus* cell walls as previously described (Tipper *et al.*, 1965). *N*-Acetylmuramic acid was isolated from this disaccharide by hydrolysis with β-*N*-acetylglucosaminidase, as previously described (Tipper *et al.*, 1965).

Procedures for the determination of reducing power, total and inorganic phosphate, free amino groups, N- and C-terminal amino acids, and total hexosamines have been described (Ghuysen *et al.*, 1966). Amino acids, glucosamine, and muramic acid contents were determined by a Beckman-Spinco amino acid analyzer, after hydrolyses under previously described conditions (Tipper and Berman, 1969). *O*-Acetyl determination, reductions with $NaBH_4$ and periodate oxidations, determination of periodate consumption, and formaldehyde production were performed as previously described (Tipper *et al.*, 1965, 1967; Leyh-Bouille *et al.*, 1966). Nmr spectra of solutions (*ca.* 20 mg/ml) in D_2O were obtained using a Varian 100-MHz spectrometer.

Results

Isolation of N-Acetylmuramyl-N-acetylglucosamine and N,O-Diacetylmuramyl-N-acetylglucosamine. Cell walls of *S. aureus* strain Copenhagen (500 mg, 230 μmoles of total glutamic acid) were incubated at 37° in 11 ml of 0.01 M K_2HPO_4 buffer (pH 7.4) with 5.3 mg of lysostaphin. Aliquots (1 μl) were removed at intervals for measurement of reducing power, which was maximal at 8 hr and equaled 156 μmoles. This amount is equivalent to release of 0.95 μmole of Mur-

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[1] D. J. Tipper and J. L. Strominger, manuscript in preparation.

[2] Abbreviations used are: Mur, muramic acid; MurNAc, *N*-acetylmuramic acid; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; MurNAc-GlcNAc, 4,*O*-β-*N*-acetylmuramyl-*N*-acetylglucosamine; MurN,O-diAcGlcNAc, 4-*O*-β-*N*,6-*O*-diacetylmuramyl-*N*-acetylglucosamine; GlcNitol, glucosaminitol.

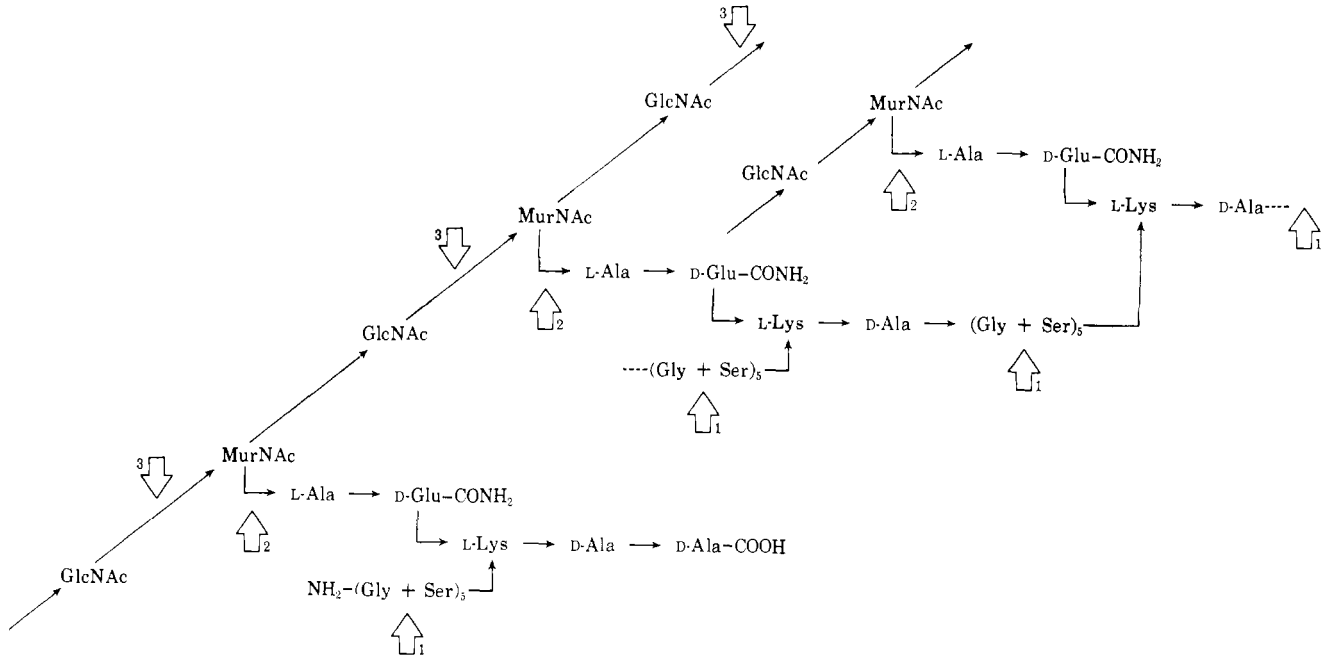

FIGURE 1: Structure of the peptidoglycan of *S. aureus* cell walls. The bonds hydrolyzed by lysostaphin are indicated by the arrows. In sequence, these are (1) glycylglycine linkages, (2) *N*-acetylmuramyl-L-alanine linkages, and (3) *N*-acetylglucosaminyl-*N*-acetylmuramic acid linkages.

