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Structure of the Cell Wall of *Staphylococcus aureus*, Strain Copenhagen. I. Preparation of Fragments by Enzymatic Hydrolysis

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The cell wall of *S. aureus*, strain Copenhagen, has been solubilized through the action of the "32 enzyme" isolated from *Streptomyces albus* G. This solubilization was the consequence of hydrolysis of acetylamino sugar linkages in the cell wall, and resulted in the formation of two compounds which were separated by gel filtration and electrophoresis, the glycopeptide and a teichoic acid–glycopeptide complex. Each of these two compounds was further hydrolyzed by a second purified enzyme from *S. albus* which cleaved the linkages between acetylmuramic acid and L-alanine in the glycopeptide. An oligosaccharide fraction and a peptide fraction, obtained from this treatment of the glycopeptide, were also separated by gel filtration. In addition to these two substances, an undegraded teichoic acid was liberated from the teichoic acid–glycopeptide complex by this enzyme. The structures of these three major components of the wall will be reported in following papers.

Bacterial cell walls are heteropolymeric substances of unusual complexity. They contain polysaccharide, polypeptide, and in some cases lipid components. Interest in their structures has been catalyzed in recent years by interest in a number of biological phenomena in which the structure or biosynthesis of cell walls is important. These include inhibition of bacterial growth by some antibacterial agents, lysis of bacteria by enzymes from a variety of sources, and specific toxic and immunological responses to bacterial infection. The structure and biosynthesis of the cell wall of *Staphylococcus aureus*, strain Copenhagen, the object of the present investigation, have been studied in connection with the mechanisms of action of some antibacterial substances (cf. Strominger, 1962). The cell wall is composed of two polymers, a glycopeptide and a teichoic acid, which are separated by trichloroacetic acid at elevated temperatures (or by prolonged extraction in the cold). It has been recognized in the course of studies of the structures of these polymers (Mandelstam and Strominger, 1961; Sanderson *et al.*, 1962) that their separation by acid is hydrolytic and that some damage to both polymers (i.e., random cleavage of chemical bonds) occurs. In the present work, therefore, wall structure has been studied following degradation with two specific, purified enzymes employed at about *p*H 5. These enzymes, purified from the culture filtrate of *Streptomyces albus* G, were isolated employing lysis of the cell walls of *Bacillus megaterium* as the assay (Welsch, 1947; Ghuysen, 1960; Ghuysen *et al.*, 1962a). One of these enzymes, amidase, cleaves the amidic linkage of acetylmuramic acid and L-alanine. The mechanism of action of the second enzyme employed, the "32 enzyme," believed to be an acetylhexosaminidase (Ghuysen *et al.*, 1962a), has been elucidated in the course of this work.

In the first paper the isolation of fragments of the cell wall following enzymatic hydrolysis will be described. In following papers the structures of the three components obtained, the oligosaccharides, the peptide, and the teichoic acid, will be reported.

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Materials and Methods

Cell Walls.—These walls were prepared from logarithmic phase cells of *S. aureus*, strain Copenhagen, by repeated disintegration with glass beads in a Nossal disintegrator. Glass beads were removed on a sintered glass filter, and the filtrate was centrifuged at 30,000 × *g*. Disintegration was repeated until a clear supernatant solution was obtained on centrifugation, and the cell wall pellet had a homogenous grayish-white appearance. This usually required three disintegrations of 4 minutes each. The pellet finally obtained was washed five times in the cold with water and then lyophilized. In some studies a glycopeptide obtained from the walls was employed. This material was obtained by hydrolysis of cell walls in 10% trichloroacetic acid at 60° for 12 hours. This is the minimum time under these conditions required to solubilize 95% of the organic phosphate (i.e., the teichoic acid). The insoluble glycopeptide was removed by centrifugation, washed with water, and dried.[1]

Enzymes.—Purification of the "32 enzyme" and of the amidase by zone electrophoresis in a sucrose gradient have been described (Ghuysen *et al.*, 1962a). The preparations employed contained 1.05 and 1.30 mg of protein per ml, respectively.

Paper Electrophoresis.—This was carried out on Whatman No. 3 MM filter paper in 0.2 M pyridine-acetate buffer, *p*H 3.8. The electrophoresis was carried out under "Varsol 3" (Enco Division, Humble Oil and Refining Co.) cooled to 0° in the 5000-v apparatus manufactured by Servonuclear Corp., N. Y.

Analytical Procedures.—Analyses were performed for

[1] We are very grateful to Mr. Rodney Wishnow who prepared the cell walls and glycopeptide used in this study.

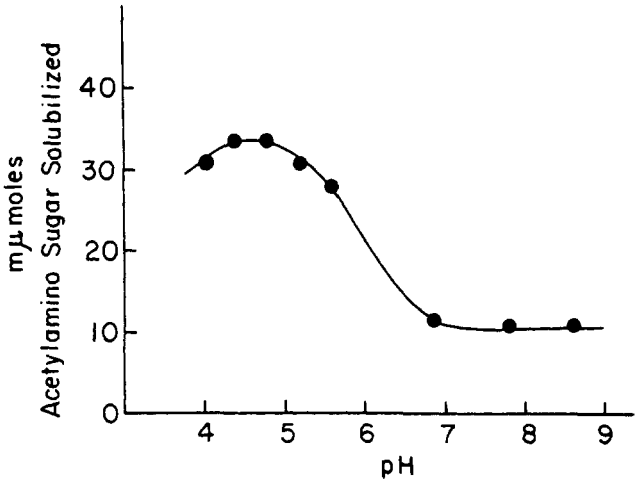

FIG. 1.—Influence of *p*H on the lytic activity of the "32 enzyme." The glycopeptide was employed as substrate. Acetate (*p*H 4.0–5.2), phosphate (*p*H 5.6–7.8) and Tris HCl (*p*H 8.6) buffers were employed, all at 0.005 M. Data are expressed relative to an acetylglucosamine standard.

total organic phosphate (Lowry *et al.*, 1954) and *reducing groups* (Park and Johnson, 1949). For the *N-acetylamino sugar* determination (Reissig *et al.*, 1955) 100 μl of 1% $Na_2B_4O_7 \cdot 10\,H_2O$ was added to the dried sample (0.01–0.05 μmole). Formation of the cyclic derivative in sodium borate buffer was carried out by placing the tubes in a boiling water bath for either 7 minutes or 30 minutes. The reaction with acetylglucosamine is maximum at 7 minutes and diminishes with further heating (Reissig *et al.*, 1955; Ghuysen and Salton, 1960); on the other hand the maximum color yield with oligosaccharides containing acetylamino sugar is obtained after 30–45 minutes of heating (Ghuysen and Salton, 1960). After addition of 500 μl of Morgan-Elson reagent (8 parts glacial acetic acid, 1 part 16% *p*-dimethylaminobenzaldehyde in 95% glacial acetic acid, 5% concd HCl) the chromogen was developed in 20 minutes at 37° and was measured at 585 mμ. *Free amino groups* were measured by reaction with dinitrofluorobenzene (DNFB).[2] To 25–125 mμmoles of sample in 25 μl was added 100 μl of 1% $Na_2B_4O_7 \cdot 10\,H_2O$ Twelve μl of 100 mM DNFB in ethanol was then added. Each tube was immediately mixed on addition of DNFB and placed in a 60° water bath for 30 minutes. After addition of 500 μl of 2 N HCl, the absorbancy at 420 mμ was read. Calculations were based on a standard of D-glutamic acid for which $A_{1\,cm}^{420\,m\mu} = 4400$ under these conditions.

