The Glycerol Teichoic Acid from the Walls of *Staphylococcus albus* N.T.C.C. 7944

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The teichoic acids in and beneath the walls of staphylococci are particularly interesting, not only with regard to structure and function but also because of their serological properties. The group-antigenic polysaccharide A from *Staphylococcus aureus* is indistinguishable from the wall teichoic acid from that organism, and a similar preparation from *S. albus* strains is serologically indistinguishable from the wall teichoic acid (Haukenes, Ellwood, Baddiley & Oeding, 1961); related serological properties have been demonstrated with other strains of staphylococci (Sanderson, Juergens & Strominger, 1961). Other wall teichoic acids have been implicated in serological reactions (Baddiley & Davison, 1961), and the intracellular teichoic acid from group D streptococci has been identified as the group antigen (Elliott, 1962; also Elliott, Wicken & Baddiley, 1963).

The teichoic acid in the walls of *S. aureus* H is a ribitol phosphate polymer with *N*-acetyl-D-glucosamine residues at position 4 of each D-ribitol 5-phosphate unit. D-Alanine ester residues are attached to position 2 or 3 of ribitol and both α - and β -glucosidic linkages occur (Baddiley, Buchanan, RajBhandary & Sanderson, 1962*a*; Baddiley, Buchanan, Martin & RajBhandary, 1962*b*). Although other strains of *S. aureus* have not been examined in such detail, it appears that their wall teichoic acid is composed of ribitol phosphate, alanine and *N*-acetylglucosamine (Armstrong *et al.* 1959; Sanderson *et al.* 1961; unpublished work by A. L. Davison).

Organisms described generally as *S. albus* strains are closely related to *S. aureus*; the one studied here is probably more accurately described as *S. lactis* (*S. albus*) N.T.C.C. 7944. A preliminary examination of walls of this and related strains by Dr J. J. Armstrong and later by Mr A. L. Davison showed that, despite the close relationship between *aureus* and *albus* strains, the latter contained glycerol teichoic acids in their walls. The more detailed examination described here has shown that the teichoic acid from strain N.T.C.C. 7944 is a glycerol phosphate polymer with D-alanine ester residues and *N*-acetylgalactosamine attached glycosidically to some of the glycerol units.

MATERIALS AND METHODS

A culture of the organism was obtained from the National Collection of Type Cultures; β -*N*-acetylglucosaminidase was kindly provided by Dr A. L. Levvy; calf-intestinal phosphatase was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.; crude hexokinase was a gift from Sigma Chemical Co.; ATP was purchased from Pabst Laboratories, Milwaukee, Wis., U.S.A., and NADH from C. F. Boehringer und Soehne, Mannheim, Germany.

Analytical methods. Phosphorus was determined by the method of Chen, Toribara & Warner (1956), alanine and amino compounds by Rosen's (1957) method, reducing sugars by Park & Johnson's (1949) method, periodate oxidation by Dixon & Lipkin's (1954) method and formaldehyde by Hanahan & Olley's (1958) method.

Paper chromatography. Whatman no. 1 and no. 4 papers, previously washed with 2*N*-acetic acid and then water, were used with these solvent systems: (A) propan-1-ol-aq. ammonia (sp.gr. 0.88)-water (6:3:1, by vol.) (Hanes & Isherwood, 1949); (B) propan-2-ol-conc. hydrochloric acid-water (65:17:18, by vol.) (Smith & Wyatt, 1951); (C) butan-1-ol-ethanol-water-aq. ammonia (sp.gr. 0.88) (40:10:49:1, by vol.; organic phase) (Foster, Horton & Stacey, 1957); (D) butan-1-ol-pyridine-water (6:4:3, by vol.) (Jeanes, Wise & Dimler, 1951); (E) pyridine-ethyl acetate-water-acetic acid (5:5:3:1, by vol.) (Fischer & Nebel, 1955).

Solvents (A) and (B) were used for ascending chromatography on Whatman no. 4, solvents (D) and (E) for descending chromatography on Whatman no. 1, and solvent (C) for descending chromatography on Whatman no. 4. Products were detected where appropriate by the periodate-Schiff reagents for glycols (Buchanan, Dekker & Long, 1950; Baddiley, Buchanan, Handschumacher & Prescott, 1956), ninhydrin reagent for amino acids and amino sugars (Consden & Gordon, 1948), molybdate-perchloric acid for phosphates (Hanes & Isherwood, 1949), aniline phthalate reagent for reducing sugars (Partridge, 1949), alkaline silver nitrate reagent for reducing compounds (Trevelyan, Procter & Harrison, 1950), ferric chloride reagent for hydroxamates (Fink & Fink, 1949), Elson-Morgan reagent for amino sugars (Partridge, 1949) and modified alkaline silver nitrate reagents for glycosides (Wickberg, 1958).

The observed chromatographic properties of compounds obtained or examined in this work are given in Table 1.

Preparation of walls. The organism was grown in batches (15 l.) under the same conditions and in the same medium as described for *S. aureus* (Baddiley *et al.* 1962*a*). The yield was 5 g. (wet wt.)/l. Walls were prepared with an Inter-

national centrifuge shaker head (Shockman, Kolb & Toennies, 1957) or a Mickle disintegrator as described for *S. aureus*, and preparations washed with 1% sodium chloride solution were examined as a routine by electron microscopy. Teichoic acid preparations used in this work were extracted from walls prepared from cultures of cells grown for 16 hr., but some studies were made with 40 and 70 hr. cultures.

Intracellular teichoic acid. In the above procedure walls and a few whole cells were removed from preparations of disrupted organisms by centrifuging at 66 000g for 30 min. in a Spinco ultracentrifuge. The slightly cloudy supernatant was then centrifuged at 100 000g for 1 hr. The gelatinous sediment was resuspended in water and freeze-dried. The sediment contained much polynucleotide (RNA), some protein and nearly all the intracellular teichoic acid (Found: P, 4.5%). On acid hydrolysis (with 2*N*-hydrochloric acid for 3 hr. at 100°) and examination in solvent (A) it yielded mainly hydrolysis products of RNA, amino acids and some glycerol and its mono- and di-phosphates.

A sample (3.0 g.) of the sedimented material was stirred with 10% (w/v) trichloroacetic acid (100 ml.) at 4° for 70 hr. After centrifuging, acetone (90 ml.) was added to the supernatant, which was kept at 4° for 24 hr. The teichoic acid (315 mg.) was centrifuged, washed with acetone and ether, dried, redissolved in water (5 ml.), centrifuged and the supernatant was freeze-dried. The white solid (190 mg.) gave on acid hydrolysis glycerol and its mono- and di-phosphates, inorganic phosphate, smaller amounts of products of RNA hydrolysis, alanine and some other amino acids, galactosamine and faint traces of other sugars.

Isolation of teichoic acid from walls. The walls (4.0 g.) were suspended in water (100 ml.), trichloroacetic acid (10 g.) was added and the suspension was stirred at 4°. Samples (1 ml.) were removed at intervals, diluted with water (5 ml.), centrifuged at 50 000g for 10 min. and the residue was washed with acetone and then ether. Initially the phosphorus content of the walls was 2.6%; after extraction

for 24 hr. the phosphorus content was 1.9%, and after 48 hr. or 70 hr. it was 1.5%. Walls were removed after 70 hr. by centrifuging for 30 min. at 80 000g and washed with 10% trichloroacetic acid solution (20 ml.). Combined supernatant and washings were mixed with acetone (94 ml.) and the mixture was kept at 0° for 24 hr. The gummy precipitate (307 mg.) was centrifuged, washed with acetone and ether, and then dried over phosphoric oxide. The residue was dissolved in water (1 ml.), and the solution was kept at 4° for 1 hr., centrifuged at 2000g and the supernatant was freeze-dried. The teichoic acid (230 mg.) was a white solid. Teichoic acid was prepared in a similar manner from walls that had been obtained from organisms grown for 40 and 70 hr. The walls from 40 hr. cultures contained 1.5% of phosphorus and those from 70 hr. cultures contained 1.23% of phosphorus before extraction.

