2. A flavan present in *E. astringens* bark and *E. marginata* heartwood has the expected chromatographic properties and colour reactions of leucodelphinidin but does not yield delphinidin when heated with acid.

3. The major stilbenes in the bark of *E. astringens* appear to be the previously unreported *cis–trans* isomers of a glucoside of 3,5,3′,4′,5′-pentahydroxystilbene. The aglucone of these stilbenes appears to be present in the heartwood of this species.

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Teichoic Acid from the Walls of *Staphylococcus aureus* H

STRUCTURE OF THE *N*-ACETYLGLUCOSAMINYLRIBITOL RESIDUES

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The teichoic acid in the walls of *Staphylococcus aureus* H is a ribitol phosphate polymer containing alanine and *N*-acylglucosamine residues (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958). Preliminary studies suggested that this compound resembles the teichoic acids in the walls of *Lactobacillus arabinosus* 17–5 (Armstrong *et al.* 1958; Archibald, Baddiley & Buchanan, 1961*b*) and *Bacillus subtilis* (Armstrong, Baddiley & Buchanan, 1960, 1961), in both of which D-alanine is attached through its carboxyl group in remarkably labile ester linkages, and the sugar residues are attached as glycosides to ribitol.

The structure of this teichoic acid is of particular interest, in view of the extensive investigations on the walls of this and related strains of *S. aureus* in connexion with mucopeptide biosynthesis and the mechanism of penicillin action (Park & Strominger, 1957; Strominger, Park & Thompson, 1959). Moreover, teichoic acids are associated with the

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immunological properties of bacteria (Baddiley & Davison, 1961), and evidence has been obtained recently (A. R. Sanderson, W. G. Juergens & J. L. Strominger, personal communication) that serological reactions shown by walls of a strain of *S. aureus* are associated with α-*N*-acetylglucosamine residues in the teichoic acid. It has also been shown (Haukenes, Ellwood, Baddiley & Oeding, 1961) that the group-specific antigens, polysaccharides A and B of *S. aureus* and *S. albus*, are serologically identical with the wall teichoic acids. Interest in this compound also arises from earlier observations that both glycerol and an unidentified polyol were found in preparations from strains of *S. aureus* of material which was believed to represent the cell walls (Mitchell & Moyle, 1951).

This paper describes the isolation and structure of *N*-acetylglucosaminylribitol obtained by alkali hydrolysis and phosphatase action on the ribitol teichoic acid from *S. aureus* H walls.

EXPERIMENTAL AND RESULTS

Materials. Staphylococcus aureus H was grown by Mr A. L. Davison from a culture provided by Dr J. T. Park; β-*N*-acetylglucosaminidase was kindly supplied by Dr G. A. Levvy; calf intestinal phosphatase was purchased from Sigma Chemical Co., Mo., U.S.A.; prostate phosphatase was prepared from human prostate glands (Schmidt, 1955); crude yeast hexokinase was a gift from Sigma Chemical Co.; ATP was purchased from Pabst Laboratories, Wis., U.S.A.; dihydrodiphosphopyridine nucleotide was purchased from C. F. Boehringer und Soehne, Germany.

Analytical methods. Phosphate was determined by the method of Chen, Toribara & Warner (1956), and alanine by the method of Rosen (1957).

Paper chromatography. Paper chromatography was carried out on Whatman no. 1 or no. 4 paper previously washed with 2N-acetic acid and then water. The following solvent systems were used: (A) propan-1-ol–aq. ammonia soln. (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 soln. (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 paper. Descending chromatography on Whatman no. 1 paper was carried out with solvents (D) and (E) and descending chromatography on Whatman no. 4 paper with solvent (C). The products were detected by the periodate–Schiff reagents for α-glycols (Baddiley, Buchanan, Handschumacher & Prescott, 1956), ninhydrin reagent for amino acids and amino sugars (Consden & Gordon, 1948), molybdate reagent for phosphate esters (Hanes & Isherwood, 1949), aniline phthalate reagent for reducing sugars (Partridge, 1949) and alkaline silver nitrate reagent for sugars (Trevelyan, Procter & Harrison, 1950).

With the exception of the methyl *N*-acetylglucosaminides, all compounds in Table 1 were obtained as degradation products of teichoic acid preparations examined during the course of this work.

Paper electrophoresis. Whatman no. 1 paper soaked with 0·1 M-ammonium acetate buffer, pH 3·8, was used at a voltage gradient of 4 v/cm. for 7 hr. Glucosaminylribitol phosphates moved 3·5 cm. and *N*-acetylglucosaminylribitol phosphates 11·5 cm. towards the anode.

Growth of organism. S. aureus H was grown in batches (15 l.) under forced aeration for 16 hr. at 37° in a medium containing: nutrient broth no. 2 (Oxoid), 25 g.; glucose, 10 g.; yeast extract (Oxoid), 5 g.; dipotassium hydrogen phosphate, 3 g.; water (1 l.); olive oil as an antifoam agent. The organisms were harvested in a refrigerated Sharples centrifuge, washed with cold 0·9% sodium chloride soln. (yield after washing, 5 g. wet wt. of organism/l. of culture) and defatted by successive treatment (30 sec.) in a blender with acetone, ethanol and ether (15 vol. of each) at room temperature. The defatted cells were dried *in vacuo*.

Preparation of walls. The washed cells (not defatted) were suspended in cold water (about 25 g. wet wt. in 100 ml. of water) and disrupted in a centrifuge shaker head (Shockman, Kolb & Toennies, 1957) at 1300 rev./min. for 40 min. Alternatively, portions (9 ml.) of the suspension were mixed with Ballotini beads (10 ml. of no. 11) and shaken in a Mickle disintegrator for 35–45 min. at 4°. The beads were removed by filtration through a no. 1 sintered-glass funnel.