Gel Filtration.—This was carried out on Sephadex G-25 and Sephadex G-50, medium grades, obtained from Pharmacia, Inc., Rochester, Minn. Columns 1 $cm^2 \times 30$ cm, were operated at 3° at a rate of 10 ml/hour. Fractions of 1.2–1.5 ml were collected. Water was employed to equilibrate the columns and as the eluting solvent.

RESULTS

I. *Lysis of Cell Walls by "32 Enzyme"*

Optimal Conditions for Lysis.—Preliminary experiments indicated that during solubilization of the cell wall or glycopeptide material reactive as "free" acetylamino sugar was liberated. A free aldehyde group as well as an unsubstituted C-4 in the acetylamino sugar is required for this reaction. Optimal conditions were established by measuring the solubilization of "free" acetylamino sugar employing the glycopeptide, free of teichoic acid, as substrate rather than cell wall. It is now known, however, that the acetylglucosaminidic linkages in the teichoic acid (Sanderson *et al.*, 1962) are not hydrolyzed by this enzyme.

TABLE I

INFLUENCE OF IONIC STRENGTH ON HYDROLYSIS OF THE GLYCOPEPTIDE BY "32 ENZYME"

Data are expressed as mμmoles of acetylamino sugar solubilized (relative to an *N*-acetylglucosamine standard) per mg of substrate in 3 hours. Sodium acetate was the buffer at *p*H's 4.4, 4.8, and 5.2, and sodium phosphate was used at *p*H 5.6.

	Final Molarity of the Buffer		
*p*H	0.005	0.01	0.02
4.4	34.2	34.2	30.4
4.8	34.	34.	32.
5.2	30.4	25.9	23.6
5.6	28.	24.	15.8

Six hundred forty-seven μg of glycopeptide was incubated with 0.77 μg of "32 enzyme" (expressed as protein) in a final volume of 144 μl in the presence of buffers of various *p*H's and ionic strengths. After 3 hours of incubation at 37° the suspensions were centrifuged. Aliquots of the supernatants were analyzed for "free" acetylamino sugar. Maximal activity occurred at *p*H 4.4–4.8 in sodium acetate buffer, 0.005–0.01 M (Fig. 1 and Table I).

Kinetics of Lysis.—Glycopeptide (Fig. 2) or cell wall (Fig. 3), 740 μg, was incubated with 0.7–7.7 μg of "32 enzyme" in 163 μl of 0.005 M acetate buffer, *p*H 4.4. As can be calculated from the data in the figures, the rate of solubilization of "free" acetylamino sugar from glycopeptide was 256 mμmoles per mg of enzyme protein per minute, and from cell wall, 76 mμmoles per mg per minute (expressed in each case relative to an acetylglucosamine standard). About 85% of the glycopeptide or cell wall preparation was solubilized at completion of the reaction (measured turbidimetrically at 700 mμ). In addition to "free" acetylamino sugar, reducing groups and free amino groups were released from the glycopeptide or wall, and all the organic phosphate in the wall was solubilized (Figs. 2 and 3, Table II). It is interesting that measurement of reducing groups (relative to a glucose standard) yielded considerably higher values than measurement of free acetylamino sugar, suggesting that the color yield in the acetylamino sugar reaction was not maximum.

Separation of the Products of Lysis of Cell Wall on Sephadex G-50.—One hundred mg of cell wall was incubated with 600 μg of "32 enzyme" at *p*H 4.4. After 10 hours, when 12 μmoles of acetylamino sugar had been solubilized, an insoluble residue (14 mg)[3]

[2] Modified from an unpublished procedure of O. H. Lowry. Abbreviation used throughout: DNFB, dinitrofluorobenzene.

[3] It should be noted that cell wall and glycopeptide employed had intentionally not been purified by trypsin or ribonuclease in order to preclude hydrolysis of labile linkages during such purification. The insoluble residue after treatment with "32 enzyme" could be either a contaminant of denatured cytoplasmic protein or a true cell wall protein. It had a brown, dirty appearance and paper chromatography, after HCl hydrolysis, yielded a large variety of amino acids, typical of protein rather than of wall. It has not been further investigated. These experiments have recently been repeated by Dr. Donald Tipper using walls which had been purified by treatment with trypsin and ribonuclease. In that case only 2 mg of material per 100 mg of wall remained insoluble after enzymatic lysis.

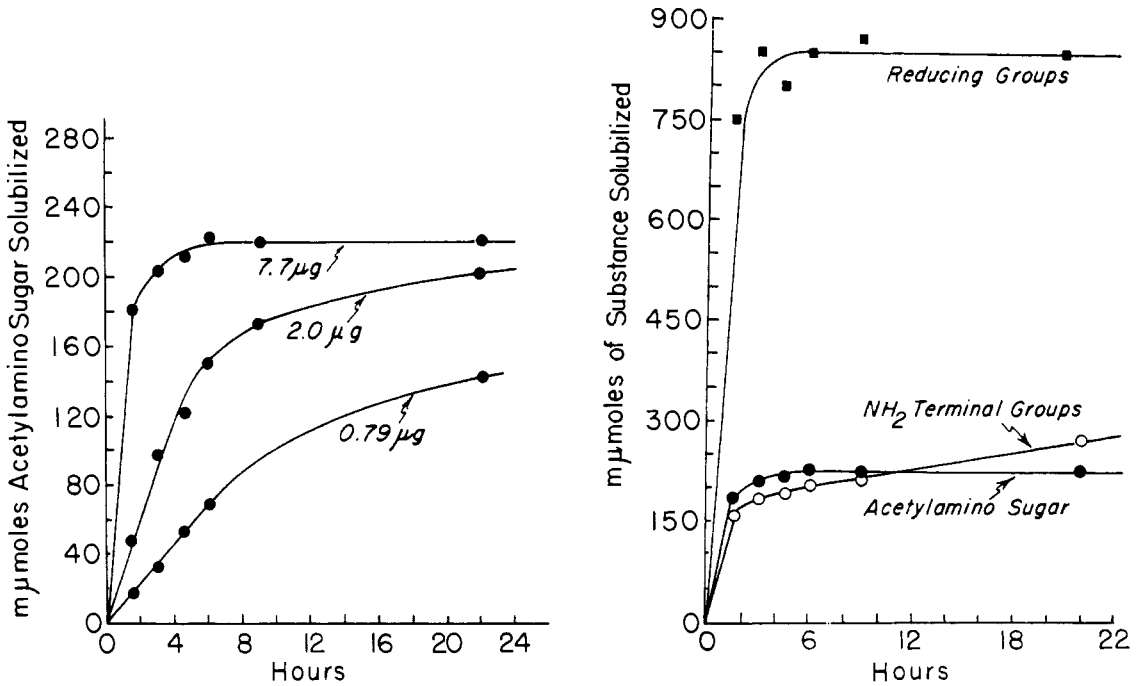

FIG. 2.—A (left). Hydrolysis of glycopeptide by different amounts of "32 enzyme." Glycopeptide (740 μg) was incubated in 163 μl of 0.005 M acetate buffer, *p*H 4.4, with different amounts of "32 enzyme." After incubation the mixture was centrifuged and the supernatant was analyzed for acetylamino sugar. Data are expressed as mμmoles of acetylamino sugar solubilized (relative to an acetylglucosamine standard) per mg of glycopeptide. B (right). Solubilization of different components of the glycopeptide by 7.7 μg of "32 enzyme."

