Structure of the Cell Wall of *Staphylococcus aureus*, Strain Copenhagen. IV. The Teichoic Acid–Glycopeptide Complex*

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ABSTRACT: A teichoic acid–glycopeptide complex has been obtained from the cell wall of *Staphylococcus aureus* after enzymatic lysis of the walls with either of two acetylmuramidases. This complex was fractionated into materials containing a glycopeptide component of varying size linked to teichoic acid. Most of the glycopeptide was removed by hydrolysis of the complexes with an acetylmuramyl-L-alanine amidase. After this treatment, a native teichoic acid with a weight-average molecular weight of about 20,000 and a number-average molecular weight of 12,000–16,000 was obtained. These data suggest that the largest molecules of the teichoic acid contain forty to fifty repeating units and that some smaller molecules also exist in the preparation. Chemical and physical analyses of native teichoic acid and various teichoic acid–glycopeptide complexes are presented.

The cell wall of *Staphylococcus aureus* is composed of two polymers. The glycopeptide has a polysaccharide backbone of alternating acetylglucosamine and acetylmuramic acid residues (Ghuysen and Strominger, 1963a). A complex peptide is linked to the polysaccharide through the carboxyl groups of acetylmuramic acid. Teichoic acid, the second polymer, is a linear polymer of 4-*O*-(*N*-acetyl-D-glucosaminyl)-D-ribitol units linked by 1,5-phosphodiester bridges; some of the ribitol residues are also esterified by D-alanine (see Salton, 1960, and Strominger, 1962, for reviews of cell-wall structure). These two polymers were first separated by means of prolonged extraction in cold 10% trichloroacetic acid, which solubilizes the teichoic acid leaving an insoluble glycopeptide (Armstrong *et al.*, 1958). In the course of study of this solubilization, it became apparent that the separation at 4° was exceedingly slow, 3 weeks being required for extraction of 95% of the teichoic acid (Mandelstam and Strominger, 1961). The separation, moreover, was markedly temperature dependent. At 60°, 95% of the teichoic acid was solubilized in 12 hours.[1] These data suggested that the separation could be caused by cleavage of covalent bonds in acid.

A second approach to the study of the polymer as it exists in the wall has therefore been employed. In these studies, the wall has been solubilized through the action of acetylmuramidases which hydrolyze all of the linkages of acetylmuramic acid and of *N*,*O*-diacetylmuramic acid in the wall. Two acetylmuramidases have been employed, the "32" enzyme from *Streptomyces albus* (Ghuysen *et al.*, 1962a) and the B enzyme from *Chalaropsis* (Hash, 1963; Tipper *et al.*, 1964b). Lysis by either of these enzymes is accompanied by formation of two major polymers, a glycopeptide and a teichoic acid–glycopeptide complex, the separation of which by paper electrophoresis has already been reported (Ghuysen and Strominger, 1963a).

The present paper is a full account of the preparation and properties of the teichoic acid–glycopeptide complex from *S. aureus*, strain Copenhagen, and of the further enzymatic hydrolysis of this complex to yield a teichoic acid. These materials have been compared with the teichoic acid obtained by acid extraction.

Methods and Materials

The methods and materials were identical to those used in previous studies of cell-wall structure from this laboratory (Sanderson *et al.*, 1962; Ghuysen and Strominger, 1963a,b; Tipper *et al.*, 1965), except as detailed in individual experiments following.

Results

Preparation of the Teichoic Acid–Glycopeptide Complex. In previous studies (Ghuysen and Strominger, 1963a) the glycopeptide and teichoic acid–glycopeptide complex present in the lysate of cell walls obtained with an acetylmuramidase (the "32" enzyme) were separated by paper electrophoresis. A detailed analysis of this separation is illustrated in Figure 1. A column of ECTEOLA-cellulose is more convenient for this purpose and is also better suited for large-scale work. When the cell-wall lysate was passed over this column, the glycopeptide came through in the water effluent. The teichoic acid–glycopeptide complex was retained on the column and was then eluted with 0.3 M LiCl (*p*H 5.5)

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[1] M. H. Mandelstam and R. Wishnow, unpublished data.

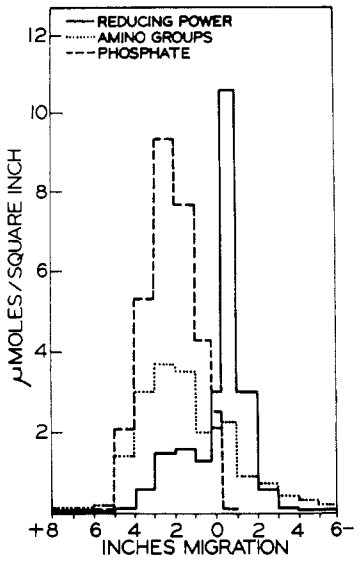


FIGURE 1: Separation of glycopeptide and teichoic acid–glycopeptide complex by paper electrophoresis. *S. aureus* strain Copenhagen cell walls (300 mg) were digested for 12 hours at 37° in 4 ml of 0.025 M acetate, *p*H 4.7, containing 0.5 mg of *Chalaropsis* B enzyme. The soluble high molecular weight fraction of the digest (254 mg) was isolated free of salt and low molecular weight material by filtration on Sephadex G-50, medium grade. This material was then applied across the width of a sheet of Whatman 3MM filter paper, and subjected to electrophoresis in 0.1 M pyridine-acetate buffer, *p*H 3.8, at 100 v/cm for 45 minutes. The paper was then air-dried and cut into strips parallel to the origin, of 13- or 26-mm. width. These strips were eluted with water, and aliquots of the eluates were analyzed for reducing power (glycopeptide), total phosphate (teichoic acid), and free amino groups (ester alanine of the teichoic acid).