NAc-GlcNAc/mole of total glutamic acid, since MurNAc-GlcNAc has a molar reducing power of 0.71 relative to the standard, *N*-acetylglucosamine (Leyh-Bouille *et al.*, 1966). Since reducing power release results from endo-*N*-acetylglucosaminidase action, and this enzyme acts on the glycan only after peptidase and amidase action have stripped it of peptide,[1] the completion of reducing power release marks the termination of release of disaccharides. After 16 hr, the lysate was fractionated in water on a column of Sephadex G-25 (50 × 2 cm). A peak of totally included inorganic phosphate was separated from a peak of partially excluded reducing power (disaccharide) and free amino groups (peptide) and also from a totally excluded peak of organic phosphate (teichoic acid). All fractions except those containing inorganic phosphate were combined. The pooled materials were fractionated on a column of Ecteola-cellulose (Bio-Rad Cellex-E, 40 × 20 cm) which had been previously washed with 0.5 M NaCl and water. Water eluted a peak containing 95% of the reducing power (disaccharide) and free amino groups (peptide). All of the organic phosphate (teichoic acid) was retained on the column and was subsequently eluted with a gradient of increasing LiCl concentration.

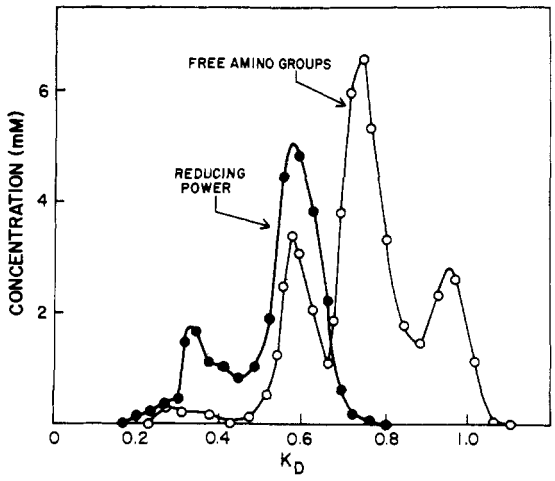

FIGURE 2: Fractionation of water eluate from Ecteola column on Sephadex G-25. The water eluate from Ecteola fractionation was concentrated to 4 ml and applied to a column of Sephadex G-25, fine grade (2 × 100 cm, V_0 = 120 ml, $V_0 + V_i$ = 240 ml; V_e = elution volume, $V_0 = V_e$ for blue Dextran (Pharmacia), $V_0 + V_i = V_e$ for NaCl, and $K_D = (V_e - V_0)/V_i$). The column was eluted with water at 0.3 ml/min. Fractions of 4 ml were collected and analyzed for reducing power and free-amino groups.

The fractions eluted with water were combined and fractionated on a column of Sephadex G-25 (Figure 2). Peptide, detected by measurement of free amino groups, was eluted in three peaks centered at K_D = 0.58, 0.74, and 0.96. Electrophoresis and detection with ninhydrin showed that the first two peaks contained basic peptides, positively charged at pH 4.8, while the third peak contained neutral peptides. A single major peak of reducing power was eluted at K_D = 0.6, almost coincident with the first peptide peak. It presumably consisted of free disaccharides. Higher molecular weight glycan components were eluted earlier at K_D = 0.2–0.45. The fractions with K_D = 0.5–0.7 were combined and fractionated on a column of CM-cellulose (Cellex CM, 30 × 15 cm) which had been previously washed with 0.4 M LiCl and water. Water eluted all of the material with reducing power and 5% of the material with free amino groups (neutral peptide), while, as expected, the bulk of the peptide (basic peptides) was retained by the column and was eluted with a gradient of increasing LiCl concentration.

The water eluate from CM-cellulose was pooled and taken to dryness (94 mg). The recovered material contained 330 μmoles of total hexosamine (equimolar glucosamine and muramic acid), 112 μmoles of reducing power, and 108 μmoles of *O*-acetyl groups, suggesting the presence of an ap-

TABLE I: Analyses of Disaccharides.[a]

	Unreduced							Reduced			
	Reducing Power	Morgan–Elson 7 min	Morgan–Elson 30 min	Morgan–Elson 60 min	*O*-Acetyl	GlcN	Mur	Reducing Power	GlcN	Mur	Glc-Nitol
MurNAc-GlcNAc	73	3	13	39	0	95	93	1	0	90	92
Mur-N,O-diAc-GlcNAc	70	3	12	37	94	107	105	2	0	101	89

[a] Data are presented as moles/100 moles of disaccharide (*i.e.*, per 200 moles of total hexosamine). The standard for reducing power and Morgan–Elson determinations (with 7-, 30-, or 60-min heating in borate) was *N*-acetylglucosamine. Samples of each disaccharide (2 μmoles of total hexosamine) were reduced in 80 μl of 0.1 M $NaBH_4$ for 18 hr at room temperature and then acidified with 1 N acetic acid (15 μl) to pH 6.5. Aliquots (10 μl) were hydrolyzed in 3 N HCl for 6 hr at 100°. Similar samples of unreduced disaccharides, hydrolyzed under the same conditions, gave the data shown. The presence, during hydrolysis, of a quantity of boric acid equivalent to that present in the hydrolysates of the reduced disaccharides did not affect these data. Hydrolysates were analyzed on a Beckman-Spinco amino acid analyzer. Glucosaminitol had a retention time and a peak area, respectively, 112 and 40% of those given by glucosamine.

proximately 1:2 mixture of MurNAc-GlcNAc and MurN,-O-diAc-GlcNAc. If pure, this mixture would weigh 90.5 mg, so the disaccharide was about 96% pure at this stage. As expected from experience with the isomeric disaccharides, GlcNAc-MurNAc and GlcNAc-MurN,O-diAc (Tipper *et al.*, 1965), the two disaccharides (detected with diphenylamine–trichloracetic acid) were well separated by paper chromatography in 1-butanol–acetic acid–water (3:1:1, v/v), MurNAc-GlcNAc having an R_F of 0.29 and a mobility (relative to *N*-acetylglucosamine) of 0.85, while MurN,O-diAc-GlcNAc had an R_F of 0.47 and a mobility of 1.48. The impurities in the preparation (residual peptide and a minor oligosaccharide component) both had low mobilities and were separated from both disaccharides by this chromatography.