was collected by centrifugation. The clear supernatant was lyophilized, dissolved in 1 ml of water, and applied to a column of Sephadex G-50. A high molecular weight fraction and a low molecular weight fraction were separated (Fig. 4). Although both fractions were obviously composed of a mixture of various compounds, the high molecular weight fraction contained all the organic phosphate. It should also be noted that lengthening the time of heating in the Morgan-Elson reaction from 7 minutes to 30 minutes enhanced the color yield of the high molecular weight fraction while it reduced the color yield of the low molecular weight fraction.

Study of the Low Molecular Weight Fraction.—The low molecular weight fraction (obtained between 35 and 48 ml, Fig. 4) was collected and lyophilized. It yielded 15 mg. The Morgan-Elson reactive material present in this fraction was free acetylglucosamine. It behaved as a neutral compound on paper electrophoresis in 1 N acetic acid at *p*H 2.25, and in pyridine-buffers at *p*H's 3.8 and 5.5. Applied to Sephadex G-25, it had the same elution volume as acetylglucosamine, a larger volume than that of diacetylchitobiose. Its R_F on Whatman No. 1 paper in *n*-butanol–acetic acid–water (3:1:1), *n*-butanol-pyridine-water (6:4:3), and in isobutyric acid–0.5 N NH_4OH (5:3) was the same as that *N*-acetylglucosamine. No oligosaccharides were present.

TABLE II
SUBSTANCES SOLUBILIZED FROM GLYCOPEPTIDE AND CELL WALL BY THE "32 ENZYME"

Data are expressed as μmoles of material solubilized from 100 mg of substrate after maximum hydrolysis. *N*-acetylamino sugar is expressed relative to an acetylglucosamine standard in the 7-minute reaction (see Methods). Reducing groups are expressed relative to glucose, and NH_2-terminal groups relative to alanine.

	Glyco-peptide	Walls
Organic phosphate	—	68.0
N-acetylamino sugar	22.0	13.0
Reducing groups	87.5	52.5
NH_2-terminal groups	21.0	40.0

The DNFB-reactive material present in this fraction was a mixture of various free amino acids and perhaps small peptides. The main fraction, positively charged, was collected after paper electrophoresis at *p*H 3.8, but by lowering the *p*H of electrophoresis to 2.25 it was separated into 3 compounds. The DNFB-reactive material may have arisen from some random degradation of the wall during its preparation, or enzymatic lysis, or from materials contaminating the wall. The free acetylglucosamine could have arisen similarly or from the ends of polysaccharide chains. Further investigation of the low molecular weight fraction has not yet been carried out.

Further Fractionation of the High Molecular Weight Fraction.—The fractions collected between 10 and 28 ml (Fig. 4) were pooled and lyophilized (yield, 57 mg). The dried residue was subjected to electrophoresis at *p*H 3.8 (see Methods). Two major zones were detected with various reagents. One zone (detected by spraying guide strips with ninhydrin and with the polyol reagents [Buchanan *et al.*, 1950; Ghuysen, 1961]) extended from 2 to 10 cm toward the anode (referred to as the negatively charged high molecular weight fraction). The second zone (which yielded a pinkish color with the diphenylamine spray [Ghuysen, 1961; Hough *et al.*, 1950] and a greenish color with the ninhydrin spray, but was insensitive to the polyol reagent) extended from 3 to 6 cm toward the cathode (referred to as the neutral high molecular weight fraction). The compounds in these zones were eluted from the paper with water.

These two materials were once again applied to the column of Sephadex G-50 and eluted (Fig. 5). In

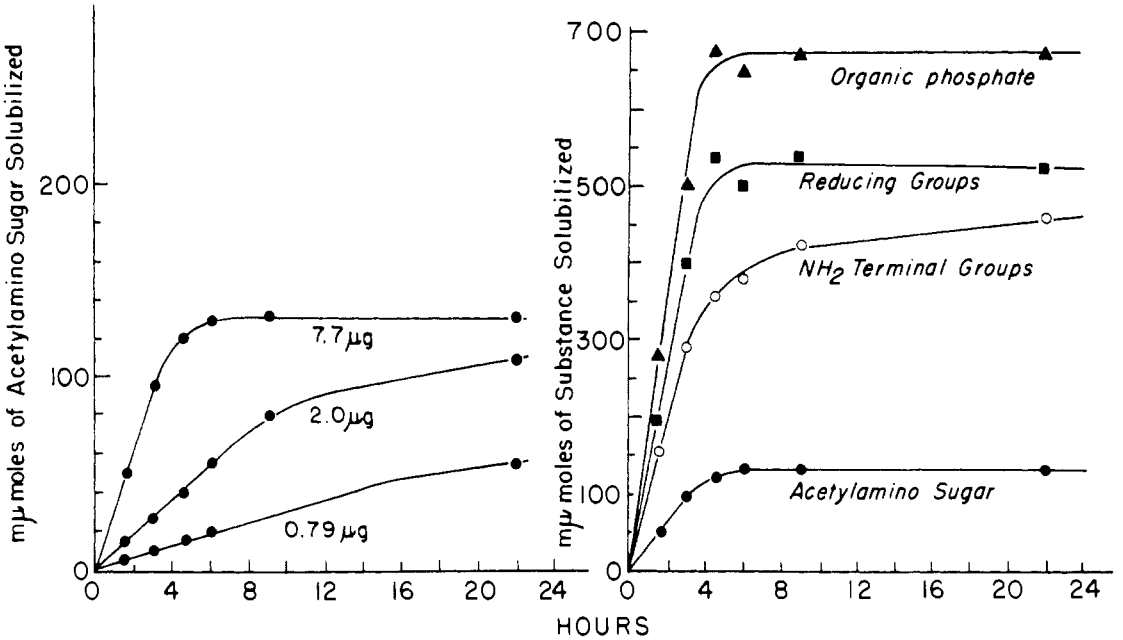

FIG. 3.—Hydrolysis of cell wall by different amounts of "32 enzyme" (left) and solubilization of different components of the wall (right). Experiments were carried out as described in the legend to Figure 2.