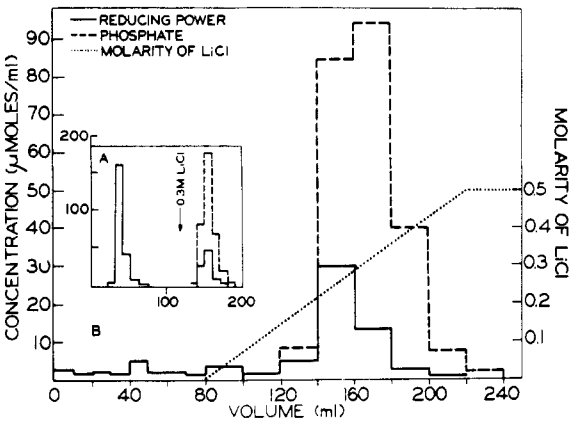


FIGURE 2: Isolation of the teichoic acid–glycopeptide complex by column chromatography. (A) Fractionation of the mixture of glycopeptide and teichoic acid–glycopeptide complex on ECTEOLA-cellulose. Cell walls of *S. aureus* strain Copenhagen (4000 mg) were incubated at 37° in 50 ml of 0.03 M triethylamine-acetate buffer, *p*H 4.7, containing 10 mg of *Chalaropsis* B enzyme. After 6 hours, insoluble material (240 mg) was removed by centrifugation, and the supernatant solution was lyophilized. A portion (10 mg) of the product (3760 mg) was dissolved in water (1 ml) and applied to a column of ECTEOLA-cellulose (1.2 × 8 cm) which had previously been equilibrated with 0.5 M LiCl, *p*H 5, and then washed with water until the eluent was salt free. The column was eluted at 0.5 ml/min with water and then with 0.3 M LiCl, *p*H 5, as indicated. Aliquots of the fractions (10 ml) were analyzed for reducing power (glycopeptide) and organic phosphate (teichoic acid). (B) Gradient elution of the teichoic acid–glycopeptide complex from ECTEOLA-cellulose. A sample (4.5 mg) of the teichoic acid–glycopeptide complex was dissolved in water (1 ml) and applied to the same column as above. After elution with water as indicated, a linear gradient of increasing LiCl concentration was started with 70 ml of water initially in the mixing vessel, and 70 ml of 0.5 M LiCl, *p*H 4.5, in the reservoir. Aliquots of the fractions (16 ml) were analyzed for reducing power (glycopeptide) and organic phosphate (teichoic acid).

(Figure 2A) or by a LiCl gradient. This procedure makes use of the strong negative charge of the teichoic acid–glycopeptide complex compared to the neutral glycopeptide, as did the separation of these polymers by electrophoresis.

When the teichoic acid–glycopeptide complex was placed on the column, both components were retained and were eluted by a LiCl gradient (Figure 2B). None of the glycopeptide was separated from the complex by this procedure. A mixture of glycopeptide (from the water effluent of the ECTEOLA-cellulose column, Figure 2A) and teichoic acid, prepared by acid extraction of cell walls, was also placed on the column. In this case the glycopeptide came through the column in the water effluent and the teichoic acid, eluted with a gradient of LiCl, retained none of it (Figure 3). It is thus apparent that the components of the teichoic acid–glycopeptide complex cannot be separated by repeated chromatography on ECTEOLA-cellulose although an artificial mixture of glycopeptide and the acid-extracted teichoic acid are readily separated by this technique. Moreover, comparison of Figures 2B and 3 indicates that complex rich in glycopeptide was eluted at lower LiCl concentrations than acid-extracted teichoic acid.

The teichoic acid–glycopeptide complex obtained through the action of the *Chalaropsis* B enzyme on cell walls followed by fractionation on ECTEOLA-cellulose was desalted first by extraction of the lyophilized solid with methanol and then by passing a solution over a column of Sephadex G-50. Fractionation of the teichoic acid-glycopeptide complex obtained by electrophoresis (Figure 1) on the same column is illustrated in Figure 4. The skewed distribution of the teichoic acid–gly-

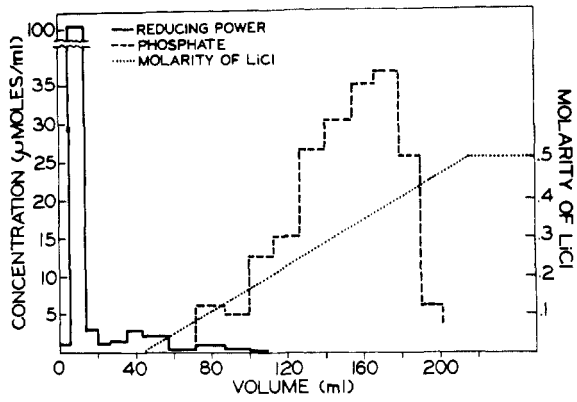


FIGURE 3: Fractionation of a mixture of glycopeptide and acid-extracted teichoic acid on ECTEOLA-cellulose. A mixture of glycopeptide (0.8 mg) and acid-extracted teichoic acid (1.0 mg) in water (1.2 ml) was applied to a column of ECTEOLA-cellulose (1.2 × 8 cm) previously equilibrated with 0.5 M LiCl, *p*H 5, and then washed with water until the eluate was salt free. After elution with water as indicated, a linear gradient of increasing LiCl concentration was started with 100 ml of water initially in the mixing vessel and 100 ml of 0.5 M LiCl, *p*H 4.5, in the reservoir. Aliquots of the fractions (13 ml) were analyzed for reducing power (glycopeptide) and organic phosphate (teichoic acid).

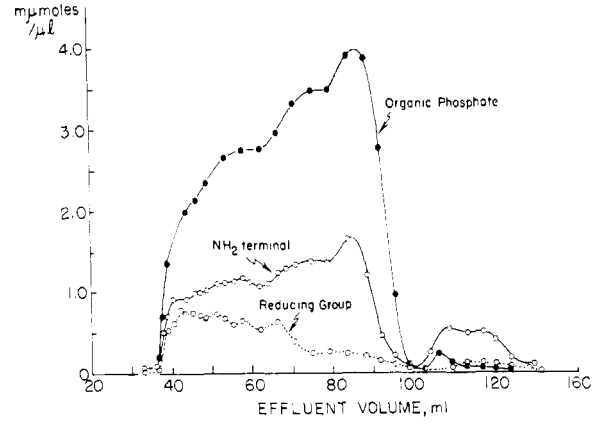


FIGURE 4: Gel filtration of the teichoic acid–glycopeptide complex. The teichoic acid–glycopeptide complex, prepared from *S. aureus* strain Copenhagen cell walls and fractionated by electrophoresis as described in Figure 1, was dissolved in water (120 mg in 10 ml) and applied to a column of Sephadex G-50, medium grade (2 × 40 cm). The column was eluted with water at 0.6 ml/min, and aliquots of the fractions (1.6 ml) were analyzed for organic phosphate (teichoic acid), reducing power (glycopeptide), and amino-terminal groups (ester alanine of the teichoic acid). On the column used, materials with K_D values of 0 and 1 would be eluted at 44 and 115 ml, respectively.

copeptide complex measured by its organic phosphate or ester alanine content in the effluent suggested that the material was heterogeneous, and measurements of reducing power or acetylamino sugar[2] indicated that the material apparently consisted of a continuous spectrum of compounds with a ratio of reducing groups to phosphate varying from 0.4 to 0.025. The material with the highest reducing power was eluted first from the column. The mixture of materials with the highest reducing power (35–68 ml, Figure 4) and that with the lowest reducing power (69–100 ml) were pooled and are referred to as *Chal.* TA-GP I and *Chal.* TA-GP II, respectively. The same continuous fractionation of glycopeptide-rich from glycopeptide-poor complex on ECTEOLA-cellulose can be seen in Figure 2B.