Table 1. *Paper chromatography of degradation products from teichoic acid*

Solvent	R_F (A)	R_F (B)	$R_{Ribitol}$ (C)	$R_{Ribitol}$ (D)	$R_{Glucosamine}$ (E)
Ribitol	0·63	0·68	1·00	1·00	1·58
Glycerol	0·72	0·80	1·53	1·50	—
Anhydroribitol	0·72	0·80	1·42	1·43	—
Glucosamine	0·63	0·45	0·78	0·61	1·00
N-Acetylglucosamine	0·67	—	1·05	1·12	1·68
Alanine	0·60	0·65	—	—	—
Glucosaminylribitol	0·54	0·24	0·47	0·53	0·83
N-Acetylglucosaminylribitol	0·54	0·62	0·47	0·68	1·26
Glucosaminylribitol phosphates	0·25	—	—	—	—
2,5-Anhydromannose	0·65	—	—	1·40	—
α-Methyl *N*-acetylglucosaminide	—	—	1·70	—	—
β-Methyl *N*-acetylglucosaminide	—	—	1·50	—	—

Walls were collected by centrifuging at 20 000*g* for 30 min. in a Spinco ultracentrifuge and the middle layer (wall fraction) was separated from cell contents and whole cells. The wall fraction was washed and centrifuged several times with cold M-phosphate buffer, pH 7·0, and water to remove cytoplasmic material and whole cells, and then freeze-dried. The walls (Found: P, 2·4%) were examined by electron microscopy and were free from whole cells and other visible contaminants.

Isolation of teichoic acid. (*a*) From whole cells. The fat-free cells (50 g.) were extracted three times with portions (250 ml.) of ice-cold 10% trichloroacetic acid in a blender for 2 min. The homogenates were centrifuged and the combined supernatants filtered once through Supercel silica and ethanol (2 vol.) was added. The cloudy solution was kept overnight at 0°, the precipitate was centrifuged and acetone (1 vol.) was added to the supernatant, which was kept at 0° for 48 hr. The material obtained initially by adding ethanol was reprecipitated from ice-cold 10% trichloroacetic acid by adding ethanol (2 vol.), and then the precipitate (0·215 g.) was centrifuged, washed with ethanol and ether, and dried *in vacuo*. It contained teichoic acid and some nucleic acid and polysaccharide. The material obtained from the acetone fraction contained very little teichoic acid and was discarded.

The cell debris from the extraction described above was homogenized for 3 min. in a blender with 5 vol. of 10% trichloroacetic acid at 0° and the resultant cream stirred in the cold for 16 hr. After centrifuging, the supernatant was passed through a bed of Supercel silica, ethanol (2 vol.) was added and the mixture was kept at 0° overnight. The teichoic acid (1·51 g.) was collected by centrifuging, washed with ethanol and ether and then dried. It was reprecipitated from cold 10% trichloroacetic acid (30 ml.) with ethanol (60 ml.). After 24 hr. the white precipitate was centrifuged, washed twice with ethanol and then ether, and dried *in vacuo*. This teichoic acid (1·06 g.) contained some nucleic acid and intracellular glycerol teichoic acid.

(*b*) From walls. The walls (3·2 g.) were stirred with cold 10% trichloroacetic acid (75 ml.) at 2° for 24 hr. A further amount (25 ml.) of 10% trichloroacetic acid was added and the extraction continued for 36 hr. The mixture was centrifuged in the cold, the residual walls were washed twice with cold 10% trichloroacetic acid (50 ml.) and the combined supernatants were diluted with ethanol (300 ml.) and kept for 18 hr. at 0°. The teichoic acid (440 mg.) was centrifuged, washed with ethanol and ether, and then dried. Acetone (300 ml.) was added to the supernatant from this precipitation, which was then kept at 0° overnight. The resulting material (30 mg.) was impure. The teichoic acid was reprecipitated from cold 10% trichloroacetic acid (15 ml.) by adding ethanol (30 ml.) and the purified material (380 mg.) was washed and dried as before.

Examination of extracted walls. Extracted walls (150 mg.) were re-extracted with cold 10% trichloroacetic acid (40 ml.) for 1 week with addition of fresh trichloroacetic acid (5 ml.) at intervals of 24 hr. The walls were centrifuged and washed repeatedly with 10% trichloroacetic acid, ethanol and ether (to remove traces of trichloroacetic acid), and dried *in vacuo* (120 mg.; Found: P, 0·4%). A sample (12 mg.) was hydrolysed with 2N-hydrochloric acid for 3 hr. at 100°; chromatography in solvents (A) and (C) showed that no anhydroribitol was present in the hydrolysate.

Teichoic acid

Analysis. Teichoic acid (380 mg.) extracted from walls had $[\alpha]_D^{20} + 6°$ (*c* 3·0 in water). A sample (3·16 mg.) was dissolved in water and 0·01N-sodium hydroxide solution added to pH 10. This solution was used for the determination of phosphorus and alanine. The teichoic acid contained 6·4% of phosphorus (Found: ratio alanine:P, 0·66:1·0).

Alkali hydrolysis and enzymic dephosphorylation; vigorous conditions. Teichoic acid (1·0 g.) from the second extraction of whole cells was hydrolysed at 100° for 3 hr. in N-sodium hydroxide solution (10·0 ml.). A small amount of a brown flocculent precipitate was centrifuged and the supernatant neutralized with 10N-acetic acid (1·0 ml.). To the clear solution was added a solution of barium acetate (850 mg.) in water (5·0 ml.), followed by ethanol (500 ml.). The suspension was kept overnight at 0° and centrifuged. The supernatant was retained for isolation of alanine (see below); the precipitate was washed with ethanol (2 × 250 ml.) and the barium salts (1·35 g.) were dried *in vacuo*. A sample (5 mg.), after passing through a small column of Dowex 50 (NH_4^+ form) resin and chromatography in solvent (*A*), was shown to contain glucosaminylribitol phosphates, a little alanine and some material which absorbed ultraviolet light.