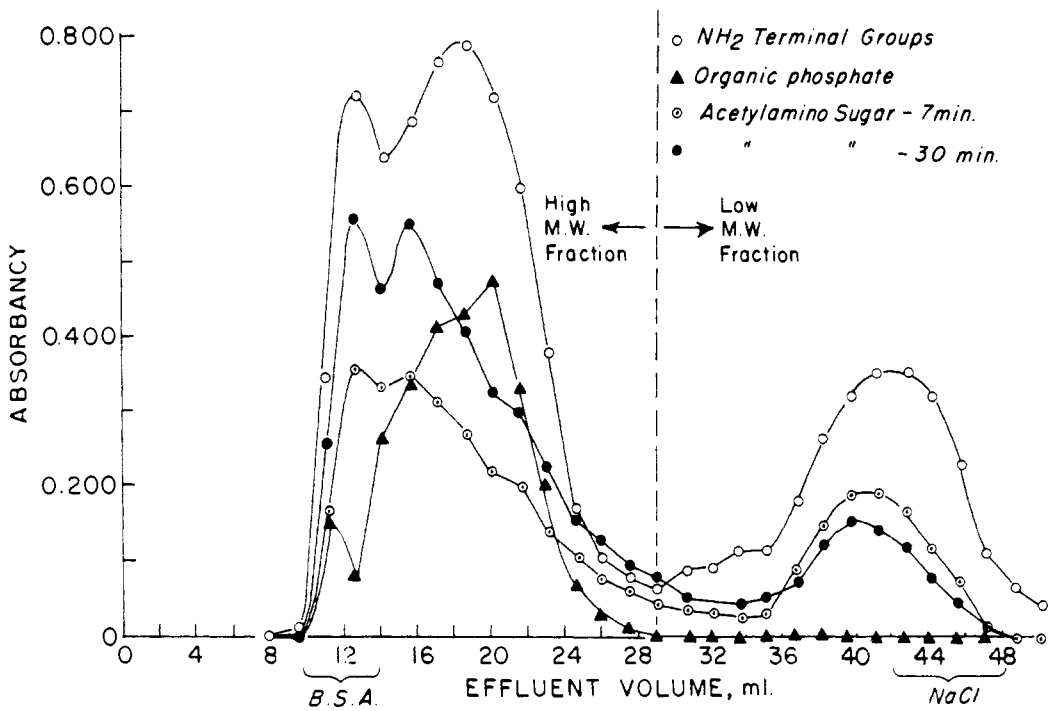

FIG. 4.—Separation on Sephadex G-50 of the "32 enzyme"-treated cell wall. Elution volumes for bovine serum albumin (B.S.A.) and NaCl are indicated.

both cases, a low molecular weight DNFB-reacting material was separated. This material, which originated from the 3MM paper used as a support for the electrophoresis, was discarded.[4] When the high molecular weight fractions were examined, it was evident that the electrophoresis had provided a clean separation of two compounds.

The negatively charged high molecular weight fraction (Fig. 5B) contained all the organic phosphate and almost all the NH_2-terminal groups (i.e., groups reactive with DNFB).[5] Moreover, the ratio of organic P/NH_2-terminal groups was the same in all the samples collected, thus indicating that the fraction was chemically homogeneous with respect to these measurements despite the fact that some physical heterogeneity was shown by the presence of two peaks, possibly a heterogeneity

[4] Elution of Whatman 3 MM paper gave similar materials. The amount present (Fig. 5) is proportional to the size of the band of paper eluted.

[5] Organic phosphate determination is the best available quantitative measure of the teichoic acid. At least 95% of the organic phosphate in these cell wall preparations is present in teichoic acid (Mandelstam and Strominger, 1961; Sanderson *et al.*, 1962). The D-alanine ester in the teichoic acid is conveniently measured by the DNFB-reaction.

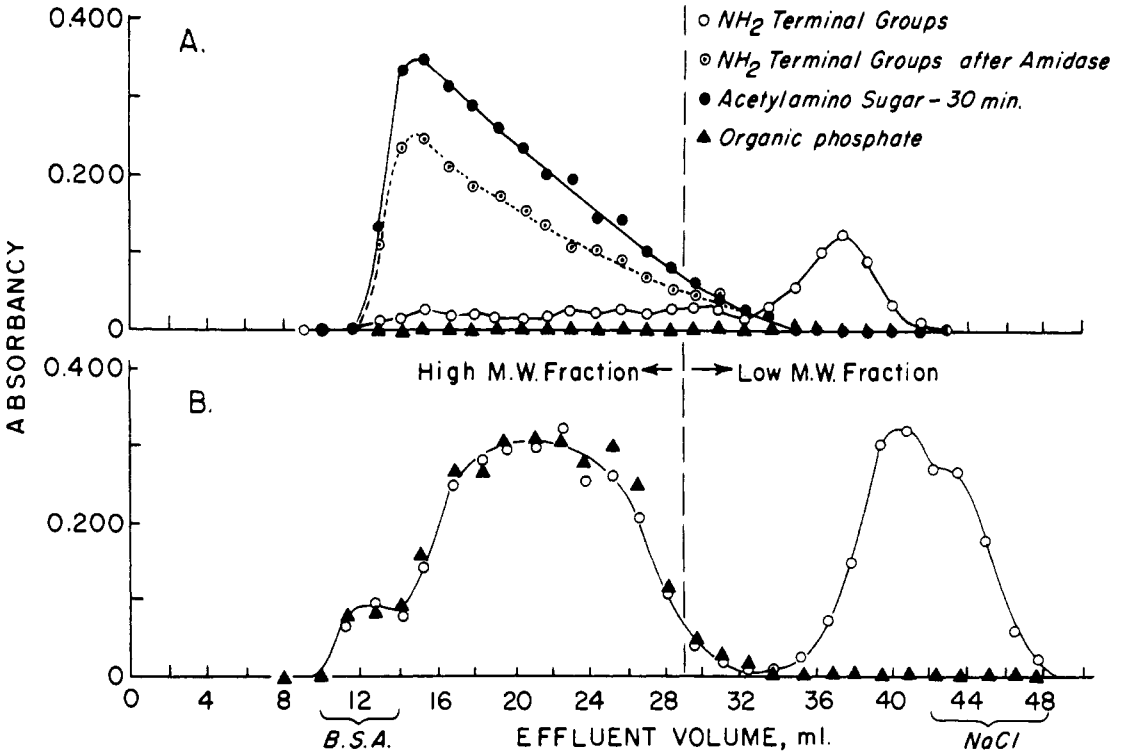

FIG. 5.—Separation on Sephadex G-50 of (A) the neutral fraction (glycopeptide) and (B) the negatively charged fraction (the teichoic acid–glycopeptide complex) obtained by paper electrophoresis of the high molecular weight material from the experiment of Figure 4. Aliquots of 32.6 μl of each sample in (A) were incubated for 1 hour at 37° with 2 μg of amidase in 70 μl of 0.025 M acetate buffer, *p*H 5.2.