The insoluble wall material recovered from the 70 hr. extraction was washed with water, acetone and ether (three lots of each), and the residual material (3.5 g.) was dried over phosphoric oxide; it contained 1.5% of phosphorus. It was re-extracted with 10% trichloroacetic acid (100 ml.) at 45° for 22 hr. Insoluble material was removed by centrifuging at 100 000g for 30 min. and washed with 10% trichloroacetic acid (25 ml.); after washing three times with ethanol and with ether, the dried material contained 0.44% of phosphorus. Combined extract and washings were mixed with an equal volume of acetone and kept at 4° for 24 hr. The precipitate (215 mg.), which was collected by centrifuging, contained teichoic acid and mucopeptide components.

A sample (10 mg.) of the insoluble residue after extraction of teichoic acid was hydrolysed in 2*N*-hydrochloric acid at 100° for 3 hr. Chromatography with solvent (A) showed the absence of teichoic acid-degradation products, and two-dimensional chromatography in butan-1-ol-acetic acid-water (8:2:1, by vol.) and then in phenol-water (organic phase) (Partridge, 1948) showed the presence of the following: glucosamine, muramic acid, alanine, glycine,

Table 1. *Paper chromatography of degradation products of teichoic acid and related compounds*

Solvent	...	...	(A) R_F	(B) R_F	(C) R_{Glycerol}	(D) R_{Glycerol}	(E) $R_{\text{Glucosamine}}$
Alanine			0.59	0.67	0.28	0.23	0.72
Alanine amide			0.70	—	—	—	—
Glycerol			0.75	0.80	1.00	1.00	—
Glycerol monophosphates			0.32	—	0.00	—	—
Glycerol diphosphates			0.13	—	—	—	—
Inorganic phosphate			0.18	—	—	—	—
Galactosamine			0.51	0.33	0.36	0.32	0.84
<i>N</i> -Acetylglucosamine			0.55	—	0.55	0.70	—
Galactosaminylglycerol			0.62	—	0.38	0.32	—
<i>N</i> -Acetylglactosaminylglycerol			0.65	—	0.53	—	—
Glucosamine			0.56	0.33	0.38	0.37	1.00
<i>N</i> -Acetylglucosamine			0.60	—	0.59	0.73	—
α -Methyl <i>N</i> -acetylglucosaminide			—	—	1.02	—	—
β -Methyl <i>N</i> -acetylglucosaminide			—	—	0.85	—	—
α -Methyl glucosaminide			—	—	0.70	0.48	—
Glucose			0.56	—	0.38	0.54	—
Ribose			—	—	0.84	—	—
Lyxose			—	—	0.78	—	—
Arabinose			—	—	0.65	—	—
Xylose			—	—	0.74	—	—
2,5-Anhydromannose			—	—	0.98	—	—
2,5-Anhydrotalose			—	—	1.00	—	—
Glyceric acid			0.57	—	—	—	—

lysine, glutamic acid and traces of leucine and valine. The material from which all teichoic acid had been removed no longer possessed the characteristic appearance of walls when examined by electron microscopy, but appeared to be granular.

Removal of alanine ester residues. For some purposes it was necessary to remove alanine ester residues from the teichoic acid without affecting other linkages. This was achieved by treatment with aq. 2*N*-ammonia solution at 37° for 3 hr. The formation of alanine and its amide were demonstrated chromatographically. Alternatively, teichoic acid (6.5 mg.) was dissolved in *N*-sodium hydroxide or *N*-lithium hydroxide (2 ml.) and the solution kept at room temperature for 3 hr. After neutralization to pH 7.0 with hydrochloric acid dialysis was carried out through Visking tubing at 4° for 24 hr. against distilled water (1 l.). Teichoic acid lacking alanine was recovered from the tube contents by freeze-drying.

Examination of teichoic acid

Analysis. Samples of ammonium salts of several preparations had $[\alpha]_D + 70^\circ$ (c 2.5 in water) after alanine had been removed.

A sample (1.0 mg.) of teichoic acid with alanine intact was hydrolysed in 2*N*-hydrochloric acid (2 ml.) for 17 hr. at 100°. The hydrolysate was neutralized (to pH 7.0) with *N*-sodium hydroxide solution and freeze-dried (Found: phosphorus:reducing sugar:amino compounds, 1:0.29:1.03). A sample from which alanine had been removed was hydrolysed similarly (Found: phosphorus:reducing sugar:amino compounds, 1:0.3:0.3).

A sample (2 mg.) was dissolved in water (0.5 ml.) and *N*-sodium hydroxide (0.5 ml.) was added. After 2 hr. at 20° the ratio phosphorus:amino compounds was 1:0.69; the amino compounds determined by this procedure were from alanine only. It follows that the ratio phosphorus:amino sugar is 1:0.34, and the ratio phosphorus:alanine is 1:0.69. Analysis gave phosphorus, 10.17%; the structure described in the Discussion section requires phosphorus, 10.57%, or, with 1 mol.prop. of water/phosphorus atom, 10.15%.

Acid hydrolysis and similar analyses of material from organisms grown for 40 hr. gave the proportions phosphorus:reducing sugar:amino compounds 1:0.39:1.25; those from organisms grown for 70 hr. were 1:0.29:0.89. Values for intracellular teichoic acid were 1:0.018:0.21.

Electrophoresis. Paper electrophoresis was carried out with 0.05*M*-ammonium acetate-acetic acid buffers at pH 3.8 or 5.5 and with a potential of 5 v/cm. for 12 hr. In each case the teichoic acid moved towards the anode as a single spot. Slight trailing was observed with material containing alanine ester residues, but not after these had been removed.

Action of ammonia. Samples of teichoic acid (0.5 mg.) and of walls (2.0 mg.) were treated with aq. 2*N*-ammonia solution (0.1 ml.) at 37° for 3 hr. Chromatography in solvent (A) indicated that alanine and its amide were formed in both cases. The positive ninhydrin reaction given by teichoic acid was no longer observed with the material at the origin of the chromatogram after treatment with ammonia.

Action of hydroxylamine. A solution of 0.5*M*-hydroxylamine was prepared from the hydrochloride with Dowex 1 (OH⁻ form) resin. The teichoic acid (1.0 mg.) and walls

(2.0 mg.) were treated with the fresh hydroxylamine solution (0.05 ml.) during 2 hr. at 37°. Products were examined by paper chromatography in propan-1-ol-water-0.5*M*-hydroxylamine (pH 7.4)-0.1*M*-EDTA (8:17:2:1, by vol.). Papers were sprayed with ferric chloride reagent. The alanine hydroxamate formed from the teichoic acid and from the walls had R_f 0.43.

Potentiometric titration. The procedure was similar to that used in work on teichoic acids by Armstrong, Baddiley & Buchanan (1960). Alanine was removed from a sample of teichoic acid (51 mg.) by treatment with ammonia followed by dialysis and freeze-drying. The product (40 mg.) in water (1.0 ml.) was passed through a column of Dowex 50 (H⁺ form) resin (5 ml.), which was washed with water until the washings reached pH 6.0. Combined eluate and washings were titrated with 0.5*N*-sodium hydroxide. A plot of $\Delta pH/\Delta V$ against *V*, where *V* is the volume of alkali used, gave two peaks corresponding to the neutralization of primary and secondary acidic groups. The ratio of primary to secondary groups was 17:1:1. Repetition on the same sample to ensure the absence of alanine ester groups gave a ratio 18:1:1.