The barium salts (1·2 g.) were mixed with Dowex 50 (H^+ form) resin (1·0 ml.) and water (5·0 ml.) and the slurry was poured on to a column (4 cm. × 3 cm.) of Dowex 50 (H^+ form) resin. The column was washed with water until the eluate was neutral (total volume of eluate, 190 ml.). Activated dry Norit charcoal, previously boiled with 4N-acetic acid and washed with water, was added in small portions to the combined acidic eluate until the extinction of the supernatant after centrifuging was below 0·4 at 260 mμ. Ethanol (9·5 ml.) was added and the solution poured through a Celite bed; the charcoal and Celite were washed repeatedly with portions (10 ml.) of 5% ethanol in 0·1N-hydrochloric acid and the combined eluate (300 ml.) was neutralized with aqueous 2N-ammonia solution to pH 7. After evaporation to 20 ml. the solution was filtered to remove traces of charcoal and the filtrate was transferred to a 50 ml. graduated flask. 0·4 M-Ammonium acetate buffer, pH 5·5 (10 ml.), and prostate phosphatase (2·0 ml.) were added, the volume was adjusted to 50 ml. and the solution was incubated at 37° for 72 hr. Portions were removed at intervals of 24 hr. for the determination of inorganic phosphate (82·7% of the organic phosphate had been hydrolysed after 72 hr.). No additional inorganic phosphate was released by

fresh enzyme (1·0 ml.) after incubation for a further period of 78 hr.

The dephosphorylated material was passed through a column (2 cm. × 8 cm.) of Dowex 1 (OH^- form) resin which was then washed with water and the combined eluate and washings were evaporated *in vacuo*. The residual glass was dissolved in water (30 ml.) and the solution passed through a column (4 cm. × 4 cm.) of Dowex 50 (NH_4^+ form) resin. The column was well washed with water and the aqueous eluates were preserved for isolation of glycerol. Material from the column was then eluted with aqueous N-ammonia solution (140 ml.). A sample of the ammoniacal eluate, on chromatography in solvent (A), was shown to contain glucosaminylribitol. The evaporated eluate was made pH 4 with 0·1 N-hydrochloric acid and the solution evaporated *in vacuo* to a syrup. The syrup was dried over phosphorus pentoxide, dissolved in a little methanol and acetone added. The white hygroscopic glucosaminylribitol hydrochloride (110 mg.) was collected by centrifuging and dried *in vacuo*; it was chromatographically homogeneous.

The Dowex 1 column was washed with *N*-hydrochloric acid (120 ml.) and the eluate rapidly evaporated *in vacuo* to about 30 ml. The concentrate was neutralized to pH 7 with N-lithium hydroxide solution and evaporated to about 2 ml. Ethanol (110 ml.) was added and the white precipitate centrifuged and washed with ethanol–ether (1:1). The lithium salts consisted mainly of orthophosphate and a small amount of glucosaminylribitol phosphate, R_P 0·27 in solvent (A).

The aqueous eluate from the Dowex 50 column was evaporated to about 10 ml. and passed through a column (2 cm. × 7 cm.) of Dowex 1 (OH^- form) resin. The column was washed with water and the eluate evaporated to dryness. The residual liquid (17·6 mg.) was identified as glycerol from a mixed m.p. (73°) of its tribenzoate with authentic tri-*O*-benzoylglycerol.

Isolation of alanine. The supernatant from the barium salt precipitation (see above) was diluted with water to 1 l. and the pH adjusted to 6·0 with N-hydrochloric acid. The solution was passed slowly through a column (5 cm. × 5 cm.) of Dowex 50 (H^+ form) resin which was then washed with water. Material was eluted with 2·5 N-hydrochloric acid, fractions (25 ml.) being collected. The contents of tubes 4–7 were combined and evaporated *in vacuo* to a small volume. Ethanol (200 ml.) was added and sodium chloride removed by centrifuging. The supernatant was evaporated to dryness, the residue dissolved in water (5·0 ml.) and the solution was passed through a column (1 cm. × 10 cm.) of Amberlite IRC-50 (H^+ form) resin. The column was washed until the washings were neutral and the combined solutions were evaporated to dryness. The residue, which still contained some sodium chloride, was neutralized to pH 7 with N-sodium hydroxide solution, diluted to 75 ml. with water and the neutral sclution passed through a column (1 cm. × 12 cm.) of Dowex 1 (OH^- form) resin.

The column was washed with water and then with N-hydrochloric acid (100 ml.). The eluate was evaporated to a white solid, which was dissolved in a little water, ethanol and ether were added and the resulting crystalline precipitate was washed with ether and then dried. The alanine hydrochloride was chromatographically homogeneous and had m.p. 194–196°.

The configuration of the alanine was established by the action of D-amino acid oxidase on the isolated sample. Details of the procedure were similar to those described by Kelemen & Baddiley (1961).

Alkali hydrolysis and enzymic dephosphorylation: mild conditions. Teichoic acid (300 mg.) from *S. aureus* H walls was dissolved in saturated (room temperature) barium hydroxide solution (14·0 ml.) and the solution kept at 100° for 30 min. A sample (0·2 ml.) was passed through a column of Dowex 50 (NH_4^+ form) resin and the eluate examined by chromatography in solvents (A) and (B). The hydrolysate contained alanine, *N*-acetylglucosaminylribitol, *N*-acetylglucosaminylribitol phosphates, glucosaminylribitol phosphates and a trace of inorganic phosphate. Electrophoresis with 0·1 M-ammonium acetate buffer, pH 3·8, for 7 hr. with a voltage gradient of 4 v/cm. gave three spots at distances 3·5, 11·5 and 19 cm. from the origin towards the anode.