A portion (53 mg, 186 μmoles of total hexosamines) of the disaccharide mixture was fractionated as a band on Whatman No. 3MM paper in the same solvent. The two disaccharides were detected on guide strips as above and eluted with water. The eluates were concentrated and separately fractionated in water on the same column of Sephadex G-25 used above. Apart from minor low molecular weight contaminants, each gave a single, sharp peak of reducing power at K_D = 0.6. These peaks were individually pooled, giving MurNAc-GlcNAc (16.6 mg) and MurN,O-diAc-GlcNAc (27.3 mg), containing 53 and 105 μmoles of total hexosamine, respectively (recovery, 85%).

Analyses of the Disaccharides: Demonstration of Sequence of Sugars. Both disaccharides contained equimolar glucosamine and muramic acid and had the same color yields, relative to *N*-acetylglucosamine, in reducing power and Morgan–Elson determinations (Table I). MurN,O-diAc-GlcNAc also contained 1 mole of *O*-acetyl. After complete reduction of both with $NaBH_4$, all of the glucosamine disappeared and was replaced by an equimolar amount of glucosaminitol in each case; both compounds are therefore disaccharides having glucosamine at the reducing end. Both compounds were free of amino acids and paper electrophoresis showed them to be negatively charged at pH 3.9 with a mobility relative to GlcNAc-MurNAc of 0.88. Thus both compounds are fully *N*-acetylated. This electrophoretic mobility was unchanged after reduction with $NaBH_4$. After treatment of reduced MurN,O-diAc-GlcNAc for 1 hr at 60° in 0.1 M carbonate–bicarbonate buffer (pH 10.5), it was free of *O*-acetyl groups and had the same chromatographic mobility as reduced MurNAc-GlcNAc. The specific rotation of MurNAc-GlcNAc was +12.4° and that of MurN,O-diAc-GlcNAc was +15.4° (at equilibrium, *c* 0.5, water). These values are low and quite similar to those found for GlcNAc-MurNAc and GlcNAc-Mur,O-diAc (+7.8 and +23.5°, respectively) (Tipper *et al.*, 1965).

Oxidation of the Disaccharides: Demonstration of the 1,4 Linkage. Neither disaccharide reduced significant amounts of 0.01 M periodate in 1 hr at room temperature, pH 4.5 (Figure 3). In contrast, after reduction, each rapidly reduced 1 mole of periodate with concomitant production of 1 mole of formaldehyde. Formaldehyde can be produced under these circumstances only from a 2-deoxy-2-acetamidohexitol with C_5 and C_6 hydroxyls unsubstituted. If the 4 position were also unsubstituted, a compound of this type would rapidly consume 2 moles of periodate under the described conditions. Both reduced disaccharides must therefore be 4-*O*-*N*-acetylmuramyl-*N*-acetylglucosaminitol derivatives, *i.e.*, both disaccharides must have a 1,4 linkage.

N-Acetylglucosamine derivatives substituted on the 4 position can form a $\Delta^{2,3}$-unsaturated derivative on heating in alkali, by elimination of hydroxyl or other C_3 substituent, but

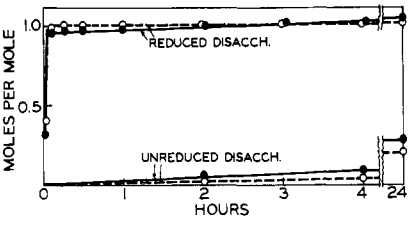

FIGURE 3: Periodate oxidation of reduced and unreduced disaccharides. Samples (0.2 μmole) of the disaccharides, reduced as described in Table I, were oxidized in 0.01 M periodate (50 μl) in 0.02 M acetate (pH 4.5) at room temperature in the dark. Residual periodate was determined from the absorbancy at 224 nM of aliquots (1 μl) diluted with water (100 μl). The differential molar extinction coefficient for the reduction of IO_4^- to IO_3^- is 8730 (Dixon and Lipkin, 1954). Formaldehyde production was determined on aliquots (3 μl) as previously described (Tipper *et al.*, 1965). (——) MurNAc-GlcNAc and (- - - - -) Mur-N,O-diAc-GlcNAc.