A second series of experiments was carried out with a teichoic acid–glycopeptide complex obtained after lysis of cell walls with the "32" enzyme and separation by paper electrophoresis. Salt, eluted from the paper strips with the complex, was removed on a Sephadex G-50 column. In this case, however, a self-fractionating system appeared to be set up, so that two components were separated with $K_D = 0$ (called "32" TA-GP I) and $K_D = 0.6$,[3] perhaps owing to heavy loading of the column (Figure 5A). Examination, however, indicated that these materials also represented an apparently continuous distribution of components varying in the ratio of acetylamino sugar with free aldehyde groups to phosphate. These components were separately placed on the Sephadex column. TA-GP I again behaved as a single component with $K_D = 0$ (Figure 5B). The material with $K_D = 0.6$, however, was again separated into two components, one with $K_D = 0$ (called "32" TA-GP II) and the other with $K_D = 0.6$ (called "32" TA-GP III) (Figure 5C). "32" TA-GP II and "32" TA-GP III were placed separately on the Sephadex column; each now behaved as a single component with $K_D = 0$ (Figures 5D and 5E). As will become evident, these three compounds ("32" TA-GP I, "32" TA-GP II, and "32" TA-GP III) are teichoic acid–glycopeptide complexes which differ strikingly in the average ratio of acetylamino sugar or amino acids to phosphate. Close examination of the data (Figure 5) indicates that each represents material with a continuous distribution of ratios of acetylamino sugar (with free aldehyde groups) to phosphate.

[2] The glycopeptide component of the complex is degraded into disaccharide units having acetylmuramic acid residues with free aldehyde groups. These disaccharides are held in a polymer by the polypeptide (see Discussion). The aldehyde groups can be measured either by reduction of ferricyanide or by the color formed in the Morgan-Elson reaction, given by acetylamino sugars with a free aldehyde group.

[3] The K_D is a measure of the distribution of the solute between the interior and exterior solvent volumes of the Sephadex gel. A substance which is completely excluded from the gel has $K_D = 0$ and a substance with the same elution volume as salt has $K_D = 1$ (see Flodin, 1961).

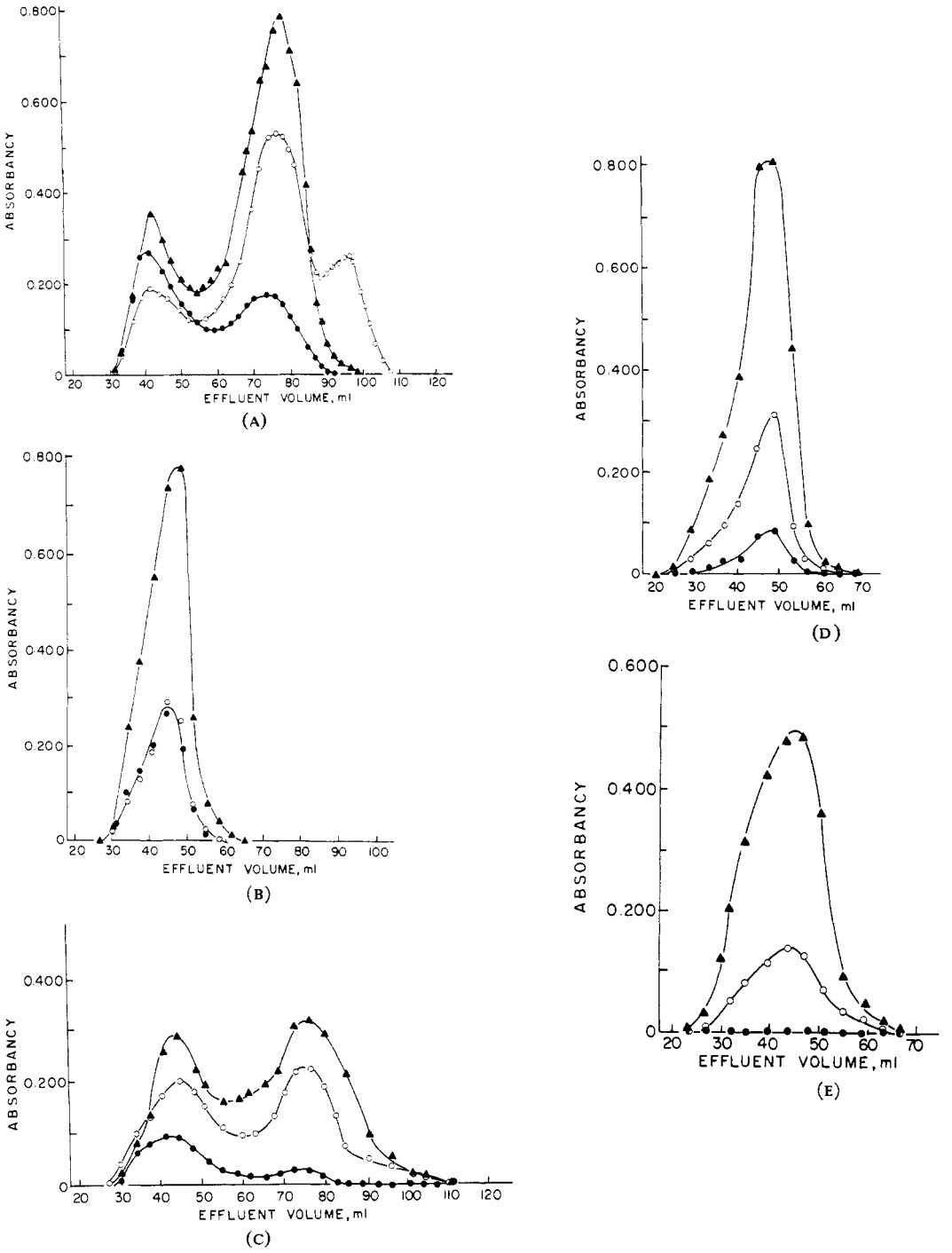


FIGURE 5: Gel filtration of teichoic acid–glycopeptide complexes. (A) The teichoic acid–glycopeptide complex (135 mg) obtained from a digest of cell wall with the "32" enzyme was applied to a column of Sephadex G-50, medium grade, 2 × 45 cm, and eluted with water. (B and C) The material in the two peaks from A were refiltered on the same column. (D and E) The material in the two peaks from C were refiltered on the same column. Measurements of organic phosphate (teichoic acid), amino-terminal groups (ester alanine of the teichoic acid), and acetylamino sugar with free aldehyde groups (glycopeptide) were carried out. Note the progressive decrease in the glycopeptide component in the three fractions, "32" TA-GP I (B), "32" TA-GP II (D), and "32" TA-GP III (E). All parts: ▲, organic phosphate; ○, NH_2-terminal groups; ●, acetylamino sugar.