The bulk of the hydrolysate was diluted to 75 ml. with water and passed through a column (2 cm. × 4 cm.) of Dowex 50 (NH_4^+ form) resin and the column washed with water until the eluate was neutral. The eluate (250 ml.) was evaporated at 35° to 50 ml., 0·1 M-ethanolamine–hydrochloric acid buffer, pH 9·5 (50 ml.), and calf intestinal phosphatase (10 mg.) were added and the solution was incubated at 37° for 36 hr. Portions were removed for phosphate determination; after 36 hr., 95% of the organic phosphate had been hydrolysed.

The solution was passed through a column (1·2 cm. × 10 cm.) of Dowex 50 (NH_4^+ form) resin which was washed with water and the ammoniacal eluate (180 ml.) was evaporated *in vacuo*. A sample, on chromatography in solvents (A), (B) and (C), contained mainly *N*-acetylglucosaminylribitol together with some glucosaminylribitol, inorganic phosphate, alanine and a trace of an organic phosphate. When the column was washed further with aqueous 2 N-ammonia solution (50 ml.) only ethanolamine (R_{Ribitol} 1·2) and a trace of glucosaminylribitol were eluted.

The residue obtained by evaporation of the aqueous eluate from the Dowex 50 column was dissolved in water (250 ml.) and passed slowly through a column (2 cm. × 13 cm.) of Norit charcoal–Celite which was then washed successively with water (240 ml.), 10% ethanol (400 ml.) and 20% ethanol (200 ml.). (The aqueous and 20% ethanol eluates, on evaporation and chromatography, contained no desired material and were discarded.) The 10% ethanol eluate was evaporated *in vacuo* until most of the ethanol had been removed, and then passed through a column (1 cm. × 5 cm.) of Dowex 3 (OH^- form) resin. The eluate and water washings (25 ml.) from the column contained *N*-acetylglucosaminylribitol and some glucosaminylribitol (chromatography in solvents A, B and C).

Th eluate (250 ml.) from the Dowex 3 resin was passed slowly through a column (1·2 cm. × 20 cm.) of Dowex 50 (NH_4^+ form) resin which was then washed with water. The combined eluate and washings were freeze-dried and the residue was dried over phosphorus pentoxide *in vacuo*. The resulting clear gum (96 mg.) was chromatographically pure *N*-acetylglucosaminylribitol (solvents A and B).

Elution of the Dowex 50 column with aqueous 2N-ammonia solution (200 ml.) and evaporation yielded a fluffy residue. This was dissolved in water (1·0 ml.), insoluble material was centrifuged and the supernatant evaporated *in vacuo*. The gum (25 mg.) consisted of chromatographically pure glucosaminylribitol (solvent B).

Glucosaminylribitol

Acid hydrolysis. (Experiment by Dr F. E. Hardy.) Glucosaminylribitol (1·3 mg.) was hydrolysed with 2N-hydrochloric acid at 100° for 18 hr. The acid was evaporated *in vacuo* and the products were chromatographed in solvents (B) and (C). The products were anhydroribitol, glucosamine, ribitol and unchanged glucosaminylribitol.

Reaction with nitrous acid. (Experiment by Dr F. E. Hardy.) Glucosaminylribitol hydrochloride (3·7 mg.) and sodium nitrite (35 mg.) were dissolved in water (2·0 ml.) and the solution was heated at 50° for 2·3 hr. It was then passed through a column of Dowex 50 (NH_4^+ form) resin and the eluate heated at 80° for 10 min., evaporated and examined in solvent (D). The products were ribitol and 2,5-anhydromannose.

Acetylation. Glucosaminylribitol hydrochloride (30 mg.) was dried *in vacuo* over phosphorus pentoxide for 2 hr. and then dissolved in anhydrous pyridine (2·0 ml.). Freshly distilled acetic anhydride (0·3 ml.) was added and the mixture kept stoppered for 18 hr. at room temperature. This solution was poured into ice-cold water (5·0 ml.) and the slightly cloudy solution extracted with chloroform (3 × 10 ml.). The chloroform extract was washed successively with 0·05N-sulphuric acid (3 × 10 ml.) water (3 × 10 ml.), 0·5N-sodium carbonate solution (3 × 10 ml.) and water (3 × 10 ml.); it was then dried over anhydrous sodium sulphate, filtered and the chloroform evaporated. The remaining gum was dissolved in hot methanol (1·0 ml.) and water (1·0 ml.) was added until cloudiness developed. The viscous solution was kept at −20° overnight. After 24 hr. the crystals were collected by centrifuging and dried over phosphorus pentoxide. The glucosaminylribitol octa-acetate (12·5 mg.) had m.p. 137–138°, $[\alpha]_D^{20} - 14°$ (*c* 1·0 in methanol) (Found: C, 48·9; H, 6·1. $C_{27}H_{39}NO_{17},H_2O$ requires C, 48·6; H, 6·1%).

N-*Acetylglucosaminylribitol*

Several attempts to crystallize this compound were unsuccessful. *N*-Acetylglucosaminylribitol (52 mg.) was dissolved in water (2·0 ml.) and this solution was used for the following experiments. The value $[\alpha]_D^{21} + 10°$, calculated for the compound on a weight basis, was corrected to $[\alpha]_D^{21} + 12°$ on the basis of quantitative determination of formaldehyde produced on periodate oxidation.

Acid hydrolysis. The *N*-acetylglucosaminylribitol solution (0·10 ml.) was evaporated to dryness and hydrolysed with 2N-hydrochloric acid at 100° for 18 hr. The hydrolysate was passed through a column of Dowex 3 (OH^- form) resin (1·0 ml.), which was then washed with water. The neutral eluate and washings were evaporated and examined in solvents (C) and (E). The products were anhydroribitol and glucosamine, together with some ribitol and glucosaminylribitol.