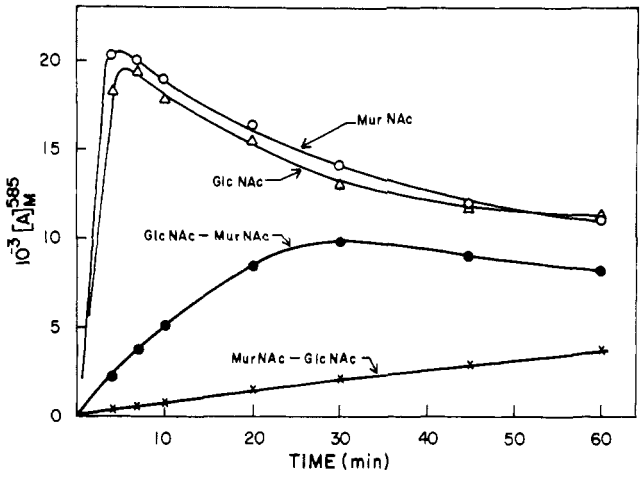

FIGURE 4: Kinetics of production of Morgan–Elson chromogen from disaccharides. Samples of disaccharides (0.03 μmole), *N*-acetylglucosamine (GlcNAc, 0.01 μmole), or *N*-acetylmuramic acid (MurNAc, 0.01 μmole) were heated in sealed tubes in a boiling-water bath for the indicated times in 40 μl of 1% $K_2B_4O_7 \cdot 5H_2O$ (0.06 M). Production of Morgan–Elson chromogen was determined by mixing with *p*-dimethylaminobenzaldehyde reagent (200 μl) and measuring absorbance at 585 nM after 20 min at 37°. Curves for MurNAc-GlcNAc and its *O*-acetyl derivative were identical. $[A]_M$ 585, molar extinction coefficient at 585 nM.

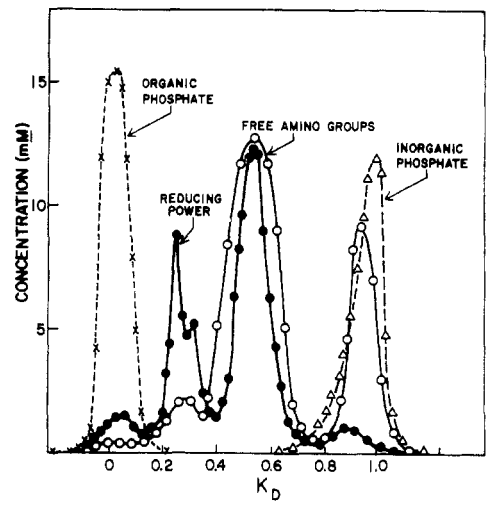

FIGURE 5: Fractionation of lysostaphin lysate of 2.5 g of *S. aureus* cell walls on Sephadex G-25. The lysate, concentrated to 20 ml, was applied to a column of Sephadex G-25 (fine grade, 5 × 75 cm, V_0 = 510 ml, $V_0 + V_i$ = 1000 ml) and eluted with 0.1 M LiCl. Aliquots of fractions (5.5 ml) were analyzed for total and inorganic phosphate, reducing power, and free amino groups.

the latter cannot then rearrange to form the five-membered ring of Morgan–Elson chromogen I (Kuhn *et al.*, 1954; Tipper, 1969). Such derivatives, therefore, give little color in the Morgan–Elson determination under the standard conditions of 7-min heating in borate. Thus the Morgan–Elson data shown in Table I support a 1,4 linkage for the disaccharides. On prolonged heating of the disaccharides in borate, a slow linear production of chromogen ensues (Figure 4), presumably due to slow elimination of the 4 substituent from the $\Delta^{2,3}$-glucoseen. At the same time, the color production from *N*-acetylglucosamine decreases, so that by 60 min the relative color production from MurNAc-GlcNAc is 38% (Table I). Relatively rapid β elimination of the D-lactic acid substituent from C_3 of the isomeric disaccharide GlcNAc-MurNAc (in contrast to the slower elimination of hydroxyl from C_3 of MurNAc-GlcNAc) leads to production of the $\Delta^{2,3}$ derivative (Tipper, 1969) and a consequent more rapid production of Morgan–Elson chromogen, maximal at 30 min (Figure 4). The relative resistance to alkaline hydrolysis of MurNAc-GlcNAc compared to its isomer, GlcNAc-MurNAc, is also the cause of its relatively low reducing power, which is only 50% of that of GlcNAc-MurNAc (Leyh-Bouille *et al.*, 1966).

Enzyme Susceptibility of MurNAc-GlcNAc and MurN,O-diAc-GlcNAc. Neither MurNAc-GlcNAc nor MurN,O-diAc-GlcNAc was hydrolyzed appreciably by any of the following endo-*N*-acetylmuramidases acting under their optimal conditions for cell wall hydrolysis: lysozyme, *Chalaropsis* B enzyme (Tipper *et al.*, 1964), *Streptomyces* "32" enzyme (Ghuysen and Strominger, 1963), or *Streptomyces* F1 enzyme (Muñoz *et al.*, 1966). They also were not hydrolyzed by pig epidydimal exo-β-*N*-acetylglucosaminidase (Findlay and Levvy, 1960).

Isolation of Larger Quantities of MurNAc-GlcNAc for Nmr Spectroscopy. The low specific rotations of the disaccharides (see above) suggest a β configuration for the glycosidic linkages. In nmr spectra, the proton on the anomeric hemiacetal carbon of sugars is readily resolved from the other protons in simple sugars because of its larger chemical shift. A clear correlation exists between the τ and J values of its adsorption peaks and the configuration of the anomeric carbon atom (Van der Veen, 1963; Lemieux and Bose, 1966). Early attempts at determining the anomeric configurations using a Varian 60-MHz nmr spectrometer supported a β linkage (Tipper and Strominger, 1966), but resolution of the single proton peaks was compromised by small available quantities of disaccharides and the resolving power of the instrument. Larger quantities of MurNAc-GlcNAc disaccharide were therefore produced for analysis on a 100-MHz nmr spectrometer. Since MurNAc-GlcNAc and MurN,O-diAc-GlcNAc both had 1,4 linkages and similar specific rotations, it was assumed that MurN,O-diAc-GlcNAc differed only in *O*-acetyl content. Cell walls were therefore hydrolyzed in alkali to remove all *O*-acetyl groups prior to lysostaphin hydrolysis, so that the MurNAc-GlcNAc disaccharide would be obtained in higher yield and would be representative of both *O*-acetylated and non-*O*-acetylated subunits of the peptidoglycan.