with respect to molecular weight. The eluate between 11 and 29 ml (Fig. 5B) was pooled and lyophilized (yield, 16.3 mg). Paper chromatography of this material after acid hydrolysis showed that this fraction was composed chiefly of alanine, ribitol, and glucosamine, in addition to phosphate, and consequently represented the teichoic acid polymer in the wall (with the ester-linked alanine responsible for the DNFB reaction). This fraction, however, also contained small amounts of muramic acid, glycine, glutamic acid, and lysine, and gave a positive Morgan-Elson reaction (Table III). For this reason it will be referred to as the teichoic acid–glycopeptide complex. The glycopeptide and the teichoic acid could not be separated from each other by electrophoresis at *p*H 2.0 or 5.8 or by repeating the electrophoresis at *p*H 3.8.[6] The material

[6] Clearly, the two components of the complex are not linked by ionic bonds dissociable at these *p*H's. The teichoic acid component is highly negatively charged. The glycopeptide component has essentially no free amino groups. On balance, it contains one free carboxyl group in each peptide segment and careful electrophoresis shows it to have a slight negative charge at *p*H 5. The occurrence of strong ionic bonds between these two negatively charged polymers is precluded, and no experimental evidence has been adduced to support the view (Archibald *et al.*, 1961; Baddiley *et al.*, 1962) that the teichoic acid is linked to the wall through ionic bonds.

TABLE III
YIELDS OF VARIOUS COMPONENTS DURING FRACTIONATION OF 100 MG OF CELL WALL

	Dry Weight (mg)	Organic P (μmoles)	NH_2-Terminal Groups (μmoles)	Acetylamino Sugar (7 min) (μmoles)[a]	Acetylamino Sugar (30 min) (μmoles)[a]
1. Original Walls	100	80.2	15.3	0	0
2. Fractions obtained after treatment with "32 enzyme" and gel filtration					
Low mw fraction (Fig. 4)	15	0	11.7	3.20	—
High mw fraction (Fig. 4)	57	56.1	29.4	8.13	15.8
3. Fractions obtained after electrophoresis and gel filtration					
Glycopeptide (Fig. 5a)	13.4	0	0.92	—	4.4
Glycopeptide after treatment with amidase (Fig. 8)	—	0	7.93	—	8.4
Teichoic acid–glycopeptide complex (Fig. 5b)	16.3	34.5	11.7	—	2.4
Teichoic acid–glycopeptide complex after treatment with amidase (Fig. 9)					
a. High mw fraction	—	27.8	13.1	—	0
b. Low mw fraction	—	0	1.1	—	2.7

[a] Acetylamino sugar was estimated relative to an acetylglucosamine standard in the 7-minute reaction. In the 30-minute reaction an extinction coefficient of 9000, the value for the disaccharides formed (see following paper), was employed for the calculation.

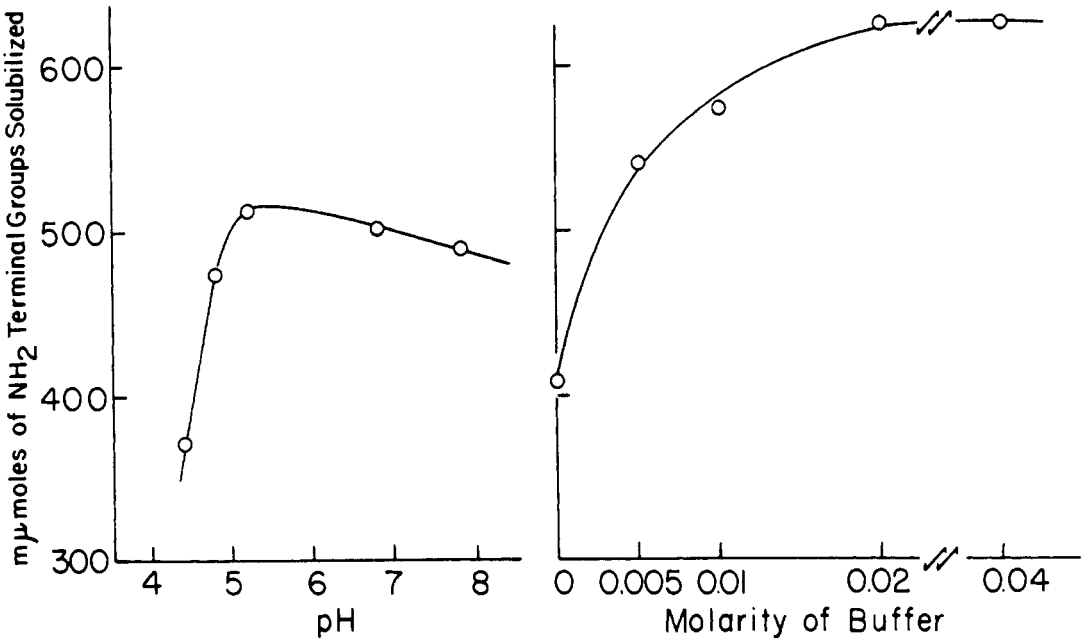

FIG. 6.—Influence of *p*H and ionic strength on activity of the amidase. The glycopeptide was the substrate and data are expressed as mμmoles of NH_2-terminal groups released per mg of substrate. Glycopeptide (79 μg) was incubated with 0.94 μg of amidase protein in a final volume of 20 μl of 0.025 M acetate (*p*H 4.0–5.2), phosphate (*p*H 5.6–7.8), or Tris (*p*H 8.6) buffer. After 15 minutes of incubation at 37° the whole incubation mixture was analyzed for free amino groups. Data are expressed as mμmoles of amino groups released per mg of substrate. The influence of ionic strength was studied in acetate buffer, *p*H 5.2.

was devoid of absorption at 260 mμ, indicating the absence of significant contamination by nucleic acid.

The neutral high molecular weight fraction (Fig. 5A) was sensitive to the Morgan-Elson reaction, but had a very low level of free amino groups and no organic phosphate. An aliquot of each of the tubes was treated with the amidase under optimal conditions (see below). The ratio, free amino groups liberated to free acetylamino sugar present in each fraction, was constant. Therefore, the neutral high molecular weight fraction also appeared to be chemically homogeneous. The asymmetry of the chromatogram (Fig. 5A) is perhaps indicative of some kind of interaction with the Sephadex. The eluate between 13 and 29 ml was pooled and lyophilized (yield, 13.4 mg). Paper chromatography after acid hydrolysis of a sample of this material showed the presence of glucosamine, muramic acid, glycine, alanine, glutamic acid, and lysine, thus identifying this fraction as the glycopeptide derived from the wall.

The yields in the various fractions both in terms of dry weight and in terms of chemical constituents are summarized in Table III. Although separation of high and low molecular weight fractions after treatment with "32 enzyme" was accomplished with yields of over 90%, extensive manipulations and analyses during separation of glycopeptide and teichoic acid–glycopeptide complex by chromatography and electrophoresis (including the cutting of guide strips after electrophoresis for locating compounds with various spray reagents) resulted in recoveries of the order of 50% for each component. No minor fractions were discarded, however, and the material obtained can be regarded as representative of the original wall itself.