Periodate oxidation and borohydride reduction. To the solution of *N*-acetylglucosaminylribitol (0·1 ml.) was added a 1% solution of sodium metaperiodate in water (0·6 ml.) and the mixture was kept at room temperature in the dark for 24 hr. Sodium borohydride (4·0 mg.) was added and the solution was kept for a further 24 hr. Concentrated hydrochloric acid (0·1 ml.) was added and the solution was kept at 100° for 2 hr. The acid was evaporated *in vacuo* and the residual gum examined in ethyl acetate–pyridine–water (7:2:1) (Viscontini, Hoch & Karrer, 1955). Glycerol was observed but no trace of ethylene glycol was detected.

Quantitative periodate oxidation. To the solution of *N*-acetylglycosaminylribitol (1·0 ml.) was added 0·3M-sodium metaperiodate solution (2·0 ml.) and the volume adjusted to 50 ml. with water. A control experiment containing no *N*-acetylglucosaminylribitol was also carried out. The oxidation mixture and control mixture were kept at room temperature in the dark. Samples (5·0 ml.) were removed for the determination of periodate and formic acid and further samples (2 ml.) for the

determination of formaldehyde (Hough, Powell & Woods, 1956). Formic acid was determined by titration of the oxidation mixture to the pH of the blank (6·4) with 0·01 N-sodium hydroxide solution. After 48 hr. the ratio periodate uptake:formaldehyde produced:formic acid produced was 3·04:1:0·62. Under alkaline conditions the oxidation mixture gave rise to acid products. Ethylene glycol (2·0 ml.) was added to a sample (5·0 ml.) of the oxidation mixture and the control solution (5·0 ml.) and the sealed samples were kept at 37° for 48 hr. to hydrolyse the formyl ester. After dilution with carbon dioxide-free water they were titrated to pH 6·4. The control solution consumed no alkali and the ratio for the oxidation mixture was 3·04:1:0·94. On the basis of the formaldehyde determination the amount of *N*-acetylglucosaminylribitol in the solution used (1·0 ml.) was 21 mg. Chromatography in solvents (A), (B), (C), (D) and (E) revealed no impurities.

Oxidation and hydrolysis to glyceric acid. The following procedure is based on that described by Archibald *et al.* (1961*b*). *N*-Acetylglucosaminylribitol solution (0·2 ml.) and α-methyl D-glucoside (4·6 mg.) were separately mixed with 0·15 M-sodium metaperiodate solution (final volume, 10 ml.) and kept at room temperature in the dark for 48 hr. The samples were passed through columns of Dowex 50 (H^+ form) (0·6 cm. × 4 cm.) and Dowex 2 (acetate form) (0·6 cm. × 4 cm.), which were then washed with water. The eluates (12·0 ml.) were freeze-dried, kept *in vacuo* for 3 hr. and dissolved in 1% barium benzoate solution (4 ml.). The solutions were cooled in ice and bromine (0·05 ml.) was added. The tubes were stoppered, shaken repeatedly in the cold for 1 hr. and kept at room temperature for 80 hr. Excess of bromine was removed by passing air through the solutions, which were then freeze-dried. Water (2·0 ml.) was added and the benzoic acid removed by centrifuging and washed with water (1·0 ml.). The supernatant and washings were concentrated to 1·0 ml., centrifuged and the supernatants hydrolysed with 6 N-sulphuric acid at 100° for 2 hr. The hydrolysates were neutralized by the addition of small portions of barium carbonate (total, 60 mg.) with shaking at intervals for 12 hr. Insoluble barium salts were centrifuged and washed with water. The supernatants and washings were passed through columns of Dowex 50 (NH_4^+ form) resin (1·0 ml.) and the eluate and washings were freeze-dried. The glyceric acid (R_F 0·53) was separated from traces of other products, e.g. glyceric acid amide (R_F 0·75), by chromatography in solvent (A). Appropriate strips were cut out and the product was eluted and rechromatographed in solvent (C). Paper strips containing glyceric acid ($R_{Ribitol}$ 0·66) were cut out and the material was eluted with a few drops of water. Control areas of paper were treated similarly.

The eluates were diluted to 2·0 ml. with water. Portions (0·2 ml.) were oxidized with 0·1 mM-sodium metaperiodate solution (Dixon & Lipkin, 1954) for determination of glyceric acid. Oxidation was complete in slightly more than 30 min., 1 mole of periodate being consumed by 1 mole of glyceric acid. Further portions (0·2–0·25 ml.) were diluted to 0·5 ml. with water and mixed with 3·5 ml. of a solution containing 0·01 M-ATP (0·2 ml.), rabbit-muscle enzyme (10 mg.) and yeast hexokinase (12 mg.).

Oxidation of the DPNH was followed spectrophotometrically at 340 mμ and was complete within 30 min. The glyceric acid from the α-methyl D-glucoside oxidized 1·85 mol.prop. whereas that from *N*-acetylglucosaminylribitol oxidized 1·87 mol.prop. of the nucleotide.

Acetylation. *N*-Acetylglucosaminylribitol (40 mg.) was dissolved in anhydrous pyridine (2·0 ml.) and freshly distilled acetic anhydride (1·0 ml.) added. After 48 hr. at room temperature the mixture was subjected to a procedure similar to that described previously for the isolation of the crystalline octa-acetate. The crystalline product (14 mg., needles) had m.p. 137–138° and was identical with the octa-acetate obtained from glucosaminylribitol (mixed m.p. 137°). The infrared spectra of the two substances were indistinguishable. The mother liquor was evaporated, the residue was dissolved in hot methanol (0·5 ml.) and water (3·0 ml.) added. After 48 hr. a further amount (2·5 mg.) of crystalline octa-acetate was collected by centrifuging. The mother liquor was evaporated to dryness and the residual gum (15 mg.) was dissolved in chloroform; insoluble material (3·0 mg.) was removed by centrifuging and the supernatant evaporated to a gum (12 mg.) and dried *in vacuo*. The gum had $[\alpha]_D^{20} + 17·5°$ (*c* 2·0 in methanol).