S. aureus Copenhagen cell walls (2.5 g) were first boiled in deionized water (100 ml) for 20 min to inactivate autolytic enzymes. The walls were then incubated for 1.5 hr at 60° in 0.1 M carbonate–bicarbonate buffer (pH 10.5). The pH remained approximately constant and the hydrolyzed walls were washed four times with water, resuspended in 0.01 M K_2HPO_4 buffer (pH 7.5) (60 ml), and treated with lysostaphin (12.5 mg) at 37°. Release of free amino groups reached a total of 2 moles/mole of glutamic acid at 18 hr, and reducing power reached a maximum of 0.65 mole/mole at 24 hr. Additional lysostaphin (5 mg) was added, and the incubation was terminated at 35 hr without further increase in reducing power.

The entire hydrolysate was fractionated on a column of Sephadex G-25 in 0.1 M LiCl (Figure 5). All of the organic phosphate (teichoic acid) was eluted in an excluded peak at K_D = 0, associated with small amounts of reducing power and peptide. As in the previous hydrolysate (Figure 2), reducing power was eluted in a major peak at K_D = 0.52 (disaccharide), and in two minor peaks of higher molecular weight (K_D = 0 and 0.25). A peak consisting mostly of diglycine was eluted near K_D = 1 with the inorganic phosphate, but almost all of the basic peptide was eluted in a single peak

TABLE II: Yields and Analyses of Disaccharide Fractions from Bio-Gel P2.[a]

Fraction	Wt (mg)	Total Hexosamine (μmoles)	Disaccharide (%)	Mp (°C)
23	37	116	80	186–189 (yellowed at 176°)
24	140	502	90	192–194 (yellowed at 189°)
25	56.0	199	88	191–194 (yellowed at 182°)
26	21	62	73	187–190 (yellowed at 180°)

[a] All fractions gave colorless solids. The per cent disaccharide is based on a molecular weight of 496.5.

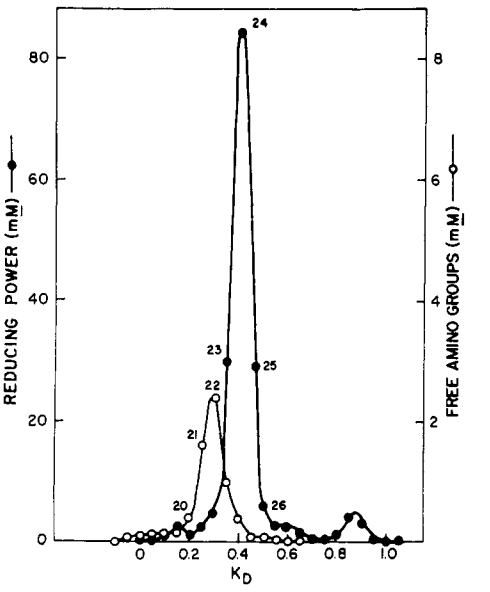

FIGURE 6: Fractionation of disaccharide on Bio-Gel P2. The disaccharide, eluted with water from CM-cellulose, was applied to a column of Bio-Gel P2 (2 × 80 cm, V_0 = 70 ml, $V_0 + V_i$ = 165 ml) in a volume of 3 ml, and eluted with water at 0.3 ml/min. Fractions (4–5 ml) were numbered as indicated.

overlapping the disaccharide peak. In the absence of salt, part of this peptide had been retarded and eluted at K_D = 0.7 (Figure 2). The fractions between K_D = 0.4 and 0.7 were combined and fractionated on a column of CM-cellulose (Bio-Rad Cellex CM, 4 × 60 cm). Water eluted a single peak of reducing power associated with a small peak of free amino groups (peptide), while most of the peptide was subsequently eluted with 0.3 M LiCl in a peak devoid of reducing power. The material eluted with water was concentrated to 3 ml and fractionated in water on a column of Bio-Gel P2 (Figure 6). A small peak of free amino groups (total 32 μmoles) was eluted in fractions 20–24, peaking in fraction 22 (K_D = 0.3). Material with reducing power was eluted in a single symmetrical peak (fractions 22–26). Fractions were individually lyophilized. Analyses (Table II) indicated that all fractions contained equimolar glucosamine and muramic acid, and after reduction with $NaBH_4$ (below), contained equimolar glucosaminitol and muramic acid. The total yield was 400 μmoles of disaccharide from 2.5 g of *S. aureus* cell walls. The purest fractions, 24 and 25, gave similar and quite sharp melting points (Table II) but contained only 90% hexosamine. The residual 10% could comprise 2–3 moles of water. There were only traces of amino acids. Materials from fractions 24 and 25 gave single spots on thin-layer chromatography on silica gel G in 1-butanol–acetic acid–water (3:1:1, v/v) with R_F 0.32 (detected with NaOH according to Sharon (1964) or with H_2SO_4 and heat). They gave single spots with R_F 0.33 on paper chromatography in the same solvent, the same R_F at GlcNAc-MurNAc. On electrophoresis in pyridine–acetic acid buffer (pH 3.9) they gave single spots of mobility +86 (relative to glutamic acid = +100, *N*-acetylglucosamine = 0, and lysine = −210). GlcNAc-MurNAc had a mobility of +98.