II. Hydrolysis of the Glycopeptide and of the Teichoic Acid–Glycopeptide Complex by Amidase

Optimal Conditions for Activity of the Amidase.—Since it was known from previous studies that this enzyme hydrolyzed the linkage between acetylmuramic acid and L-alanine (Ghuysen *et al.*, 1962a),[7] its activity was followed as the liberation of DNFB-reactive material. The optimal *p*H was 5.2 (Fig. 6). It is interesting to note that at *p*H 4.4 (which is optimal for the "32 enzyme") the amidase is almost completely inactive. It is, in fact, denaturated at *p*H's below 5. The optimal ionic strength was 0.02–0.04 M (Fig. 6). Similar results were obtained with the teichoic acid–glycopeptide complex which is also a substrate for the amidase. Acetate buffer, *p*H 5.2, 0.025 M, was adopted as the optimum condition for hydrolysis of both substrates. The preparation employed liberated 1.5–2.0 μmoles of amino groups per mg of protein per minute in this buffer.

Kinetics of Hydrolysis.—Conditions for a complete hydrolysis of the sensitive linkages present in both substrates were examined by determining conditions for maximum liberation of NH_2-terminal groups. Incubation for 30 minutes of 53 μg of either substrate in a final volume of 15 μl of 0.025 M acetate buffer, *p*H 5.2, indicated that about 1.5 μg of amidase was required for maximum liberation of amino groups under these conditions (Fig. 7).[8]

Fractionation of the Amidase-treated Glycopeptide.—After treatment of 13.4 mg of the purified glycopeptide (Fig. 5A, Table III) under the above conditions, 800 mμmoles of NH_2-terminal groups was liberated per mg of glycopeptide. This value can be compared to the

[7] The configuration of the alanine moiety which is liberated by the enzyme has not been established, but since L-alanine is the amino acid linked to acetylmuramic acid (Strominger, 1962) it is assumed that the liberated alanine has the L-configuration.

[8] With both substrates, hydrolysis by the amidase stopped after 45 minutes of incubation independent of the amount of enzyme used, suggesting that the enzyme was denatured during the incubation. The same observations were made at *p*H 6.8, however, at which *p*H the amidase is known to be very stable. Possibly some product of the hydrolysis inhibits the enzyme.

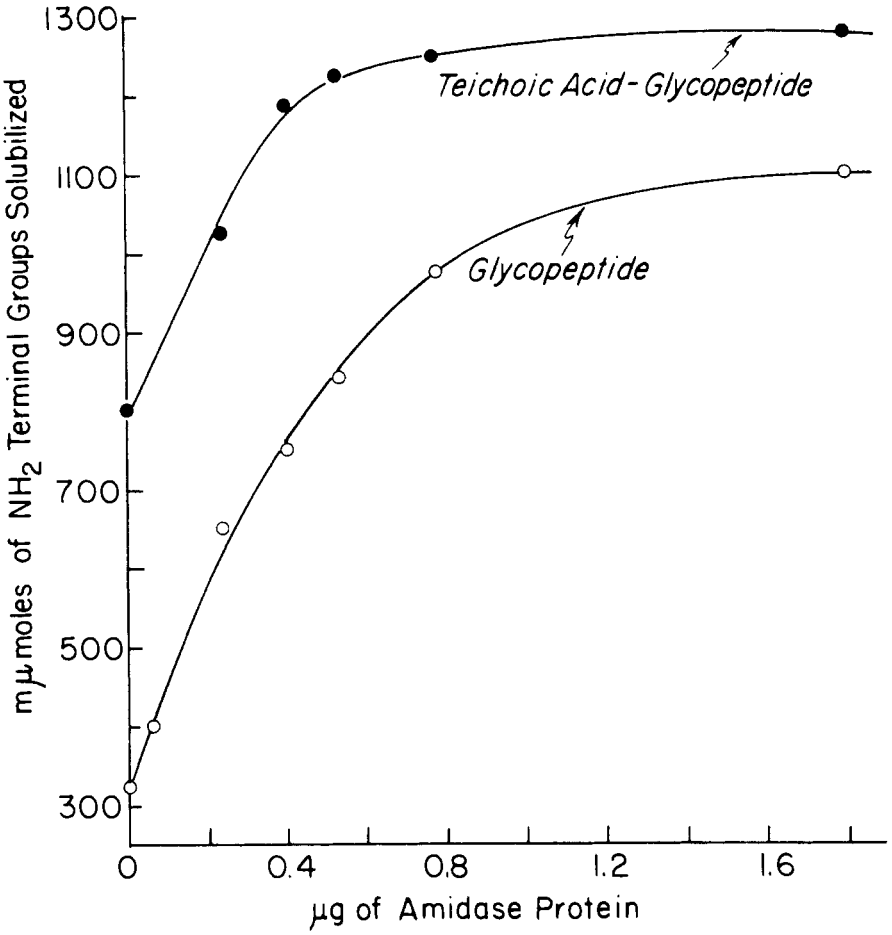

FIG. 7.—Liberation of amino groups by increasing amounts of amidase. Experiments were carried out with the glycopeptide (Fig. 5a) or with the teichoic acid–glycopeptide complex (Fig. 5b) in 0.025 M acetate buffer, *p*H 5.2, and data are expressed as mμmoles of NH_2-terminal groups released (measured with DNFB) per mg of substrate.

value of 750 mμmoles per mg calculated from the data obtained by treating separately with the amidase all the glycopeptide samples collected during the gel filtration (Fig. 5A).

The enzyme-treated mixture was applied to Sephadex G-50 and eluted (Fig. 8A). Since the separation obtained by this means was incomplete, the eluate was pooled, lyophilized, applied to Sephadex G-25, and again eluted (Fig. 8B). Analyses (Fig. 8A,B) showed that the glycopeptide was split by the amidase into its two constituents, the oligosaccharide moiety and the peptide moeity, which were separated on the columns.[9] The oligosaccharide fraction contained 8.4 μmoles of acetylhexosamine-reactive material and the peptide fraction contained 7.9 μmoles of NH_2-terminal groups (Table III).

Treatment of the Teichoic Acid–Glycopeptide Complex with Amidase.—Sixteen and three-tenths mg of the purified teichoic acid-glycopeptide complex (Fig. 5B, Table III) was treated in the same manner as the glycopeptide. An increase of 580 mμmoles of NH_2-terminal groups per mg of substrate was observed. The enzyme-treated mixture was then applied to Sephadex G-50 (Fig. 9A), which separated the high molecular weight fraction containing all the organic phosphate from a low molecular weight fraction containing the oligosaccharide and peptide moieties. The latter were then completely separated from each other on Sephadex G-25 (Fig. 9B). The high molecular weight fraction contained 27.8 μmoles of organic P and 13.1 μmoles of NH_2-terminal groups, while the low molecular weight fraction contained 1.1 μmoles of NH_2-terminal groups and 2.7 μmoles of material reactive as acetylhexosamine (Table III).