Studies with β-N-acetylglucosaminidase

Action on α- and β-methyl N-acetylglucosaminides and N-acetylglucosaminylribitol. The methyl *N*-acetylglucosaminides (2 mg.) (prepared by Mr D. C. Ellwood) were dissolved in water (0·1 ml.) and 0·1 M-citric acid–sodium hydroxide buffer, pH 4·25 (0·2 ml.), and enzyme solution (0·1 ml.) was added. The *N*-acetylglucosaminylribitol solution (0·1 ml.) was mixed with buffer (0·2 ml.) and enzyme solution (0·1 ml.), and all mixtures were incubated at 37°. The incubation mixtures were examined after 5 and 27 hr. in solvent (C). The α-methyl *N*-acetylglucosaminide, which contained a trace of the corresponding β-anomer, gave a trace of *N*-acetylglucosamine but was otherwise unchanged. The β-methyl *N*-acetylglucosaminide was completely hydrolysed to *N*-acetylglucosamine under the

conditions employed. The *N*-acetylglucosaminylribitol gave *N*-acetylglucosamine, ribitol and some unhydrolysed *N*-acetylglucosaminylribitol. Incubation for 60 hr. with additional enzyme did not affect the unhydrolysed *N*-acetylglucosaminylribitol.

Action on products from de-O-acetylation of the crystalline octa-acetate and material from mother liquors. The crystalline octa-acetate (6 mg.) and the material (6 mg.) from the mother liquors were dried, dissolved in anhydrous methanol (1·0 ml.), saturated with ammonia and the solutions were kept stoppered at room temperature for 60 hr. Ammonia and methanol were evaporated and the residual gums dissolved in water (0·2 ml.). Chromatography in solvents (B) and (C) showed that both contained *N*-acetylglucosaminylribitol and no other glucosamine or ribitol derivatives. Portions (0·1 ml.) were treated with enzyme as described above. After incubation at 37° for 16 hr., chromatography showed that hydrolysis of the material from the crystalline octa-acetate was complete, the products being ribitol and *N*-acetylglucosamine. These products were also formed from the material from the mother liquors, but even after incubation for 40 hr. about 30–40 % of the *N*-acetylglucosaminylribitol was unchanged. This mixture was passed through short columns of Dowex 3 (OH^- form) resin (0·5 ml.) and Dowex 50 (H^+ form) resin (0·5 ml.) and the columns were washed with water. The neutral eluate was evaporated to dryness and the residue examined in solvent (C).

The unhydrolysed *N*-acetylglucosaminylribitol was eluted from a paper chromatogram with a few drops of water and the eluate hydrolysed with 2N-hydrochloric acid at 100° for 24 hr. The hydrolysate was passed through a short column of Dowex 3 (OH^- form) resin (1·0 ml.) and the neutral eluate freeze-dried and chromatographed in solvent (E) for 24 hr. Glucosamine was the only hexosamine detected. Co-chromatography with glucosamine gave only one spot, whereas with galactosamine two spots were observed.

DISCUSSION

Teichoic acid is an important component (25–40 % of dry weight) of the walls of *Staphylococcus aureus*. Clean washed walls, prepared by a method based upon that described by Salton & Horne (1951), contain about 2·4 % of phosphorus, 83 % of which can be accounted for as extractable teichoic acid. The small amount of unextracted phosphate is not a teichoic acid, as acid hydrolysates of quite large samples of extracted walls do not contain detectable amounts of anhydroribitol or ribitol phosphates. The presence in comparable amounts of an insoluble organic phosphate has been noticed in the walls of many other Gram-positive bacteria and, in fact, has been encountered in the walls of all organisms studied in this Laboratory (Archibald *et al.* 1961*b*; Kelemen & Baddiley, 1961). As walls which have been extracted exhaustively are composed solely of mucopeptide (cf. Archibald, Armstrong, Baddiley & Hay, 1961*a*), we conclude that in general mucopeptide must contain a few phosphate residues.

The teichoic acid extracted from washed walls with trichloroacetic acid solution and then precipitated with acetone contained no detectable contaminants, and yielded, on acid hydrolysis, alanine, glucosamine, inorganic phosphate, ribitol phosphates, anhydroribitol and a trace of glucosaminylribitol (Armstrong *et al.* 1958). Most of the degradations described in the present work were carried out on material prepared from walls, although when the presence of impurities was relatively unimportant, material extracted from whole, defatted cells was used. These less-pure preparations contained nucleic acids and intracellular glycerol teichoic acid. It is noteworthy that, although the organism contains an intracellular glycerol teichoic acid, no traces of glycerol teichoic acid were detected in washed walls.

From the earlier work on the nature of the acid-hydrolysis products, and from the paper-chromatographic identification of a glucosaminylribitol from alkali and phosphatase hydrolysis, it was clear that the teichoic acid from *S. aureus* walls was similar to other ribitol teichoic acids in being a polymer in which ribitol phosphate units were joined together through phosphodiester linkages. The sugar residues would be attached glycosidically to ribitol hydroxyl groups, and alanine would be attached to the polymer through ester linkages involving the carboxyl groups of the amino acid residues and hydroxyl groups in the polymer. The alanine in this teichoic acid, like that in all other teichoic acids examined, has the D configuration. The elucidation of the detailed structure of the polymer necessitated the isolation of the glucosaminylribotol and its degradation. The earlier procedure was unsuitable for the preparation of isolatable amounts of this material, but a modification involving intermediate isolation of the glucosaminylribitol phosphates, and then removal of nucleotide impurities with charcoal and ion-exchange chromatography, gave reasonable amounts of pure material.