TABLE 1: Analyses of the Teichoic Acid–Glycopeptide Complexes.[a]

Preparation[7]	Ester-linked N-Terminal Ala[b]	Amino Acids of the Glycopeptide[b]				Disaccharide[c]	s°_{20} × 10^{13}	D_{20} × 10^{7} cm²/sec	Wt-Average mw	Minimal mw[d]	Minimal mw of Glycopeptide[e]	Degree of Polymerization of Teichoic Acid[f]
		Ala	Glu	Lys	Gly							
"32" TA-GP I	0.35	0.55	0.28	0.25	1.23	0.24	1.90	4.0	31,300	862	307	36
"32" TA-GP I_a	0.39	0.74	0.31	0.30	1.48	0.24	2.35	4.0	41,500	800	320	52
Chal. TA-GP I		0.31	0.24	0.16	0.73	0.17	2.08	4.4	31,800	725	190	44
"32" TA-GP II	0.41	0.36	0.14	0.14	0.75	0.10	1.94	4.0	32,000	714	142	45
Chal. TA-GP II		0.13	0.09	0.06	0.28	0.05	1.92	6.3	20,600	606	66	33
"32" TA-GP III	0.35	0.21	0.07	0.07	0.30	0.04	1.66	5.0	23,600	662	73	36
"32" TA-GP III_a	0.45	0.20	0.04	0.04	0 25	0.07	1.71	5.0	25,100	563	63	44
"32"-Amidase teichoic acid	0.32	0.16	0.09	0.02	0.15	0.01	1.80	6.3	20,400	500	40	41
Chal.-Amidase teichoic acid		0.08	0.04	0.03	0.20	0.02	1.46	5.7	18,900	485	20	39
Trichloroacetic acid–teichoic acid	0.40	0.00	0.00	0.00	0.00	0.00	1.12	12.6	5,100	450	0	(11)

[a] The compounds analyzed are described in the text. Data are expressed as moles per mole of organic phosphate. [b] Analyses were carried out in the Beckman Spinco amino acid analyzer and in some cases by a method employing paper chromatography of dinitrophenylamino acids. When both methods were employed, good agreement was obtained. The alanine values for the glycopeptide were obtained by subtracting the ester-linked N-terminal alanine (measured with dinitrofluorobenzene) from the total alanine. [c] Disaccharides, expressed as moles per mole of organic phosphate, were measured by the Morgan-Elson reaction after heating for 30 minutes in borate buffer with reference to a standard of purified disaccharide. Under these conditions the extinction coefficient of the isolated disaccharide was 9000 (Ghuysen and Strominger, 1963b). Muramic acid values, obtained from the amino acid analyzer, were in agreement with these values. [d] Grams of material containing 1 mole of organic phosphate. [e] Grams of glycopeptide component (calculated from amino acid and disaccharide values) per mole of organic phosphate. [f] The number of repeating units in the teichoic acid is obtained by dividing the weight-average molecular weight by the minimal molecular weight. The average of these values, excluding trichloroacetic acid–teichoic acid, is 41 and, since the calculated molecular weight of the repeating unit is 452, the molecular weight of the teichoic acid in the complexes, calculated this way, is 18,500. The approximate degree of polymerization of the glycopeptide moiety is obtained by multiplying its minimal molecular weight by the degree of polymerization of the teichoic acid, and dividing by the calculated molecular weight of one glycopeptide unit, 1180.

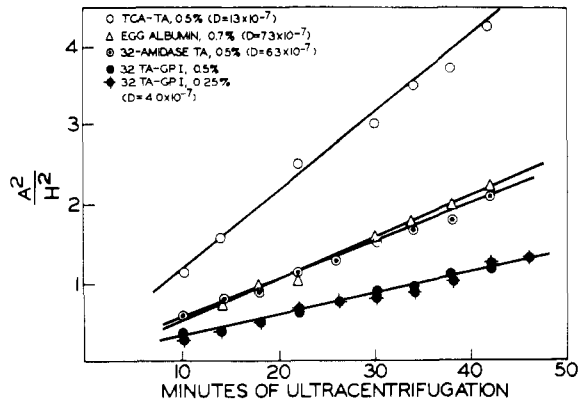


FIGURE 6: Determination of diffusion constants of various teichoic acids. Here A = area, and H = height of the peak in the ultracentrifuge diagram. Measurements were made at 0.25% and 0.5% in 0.1 M phosphate, *p*H 7. The D is calculated from the slopes of the lines.

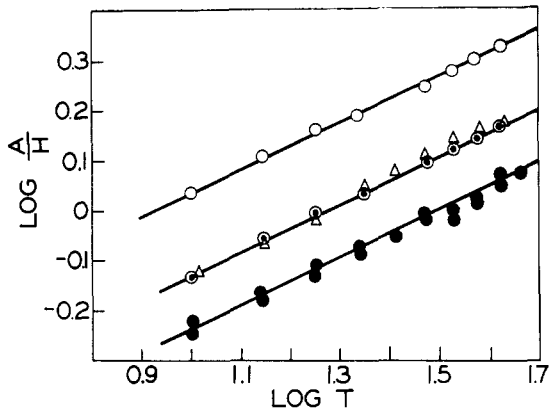


FIGURE 7: Validation of determination of diffusion constants. The slope of each of the lines is equal to 0.5 (see Dieu and Oth, 1954). The symbols are the same as those used in Figure 6.

Separation of the Teichoic Acid from the Complex by the Action of Acetylmuramyl-L-alanine Amidase. After treatment of *Chal.* TA-GP or "32" TA-GP with this enzyme, three components were formed: a teichoic acid, a peptide fraction, and a disaccharide fraction (see Discussion). When the enzymatic digests were placed on a column of Sephadex G-25, the teichoic acid and peptide were eluted together with $K_D = 0$ and the disaccharide fraction was eluted later (see Ghuysen and Strominger, 1963a, Figure 9). Separation of the peptide and teichoic acid was then effected by chromatography on ECTEOLA-cellulose (carried out as described in Figure 2). The peptide fraction came through the column in the water effluent and the teichoic acid was eluted with LiCl.[4] Finally, these teichoic acid preparations ("32"-amidase teichoic acid and *Chal.*-amidase teichoic acid, also referred to as native teichoic acids) were desalted on a column of Sephadex G-25, and then lyophilized.

Analyses of the Teichoic Acid–Glycopeptide Complexes. Several methods have been employed to measure the amino acid and amino sugar content of various preparations (Table I). It is apparent that the samples with a high ratio of Morgan-Elson color to total phosphate contained a large fragment of glycopeptide component. After treatment with the acetylmuramyl-L-alanine amidase, the teichoic acid was virtually free of glycopeptide.[5] The analyses indicate that the glycopeptide moieties of the teichoic acid–glycopeptide complexes had virtually the same composition as that of the glycopeptide itself, i.e., the ratio Glu:Lys:Ala:Gly was about 1:1:2:4.6. The content of ester D-alanine in the complexes was about 0.4 residue per phosphate, about the same as found in the acid-extracted teichoic acid.

Physical Studies of the Teichoic Acid–Glycopeptide Complexes. In order to estimate the molecular weights of these complexes, sedimentation constants have been estimated using a synthetic boundary cell (Table I). The components sedimented as single symmetrical peaks. From the spreading of the boundaries, the diffusion constants have also been estimated (see Svedberg and Pederson, 1940) (Figure 6, Table I).[6] This method is valid when the material is a single compound. If, however, as in this case, the material is polydisperse, it is necessary to ascertain that the broadening of the peak is caused only by diffusion and not by any separation of the components of the system by the centrifugal force. When log [area (A)/height (H)] of the peaks was plotted against log time, a straight line was obtained with slope 0.50 for all of the compounds examined (Figure 7). If the broadening of the peak had been caused by centrifugal separation of the components, then the slope would have gradually increased to approach 1.00 (Dieu and Oth, 1954).