It may be noted that a portion of the teichoic acid contained an excess of free amino groups (Fig. 9A) and that the yield of peptide was lower than the yield of oligosaccharides (Table III). Moreover, this teichoic acid fraction contained all of the amino acids found in the original glycopeptide (examined after hydrolysis in 6 N HCl). In contrast to the teichoic acid–glycopeptide complex, however, a peptide in this fraction was readily separated by electrophoresis at *p*H 2, 3.8, or 5.8 from a teichoic acid.[10] The only amino acid present in the teichoic acid after electrophoresis was alanine, while the peptide contained alanine, glutamic acid, glycine, and lysine. Two possible explanations of this phenomenon exist. Conceivably, the peptide (which contains free amino groups as a consequence of removal of the oligosaccharides) and the negatively charged teichoic acid form an ionic association which behaves as a single compound on Sephadex. It is also possible that the teichoic acid in the mixture protected the high molecular weight peptide from adsorption (and anomalous retardation) on Sephadex.

[9] Oligosaccharides and peptide moieties were finally completely separated from each other by a third run on Sephadex G-25.

[10] The teichoic acid was negatively charged at all *p*H's. The peptide (which contains approximately equal numbers of free amino and free carboxyl groups) was neutral at *p*H 3.8 or 5.8, but strongly positively charged at *p*H 2.

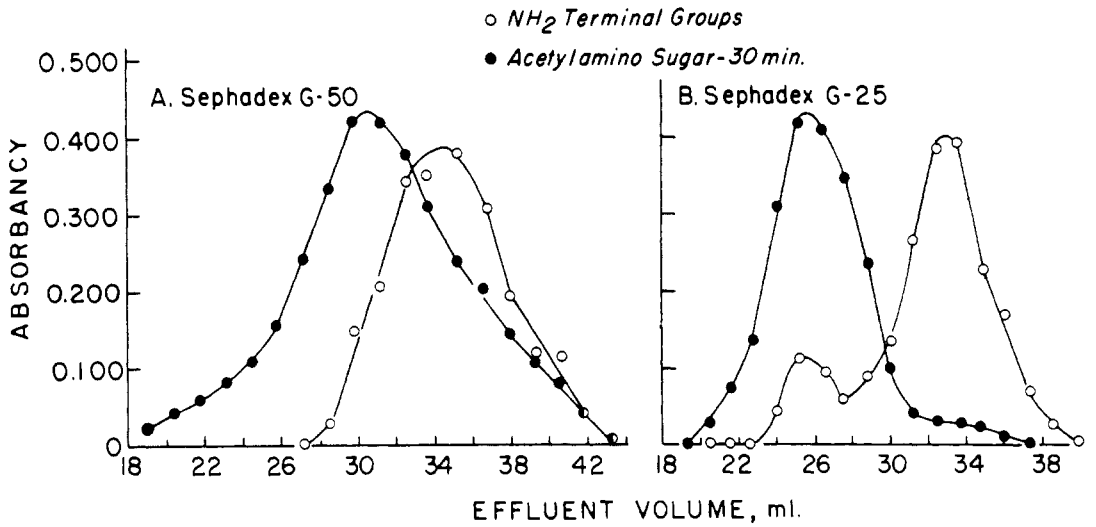

FIG. 8.—Separation on Sephadex G-50 (A) and on Sephadex G-25 (B) of fragments from the glycopeptide (Fig. 5a) after further hydrolysis with amidase. Other studies have shown that the peptide is high molecular weight and that it is therefore in an anomalous position in the effluents, presumably due to some interaction of this molecule, which contains a large number of free amino groups, with the Sephadex (cf. Glazer and Wellner, 1962).

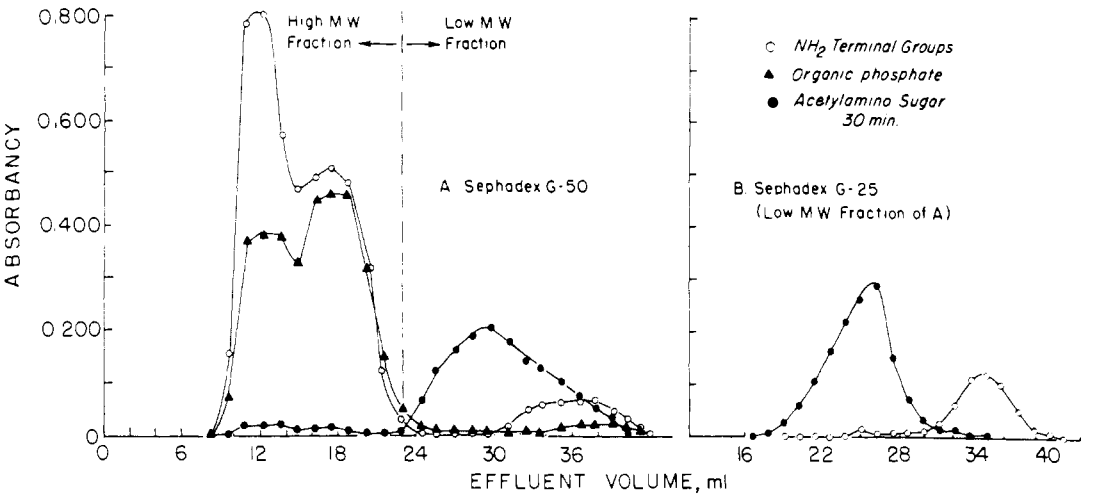

FIG. 9.—Separation on Sephadex G-50 (A) and on Sephadex G-25 (B) of fragments from the teichoic acid–glycopeptide complex (Fig. 5b) after further hydrolysis with amidase. Also see legend to Figure 8.

In another experiment, the components of the amidase-treated teichoic acid–glycopeptide complex were separated by electrophoresis at *p*H 3 prior to purification on Sephadex. Three fractions were obtained by electrophoresis: the teichoic acid (mobility: 8 cm+), the oligosaccharides (mobility: 2.8 cm+) and the peptide (mobility: 9 cm−). These were eluted from the paper and then passed over a column of Sephadex G-50 separately (Fig. 10). Under these conditions 40 μmoles of teichoic acid phosphate, 4.0 μmoles of oligosaccharides, and 3.2 μmoles of peptide amino groups were obtained. Considering the manipulations involved, the peptide amino groups may be considered as equimolar to the oligosaccharides.

DISCUSSION

Solubilization of the cell wall or glycopeptide from *S. aureus*, strain Copenhagen, by the "32 enzyme" from *Streptomyces albus* G was accompanied by liberation of phosphate, "free" acetylamino sugar, reducing groups, and NH_2-terminal groups at the same rates. It is probable, therefore, that the solubilization is catalyzed by a single enzyme, an acetylhexosaminidase, the specificity of which will be defined in the following paper. It may be noted (Figs. 2 and 3) that, after an initial rapid liberation of NH_2-terminal groups, a slower secondary increase of NH_2-terminal groups occurred, not seen with the other substances measured. This slow secondary reaction could be due to minor contamination of the "32 enzyme" with some peptidase.