Preparation of N-Acetylglucosaminitol and Reduced MurNAc-GlcNAc. *N*-Acetylglucosamine (0.5 mmole) was reduced with $NaBH_4$ (1.0 mmole) in water (15 ml) at 0° for 1 hr, then at 20° for 2 hr. After acidification with 1 M acetic acid to pH 5, residual reducing power was found to be less than 0.2 μmole. The mixture was concentrated to 2 ml and fractionated in water on a column of Bio-Gel P2 (1.5 × 60 cm). Aliquots (10 μl) of fractions (3 ml) were hydrolyzed for 30 min at 100° in 6 N HCl, lyophilized, and assayed with ninhydrin. A single peak of ninhydrin-positive material (devoid of reducing power) was found well separated from boric acid and acetate. After pooling and lyophilization, it was found to have a melting point of 108–118°.

MurNAc-GlcNAc (0.1 mmole, 49.6 mg) was reduced with $NaBH_4$ (1 mmole) in water (10 ml) at 0° for 1 hr, then at 20° for 3 hr, after which reducing power could not be detected in acidified samples. The mixture was fractionated on Bio-Gel P2, and the fractions were again assayed with ninhydrin after hydrolysis. A single sharp peak was found, well separated from inorganic material. After lyophilization, this material melted at 180 to 185° with some decomposition (foaming).

Nmr Spectra. Nmr spectra of *N*-acetylglucosamine (62 mg), *N*-acetylglucosaminitol (42 mg), MurNAc-GlcNAc (59 mg), and reduced MurNAc-GlcNAc (40 mg) were performed in D_2O (0.6 ml each). In the nmr spectra of neutral sugars, α-anomeric protons (equatorial in the C_1 conformation) absorb in a region near τ 4.9 with a low *J* of about 3 cps, β-anomeric protons (axial in the C_1 conformation) absorb in a region near τ 5.6 with a higher *J* of about 7 cps. Van der Veen (1963) found that *N*-acetylglucosamine, in D_2O solution, at equilibrium, exists mostly in the α configuration, presumably due to intramolecular hydrogen bonding of the C_1 hydroxyl to the acetamido group. This was confirmed by the data presented in Table III. *N*-Acetylglucosamine, assumed to contain 3 acetamido protons (at τ 8.02), was found to contain 0.8 proton in the α-anomeric region (J = 3.0), none detectable in the β-anomeric region, and 6.1 other protons (theoretically 6.0). After reduction, the protons in the α-anomeric region completely disappeared, and two additional protons were found as predicted.

MurNAc-GlcNAc, assumed to contain 6 acetamido protons in two barely separated peaks of equal size at τ 8.01 and 8.03, contained 2.8 protons at τ 8.74, J = 6.9, which must correspond to the lactic acid CH_3 group. It also contained 0.8 proton in the α-anomeric region (τ 4.88, J = 2.5), and two separate doublets in the β-anomeric region, 0.9 and 1.0 proton each (Table III, Figure 7). One of these should belong to the

TABLE III: Data from Nmr Spectra.[a]

Sample	Lactyl CH_3			Acetamido CH_3		Other Protons		Lactyl C(=O)C(O)H			β Anomeric			α Anomeric		
	τ	J	A	τ	A	τ	A	τ	J	A	τ	J	A	τ	J	A
GlcNAc			0	8.03	(3.0)	6–6.7	6.1			0			0	4.88	3.0	0.8
Reduced GlcNAc			0	8.01	(3.0)	6–6.7	7.9			0			0			0
MurNAc-GlcNAc	8.74	6.0	2.8	8.01	(6.0)	6–6.7	12.2	5.83	6.7	0.9	5.49	7.5	1.0	4.88	2.5	0.8
Reduced MurNAc-GlcNAc	8.75	6.7	2.9	8.02	(6.0)	6–6.7	13.8	5.83	6.6	1.3	5.47	7.5	1.1			0

[a] The standard for the peak area integrals (A) was the acetamido CH_3 peak, taken to equal 3.0 for reduced and unreduced *N*-acetylglucosamine (GlcNAc) and 6.0 for reduced and unreduced MurNAc-GlcNAc. J in hertz.

proton on the β carbon of the lactate moiety, whose chemical shift should be quite similar to that of a hemiacetal proton. Irradiation at the average frequency of the lactyl CH_3 doublet (τ 8.75) decoupled the doublet at τ 5.83, and conversely, irradiation at frequency of the τ 5.83 doublet decoupled the lactyl CH_3 doublet (Figure 7). The τ 5.83 doublet is therefore identified as the β-carbon proton of the lactate moiety, and the doublet at τ 5.49, J = 7.5, is due to a β-anomeric proton. A total of 12.2 additional protons were found (theoretically 12.0).

The nmr spectrum of the reduced disaccharide (MurNAc-GlcNitol) (Figure 8) revealed that the doublet in the α-anomeric region had disappeared (Figure 8, Table III). The number of additional protons increased to 13.8 (theoretically 14). The doublet at τ 5.47 (J = 7.5, 1.1 proton) persisted. Again, irradiation at the average frequency of the 1.3 proton doublet at τ 5.83 decoupled the 2.9 proton doublet at τ 8.75 and *vice versa*, identifying these as the lactate protons. The proton peak at τ 5.47 thus shows that at least 90% of the glycosidic linkages in MurNAc-GlcNAc have the β configuration. At equilibrium in D_2O, MurNAc-GlcNAc exists at least 80% as the α anomer, as does free *N*-acetylglucosamine. This is predictable, since glycosylation at C_4 would not be expected to affect hydrogen bonding of the C_1 hydroxyl to the adjacent acetamido group.