Periodate oxidation. (a) With dilute periodate. Teichoic acid (1.7 mg.) from which alanine had been removed was dissolved in water (5 ml.) and its phosphorus content was determined. Samples (0.5 ml.) were mixed with 0.2 *mm*-sodium metaperiodate solution (1.5 ml.) and water (1.5 ml.), and the consumption of periodate was measured spectroscopically. The change in extinction with time is given in Fig. 1. Extrapolation of the curve to zero time gives the ratio phosphorus:periodate consumed by terminal glycol residue (18:0:1).

(b) With more concentrated periodate. Teichoic acid (47.8 mg., without alanine residues) in water (5 ml.) was added to 0.1*M*-sodium metaperiodate (1 ml.) and the volume adjusted to 10 ml. with water. A control experiment without teichoic acid was also carried out. The solutions were kept at 4° in the dark and samples (0.05 ml.) were withdrawn at intervals for periodate measurement. When oxidation was complete (90 hr.) samples were removed for the determination of formaldehyde and of phosphorus; the pH of the solution was unchanged throughout the oxidation period. The ratio phosphorus:formaldehyde produced was 18:1:1. From the total periodate consumed and the formaldehyde produced, the amount of periodate consumed by the sugar residues can be calculated; the ratio phosphorus:periodate consumed by sugar was 1:0.33.

A sample (0.1 ml.) of the oxidation mixture was heated

Fig. 1. Periodate oxidation of teichoic acid. Oxidation was carried out with dilute periodate as described in the text. Alanine had been removed from the polymer with ammonia.

for 6 hr. at 100° with 10N-sulphuric acid, neutralized (bromothymol blue) with barium hydroxide, and the precipitate removed by centrifuging. Supernatant and water washings were combined and evaporated, then examined by paper chromatography with solvents (A), (D) and (E); no amino sugar was detected. The remaining solution of oxidized teichoic acid was used for characterization of the amino sugar by degradation to serine (see below).

Action of phosphomonoesterase. Teichoic acid (13 mg.) without alanine residues was dissolved in 0.05M-ethanolamine-hydrochloric acid buffer (10 ml.), pH 9.5, and calf-intestinal phosphomonoesterase (0.2 mg.) was added. The solution was kept at 37° under toluene and samples (0.1 ml.) were removed at intervals. Hydrolysis to inorganic phosphate was complete after about 4 hr. No further amounts were produced after 24 hr., even when fresh enzyme was added. The ratio total phosphorus:inorganic phosphate formed was 18.3:1.

Hydrolysis products of teichoic acid

Acid hydrolysis. The teichoic acid (2.0 mg.) was heated at 100° with 2N-hydrochloric acid (0.2 ml.) for 3 hr. After evaporation *in vacuo* the residue was dissolved in water and examined by paper chromatography with solvents (A), (B), (C) and (D). Products are given in Table 2 and their chromatographic properties are listed in Table 1.

Identification of galactosamine. Dialysed teichoic acid (25 mg.) from which alanine had been removed was hydrolysed with 6N-sulphuric acid (2 ml.) in a sealed tube at 100° for 6 hr. After neutralization (bromocresol green) with barium hydroxide solution, the precipitated barium sulphate was centrifuged and washed with water (2 ml.). Combined supernatant and washings were evaporated *in vacuo* to 0.5 ml. A sample (0.1 ml.) examined by chromatography with solvents (D) and (E) gave only one reducing product with R_F corresponding to galactosamine. On co-chromatography in solvent (E) with glucosamine and galactosamine it was indistinguishable from the latter, but differed from glucosamine.

The ninhydrin degradation to a pentose was followed according to the method of Stoffyn & Jeanloz (1954). A sample (0.2 ml.) of the acid hydrolysate was chromatographed as a band with solvent (D). The hexosamine

(R_{Glucose} 0.67) was eluted with water and the solution was freeze-dried. The residue was dissolved in a 2% solution (0.1 ml.) of ninhydrin containing 4% of pyridine. Samples of glucosamine and galactosamine were similarly treated. Solutions were heated for 30 min. at 100° and products were examined by chromatography with solvent (D). The pentose obtained from the hexosamine of the teichoic acid was indistinguishable from lyxose obtained from authentic galactosamine, but differed from arabinose derived from glucosamine.

The acetamido sugar was obtained from a cautious acid hydrolysis. The teichoic acid (10 mg.) from which alanine had been removed was hydrolysed at 100° for 30 min. in 0.1N-sulphuric acid (0.5 ml.). The solution was adjusted to pH 7.0 with barium hydroxide solution and barium sulphate removed by centrifuging. The supernatant was passed through columns of Dowex 50 (NH_4^+ form) resin (1 ml.) and Dowex 2 (HCO_3^- form) resin (1 ml.). Eluate and washings were combined and evaporated *in vacuo* to 0.5 ml. The products were examined by chromatography with solvent (D). A *N*-acetylhexosamine indistinguishable from *N*-acetylgalactosamine was observed.

Identification of D-alanine. Teichoic acid (428 mg.) was dissolved in N-lithium hydroxide (10 ml.) and kept at 23° for 4 hr. The solution was adjusted to pH 7.0 with hydrochloric acid and dialysed with stirring in a Visking tube for 12 hr. at 4° against distilled water (4 l.). Dialysis was repeated with two further lots of water. The material in the tube (teichoic acid lacking alanine) was recovered by freeze-drying and used in other experiments. The diffusate (total 12 l.) was passed slowly through a column of Dowex 50 (H^+ form) resin (50 ml.). After washing the column with water (500 ml.), the alanine was eluted with 2N-hydrochloric acid, fractions (10 ml.) being collected automatically. Appropriate fractions were combined and evaporated to dryness *in vacuo*, the residual solid was dissolved in water (1 ml.) and ethanol (5 ml.) was added. Careful addition of ether gave a cloudy solution from which crystals were obtained by cooling to -20°. Alanine hydrochloride was filtered, washed with ether and dried. It was purified further by chromatography on Whatman 3 MM paper with solvent (A) and then solvent (D). The appropriate area was eluted with water and the solution was freeze-dried.

The configuration of the alanine was established by the actions of L-alanine transaminase and D-amino acid oxidase, under the conditions described by Kelemen & Baddiley (1961). No reaction was observed with the transaminase, whereas the alanine was fully oxidized by the oxidase at the same rate as an authentic sample of D-alanine.

Alkali hydrolysis. Teichoic acid (2 mg.) was hydrolysed in N-sodium hydroxide (0.1 ml.) for 3 hr. at 100°. The hydrolysate was passed through a column of Dowex 50 (NH_4^+ form) resin (1 ml.), and the combined filtrate and water washings (5 ml.) were evaporated *in vacuo*. Products (see Table 2) were examined by chromatography with solvents (A), (C) and (D). They were identified from R_F values (Table 1) and by the degradation procedures described below.

Isolation of galactosaminylglycerol. Teichoic acid (200 mg.) from which alanine had been removed was hydrolysed with N-potassium hydroxide (5 ml.) at 100° for 24 hr. After adjusting to pH 7.0 with 72% perchloric acid the solution was kept at 4° for 4 hr. Potassium perchlorate was removed

Table 2. *Acid and alkali hydrolysis products of teichoic acid*

Conditions of hydrolysis are given in the text and R_F values are given in Table 1.