The "32 enzyme" liberated from the walls two major components, a glycopeptide and a teichoic acid–glycopeptide complex. The glycopeptide in each of these is a degraded compound in that it contains a large number of acetylamino sugar residues with free aldehyde groups. Further treatment of these two fractions with an amidase, also purified from *Streptomyces albus* G, which splits the linkage between acetylmuramic acid and L-alanine, resulted in formation from the glycopeptide of oligosaccharides and of a peptide and from the teichoic acid–glycopeptide complex of a teichoic acid, as well as an oligosaccharide and a peptide.[11] The structures of these three major fragments,

[11] A mechanism by which hydrolysis of the teichoic acid–glycopeptide complex by a single enzyme, amidase, would result in formation of three fragments is suggested by Strominger and Ghuysen (1963).

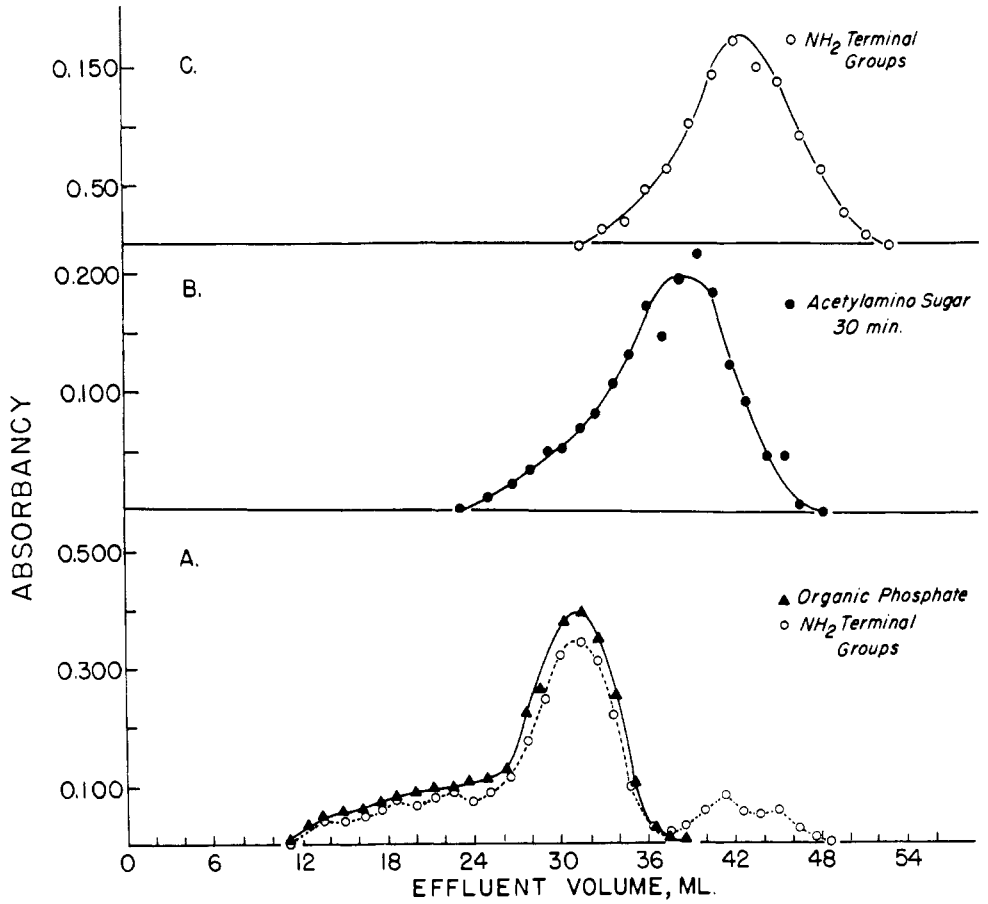

FIG. 10.—Filtration on Sephadex G-50 of the components of the amidase-treated teichoic acid–glycopeptide complex after prior separation by paper electrophoresis; A, the teichoic acid; B, the oligosaccharides; C, the peptide.

the teichoic acid, the oligosaccharides, and the peptides, will be reported in detail in following papers.

Most of the DNFB-reactive material in the wall was accounted for as the ester-linked D-alanine of the teichoic acid, and accompanied the organic phosphate during the fractionation. A small percentage of the DNFB-reactive material was due to low molecular weight substances, probably contaminating the wall preparation and removed during the fractionation. The glycopeptide itself contained only a few NH_2-terminal groups. Following hydrolysis by the amidase and separation of the oligosaccharide, the peptide contained about one free amino group per repeating unit. This amino group was solely an alanine residue.[12] The peptide is a high molecular weight substance, and the question of its homogeneity remains to be established. The oligosaccharide fraction has been separated into two major components, described in the following paper.

The teichoic acid, on liberation from the cell wall through enzymatic hydrolysis, was wholly associated with a fraction of the glycopeptide in a linkage which could not be dissociated on electrophoresis at several *p*H's or by chromatography on a column of Ecteola-cellulose. Moreover, the teichoic acid prepared after hydrolysis of cell wall in trichloroacetic acid had a faster electrophoretic mobility than the teichoic acid–glycopeptide complex.[13] The complex itself contained no material with the mobility of the acid-extracted teichoic acid. It may therefore be concluded, as previously suggested (Mandelstam and Strominger, 1961; Strominger, 1962), that all the teichoic acid is associated in a covalent linkage with a fraction of the glycopeptide. Haukenes (1962) has prepared a similar teichoic acid from the cell walls of *S. aureus* after the action of autolytic enzymes, but he obtained only a small fraction of the teichoic acid in this form. It is clear from our studies that all the teichoic acid in the cell wall is present as the teichoic acid–glycopeptide complex (called polysaccharide A by Haukenes, 1962). The situation is therefore analogous to that which exists in Groups A and C *Streptococcus hemolyticus* (Krause and McCarty, 1961; Krause and McCarty, 1962) and in *B. megaterium* (Ghuysen, 1961; Ghuysen *et al.*, 1962b). On enzymatic hydrolysis of the cell walls of these organisms the group-specific carbohydrates in the first case and a polyol phosphate polymer in the second case remained covalently linked to a fraction of the glycopeptide. Similarly, the teichoic acid, which is an immunological determinant in this (Juergens *et al.*, 1960; Sanderson *et al.*, 1961) and other (Haukenes *et al.*, 1961; Morse, 1962; Nathenson and Strominger, 1962) strains of *S. aureus*, appears to be a covalently linked component of the cell wall (Strominger and Ghuysen, 1963).

[12] Unpublished data.

[13] A preliminary account of the analysis and properties of the teichoic acid–glycopeptide complex has appeared (Strominger and Ghuysen, 1963) and full details will be published shortly. The column chromatography was carried out by Dr. Donald Tipper.

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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.