The glucosaminylribitol gave a solid hygroscopic hydrochloride which readily yielded a crystalline octa-acetate, m.p. 137–138°. Previous observations on its acid hydrolysis were confirmed; it was hydrolysed only slowly under vigorous conditions, giving glucosamine and anhydroribitol. On the

other hand, it reacted readily with nitrous acid in the manner described by Foster, Martlew & Stacey (1953) for glucosaminides. The products were identified chromatographically as ribitol and 2,5-anhydromannose (chitose), thus confirming that the compound is a ribitol glucosaminide.

Less vigorous alkali hydrolysis followed by phosphatase treatment gave a mixture containing *N*-acylglucosaminylribitol and a small amount of glucosaminylribitol. Although the acyl compound was isolated in pure form by ion-exchange chromatography it did not crystallize. However, it yielded a crystalline acetyl derivative which was identical with the octa-acetate obtained from the glucosaminylribitol. It follows that the *N*-acyl residues in the teichoic acid are acetyl.

Acid hydrolysis of the *N*-acetyl compound gave glucosamine and anhydroribitol, together with small amounts of ribitol and glucosaminylribitol. The *N*-acetylglucosaminide was considerably more labile towards acid than was glucosaminylribitol, an observation in agreement with hydrolysis characteristics reported for a variety of glucosaminides and their *N*-acyl derivatives (Foster *et al.* 1957).

The *N*-acetylglucosaminide was oxidized with periodate and the resulting trialdehyde reduced with sodium borohydride. Hydrolysis of the reduction product with acid and paper chromatography of the hydrolysate gave glycerol but not ethylene glycol. This indicates a pyranose structure in the acetylglucosamine residue and precludes the 1 and 5 positions in the ribitol for the location of the glycosidic linkage.

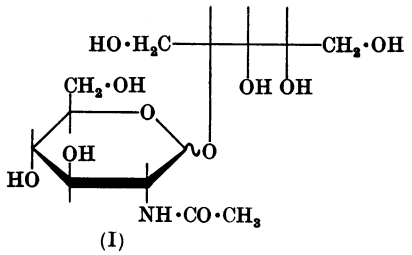
The *N*-acetyl compound consumed 3 mol.prop. of periodate and yielded 1 mol.prop. of formaldehyde and 1 mol.prop. of formate. It follows that the glucosaminyl residue possesses a pyranose structure and must be attached to the 2 or 4 hydroxyl group of the ribitol as shown in (I) and (II).

A decision between these alternatives was made by oxidation studies analogous to those described for ribitol glucosides obtained from teichoic acids (Armstrong *et al.* 1960; Archibald *et al.* 1961*b*). The trialdehyde from the periodate oxidation of the *N*-acetylglucosaminide was oxidized with bromine water to a tricarboxylic acid. Acid hydrolysis of this gave glyceric acid, together with other products. The configuration of this glyceric acid was determined by conversion of a known amount into its 3-phosphate by the action of ATP and a kinase, then determination with DPNH and a rabbit-muscle-enzyme preparation. Glyceric acid 3-phosphate determined in this way must possess the D configuration. It was found that all the glyceric acid formed through oxidation of the ribitol derivative had the D configuration. Structure (I) would yield 2 mol.prop. of D-glyceric acid whereas structure (II) would give 2 mol.prop. of DL-glyceric acid; consequently structure (I) correctly represents the *N*-acetylglucosaminylribitol from the *S. aureus* teichoic acid.

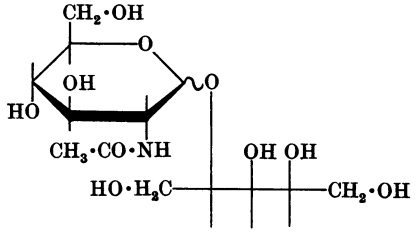
The *N*-acetylglucosaminide is a 4-substituted D-ribitol derivative (equivalent to a 2-substituted L-ribitol derivative), and thus corresponds to the monoglucosides of ribitol obtained from the wall teichoic acids of *B. subtilis* (Armstrong *et al.* 1960) and *L. arabinosus* (Archibald *et al.* 1961*b*).

The rotation of the crystalline octa-acetate, $[\alpha]_D - 14°$, obtained either by acetylation of glucosaminylribitol or its *N*-acetyl derivative, is consistent with a β configuration for the glycosidic linkage. However, the corresponding values for the non-crystalline *N*-acetylglucosaminylribitol, $[\alpha]_D + 12°$, and for the teichoic acid itself, $[\alpha]_D + 6°$, suggest that both α and β linkages may be present. If the crystalline octa-acetate possesses the β configuration it was likely that selective crystallization of this enantiomorph had occurred. This was supported by the rotation, $[\alpha]_D + 17{\cdot}5°$, of the gummy residue from the crystallization mother liquors, which would appear to contain both α and β forms.

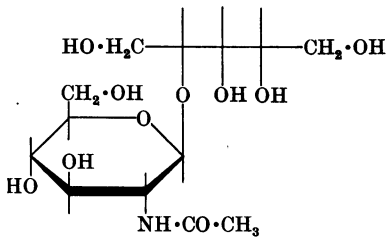
The presence of α and β linkages was confirmed by studies with a β-*N*-acetylglucosaminidase. The specificity of this enzyme for the hydrolysis of β linkages was confirmed through control experiments with α- and β-methyl *N*-acetylglucosaminides. Visual estimation by paper chromatography of products indicated that the *N*-acetylglucosaminylribitol was only partially hydrolysed by the enzyme to ribitol and *N*-acetylglucosamine under



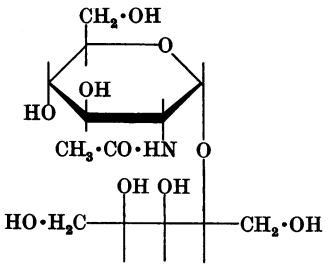
(I)



(II)



(III)



(IV)

conditions which effected complete hydrolysis of the β-methyl compound. Moreover, prolonged treatment of the unhydrolysed part with fresh enzyme was ineffective. It follows that both α and β linkages are present in the preparation.