In addition, the partial specific volume of a mixture of *Chal.* TA-GP I and *Chal.* TA-GP II was measured by pycnometry ($\bar{v} = 0.63$). From these data, the molecular weights of the various teichoic acid–glycopeptide complexes was estimated using the relationship, $mw = RTS/D(1 - \bar{v}\rho)$.

The validity of the method was tested using crystalline egg albumin. The diffusion constant in 0.2 M acetate buffer, *p*H 4.5, was found to be 7.3×10^{-7} cm²/sec (Figures 6 and 7), the same value as that found by Lamm and Polson (1936) by other methods. The measured sedimentation constant was $s^{\circ}_{20} = 2.80$ and, together

[4] These components have also been separated by paper electrophoresis at *p*H 3.8 (Ghuysen and Strominger, 1963a).

[5] This material did, however, contain a small amount of residual glycopeptide. Two preparations were made (Table I). One of these was treated a second time with amidase, but this failed to remove these traces of glycopeptide.

[6] It has been shown by Lamm (1929) that a small correction is necessary to obtain the true diffusion constant from the diffusion constant obtained on sedimentation, $D = D_{sed}(1 - \omega^2 st.)$. In the present work, however, this correction was less than 0.001 and has been ignored.

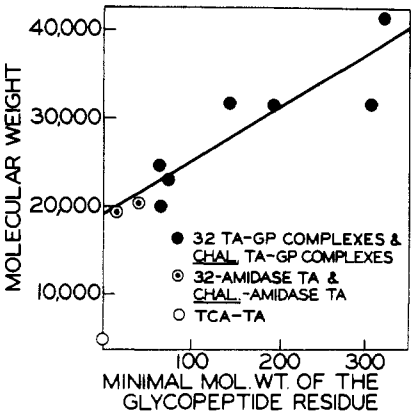


FIGURE 8: Relationship of molecular weight of various teichoic acid preparations to the size of the glycopeptide component. The values plotted are from Table I.

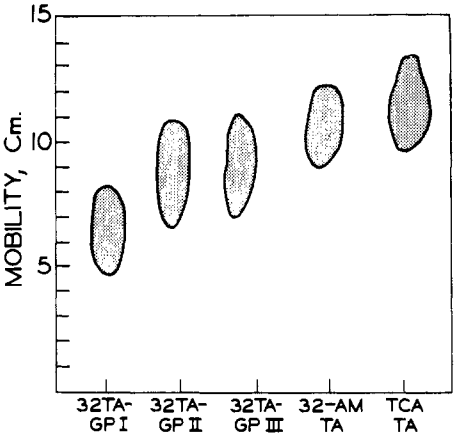


FIGURE 9: Paper electrophoresis of alanine-free teichoic acid preparations. Samples were subjected to a potential gradient of 20 v/cm for 90 minutes in the Electrorheophor apparatus (Pfleuger, Antwerp) in pyridine-acetate buffer, *p*H 5.8, and were detected by the periodate-Schiff spray.

with the diffusion constant and partial specific volume (0.76), a mw of 43,100 was calculated.

The weight-average molecular weights of the complexes were related to the amount of the glycopeptide component, varying between 18,900 (for a preparation obtained after treatment with the amidase) to 41,500 (for the largest of the teichoic acid–glycopeptide complexes). When molecular weight was plotted against the minimal molecular weight of the glycopeptide component (calculated from amino acid and disaccharide content, Table I) a line was obtained which extrapolated to a value for the weight-average molecular weight of 20,000 for the teichoic acid itself (Figure 8), corresponding to an average number of repeating units in the teichoic acid of about 40.

Electrophoretic Mobility. Further evidence for the nonseparability of the components of the teichoic acid–glycopeptide complexes by physical methods was obtained by paper electrophoresis at *p*H 2.2, 3.7, 5.8, 7.8, and 9.1. Moreover, to ensure that the ester-linked alanine played no role in the linking of the two compo-

TABLE II: Electrophoretic Mobilities of Various Teichoic Acids at Different *p*H Values.[a]

Compound	*p*H 2.2	*p*H 3.7	*p*H 5.8	*p*H 7.8	*p*H 9.1	Average
"32" TA-GP I, II, and III (mixture)	0.132	0.119	0.150			0.134
"32"-Amidase teichoic acid	0.198	0.168				0.180
Trichloroacetic acid–teichoic acid	0.156	0.132	0.162			0.150
Ester alanine-free compound						
"32" TA-GP I	0.276	0.288	0.222	0.284	0.258	0.266
"32" TA-GP II	0.372	0.300	0.306	0.318	0.324	0.324
"32" TA-GP III	0.372	0.318	0.306	0.324	0.366	0.331
"32"-Amidase teichoic acid	0.372	0.312	0.372	0.366	0.372	0.359
Trichloroacetic acid–teichoic acid	0.396	0.360	0.372	0.396	0.384	0.382

[a] The buffers employed were 0.2 N formic acid (*p*H 2.2), pyridine–acetic acid–water (2:10:1000, *p*H 3.7; or 7:1:1000, *p*H 5.8), collidine–acetic acid–water (7:1:1000, *p*H 7.8), and 0.02 N ammonium acetate, *p*H 9.1. Electrophoresis was carried out in the Electrorheophor apparatus, Pfleuger, Antwerp, at a potential gradient of 20 v/cm for 90–120 minutes. Data are expressed as mobility toward the anode in cm/hr per volt per cm.

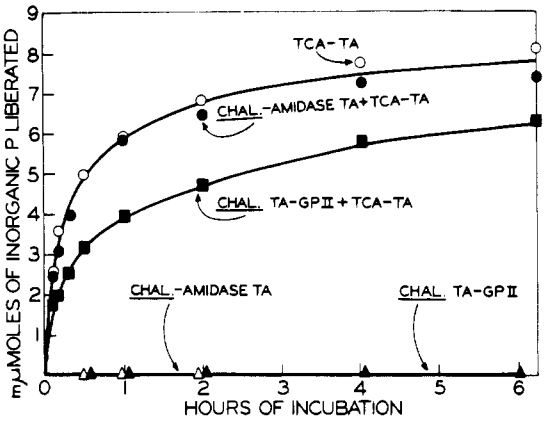


FIGURE 10: Measurement of phosphomonoester end groups in various teichoic acids. Samples of acid-extracted teichoic acid (trichloroacetic acid–teichoic acid (TCA–TA), 440 mμmoles as organic phosphate) or of teichoic acid–glycopeptide complex (*Chal.* TA-GP II, 1400 mμmoles) or native teichoic acid (*Chal.*-amidase teichoic acid, 1050 mμmoles), or mixtures of these were incubated at 37° with *E. coli* phosphomonoesterase (20 μg, obtained from Sigma Chemical Co., St. Louis) in 0.04 M Tris, *p*H 8.5 (125 μl). At the indicated intervals, aliquots (11 μl) containing 39 mμmoles of trichloroacetic acid–teichoic acid, 123 mμmoles of *Chal.*-amidase teichoic acid, 163 mμmoles of *Chal.* TA-GP II, or mixtures of these were removed and frozen. They were subsequently analyzed for inorganic phosphate; 0.2 mμmole could be measured easily under the conditions used.