Earlier nmr spectra at 60 MHz had shown that the isomeric disaccharide, GlcNAc-MurNAc, gave spectra consistent with the above data and interpretation. It contained 0.7 α-anomeric proton, presumably due to the *N*-acetylmuramic acid C_1 proton in the α configuration, and two single proton doublets at τ 5.5 and 5.8, both with J about 7, corresponding to the lactate β-carbon proton and the β-*N*-acetylglucosaminyl C_1 proton. The β configuration of this linkage was also indicated by its susceptibility to the specific pig epidydimal β-*N*-acetylglucosaminidase (Findlay and Levvy, 1960).

Discussion

MurNAc-GlcNAc and MurN,O-diAc-GlcNAc have been isolated from *S. aureus* cell walls in a 1:2 molar ratio and in 85% overall yield following hydrolysis with lysostaphin and shown to be 4-*O*-*N*-acetylmuramyl-*N*-acetylglucosamine derivatives. MurNAc-GlcNAc was also isolated in large amounts from de-*O*-acetylated *S. aureus* cell walls and shown to be β linked. These β linkages are hydrolyzed in the intact wall by endo-*N*-acetylmuramidases such as lysozyme, *Chalaropsis* B enzyme, and *Streptomyces* "32" and "F1" enzymes, al-

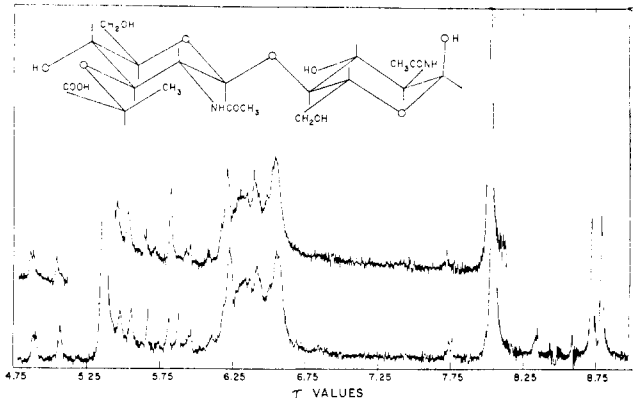

FIGURE 7: Nmr spectrum of MurNAc-GlcNAc. The structure of the disaccharide with β-1,4 linkage and both sugars in the *C*-1 conformation is shown at the top. The upper tracing is for the sample irradiated at τ8.75 while the lower tracing is the unirradiated sample. The peak at τ 5.36 is due to HDO, with side bands of τ 5.05 and 5.66.

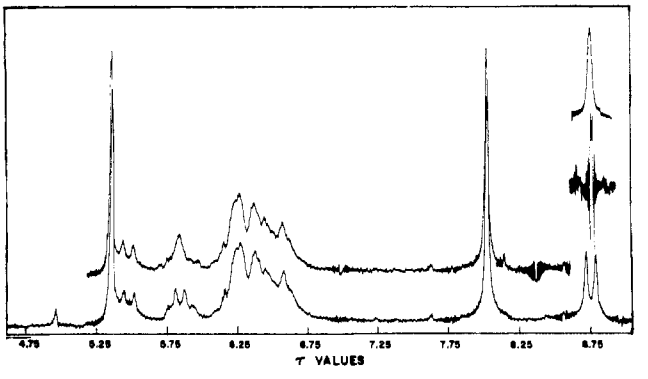

FIGURE 8: Nmr spectrum of reduced MurNAc-GlcNAc. The upper tracings are for the sample irradiated at τ 8.75 and 5.83, and the lower tracing is for the unirradiated sample. The HDO peak and its side bands are at τ 5.36, 4.96, and 5.78.

though these enzymes are inactive on isolated MurNAc-GlcNAc. If these enzymes are specific for this linkage, then all cell walls which are hydrolyzed by them must contain peptidoglycan with this linkage, and this includes walls of all bacteria tested, with the exception of *Mycobacteria* (Muñoz *et al.*, 1966) (see below). MurNAc-GlcNAc has also been isolated following lysostaphin hydrolysis of cell walls or glycan from cell walls of several organisms and shown to have a 1,4 linkage. These organisms include *Micrococcus lysodeikticus* (Leyh-Bouille *et al.*, 1966), *Staphylococcus epidermidis* (Tipper, 1969), *Lactobacillus casei* (Hungerer *et al.*, 1969), and *Bacillus sphaericus* (Hungerer and Tipper, 1969). The tetrasaccharide, GlcNAc-MurNAc-GlcNAc-MurNAc, was also isolated following lysozyme hydrolysis of *M. lysodeikticus* cell walls; its glycosidic linkages are all 1,4 (Leyh-Bouille *et al.*, 1966).