The crystalline octa-acetate and the gummy acetate from the mother liquors were de-*O*-acetylated with ammonia, and the resulting samples of *N*-acetylglucosaminylribitol were subjected to hydrolysis with the enzyme. That from the crystalline octa-acetate was completely hydrolysed, whereas only 60–70% of that from the non-crystalline acetate was hydrolysed. *N*-Acetylglucosaminylribitol which resisted enzymic hydrolysis was isolated from the incubation mixture and shown to contain glucosamine and no other sugar.

It is clear that the *N*-acetylglucosaminylribitol obtained from this teichoic acid is a mixture of 4-*O*-β-(*N*-acetyl-D-glucosaminyl)-D-ribitol (III) and 4-*O*-α-(*N*-acetyl-D-glucosaminyl)-D-ribitol (IV). It has been suggested that partial inversion of configuration of the glycosidic linkage can occur during de-*O*-acetylation of fully acetylated glucosaminides under alkaline conditions (Hough & Taha, 1956; Leaback & Walker, 1957). If this suggestion were correct, the isolation of both α- and β-glucosaminides and their *N*-acetyl derivatives by alkali hydrolysis of a teichoic acid would not necessarily imply the presence of both types of linkage in the teichoic acid polymer. However, it has been shown by Dr F. E. Hardy that the glycosidic linkage in α- and β-methyl *N*-acetylglucosaminides is unaffected by alkali under conditions similar to and even more vigorous than those used in this work. Experiments to be described elsewhere have shown that the earlier suggestion is incorrect, and no inversion of configuration of the glycosidic linkage occurs during alkali de-*O*-acetylation of glucosaminides.

The isolation of a mixture of α- and β-*N*-acetylglucosaminylribitol and the rotation of the teichoic acid indicate that both types of linkage must occur in the polymer. However, the rotation of samples of teichoic acid prepared from different batches of *S. aureus* H cells and walls has varied, and recent samples, e.g. one examined by Dr R. O. Martin with $[\alpha]_D - 15{\cdot}6°$, do not contain detectable amounts of α linkages. These variations are reflected in the content of α anomers in the glucosaminylribitol phosphates obtained by alkali hydrolysis of different samples of teichoic acid (unpublished observations by Dr R. O. Martin). It is not yet known what are the factors concerned in determining the presence and proportion of α linkages in this teichoic acid. No obvious mutation has been detected, as the phage type pattern and behaviour towards different antibiotics have remained unaltered during the period when change in the composition of the teichoic acid was observed (we thank the Department of Bacteriology, Royal Victoria Infirmary, Newcastle upon Tyne, for phage and antibiotic type determination).

The teichoic acid from walls of *S. aureus* H contains *N*-acetyl-D-glucosamine and D-alanine. It follows that structures of the mucopeptide component cannot be directly derived from amino sugar and amino acid ratios in unextracted walls (Park & Strominger, 1957; Strominger, Park & Thompson, 1959). In this we agree with Janczura, Perkins & Rogers (1961). Evidence has been presented by Archibald *et al.* (1961*a*) that the alanine of *S. aureus* H teichoic acid is not derived from a terminal D-alanine residue of mucopeptide in covalent ester linkage with the teichoic acid in the wall.

SUMMARY

1. The ribitol teichoic acid has been isolated from the walls of *Staphylococcus aureus* H, where it accounts for nearly all the organic phosphorus and comprises 25–40% of the dry weight of the walls.

2. The general structure of this material, which is a ribitol phosphate polymer containing *N*-acetylglucosamine residues and labile D-alanine ester groups, has been confirmed.

3. Alkali hydrolysis followed by phosphatase treatment gives glucosaminylribitol, whereas similar treatment under less vigorous conditions gives *N*-acetylglucosaminylribitol. Both these glycosides are mixtures of α and β anomers.

4. The structures (III) and (IV), in which the α- or β-*N*-acetylglucosaminyl residue occupies the

4-position of D-ribitol, have been established by periodate oxidation, oxidation and hydrolysis to D-glyceric acid and from optical-rotation studies.

5. Studies with β-*N*-acetylglucosaminidase confirm the presence of α and β linkages in the acetylglucosaminides (III) and (IV), and it is concluded that both types of glycosidic linkage occur in teichoic acid preparations from some batches of the organism.

6. The proportion of α and β linkages in the teichoic acid has varied in samples prepared from different batches of organism, and batches produced recently yield a teichoic acid which apparently does not contain α linkages.

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Amino Acid Transfer from Aminoacyl-Ribonucleic Acid to Serum Albumin by the Microsome Fraction from Rat Liver

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In 1958 it was reported that the soluble cytoplasm of rat liver contained a ribonucleic acid fraction, to which amino acids could be bound in the presence of adenosine triphosphate (Hoagland, Stephenson, Scott, Hecht & Zamecnik, 1958). Amino acids attached to this nucleic acid were transferred to protein when rat-liver microsomes were incubated in the presence of an energy-generating system. The ribonucleic acid fraction involved in these reactions has been designated soluble or transfer ribonucleic acid. It is now known that the carboxyl group of the amino acid is linked to the 2′,3′-position of the terminal ribose of the nucleic acid (Zachau, Acs & Lipmann, 1958; Preis, Berg, Ofengand, Bergmann & Dieckmann, 1959). Hence the complex is an aminoacyl-ribonucleic acid.

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