	Acid hydrolysis	Alkali hydrolysis
Glycerol	+	+
Glycerol monophosphates	+	+
Glycerol diphosphates	+	+
Inorganic phosphate	+	Trace
Alanine	+	+
Galactosamine	+	-
<i>N</i> -Acetylgalactosamine	+	-
<i>N</i> -Acetylgalactosaminylglycerol	-	Trace
Galactosaminylglycerol	Trace	+
Galactosaminylglycerol phosphates (low R_F)	-	+
Diglycerol triphosphate	-	+

* Cautious acid hydrolysis only.

by centrifuging, washed with 2 ml. of ice-cold water (three times) and the supernatant and washings were combined and ammonium carbonate (250 mg.) was added. The pH was adjusted to 9.5 with aq. ammonia solution and the volume made to 25 ml. Samples (0.1 ml.) were removed for determination of phosphorus and inorganic phosphate. Phosphomonoesterase (2 mg.) was added and the solution was kept under toluene at 37°. Samples were removed at intervals for determination of inorganic phosphate. After 24 hr., 15% of the total phosphorus was organically bound; further treatment with fresh enzyme (2 mg.) for 24 hr. produced an additional 2% of inorganic phosphate, but reaction then ceased. The solution was evaporated *in vacuo*, the residue was dissolved in water (10 ml.) and evaporation was repeated. This procedure for the removal of ammonium carbonate was repeated five times. Paper chromatography with solvents (A), (B), (C) and (D) revealed the presence of glycerol, galactosaminylglycerol, inorganic phosphate, phosphodiester with low R_F values and a trace of *N*-acetylgalactosaminylglycerol.

The mixture of products was heated at 100° for 6 hr. in *n*-potassium hydroxide (5 ml.), the solution neutralized (pH 7.0) with 72% perchloric acid and potassium perchlorate removed as described above. Paper chromatography of the products (180 mg.) with solvent (A) showed that phosphodiester were absent and glycerol phosphates were present. Analysis indicated that 11% of the total phosphorus was organically combined, and phosphatase liberated all of this as inorganic phosphate in 4 hr. at 37°.

The products from this second alkali hydrolysis were dissolved in water (5 ml.) and applied to a column of Dowex 50 (H^+ form) resin (20 ml.). The column was washed with water (200 ml.) and combined filtrate and washings were evaporated to dryness *in vacuo*. The residue contained glycerol, glycerol phosphates, inorganic phosphate and a trace of *N*-acetylgalactosaminylglycerol. Material was eluted from the column with 2*N*-hydrochloric acid (200 ml.) and the eluate was evaporated to 10 ml. *in vacuo*; water (25 ml.) was added and the evaporation was repeated. The procedure was repeated five times and the solution was then evaporated to dryness. The residue (65 mg.) contained potassium chloride and galactosaminylglycerol.

The galactosaminylglycerol could be isolated as its hydrochloride from the above-mentioned residue by dissolving in water (5 ml.) and applying to a column of Ultrasorb charcoal (5 g.) mixed with Celite (5 g.). After the column was washed with water (500 ml.), traces of glycerol were removed with water (250 ml.) containing 1% of ethanol and 1% of 1 *M*-hydrochloric acid. The hydrochloride of galactosaminylglycerol was eluted with 5% ethanol (250 ml.) followed by 10% ethanol (250 ml.), both containing 1% of 1 *M*-hydrochloric acid. The substance was recovered by evaporating the solvent *in vacuo*; its homogeneity was demonstrated by chromatography with solvent (C).

The galactosaminide was isolated more conveniently as its *N*-acetyl derivative. A solution of the residue (65 mg.) containing potassium chloride (see above) in water (20 ml.) was mixed with Dowex 2 (CO_3^{2-} form) resin (10 ml.) and methanol (1 ml.) and then cooled to 0°. Acetic anhydride (0.3 ml.) was added and the mixture was stirred at 4° for 90 min. The resin was removed by filtration and washed with water (50 ml.). Combined filtrate and washings were

evaporated *in vacuo* to 1 ml. and passed rapidly through a column of Dowex 50 (H^+ form) resin (10 ml.). Eluate and water washings (50 ml.) were evaporated *in vacuo* to dryness and the residue (17 mg.) was dissolved in a little water and then freeze-dried. It crystallized from anhydrous methanol-ether mixtures, but the crystals were too deliquescent for satisfactory isolation; it had $[\alpha]_D + 112 \pm 20^\circ$ (*c* in water 0.3). It was examined chromatographically with solvents (C) and (D).

Acid hydrolysis of galactosaminylglycerol and its N-acetyl derivative

The hydrochloride was hydrolysed in 6*N*-sulphuric acid at 100° for 18 hr. After neutralization with barium hydroxide solution and centrifuging of barium sulphate, the combined supernatant and water washings were evaporated *in vacuo*. Chromatography was carried out with solvents (A), (C), (D) and (F). Products were glycerol and galactosamine, but unhydrolysed material was also observed.

Similar hydrolysis of the *N*-acetyl derivative gave glycerol, galactosamine and a little galactosaminylglycerol.

Action of nitrous acid on galactosaminylglycerol. To a solution of the hydrochloride (1 mg.) in water (0.5 ml.) was added sodium nitrite (50 mg.), the pH was adjusted to 5.6 with 0.01 *N*-acetic acid and the mixture was heated for 6 hr. at 60°. The solution was applied to a column of Dowex 50 (NH_4^+ form) resin (5 ml.), which was then washed with water (25 ml.). Combined eluate and washings were evaporated to dryness *in vacuo* and the residue was examined chromatographically with solvents (A), (C) and (D). Products were glycerol and a compound giving an elongated yellow spot with the periodate-Schiff reagents that was indistinguishable from 2,5-anhydrotalose obtained from galactosamine. This anhydro sugar differed slightly in R_F from 2,5-anhydromannose derived similarly from glucosamine (cf. Baddiley *et al.* 1962*a*).

A similar procedure was carried out with glucosamine, galactosamine and the glycerol derivative (1 mg. of each), and the products were reduced with sodium borohydride (10 mg.) in water (1 ml.) at room temperature for 12 hr. Acetic acid (0.1 ml.) was added and after 30 min. the solutions were passed through columns of Dowex 2 (HCO_3^- form) resin (2 ml.) and Dowex 50 (H^+ form) resin (2 ml.). Eluates and washings were evaporated to dryness *in vacuo* and boric acid was removed by repeated (three times) evaporation from methanol (1 ml.) and 2*N*-hydrochloric acid (0.1 ml.). Ions were again removed by passage of an aqueous solution through the two ion-exchange columns, and eluates and washings were evaporated to dryness *in vacuo*. The residues were examined by electrophoresis on Whatman no. 1 paper moistened with 0.05 *M*-sodium borate, pH 9.2, at a potential of 7.5 v/cm. for 6 hr. The paper was sprayed with alkaline silver nitrate by the technique of Frahn & Mills (1959). Several spots were observed in all cases but the main component from galactosamine was indistinguishable from that from the glycerol derivative; it moved rapidly, showing the presence of a *cis* glycol grouping, and was presumably 2,5-anhydrotalitol. Glucosamine gave a slower-moving main product, presumably 2,5-anhydromannitol, which possesses a *trans* glycol grouping.