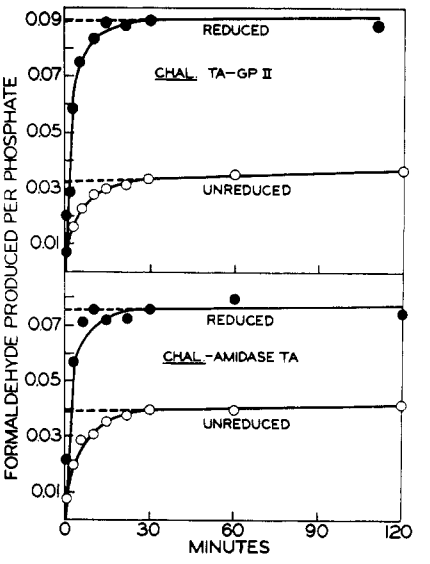


FIGURE 11: Measurement of formaldehydogenic end groups in teichoic acids. The formaldehyde produced during periodate oxidation was measured on unreduced and reduced samples of *Chal.* TA-GP II, *Chal.*-amidase teichoic acid, and "32"-amidase teichoic acid. The latter two preparations gave nearly identical results and only one of them is shown. Samples (containing 500 mμmoles of phosphate) were reduced at room temperature in 0.25 M $NaBH_4$ (30 μl). After 5 hours, 1.0 M acetic acid (15 μl) was added, giving a final *p*H of 4.5. Samples of these reduced teichoic acids (containing 250 mμmoles of phosphate) and the unreduced teichoic acids (containing 500 mμmoles of phosphate) were oxidized at room temperature in 0.001 M periodate (200 μl) containing 0.01 M acetate, *p*H 4.5. At the indicated intervals, aliquots (20 μl) were removed for formaldehyde determination. Under these conditions, the production of formaldehyde from 4-*O*-*N*-acetyl-D-glucosaminyl-D-ribitol and *N*-acetylmuramitol is complete in 30 and 10 minutes, respectively. After 30 minutes, formaldehyde production proceeded at a barely perceptible rate, presumably owing to some overoxidation. Values were obtained by extrapolation of the plateaus to zero time. The reciprocal of the intercept is the number of phosphates per formaldehydogenic end group. The importance of careful kinetic analysis to obtain the correct end point cannot be overemphasized. In 0.01 M periodate, at room temperature, the increased rate of overoxidation was sufficient to give erroneously high results unless a careful kinetic study was made. In 0.001 M periodate at 2°, oxidation was slow and erroneously low results would be obtained if oxidation were not continued for 4–6 hours.

nents of the complexes, the electrophoretic behavior after removal of the ester alanine (by treating solutions of the complexes with an equal volume of concentrated NH_4OH at room temperature for 30 minutes) was also examined. Removal of the ester alanine also greatly increased the mobilities of the compounds and hence enhanced the differences between them. The mobilities of the various compounds were related to their content of glycopeptide, that with the highest content having the lowest mobility at every *p*H and that containing only a trace of glycopeptide (obtained after amidase treatment) having virtually the same mobility as the material obtained by acid extraction (trichloroacetic acid–teichoic acid). Each of the compounds was eluted from the paper after electrophoresis at all *p*H values employed and subjected to hydrolysis in 6 N HCl at 100° overnight. The amino acid components were then examined by two-dimensional paper chromatography in 1-butanol–acetic acid–water (3:1:1) and pyridine-water (4:1). In every case the amino acid components of the glycopeptide had not been separated from the teichoic acid. The electrophoretic mobilities are given in Table II and are illustrated in Figure 9. Since the mobilities were not markedly *p*H dependent in the range employed, the average mobilities have also been calculated (Table II).

Phosphomonoester End-Group Determinations. A characteristic property of the acid-extracted teichoic acid is its content of phosphomonoester end groups. The determination of these end groups has in fact been used to estimate the molecular weight of the acid-extracted teichoic acids. However, all of the teichoic

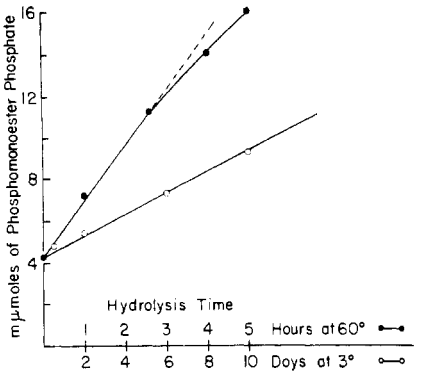


FIGURE 12: Hydrolysis of phosphodiester bonds of teichoic acid in 10% trichloroacetic acid at 3° and at 60°. A preparation of teichoic acid extracted with trichloroacetic acid and containing 1 phosphomonoester end group per 7 phosphates was incubated in 10% trichloroacetic acid at 3° or 60°. Aliquots containing 29 mμmoles of organic phosphate were removed at intervals. After incubation with *Escherichia coli* phosphomonoesterase at 38° for 1 hour at *p*H 7.5, inorganic phosphate was measured.

acid–glycopeptide complexes and the teichoic acid prepared from these complexes by treatment with amidase were totally devoid of detectable phosphomonoester groups (Figure 10). By the method employed, one phosphomonoester group per 500 organic phosphate residues would have been readily detected. As previously reported (Sanderson *et al.*, 1962) the teichoic acid extracted from cell walls of *S. aureus* by trichloroacetic acid had one phosphomonoester group per fourteen organic phosphate residues.

The phosphomonoester end group of the acid-extracted teichoic acid was readily measured by the method employed. In order to ensure that the presence of glycopeptide in the complexes did not significantly inhibit the phosphomonoesterase, a sample of the teichoic acid–glycopeptide complex and of acid-extracted teichoic acid was mixed and treated with phosphomonoesterase. Again the phosphomonoester was liberated from the acid-extracted teichoic acid and no additional inorganic phosphate which could have been derived from the complex was produced (Figure 10). It was also established that none of the enzymes employed in preparation of the various teichoic acids (*Chalaropsis* B enzyme, "32" enzyme, and acetylmuramyl-L-alanine amidase) had detectable phosphomonoesterase activity when tested on the acid-extracted teichoic acid as substrate. Thus a phosphomonoester group could not have been removed by these enzymes during preparation of the various teichoic acids.