The isomeric disaccharide, GlcNAc-MurNAc, and its *O*-acetyl derivative were previously isolated in high yield from *S. aureus* cell walls and shown to be β-1,4 linked (Tipper *et al.*, 1965) and this has also been demonstrated in *M. lysodeikticus* (Leyh-Bouille *et al.*, 1966), *Micrococcus roseus* (Petit *et al.*, 1966), *Streptococcus pyogenes* (Muñoz *et al.*, 1967), *Streptococcus faecium* (Shockman *et al.*, 1967), *Bacillus megaterium* (Bricas *et al.*, 1967), *Bacillus licheniformis* (Mirelman and Sharon, 1966), *S. epidermidis* (Tipper, 1969), *L. casei* (Hungerer *et al.*, 1969), *B. sphaericus* (Hungerer and Tipper, 1969), and *Escherichia coli* (Van Heijenoort *et al.*, 1969). It seems probable that all of these organisms have a glycan with a fully *N*-acetylated, β-1,4-linked structure. This structure is therefore a substituted chitin, in which every other residue of *N*-acetylglucosamine is substituted by 3-*O*-D-lactyl peptide. A variant of this structure is known to exist in cell walls of *Mycobacteria*, where *N*-acetylmuramic acid is replaced by its oxidized derivative, *N*-glycolylmuramic acid, though the glycosidic linkages are probably still 1,4 (Petit *et al.*, 1969). The same variation occurs in *Nocardia* (Guinand *et al.*, 1970). Other known variants include the occurrence, in *Bacillus cereus* cell walls, of a glycan in which many of the glucosamine residues do not have any substituent on the amino group (Araki *et al.*, 1971a,b) and in the cell walls of *Bacillus subtilis* spores of a glycan in which many of the muramic acid residues occur as the nonacetylyated internal amides (Warth and Strominger, 1969).

Chitin has a preferred linear conformation which is stabilized by hydrogen bonds between the C_3 hydroxyl and the C_5 ring oxygen of adjacent residues, as in cellulose (*cf.* Carlstrom, 1957). The D-lactyl groups in the glycan of peptidoglycan halve the possible number of these hydrogen bonds, but can be seen in models to provide a possible alternative between the lactyl carbonyl groups and the C_6 hydroxyls on adjacent residues. If these do exist, the glycan would maintain a chitin-like conformation, forming short (Figure 1), but relatively rigid linear backbones on which lactyl peptide side chains are aligned in parallel (Tipper, 1971). In this conformation, the C_6 hydroxyls of *N*-acetylmuramic acid residues are relatively unhindered and would be available for substitution. These groups are acetylated in *S. aureus*, and phosphodiester linked to other polymers in *L. casei* (Knox and Holmwood, 1968; Hungerer *et al.*, 1969), *Streptococcus pyogenes* (Muñoz *et al.*, 1967), and *Staphylococcus lactis* (Button *et al.*, 1966). Pure chitin forms sheets by further hydrogen bonding between acetamido groups, forming a rather rigid semicrystalline material. Chitin, in the functional state, is always found in association with a ground substance, containing large amounts of protein where flexibility is required (Rudall, 1967). In peptidoglycan, this relationship between a potentially linear glycan and peptide is formalized by covalent linkage, and this peptide may contribute flexibility to the polymer. The flexibility of *S. aureus* peptidoglycan has been investigated by Ou and Marquis (1970). The conformational properties of a β-1,4-linked glycan may be essential to the structural function of cell wall peptidoglycan, and so may ensure that the glycan structure is invariant. A modified peptidoglycan, possibly with a modified function, occurs in the cortex of bacillus spores (Warth and Strominger, 1969). Only 30% of the muramic acid residues are substituted by peptide, which is itself only 20% cross-linked. Another 20% of the muramic acid residues are substituted by C-terminal L-alanine residues, and the remaining 50% exist as the internal amides of muramic acid, possibly introducing more flexibility to the glycan by eliminating 50% of the hydrogen bonding. The long, loosely cross-linked peptidoglycan chains carry many carboxyl groups and may have marked contractile properties in the presence of salts, especially divalent cations. This may function in the dehydration of the spore, whose chief protection from the environment is provided by its polypeptide coats (Lewis *et al.*, 1960; Warth and Strominger, 1969).

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Isolation and Characterization of Carboxypeptidases A and B from Activated Pancreatic Juice*

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ABSTRACT: A procedure utilizing affinity chromatography as well as conventional techniques is outlined in detail for the efficient, large-scale purification of bovine carboxypeptidases A and B from activated pancreatic juice. Carboxypeptidase A thus prepared is in the β form of the enzyme. Three forms of carboxypeptidase B are found. The minor form, accounting for about 10% of the total enzyme, is a single-chain protein comparable to carboxypeptidase B isolated after tryptic activation of partially purified procarboxypeptidase B. The two predominant forms of carboxypeptidase B are two-chain proteins. The molecular weights of the component chains of each enzyme are 10,000 and 25,000. The amino acid compositions and enzymatic properties of the two-chain enzymes appear to be the same as that of the single-chain protein. A method of separating the chains of these proteins is also presented.

Studies of the structure and function of bovine carboxypeptidase B have been limited by the difficulties of its preparation. The standard procedure for the isolation of the enzyme involves the partial purification of the zymogen, procarboxypeptidase B, from pancreatic juice or extract (Wintersberger *et al.*, 1962; Kycia *et al.*, 1968). After activation by exogenous trypsin, carboxypeptidase B is crystallized. This procedure depends on the supply of unactivated pancreatic juice or extract and the prevention of activation during the preparation of the zymogen.

In view of the difficulties of avoiding spontaneous activation, we have developed an alternate route to the isolation of carboxypeptidase B from activated pancreatic secretions (Reeck *et al.*, 1971). The present paper describes the purification procedure in detail, as well as the characterization of the resulting enzymes. As a by-product of the present procedure, a method has also been developed for the isolation of pure carboxypeptidase A_β.

Experimental Procedure

Materials

DE-52 cellulose was obtained from Whatman Reeve Angel and Sephadex G-75 and Sepharose 4B from Pharmacia Fine Chemicals.

Bovine pancreatic juice was collected at the School of Veterinary Science, Washington State University, Pullman, Wash., using the general procedure of Keller *et al.* (1958). Despite antiseptic precautions and rapid freezing of the collected juice, the zymogens were fully activated on arrival at the University of Washington.

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