Periodate oxidation of N-acetylgalactosaminylglycerol. The acetyl derivative (1.85 mg.) was weighed rapidly, dissolved

in water (2.0 ml.) and 8 mM-sodium metaperiodate (2.0 ml.) was added; control experiments without sugar derivatives and with α -methyl *N*-acetylglucosaminide were also carried out. Solutions were kept at 4° in the dark and samples (0.1 ml.), diluted with water (3.9 ml.), were examined spectroscopically at intervals. When oxidation had ceased (48 hr.), formaldehyde was determined in the oxidation mixture; the pH was unchanged throughout the experiment (1.02 mol.prop. of periodate was consumed and 0.03 mol.prop. of formaldehyde was produced; the glucosaminide consumed 0.99 mol.prop. of periodate and yielded no formaldehyde without change in pH).

Configuration of galactosamine

D-Glyceric acid from C-4-C-6. The mixture from oxidation of *N*-acetylglactosaminylglycerol (1.85 mg.) with periodate by the method described above was treated with ethylene glycol (50 μ moles) at 4° for 24 hr. Removal of ions and further oxidation with bromine were effected by methods similar to those described by Archibald, Baddiley & Buchanan (1961b). Hydrolysis was carried out with 2*N*-hydrochloric acid at 100° for 2 hr. and mineral acid was removed by evaporation. Isolation of glyceric acid and determination of its configuration by phosphorylation and enzymic assay with NADH and a preparation of rabbit-muscle enzymes were carried out by methods described by Archibald *et al.* (1961b). The glyceric acid from α -methyl *N*-acetyl-D-glucosaminide oxidized 1.98 mol.prop. of NADH, and that from *N*-acetylglactosaminylglycerol oxidized 2.08 mol.prop. of NADH.

D-Serine from C-1-C-3. The solution remaining from the oxidation of teichoic acid with periodate by method (b) was diluted with water (10 ml.), sodium borohydride (50 mg.) was added and the mixture was kept at room temperature for 17 hr. Acetic acid (0.1 ml.) was added and after 2 hr. the solvent was evaporated *in vacuo*. After addition of methanol (2 ml.) and 2*N*-hydrochloric acid (0.1 ml.) solvent was again evaporated; this procedure was repeated three times to remove boric acid. The residue was dissolved in 0.1*N*-sulphuric acid (10 ml.) and bromine (1 ml.) was added, followed after 17 hr. at room temperature by barium benzoate (200 mg.). The mixture was shaken for 1 hr. and kept for a further 60 hr. Solid was removed by centrifuging, washed with water (5 ml.) and bromine was removed from the supernatant and washings by aeration until colourless. 10*N*-Sulphuric acid (1.0 ml.) was added and the solution was heated at 100° for 2 hr. After neutralization (to pH 7.0) with barium hydroxide solution, solid was removed by centrifuging. The clear solution was passed through a column of Dowex 50 (H⁺ form) resin (10 ml.), which was then washed with water (100 ml.). Serine was eluted with 2*N*-hydrochloric acid (100 ml.) and recovered by evaporation *in vacuo* and drying over phosphoric oxide and potassium hydroxide. It was purified by chromatography with solvent (A) and was indistinguishable from authentic serine (R_F 0.41); the expected reactions were observed with ninhydrin and periodate-Schiff reagents. The material was extracted from the paper with water and rechromatographed with solvent (A) alongside authentic D- and L-serine. The paper was sprayed with 1% D-amino acid oxidase in 0.05*M*-pyrophosphate buffer, pH 8.5, kept in an atmosphere of moist oxygen at 37° for 2 hr. and then dried (Jones, 1948; Salton, 1957). Although a spot corresponding to L-serine was readily observed with

ninhydrin, no spots were observed with authentic D-serine or that from the teichoic acid. On the other hand, when a chromatogram paper, after treatment with D-amino acid oxidase, was sprayed with 2% 2,4-dinitrophenylhydrazine hydrochloride in 2*N*-hydrochloric acid and examined in ultraviolet light (max. about 260 $m\mu$), the yellow spots (R_F 0.41) given by the product from D-serine and by material from the teichoic acid were strongly fluorescent. The L-serine was unchanged in these procedures.

Phosphodiester from alkali hydrolysis of teichoic acid

Hydrolysis and separation of products. Teichoic acid (217 mg.) from which alanine had been removed was heated at 100° for 6 hr. in *N*-potassium hydroxide (5 ml.). A small amount of insoluble inorganic material was removed by centrifuging and the supernatant was adjusted to pH 7.0 with 72% (w/v) perchloric acid then cooled to 0°. Potassium perchlorate was centrifuged and washed with a little ice-cold water. The combined supernatant and washings were evaporated to 1 ml. *in vacuo* and passed through a column of Dowex 2 (HCO₃⁻ form) resin (50 ml.). The column was washed with water (500 ml.) and combined eluate and washings were evaporated to dryness *in vacuo*. This represented the 'non-acidic' fraction of the hydrolysate, and contained glycerol, galactosaminylglycerol and some *N*-acetyl derivative, and not more than 2% of the total phosphorus in the hydrolysate.

Acidic material was eluted from the column with 10% (w/v) ammonium carbonate (250 ml.) followed by water (100 ml.). Combined eluate and washings were evaporated to dryness *in vacuo*; the residue was dissolved in water (10 ml.), which was again evaporated. This was repeated five times to remove ammonium carbonate, and the material (80 mg.) was recovered finally by freeze-drying. It contained 85% of the phosphorus present in the hydrolysate.

Glycerol and galactosaminylglycerol from the non-acidic fraction

Material that had passed through the Dowex 2 column (see above) was dissolved in water and applied to a column of Dowex 50 (H⁺ form) resin (25 ml.), which was then washed with water (250 ml.). Combined eluate and washings were evaporated *in vacuo* to a small volume.

The neutral eluate and washings were chromatographed as a band on Whatman no. 3 MM paper with solvent (C). A band with the R_F of glycerol was cut out and the material was eluted with water (1 ml.). Evaporation of the water yielded a residue (25 mg.), which gave a benzoyl derivative, m.p. 73°, undepressed on admixture with authentic tri-*O*-benzoylglycerol; its infrared spectrum was also identical with that of authentic benzoylglycerol. Material (2.2 mg.) with the chromatographic properties of *N*-acetylglactosaminylglycerol was also eluted from the paper; it gave glycerol and galactosamine on hydrolysis in 6*N*-sulphuric acid for 6 hr. at 100° [chromatography with solvents (A), (C), (D) and (E) after neutralization with barium hydroxide solution and centrifuging].

Galactosaminylglycerol was isolated as its *N*-acetyl derivative by elution from the Dowex 50 column with 2*N*-hydrochloric acid (150 ml.), followed by evaporation to dryness *in vacuo* and *N*-acetylation.

Phosphodiester fraction. The acidic material eluted from the Dowex 2 resin column after alkali hydrolysis of teichoic acid (see above) was examined by chromatography with solvent (A). It contained glycerol mono- and diphosphates together with material that did not move significantly from the origin. Paper electrophoresis (5v/cm.) in 0.05M-potassium hydrogen phthalate-hydrochloric acid (pH 2.2 and 6.5) or 0.05M-ammonium acetate (pH 3.8) buffers did not effect satisfactory separation of the higher phosphates.