Formaldehydogenic End-Group Determination. The nonphosphorylated end of the teichoic acid is a 4-*O*-glycosyl-D-ribitol-5-phosphate which may also be substituted on the 2- or 3- position of the ribitol by an alanine ester. If the ester alanine is removed in dilute alkali, periodate will rapidly oxidize the C_1-C_2 diglycol of the end-group ribitol, producing formaldehyde. Measurement of this group has been used to estimate the chain length of teichoic acids. No other primary hydroxyls which could yield formaldehyde are present in the molecule.

A second source of formaldehydogenic end groups is present, however, in the teichoic acid–glycopeptide complexes or in the native teichoic acid obtained from these materials after treatment with amidase. The disaccharides present in the glycopeptide or the single disaccharide unit presumed to remain in the native teichoic acid (see discussion) do not yield formaldehyde under the conditions of periodate oxidation used here since the pyranose ring of the reducing end group (acetylmuramic acid) is stable under these conditions. Reduction with $NaBH_4$, however, opens the pyranose ring. Periodate then produces formaldehyde from oxidation of the diglycol at C_5-C_6 in the reduced acetylmuramic acid (see Tipper *et al.*, 1965). It is therefore possible to measure two formaldehydogenic end groups in the teichoic acid complexes. One of these is derived from the ribitol unit at the nonphosphorylated end of the teichoic acid molecule and the other, measured after $NaBH_4$ reduction, is derived from the acetylmuramic acid residues in the disaccharides.

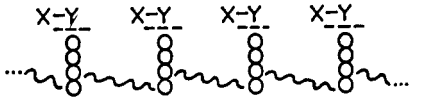
The formaldehydogenic end groups of *Chal.* TA-GP II, *Chal.*-amidase teichoic acid, and "32"-amidase teichoic acid have been measured (Figure 11). Before reduction, these preparations yielded 1 mole of formaldehyde per 31, 26, and 26 phosphates, respectively, presumably a measure of the ribitol end groups in the teichoic acids. After reduction, the *increase* in formaldehyde production due to the disaccharide units was 1 per 17, 27, and 33 phosphates for the three compounds, respectively. *Chal.* TA-GP II contains an average of approximately *two* disaccharide-peptide units in the glycopeptide moiety of each teichoic acid molecule (Table I). Therefore, on this basis, the value of 1 disaccharide end group per 17 phosphates is equivalent to 1 glycopeptide moiety per 34 phosphates for this preparation. The virtual equivalence of the ribitol end group and the disaccharide end group in *Chal.*-amidase teichoic acid and "32"-amidase teichoic acid lends strong support to the concept that the native teichoic acid is substituted at one end by a single disaccharide unit. These end-group determinations (1 per 26–34 phosphates) yield number average molecular weights of 12,000–16,000.

Degradation of Teichoic Acid in Trichloroacetic Acid. The absence of phosphomonoester groups in the teichoic acid prepared after enzymatic lysis of cell walls suggested that their presence in the acid-extracted material might have been owing to cleavage of phosphodiester linkages in the native teichoic acid. In order to examine this point, a sample of teichoic acid which had been obtained by acid extraction was exposed to 10% trichloroacetic acid at 3° and at 60°. At both these temperatures the number of phosphomonoester groups increased progressively with time (Figure 12), indicating that phosphodiester bonds were being cleaved under these conditions, the same conditions as those usually

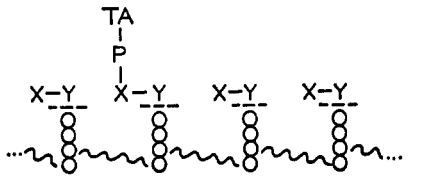
employed for "extraction" of teichoic acid from the cell wall.

Discussion

The isolation of the various compounds described here may be visualized through a diagrammatic representation of the structure of the wall (Figure 13). The wall is represented here as a series of polysaccharide chains, the acetylmuramic acid residues of which are linked to tetrapeptides (Ghuysen *et al.*, 1964). The tetrapeptides are in turn linked to each other by polyglycine bridges, resulting in a two-dimensional network. An important point in consideration of this structure is the fact that analyses (to be published in a following paper) indicate that the various polymers which comprise this structure have natural breaks in them, perhaps random in nature. Conceivably, these are the consequence of the natural biosynthetic process, but they may also be the consequence of action of autolytic enzymes during preparation of the walls. Thus, it may not be possible to obtain undegraded walls by the procedures employed.[7] After hydrolysis of the acetylmuramyl-acetylglucosamine linkages by the acetylmuramidases, the wall is degraded into disaccharides held into a polymer by the cross-linked peptides. The size of this polymer will depend on the integrity of the polypeptide chain involving the glycine cross-bridges. This degraded glycopeptide may be represented as



To some of these degraded glycopeptide molecules teichoic acid may be attached, similarly represented as



The size of the glycopeptide moiety of this teichoic acid–glycopeptide complex will again vary, as already described. Treatment of the complex with amidase results in cleavage, represented by dashed lines, as a consequence of hydrolysis of the bonds indicated. The products are disaccharide, polypeptide, and a native teichoic acid in which one disaccharide blocks the phosphomonoester end. Only one such disaccharide exists per 30–35 phosphate residues (about 2% of the weight). A small amount of disaccharide was detected by chemical analysis (Table I) and its presence was confirmed by the appearance of new formaldehydogenic end groups after $NaBH_4$ reduction.

The teichoic acid obtained after enzymatic lysis of cell walls differs markedly from the preparation which is obtained by solubilization with trichloroacetic acid. The enzymatically prepared material is a higher molecular weight substance associated with a fraction of the glycopeptide from which it cannot be disassociated by several physical methods: chromatography on ECTEOLA-cellulose, electrophoresis, and ultracentrifugation. An artificial mixture of the acid-extracted teichoic acid and glycopeptide is, however, readily separated by the first two of these techniques. These data suggest that the two components of the complex are linked by forces stronger than ionic bonds, presumably by a covalent linkage.

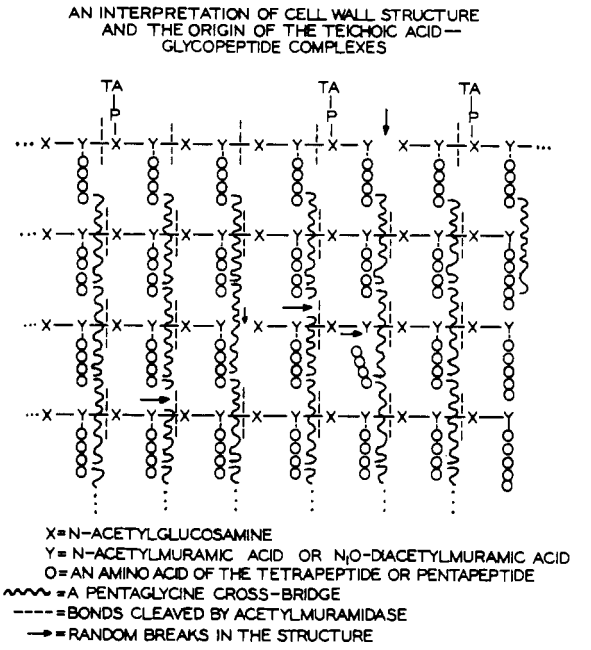


FIGURE 13: A representation of a possible structure of the cell wall of *S. aureus* and of the origin of the teichoic acid–glycopeptide complexes. It should be emphasized that this structure is a working hypothesis which is compatible with the known facts. It may have to be modified as additional information is obtained. The teichoic acid is shown linked to acetylglucosamine for the purpose of this illustration, but data presently available do not indicate to which of the sugars it is linked or how such linkages are distributed in the cell wall. Studies of the peptide structure will be presented in following papers. No attempt has been made to represent a three-dimensional structure but one could be built up by polyglycine cross-bridges extending to a plane in front of or behind this page.