(a) Treatment with nitrous acid and then phosphomonoesterase. The acidic material (20 mg.) was dissolved in water (0.5 ml.), sodium nitrite (50 mg.) was added, pH was adjusted to 5.6 with 0.1N-acetic acid and the solution was heated at 60° for 6 hr. It was then passed through a column of Dowex 50 (NH_4^+ form) resin (5 ml.), which was subsequently washed with water (25 ml.). Combined eluate and washings were evaporated to dryness *in vacuo*. Chromatography with solvents (A), (C) and (D) revealed the formation of 2,5-anhydrotalose and a trace of glycerol; no diglycerol phosphate was detected. The products were dissolved in water (5 ml.) and applied to a column of Dowex 2 (HCO_3^- form) resin (20 ml.). After the column had been washed with water (100 ml.), products were eluted with 10% ammonium carbonate (200 ml.) and then water (100 ml.). Combined eluate and washings were evaporated to dryness *in vacuo*, the residue was dissolved in water (5 ml.), pH adjusted to 9.5 with aq. 10N-ammonia solution and intestinal phosphomonoesterase (2 mg.) was added. Incubation was carried out for 48 hr. at 37° under toluene in a sealed tube. Ammonium carbonate was removed by evaporation to dryness several times and products were examined chromatographically with solvents (A) and (C). The main product was diglycerol phosphate; a small amount of a phosphate with R_F 0.0-0.15, giving a rapid reaction with the periodate-Schiff reagent was probably triglycerol diphosphate; a trace of glycerol was detected.

(b) Treatment with phosphomonoesterase and then alkali or nitrous acid. A sample (50 mg.) of the acidic fraction was dissolved in water (10 ml.), ammonium carbonate (250 mg.) was added, the pH was adjusted to 9.5 with aq. 10N-ammonia solution and intestinal phosphomonoesterase (2 mg.) was added. A little toluene was added, the tube was sealed with Parafilm and incubation was carried out at 37°. After 48 hr., 15% of the total phosphorus was still organically bound and this was not reduced by further incubation with fresh enzyme. The solution was evaporated to dryness *in vacuo* and ammonium carbonate was removed from the residue by repeated (five times) evaporation with water. The freeze-dried product (70 mg.) was examined chromatographically with solvent (A). In addition to a small amount of glycerol and a trace of galactosaminylglycerol, the main products were inorganic phosphate, diglycerol phosphate (R_F 0.55), isomeric galactosaminyl diglycerol phosphates (XII and XIII) (R_F 0.30), and galactosaminyl triglycerol diphosphate (XIV) (R_F 0.0-0.15). All the phosphodiester reacted rapidly in the periodate-Schiff spray procedure.

A sample of the mixture after the enzyme treatment was fractionated by paper chromatography as a band on Whatman 3MM paper with solvent (A). Appropriate areas were cut out and products eluted with water and freeze-dried. Hydrolysis was carried out with N-sodium

hydroxide (0.1 ml.) or 0.5N-barium hydroxide (0.2 ml.) at 100° for 3 hr. After passage through columns of Dowex 50 (NH_4^+ form) resin (1 ml.) and washing with water (5 ml.) the solutions were concentrated *in vacuo* and examined chromatographically with solvent (A). The diglycerol phosphate gave glycerol and its monophosphates; the isomeric galactosaminyl diglycerol phosphates gave galactosaminylglycerol and glycerol monophosphates. Visual examination indicated that for corresponding galactosamine contents, the triglycerol compound gave twice as much glycerol monophosphates as did the isomeric diglycerol compounds.

A sample (20 mg.) of the mixture after enzyme treatment was dissolved in water (0.5 ml.), sodium nitrite (50 mg.) was added and reaction was carried out as described above. Products were prepared for chromatography with solvents (A), (C) and (D) as before. An increased amount of diglycerol phosphate was observed, and a compound with R_F 0.0-0.15 (probably triglycerol diphosphate) was detected; 2,5-anhydrotalose was also formed.

Enzymic hydrolysis of galactosaminides

Methyl α - and β -N-acetylglucosaminides were prepared from N-acetylglucosamine (Zilliken, Rose, Braun & György, 1955) and separated by paper chromatography (solvent C). The α compound was also prepared by N-acetylation of methyl α -glucosaminide (Roseman & Ludowieg, 1954). The α compound had m.p. 187° and $[\alpha]_D + 128$ –131° (c in water 3.0) and the β compound had m.p. 197° and $[\alpha]_D - 40$ ° (c in water 3.0).

A sample of methyl N-acetyl galactosaminide was prepared from the acetamidogalactosamine in a similar manner. It had $[\alpha]_D + 139$ ° in water, whereas pure α compound has $[\alpha]_D + 170$ ° (Stacey, 1944). From the reported value for the β compound, $[\alpha]_D - 12$ °, it is concluded that the preparation contained about 20% of the β compound.

In the enzyme experiments samples of substrates (0.5 mg.) were mixed with M-sodium chloride (0.1 ml.), 0.1% bovine plasma albumin (L. Light and Co. Ltd., Colnbrook, Bucks.) (0.1 ml.), 0.5M-citric acid buffer, pH 4.3 (0.1 ml.), water (0.7 ml.) and β -N-acetylglucosaminidase solution (0.1 ml.). Solutions were kept at 37° under toluene for 24 hr. After evaporation of toluene, the solutions were passed through columns of Dowex 50 (NH_4^+ form) resin (3 ml.) and then Dowex 2 (HCO_3^- form) resin (3 ml.). Eluates and washings were evaporated *in vacuo* and examined with solvent (C). Products were estimated visually after spraying the paper with silver nitrate reagent (Wickberg, 1958). Results are given in Table 3.

Table 3. *Enzymic hydrolysis of glucosaminides and galactosaminides*

Hydrolysis with pig-epididymis enzyme was carried out for 24 hr. as described in the text.

	Acetamidogalactosaminide sugar produced
α -Methyl N-acetylglucosaminide	Trace
β -Methyl N-acetylglucosaminide	+++
Methyl N-acetyl galactosaminide	+
N-Acetyl galactosaminyl glycerol	+
Teichoic acid (<i>S. albus</i>)	+

DISCUSSION

Walls were prepared and teichoic acid was extracted from them by procedures similar to those used with other organisms. Despite the rather prolonged treatment required for complete extraction from these walls, there is no evidence for covalent linkage between the mucopeptide and teichoic acid. In fact, it was shown that ammonia and dilute hydroxylamine at room temperature remove the alanine from teichoic acid in the walls of this organism, giving alanine amide and hydroxamate respectively. This is consistent with the conclusion from studies with other organisms that teichoic acids in the walls are held by salt linkages and hydrogen bonds and not by covalent linkages involving alanine (Archibald, Armstrong, Baddiley & Hay, 1961*a*; Baddiley *et al.* 1962*a, b*; Critchley, Archibald & Baddiley, 1962). After complete extraction of teichoic acid much of the original wall structure had been lost, but the residual material still contained 0.44% of phosphorus. Analysis indicated that this insoluble residue was a mucopeptide containing glucosamine, muramic acid, alanine, glycine, lysine, glutamic acid and traces of leucine and valine. The unextractable phosphorus is presumably bound chemically in a manner similar to that found in all mucopeptide preparations studied in this Laboratory (e.g., Kelemen & Baddiley, 1961; Archibald *et al.* 1961*b*; Baddiley *et al.* 1962*a, b*).

Walls were separated from cell contents by centrifuging, and the intracellular teichoic acid was isolated from the supernatant by ultracentrifuging. The gelatinous sediment obtained at 100 000*g* contained the intracellular teichoic acid, RNA and some protein. The interesting affinity between the relatively low-molecular-weight teichoic acid and the much larger RNA and protein of the ribosomal cell fraction was first described in *Lactobacillus arabinosus* (Critchley *et al.* 1962), but has been observed since then with many organisms (unpublished work by A. L. Davison, A. J. Wicken and others). The significance of the association is not known, particularly as studies with protoplasts suggest that intracellular teichoic acid is probably located between the wall and protoplast membrane, at least in the bacteria examined so far in this connexion (Hay, Wicken & Baddiley, 1963), and so may not be in direct association with RNA in the cell.

The structure of the intracellular teichoic acid has not been examined in detail. Acid hydrolysis indicated that it is a glycerol phosphate polymer containing alanine ester residues and acetyl-galactosamine. However, the proportions phosphorus:amino sugar:total amino compounds were 1:0.018:0.21, indicating a much lower proportion

of amino sugar than was found in the wall teichoic acid. The low value for total amino compounds suggests loss of some of the alanine ester residues during isolation.

The wall teichoic acid gave, on acid hydrolysis, glycerol and its mono- and di-phosphates, alanine and an amino sugar that differed from glucosamine but was chromatographically indistinguishable from galactosamine. The formation of both glycerol and its diphosphates indicates that, like other glycerol teichoic acids, it is a glycerol phosphate polymer in which adjacent glycerol units are joined through phosphodiester linkages. Analysis gave the proportions phosphorus:amino sugar:alanine 1:0.34:0.69, and these figures were unchanged by dialysis (the glycerol teichoic acids do not pass through the usual Visking membranes) or by chromatography on ECTEOLA-cellulose columns. In this work organisms were harvested and teichoic acid was extracted after growth for 16 hr.; growth for 40 or 70 hr. did not alter much the proportions of components in the teichoic acid, although that from old cells contained rather less alanine and may have had a somewhat lower molecular weight. The optical rotation, $[\alpha]_D + 70^\circ$, was more or less unaffected by age, indicating no appreciable alteration in the proportion of α - to β -glycosidic linkages. The content of phosphorus of walls from older cells suggested some decrease in the amount of teichoic acid with age.

The alanine was completely oxidized by a D-amino acid oxidase after removal from the teichoic acid, and so must have the D-configuration. This, and the very ready reaction between these ester residues and ammonia or hydroxylamine to give alanine amide or hydroxamate, are typical characteristics of the alanine residues in other teichoic acids; the lability of the ester groups is a consequence of the presence of neighbouring phosphate groups (Shabarova, Hughes & Baddiley, 1962). In other cases it is concluded that alanine is attached to glycerol rather than to sugar residues (Kelemen & Baddiley, 1961; Critchley *et al.* 1962); the high alanine content and smoothness of its removal with bases indicate that in the *S. albus* wall teichoic acid the alanine is attached to glycerol. Analysis shows that, if those glycerol units that possess sugar do not also possess alanine, then all the remaining sugar-free glycerol units must have alanine attached to them.

The wall teichoic acid from *S. albus* is a glycerol phosphate polymer to which are attached acyl-amino sugar residues and labile D-alanine ester residues. Thus it resembles other teichoic acids in its general structure (for a review see Baddiley, 1962). The structural features on which more exact information was required were: an unequivocal identification of the acylamino sugar and its

method of attachment to the polymer, the distribution of sugar residues along the chain and the location of the phosphodiester linkages, the length of the polymer chain.

Confirmation of the identity of the amino sugar was obtained in several ways. Gentle acid hydrolysis of the teichoic acid gave, in addition to some free amino sugar, an acylamino hexose that was chromatographically indistinguishable from *N*-acetyl galactosamine. Moreover, removal of C-1 from the amino sugar with hot ninhydrin solution (Stoffyn & Jeanloz, 1954) gave lyxose; this degradation establishes the configuration of C-3–C-6 as that of galactosamine, but does not clarify the stereochemistry at C-2. The stereochemistry at C-2 was demonstrated by periodate oxidation of the polymer after removal of alanine with ammonia. All the acetamido sugar residues were oxidized in this way, and the resulting aldehydic polymer (I) was reduced with sodium borohydride. Acid hydrolysis followed by oxidation with bromine water gave an amino acid, identified chromatographically as serine (II). The carboxyl group in this serine represents C-1 of the amino sugar, and the terminal carbon atom bearing the hydroxyl represents C-3. The serine would have the *D*-configuration if the amino sugar were *D*-galactosamine or *L*-talosamine, and the *L*-configuration if it had been obtained from *L*-galactosamine or *D*-talosamine. It is shown below that the amino sugar is in the *D*-series; thus the configuration of the serine would finally establish the structure of the amino sugar. The small scale of the experiments

precluded direct measurement of optical rotation, but as the amino acid was readily oxidized by a specific *D*-amino acid oxidase it follows that it had the *D*-configuration; consequently, the amino sugar must be galactosamine.

Alkali hydrolysis of the teichoic acid gave a complex mixture of organic phosphates, alanine, glycerol, a galactosaminylglycerol and a trace of its *N*-acetyl derivative (see Table 2); the yield of galactosaminylglycerol was increased by the action of phosphatase followed by alkali on the hydrolysate. Glycerol was identified as its crystalline tri-*O*-benzoyl derivative, and the galactosaminide by its slow hydrolysis in 6*N*-sulphuric acid to glycerol and galactosamine. The *N*-acetyl derivative was identical with the compound obtained by *N*-acetylation of the galactosaminylglycerol, thus proving that the acyl group on the amino sugar in the teichoic acid is acetyl. Unfortunately, neither the galactosaminylglycerol nor its *N*-acetyl derivative could be obtained in stable crystalline forms, and comparisons were based on chromatography and behaviour on hydrolysis. The relative stability towards acid of glycosides of amino sugars, and the ease of hydrolysis of their *N*-acetyl derivatives, is general (Foster *et al.* 1957) and has been noted in studies on ribitol glucosaminides and their *N*-acetyl derivatives from another teichoic acid (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958; Baddiley *et al.* 1962*a*). The general structure of the galactosaminylglycerol was confirmed by its reaction with nitrous acid; the products were glycerol and 2,5-anhydro-

Fig. 2. Formation of *D*-serine from galactosamine residues.

talose (IV), the reaction being analogous to the formation of ribitol and 2,5-anhydromannose from ribitol glucosaminides (Baddiley *et al.* 1962*a*). The anhydro sugar was further characterized by reduction with sodium borohydride to 2,5-anhydrotalitol. These reactions, and the action of periodate, are outlined in Fig. 3.

The *N*-acetylgalactosaminylglycerol consumed 1 mol.prop. of periodate, giving neither formic acid nor formaldehyde. Hydrolysis of the oxidation product gave glycerol but no amino sugar, and it follows that the glycoside is correctly represented as 2-*O*-(*N*-acetyl-D-galactosaminyl)glycerol (III; R = acetyl). The sugar possesses the D-configuration, as its periodate oxidation product (V), when oxidized further with bromine water and hydrolysed with acid, yielded D-glyceric acid; the configuration of the glyceric acid was established by conversion into its 3-phosphate with a kinase, and determination of the ability of the phosphate to cause oxidation of NADH with a preparation of rabbit-muscle enzymes (Armstrong *et al.* 1960; Archibald *et al.* 1961*b*).

The teichoic acid has $[\alpha]_D + 70^\circ$ and the *N*-acetylgalactosaminylglycerol has $[\alpha]_D + 112^\circ$. These high positive rotations suggest that most of the glycosidic linkages have the α -configuration (as shown in III), since α -methyl *N*-acetylgalactosaminide has $[\alpha]_D + 170^\circ$ (Stacey, 1944), whereas the β -methyl derivative has $[\alpha]_D - 12^\circ$ (Tarasiejska & Jeanloz, 1958). The rotation of the *N*-acetylgalactosaminylglycerol suggests the presence of a small proportion of β -linkages, but the hygroscopic nature of this substance might account, at least in part, for the specific rotation appearing to be lower than expected for a pure α compound. As no *N*-acetylgalactosaminidase with established specificity was available, enzymic methods for settling this point were not entirely satisfactory. However, an enzyme preparation from pig epididymis, which readily hydrolysed β - but not α -methyl *N*-acetylglucosaminide, had a slight action on *N*-acetylgalactosaminylglycerol and on the teichoic acid. A sample of α -methyl *N*-acetylgalactosaminide, which, from its rotation, contained 20% of the β -isomer, was hydrolysed to the extent of about 20%

Fig. 3. Reactions of galactosaminylglycerol and its *N*-acetyl derivative.