[7] The possibility was considered that the varying size of the glycopeptide portion of the complexes was caused by contamination of the acetylmuramidases employed to lyse the walls by a peptidase splitting the glycine cross-bridges. Two different acetylmuramidases, the "32" enzyme from *Streptomyces* and B enzyme from *Chalaropsis*, have yielded identical products, however, and would thus have to be contaminated to the same extent by a peptidase. Moreover, the teichoic acid–glycopeptide complexes have also been isolated after brief and incomplete lysis of cell walls by the "32" enzyme. These complexes were separated into two components and had the same properties as those prepared after prolonged lysis. They are indicated in Table I as 32 TA-GP I_a and 32 TA-GP III_a.

The molecular weight of the separated complexes depends largely on the size of the glycopeptide components. The teichoic acid itself may also vary somewhat in size, however. Its weight-average molecular weight was about 20,000 while the number-average molecular weight was 12,000–16,000. These data suggest that the maximum size of the teichoic acid molecule may correspond to forty to fifty units but that some smaller molecules must also be present. Since the cell walls used in these studies were obtained from cultures in the logarithmic phase of growth, these could represent molecules in the process of assembly.

Earlier analyses of cell walls (Mandelstam and Strominger, 1961) had suggested, *as one possibility* for the linkage, a peptide bond between the amino group of the ester alanine of the teichoic acid and the free carboxyl group at the end of the peptide chain linked through its amino end to acetylmuramic acid. This possibility had been suggested because of some anomalies in the overall analyses of cell walls. It is clear, however, from these studies, as well as those of others (Armstrong *et al.*, 1961), that most, at least, of the amino groups of the ester alanine are free and hence could not be involved in a covalent linkage. The conditions of enzymatic lysis of the cell walls (*p*H 5) could not be expected to break peptide bonds, freeing the amino group of alanine, and there is no evidence for the occurrence of a specific peptidase in the acetylmuramidases employed. The fact that the teichoic acid remains linked to the glycopeptide, after removal of the ester alanine in the complexes by treatment with ammonia, also argues against involvement of the ester alanine in the linkage to the glycopeptide.

The present data, moreover, strongly suggest another possibility. The repeating unit of the teichoic acid is a substituted D-ribitol-5-phosphate, and the polymer should therefore end in a phosphomonoester group. The absence of such a phosphomonoester suggests that this end of the molecule may be involved in a phosphodiester linkage to some group in the glycopeptide. Other possibilities to explain the lack of a phosphomonoester, although they are not excluded by the present data, seem unlikely. For example, the teichoic acid might be synthesized normally without a phosphomonoester end; it could be a cyclic ribitol phosphate polymer, or the end-group phosphomonoester phosphate could be removed in a normal biosynthetic process, or the end group itself could be a cyclic phosphate. As synthesized, the end group might also be blocked by some hitherto undetected small molecule. None of these possibilities seems as likely an explanation, however, as the hypothesis that the end-group phosphate is involved in a phosphodiester linkage to the glycopeptide.

The presence of phosphomonoester groups in the teichoic acid, prepared by acid extraction, is clearly the consequence of hydrolysis of phosphodiester bonds in acid. The lability of such bonds to acid may be further support for the hypothesis that the teichoic acid is linked to the glycopeptide by a phosphodiester. Possibly the phosphodiester linking the teichoic acid to the glycopeptide is especially acid labile. However, the number of phosphomonoester groups in a teichoic acid is directly related to the time employed in acid extraction. A small amount of teichoic acid can be solubilized by exposure to cold trichloroacetic acid for 2–3 minutes. About 2 mg of this material was obtained from 200 mg of cell walls. It contained low but measurable phosphomonoester groups (1 per 120 organic phosphate residues) (Sanderson *et al.*, 1962). After hydrolysis in 6 N HCl it was, moreover, found to contain all of the amino acids which are characteristic of the glycopeptide (Ala, Glu, Lys, and Gly) and presumably was primarily teichoic acid–glycopeptide complex extracted by acid. Exposure to 10% trichloroacetic acid for 6 hours at 60° solubilized about three-fourths of the teichoic acid. This material had one phosphomonoester group per 10–20 phosphates and had a molecular weight of about 8000 ($s^{\circ}_{20} = 0.79$, $D_{20} = 14 \times 10^{-7}$ cm²/sec). The material obtained between 6 and 12 hours at 60° had one phosphomonoester group per 7–10 phosphates and a molecular weight of about 5000 (Table I). Still further exposure to acid (Figure 12) resulted in cleavage of virtually all of the phosphodiesters in the molecule.

It has been repeatedly suggested that the teichoic acid is held to the cell wall of *S. aureus* by an ionic linkage (Archibald *et al.*, 1961; Baddiley *et al.*, 1962; Critchley *et al.*, 1962). No data, however, have been produced to support this assumption. Moreover, the glycopeptide is a neutral molecule, containing few positively or negatively charged groups. It is difficult to imagine that so strong an affinity as is present in the teichoic acid–glycopeptide complex could be brought about only through the agency of electrostatic forces. Final proof of the covalent linkage, however, depends on the isolation of the "linking group," i.e., the phosphodiester which is presumed to link ribitol to some group in the glycopeptide and represents only 1–2% of the weight of the cell wall. This goal has recently been accomplished through a series of degradative procedures (Tipper *et al.*, 1964a; Ghuysen *et al.*, 1964) and full details will be published when the precise nature of this link compound has been elucidated.

Similar complexes of glycopeptides with polysaccharides or teichoic acids have been obtained by enzymatic lysis of the cell walls of other bacteria. These include a glycopeptide–teichoic acid complex from *Bacillus subtilis* (Young *et al.*, 1964), a polysaccharide-glycopeptide complex from *Streptococcus hemolyticus* (Krause and McCarty, 1961, 1962), and a glycerophosphate polymer linked to the glycopeptide in *Bacillus megaterium* (Ghuysen *et al.*, 1962a; Ghuysen, 1964). The covalent linkage of these components to glycopeptide appears to be a general feature of the architecture of bacterial cell walls.

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