(visual estimation) with this enzyme. Although this evidence is not conclusive, it appears that the enzyme is specific for β -acetylgalactosamine residues, and its action on the glycerol compound and the teichoic acid supports the view that some β -linkages may occur in these compounds. Cross-reactions of *S. albus* immune sera with *S. aureus* teichoic acid (containing only β -*N*-acetylglucosamine residues) may be related to the presence of a few β linkages in the *S. albus* teichoic acid (Haukenes *et al.* 1961).

The *N*-acetylglactosaminylglycerol units in the teichoic acid can possess phosphodiester linkages at positions 1 and 3 only. On the basis of a regular phosphodiester structure for this polymer, and from the analytical data, the formula (VI) is proposed for this teichoic acid (β -linkages omitted).

The alkali hydrolysis of a compound with the structure (VI) would involve the intermediate formation of cyclic phosphates of glycerol. Cyclization to a 5-membered phosphate could not occur on those glycerol units, that possess a sugar substituent at position 2; consequently galactosaminylglycerol monophosphate would be expected amongst the hydrolysis products only if such a grouping were to represent the terminal phosphorus end of the polymer. The absence of an appreciable amount of this phosphate indicates that few, if any, of these groups occur in the teichoic acid.

The presence of glycerol and its diphosphates in alkali hydrolysates shows that cyclic phosphate formation is not unidirectional, but can occur at either or both ends of a glycerol unit, provided that cyclization can occur on neighbouring glycerol residues (Kelemen & Baddiley, 1961). As with other teichoic acids, alkali hydrolysis should give small amounts of the alkali-stable diglycerol triphosphate (VII). This product, which is diagnostic for the presence of 1,3-phosphodiester linkages in the polymer, arises through cyclization occurring in opposite directions along the chain. However, the mixture of alkali-stable phosphodiesters from this teichoic acid was complex, and the satisfactory separation of all products was not achieved. In addition to (VII), the compounds (VIII) to (X) would be expected from this polymer, their occurrence being the result of hydrolysis of inter-

mediate cyclic phosphates in a manner very similar to that postulated for the formation of diglycerol triphosphate itself. The presence of all these phosphates in the phosphodiester fraction was demonstrated in the following manner.

The phosphodiesters were treated with intestinal phosphomonoesterase; products were inorganic phosphate and phosphodiesters, which separated on paper as three spots, all giving a rapid reaction with the periodate-Schiff reagent and thus containing glycerol residues that yield formaldehyde on oxidation. The product with the highest R_F (0.55) was diglycerol phosphate (XI), identified chromatographically (Kelemen & Baddiley, 1961; Critchely *et al.* 1962) and by further alkali hydrolysis to a mixture of glycerol and its monophosphates; this establishes the presence of di-

glycerol triphosphate (VII) in the teichoic acid hydrolysate. Material with a lower R_F (0.3) was a mixture of the two isomeric galactosaminyldiglycerol phosphates (XII) and (XIII), which gave on alkali hydrolysis galactosaminylglycerol and glycerol monophosphates. The material with the lowest R_F (0.0–0.15) was presumably the galactosaminyltriglycerol diphosphate (XIV), as it was hydrolysed with alkali to galactosaminylglycerol and a larger amount of glycerol monophosphates. Moreover, the mixture of products after hydrolysis with the phosphomonoesterase was treated with nitrous acid; the compounds (XII), (XIII) and (XIV) were destroyed, giving 2,5-anhydrotalose, diglycerol phosphate and a slow-moving compound believed to be triglycerol diphosphate. In addition, the original phosphodiesters (VII) to (X) were treated first with nitrous acid and then with the phosphatase; products were the anhydro sugar, much diglycerol phosphate (XI) and a little triglycerol diphosphate.

Three independent methods have been used for determining the chain length of the teichoic acid after removal of alanine. Potentiometric titration gave the ratio of primary to secondary phosphate groups, from which it was concluded that between 17 and 18 glycerophosphate units are present if the teichoic acid resembles others in possessing a phosphomonoester group at one end and a glycol at the other. These conclusions were supported by studies

Fig. 4. Phosphodiester from alkali hydrolysis and their dephosphorylation with a phosphomonoesterase. GalNH₂, Galactosaminyl.

with the phosphomonoesterase; the ratio inorganic phosphate formed to total phosphorus was 1:18.3. Oxidation with very dilute periodate did not give a sharp end point, presumably through oxidation of sugar residues and other degradative changes (Critchley *et al.* 1962). By extrapolation to zero time (see Fig. 1) the ratio periodate consumed by the terminal glycol group to total phosphorus was 1:18. Moreover, with more concentrated periodate it was shown that under conditions which cause complete oxidation of all sugar residues the ratio formaldehyde produced to total phosphorus was 1:18.1. The absence of detectable amounts of phosphodiester containing more than one amino sugar residue shows that at no part in the polymer chain are these residues attached to adjacent glycerol units.

SUMMARY

1. A glycerol teichoic acid has been isolated from the walls of *Staphylococcus albus* N.T.C.C. 7944. Experiments with ammonia and hydroxylamine on walls show that this polymer, like all others examined, is not associated covalently with mucopeptide.

2. The 'intracellular' teichoic acid was isolated from the supernatant, after removal of walls by

centrifuging at 100 000g, whereupon it sedimented with ribosomal material.

3. Acid hydrolysis of the wall teichoic acid gave galactosamine or, under milder conditions, *N*-acetylgalactosamine, together with glycerol and its mono- and di-phosphates and alanine.

4. The alanine has the D-configuration and is joined to hydroxyl groups in glycerol through labile ester linkages.

5. Galactosamine was identified by chromatography, by degradation with ninhydrin to lyxose and by periodate oxidation of the teichoic acid followed by reduction with borohydride and acid hydrolysis, whereupon D-serine was obtained (Fig. 2).

6. 2-O-D-Galactosaminylglycerol (III; R = H) and a little *N*-acetyl derivative (III; R = acetyl) were formed by hydrolysis with alkali. Their structures were established by hydrolysis, periodate oxidation and reaction with nitrous acid (Fig. 3). Oxidation of the *N*-acetyl derivative with periodate and then bromine, followed by acid hydrolysis, gave D-glyceric acid; this showed that the amino sugar was in the D-series.

7. From optical rotation and enzyme studies it is concluded that most of the glycosidic linkages have the α-configuration, but a small proportion of β-linkages may occur.

8. Amongst the products of alkali hydrolysis of the teichoic acid were several stable phosphodiester. These included the diglycerol triphosphate (VII), isomeric galactosaminyldiglycerol diphosphates (VIII) and (IX) and the galactosaminyltriglycerol tetraphosphate (X). The structures assigned to these compounds were deduced from a combination of phosphatase action and treatment with nitrous acid, followed by alkali hydrolysis (Fig. 4).

9. The structure (VI) is assigned to this teichoic acid. Titration, phosphatase action and periodate oxidation indicate a chain comprising about 18 glycerol phosphate units; approximately every third glycerol bears a *N*-acetylgalactosaminyl residue.

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